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Please address all inquiries to the Publishers:
Eurekah.com / Landes Bioscience, 810 South Church Street
Georgetown, Texas, U.S.A. 78626
Phone: 512/ 863 7762; FAX: 512/ 863 0081
www.Eurekah.com
www.landesbioscience.com

ISBN: 1-58706-203-8

Library of Congress Cataloging-in-Publication Data

Klionsky, Daniel J.
Autophagy / Daniel J. Klionsky.
p. ; cm.
Includes bibliographical references and index.
ISBN 1-58706-203-8
1. Cell death. 2. Apoptosis. 3. Homeostasis. I. Title.
[DNLM: 1. Autophagocytosis. 2. Cell Death. 3. Homeostasis. 4. Lysosomes--physiology. 5. Vacuoles--physiology. QH 603.L9 K65a 2003]
QH671.K565 2003
571.9'36--dc22

2003021921

Dedication

This book is dedicated to my favorite editor, Lisa.

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PREFACE

After decades of research in the field of autophagy the first Gordon Research Conference on "Autophagy in Stress, Development and Disease" was held in June, 2003. This meeting led me to consider whether this is the right timing for a book on the topic of autophagy (pronounced \ȯt-ō-fā'-jē\) The success of the meeting—the diverse and interesting talks and active audience participation—indicated that the answer is obviously "yes." This is a very exciting time for autophagy research as tremendous advances are being made while some fundamental questions remain to be addressed—a situation that is very inviting for new scientists entering the field.

As the conference chair Beth Levine pointed out, some of the most intriguing questions facing autophagy researchers were proposed nearly 40 years ago in a review article written by de Duve and Wattiaux (Annual Review of Physiology 1966; 28:435-492): For example, concerning the source of the sequestering membrane, the hallmark of macroautophagy, they wrote "The origin of the membrane surrounding autophagic vacuoles has given rise to many speculations" including de novo formation, the endoplasmic reticulum, Golgi complex and endocytic vacuoles. Furthermore, regarding the specificity of macroautophagy, de Duve and Wattiaux questioned whether the process is "…essentially blind and accidental or discriminating and directed…a much more sophisticated phenomenon capable…of discriminating between normal and abnormal cellular constituents…"

The data that led to de Duve's astute questions, however, were based almost entirely on morphological observations. Fortunately, researchers in the autophagy field are now able to employ genetic, molecular genetic and biochemical approaches along with sophisticated microscopy techniques to address these problems. I think the following chapters of this book will make it clear that while many questions still remain, we have made tremendous advances in understanding the molecular basis of autophagy. Approximately 27 genes have been identified that appear to have exclusive roles in autophagy or autophagy-like processes in yeast, and at least an additional 22 genes have been implicated in the process. This fact alone has opened up tremendous lines of research, allowing advances in several model systems as homologues of most of these genes are being identified in higher eukaryotes. Research in systems other than yeast will soon allow advances in new areas of autophagy such as its molecular role in organismal development. Increasing connections between autophagy and cell physiology as well as the role of autophagy in human disease will continue to make this an exciting and relevant area of research.

Would I undertake this type of editing project again? Although it turned out to be much more work than I anticipated, the answer is still "yes"—the opportunity to interact closely with so many colleagues who have been

extremely generous with their time and efforts has been a wonderful experience. The autophagy community continues to be open and friendly and the Gordon Research Conference was marked by the presence of many researchers who are new to the field. We can all look forward to the addition of new chapters from these scientists when it is time to prepare the second edition of this book.

As a final but important note, researchers in the yeast autophagy community have recently agreed on a common nomenclature for the various genes that are involved in autophagy, pexophagy (pronounced \pex-ō-fā'-jē\) and the cytoplasm to vacuole targeting pathway (Table 1). The adoption of a common nomenclature will be of tremendous help to newcomers who will no longer have to remember that, for example, *APG9* = *AUT9* = *CVT7* = *GSA14* = *PAZ9*. Unfortunately, this consensus was reached much too late to modify the chapters in this book. This will have to be corrected in the second edition…

Daniel J. Klionsky, Ph.D.

Table 1. New nomenclature for autophagy-related genes

Gene Designation[a]						
Current	**Former**					
ATG	***APG***	***AUT***	***CVT***	***GSA***	***PAZ***	***PDD***
1	1	3	10	10	1	7
2	2	8	—	11	7	—
3	3	1	—	20	—	—
4	4	2	—	—	8	—
5	5	—	—	—	—	—
(6)[b]	6	—	—	—	—	—
7	7	—	2	7	12	—
8	8	7	5	—	2	—
9	9	9	7	14	9	—
10	10	—	—	—	—	—
11	—	—	9	9	6	18
12	12	—	—	—	—	—
13	13	—	—	—	—	—
14	14	—	12	—	—	—
15	—	5	17	—	—	—
16	16	—	11	—	3	—
17	17	—	—	—	—	—
18	—	10	18	12	—	—
19	—	—	19	—	—	—
20	—	—	20	—	—	—
21[c]	—	—	21	—	—	—
22	—	4	—	—	—	—
23	—	—-	23	—	—	—
(24)[d]	—	—	13	—	16	—
25	—	—	—	—	—	4
26[e]	—	—	—	—	4	—
27[f]	—	—	24	—	—	—

[a] See Klionsky DJ, Cregg JM, Dunn Jr WA et al. A unified nomenclature for yeast autophagy-related genes. Dev Cell 2003; 5:539-545 for more detailed information.
[b] The standard name for this gene is *VPS30*.
[c] This gene was originally named *MAI1*.
[d] The standard name for this gene is *SNX4*.
[e] This gene was originally named *UGT51*.
[f] This gene was originally named *ETF1*.

CHAPTER 1

Autophagy: An Overview

Daniel J. Klionsky

Autophagy has been a focus of research for over half a century. Based on the increased number of publications, range of model systems and variety of topics being studied in regard to autophagy, however, research into this topic has increased and continues to increase tremendously starting within the last five years. There are various reasons for this increased focus including the identification of the molecular components of the autophagic machinery as well as the correlation between autophagy and various human diseases. With a greater number of researchers entering into the field, I thought it would be useful to have a text that provides a summary of the current state of knowledge as well as presents some of the future directions for autophagy research. These are the goals of this book.

Most of the focus in cell biology concerning organelles has been on protein biosynthesis and organelle biogenesis. While these are important topics, this focus has largely ignored the fact that there is a homeostasis that involves continuous adaptation by both synthesis and degradation. With this realization, the lysosome and the plant and yeast vacuole would seem to be obvious candidates for cell biology research. Unfortunately, these organelles have often been overlooked due to the commonly held view that they act primarily as cellular garbage disposals. As we now know, these organelles play critical roles in a variety of cellular processes that go well beyond the concept of general degradation. In particular, the yeast vacuole carries out a range of physiological functions including cytosolic ion and pH homeostasis and, similar to the plant vacuole, has roles in osmoregulation and metabolite storage. Furthermore, as will become clear from this book, these organelles are a vital part of the dynamic cellular processes that are grouped under the name "autophagy." What is autophagy? In selecting chapters for this book, I have been liberal with the use of this term. Most researchers think of autophagy as a degradative process occurring within the cell, so called "self eating," and indeed this is one of the primary purposes of the autophagic process. However, there are now examples of specific autophagy that is involved in biosynthesis (the Cvt pathway), prevention of disease and aging (including removal of damaged organelles), regulation of metabolism (through the elimination of specific enzymes) as well as developmental pathways (such as the death of sympathetic neurons). Thus, this book includes chapters on sorting and degradative processes (for example, chaperone-mediated autophagy, vacuolar import and degradation, and the multivesicular body pathway) that may not fit within some people's definition of this term.

As a quick overview, this book has been divided into six main sections. The first section deals with macroautophagy in mammalian cells with a focus on regulation. There is a transition chapter that covers regulation in both mammalian cells and yeast, while the second section of the book is on macroautophagy in baker's yeast. This part of the book has a focus on components of the autophagic machinery. Continuing with yeasts, the third section discusses microautophagy. This includes a chapter about a new process, piecemeal microautophagy of the nucleus, and then moves into a well described system, peroxisome degradation, pexophagy, which occurs through both micro- and macroautophagic processes. Concluding the yeast section (and going back to mammalian cells), the fourth part of the book describes additional

Autophagy, edited by Daniel J. Klionsky.

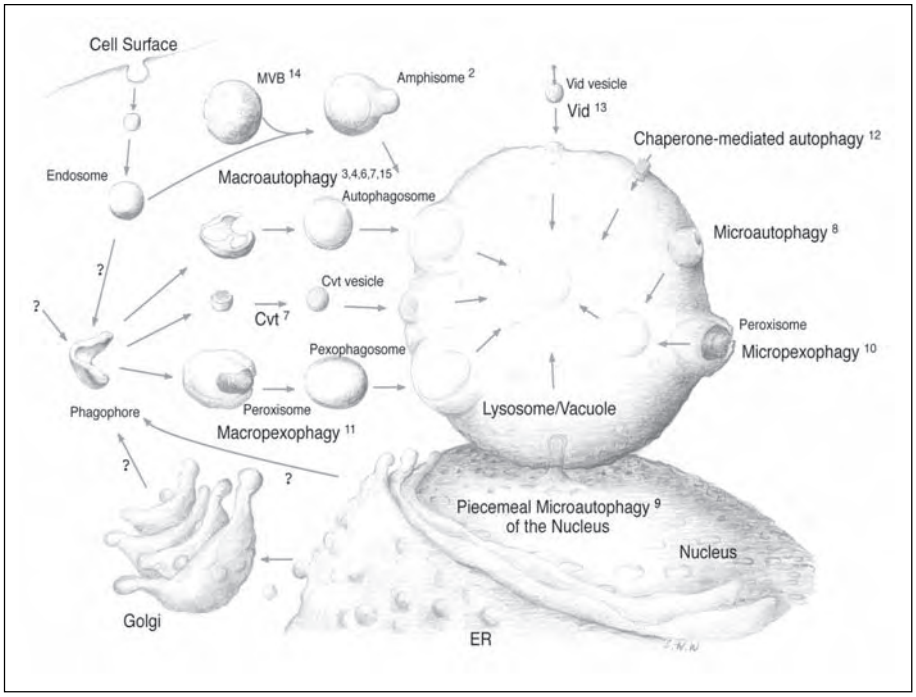

Figure 1. Lysosomal/vacuolar degradation pathways. Membrane-bound intermediates, pathways and organelles are labeled. The relevant chapters are indicated in superscript. See the text for details.

degradative processes that involve the vacuole. While these processes are "autophagic" in the sense that they involve degradation of parts of the cell, they are all specific processes that do not appear to utilize components of the autophagy pathways that have been identified as playing a role in micro- or macroautophagy. The fifth section primarily discusses autophagy in additional model systems. This includes the molecular analysis of autophagy in mammalian cells, and autophagy in plants, *C. elegans* and *Drosophila*. Finally, the sixth section of the book examines the connections between autophagy and disease.

As can be seen in the above paragraph, there are many types of degradative processes. These are described in detail in the following chapters. For a brief overview, I refer the reader to (Fig. 1), which shows the various pathways used for delivery of proteins and organelles to the lysosome or the analogous yeast organelle, the vacuole. The best characterized of these pathways is macroautophagy, which typically involves the formation of a double-membrane cytosolic vesicle. Through most of the book, the term "autophagy" is used synonymously with macroautophagy except where otherwise specified. The degradation of organelles such as peroxisomes, by a process termed pexophagy, can occur by a macroautophagy-like process. However, organelle degradation, and possibly that of bulk cytosol, can also occur through a microautophagic process. In this case, uptake occurs at the surface of the lysosome/vacuole and presumably does not involve a separate membrane (although the role of an additional membrane source cannot be ruled out). As described below, other degradative processes involve direct translocation across the lysosome membrane or sequestration within single-membrane cytosolic vesicles. Thus, there are many variations on the mechanisms used for subcellular degradation.

In chapter 2, Per Seglen and colleagues start with an introduction to the terminology of the autophagic structures. This chapter discusses one of the most prominent questions in the field—what is the origin of the sequestering membrane. This chapter also raises issues concerning the

formation of the autophagosome such as the role of cytoskeletal elements. One of the main advantages of studying autophagy in mammalian cells versus yeast is that the morphology of the subcellular organelles and autophagic intermediates is distinct. Nonetheless, the complexity of autophagy has made the morphological analysis problematic. Seglen and colleagues point out the difficulties with some of the autophagy nomenclature (e.g., autophagic vacuole versus autophagosome). However, I must note that there is not a general agreement in the autophagy field about these terms. Furthermore, some terms derive from strictly morphological observations; and in some cases, morphological observations are the primary means of analysis (for example, for diagnosis of diseases where small sample sizes are available, or in systems where biochemical or molecular genetic approaches are not practical). In the absence of specific markers that can be used to define each type of compartment the main discriminating features are the type of limiting membrane and the nature of the lumenal contents. Another point of difference with regard to nomenclature concerns the particular model system being studied. For example, because the lysosome-like compartment in yeast is referred to as the "vacuole," yeast researchers use the term "autophagosome" rather than "autophagic vacuole." Accordingly, in chapters that deal specifically with yeast, "vacuole" refers to the lysosome-like organelle. In other chapters, I have attempted to include the modifier "yeast" when referring specifically to the yeast vacuole.

I think it is accurate to say that autophagy research in higher eukaryotes has been hindered by the difficulty of applying genetic approaches. This has made it problematic to identify the molecular components of the autophagic machinery. However, regulation of autophagy is one area in particular where the research in mammalian cells has provided the most insight. Chapters 3 and 4, by Patrice Codogno and Fred Meijer, and by Per Seglen and colleagues discuss the roles of amino acids, lipid kinases, G proteins, and protein kinases and phosphatases in controlling autophagy. It is important to stress that the study of the control of autophagy has shed light on the role of amino acids as signaling molecules. Furthermore, several signaling pathways engaged in the control of autophagy also control cell death and are frequently altered during tumor growth. One point to note is that autophagy in mammalian cells appears to be constitutively active and is subject to suppression. In contrast, autophagy in yeast is inducible. This probably reflects the different roles of autophagy in yeasts versus higher eukaryotes. In yeasts, autophagy is primarily a starvation response, while in higher eukaryotes it plays a significant developmental and homeostatic role. The one area of regulation that has seen significant progress in the yeast system concerns the function of the Tor kinase. In chapter 5, Hagai Abeliovich discusses the current understanding of the yeast and mammalian Tor proteins and how they regulate autophagy.

In contrast to higher eukaryotes, the single biggest advantage of yeasts is the facility of applying genetic and molecular genetic techniques. That is not to say that morphological studies in yeasts have not also been important. In fact, morphological analyses first established the fact that yeasts carry out an autophagic process that appears to be similar to that in mammalian cells. However, as a result of genetic and molecular genetic screens, the ease of gene disruptions in a haploid background, the complete sequencing of the yeast genome and the application of genomic/proteomic approaches, many components of the autophagic machinery have been first identified in yeasts. A major breakthrough in identifying the proteins that play a role in autophagy came from work in Yoshinori Ohsumi's lab, beginning with the identification and characterization of the first autophagy gene, Apg1. Chapter 6, by Takeshi Noda and Yoshinori Ohsumi, describes our current knowledge of this and many other components that have since been identified in their lab, that of Michael Thumm and my own. I would like to offer some brief comments about terminology in the yeast and mammalian systems. In yeast, wild type gene names are designated by three letters and a number in upper case, italics (*CVT19*); mutant genes are in lower case, italics (*cvt19*; sometimes with a hyphenated number to indicate a particular allele); proteins are designated with three letters, only the first letter capitalized, roman (Cvt19; throughout the text, I have omitted the suffix "p" that is sometimes used to designate a protein); pathways are typically denoted by three letters, only the first being capital-

ized, without a number (Cvt). In mammalian cells both wild type and mutant genes are written in lower case, italics. Proteins are written in nonitalics, with the first letter upper case and all other letters lower case. While these rules are followed quite closely in the yeast field, they are not as closely observed by mammalian cell researchers. Finally, because the autophagy genes were first identified in *Saccharomyces cerevisiae* the prefix "Sc" is not usually used to denote genes from this organism. In the absence of any modifier, it should be assumed that the gene being referred to is in yeast. Otherwise, when referring to homologues this book uses the first letter of the genus and species to denote the organism. For example, *AtAPG1* and AtApg1 refer to the homologues of the yeast *APG1* gene and the Apg1 protein in *Arabidopsis thaliana*.

As described in chapter 7 by Per Stromhaug and myself, simultaneous with the studies of autophagy in yeast, my lab was examining the novel targeting mechanism used to transport the resident hydrolase aminopeptidase I to the vacuole; delivery occurs essentially independent of the secretory pathway used by most resident hydrolases and the process has been termed the cytoplasm to vacuole targeting (Cvt) pathway. The protein components of the Cvt pathway overlap extensively with those of autophagy (a complete listing of the genes involved in the autophagy, Cvt and peroxisome degradation pathways in yeasts can be found through the following URL: http://www.biology.lsa.umich.edu/research/labs/klionsky/klionskylab.html). At present, there is no evidence for the Cvt pathway outside of baker's yeast. However, there are two major avenues opened by these studies. First, as mentioned above, autophagy in yeast is inducible while the Cvt pathway appears to be constitutive. Thus, regulation of the conversion between the two pathways can be conveniently studied in this system. A second and related topic concerns the observation that in *Saccharomyces cerevisiae* there are components that are specific for the Cvt pathway or autophagy. For example, the import of aminopeptidase I occurs by both the Cvt pathway and autophagy. Under starvation conditions, this process represents a specific or selective type of autophagy. Selective autophagy is an important topic as it pertains to the degradation of particular organelles as described below. Thus, autophagy can be nonspecific (for example, in response to nutrient deprivation) or specific (for example, in the case of organellar turnover). These basic differences lead to speculation on the origin of these pathways. Was bulk (that is, nonspecific) autophagy the primordial pathway and did cells adapt this process to allow specific types of degradation with the addition of a few components to the autophagic machinery?

The topics in this book have so far focused on macroautophagy, yet many people new to the field are quick to ask about microautophagy. Relatively little is known about nonspecific microautophagy. Chapter 8 by Chao-Wen Wang and myself gives a brief summary of the state of research on this topic. Microautophagy is most easily distinguished from macroautophagy based on morphological criteria (Fig. 2). As noted above, macroautophagy involves sequestration by membrane of nonlysosomal/vacuolar origin that results in the formation of a cytosolic multi- or double-membrane vesicle. The vesicle contents are subsequently delivered to the lumen of the degradative compartment following vesicle fusion with the lysosome/vacuole. In contrast, during microautophagy the sequestration is initiated directly at the lysosome/vacuole surface and at least in large part does not involve a separate membrane donor. The topic of microautophagy brings up a point that deserves consideration. That is the question of differentiation of the sequestering membrane from that of the degradative organelle in both micro- and macroautophagy. For example, there is no evidence at present that there are structural differences between the two sides of the phagophore membrane. Following fusion with the lysosome/vacuole, the outer membrane of the autophagosome, as well as the amphisome membrane, becomes part of the lysosome/vacuole limiting membrane. The autophagosome inner membrane is delivered into the lysosome/vacuole lumen where it is destined for degradation. It is necessary to prevent damage to the portion of the amphisome and autophagosome membrane that is now contiguous with the lysosome/vacuole, because loss of organelle integrity would allow the deleterious release of hydrolases. It is not clear whether this membrane is modified during the formation of the sequestering vesicle or rapidly following fusion with an

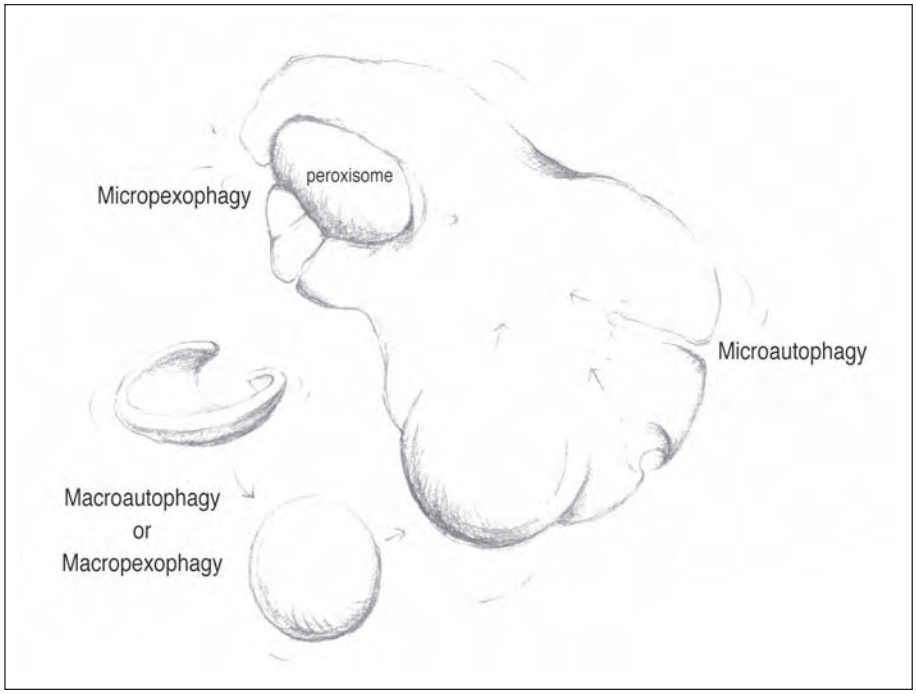

Figure 2. Macroautophagic versus microautophagic delivery pathways. Schematic illustration of the differences between micro- and macroautophagic processes. In microautophagy or micropexophagy, cytosol or peroxisomes, respectively, are taken up directly at the surface of the lysosome or vacuole. The sequestering membrane derives directly from the degradative organelle. Sequestration may occur by invagination or protrusion and may involve septation of the sequestering membrane. During macroautophagy or macropexophagy, the initial sequestration event occurs away from the lysosome/vacuole and presumably involves a membrane that does not originate from this organelle. The origin of the sequestering membrane has not been definitively identified.

endosome or with the lysosome such that it is not a substrate for lysosomal/vacuolar lipases and proteases. Alternatively, this membrane may be invaginated through a microautophagic process and subsequently degraded in the lumen. However, in the case of microautophagy the invaginating membrane is of lysosomal/vacuolar origin. How does the cell distinguish this membrane following scission from the limiting membrane, so that it is now subject to degradation?

Piecemeal microautophagy of the nucleus (Pmn) represents a new area of autophagic research in yeast. In this process, described by David Goldfarb in chapter 9, parts of the nuclear membrane and nucleoplasm are sequestered into the vacuole at sites of nucleus-vacuole junctions. The resulting membrane blebs are scissioned from the nuclear envelope and degraded. The physiological role of Pmn is not clear. It may play a homeostatic role in removing damaged nuclear components and/or may be a starvation response. One interesting point, however, is that this process appears to occur independent of the characterized macroautophagy proteins. Pmn occurs at a topologically distinct location from the cytosol. Thus, it does not have obvious access to the protein machinery of macroautophagy and may require a distinct set of components.

As with other aspects of cell biology, peroxisome biogenesis has been a focus of extensive research for many years, while the study of peroxisome degradation, pexophagy, has lagged

behind. Degradation of peroxisomes is one of the best-characterized examples of specific autophagy, and along with Pmn, of organelle degradation. Pexophagy has been best studied in the model yeasts *Hansenula polymorpha* and *Pichia pastoris* due in part to the relatively large size of the organelle in these organisms. Relatively less is known about pexophagy in *Saccharomyces cerevisiae* but a comparison among these systems is quite interesting. First, there is a large overlap among the pexophagy genes in all three organisms. Second, *P. pastoris* and *H. polymorpha* in particular provide an interesting situation where both micro- and macropexophagy operate depending on the nutrient conditions. Thus, these systems offer the possibility of studying the regulation of the conversion between these two processes. Third, while there is substantial overlap among the Apg/Cvt proteins in *S. cerevisiae*, the Gsa and Paz proteins in *P. pastoris*, and the Pdd proteins in *H. polymorpha*, there are some specific differences in the localization and possibly function of these proteins. This situation provides one good example of why it is not sufficient to study a complex process such as pexophagy in only one organism. This overlap among the protein components also brings up another interesting question—why is there a tremendous overlap between macroautophagy proteins and those involved in micropexophagy? One possibility is that the two processes are quite similar, involving membrane deformations, and that the main difference simply concerns the site of sequestration. It is also possible, however, that there are substantial mechanistic differences between these two processes and the macroautophagy proteins could play a very defined role in one particular stage of micropexophagy.

In chapter 10, Bill Dunn describes work that has been carried out in the methylotrophic yeast *Pichia pastoris* (in his lab as well as those of Suresh Subramani and Yasuyoshi Sakai). In this organism, fluorescent and electron microscopy studies have demonstrated two modes of peroxisome degradation; macro- and micropexophagy. As stated above, most of the characterized genes involved in micropexophagy in *P. pastoris* are homologous to *APG*, *AUT* and *CVT* genes that are required for macroautophagy and the Cvt pathway in *Saccharomyces cerevisiae*. An important question is what allows pexophagy to be specific. This presumably involves tags on the organelles as well as regulatory machinery that activates these tags and/or recognition components of the degradative machinery. The phenomenon of selective peroxisome degradation was first described in *H. polymorpha*. In chapter 11, Jan Kiel and Marten Veenhuis describe macropexophagy in *H. polymorpha* and discuss the current understanding of the selectivity mechanism.

In the previous sections of the book, the lysosomal/vacuolar membrane or membranes of a distinct origin undergo dynamic changes to sequester cytoplasm. In contrast, chaperone-mediated autophagy (Cma) involves direct translocation of proteins across the lysosomal membrane. Rather than relying on changes in the membrane, Cma requires the transported protein to be in an unfolded state and hence, is highly dependent on molecular chaperones. The translocation process involves the receptor lamp2a that may serve in a double role as a translocon. An unusual feature of this receptor is that it can be reinserted into the lysosomal membrane following its own translocation into the lumen. Cma appears to be a secondary response to starvation and is induced after macroautophagy. Rates of Cma decrease with age suggesting an interesting avenue for future research concerning the aging process. One of the interesting aspects of Cma is that it brings the lysosome into the specialized group of organelles that have translocation capacity. This was unexpected because proteins typically reach the lysosome through a portion of the secretory pathway. Thus, it might be expected that the translocation competency of the endoplasmic reticulum would suffice for all "downstream" organelles. Chapter 12 by Dice and colleagues provides a summary of our current knowledge and also an interesting history of this subject. One final point worth mentioning is that the membrane receptor lamp2a is one of three lamp2 proteins expressed in mammalian cells. The lamp2b protein will be mentioned in the chapter on neuromuscular diseases as a defect in this protein is the cause of Danon's disease.

An analogous system to Cma has not been demonstrated in yeast, although the vacuole import and degradation (Vid) pathway may be related. The Vid pathway is used for degrada-

tion of the gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase) and may be similar to Cma in that the cargo is thought to translocate across a membrane, in this case, a completed cytosolic single-membrane vesicle. That is, there are no postulated dynamic membrane rearrangements to enwrap FBPase and there presumably must be some type of translocation apparatus to achieve this localization. In addition, the Vid pathway is dependent upon molecular chaperones suggesting that the target protein(s) must be in an unfolded state for sequestration. There has been some controversy regarding the localization and mechanism of FBPase degradation; evidence has been presented for both a proteasome- and vacuole-dependent process. One explanation is simply that FBPase is degraded by two different methods. One interesting aspect of the Vid pathway, as described by Randy Brown and Hui-Ling Chiang in chapter 13, is that it points out a regulatory role for autophagic processes in yeast. Rather than being simply a degradative process that responds to starvation signals, the Vid pathway operates under nutrient-rich conditions to prevent futile cycling between glycolysis and gluconeogenesis.

In chapter 14, David Katzmann discusses the multivesicular body (Mvb) pathway, another example of a process that is closely tied in with regulation of cellular physiology. For example, the level of cell surface receptors must be precisely controlled. In part, this is achieved through their degradation and these proteins are accordingly removed from the plasma membrane by an internalization process. However, how does a cell degrade an integral membrane protein? One mechanism is to deliver it to the endosome and subsequently sequester it within intralumenal vesicles of the multivesicular body. Fusion with the lysosome/vacuole will ultimately result in degradation of the entire receptor within the lumen. It is worth noting that the Mvb pathway is an example of a process that is both biosynthetic and degradative. This is reminiscent of autophagy in yeast where certain zymogen hydrolases are packaged along with bulk cytoplasm for vacuolar delivery under starvation conditions.

The next part of the book describes studies of autophagy in "newly emerging" systems. I put the term "newly emerging" in quotes because autophagy has been studied for many years in various plant systems as well as in worms (and needless to say in mammalian cells). However, the identification of homologues to the yeast autophagy genes is certain to accelerate the advances in understanding autophagy in these organisms. One point I would like to make is that the analysis of autophagy in *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana* and mammalian cells opens a line of investigation that is essentially not available, or at least is limited, in yeast. That is, what are the developmental roles of autophagy? In chapter 15 by Noboru Mizushima and Tamotsu Yoshimori, it becomes clear that one of the most unique aspects of the yeast autophagy machinery—the use of two distinct conjugation systems involving ubiquitin-like proteins—is conserved in humans. The succeeding chapter by Yuji Moriyasu and myself points out that the autophagy genes are also conserved in plants and that autophagy plays a developmental role in this system. In addition, one feature of autophagy in plants that has not been considered in other organisms, is that it contributes to the biogenesis of organelles, in particular the vacuole. Because the vacuole plays a central role in plant physiology and morphogenesis, autophagy can similarly be considered to play a morphogenetic or structural role in whole plants, yet one more attribute of this complex process.

While the *C. elegans* model system has attracted widespread attention for various topics, it has not been used extensively for studying autophagy, as pointed out by Attila Kovacs and colleagues in chapter 17 (although they have been using this system for many years). In some ways, the analysis of autophagy in worms has mirrored that in mammalian cells. That is, most of the previous research has focused on morphological analyses. As with the mammalian system, the identification of homologues to the yeast autophagy genes promises more rapid advances in *C. elegans*. As discussed in this chapter, however, one interesting aspect suggested by preliminary studies is that there may be substantial differences in the regulation of autophagy between yeasts and worms.

Chapter 18 by Thomas Neufeld demonstrates that autophagy research in *Drosophila* is also a newly emerging area. Again, with the identification of yeast homologues, this field is poised to advance more rapidly and the current data reveal that this will be a good system to study developmental issues. In fact, studies in insect systems provide some of the best data for the role of autophagy and autophagic cell death (see below) in various stages of development. As with mammalian and yeast cells, the Tor kinase is a central player in the reguatory process. However, similar to the case with *C. elegans*, some of the key components identified in yeast as interacting with the Apg1 kinase, that is indirectly regulated through Tor, do not appear to have homologues in *Drosophila*. Thus, some aspects of the conserved machinery may be regulated in unique ways. It is also interesting to note that there are multiple loci for many of the autophagy genes in higher eukaryotes, suggesting tissue-specific or developmental stage-specific roles for some of these gene products.

Up to this point, the book has focused on the role of autophagy in adapting to stress conditions such as starvation, in cellular remodeling during development and in biosynthetic delivery of proteins and organelle biogenesis. The last section of this book deals with the evasion of host defenses by pathogenic microbes, the role of autophagy in preventing disease and aging, and the consequences of autophagic dysfunction. In chapter 19, Bill Dunn and colleagues describe how certain bacterial pathogens are able to enter the autophagic pathway and manipulate the environment of the autophagosome to escape host defense mechanisms. It is possible that some microbial pathogens actually induce autophagy to provide an appropriate environment for intracellular reproduction. This is an area that will continue to gain interest as it appears that some viruses may employ similar means to evade the host immune response. An exciting breakthrough in autophagy research came with the realization by Beth Levine's lab that the yeast autophagy gene *APG6* was homologous with the mammalian *beclin 1* gene. Beclin 1 has tumor suppressor activity, providing a correlative link between autophagy and cancer. While the connections between autophagy and cancer need further experimental verification, the current model suggests that autophagy may be involved in tumor suppression. Similarly, autophagic defects may lead to tumorigenesis. Conversely, there is some evidence that cancer cells may utilize autophagy to survive the conditions of high-density and subsequent low nutrient availability that exist in tumors. Chapter 20 by Beth Levine and colleagues presents an up to date summary of the data that link cancer and autophagy.

Apoptosis, a term that has been synonymous with programmed cell death (PCD), became a major focus of research when it started to become clear that this process is involved in many developmental pathways. More recently, researchers have come to realize that PCD can occur by more than one pathway. In particular, autophagy is characterized as type II PCD. In this capacity it can play a developmental role, but can also protect an organism by eliminating damaged cells that could otherwise be harmful to the surrounding tissue. Chapters 21 and 22 by Aviva Tolkovsky and by Takashi Ueno, Isei Tanida and Eiki Kominami, respectively, focus on neuronal cell death during development and on neuromuscular diseases that are associated with autophagic dysfunction. One of the exciting aspects of the work described here is the genetic analyses of various human diseases that offers the prospect of specific diagnoses and treatments in the near future. There are at least two reasons why apoptosis has dominated the PCD field. First, researchers initially focused on the morphology of apoptotic PCD. Second, apoptosis and autophagy can occur at the same time in tissues, and dying cells can switch from the autophagic to the apoptotic mode. The pathway that is chosen appears to depend on the specific "death signal." As described in chapter 23 by Wilfried Bursch and colleagues, apoptosis is marked by condensation of cytoplasm and chromatin. In the final stage, apoptotic cells are phagocytosed by neighboring cells to prevent tissue damage. In autophagic PCD there is degradation of cytoplasmic components and the accumulation of autophagosomes prior to degradation of the nucleus. One interesting suggestion is that autophagy may initially be an adaptive response to stress but that if it is unsuccessful, it may then be used alone or in combination with apoptosis to cause cell death.

There are additional topics in autophagy that have not been covered in this book. Also, the future of autophagy research may change substantially in the next few years. For example, now that the majority of the autophagy proteins have been discovered in yeast, continued research will likely focus on the mechanistic aspects of the process. In higher eukaryotes, homologues to the yeast proteins will continue to be identified and characterized. In addition, we are likely to learn more about the developmental and cell cycle-dependent roles of autophagy. Finally, further studies will clarify the connections between autophagy and human disease promising the possibility of specific treatments that build upon the years of basic reasearch that have gone into studying the complex and fascinating process of autophagy.

Commonly Used Terms

Amphisome: Intermediate compartment formed by the fusion of an autophagosome with an endosome (this compartment can be considered a type of autophagic vacuole and may be equivalent to a late autophagosome); this compartment has not yet fused with a lysosome.

Autolysosome: A degradative compartment formed by the fusion of an autophagosome (or autophagic vacuole) or amphisome with a lysosome (also called degradative autophagic vacuole or AVd). Upon completion of degradation this compartment again becomes a lysosome or residual body.

Autophagosome: A cytosolic membrane bound compartment denoted by a limiting double membrane (also referred to as initial autophagic vacuole, AVi, or early autophagosome), or single membrane (also referred to as an intermediate autophagic vacuole, AVi/d, or late autophagosome). The early autophagosome in particular contains cytoplasmic inclusions and organelles that are morphologically unchanged because the compartment has not fused with a lysosome and lacks proteolytic enzymes.

Autophagy (Apg): A process in which the cell typically undergoes membrane rearrangements to sequester a portion of cytoplasm, deliver it to a degradative organelle and recycle the macromolecular constituents.

Chaperone-mediated autophagy (Cma): A degradative process in mammalian cells by which proteins containing a particular pentapeptide motif related to KFERQ are transported across the lysosomal membrane and degraded. The translocation process requires the action of the integral membrane protein lamp2a and both cytosolic and lumenal hsc73.

Cytoplasm to vacuole targeting (Cvt): A biosynthetic pathway in yeast that transports resident hydrolases to the vacuole through a selective autophagy-like process.

Lysosome: A degradative organelle in higher eukaryotes that compartmentalizes a range of hydrolytic enzymes and maintains a highly acidic pH.

Macroautophagy: An autophagic process involving the formation of a double- or multiple-membrane cytosolic vesicle of nonlysosomal/vacuolar origin.

Microautophagy: An autophagic process involving direct uptake of cytosol, inclusions (e.g., glycogen) and organelles (e.g., ribosomes, peroxisomes) at the lysosome/vacuole by protrusion, invagination or septation of the sequestering organelle membrane.

Multivesicular body (MVB): An endosome containing multiple 50-80 nm vesicles that are derived from invagination of the limiting membrane. Under some conditions the MVB contains hydrolytic enzymes in which case it may be considered to be a lysosome or autolysosome with ongoing microautophagy.

Multivesicular body (Mvb) sorting pathway: A process in which proteins are sequestered into vesicles within the endosome through the invagination of the limiting membrane. This process is usually, but not always, dependent upon ubiquitin tags on the cargo and serves as one means of delivering integral membrane proteins destined for degradation into the vacuole lumen.

Pexophagy: A selective type of autophagy involving the sequestration and degradation of peroxisomes; can occur by a micro- or macroautophagic process.

Piecemeal microautophagy of the nucleus (Pmn): A process in which portions of the yeast nuclear membrane and nucleoplasm are invaginated into the vacuole, scissioned off from the remaining nuclear envelope and degraded within the vacuole lumen.

Phagophore: Membrane cisterna that has been implicated in an initial event during formation of the autophagosome. Also referred to as the "isolation membrane" or the "cup-shaped structure."

Phosphatidylinositol (PtdIns) 3-kinase: A family of enzymes that add a phosphate group to the 3' hydroxyl on the inositol ring of phosphoinositides. The class III PtdIns 3-kinases are stimulatory for autophagy while class I enzymes are inhibitory.

Preautophagosomal structure (PAS): a perivacuolar compartment or location that is involved in the formation of Cvt vesicles and autophagosomes in yeast. The PAS may supply membranes during the formation process or may be an organizing center where most of the autophagic machinery resides at least transiently.

Programmed cell death (PCD): Regulated self-destruction of a cell. Type I is associated with apoptosis and is marked by cytoskeletal breakdown and condensation of cytoplasm and chromatin followed by fragmentation. Type II is associated with autophagy and is characterized by the presence of autophagic vacuoles (autophagosomes) that sequester organelles. Type III is marked by the absence of condensation, and does not involve the lysosomal system but rather is proteasome-dependent.

Vacuole: The yeast equivalent of the lysosome; this organelle also carries out storage and osmoregulatory functions.

Vacuole import and degradation (Vid): A degradative pathway in yeast in which a specific protein(s) is sequestered into small single-membrane cytosolic vesicles that fuse with the vacuole allowing the contents to be degraded in the lumen. This process has been characterized for the gluconeogenic enzyme fructose-1,6-bisphosphatase in the presence of glucose, and sequestration is thought to involve translocation into the completed vesicle.

Acknowledgements and Excuses

I would like to thank all of the authors who have generously contributed to this book, in many cases providing information prior to publication, and offering helpful discussions about many aspects of autophagy. Regarding this chapter I would like to thank Drs. Randell Brown, Patrice Codogno, J. Fred Dice, William Dunn Jr., David Goldfarb, Attila Kovacs, Beth Levine, Fred Meijer, Per Seglen, Takashi Ueno and Marten Veenhuis for reading and/or making helpful comments, and Chao-Wen Wang for the illustrations. In this book I have attempted to bring together many researchers from around the world, working in various model systems and on many different problems. Each person brings their own viewpoint to the topic of autophagy. I have tried to allow those differing viewpoints to be expressed while maintaining a certain consistency throughout the text. I apologize for any misrepresentations or errors that have resulted from the editing of the text. Finally, I offer my apology to any authors who were not able to contribute chapters or whose work was not cited in individual chapters due to space limitations. This work was supported by National Institutes of Health Public Health Service grant GM53396.

CHAPTER 2

Structural Aspects of Mammalian Autophagy

Monica Fengsrud, Marianne Lunde Sneve, Anders Øverbye and Per O. Seglen

Abstract

The initial event in mammalian autophagy, triggered, for example, by amino acid starvation, is the sequestration and enclosure of a piece of cytoplasm by one or more specialized membrane cisternae of uncertain origin, called phagophores. The resulting cytoplasm-filled vacuolar organelle, known as an autophagosome, is delimited by a double or multiple membrane layer derived from the sequestering phagophore(s). These delimiting membranes are characteristically lipid-rich and protein-poor, being almost devoid of transmembrane proteins as indicated by freeze-fracture electron microscopy. However, several cytoplasmic proteins appear to be partially associated, as peripheral membrane proteins, with the autophagosomal delimiting membranes. Among these, LC3-II, a mammalian homologue of the yeast autophagy protein Aut7, is thought to exert an essential function in the autophagic sequestration process. Autophagosomes can fuse with early or late endosomes to form amphisomes, recognizable ultrastructurally as autophagic organelles containing endosomal markers and undegraded cytoplasm, delimited by a single membrane. The cytoplasmic contents of the amphisome are sometimes slightly denatured, probably due to acidity generated by proton pumps contributed by the endosomal fusion partner. An ATPase, Skd1, seems to be required for autophagosome-endosome fusion. The amphisomes (and probably some of the autophagosomes) in turn fuse with lysosomes, in a process requiring the lysosomal membrane protein, LAMP-2. Inside the lysosomes, the autophagocytosed cytoplasm is degraded by lysosomal hydrolases. All lysosomes are apparently capable of engaging simultaneously in autophagy and endocytosis.

Introduction: Terminology of Autophagic Organelles

Autophagy (from Greek *auto*, self, and *phagos*, to eat) was first described ultrastructurally as the sequestration of cytoplasm into closed, membrane-delimited vacuoles called cytosegresomes[1] or *autophagic vacuoles.*[2] The sequestration is performed by morphologically distinctive membrane cisternae that have been given a specific name, *phagophores* (from Greek, *phagos* to eat, and *phores*, to carry), to emphasize their uniqueness (see Fig. 1).[3,4] As it became clear that autophagy is a stepwise process for the delivery of autophagocytosed cytoplasm to lysosomes, the term *autophagosome* was introduced to describe the initial, prelysosomal autophagic organelle, whereas the term *autolysosome* was used to denote an autophagic organelle that had acquired lysosomal enzymes by fusing with a lysosome.[1,2] Later studies have indicated that all lysosomes can engage in the simultaneous degradation of autophagocytosed and endocytosed material,[5,6] making terms like autolysosomes and heterolysosomes (lysosomes containing exogenous material received through heterophagy or endocytosis) somewhat redundant. In addition to the phagophore, the autophagosome and the lysosome, the autophagic organelle family includes an intermediate compartment, the *amphisome* (from Greek *amfi,* both, and *soma,*

Autophagy, edited by Daniel J. Klionsky.

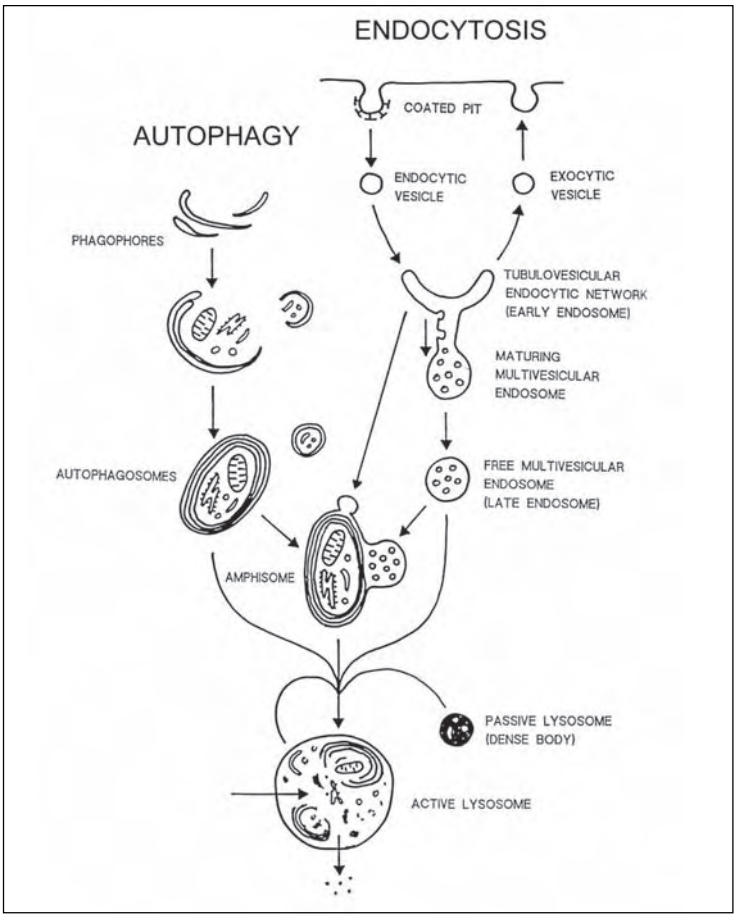

Figure 1. Autophagic-endocytic-lysosomal interactions. See text for details. Modified from ref. 8.

body), which forms by fusion between an autophagosome and an endosome, i.e., prior to fusion with the lysosome.[7,8]

The switch of the term "autophagic vacuole" from a specific to a collective denotation has not been entirely painless: the term is still sometimes used as a synonym for autophagosomes, which may create considerable confusion. In addition, the use of the word "vacuole" is problematic because this term denotes the yeast organelle that is equivalent to the lysosome, the terminal acceptor compartment of autophagy. The situation is not made easier by a current terminology that subclassifies the autophagic vacuoles (AVs) into initial AVs (AV_i), which correspond to autophagosomes; intermediate AVs ($AV_{i/d}$), most of which may be amphisomes, and degradative AVs (AV_d), corresponding to lysosomes.[9] It would probably be advisable to avoid the AV term altogether, or at least to use it only in the collective sense, and in cases where a subclassification cannot be made with certainty.

The Phagophore

The origin and nature of the autophagic sequestering cisterna (isolation membrane; phagophore) has been a matter of debate ever since the autophagosomes were first discovered. Two major alternatives may be considered: either the cisternal membrane is formed by de novo

synthesis, or by utilization of preexisting cytoplasmic membranes. In the latter case, any of the cell's membranes could in principle be used for phagophore formation; one should also bear in mind the possiblity that more than one membrane type may contribute.[10] Cisternal organelles such as the endoplasmic reticulum (ER)[1,11-14] and Golgi[15-17] have been suggested to be the membrane source, as have post-Golgi compartments[18] as well as the plasma membrane.[19-21]

Several studies have reported the presence of ER marker proteins (but not Golgi, plasma membrane or endosome markers) in the membranes or lumen of the phagophores and in the delimiting cisternae of early autophagic organelles.[11,22,23] However, other reports have shown that the early autophagic structures lack ER markers;[24-26] furthermore, purified autophagosomes show no enrichment in ER proteins.[27] Autophagosomal delimiting membranes also differ from ER membranes in having a low cholesterol content (labelling poorly with filipin),[24] and different surface charge characteristics, as indicated by their selective binding of cationized ferritin.[28] In some of the marker studies only a fraction of the presumptive early autophagic organelles were found to carry ER markers,[22,29,30] suggesting the possibility that such markers may be secondary acquisitions following the fusion of autophagosomes with other vacuolar/cisternal compartments. This appears to be the case with the lysosomal/endosomal marker enzymes sometimes reported to reside in the delimiting cisternae of autophagic organelles.[16,23,26,31] It should, furthermore, be kept in mind that a section through any curved membrane cisterna can easily be misinterpreted as a phagophore or an autophagosome. Apart from the truism that all membranes ultimately derive from the ER, the case for phagophores being derived from smooth or degranulated ER cisternae does not seem very strong based on morphological and cytological studies. In yeast, recent molecular genetic studies suggested a role for the ER in autophagy (see chapters 6 and 7).[32] However, it is not clear whether ER membrane directly contributes to the phagophore or if protein components transiting through the ER have a function during autophagosome formation.

Curving cisternae deriving from the plasma membrane,[20] or containing demonstrable plasma membrane marker enzymes[21] have been thought to perform autophagic sequestration, but these structures are probably unrelated to autophagy. Several studies have in fact failed to detect plasma membrane markers in early autophagic organelles.[11,29,33] The plasma membrane can, therefore, most likely be excluded as a source of material for phagophore formation.

Like in the case of ER markers, the evidence for an association of Golgi markers with phagophores and other early autophagic organelles is rather ambiguous. Some studies have reported the presence of Golgi marker enzymes in these organelles;[15-17] other studies have failed to detect such markers.[11,22,23] One of the most characteristic cytochemical properties of phagophores and autophagosomal delimiting cisternae, i.e., their heavy staining by hot, unbuffered or imidazole-buffered osmium tetroxide,[33] is shared by the outer saccules of the *cis*-Golgi network, which are negative for standard Golgi markers like thiamine pyrophosphatase.[16] The observation that certain plant lectins which detect non-Golgi glycosylations bind specifically to the edges of the phagophores,[18] is also consistent with a derivation of these organelles from the vesicles/saccules of the *cis*-Golgi region.

The heavy osmium staining of the phagophores and autophagosome walls (Fig. 2A,B) indicates that they are equipped with exceptionally lipid-rich, protein-poor membranes, with a high content of unsaturated fatty acids.[24] Freeze-fracture studies of early autophagic organelles in situ suggested that their surface membranes had relatively few transmembrane proteins compared to other organelles,[35,36] and similar studies of purified autophagosomes and phagophores (Fig. 2C,D) revealed that they were almost devoid of transmembrane proteins.[4] Whatever their origin, it is thus clear that the phagophores differ structurally from other cisternal organelles.

In yeast cells, phagophores are apparently formed de novo during adaptation to nitrogen starvation or to novel energy substrates, the induction of autophagy being blocked by inhibitors of protein synthesis such as cycloheximide.[37] A score of *APG/AUT* genes have been shown to be required for yeast autophagy;[38,39] most of these colocalize to cytoplasmic "phagophore factories" (pre-autophagosomal structures; see chapters 6 and 7).[40,41] In mammalian cells, au-

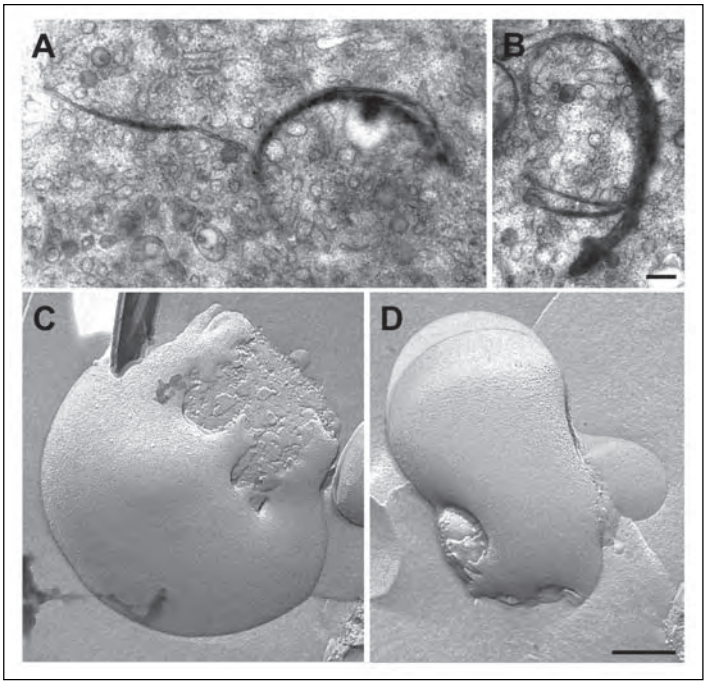

Figure 2. Phagophores visualized by electron microscopy. Isolated rat hepatocytes were processed for conventional (epon) electron microscopy (A and B), or purified hepatocytic autophagosomes were freeze-fractured and shadowed with platinum (C and D). Phagophores in the process of engulfing cytoplasm could be found in both preparations, although they are a rare occurrence among the purifed autophagosomes. Bar length, 150 nm (A,B) or 250 nm (C,D). B-D, modified from refs. 6 and 4.

tophagy is a constitutive rather than an inducible process, regulated on a short-term basis by changes in activity. Since autophagy can be switched on and off in the presence of cycloheximide, i.e., in the absence of new protein synthesis,[11,42,43] a pool of preformed phagophores, or phagophore protein, would seem to be present in the cell. The fact that autophagy can go on for hours without protein synthesis,[42,43] despite the rapid turnover (~10-min half-life) of autophagosomes,[44] would suggest that the pool of phagophore protein is rather large, or that phagophores/proteins are extensively reutilized. However, although lipid-rich (osmiophilic) structures traversing the lysosomal membrane have been observed,[3] questions as to how phagophore components recycle, and where they reside during periods of low autophagic activity, remain to be resolved.

The mammalian homologue of yeast Apg5 has been shown to localize to the membranes of nascent phagophores, where its conjugation to Apg12 is needed for phagophore elongation (see chapter 15).[45] This Apg12-Apg5 conjugate is required for subsequent binding of a processed isoform (LC3-II) of LC3 (microtubule-associated protein light chain 3), the mammalian homologue of Aut7/Apg8. Apg12-Apg5 and some of the LC3-II detach from the phagophore upon autophagosome closure, while some of the LC3-II remains associated with the autophagosomal delimiting membrane.[46] The formation of LC3-II (16 kDa) from its larger precursor, LC3-I (18 kDa), is strongly correlated with autophagic activity, suggesting that LC3 processing may be a rate-limiting factor in autophagy. There is some evidence that autophagosomal LC3-II may recycle (along with the phagophore?), and even be reprocessed to LC3-I by the aid of the mammalian homologue of Aut2/Apg4.[47]

The Role of the Cytoskeleton in Autophagic Sequestration

It is difficult to imagine how the phagophore can fold up to envelop a large volume of cytoplasm without the aid of some mechanical support, e.g., in the form of a cytoskeletal scaffold. All the major cytoskeletal elements have, accordingly, been examined with this possibility in mind. Microtubules are clearly not required for phagophore function, since microtubule-depolymerizing drugs like vinblastine and nocodazole fail to prevent autophagic sequestration and autophagosome formation.[6,48-51] In fact, these drugs cause the accumulation rather than the disappearance of autophagosomes[52] by virtue of their ability to suppress the fusion of the latter with endosomes and lysosomes.[6,48,49] These fusion processes are thus apparently microtubule-dependent, making the microtubule-depolymerizing drugs strong inhibitors of the overall autophagic degradation process.[48,53]

The actin-depolymerizing drugs, cytochalasin B and D, were reported to inhibit the formation of autophagosomes in the NRK rat kidney cell line,[50] suggesting a role for actin microfilaments in autophagic sequestration. However, cytochalasin B did not prevent autophagy in Ehrlich ascites cells,[52] nor did cytochalasin D have much effect on autophagic sequestration[51] or overall protein degradation[54] in isolated rat hepatocytes at concentrations where the cellular actin network was disrupted. The reported suppression of autophagic-lysosomal flux in liver cells in vivo after administration of a microfilament poison, phalloidin,[55] is thus likely to be an indirect effect. While an autophagic involvement of actin microfilaments in specific cell types cannot be excluded, this cytoskeletal element would not seem to be required for autophagy in general.

Suppression of hepatocytic autophagy by the protein phosphatase inhibitor, okadaic acid, was found to be accompanied by fragmentation of the keratin intermediate filament network, whereas microtubules and actin microfilaments were unaffected.[51] The intracellular network of plectin, a cytoskeletal cross-linking protein that links intermediate filaments to each other and to actin filaments, was similarly fragmented by okadaic acid, accompanied by overphosphorylation of plectin[56] as well as of keratin.[57] Although these data do not constitute evidence for the involvement of intermediate filaments or plectin in autophagic sequestration, they are at least compatible with such a possibility. It has previously been speculated that the intermediate filaments present in cytoplasmic inclusion bodies (protein aggregates) might serve as a signal for their autophagy,[58] but no selective autophagic sequestration of such bodies has been documented. In fact, keratin and other cytoskeletal proteins (actin, tubulin) are present only in very low amounts in purified autophagosomes,[27] and we have recently found this to be the case with plectin as well (our unpublished results). Cytoskeletal elements thus seem to be selectively excluded from the forming autophagosome, making it tempting to speculate that the sequestering phagophore is sterically guided by an external keratin-plectin scaffold which is detached upon closure of the autophagosome, or stripped off during autophagosome purification.

Structure of the Autophagosome

When the autophagic sequestration process is completed by autophagosomal closure, the sequestering phagophore becomes the delimiting cisterna of the newly formed autophagosome. The characteristics of the autophagosomal delimiting cisterna are thus identical with those of the phagophore (at least initially), i.e., a highly osmiophilic structure made up of protein-poor, smooth membranes with a high content of unsaturated fatty acids.[4,24,35,36] In transmission electron micrographs, autophagosomes can often be easily recognized by their thick, osmiophilic outline, reflecting osmium deposition both at the membranes and in the intracisternal cleft (Fig. 3A). In cryo-electron microscopy, on the other hand, the autophagosomal membranes are poorly preserved,[6,59,60] probably because their lack of protein precludes a satisfactory fixation by proteophilic fixatives like formaldehyde and glutaraldehyde.

Cellular membranes vary in thickness, and can roughly be divided into two groups: those with thin (60-70 Å) membranes and those with thick (90-100 Å) membranes. The

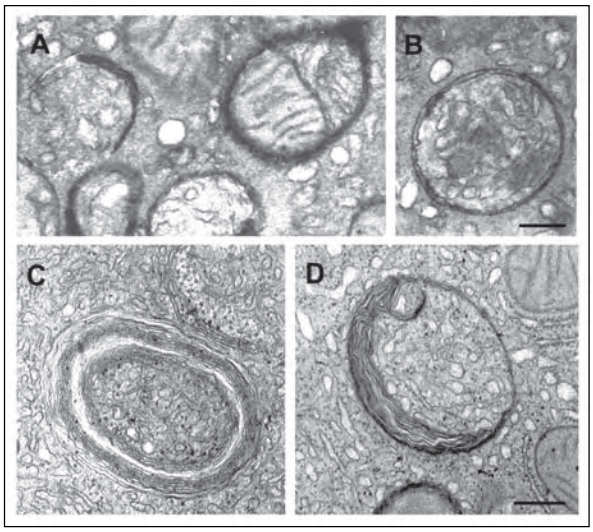

Figure 3. Autophagosomes in situ. Isolated rat hepatocytes, incubated for 2 h at 37°C in the absence of amino acids to induce maximal autophagic activity, were processed for conventional electron microscopy. Bar length, 250 nm.

autophagosomal delimiting membranes are of the thin type, as are the ER membranes, the *cis*-Golgi membranes, the mithochondrial inner and outer membranes, and the nuclear envelope membranes. In contrast, late autophagic organelles have a single thick membrane, characteristic of lysosomes (they are in fact lysosomes), the plasma membrane and the major part of the Golgi apparatus.[11] Filipin, a cholesterol binding probe, did not detectably bind to autophagosomes in situ, consistent with the fact that thin membranes have a lower content of free cholesterol than thick membranes.[36]

There is no evidence for any structural differentiation between the outer and inner membrane of the curving phagophore, either before or after autophagosome formation (when the phagophore turns into the autophagosomal delimiting cisterna). However, following fusion with an endosome or a lysosome, the outer membrane of the autophagosome becomes part of the amphisomal or lysosomal delimiting membrane, and may acquire the characteristics of the latter, including a resistance against degradation by the lysosomal lipases and proteases. Alternatively, the autophagosomal outer membrane elements in the hybrid organelle may be selectively internalized by a microautophagic invagination process, thus becoming part of the lumenal contents of the organelle, like the inner autophagosomal membrane (see chapter 8). To what extent the autophagosomal membrane components are eventually degraded or recycled, is not clear.

The availability of highly purified autophagosomes[27] has greatly facilitated the analysis of their structure and composition. Autophagic organelle preparations previously described have been complex mixtures of autophagosomes and (auto)lysosomes, with a high content of lysosomal marker enzymes.[61-64] However, the selective destruction of lysosomes by substrate-induced osmotic disruption[65] has allowed lysosome-free autophagosome preparations to be made.[27]

Autophagosomes are often described as cytoplasmic organelles delimited by a double membrane (Fig. 3B), but multilayered delimiting membranes and phagophores are also frequently observed (Fig. 3C).[6] An analysis of purified autophagosomes showed them to have the same membrane characteristics as in situ, about 85% being delimited by a double membrane (a single cisterna), while 10-15% had multiple delimiting membranes derived from two or more stacked cisternae.[4] The autophagosome preparations also included a few amphisomes, with a

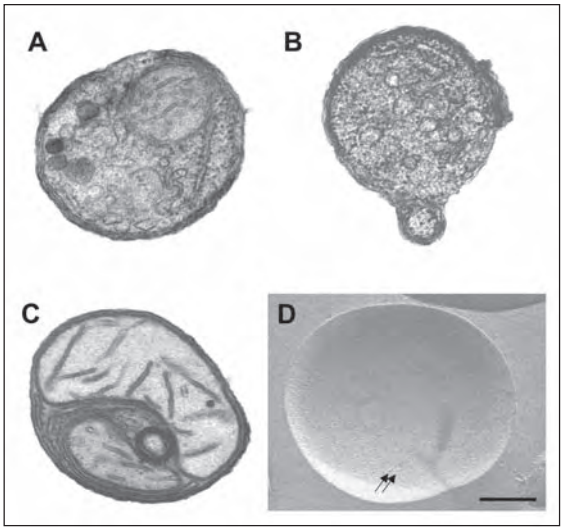

Figure 4. Isolated autophagosomes. Purified autophagosomes from rat hepatocytes[27] were processed for conventional (A-C) or freeze fracture electron microscopy (D). The autophagosomes contain normal cytoplasm, including mitochondria (A,C) and rough endoplasmic reticulum (A,B). Autophagosomal delimiting membranes carry few and small intramembrane particles (D, arrows). Bar length, 250 nm. D, modified from ref. 4.

single delimiting membrane (see below). Freeze-fracture studies revealed that all membranes of multilayered autophagosomes were equally smooth (particle-free, indicating the absence of transmembrane proteins), with no detectable difference between the inner and outer membrane of a given cisterna.[4]

The contents of the autophagosome is, in general, completely normal cytoplasm (Fig. 4A-C), the nuclei usually (but not always, cf. ref. 66) being excluded. About 30% of rat liver autophagosomes contain mitochondria, about 70% contain rough ER and about 4% contain peroxisomes.[27] Cytosolic enzymes are present at the same concentrations in autophagosomes as in cytoplasm,[6,59,60,67] as are most other cellular proteins,[27] consistent with autophagic sequestration being basically a bulk uptake mechanism. However, as discussed in the previous section, there is a notable deficiency in cytoskeletal proteins (actin, tubulin, keratin, plectin) among the autophagosomal contents.[27,56] The sequestering phagophores would thus seem to steer away from solid cytoskeletal structures as they cut through the cytoplasm. The presence of small amounts of tubulin[27] could reflect the fact that autophagosomes are routinely purified from vinblastine-treated cells, which have a disrupted microtubular network. Most of the tubulin is, however, in aggregates[51] which are probably too large to be autophagocytosed.

Autophagosomal Membrane Proteins

Freeze-fracture studies of purified rat liver autophagosomes have shown that their delimiting membranes carry extremely few intramembrane particles compared to other organelles like lysosomes and mitochondria, indicating a relative paucity of transmembrane proteins (Fig. 4D). Inner and outer cisternal membranes appear to be equally smooth, as are the individual membranes of multilayer-delimited autophagosomes.[4] In previous studies, mammalian autophagic organelles freeze-fractured in situ or after isolation had rather more intramembrane particles (albeit still much fewer than other organelles), probably because these organelle populations included amphisomes and lysosomes as well as autophagosomes.[36] Autophagosomes from yeast were also shown to have very few intramembrane particles.[68] Phagophore and

Table 1. Autophagosomally enriched membrane proteins

Protein	Autophagosomal Enrichment
3-α-Hydroxysteroid dehydrogenase	> 25
Betaine homocysteine methyltransferase	> 25
Carbonic anhydrase III	> 25
Glucose-regulated protein 78	22
Glyceraldehyde 3-phosphate dehydrogenase	15
Thiosulfate sulfurtransferase	10
Argininosuccinate synthase	6
Short-chain 2-enoyl-CoA hydratase	ND
Microtubule-associated protein light chain 3	ND

Membrane proteins from purified autophagosomes[27,69] were separated on pI 4-7 and pI 6-9 2D-gels, silver-stained and compared with proteins from total (postnuclear supernatant) cellular membranes. Proteins with a statistically significant (n = 3; $p < 0.05$), >5x autophagosomal enrichment were identified by MALDI-TOF MS tryptic fingerprinting.[55] The last two proteins were observed on pI 3-10 2D-gels and 1D-gels, respectively, and were not quantified (ND, not determined).

autophagosome proteins involved in autophagic function are, therefore, likely to be peripherally attached membrane proteins rather than transmembrane proteins, as is the case with Apg5, Apg12 and Aut7 as discussed above.

Few of the yeast proteins implicated in autophagy have transmembrane domains, and none of those seem to associate with the completed autophagosome.[69] Among the yeast autophagy proteins and their mammalian homologues, only Aut7 and its homologue, LC3-II, are associated with autophagosomes (see chapters 6, 7 and 15).[46] LC3-II is bound as a peripheral membrane protein both to the inner and outer surface of the autophagosomal delimiting cisterna, possibly through a phosphatidylethanolamine anchor as in yeast.[70] In addition, variant isoforms of the enzymes glyceraldehyde 3-phosphate dehydrogenase, argininosuccinate synthase and short-chain 2-enoyl-CoA hydratase were found to bind selectively to the inner surface of the autophagosomal delimiting cisterna.[71] By using an expanded proteomic approach (2-D gels isoelectrofocused over narrow pI ranges combined with mass spectrometric identification), an additional five proteins associating with autophagosomal membranes have been identified (Table 1): 3-α-hydroxysteroid dehydrogenase (3αHSDH), betaine homocysteine methyltransferase (BHMT), carbonic anhydrase III (CA-III), glucose-regulated protein 78 and thiosulfate sulfurtransferase. They all thus seem to represent membrane-binding isoforms of abundant cytoplasmic enzymes. A 35 kDa BHMT isoform has previously been shown to associate with the outer surface of purified autolysosomes; this protein was suggested to be a proteolytic degradation product generated secondarily during organelle purification.[72] It is, therefore, clear that the membrane localization and possible artificial generation of the novel autophagosome-associated proteins needs to be carefully checked (although our autophagosomal BHMT is an approximately full-length 44 kDa isoform). Furthermore, the propensity of an abnormal protein to bind selectively to autophagic organelle membranes in vitro may well have its physiological counterpart in intact cells; additional experimentation is, therefore, required to tell whether the autophagosome-associated enzyme isoforms perform an autophagic function, or whether they represent proteins en route to the lysosomes for degradation. In any case, several of these proteins (LC3-II, 3αHSDH, BHMT, CA-III) may be useful autophagosomal markers, hopefully aiding the purification of autophagosomal delimiting membranes and phagophores.

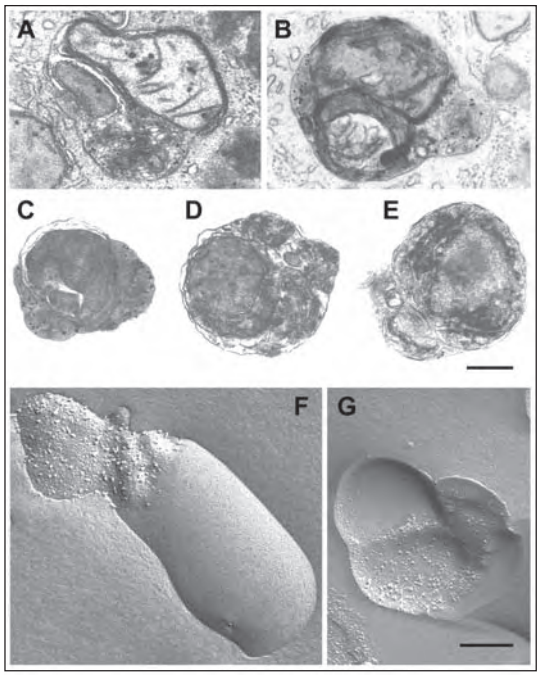

Figure 5. Amphisomes. Rat hepatocytes were incubated for 3 h with asialoorosomucoid-coated, 10-nm gold particles (small black dots) as an endocytic marker. A,B, Amphisomes in situ (in intact cells); C-E, purified amphisomes[8]; F,G, purified amphisomes, freeze-fracture preparation. Bar length, 250 nm. F and G, modified from ref. 4.

The Amphisome

Amphisomes are defined as organelles formed by the fusion of autophagosomes with endosomes or heterophagosomes. Amphisomes can thus be classified as either autophagic or heterophagic/endocytic organelles. The existence of these hybrid organelles was first indicated by biochemical experiments that used electroinjected, radiolabelled lactose to investigate the autophagic-lysosomal pathway. Cytoplasmic lactose was found to be taken up into autophagosomes by autophagy, but was degraded by the lysosomal α-galactosidase upon delivery of autophagocytosed material to the lysosome.[49] However, high concentrations of asparagine inhibited the autophagic-lysosomal flux, causing lactose to accumulate in prelysosomal autophagic organelles. The surprising discovery was that this prelysosomal lactose could be degraded by endocytosed α-galactosidase, suggesting that it resided in a vacuolar compartment accessible to both autophagic and endocytic inputs, i.e., an amphisome.[7,73] Subsequent studies showed that amphisome accumulation could also be induced by leupeptin treatment, apparently due to the relative fusion incompetence of degradation-inhibited lysosomes filled up with autophagocytosed cytoplasm.[8] A large fraction of the "autolysosomes" isolated after leupeptin treatment[63] would thus actually be amphisomes. The amphisomes could be physically separated from lysosomes on density gradients, and eventually purified by a modification[8] of the procedure used for autophagosome preparation,[27] where lysosomes are selectively removed by substrate-induced osmotic disruption.[65]

Amphisomes can be recognized ultrastructurally as autophagic organelles containing both autophagocytosed cytoplasm and endocytosed markers (e.g., asialoglycoprotein-gold conjugates, Fig. 5A-E), but lacking lysosomal markers.[6] They have an easily recognizable, but often slightly denatured, cytoplasmic contents, and are usually delimited by a single membrane, which can sometimes be partially or completely double, depending on the extent of autophagosome-endosome fusion and on the plane of section.[8] The amphisomes are often complex, clearly being the result of multiple fusion events involving several autophagosomes as

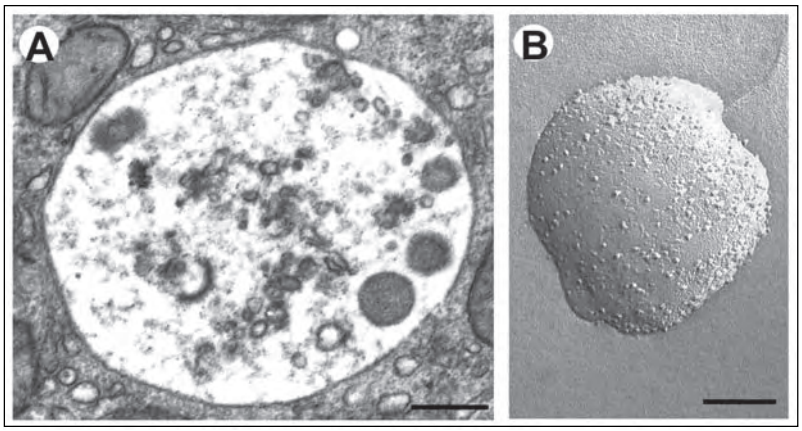

Figure 6. Lysosomes. Rat hepatocytes, incubated for 2 h at 37°C under autophagic conditions, were processed for conventional electron microscopy (A), or used to prepare purified lysosomes[8] subjected to freeze-fracture electron microscopy (B).[4]

well as several endosomes (Fig. 5B). In freeze-fracture images, the fusion between particle-rich endosomes and smooth autophagosomes is well visualized (Fig. 5F,G).[4] Eventually, the endosomal intramembrane particles (transmembrane proteins) diffuse out and distribute evenly over the merged membrane of the amphisome, giving the amphisomes an average particle density of about 90 particles/mm^2, i.e., 4-5x higher than the average autophagosome (about 20 particles/mm^2), but still considerably lower than the average lysosome (Fig. 6B; about 2000 particles/mm^2).[4]

Amphisomes have been shown to have an acidic interior,[74] suggesting that the observed denaturation of contents may be due to acidification by a proton pump, brought in by the endosomal fusion partner. Most of the autophagic organelles of intermediate morphology (contents denatured, but not degraded) described and classified as $AV_{i/d}$, are thus likely to be amphisomes.[9,60,75] The presence of endocytic markers cleanly distinguishes amphisomes from autophagosomes, but the distinction versus lysosomes can be more problematic, because the endosomal fusion partner may furnish the amphisome with a variable portifolio of lysosomal enzymes.[76] For example, acid phosphatase was found to be present in the majority of pancreatic amphisomes (type I AVs), whereas cathepsins B and D, the lysosomal membrane glycoprotein Lgp120 and the cation-independent mannose 6-phosphate receptor were found exclusively in lysosomes (type II AVs).[75] In rat hepatocytes, cathepsin D and Lgp120 were similarly confined to lysosomes,[6,60] and would appear to be suitable as lysosomal markers.

Is amphisome formation an obligatory step in the autophagic-lysosomal pathway, required to make prelysosomal autophagic organelles competent for fusion with lysosomes? In yeast cells, the ATPase Vps4, required for endosomal translocation and multivesicular body formation (see chapter 14), was found to be necessary for autophagy as well.[77] Mutations in its mammalian homologue, Skd1, prevented amphisome formation and caused an accumulation of autophagosomes in HeLa cells.[78] Starvation-induced protein degradation was strongly suppressed under these conditions, suggesting that the autophagic-lysosomal flux proceeded predominantly through amphisome formation. However, the possibility should also be considered that Skd1 might be directly involved in autophagosomal translocation, thereby preventing autophagosome-lysosome fusion independent of its effect on endosomes (see chapter 15).

In isolated rat hepatocytes, an ultrastructural study of vacuole fusion profiles indicated that about 80% of the autophagosomal fusion events were with endosomes, and that 20% represented direct autophagosome-lysosome fusion.[60] The amphisomal pathway would thus seem

to be predominant, but not obligatory. Apparently, both early and late endosomes can fuse with autophagosomes to form amphisomes.[8] Although one report observed autophagosome-lysosome fusion only, this was probably due to a very low rate of marker endocytosis in the cell line studied, with negligible endocytic flux into either amphisomes or lysosomes.[79] In hepatocytes, there was rapid delivery of endocytosed material both to amphisomes and directly to lysosomes.[80] The endocytic-lysosomal pathway was operative even if autophagy had been blocked by 3-methyladenine,[81] i.e., amphisome formation is not an obligatory step in this pathway either.[80] In vivo, hepatic endocytosis was found to proceed mainly along the direct pathway to the lysosome, with little amphisome formation.[82] Convergence of the endocytic and autophagic pathways at the amphisome level would thus seem to represent a condition-dependent, probably regulated event.

The Delivery of Autophagocytosed Cytoplasm to the Lysosome

Autophagocytosed material is eventually degraded following the acquisition of hydrolytic enzymes by the autophagic organelles. It has been suggested that such enzymes could be delivered directly to the autophagic compartment by post-Golgi vesicles carrying mannose 6-phosphate receptors,[59,83] or by endosomes containing lysosomal enzymes.[75,83-85] However, although several modes of delivery may operate, most of the evidence indicates that lysosomal enzymes are mainly delivered to autophagic organelles by fusion of these organelles with preexisting lysosomes,[1,79,86] turning the autophagic organelle itself into a lysosome. All lysosomes are apparently capable of engaging simultaneously in autophagy and endocytosis.[5,6,60]

Lysosomes can fuse with either endosomes, autophagosomes or amphisomes, and by the use of suitable drugs some of these fusions can be studied independently. For example, lysosomal neutralization and swelling induced by propylamine or ammonia seems to suppress the ability of lysosomes to fuse with endosomes.[8] However, autophagocytosed material is still delivered to the lysosomes, and accumulates intralysosomally due to the impairment of degradation by the amines.[73] Similar results have been obtained with bafilomycin, a vacuolar proton pump inhibitor.[87] Both bafilomycin[88] and propylamine[48] may induce some accumulation of prelysosomal autophagic organelles as a secondary consequence of the lysosome affliction, but most of the autophagic flux clearly goes all the way to the lysosomes.[73,87]

In contrast to amines and bafilomycin, asparagine and leupeptin do not seem to affect direct endosome-lysosome fusion,[8,73] but alter the lysosomes in such a way as to make them selectively incompetent to fuse with amphisomes, thereby causing accumulation of the latter[6,8] as discussed in the previous section. Both of these agents also induce some autophagosome accumulation, probably as a secondary effect of the amphisome accumulation.[6,8]

Vinblastine and other microtubule disruptants appear to be less selective: they prevent the fusion of endosomes with autophagosomes or lysosomes[6,80] as well as the fusion of amphisomes with lysosomes,[73] i.e., all fusions involving endosomal components seem to be dependent on microtubular translocation. Furthermore, the extensive inhibition of the overall autophagic-lysosomal flux by vinblastine[7,48,49] suggests that any direct autophagosome-lysosome fusion is suppressed as well. The alternative interpretation, i.e., that all autophagic-lysosomal flux occurs via amphisome formation and thus is entirely dependent on vinblastine-sensitive endosome translocation, cannot be completely excluded, since data pertinent to this point are somewhat conflicting, as discussed in the previous section. Another issue regarding the effect of vinblastine is whether the autophagosome accumulation induced by this drug is due to inhibition of autophagosome disposition (fusion) only,[7,48,73] or whether vinblastine can also stimulate autophagosome formation as originally believed.[89] Clearly, vinblastine does not stimulate autophagy in isolated hepatocytes,[49] but there is some evidence that it may do so in vivo,[90] possibly by an indirect effect on hormone levels.[91] Because the fusion-inhibitory effect of vinblastine appears to be somewhat transient,[73,92] the net drug response observed may depend on the time frame of the experiment.[90] It is also noteworthy that the inhibition of autophagic flux (amphisome formation) caused by an Skd1 mutation seemed to elicit a secondary stimulation

of autophagosome formation,[78] but this feedback mechanism would not seem to operate in the case of vinblastine, at least not in isolated hepatocytes.[73,80]

Little is known about the molecular requirements for fusion of autophagic organelles with lysosomes. In yeast, the Mon1 and Ccz1 proteins are essential; these cytosolic proteins probably bind to autophagosomes prior to fusion (see chapter 7).[93-95] Furthermore, proteins in the SNARE family seem to be involved, as in endosomal fusions.[32] In mammalian cells, a deficiency in the lysosomal membrane protein Lamp-2 causes autophagic organelle accumulation, indicating that this protein is required for lysosomal fusion competence.[96] There may also be specific proteins involved in the intralysosomal permeabilization and/or breakdown of the autophagosomal delimiting membranes: in yeast, both a putative permease (Aut4) and a lipase (Cvt17/Aut5) have been implicated at this stage.[97-99] The final degradation of autophagocytosed cytoplasmic material is presumably performed by the same lysosomal enzymes that degrade endocytosed material; however, a discussion of these enzymes is outside the scope of the present review.

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CHAPTER 3

Signaling Pathways in Mammalian Autophagy

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Abstract

Macroautophagy is a major catabolic process conserved from yeast to human. The formation of autophagic vacuoles is stimulated by a variety of intracellular and extracellular stress situations including amino acid starvation, aggregation of misfolded proteins, and accumulation of damaged organelles. Several signaling pathways control the formation of autophagic vacuoles in mammalian cells. As some of these signaling pathways are engaged in either the control of protein synthesis or cell survival this suggests that macroautophagy is intimately associated with the execution of cell proliferation and cell death programs.

Introduction

Autophagy Is a Regulated Process

At steady state, rates of protein synthesis and degradation in the cell must balance. In some cell types (e.g., the liver) protein degradation primarily occurs by autophagy and in these cells autophagy is, from a quantitative point of view, as important as protein synthesis in the control of the cellular protein content. Autophagy is extremely important for proper functioning and survival of the cell. The process not only takes care of the degradation of proteins with a relatively long half-life but also eliminates organelles that are either functionally redundant or damaged to such an extent that they become a threat to cell functioning. It has become more and more clear in recent years that autophagy is involved in the execution of a type of programmed cell death because it provides the cell with a mechanism to digest its interior while leaving the plasma membrane intact (see chapter 23). And last, but not least, as we will see, more and more evidence has become available indicating that suppressed autophagy is intimately associated with tumor cell growth (see chapter 20).

Although autophagy consists of both micro- and macroautophagy, in this review we will confine ourselves to a discussion of macroautophagy. In contrast to macroautophagy, microautophagy in mammalian cells, defined as the sequestration of portions of the cytoplasm by invaginations of the lysosomal membrane, is quantitatively far less important than macroautophagy and does not seem to be subject to metabolic regulation (see ref. 1 for review, also see chapter 8). In order to avoid confusion, in this review we will use the term "autophagy" to denote "macroautophagy."

In times of food restriction, autophagy in mammalian cells becomes activated to provide the body with amino acids for gluconeogenesis, for enzyme synthesis, and for the synthesis of essential nitrogen-containing compounds. Under those conditions, autophagy is largely nonselective and cell components are sequestered by the autophagic system in the same ratio as they occur in the cytoplasm.[2,3] Although the volume percentage of autophagosomes is low

Autophagy, edited by Daniel J. Klionsky.

(e.g., in hepatocytes, 0.2% in the fed state and 1-1.5% of total cell volume in the fasted state[4]), the turnover of these organelles is high ($t_{1/2}$ about 8 min).[4,5] Rates of starvation-induced autophagy in liver are high and can reach values of 2% of cell protein per hour in vivo, at least in rats and mice.[6,7] Because of ongoing protein synthesis, net liver-protein loss is less but still considerable (about 1%/h). These values stress the importance of autophagy in the control of nitrogen economy and, indeed, variations in autophagic flux during the fed-fasted transition (and vice versa) proved to be quantitatively more important than changes in the rate of protein synthesis, and primarily determine the amount of liver-cell protein.[8] Autophagy is, in fact, a constitutive process and variations in its rate are caused by variations in inhibitory effectors of the process. A complete understanding of autophagy at the molecular level requires both the identification of the signaling pathways that transmit stimuli to trigger the formation of autophagic vacuoles, and the characterization of the molecular machinery responsible for the formation and maturation of autophagosomes.

The Origin of the Autophagosomal Membrane and Maturation of the Autophagosome

There is still considerable debate about the origin of the autophagosomal membrane of mammalian cells. Because this topic is discussed in chapter 2, we will confine ourselves to some general remarks. One possibility is that autophagosomes are formed by invaginations of the ribosome-free parts of the endoplasmic reticulum.[9-11] Post-Golgi membranes and Golgi membranes have also been proposed as origins of the autophagosomal membrane.[12,13] According to Seglen and coworkers, formation of autophagosomes occurs from preexisting membrane structures, which they called phagophores.[14] Recent data suggest that the mobilization of membrane pools from the endoplasmic reticulum[15] and Golgi/post Golgi[16,17] can contribute to the formation of the autophagosome. This would agree with the original nature of the phagophore and also conciliates the different origins reported in the literature for the autophagosomal membrane.

The initial autophagosomes, once formed, mature in a stepwise manner into degradative autophagosomes: they acquire lysosomal membrane proteins by fusion with vesicles deficient in hydrolytic enzymes, followed by acidification and fusion with existing lysosomes for acquisition of hydrolytic enzymes so that macromolecular material sequestered in the autophagosomes can be degraded.[10,18] Hepatocytic autophagosomes may also become acidic by fusion with early endosomes to form so called amphisomes.[14] Ubiquitin and ubiquitin-like proteins may participate in the process of autophagosome maturation (see chapters 6, 7 and 15).[19,20]

Regulation of Autophagy by Amino Acids

Because amino acids are the end products of autophagic protein degradation, it is not surprising that they are effective inhibitors of the process. The process is also hormonally controlled, with insulin (inhibitory) and glucagon (stimulatory) as some of the key players.[6,21] Interestingly, in the liver, insulin and glucagon only exert their effect on autophagy at intermediate, but not at either high or very low amino acid concentrations when autophagy proceeds at minimal or maximal rates, respectively.[4,6] As discussed below, these effects parallel the effects of the two hormones and amino acids on signal transduction.

There is no doubt that most of the inhibitory effect of amino acids on flux through the autophagic system is due to inhibition of the first step in autophagy, i.e., sequestration,[6,21] but post-sequestrational inhibition of autophagy can also not be excluded. For example, asparagine, when used at high concentrations in hepatocytes, primarily interfered with the fusion between initial autophagosomes and lysosomes.[14,22] It is unclear, however, whether this was due to asparagine itself or whether it was caused by one or more of its catabolites, such as aspartate, alanine and (acidotropic) ammonia, because the incubation medium contained high concentrations of pyruvate and was devoid of HCO_3^-/CO_2, and urea synthesis may have been low under these conditions. High concentrations of leucine have been shown to increase the lysosomal pH, presumably by direct inhibition of the lysosomal proton pump.[23]

The specificity of amino acid inhibition of autophagic sequestration has been intensively studied. Based on experiments with the perfused rat liver, Mortimore and colleagues defined a regulatory group of amino acids consisting of leucine, phenylalanine, tyrosine, glutamine, proline, histidine, tryptophan and methionine; together with alanine as a synergistic coregulator, this group of amino acids was able to mimic the inhibitory effect on autophagy of a complete mixture of amino acids at near-physiological concentrations.[24-26] In isolated rat hepatocytes, high concentrations of leucine together with either glutamine or histidine acted in synergy and were particularly effective in inhibiting autophagic sequestration of electro-injected cytosolic [^{14}C]sucrose.[27] A combination of near-physiological concentrations of leucine with either alanine, glutamine, proline or asparagine was also extremely effective in inhibiting autophagic proteolysis in hepatocytes,[28,29] and the same was true for leucine and alanine in the perfused liver.[25] Presumably, leucine in combination with intracellular glutamate and/or aspartate, derived from catabolism of other amino acids, is sufficient to inhibit autophagy maximally.[29] It is important to note that leucine, in contrast to the other amino acids mentioned, is hardly catabolized in hepatocytes because of the low activity of leucine aminotransferase in these cells.[30] Analysis of the relationship between extra- and intracellular amino acid concentrations with varying rates of autophagic protein degradation in cultured hepatocytes derived from rats at various stages of development showed that leucine, phenylalanine, tyrosine and lysine were possible regulators of autophagy.[31]

From this brief survey on liver data it appears that leucine, phenylalanine and tyrosine, in combination with a few other amino acids (e.g., alanine, glutamine), is sufficient to inhibit autophagy in hepatocytes. Interestingly, leucine and glutamine are also known as potent inhibitors of proteolysis in skeletal muscle (see ref. 1 for literature).

An important step forward in our understanding of the mechanism by means of which certain amino acids inhibit protein degradation was the discovery, initially made in liver but also valid for other cell types, that an increase in cell volume mimics many of the anabolic effects of insulin, including inhibition of proteolysis and stimulation of protein synthesis (for review see ref. 32). An increase in cell volume may occur not only by hypo-osmolarity of the extracellular environment but can also be the result of concentrative, Na^+-dependent, amino acid transport across the plasma membrane. Intracellular accumulation of impermeant amino acid catabolites (e.g., glutamate, aspartate) may further enhance intracellular osmolarity and contribute to cell swelling. Cells can compensate, albeit not completely, for the initial cell-volume increase by a mechanism known as "regulatory volume decrease", for example, by releasing KCl in many cell types.[32] Stimulation of the synthesis of macromolecules (and inhibition of their degradation) by an increase in intracellular osmolarity relative to that in the extracellular environment can be envisaged as an additional mechanism by which the cell tries to counteract an increase in intracellular osmolarity; this closely resembles similar mechanisms in microorganisms when they have to adapt to changes in osmotic stress.[33]

In hepatocytes, hypo-osmotically induced cell swelling increases the sensitivity of autophagic proteolysis by low concentrations of amino acids, but has no effect by itself.[34-36] This resembles the effect of insulin,[6] as discussed above, but is at variance with data by Häussinger and colleagues,[37] who did observe inhibition of hepatic proteolysis by hypo-osmotically induced cell swelling in the flow-through perfused rat liver also in the absence of amino acids (see refs. 38, 39 for a discussion of this difference in results). An intact cytoskeleton is not only required for the autophagic machinery (for review see ref. 1 and chapter 2) but also for control of autophagy by changes in cell volume.[40,41] It must be pointed out that leucine (like tyrosine and phenylalanine) is not transported by Na^+-coupled systems and is not accumulated by cells, but rather equilibrates across the plasma membrane. Synergy between leucine and alanine (or glutamine) with regard to their inhibition of autophagy may, as discussed above, be explained by the fact that an increase in cell volume caused by Na^+-driven influx of alanine (or glutamine) potentiates the specific effect of leucine on the sequestration step, which cannot be ascribed to a swelling-induced increase in permeability of the plasma membrane for leucine (AJ Meijer,

unpublished data). As we will discuss below, these effects parallel the interaction of the amino acids on signal transduction.

Amino Acid-Dependent Signaling and Control of Autophagy

Initial indications that signal-transduction pathways are involved in the control of autophagy came from studies showing that inhibitors of tyrosine kinases and of protein phosphatases strongly inhibited autophagy in hepatocytes (see chapter 4).[42-44]

The ability of leucine to inhibit autophagic proteolysis (see previous section) was of interest because leucine had been known for a long time as an effective stimulator of protein synthesis not only in liver but also in muscle cells.[45] Progress in our understanding of how amino acids may affect protein degradation and synthesis (apart from their effect as substrates) was made when it was discovered that in hepatocytes administration of a complete, physiological mixture of amino acids resulted in strong and rapid ($t_{1/2}$, 10 min) phosphorylation of ribosomal protein S6.[35,46] This protein has five phosphorylation sites, is a component of the 40S ribosomal subunit and its phosphorylation is required for the translation of the terminal oligopyrimidine ("TOP") family of mRNA molecules, which contain an oligopyrimidine tract upstream of their transcription-initiation site and which encode proteins belonging to the protein-translational machinery.[47] Phosphorylation of S6 by addition of amino acids was prevented by rapamycin, indicating that the serine/threonine protein kinase mTOR (mammalian target of rapamycin) was involved in the process (see chapter 5). Addition of insulin in the absence of amino acids did not affect phosphorylation of S6 but potentiated the effect of low, but not of high, amino acid concentrations. In contrast to insulin, glucagon decreased the phosphorylation of S6 at low but not at high amino acid concentrations. Hypo-osmotically-induced cell swelling completely mimicked the effect of insulin. In addition, the effect of a complete mixture of amino acids could be mimicked by combination of low concentrations of leucine, phenylalanine and tyrosine and either insulin or hypo-osmotically induced cell swelling. These effects closely resembled the effects of amino acids, insulin and cell swelling on autophagy, as discussed earlier. Indeed, under a wide variety of conditions, there was an approximately linear relationship between the degree of phosphorylation of S6 and the percentage of inhibition of autophagic proteolysis.[46] It must be pointed out that these experiments were carried out in the presence of cycloheximide to inhibit protein synthesis to ensure that the effects on proteolysis were not caused by amino acid effects on protein synthesis. Interestingly, rapamycin could, albeit not completely, prevent the inhibition of autophagic proteolysis by amino acids. It was concluded that the same mechanism perhaps controls (global) protein synthesis and degradation in opposite directions, because in the absence of cycloheximide rapamycin inhibited protein synthesis only partially, which would be very efficient from the point of view of metabolic regulation.[46,48,49] The observation that rapamycin can stimulate autophagy was confirmed in yeast (see chapter 6).[50,51]

If ribosome-free parts of the endoplasmic reticulum are the origin of the autophagosome membrane, an attractive hypothesis would be that the degree of S6 phosphorylation may determine the degree of occupancy of the endoplasmic reticulum by ribosomes, and thus determine the rate of autophagic sequestration and the rate of ER-linked protein synthesis.[46] Indeed, in hepatocytes, synthesis of export proteins, but not of house-keeping proteins, declines after amino acid deprivation.[52] If the hypothesis is correct, it would mean that autophagy is a constitutive process, unless its rate becomes inhibited because of a decrease in the surface area of ribosome-free endoplasmic reticulum. The previous findings in hepatocytes that the degree of phosphorylation of S6, when bound to the ER, was higher than that of free ribosomes would support this.[1]

Since its discovery in 1994, the existence of amino acid-dependent signaling has been confirmed for many insulin-sensitive cell types, including muscle cells, adipocytes, hepatoma cells, CHO cells, HT-29 cells and pancreatic β-cells (for review see refs. 53, 54). Because it is not possible to discuss the available literature on amino acid signaling at great length, we will only

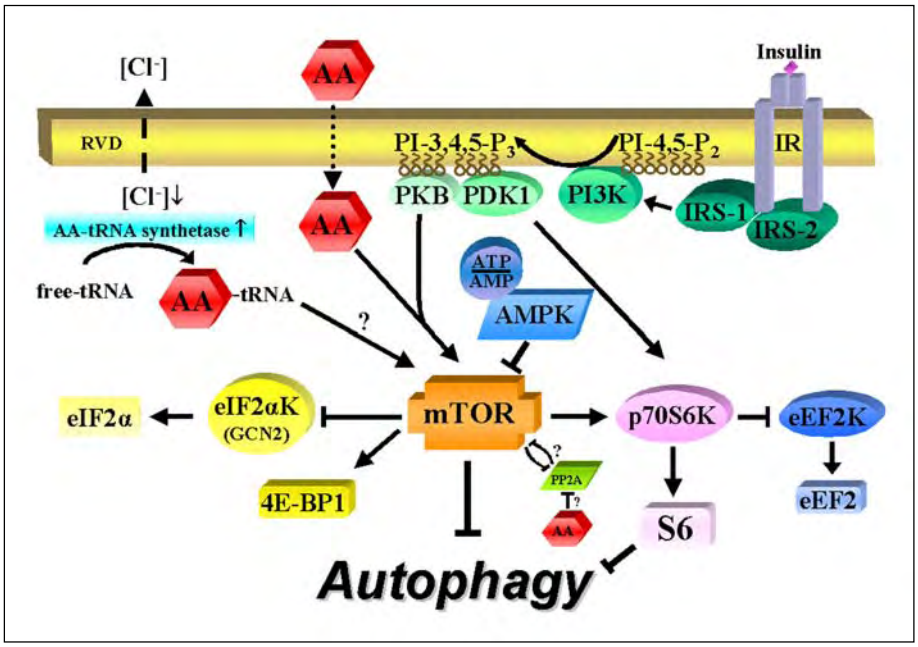

Figure 1. Amino acid-dependent signal transduction. Abbreviations: IR, insulin receptors; IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; PDK1, phosphoinositide-dependent kinase 1; PKB, protein kinase B; AA, amino acids; RVD, regulatory volume decrease. For other abbreviations and further explanation, see text.

briefly summarize the main features of the process. As in hepatocytes, in most cell types leucine appeared to be the most effective amino acid. This demonstrates that the same mechanism must be involved in amino acid signaling, independent of cell type. Although additional components cannot be excluded, the amino acid-dependent signaling pathway to S6 includes mTOR and p70S6 kinase; other endpoints are also possible.[53,54] Thus, mTOR-mediated phosphorylation of 4E-BP1 and of eIF2α kinase (the equivalent of Gcn2 in yeast), and mTOR and p70S6 kinase-dependent phosphorylation of eEF2 kinase (resulting in the stimulation of mRNA translation-initiation, initiation of protein synthesis and elongation, respectively) are all dependent on the presence of amino acids. Although insulin alone stimulated both phosphatidylinositol (PtdIns) 3-kinase and protein kinase B activity in all these cell types, the hormone was not able to stimulate mTOR-dependent processes unless low concentrations of amino acids were also present. In cases where insulin was effective on its own this was because amino acids were generated by autophagy in the course of the experiment.[55]

There is general agreement that amino acids, in contrast to insulin, are not able to stimulate protein kinase B phosphorylation and activity.[53,54] Controversy, however, exists as to whether or not amino acids can stimulate PtdIns 3-kinase activity, because direct attempts to show this failed[55-57] or the observed stimulation was transient.[58,59] By contrast, amino acid-dependent, mTOR-mediated protein phosphorylation was sensitive to PtdIns 3-kinase inhibitors, as first shown by Blommaart et al[60] and later confirmed by others (for review see refs. 53, 54). Although it has been argued in the literature that PtdIns 3-kinase inhibitors are not specific and may also inhibit mTOR directly, this was considered unlikely.[54] Rather, it may be that mTOR-mediated signaling and PtdIns 3-kinase-mediated signaling (perhaps via PDK1) are on parallel pathways and that both pathways are required to phosphorylate downstream targets, such as p70S6 kinase, at different positions before full activation is obtained.[61-63] Because

in most cell types high concentrations of amino acids alone (i.e., in the absence of insulin) stimulate mTOR-downstream targets in a wortmannin-sensitive or LY294002-sensitive manner, one has to assume that either basal activity of PtdIns 3-kinase, or a slight stimulation of PtdIns 3-kinase by amino acids is sufficient. In addition, amino acids can directly activate mTOR; both pathways, then, separately feed into p70S6 kinase (Fig. 1).

Apart from the fact that amino acid-dependent signaling is rapamycin-sensitive, there is also direct evidence that amino acids do increase mTOR phosphorylation and/or activity in intact cells. Thus, mTOR isolated from amino acid-stimulated Jurkat cells could phosphorylate the protein phospatase PP2A in vitro.[64] In HEK293 cells, mTOR could be phosphorylated by protein kinase B in vitro but only when mTOR was immunopurified from amino acid-treated cells.[65]

Amino Acids, Protein Phosphatases and Autophagy

The mechanism by which amino acid-dependent signaling affects autophagy is unknown. An attractive possibility is that amino acid signaling and autophagy are linked by a protein phosphatase, presumably PP2A. In hepatocytes, for example, inhibition of PP2A by okadaic acid strongly inhibits autophagy (see chapter 4).[42] In yeast, autophagy also parallels PP2A activity. Interestingly, the rapamycin-sensitive mTOR proteins in yeast affect PP2A activity by modulating its association with the Tap42 protein. Under nutrient-rich conditions Tap42 is phosphorylated, associates with PP2A, and inhibits phosphatase activity. Nutrient deprivation or rapamycin addition reverses these events and autophagy becomes activated, although there is evidence from studies in yeast suggesting that activation of autophagy is independent of Tap42.[51,61] In mammalian cells, association of mTOR with PP2A and control of its activity by mTOR seems likely.[64,66] In this view, amino acids control mTOR activity which, in turn, simultaneously controls both PP2A activity and the degree of S6 phosphorylation (Fig. 1). In hepatocytes, a possible target of PP2A may be the cytokeratin filaments.[67]

Amino Acid Sensor

Although amino acids appear to activate mTOR, the mechanism by which this occurs is not yet clear. Originally, it was thought that amino acids (leucine in particular) inhibited autophagy by binding to a receptor protein on the plasma membrane and that the signal is somehow transmitted to the cell interior.[68] However, the evidence in favor of a plasma-membrane amino acid receptor was not very strong.[53,69] Because inhibition of plasma membrane amino acid transport prevented activation of p70S6 kinase by amino acids, it is more likely that amino acids act intracellularly.[70,71] Direct activation by amino acids of mTOR kinase activity in vitro was ruled out.[72]

An attractive, albeit hypothetical, mechanism is one in which amino acid starvation is sensed by the degree of amino acid charging of tRNA. This hypothesis is based on data in yeast which show that uncharged tRNA strongly binds to, and activates, the protein kinase Gcn2 (controlled by mTOR; see *Amino Acid-Dependent Signaling and Control of Autophagy*); this results in derepression of *GCN4* mRNA translation. This transcriptional activator promotes the transcription of many genes involved in nitrogen metabolism, that do not only participate in amino acid biosynthesis but also in autophagy.[73] In mammalian cells, the eIF2α kinase PKR (double-stranded RNA-dependent protein kinase), the equivalent of Gcn2, and its target eIF2α also participate in the control of autophagy, and PKR can rescue starvation-induced autophagy in *GCN2*-disrupted yeast.[74] The issue of whether uncharged tRNA acts as a sensor in amino acid signaling and controls autophagy in mammalian cells has not yet been resolved. CHO cells increase their rate of protein degradation when the degree of charging of histidinyl-tRNA declines.[75] On the other hand, controversial results have been obtained with amino alcohols, inhibitors of aminoacyl-tRNA synthetases. In Jurkat cells these compounds were shown to prevent amino acid-dependent p70S6 kinase activation,[70] but not in adipocytes.[76] Moreover, in HEK-293 cells the level of aminoacylated tRNA did not correlate with signaling.[72] Amino alcohols inhibited (rather than stimulated) autophagic proteolysis in hepatocytes.[77] Although

one may consider this as evidence against a role of uncharged tRNA in autophagy, interpretation of such data may be difficult because of lack of specificity of these compounds, for example, because some increase the intralysosomal pH.[77] Uncharged tRNA as amino acid sensor would provide a mechanism accounting for the inhibition of autophagy, and promotion of amino acid signaling by cell swelling: chloride ions inhibit aminoacyl tRNA synthetases and the fall in intracellular chloride accompanying regulatory volume decrease (see *Regulation of Autophagy by Amino Acids*) may be sufficient to stimulate the charging of tRNA.[53]

Regulation of Autophagy by Energy

Nucleotide-Dependent Control

Autophagy is not only controlled by amino acids but is also energy-dependent.[78,79] In hepatocytes, the process is sensitive to even relatively small variations in ATP and becomes inhibited when ATP falls, because ATP is required for autophagic sequestration, fusion of autophagosomes and lysosomes, and for lysosomal proton pumping.[80] Inhibition of autophagy by a fall in ATP was also observed in rat liver in vivo.[81] However, because decreases in ATP production are often accompanied by a rise in AMP via the adenylate kinase equilibrium, it is possible that activation of the AMP-dependent protein kinase may have contributed to the inhibition of autophagy by a fall in ATP, especially because activity of this protein kinase is controlled by the cytosolic AMP/ATP ratio.[82] Seglen and colleagues observed strong inhibition of autophagy in hepatocytes by the adenosine analogue AICAriboside (AICAR), a compound which, after its intracellular phosphorylation, activates AMP-dependent protein kinase, which supports this view. Likewise, pharmacological manipulations meant to increase intracellular AMP levels also inhibited autophagy.[83,84]

mTOR As a Sensor of ATP and AMP

Apart from the fact that mTOR acts as a switch point controlling protein synthesis and autophagic protein degradation, and, presumably indirectly, senses the level of amino acids in the cell, it has recently been proposed that mTOR may also be a sensor of intracellular ATP.[72] This proposal was based on the finding that, in vitro, the affinity of mTOR for ATP was much lower than that of other protein kinases and that inhibition of ATP production in intact HEK293 cells inhibited mTOR activity in situ. An additional control mechanism may be that mTOR is controlled by AMP-dependent protein kinase which is determined by the AMP/ATP ratio (see previous paragraph). This idea was supported by the finding that addition of AICAR inhibited amino acid-stimulated p70S6 kinase phosphorylation in hepatocytes without affecting insulin-stimulated protein kinase B phosphorylation.[54,85] In this context, the recent observation that mTOR and p70S6 kinase are both associated with the mitochondrial outer membrane[86,87] is highly relevant, because adenylate kinase is predominantly located in the mitochondrial intermembrane space, and is not present in the mitochondrial matrix.[88] mTOR is thus in a perfect position to be controlled by changes in the AMP/ATP ratio via AMP-dependent protein kinase.

Because activation of AMP-dependent protein kinase prevents amino acid signaling, the inhibition of autophagy by activation of AMP-dependent protein kinase (see previous section) is unexpected. The reason for this is not yet known. However, in yeast, Snf1, the homologue of AMP-dependent protein kinase, does activate autophagy.[89]

The Role of Phosphatidylinositol 3-Kinases and Lipid Phosphatases in Autophagy

As indicated above (see *Amino Acid-Dependent Signaling and Control of Autophagy*), amino acid-dependent phosphorylation of S6 in hepatocytes was not only inhibited by rapamycin but also by the PtdIns 3-kinase inhibitors wortmannin and LY294002.[60] Unexpectedly, however, and in contrast to stimulation of autophagy by rapamycin, interruption of amino acid signal-

ing by wortmannin and LY294002 strongly inhibited autophagic sequestration and did not reverse the inhibition by amino acids. In order to account for this apparent anomalous result it was assumed that constitutively produced PtdIns(3)P may be essential for autophagosome formation, whereas PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 inhibit the process.[60,90] Inhibition of PtdIns(3)P production by wortmannin and LY294002 would then explain the observed inhibition of autophagy. The idea that PtdIns(3)P might be essential for autophagy was based on the fact that yeast cells, being very active in autophagy under nutrient-poor conditions, can only produce PtdIns(3)P but not PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (see below).

Further support in favor of a role of PtdIns 3-kinase in the control of autophagy was obtained by the discovery that 3-methyladenine, the classical inhibitor of autophagic sequestration,[91] proved to be an inhibitor of PtdIns 3-kinase.[60,90] This provided a satisfactory explanation for its mechanism of action. Likewise, the cAMP phosphodiesterase inhibitor theophylline is also an inhibitor of PtdIns 3-kinase[92] which may account for its ability to inhibit autophagy in hepatocytes.[93]

Phosphatidylinositol 3-Kinases

PtdIns 3-kinase is a family of enzymes that phosphorylate the 3'-hydroxyl group on the inositol ring of phosphoinositides.[94,95] These phospholipids are involved in a large array of signal-transduction pathways controlling mitogenic responses, differentiation, apoptosis, cytoskeletal organization and membrane flow along the secretory and endocytic pathways.[96-99] PtdIns 3-kinases are classified into three classes according to their enzymatic properties. Class I enzymes are composed of catalytic p110 subunits and p85 adaptors. The SH2 motif contained in p85 adaptors bind to phosphorylated Tyr residues, thereby linking the catalytic subunit to the Tyr kinase signaling pathway (class I_A). A member of the class I PtdIns 3-kinase family (i.e., class I_B, p110γ/p101) is activated by subunits of trimeric G proteins.[100,101] Class I PtdIns 3-kinases phosphorylate PtdIns, PtdIns(4)P and PtdIns(4,5)P_2, but in vivo PtdIns(4,5)P_2 is likely to be the favorite substrate. Class II enzymes are large enzymes (>200 kDa) characterized by a carboxyl terminus containing a C2 domain.[102-104] In vitro these enzymes phosphorylate PtdIns and PtdIns(4)P, but not PtdIns(4,5)P_2. Class III enzymes are homologous to the archetypal Vps34 characterized in *Saccharomyces cerevisiae* which only produce PtdIns(3)P.[105] Vps34 function requires its association with a myristylated serine kinase Vps15.[106] The complex Vps15/Vps34 is of fundamental importance in controlling vesicular transport to the yeast vacuole (reviewed in ref. 98). The human homologue of Vps34 has been shown to be associated with a p150 myristylated protein kinase.[107]

PtdIns 3-Kinase in Autophagy

At least two different types of PtdIns 3-kinase are involved in the formation of the autophagosome. They control either a signaling pathway emanating from the cell surface (PtdIns 3-kinase class I) or membrane remodeling associated with the sequestration step (PtdIns 3-kinase class III).[90]

PtdIns 3-Kinase Class III

Both the PtdIns 3-kinase class III and its membrane adaptor p150 are mandatory for the formation of autophagic vacuoles.[90] In yeast, the *Saccharomyces cerevisiae* homologues of Vps15 and Vps34 have been shown to control the autophagic degradation in *Pichia pastoris*[108] and *Hansenula polymorpha*,[109] respectively. Recently, Ohsumi's lab provided some clues to the mechanism by which Vps34-Vps15 control autophagy in *Saccharomyces cerevisiae* (see chapter 6).[17] Vps34-Vps15 exist in two complexes which control different membrane-transport processes. Complex I which contains Apg14 and Apg6/Vps30 controls autophagy, and complex II, composed of Vps38 and Apg6/Vps30, governs the vesicular transport to the vacuole (yeast lysosome). In mammalian cells, PtdIns 3-kinase class III/p150 is associated with the protein Beclin 1.[16] Beclin 1, the yeast functional homologue to Apg6, induces autophagy in cultured breast cancer cells and inhibits their tumorigenicity.[110] The discovery of Beclin 1 which has

tumor-suppressor properties indicates a potential link between autophagy and tumor progression (see chapter 20). Another link between autophagy and cancer is indicated by the role of the PtdIns 3-kinase class I signaling pathway in autophagy.

PtdIns 3-Kinase Class I

PtdIns 3-kinase class I enzymes phosphorylate PtdIns(4)P and PtdIns(4,5)P_2 to produce PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 which bind via PH (Pleckstrin Homology) domains to protein kinase B (Akt/PKB) and its activator PDK1 (phosphoinositide-dependent kinase 1).[94,111] PDK1 phosphorylates other kinases including p70S6 kinase.[112] Stimulation of the PtdIns 3-kinase class I signaling pathway in human colon cancer HT-29 cells by the cytokine interleukin-13 inhibited autophagy.[90] Accordingly, expression of a constitutive active form of either Akt/PKB[113] or PDK1 (our unpublished results) reduced autophagic capacity in these cells. Several lines of evidence indicate that among the known targets of Akt/PKB, mTOR occupies a central position in the signaling cascade of autophagy in eukaryotic cells (see *Amino Acid Signaling and Control of Autophagy*).[46,50,114] As discussed above, amino acids can control mTOR in a PKB-independent manner. Alternatively we cannot exclude the possibility that a PDK1 signaling pathway to p70S6 kinase that does not encompass Akt/PKB could regulate autophagy.

Lipid Phosphatases

Control of the phosphorylation of the 3'-position of PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 by the action of phosphatases offers potential control of the signaling as well as of the membrane remodeling that occurs during autophagy.[115,116] The level of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 is controlled by the activity of PtdIns 3-kinase and by the lipid phosphatase activity of PTEN (phosphatase and tensin homologue deleted from chromosome 10) which specifically dephosphorylates the 3'-position of PtdIns(3,4,5)P_3.[117] *PTEN* is a tumor-suppressor gene frequently mutated in a large number of cancers, including glioblastomas, endometrial cancer and prostate cancer, and in rare autosomal dominant cancer predisposition syndromes.[118] PTEN is a dual phosphatase with a protein phosphatase activity and a lipid phosphatase activity.[116] The inactivation of the lipid phosphatase activity of PTEN leads to inhibition of autophagy by exacerbating the inhibitory effect of the PtdIns 3-kinase/PKB pathway.[113]

Myotubularin (MTM) and related proteins (MTMR) form a family of dual protein-tyrosine phosphatases.[115,116] Several members of this family have been shown to utilize PtdIns(3)P as substrate; these different forms can be discriminated by their cellular and tissue expression and their intracellular localization.[119] Mutations in two human genes MTM1 and MTMR2 have been shown to cause X-linked myotubular myopathy[120] and type 4B Charcot-Marie-Tooth syndrome,[121] respectively. Recently, the expression of a catalytically inactive mutant of myotubularin-related protein 3 (MTMR3), which can hydrolyze the PtdIns(3)P in mammalian cells, was characterized by the accumulation of autophagic vacuoles,[122] giving credence to the role of these phosphatases in the control of the formation of autophagosomes.

The intracellular localization of other phosphoinositide 3-phosphatases such as PTEN2[123] and TPIP[124] on the Golgi apparatus and endoplasmic reticulum makes them possible candidates to regulate intracellular pools of PtdIns(3)P in cells where they are expressed.

G Proteins

Trimeric G Proteins

The role of heterotrimeric G proteins ($\alpha\beta\gamma$) as signal transducers is well documented.[125-127] Their interaction with activated receptors triggers guanine nucleotide exchange on the α subunit. After dissociation from the $\beta\gamma$ dimer, the activated α-subunit (α-GTP) acts on the appropriate downstream effector. Likewise the $\beta\gamma$ dimer can also act on specific effectors.[128] During the past few years the function of heterotrimeric G proteins in membrane transport along the exo/endocytotic pathways has been recognized. This includes the transport from endoplasmic

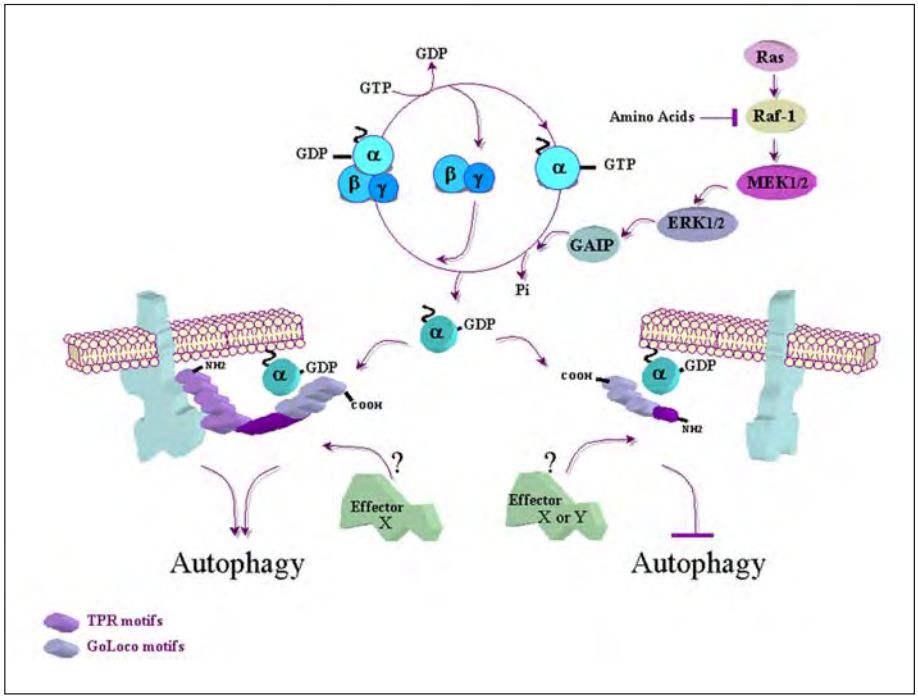

Figure 2. Control of autophagy by the trimeric G_{i3} protein signaling pathway. The GTPase Activating Protein activity of GAIP is stimulated by phosphorylation on Ser151. Phosphorylation of this site is dependent on the MAP kinases ERK1/2, the activity of which, in turn, is controlled by amino acids. Amino acids interfere with the ERK1/2 signaling pathway by favoring phosphorylation on Ser259 of Raf-1 which reduces its kinase activity toward MEK1/2, the upstream kinase activators of ERK1/2. The mechanism by which amino acids increase phosphorylation on Ser259 could be via PP2A (Fig. 1) The long form of AGS3 (seven TPR N-terminal repeats linked to four GoLoco C-terminal domains by a linker sequence) stimulates autophagy by stabilizing the GDP-bound form of the α-subunit of the G_{i3} protein ($G_{\alpha i3}$ is a lipid-tagged protein that interacts with the lipid bilayer) independently of the βγ dimer. As the AGS3-long form interacts with membranes in a TPR-dependent manner it is speculated that both N- and C-terminal halves of AGS3 are required to stimulate autophagy. The N-terminal half can interact with a membrane partner. The recruitment of cytosolic effectors (X) by the GDP-bound form of the α_{i3} subunit cannot be excluded. In contrast, a short form of AGS3 with an N-terminal truncation inhibits autophagy. Long and short forms of AGS3 cannot be discriminated by their GDI (GDP Dissociation Inhibitor) activity toward the $G_{\alpha i3}$ protein. However, they differ in their localization in that the short form is cytosolic and the long form is associated with as yet unidentified cytoplasmic membranes. The short form is unable to interact with a membrane-bound partner via its N-terminal part. In addition, this form could also recruit cytosolic effectors similar or different from the AGS3-long form (X or Y).

reticulum to Golgi[129,130] and intra-Golgi transport,[131] constitutive and regulated vesicle budding from the trans-Golgi network,[132] control of exocytosis,[133,134] formation of endocytic vesicles[135] and endosome fusion.[136] In polarized cells different trimeric G proteins were shown to control the sorting of proteins: G_s protein governs the apical sorting from the trans-Golgi network[137] and basolateral to apical transcytosis[138] and G_{i3} protein is involved in the basolateral sorting of proteins.[131,137]

The first evidence for a role of GTPases in the control of autophagy came from experiments showing that in permeabilized rat hepatocytes nonhydrolyzable analogs of GTP inhibited the de novo formation of autophagic vacuoles.[139] Thereafter we showed that autophagic sequestra-

tion is controlled by the trimeric G_{i3} protein in the human colon carcinoma HT-29 cell line.[140] An important step towards understanding the control of autophagy is to identify the state of activation of the G_{i3} protein (i.e., nucleotide binding) that regulates autophagic sequestration. Using site-directed mutagenesis and stable cell transfections, we demonstrated that autophagic sequestration is switched on when the G_{i3} protein is bound to GDP[141] and that, in contrast, autophagic sequestration is inhibited when the G_{i3} protein is bound to GTP. Autophagy is also reduced when dissociation of the $\beta\gamma$ dimer from the GTP-bound $G_{\alpha i3}$ protein is inhibited (Fig. 2). The localization of the $G_{\alpha i3}$ protein is another important element to consider for its role in autophagy. The $G_{\alpha i3}$ protein must be associated with cytoplasmic membranes, i.e., Golgi apparatus and/or endoplasmic reticulum to control autophagic sequestration, but when it is ectopically expressed at the plasma membrane, $G_{\alpha i3}$ protein no longer acts on autophagy.[142] However, destabilization of the Golgi apparatus following treatment with the fungal metabolite brefeldin A[143] did not inhibit the formation of autophagosomes and lysosomal breakdown of material sequestered in autophagic vacuoles.[144,145] These results suggest that the integrity of the Golgi apparatus is not required for the formation of autophagic vacuoles. In addition, they point to the independence of autophagy toward GTPases of the ARF family (ADP-ribosylation factors) which use a GEF (guanine nucleotide exchange factor) sensitive to brefeldin A.[146,147]

A better understanding of the function of the $G_{\alpha i3}$ protein in autophagy would require the characterization of elements acting upstream and downstream of the $G_{\alpha i3}$ protein and of elements which control the GTP cycle on the $G_{\alpha i3}$ protein.

G_{α}-Interacting Protein (GAIP)

GAIP belongs to the protein family of regulators of G protein signaling (RGS).[148] RGS are GTPase-activating proteins (GAP) which increase the rate of GTP hydrolysis by $G_{\alpha o/i}$.[149-152] GAIP, which interacts with the $G_{\alpha i3}$ protein, has been shown to favor autophagic sequestration by accelerating GTP hydrolysis by the $G_{\alpha i3}$ protein.[153] Interestingly the lower expression of GAIP in differentiated HT-29 cells than that in the undifferentiated counterparts is correlated with the differentiation-dependent autophagic capacity of these cells.[153,154] More recently we showed that the phosphorylation of a conserved serine residue in the RGS domain of GAIP stimulates its GAP activity, and consequently the autophagic pathway.[155] This phosphorylation is dependent upon the activity of the MAP kinases ERK1/2. This signaling pathway is turned off in the presence of amino acids. The GTPase Activating Protein activity of GAIP is stimulated by phosphorylation on Ser151. Amino acids interfere with the ERK1/2 signaling pathway by favoring phosphorylation on Ser259 of Raf-1,[155a] which reduces its kinase activity towards MEK1/2, the upstream kinase activators of ERK1/2 (reviewed in ref. 156). These results indicate a molecular connection between amino acid signaling and the control of autophagy by trimeric G proteins (see Figs. 1 and 2). As the phosphorylation of Ser259 is dependent on the activity of PP2A,[157] it can be speculated that amino acids can regulate the p70S6 kinase and the G protein pathways by similar mechanisms with a common outcome on autophagy. Studies are in progress to investigate whether the amino acid-dependent phosphorylation of Raf-1 can be regulated by mTOR similarly to that of p70S6 kinase (see Fig. 1).

Activators of G Protein Signaling (AGS)

Recently, a novel group of proteins containing shared sequence repeats named GoLoco or G protein regulators (GPR) has been shown to interact with $G_{\alpha i}$ and $G_{\alpha t}$ proteins.[158,159] Several of these proteins, AGS3, LGN, Pcp2 and Rap1GAP acted as a guanosine nucleotide dissociation inhibitor (GDI) towards $G_{\alpha i}$, including the $G_{\alpha i3}$ protein.[160] AGS3 is a bimodular protein:[161] its amino terminal half contains seven tetratricopeptides (TPR) repeats which are protein-protein interaction modules,[162] and the carboxyl terminal half contains four GoLoco motifs.[158] Goloco motifs interact with the GDP-bound form of the $G_{\alpha i3}$ protein and inhibits AGS3 association with the $\beta\gamma$ dimers.[160,163,164] Recently we found that according to its nucleotide-binding property, the full-length ASG3 stimulates the autophagic pathway.[164a] In contrast, expression of the C-terminal part of AGS3 containing GoLoco domains inhibits

autophagy, but the N-terminal part containing TPR domains has no effect (Fig. 2). The full-length AGS3 and the AGS3-4 GoLoco which are not discriminated by their GDP-binding properties have distinct localizations. The AGS3-4 GoLoco is cytosolic and the full-length AGS3 is associated with cytoplasmic membranes not yet identified. Interestingly, two forms of AGS3 resulting either from alternative gene splicing or trans-splicing, or from alternative promoters have a differential tissue distribution.[165] An N-terminal truncated short form containing GoLoco motifs has been shown to be the major AGS3 form present in heart, whereas the long form (7 TPR + 4 GoLoco) is more widely distributed. In intestinal cells, mRNAs encoding both forms have been detected which suggests that AGS3 can modulate autophagy in opposite directions depending upon the $G_{\alpha i3}$-dependent recruitment of either the long or the short form. The identification of the mechanism that controls this differential recruitment would help in the understanding of the $G_{\alpha i3}$ protein-dependent autophagy.

How the G_{i3} protein controls autophagy remains to be elucidated. However, one should keep in mind that GAIP and AGS3 are recruited from the cytosol by the $G_{\alpha i3}$ protein. The $G_{\alpha i3}$ protein is probably able to recruit other proteins that could be potential candidates to control autophagy (Fig. 2). Calnuc (nucleobindin) is a Ca^{2+} sensor that is recruited to Golgi membranes in a $G_{\alpha i3}$ protein-dependent manner.[166-168] Calnuc was also found to be associated with the endoplasmic reticulum.[169] Interestingly, the release of calcium stores from membrane bound compartments proved to be important in the control of autophagic sequestration.[170] Whether or not $G_{\alpha i3}$ protein-dependent recruitment of calnuc, or of other cytosolic proteins, to membranes is instrumental in autophagy is a challenge for further studies.

Monomeric G Proteins

Rab proteins are a family of monomeric GTPases necessary for vesicular transport along the exo/endocytic pathway (for review see refs. 171, 172). Rab22a is associated with early and late endosomes, but a mutant with low GTPase activity (Rab22aQ64L) colocalized with autophagic vacuoles.[173] Interestingly, Rab24 with a localization compatible with a role along the autophagic pathway[174,175] has been shown to be preferentially in a GTP-bound state when expressed in cultured cells.[176] The GTP membrane-bound forms of Rab proteins are able to recruit cytosolic proteins which target vesicles to appropriate sites on the acceptor membrane.[171,172] Whether or not effectors recruited by the GTP-bound forms of Rabs are mandatory for membrane fusion or transport along the autophagic pathway remains to be investigated. In yeast, the fusion of the autophagosome with the vacuole is probably dependent upon the Rab homolog Ypt7.[177] According to the paradigm of vesicular transport,[178] other proteins such as the NSF protein[15] and vacuolar members of the transmembrane SNARE protein family are involved in completing the fusion between the autophagosome and the vacuole.[15,179,180] Although not yet reported, mammalian homologues of yeast NSF and SNARE proteins should also contribute to the maturation of autophagic vacuoles.

A large-scale two-hybrid experiment in the yeast *S. cerevisiae* has suggested that Apg17 (a protein associated with the Apg1 kinase complex that controls the formation of the autophagosome; reviewed in ref. 181 and see chapters 6 and 7) can be a partner of monomeric Rho1 and Rho2 GTPases.[182] These results suggest that autophagy could in part be regulated by Rho proteins. Because in mammalian cells members of the Rho-family are involved in the control of cytoskeleton organization, intracellular trafficking, cell cycle progression, and tumor progression,[183,184] it would be important to determine whether or not members of the Rho-family could be involved in the control of autophagy.

Selectivity of Autophagy

Autophagy has long been considered to be a nonspecific process in which cytoplasmic structures or macromolecules are randomly sequestered.[2,3] This is not always correct, however, and we now know that autophagy can be selective under certain conditions. This was first described for yeast, with the specific autophagic elimination of peroxisomes when the cells were switched to culture media in which peroxisomal function was no longer required for growth.[185] Specific

removal of peroxisomes by autophagy was also demonstrated in rat hepatocytes, both in vivo and in vitro, under conditions where peroxisomes are functionally redundant.[11,186-191] The molecular machinery involved in the process of yeast "pexophagy" is gradually being unravelled (see chapters 10 and 11). The signal(s) protecting peroxisomes from removal by autophagy are still unknown. One possibility is that long-chain acylation of a peroxisomal membrane protein may be involved in the protection mechanism.[1] Because long-chain fatty acids are oxidized by peroxisomes, this would provide the cell with an elegant mechanism from a physiological point of view.

Another example of selective autophagy, in this case of the elimination of smooth endoplasmic reticulum, was observed in rats, after prior treatment of the animals with phenobarbital followed by its removal.[192] Again, the recognition mechanism responsible for this process is not known.

According to Lardeux and Mortimore[193], autophagic breakdown of protein, but not of ribosomal RNA, is accelerated by glucagon. This may be interpreted as a means to protect hepatocytes against excessive degradation of ribosomes in order to maintain the capacity to synthesize house-keeping proteins during starvation.

More and more evidence is now accumulating which indicates that specific autophagic elimination of mitochondria is also possible. Some liver mitochondrial proteins have half-lives of as long as one week.[194] This leads one to conclude that under normal conditions mitochondria are perhaps excluded from breakdown by autophagy unless their presence becomes a threat to cell survival. Indeed, mitochondria with a low membrane potential are specifically eliminated by autophagy in order to prevent cells from entering apoptosis, because these mitochondria produce oxygen radicals at high rates (see chapter 23).[195-197] It is of interest that compounds shown to be involved in the pathogenesis of Reye syndrome (e.g., salicylate, the hydrolysis product of aspirin) caused opening of the liver mitochondrial membrane pore[198] and that, indeed, selective autophagic degradation of damaged mitochondria was found in liver biopsies of patients with Reye syndrome,[199] and also in the liver of an influenza B virus mouse model of Reye syndrome.[200] As with autophagy of peroxisomes, nothing is known about the molecular signal(s) that destine mitochondria for autophagic sequestration. One may speculate, however, that the interaction between Bcl2 (which may be part of the mitochondrial membrane-pore complex[201]) and beclin is involved in this mechanism. The fact that, in addition, PtdIns 3-kinase class III is associated with beclin[16] and that mTOR is associated with the mitochondrial outer membrane,[86] as we have seen earlier, may also be of significance in this regard.

Another interesting example of specific autophagy can be found during maturation of erythroid cells when several cellular structures, including mitochondria and ribosomes, disappear by autophagy.[202-204] Again, the underlying signals are unknown.

Conclusion

Investigations of the signaling pathways controlling autophagy clearly show overlap with signaling pathways involved in the control of protein synthesis, mitogenesis and cell survival. There is increasing evidence in support of a role of autophagy in cell death. It is perhaps no accident that signaling components controlling the autophagic pathway are also associated with mitochondria (mTOR and p70S6 kinase). There is also mounting evidence in support of a relationship between autophagy and tumorigenesis.[205-207] The PtdIns 3-kinase, mTOR signaling pathway is frequently altered in cancer either by gene amplification or by activation of oncoproteins.[208-212] In addition, other proteins with tumor suppressor (Beclin 1)[110] and proto-oncogene (Ras)[213] properties are also engaged in the control of autophagy. Considering the growing evidence supporting a role of autophagy in pathophysiology, the elucidation of the molecular controls of autophagy will allow new therapeutical approaches to human disease.

Another challenge for the future would be to unravel the signaling mechanisms involved in the recognition of specific cellular structures destined for autophagic removal. In the case of mitochondria this would directly address the role of autophagy in cell death, ageing and mitochondrial pathologies.

Acknowledgements

A.J. Meijer is grateful to the Dutch Diabetes Fund for financial support of his research (grant 96.604). Work in P. Codogno's laboratory is supported by institutional funding from The Institut National de la Santé et de la Recherche Médicale (INSERM) and grants from the Association pour la Recherche sur le Cancer (grant 5831). The valuable discussions with P.F. Dubbelhuis and E. Ogier-Denis are gratefully acknowledged.

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CHAPTER 4

Regulation of Mammalian Autophagy by Protein Phosphorylation

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Abstract

Mammalian autophagy is subject to regulation by a variety of protein kinases and phosphatases. Long-term control of autophagic capacity seems to be mediated by transcriptional effect(s) of eIF2α kinases, whereas a signaling pathway initiated by the AMP-activated protein kinase (AMPK) and its upstream activating kinase (AMPKK) may be central in the short-term regulation of autophagic activity in isolated rat hepatocytes. The AMPKK/AMPK pathway is activated by the autophagy-suppressive adenosine analogue, AICAriboside (AICAR), as well as by okadaic acid and other autophagy-suppressive algal toxins which inhibit protein phosphatases of type 2A or type 1. Naringin, a flavonoid antagonist of okadaic acid-induced autophagy suppression, inhibits cellular AMPKK activity and blocks downstream protein phosphorylations in intact cells. Proteins subject to okadaic acid/AICAR-induced, naringin-sensitive phosphorylation include the stress-activated protein kinases SEK1 and JNK, which are likely to be downstream elements in the AMPKK/AMPK-initiated signaling pathway. Hepatocytic p70S6 kinase is phosphorylated in a naringin-sensitive manner in its tail region (T421/S424) after okadaic acid or AICAR treatment, and in a rapamycin-sensitive manner (suggesting mediation by the protein kinase mTOR) after incubation with an autophagy-suppressive amino acid mixture. Tail-phosphorylated p70S6 kinase could thus conceivably be a final common autophagy inhibitor. Such a function would have to be independent of p70S6 kinase catalytic activity, since AICAR prevented the activating phosphorylation of the enzyme at T389 (probably by an AMPK-independent mechanism) as well as its ability to phosphorylate intracellular S6. In other cell types, such as intestinal cells, the autophagy-suppressive effect of amino acids may be mediated by MAP kinases, whereas cytokine-induced autophagy suppression may be mediated by the phosphatidylinositol (PtdIns) 3-kinase/protein kinase B pathway. Analogues of cAMP, and drugs which elevate cAMP levels, are strongly autophagy-suppressive, suggesting a regulatory role for the cAMP-dependent protein kinase, possibly in part through the AMPKK/AMPK pathway. Ca^{2+}/calmodulin-dependent kinase II, cyclin-dependent protein kinases and protein-tyrosine kinases have also been implicated in autophagy control, but their postulated involvement rests on inhibitor experiments only.

Introduction

The Fundamental Role of Protein Phosphorylation in Autophagy

The study of yeast mutants has uncovered three basic elements required for autophagy: a receptor complex, a lipid kinase complex, and a protein kinase complex (see chapters 6 and 7).[1,2] The central component of the latter is Apg1/Aut3, an autophagy-stimulatory

Autophagy, edited by Daniel J. Klionsky.

Ser/Thr-protein kinase that becomes recruited upon nitrogen starvation.[3,4] A second autophagy-stimulatory Ser/Thr-protein kinase in yeast, Snf1 (a homologue of the mammalian AMP-activated protein kinase), is activated by nutrient deprivation in general.[5] A third yeast Ser/Thr-protein kinase, the eIF2α kinase, Gcn2, apparently promotes autophagy by activating Gcn4, a transcriptional activator of starvation-induced genes.[6]

Apg1/Aut3 is inactivated by an upstream kinase,[4] suggesting it to be part of an autophagy-regulatory kinase cascade. One candidate for negative Apg1/Aut3 regulation could be the autophagy-suppressive, cyclin-dependent Ser/Thr-protein kinase Pho85.[5] Pho85 antagonizes all metabolic effects of Snf1, indicating that the two kinases reside in the same signaling network. Apg1/Aut3 activity is, furthermore, dependent on a binding protein, Apg13, which can be inactivated by phosphorylation mediated by the rapamycin-sensitive, autophagy-suppressive protein/lipid kinase TOR (see chapter 5).[7,8] Rapamycin can thus induce autophagy in yeast by alleviating the TOR-dependent suppression of Apg1/Aut3 activity.

Mammalian cells incubated in nutrient-free saline media tend to exhibit maximal autophagic activity independent of de novo protein synthesis.[9] In contrast to the inducible yeast autophagy, mammalian autophagy thus appears to be constitutively active and regulated mainly by suppressive mechanisms. Protein phosphorylation, accordingly, has a predominantly negative control function in mammalian autophagy, as illustrated by the ability of hyperphosphorylation-inducing protein phosphatase inhibitors to suppress autophagy virtually completely.[10,11] Mammalian protein kinases implicated in autophagy are generally inhibitory,[12] a notion emphasized by the fact that no clear mammalian homologue of the autophagy-stimulatory yeast protein kinase, Apg1/Aut3, has been found. The demonstrated mammalian requirement for eIF2α kinase function[6] may, like in yeast, relate to the long-term maintenance of autophagic capacity rather than to the short-term regulation of autophagic activity.

In mammalian cells, the role of phosphorylation in the regulation of autophagy has been studied mainly by biochemical rather than by genetic methods. Various activators and inhibitors of protein kinases and protein phosphatases have been shown to have strong effects on autophagic activity, making it clear that this process is controlled by complex intracellular signaling networks.[12] There is thus some evidence for the involvement of an AMP-activated protein kinase (AMPK),[13,14] a Ca^{2+}/calmodulin-dependent protein kinase (CaMK-II),[10] cAMP-and cGMP-dependent protein kinases (PKA, PKG),[15,16] a cyclin-dependent protein kinase (CDK),[12] stress-activated protein kinases (SAPKs),[17,18] eIF2α kinases[6] and p70S6 kinase.[19] Although the complete characterization of these networks and their molecular connection to autophagic sequestration still remains a subject of future research, the present review will attempt to summarize current knowledge about the various network elements and their possible interrelationships.

Protein Phosphatases

Inhibitors of pSer/pThr-protein phosphatases strongly inhibit autophagy in isolated rat hepatocytes.[10,11] A comparison of the dose-response characteristics of various protein phosphatase inhibitors (okadaic acid, microcystin, calyculin A) shows that okadaic acid is much more potent than the other inhibitors, suppressing autophagy at such low concentrations (Fig. 1A) as to provide unequivocal evidence for the involvement of a type 2A protein phosphatase (PP2A) in the maintenance of autophagy. The other phosphatase inhibitors could conceivably exert their effects through inhibition of either PP2A or a type 1 enzyme (PP1). The large number of autophagy-regulatory protein kinases leaves ample opportunity for other phosphatases as well: for example, the autophagy-suppressive kinase AMPK[13] appears to be uniquely dephosphorylated by a PP2C type enzyme,[20] implicating an autophagy-regulatory role for this protein phosphatase. In fission yeast, a PP2C-like enzyme (Ptc4) seems to be involved in vacuole fusions accompanying starvation-induced autophagy.[21]

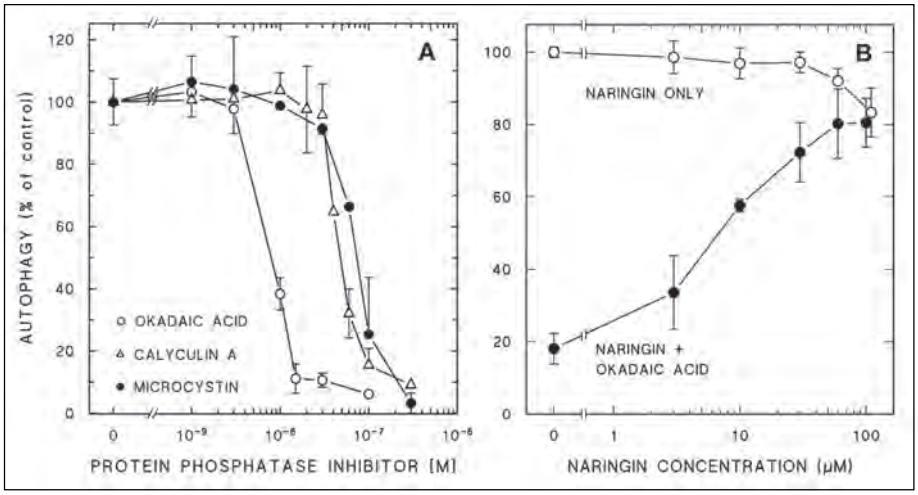

Figure 1. Naringin-sensitive suppression of autophagy by protein phosphatase inhibitors. Freshly isolated rat hepatocytes were incubated for three hours at 37°C with the indicated concentration of (A), okadaic acid (open circles), calyculin A (open triangles) or microcystin (closed circles), or (B), naringin in the absence (open circles) or presence (closed circles) of 15 nM okadaic acid. The autophagic activity during this period was measured and expressed as percent of the activitiy in the absence of inhibitors (~3.5%/h). Modified from refs. 11 and 26.

Autophagy can be strongly suppressed by sodium orthovanadate, an inhibitor of pTyr protein phosphatases.[22,23] However, vanadate tends to inhibit many ATP-dependent enzymes;[24] it would, therefore, seem premature to implicate pTyr protein phosphatases in the regulation of autophagy. In isolated hepatocytes, the autophagy-suppressive effect of vanadate would rather seem related to the osmotic regulation of Ca^{2+} stores[23] known to bee needed for autophagy.[25]

The effective suppression of autophagy by okadaic acid offers a general strategy for the identification of autophagy-regulatory protein kinases: if a specific protein kinase inhibitor is able to antagonize okadaic acid, an involvement of the respective protein kinase would be indicated. The principle is illustrated in Figure 1B, where the flavonoid naringin is shown to abolish the autophagy-suppressive effect of okadaic acid. This would suggest the existence of a PP2A-antagonistic, naringin-sensitive protein kinase, capable of inhibiting hepatocytic autophagy.[26] Naringin is also able to protect hepatocytes against okadaic acid-induced cell death,[27] indicating the putative naringin-sensitive kinase to be part of a regulatory signaling network shared by autophagy and apoptosis.

Protein Kinases

AMP-Activated Protein Kinase (AMPK)

Hepatocytic autophagy is feedback-inhibited by adenosine, a product of autophagic RNA degradation.[28,29] This effect of adenosine is potentiated by deoxycoformycin, an adenosine deaminase inhibitor, indicating that the efficacy of adenosine is curtailed by its intracellular metabolism to inosine.[13] Non-metabolizable adenosine analogues are, accordingly, more potent autophagy suppressants than adenosine itself (Fig. 2). Because the effect of adenosine is completely abolished by iodotubercidin, an adenosine kinase inhibitor that prevents the phosphorylation of adenosine to AMP, autophagy suppression would seem to be mediated by AMP.[14] The adenosine analogue, AICAR, which is phosphorylated intracellularly to the AMP ana-

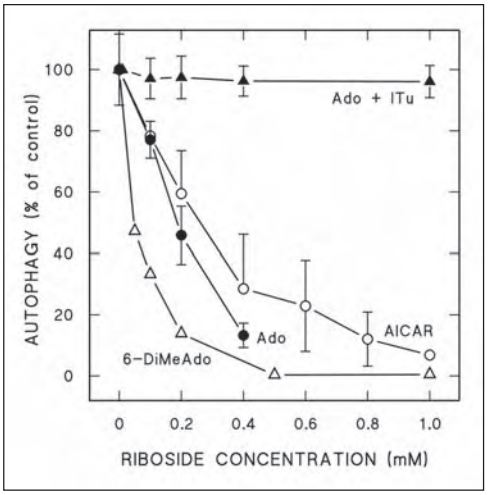

Figure 2. Inhibition of autophagy by adenosine and adenosine analogues. Rat hepatocytes were incubated for two hours at 37°C with the indicated concentration of 5-aminoimidazole-4-carboxamide riboside (AICAR, open circles), N^6,N^6-dimethyladenosine (6-DiMeAdo, open triangles), adenosine (closed circles), or adenosine *plus* 10 mM 5-iodotubercidin (ITu, closed triangles). 2'-deoxycoformycin (50 mM) was administered along with adenosine to prevent deamination of the latter. Autophagic activity was measured and expressed as percent of the activitiy in the absence of inhibitors. Modified from refs. 13 and 14.

logue ZMP, a potent and specific stimulant of AMPK activation, similarly inhibits autophagy strongly (Fig. 2). These results suggest the possibility that AMPK may function as an autophagy-suppressive protein kinase.[13]

AMPK plays an important role as a sensor of metabolic stress: when intracellular ATP levels fall low and AMP levels rise, AMP allosterically sensitizes AMPK to an activating phosphorylation by an upstream AMPK kinase, AMPKK (an effect mimicked by AICAR/ZMP).[30] The activated AMPK helps to conserve ATP by shutting down energy-requiring processes involved in lipid synthesis and other anabolic pathways.[31]

By immunoblotting with a phosphospecific antibody against the AMPK site (T172) phosphorylated by AMPKK, it could be shown that both AICAR and okadaic acid induced a naringin-sensitive AMPK phosphorylation (Fig. 3A,B). Because there is no kinase upstream of AMPKK,[31] the latter would seem likely to be the naringin-sensitive protein kinase.[32] This notion is supported by the ability of naringin to block the autophagy-suppressive effect of AICAR (unpublished results).

How okadaic acid causes AMPK activation is not clear. AMPK dephosphorylation at T172 is thought to be performed by a type 2C rather than by a type 2A phosphatase, and is not affected by okadaic acid in intact hepatocytes.[33] However, because AMPK apparently is phosphorylated at several sites,[34] it is possible that okadaic acid-induced overphosphorylation at one or more of these sites may have a permissive effect on T172 phosphorylation.

Autophagy in hepatocytes is suppressed under conditions of energy deprivation, suggesting an ATP requirement.[35,36] However, AMPK activation would also be expected under these conditions, making the interpretation of the energy depletion experiments somewhat ambiguous. In a recent study, homocysteine was found to induce hepatocellular ATP depletion and inhibition of autophagy in the absence of AMPK activation (phosphorylation) (Møller et al., manuscript submitted). These results support the contention that autophagic activity may be regulated by ATP independently of AMP and AMPK.

Whereas AMPK is a negative regulator of autophagy in mammalian cells, its yeast homologue, Snf1, promotes autophagy.[5] These opposite effects may reflect different survival strategies in response to metabolic stress: yeast cells apparently attempt to utilize all nutrient-providing options, including autophagy; in contrast, mammalian cells seek to conserve energy by turning off autophagy and other processes with a short-term ATP requirement, in addition to enhancing their glucose and fatty acid utilization.[31]

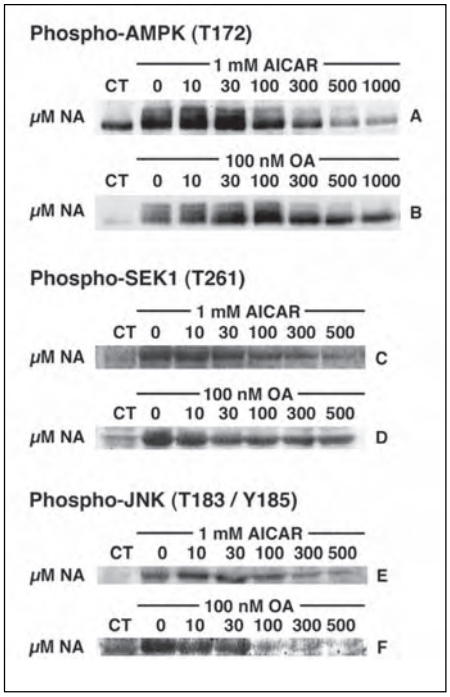

Figure 3. Naringin-sensitive activation of protein kinases by AICAR and okadaic acid. Rat hepatocytes were incubated for one hour at 37°C with 1 mM AICAR (A, C, E) or 100 nM okadaic acid (OA; B, D, F), and naringin (NA) at the concentration indicated. CT, control (no additions). The cells were lysed, and the lysates immunoblotted with phosphospecific antibodies against activated AMPK (A-B), activated SEK1 (C-D) or activated JNK (E-F). Modified from ref. 31.

Stress-Activated Protein Kinases (SAPKs)

AMPK is activated in response to a variety of stress stimuli other than metabolic stress, including heat shock, hypoxia, oxidative stress and exposure to toxicants like arsenite and cadmium, all of which cause a fall in ATP and an increase in AMP levels.[37,38] If AMPK functions as a general stress sensor, it is perhaps not surprising that it has been reported to signal through a downstream stress-activated protein kinase (SAPK) like p38.[39] In the perfused liver, p38 was implicated as a mediator of autophagy suppression caused by hypotonic stress, based on the ability of the p38 inhibitor, SB-203580, to prevent the suppression.[18]

In isolated rat hepatocytes, the SAPK kinase SEK1/MKK4 was found to be activated (as detected with a phosphospecific antibody) after treatment with AICAR or after okadaic acid treatment,[32] in a naringin-sensitive manner (Fig. 3C,D). The downstream SAP kinase JNK was similarly subject to naringin-sensitive phosphorylation after treatment with AICAR or okadaic acid (Fig. 3E,F), as were the JNK substrates, c-Jun and ATF-2 (tested after okadaic acid treatment only). The naringin-sensitive AMPKK would, therefore, seem to activate a downstream SEK1-JNK pathway. Although some p38 phosphorylation could be detected after okadaic acid treatment, this effect was naringin-insensitive and thus unlikely to be a direct consequence of AMPKK activation.

Most of the JNK inhibitors tested on hepatocytes have either been unable to inhibit JNK, or have shown autophagy-suppressive effects on their own. However, SB-203580, used as a specific p38 inhibitor at low concentrations, is able to block the SEK1-JNK pathway at higher concentrations,[40] and to antagonize the autophagy-suppressive effect of AICAR (unpublished results). Stress-activated protein kinases would thus seem likely to play a part in mediating autophagy-suppressive toxin effects, probably by acting as downstream elements in the AMPKK/AMPK-initiated signaling pathway.

We have previously shown that okadaic acid-induced apoptosis in normal hepatocytes is naringin-sensitive and thus likely to involve the AMPKK/AMPK pathway.[27] Recently Meisse

et al.[41] suggested the involvement of JNK in AICAR-induced hepatoma cell apoptosis, thus supporting the existence of a hepatocellular signaling pathway comprising both AMPK and stress-activated protein kinases, apparently involved in the regulation of apoptosis as well as of autophagy.

Mitogen-Activated Protein Kinases (MAPKs)

The MAP kinases (Erk1 and Erk2) are closely related to the SAP kinases; some terminologies in fact include the SAPKs among the MAPKs. The MAPK signaling pathway can be effectively disrupted by PD-98059, a specific inhibitor of the upstream MAP kinase kinases MEK1 and MEK2. In the HT-29 intestinal cell line, PD-98059 was found to suppress amino acid starvation-induced autophagy and the MAPK-dependent phosphorylation and activation of GAIP, a protein that promotes the dissociation of GTP from, and hence inhibits the autophagy-suppressive effect of, the $G_{\alpha i3}$ protein (see chapter 3).[42] Amino acids, the major physiological autophagy suppressants, may possibly exert their effect by inducing a reduction in MAPK activity and MAPK-dependent GAIP phosphorylation in HT-29 cells.[42] However, in rat hepatocytes, PD-98059 has no autophagy-suppressive effect alone, nor is it able to antagonize the effects of okadaic acid or amino acids (our unpublished results). PD-98059 is also unable to prevent hypotonic stress-induced autophagy suppression in the perfused liver.[18] The possibility must, therefore, be considered that MAPKs regulate intestinal autophagy in a tissue-specific manner, being functionally replaced by other enzymes in tissues such as the liver.

Protein Kinase B (PKB/Akt)

Autophagy in HT-29 intestinal cells is controlled not only by amino acid-induced MAPK inhibition, but also by the phosphatidylinositol (PtdIns) 3-kinase/protein kinase B (PKB) pathway, independently of amino acids.[43] Activators of class I PtdIns 3-kinases, such as interleukin-13, can thus suppress autophagy in these cells, whereas hyperactivity of the PtdIns 3-kinase-antagonistic phosphoinositide phosphatase, PTEN, stimulates autophagy (and PTEN-inactivating mutations are autophagy-suppressive). PtdIns phosphates generated by class I PtdIns 3-kinases, e.g., PtdIns(3,4,5)P_3, inhibit autophagy by inducing PKB activation (inactivating and activating PKB mutations causing stimulation and suppression of autophagic activity, respectively).[44]

The autophagy-suppressive PtdIns 3-kinase-PKB signal is part of a PKB-mediated cell survival response that also includes inhibition of apoptosis.[44] By shutting down autophagy, cells preserve their mass and improve their survival.[45] Cancer cells often have reduced autophagic activity and thus better survival abilities,[45,46] which could well be due to inactivating PTEN mutations, frequently seen in cancer.[47]

It should be noted that PtdIns(3)P, generated by class III PtdIns 3-kinases, activates autophagy (see chapter 3),[48] resulting in an overall autophagy-suppressive effect of PtdIns 3-kinase inhibitors such as 3-methyladenine, wortmannin and LY-294002.[12,49,50] The mechanism of this PtdIns(3)P-mediated autophagy control is not known, but its affiliation with the lipid kinase module in yeast[1,2] may suggest a type of control not directly connected to, but probably preceding, autophagy-regulatory protein phosphorylation.[51]

cAMP-Dependent Protein Kinase (PKA)

Liver autophagy in vivo is subject to regulation by the pancreatic hormones, insulin and glucagon, which suppress and stimulate autophagy, respectively, probably through modulation of amino acid transport or metabolism.[52-54] In isolated hepatocytes, insulin and glucagon similarly potentiate and antagonize, respectively, the autophagy-suppressive effect of amino acids, while having little effect on their own.[55-57]

Hepatocytic autophagy is also suppressed by adrenergic hormones, acting through α1 receptors.[57] The α1-adrenergic pathway promotes an IP_3-mediated release of Ca^{2+} from intracellular stores; such loss of sequestered Ca^{2+} has been shown to impede autophagic activity.[25]

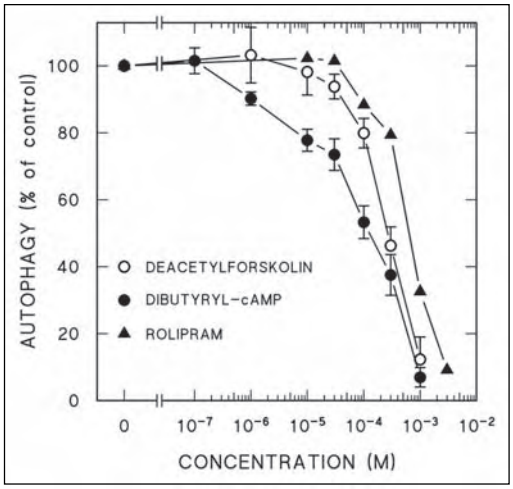

Figure 4. Suppression of autophagy by cAMP-elevating agents and the cAMP analogue, dibutyryl-cAMP. Rat hepatocytes were incubated for three hours at 37°C with dibutyryl-cAMP (closed circles), the adenylate cyclase activator, deacetylforskolin (open circles) or the cAMP-phosphodiesterase inhibitor, rolipram (closed triangles) at the concentration indicated, and autophagic activity during the incubation period was measured. Modified from ref. 16, with additions.

Since no β-adrenergic (i.e., cAMP/PKA-mediated) regulation of autophagy can be detected, and since glucagon (signaling mainly through cAMP/PKA) alone has little effect on autophagy,[16,57] one might not expect PKA to regulate hepatocytic autophagy except through modulation of amino acid suppression. However, an adenylate cyclase activator (deacetylforskolin), dibutyryl-cAMP (DB-cAMP) and several other cAMP analogues, as well as a variety of cAMP phoshodiesterase (PDE) inhibitors (which cause sustained increases in intracellular cAMP levels), strongly suppress autophagy in isolated hepatocytes.[15,16] The effective autophagy suppression by rolipram, a specific PDE4 inhibitor (Fig. 4), may suggest that PDE4 is engaged in localized subcellular PKA regulation related to autophagy.[58] Altogether, these data make a good case for an involvement of PKA as a negative autophagy regulator. Interestingly, DB-cAMP-induced autophagy suppression can be partially (35-50%) antagonized by naringin,[16] perhaps indicating an interaction of PKA with the AMPKK/AMPK pathway.

Most autophagy-suppressive PDE inhibitors raise the hepatocellular levels of cGMP as well as of cAMP, and DB-cGMP itself can inhibit autophagy.[57] However, inhibitors of the more cAMP-specific PDEs tend to have the largest effects on autophagy.[15] An involvement of PKG in autophagy control is thus not well supported; it is more likely that DB-cGMP may affect local cAMP levels, e.g., through PDE3 inhibition.[59]

p70S6 Kinase

Amino acids are the major physiological regulators of autophagy,[60,61] but their mechanism of action has proven hard to unravel. The evidence that amino acids may suppress an autophagy-activating function of MAPK in intestinal cells,[42] but not in hepatocytes, has been discussed above. In hepatocytes, amino acids have been suggested to signal through the rapamycin-sensitive protein kinase mTOR (mammalian target of rapamycin), p70S6 kinase and phosphorylation of the ribosomal protein S6.[19] We have confirmed, using phosphospecific antibodies, that an autophagy-suppressive amino acid mixture[62] elicits rapamycin-sensitive S6 phosphorylation in hepatocytes.[33] In addition, amino acids induce rapamycin-sensitive (but naringin-resistant) phosphorylations of p70S6 kinase at both the activating site (T389) and in the tail region (T421/S424). Amino acids have no effect on the phosphorylation of AMPK, SEK1 or JNK (our unpublished results). The p70S6 kinase tail (but not the T389 site) is also phosphorylated, in a naringin-sensitive but rapamycin-resistant manner, after treatment with okadaic acid or AICAR.[33] Whether SEK1/JNK is involved in the latter p70S6 kinase tail phosphorylation remains to be elucidated. The p70S6 kinase tail might thus qualify as a possible common effector for several autophagy inhibitors, being phosphorylated both through an

AMPKK/AMPK pathway (AICAR, okadaic acid) and through an mTOR pathway (amino acids). An autophagy-suppressive function of the phosphorylated p70S6 kinase tail would have to be independent of p70S6 kinase catalytic activity, because AICAR abolishes both the activating phosphorylation at T389 and the consequent phosphorylation of S6,[33,63,64] apparently by an AMPK-independent (naringin-resistant) mechanism (our unpublished results).

Ca^{2+}/Calmodulin-Dependent Protein Kinase

The autophagy-suppressive effect of okadaic acid is effectively antagonized by KN-62, an allegedly specific inhibitor of CaMK-II.[10] Other protein kinase inhibitors, like KT-5720, KT-5823 and KT-5926, are similarly effective okadaic acid antagonists[11] as well as potent CaMK-II inhibitors (our unpublished results). These results would seem to be compatible with a role for CaMK-II in autophagy control. However, it may be presumptuous to implicate CaMK-II on the basis of inhibitor data alone: the CaMK-II inhibitors KT-5720, KT-5823 and KT-5926 have, after all, been marketed as inhibitors of PKA, PKG and myosin light chain kinase, respectively, but they are clearly not very specific. The possibility thus cannot be excluded that the CaMK-II inhibitors, including KN-62, may affect other relevant kinases; e.g., AMPKK.

Cyclin-Dependent Protein Kinase (CDK)

In yeast, the cyclin-dependent protein kinase Pho85 inhibits autophagy, antagonizing the autophagy-stimulatory effect of Snf1/AMPK.[5] In mammalian cells, a CDK regulation of autophagy would apparently have to be in the opposite direction (as is the case with AMPK), since the CDK inhibitor, olomoucine, has been shown to suppress autophagy in rat hepatocytes.[12] However, as discussed above, the specificity of an enzyme inhibitor is rarely (if ever) absolute; additional studies are, therefore, required to assess a possible CDK involvement in mammalian autophagy.

eIF2α Kinases

Genetic and morphological studies have shown that eIF2α phosphorylation by Gcn2, an eIF2α kinase, is required for starvation-induced autophagy in yeast.[6] Phosphorylation of eIF2α is necessary for effective translation of mRNAs encoding Gcn4,[65] a transcriptional activator of several autophagy genes.[66] In mammalian cells (fibroblasts), eIF2α phosphorylation (at Ser51) has similarly been shown to be required for amino acid starvation- or virus-induced autophagy.[6] It is likely that eIF2α and eIF2α kinases serve a long-term function in both mammalian and yeast cells, helping to maintain the synthesis of protein components involved in the autophagic machinery. In the short-term regulation of hepatocytic autophagy, eIF2α phosphorylation is associated with suppression of autophagy (e.g., after okadaic acid treatment) rather than with stimulation of autophagy (our unpublished results).

Protein Tyrosine Kinases (PTKs)

Several tyrphostins, inhibitors of protein tyrosine kinases, are able to suppress hepatocytic autophagy,[67] perhaps indicating an autophagy-stimulatory role for some member(s) of this protein kinase family. However, as in the case of other protein kinase candidates nominated purely on the basis of inhibitor studies, additional research is required to support this possibility.

Conclusions

The strong and consistent autophagy-suppressive effects of protein phosphatase inhibitors clearly demonstrate the importance of protein phosphorylation in the regulation of mammalian autophagy.[11] However, our understanding of the protein kinase networks involved in this regulation is still somewhat sketchy. In long-term autophagy control, the case for involvement of eIF2α and eIF2α kinase at the level of gene/mRNA expression is rather strong.[6] In the short-term regulation of autophagy, different autophagy-suppressive or -stimulatory conditions may utilize different kinase signaling pathways, such as the MAPK and SAPK cascades,[18,42] perhaps in a cell/tissue-specific manner.

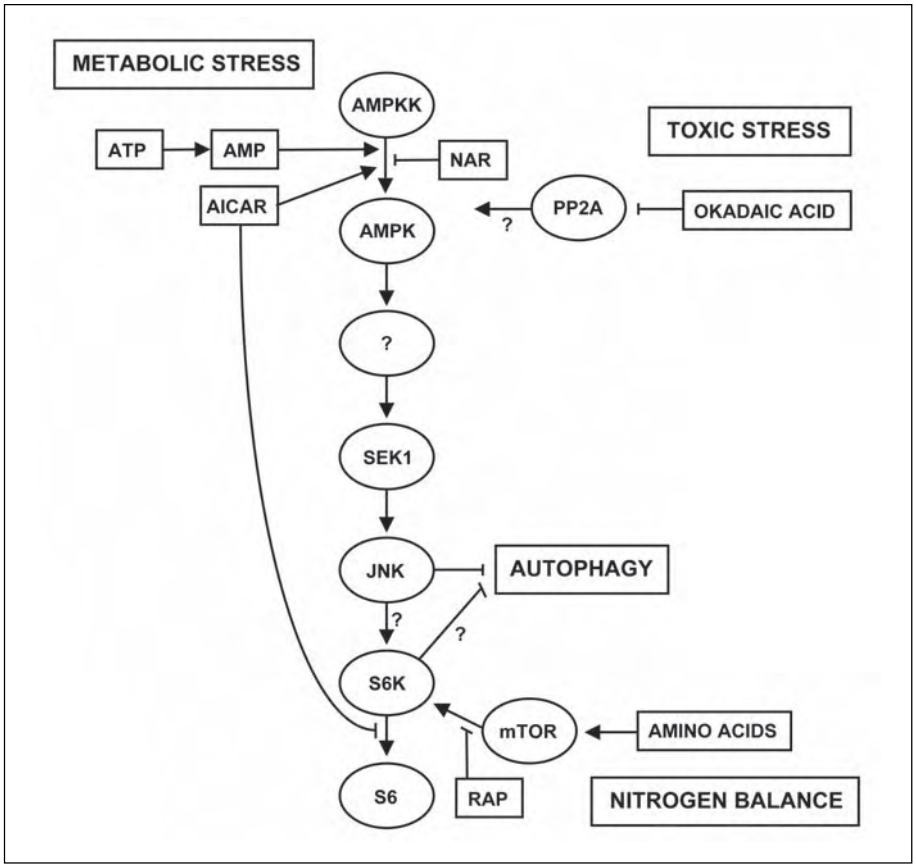

Figure 5. A putative autophagy-suppressive phosphorylation pathway in rat hepatocytes. AMPK, AMP-activated protein kinase; AMPKK, AMPK kinase; JNK, c-Jun NH_2-terminal kinase; mTOR, mammalian target of rapamycin (a rapamycin-sensitive protein kinase); NAR, naringin; OA, okadaic acid; PP2A, protein phosphatase 2A; RAP, rapamycin; S6, ribosomal protein S6; S6K, S6 kinase; SEK1, stress-activated protein kinase/extracellular signal-regulated kinase kinase 1. It should be emphasized that the pathway must be regarded as a working hypothesis, in need of further experimental verification and elaboration at every step.

In hepatocytes, the common ability of several strong autophagy suppressants (AICAR; protein phosphatase inhibitors) to promote an activating phosphorylation of AMPK by AMPKK, characteristically antagonized by naringin, would strongly implicate AMPK/AMPKK in the regulation of autophagy.[13,14] The parallel activation of a SAPK relay (SEK1-JNK) is compatible with a position of the latter downstream of AMPKK/AMPK (Fig. 5), a contention supported by the autophagy-protective effect of a JNK inhibitor. Whether the p70S6 kinase tail phosphorylation is part of this autophagy-suppressive signaling pathway, or represents a separate AMPKK/AMPK-dependent signal, remains to be shown.

The autophagy-suppressive effect of cAMP/PKA may at least be partially mediated by AMPKK/AMPK, as indicated by the partial naringin antagonism of the DB-cAMP effect.[16] For CaMK-II, CDKs and PTKs, studies beyond inhibitor experiments are needed to substantiate a participation of these enzymes in the regulation of autophagy.

Amino acids, the major physiological regulators of autophagy, apparently signal through mTOR rather than through AMPKK/AMPK in hepatocytes, but the two pathways may, con-

ceivably, converge upon the p70S6 kinase tail as a common autophagy-suppressive element. Although a MAPK cascade may be involved in amino acid signaling in intestinal cells,[42] this is not the case in hepatocytes. The role of protein phosphorylation in the control of autophagy by amino acids thus remains obscure.

How autophagy-regulatory signals, such as the AMPKK/AMPK pathway, eventually interact with the autophagic sequestration machinery is not known. The delimiting membranes of mammalian phagophores and autophagosomes appear to be extremely protein-poor;[68] very few proteins (and so far, no phosphoproteins) having been found associated with them (see chapter 2).[69-71] Signaling pathways might well affect autophagy indirectly: for example, the naringin-sensitive AMPKK/AMPK pathway causes phosphorylation of cytoskeletal proteins like keratin and plectin.[27,33] The autophagy suppression mediated by this pathway could, therefore, conceivably reflect the need for an intact intermediate filament cytoskeleton in autophagic sequestration.[72] Clearly, a lot of additional experimental work is needed to chart autophagy-regulatory phosphorylation pathways and to find out how they eventually control the activation and suppression of autophagy.

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CHAPTER 5

Regulation of Autophagy by the Target of Rapamycin (Tor) Proteins

Hagai Abeliovich

Abstract

Administration of the small macrolide antibiotic rapamycin to eukaryotic cells results in physiological responses that mimic nutrient starvation, and in many ways resembles nitrogen starvation. The target for rapamycin action in these cells is a family of conserved kinases known as TOR (target of rapamycin). Tor kinases are essential for the normal function of eukaryotic cells and participate in the integration and control of nutrient-sensing signals. Inhibition of Tor by rapamycin triggers a variety of molecular responses. These include global changes in gene expression, which are mediated by effects on both transcription and translation, as well as direct effects on other cellular processes. Within this context, inhibition of TOR has a number of effects on membrane trafficking, including the induction of autophagy. This chapter represents a compendium of our current state of knowledge on the role of TOR family proteins, their architecture and molecular interactions, as well as the mechanisms by which inhibition of TOR leads to various cellular responses. This general perspective is aimed at understanding the relative role of the induction of autophagy within the broader response of cells to rapamycin and starvation.

The Target of Rapamycin (TOR) Proteins: Integrators of Cellular Nutrient Responses in Eukaryotes

Rapamycin is a macrolide antibiotic isolated from certain strains of *Streptomyces hygroscopicus*. In eukaryotic cells, administration of rapamycin generally invokes starvation responses, cell cycle arrest, and inhibition of cell growth.[1-4] The intracellular receptor for rapamycin in all eukaryotes is a small, ubiquitous protein termed FKBP (FK506 binding protein). The rapamycin-FKBP12 complex then specifically interacts with the evolutionarily conserved TOR proteins, to potently inhibit signaling to downstream targets.[5] Two *Saccharomyces cerevisiae* TOR genes, *TOR1* and *TOR2*, code for a pair of large molecules of 2470 and 2473 amino acid residues, while metazoans such as *Drosophila*, human and mice seem to possess only one Tor gene.[6,7] Mammalian Tor is variously known as mTor, FRAP, RAFT, SEP, or RAPT. In this chapter, we will adopt the simplifying convention of using the term mTOR for the mammalian protein.

Tor proteins belong to a family of proteins called phosphatidylinositol (PtdIns) kinase related kinases, or PIKKs.[8] This group of proteins also includes the ataxia-telangiectasia mutated (ATM) protein, ATR/FRP (ataxia-telangiectasia – and rad3- related/FRAP related protein), and DNA-dependent protein kinase (DNA-PK), as well as Rad53, Mec1 and Tel1 from yeast.[9] Although members of this protein family possess homology to phosphatidylinositol kinase catalytic domains, they do not exhibit lipid kinase activity themselves. Rather, most of these proteins have been demonstrated to carry out in vitro protein phosphorylation at serine or

Autophagy, edited by Daniel J. Klionsky.

threonine residues.[10,11] While the rapamycin-FKBP12 complex specifically affects the function of Tor proteins, this effect is not synonymous with total inactivation but rather appears to affect distinct functional aspects of the protein, and may reflect a change in substrate specificity.[12]

General Architecture of PIKK Family Proteins and TOR Structure/Function Relationships

By definition, all PIKK proteins contain a PtdIns kinase catalytic domain. In all these proteins, the PtdIns kinase homology resides very near the C terminus of the protein and this domain takes up only ~300 amino acids within the 2000-4000 amino acid sequences of these large proteins. In addition to this defining feature, the PIKK family proteins contain an additional conserved module at the C terminus itself, adjacent to the PtdIns kinase domain, called the FATC domain, and a third conserved region near the center of the molecule, termed the FAT domain.[13] While the PtdIns kinase domain has been shown to be required for kinase activity, the role of the other two conserved domains remains unclear. Mutations that confer rapamycin resistance in yeast Tor map outside the kinase domain, suggesting that the effect of the FKBP12-rapamycin complex is not a simple inhibition of catalytic activity. Indeed, this idea is supported by the finding that rapamycin selectively interferes only with a subset of the functions of Tor in yeast.[14] The sensitivity to rapamycin appears to depend on the phosphorylation state of yeast Tor at Ser1972 in Tor1 or Ser1975 in Tor2 (located between the FAT domain and the PtdIns kinase domain), and substitution of this residue with bulky side chains also results in rapamycin resistance. It was therefore suggested that this region, called the FRB (FKBP12-rapamycin binding) domain, binds the FKBP-rapamycin complex, and this was corroborated by crystallographic analysis of the FKBP12-rapamycin-mTOR FRB complex.[15] A fourth structural signature that occurs in the N-terminal part of TOR proteins is the presence of at least 16 repetitions of a small domain called HEAT, named after the proteins in which it is found: Huntingtin, elongation factor 3, the A subunit of type 2A protein phosphatase (PP2A) and TOR. The repeats are 36-47 amino acids long and are characterized by a series of spaced hydrophobic amino acid residues, potentially folding into an antiparallel α-helical motif.[16] These characteristics of HEAT domains were suggested by some authors to play a role in protein-protein interactions. Tor proteins are peripheral membrane proteins and Kunz et al found that mutations in the HEAT domains impair membrane association of Tor in yeast, implying that these modules are required for an interaction with specific factors that mediate Tor localization.[8]

Protein-Protein Interactions of TOR Proteins

In both mammalian cells and in yeast, Tor has been shown to function in complex with other proteins. mTor forms a detergent-sensitive complex with a protein called raptor in mammalian cells. Small-interfering RNA (siRNA) technology knockdowns of raptor expression revealed phenotypes similar to inhibition or downregulation of mTOR. It was also shown that raptor binds directly to known mTOR substrates, 4E-BP and S6K1, leading to a model in which raptor is an adaptor molecule that tethers mTOR to its substrate(s).[17,18]

Affinity purification and mass-spectrometric analysis of Tor binding proteins in yeast revealed the existence of two Tor-containing protein complexes, TORC1 and TORC2 (see chapters 6 and 7).[19] TORC1 contains either Tor1 or Tor2, together with proteins called Kog1 (the yeast raptor homolog) and Lst8. The TORC1 complex is capable of binding the FKBP12-rapamycin complex and is thought to carry the rapamycin-sensitive nutrient signaling cascade. TORC2 contains Tor2 exclusively and has three additional unique subunits, Avo1, 2, and 3. Intriguingly, TORC2 is incapable of binding the FKBP12-rapamycin complex, mirroring the fact that Tor2 has rapamycin-insensitive functions that relate to the actin cytoskeleton in yeast.[20] Indeed, mutations in TORC2 members result in cytoskeletal defects.

Signaling Pathways Involving Tor in Yeast and Mammalian Systems

Tor signaling is thought to be effected through a combination of direct phosphorylation of downstream targets and repression of phosphatase activity. Protein phosphatases play a major role in Tor signaling activity. Many processes that depend on Tor inactivation are inhibited by the phosphorylated form of a protein called Tap42, which is a substrate for Tor and a regulatory subunit of a number of PP2A-like catalytic subunits.[21-24] In the absence of Tor activity, Tap42 is dephosphorylated and uncouples from the catalytic PP2A subunits which then associate with different regulatory subunits, leading to changes in activity that mediate downstream events. There are at least four structural genes for PP2A-related catalytic subunits in yeast: *PPH21*, *PPH22*, *PPH3*, and *SIT4*. Each of these is associated with a set of regulatory subunits, which have the potential to displace Tap42 from the catalytic subunit, allowing a wide spectrum of combinatorial possibilities for physiological regulation (Fig. 1). Tap42 has a mammalian ortholog, α4, which is also phosphorylated in an mTOR-dependent fashion and shows a TOR-dependent interaction with PP2A catalytic subunits.

In mammalian cells, Tor is thought to be part of a complex signaling network that includes the type I PtdIns 3-kinase, the tyrosine phosphatase PTEN, protein kinase B (PKB), and Akt, all of which have been suggested to act upstream of Tor (Fig. 2).[25] While these factors may indeed regulate mTOR or Tor in a linear signaling pathway, other data indicates that they act in parallel with mTOR in a joint regulatory network that impinges on common downstream substrates.[4,26,27]

Regulation of Transcription and Translation by Tor Proteins in Yeast and Mammalian Cells

Nitrogen limitation and inhibition of Tor result in activation of Gln3 and Gat1, two GATA transcription factors. This activation is achieved by releasing these proteins from a cytosolically sequestered state and subsequent transport into the nucleus where they activate transcription of numerous genes that contain GATA sequences within their promoter regions. [21,28-30] This change in transcription profile is required for the proper physiology of yeast under poor nitrogen conditions. Cytosolic sequestration of Gln3 and Gat1 is achieved, at least in part, through an interaction with Ure2, a preprion protein that binds Gln3, and possibly Gat1, in the presence of a good nitrogen source but releases them in response to starvation cues. The signal that controls the release of Gln3 from Ure2-dependent sequestration correlates with changes in the phosphorylation state of both proteins and depends on Tap42 and phosphatase activity.[21,28,29]

In both yeast and mammalian cells, inhibition of Tor has global effects on the specificity and amount of translational initiation of mRNA. In yeast, this results in a general decrease of translation activity, while specific proteins are upregulated. The mechanism for this overall decrease in translation activity is poorly understood but may be due to degradation of initiation factor eIF4G.[31] A potential regulator of yeast eIF4E called Eap1 has also been implicated in this process, as *EAP1* gene disruption leads to partial rapamycin resistance.[32] In addition, it was found that treatment of yeast with rapamycin results in inhibition of ribosomal RNA synthesis and rapid degradation of ribosomal RNA.[33]

In mammalian cells, rapamycin treatment inhibits the translation of specific classes of mRNAs. This is thought to be mediated by Tor-dependent changes in the phosphorylation state of two proteins: ribosomal protein S6, and eukaryotic translation initiation factor 4E binding protein (eIF4E-BP).[34] The phosphorylation of ribosomal protein S6, catalyzed by the S6 kinases S6K1 and S6K2 (referred to as p70S6 kinases), is required for the translation of a group of mRNAs possessing a 5' terminal oligopyrimidine tract (5'TOP).[35] This is a stretch of 4-14 pyrimidines found at the extreme 5' end of mRNAs that code for ribosomal proteins and other components of the translational machinery. When nutrient levels are low, translation of 5'TOP mRNAs is repressed, and this is mimicked by rapamycin treatment. Inhibition of Tor, as well as starvation, leads to the dephosphorylation of S6 by downregulating the activity of the

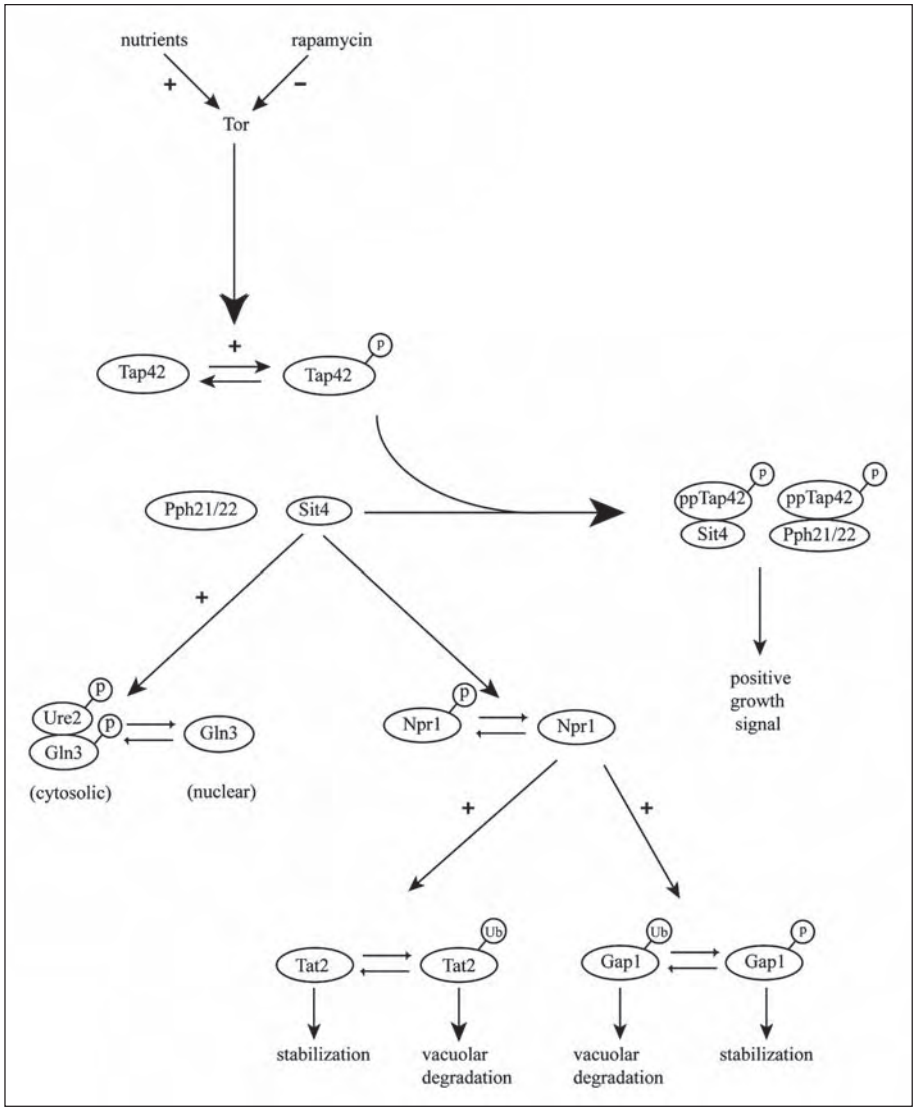

Figure 1. A partial model for Tap42-mediated regulation of Gln3-dependent transcription and amino acid permease trafficking by the TOR pathway in yeast. Tor phosphorylates Tap42, and phospho-Tap42 associates with PP2A-like catalytic subunits, such as Pph21, Pph22 and Sit4. The accumulated complexes then exert a positive growth signal.[22] In the absence of Tor activity, as in the presence of rapamycin, unphosphorylated Tap42 dissociates from the catalytic subunits and allows their association with other regulatory subunits. In this form, the phosphatases affect the phosphorylation state of a complex composed of Ure2 and Gln3. This change in phosphorylation causes the dissociation of the complex and allows nuclear import of the GATA transcription factor, Gln3, which effects nitrogen limitation-specific gene transcription. In parallel, Sit4 mediates the dephosphorylation of Npr1, activating its kinase activity. Npr1-dependent phosphorylation of the general amino acid permease Gap1 protects it from ubiquitination by the ubiquitin ligase Npi1. Conversly, Npr1 activity promotes the Npi1-dependent ubiquitination of the tryptophan permease Tat2. Ubiquitination of either permease dictates its delivery into the vacuolar lumen and leads to degradation.

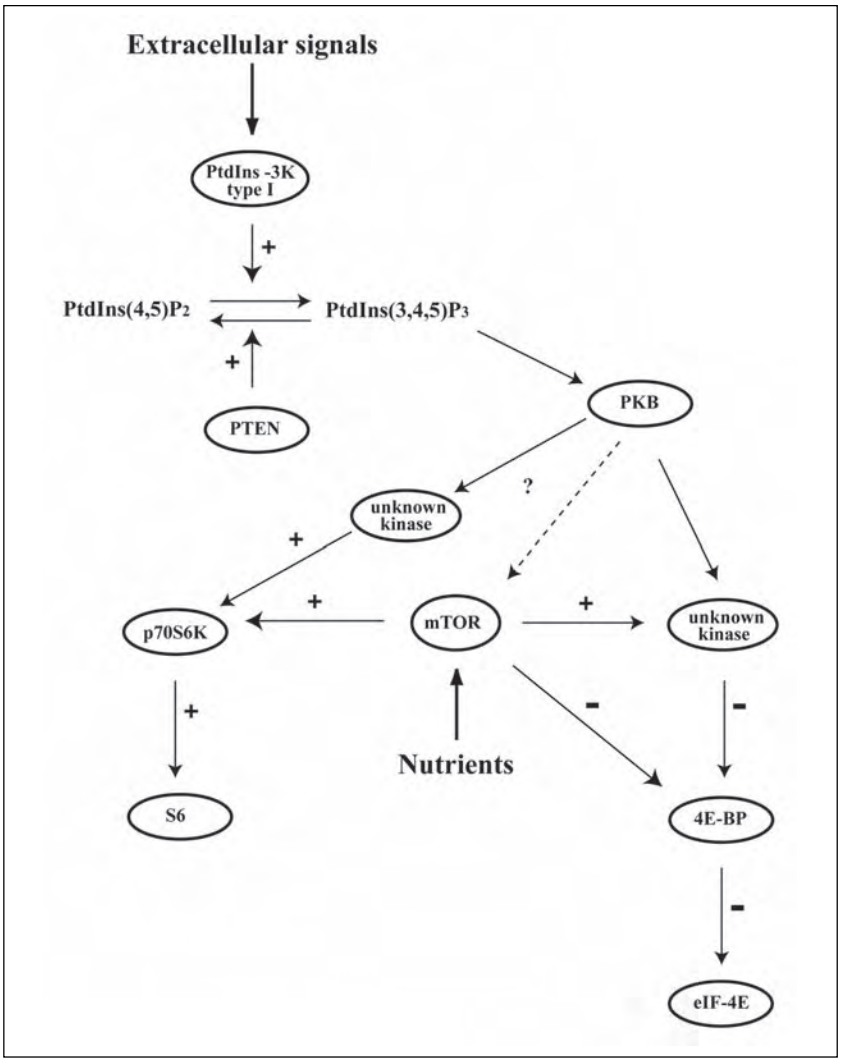

Figure 2. Model for convergent signaling of PtdIns(3,4,5)P_3 and mTOR-dependent nutrient sensing in the regulation of translation initiation in mammalian cells. mTOR phosphorylates p70S6 kinase and eIF4E-BP, thus affecting the efficiency of translation initiation. This regulation converges with signals that depend on the activities of the type I PtdIns 3-kinase, which synthesizes PtdIns(3,4,5)P_3, and PTEN, a dual specificity protein/lipid phosphates that catalyzes the reverse reaction. The levels of PtdIns(3,4,5)P_3 dictate the localization and activity of Akt/PKB, a protein kinase that contains a pleckstrin homology domain. PKB in turn regulates the phosphorylation state of p70S6 kinase and 4E-BP in ways that can be experimentally distinguished from Tor signaling (see text). The activities of 4E-BP and p70S6 kinase then regulate translation initiation via effects on eIF-4E and ribosomal protein S6, respectively.

p70S6 kinases, which are phosphoproteins whose activity and phosphorylation state depend on Tor, and are known in vitro substrates for Tor phosphorylation (see chapters 3 and 4).[36] The p70S6 kinase activity is inhibited by rapamycin, but the same effect can be achieved with PtdIns 3-kinase inhibitors. It was recently shown that these effects on p70S6 kinase activity can

be experimentally distinguished: N-terminal truncation of S6K1 confers rapamycin resistance while preserving sensitivity to PtdIns 3-kinase inhibition, leading to the conclusion that PtdIns 3-kinase and mTOR function in distinct but convergent pathways that regulate p70S6 kinase.[26,27]

In addition to these effects of Tor on p70S6 kinase activity, Tor is known to phosphorylate 4E-BP, a regulator of eIF4E activity. Most eukaryotic mRNAs contain a specialized chemical structure at the 5'-terminus called the "cap" (a modified guanine residue with the structure m7GpppN in which m is a methyl group and N is any nucleotide). The cap structure is recognized by the eukaryotic initiation factor 4E (eIF4E). eIF4E, together with large scaffolding proteins (either eIF4GI or eIF4GII) directs the ribosomal machinery to the translation initiation site on the mRNA. The interaction between eIF4E and eIF4G is antagonized by binding and sequestration of eIF4E by 4E-BP. 4E-BP is a phosphoprotein and binds to eIF4E in the hypophosphorylated form, and phsophorylation of 4E-BP is Tor-dependent and rapamycin sensitive.[34,37,38] Thus, TOR function is essential for efficient cap-dependent translation in eukaryotic cells.

Tor-Dependent Regulation of Nonautophagic Membrane Trafficking in Yeast

The quality of the nitrogen source determines the type and levels of amino acid permeases that are expressed at the cell surface of yeast cells.[39] Under poor nitrogen conditions (e.g., urea or proline, or starvation conditions), cells express a general amino acid permease with broad specificity and relatively low affinity, called Gap1. In the presence of a preferred nitrogen source such as glutamine or ammonium, Gap1 is shunted into the vacuole (the yeast equivalent of the lysosome) and degraded there. Conversely, in the presence of a good nitrogen source yeast up-regulate high-affinity amino acid permeases that are specific for a single amino acid or for a set of structurally related amino acids, such as the histidine permease Hip1 and the tryptophan permease Tat2. In poor nitrogen conditions, these specific transporters are degraded, in effect behaving as a mirror image of the regulation of Gap1.[23,40] Degradation of both Gap1 and Tat2 is dependent on ubiquitination and vacuolar proteases, and is inversely regulated by a protein kinase called Npr1.[41,42] Npr1 is a protein kinase that mediates phosphorylation of Gap1 and thereby protects it from ubiquitination and degradation. At the same time, Npr1 activity promotes Tat2 degradation. The differential stabilization of these proteins reflects an inverse regulation of their trafficking patterns: When one is expressed at the cell surface, the other is ubiquitinated and routed to the vacuole for degradation. The vacuolar degradation of nitrogen permeases does not depend on autophagy, but relies on classical vesicular trafficking pathways from the ER through the Golgi complex and the endosomal system.

The regulation of permease trafficking patterns is mimicked by rapamycin-mediated inhibition of Tor. It was shown that Npr1 is phosphorylated, and that Tor and Tap42 regulate its level of phosphorylation. When Tor is inactivated, Npr1 is rapidly dephosphorylated and Tat2 is subsequently degraded. At the same time, Npr1-dependent Gap1 phosphorylation is increased, promoting stabilization of Gap1.[23,40]

Induction of Autophagy by Rapamycin in Mammalian and Yeast Cells

Autophagy in mammalian cells is potently inhibited by amino acids (see chapter 3).[43,44] Blommaart et al found that this inhibition correlates with the phosphorylation of a 31 kDa protein which they identified as ribosomal protein S6. They consequently showed that rapamycin, which is known to induce dephosphorylation of S6 through inactivation of p70S6 kinase (see above), can bypass the amino acid-mediated inhibition of autophagy, that is, administration of rapamycin results in induction of autophagy in the presence of a good nitrogen source.[45] Noda and Ohsumi then went on to demonstrate that this is true in yeast as well (see chapter 6).[46] In yeast, however, the preeminence of molecular genetic analysis coupled with several quantitative biochemical assays for autophagy opened the door to a much better under-

standing of the underlying molecular mechanisms.[47,48] Noda and Ohsumi went on to show that cell cycle arrest at G1(a hallmark of the response to rapamycin) was not required for the induction of autophagy, and that autophagy could also be induced without changes in the intracellular cyclic AMP levels. This last result is consistent with the fact that cAMP levels do not change in response to changes in nitrogen source availability.

Molecular Mechanism of Induction of Autophagy by Rapamycin in Yeast: Data and Hypotheses

Our understanding of the molecular mechanism by which inhibition of Tor causes induction of autophagy has relied predominantly on the characterization of the gene products that were identified as essential for autophagy by the laboratories of Yoshinori Ohsumi and Michael Thumm as well as the closely related cytoplasm to vacuole targeting pathway, which was discovered and characterized by Klionsky and coworkers (see chapters 6 and 7).[49-52]

One basic question regarding the induction of autophagy relates to the level at which different cellular control mechanisms impinge on its regulation. For example, it is widely known (see above) that inhibition of Tor results in global changes in the transciption and translation profile of cells. Since some of these changes affect genes that are directly involved in autophagy, such as up-regulation of *AUT7/APG8* transcription in response to starvation or rapamycin, it is conceivable that these changes in gene expression constitute the signal to induce autophagosomes.[53] To test this hypothesis, Abeliovich et al carried out pulse-chase experiments which tested the ability of cells to carry out autophagic trafficking in the absence of de novo protein synthesis. It was found that the induction of autophagy could be dissected into at least two components: An initiation step, which is the result of a direct signal transduction mechanism that does not depend on changes in gene expression, and a secondary expansion step that requires the upregulation of starvation genes such as *AUT7*. This secondary level of regulation determines the ultimate volume of cytosolic material that is recycled, allowing more flexibility in the cellular response.[48]

Kamada et al concentrated on understanding the role of an autophagy-dedicated protein kinase called Apg1. These workers found that Apg1 could be coprecipitated with a phosphoprotein called Apg13, and that this interaction was enhanced by treating the cells with rapamycin prior to preparation of extracts. In addition, these immunoprecipitates contained Apg1-dependent kinase activity towards an artificial in vitro substrate (myelin basic protein, MBP) and this activity increased in response to rapamycin. These changes also correlated with a rapid dephosphorylation of Apg13, which did not depend on de novo protein synthesis, implicating these events as participating in the direct signal transduction mechanism that regulates the initiation of autophagosome formation. Defects in Apg13 can be overcome by overexpressing Apg1, suggesting an upstream regulatory role for Apg13.[54] These findings led to the following model: Apg1 kinase activity is a parameter that directly regulates the induction of autophagy. Inhibition of Tor results in a signaling cascade that dephosphorylates Apg13, leading to an increase in its association with Apg1 and a subsequent upregulation of kinase activity that leads to induction of autophagy.

There are several problems with this model. First, prior studies by Matsuura et al showed that Apg1 has autophosphorylation activity, and that this activity is downregulated, not upregulated, in response to starvation.[55] Furthermore, since MBP is an artificial, exogenous substrate, an increase in its phosphorylation may not necessarily reflect an increase in Apg1 kinase activity towards a physiological substrate. It could, for example reflect a change in the quaternary structure of an Apg1 complex that renders the active site more accessible to noncognate substrates. Indeed, a more recent study by Abeliovich et al showed that such a conformational change does indeed occur in Apg1.[56]

Moreover, specific inhibition of Apg1 kinase activity using the chemical genetic approach of Shokat and colleagues resulted in complete inhibition of the Cvt pathway, but not in inhibition of autophagy, suggesting that the in vitro data with MBP, while possibly reflecting an actual in vivo process, are not a faithful measure of native Apg1 kinase activity and its role.[56]

What is the signal that causes changes in the molecular behavior of Apg1 and other autophagy proteins? The dual regulation of autophagy by transcription-dependent and transcription-independent mechanisms is reminiscent of the dual regulation of amino acid permeases by Tor and nitrogen sources. Nitrogen sources determine permease expression by controlling transcription through the nitrogen catabolite repression mechanism that involves Ure2 function, but also independently regulate the trafficking of permease molecules, directing them either to the plasma membrane or to the vacuole for degradation, depending on the physiological requirements. Nonetheless, Tap42, a central player in these Tor-dependent phenomena, does not play a role in the induction of autophagy.[54] Similarly, preliminary studies on the role of Npr1, a protein kinase that regulates permease trafficking, failed to show a role in regulation of autophagy (H. Abeliovich, unpublished data).

The lack of evidence for involvement of Tap42 and Npr1 implies that autophagy is induced by a novel Tor-dependent signaling pathway that impinges on Apg1 function. The present state of our knowledge does not allow a clear mechanistic model. However, the recent findings by Wang et al that Apg1 and Apg13 are multicopy suppressors of *snf1* mutants, and that Snf1, a protein kinase that is also required for glucose derepression is required for efficient autophagy, may imply a role for these molecules as upstream regulators of the pathway, although whether they function upstream, downstream, or in parallel with Tor remains to be established.[57]

Finally, both Apg1 and Apg13 are phosphoproteins that undergo dephosphorylation in response to rapamycin and starvation. Since Tor is a known regulator of phosphatase signaling, an appealing hypothesis is that changes in phosphatase activity contribute to the regulation of autophagy. The identification of such a Tor-dependent protein phosphatase that is required in autophagy remains a goal for future research.

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CHAPTER 6

Macroautophagy in Yeast

Takeshi Noda and Yoshinori Ohsumi

Abstract

The discovery of the occurrence of autophagy in the yeast *Saccharomyces cerevisiae*, and the subsequent isolation of autophagy defective mutants provided the first opportunity to understand the details of the molecular mechanism involved in this process. In this chapter, we provide a brief history of the study of autophagy using yeast cells. Following an overview of the entire autophagy process, the detailed molecular nature of each of the characterized Apg/Aut/Cvt proteins that are involved in autophagy is depicted. The unusual features of these proteins highlight the uniqueness of the autophagic process among the entire range of intracellular membrane dynamics.

Introduction

The yeast *Saccharomyces cerevisiae* has played an important role as a model system for studying fundamental problems in biology, such as cell cycle and membrane trafficking. Recently, studies on yeast autophagy have also led to many important breakthroughs in this field. Genetic analyses in yeast have allowed the identification of many of the molecular components of the autophagy pathway. Because these proteins have homologues in mammalian cells, the information gained from studying autophagy in yeast will be applicable to other systems. The hallmark of autophagy is the production of sequestering vesicles that transport cytoplasmic material into the vacuole. Currently, the protein machinery of autophagy can be grouped into four classes based on the stage at which each protein functions during vesicle biogenesis: Induction, formation, fusion and disintegration.

The Vacuole As a Lytic Compartment

For a long time it has been postulated that the vacuole is a lytic organelle in yeast. It was well known that yeast cell lysate contains strong proteolytic activities derived from vacuoles. Major hydrolases in yeast vacuoles are listed in (Table 1). Genetic approaches to identify and characterize vacuolar proteinases were undertaken primarily by two groups, that of E. Jones in the United States and D. Wolf in Germany.[1,2] The enzymology and biosynthesis of vacuolar enzymes have been studied quite extensively. These hydrolases play a critical role in various physiological processes. For example, it was shown that bulk protein turnover induced by nitrogen starvation is dependent upon vacuolar enzyme activities.[3,4] However, nothing was known about the kinds of substrates that are degraded in the vacuole or the mechanism of sequestration of these substrates.

Discovery of Autophagy in Yeast

In early 1990 Ohsumi and colleagues found that the yeast cell induces autophagy under various nutrient starvation conditions.[5] When vacuolar proteinase-deficient mutants grown in a rich medium were shifted to nitrogen-depleted medium, spherical structures appeared in the vacuole after 30 minutes. These structures accumulate and almost fill the vacuole within ten

Autophagy, edited by Daniel J. Klionsky.

Table 1. Proteases in the yeast vacuole

Enzyme	Gene	Type
Proteinase A	*PEP4*	Aspartic endoproteinase
Proteinase B	*PRB1*	Serine endoproteinase
Carboxypeptidase Y	*PRC1*	Serine carboxypeptidase
Carboxypeptidase S	*CPS1*	Metallo (Zn^{2+}) carboxypeptidase
Aminopeptidase I	*APE1*	Metallo (Zn^{2+}) aminopeptidase
Aminopeptidase Y	*APE3*	Metallo (Co^{2+}) aminopeptidase

hours. These structures, named autophagic bodies, are mostly single membrane-bound structures containing a portion of cytoplasm. Further electron microscopy (EM) studies revealed the presence of double membrane structures, autophagosomes, in the cytosol of the starved cells.[6] Autophagosomes in yeast are approximately 300-900 nm in diameter and contain cytosolic enzymes, ribosomes and various other cellular structures such as rough endoplasmic reticulum. The autophagosomal membrane appears to be thinner than any other membrane and the outer and inner membranes stick together and have mostly no lumenal space. Freeze fracture images revealed that the inner membrane lacks intramembrane particles, while the outer membrane contains sparse but significant particles.[7] These studies indicate that both membranes are differentiated and that the autophagosomal membrane is specialized for delivery to the lytic compartment.

Biochemical and immunoEM analyses clearly indicated that autophagy in yeast induced by starvation is a process for nonselective degradation of cytoplasmic components.[5] Organelles such as mitochondria are taken up by chance into autophagososomes and delivered to the vacuoles. Several carbon metabolism enzymes such as alcohol dehydrogenase, glucose 6-phosphate dehydrogenase, and phosphoglycerate kinase are nonselectively sequestered within the vacuole via autophagosomes. Mitochondria and rough endoplasmic reticulum are also taken up by autophagosomes indicating the nonselective nature of the degradation process. However, it has not been excluded that there is a selective aspect to the sequestration of certain molecules. In fact, aminopeptidase I (Ape1) and α-mannosidase have been shown to be selectively enwrapped in autophagosomes under starvation conditions (see chapter 7), although in this case sequestration is not for the purpose of degradation but rather is part of a biosynthetic pathway. Following sequestration of the cargo, the outer membrane of the completed autophagosome fuses with the vacuolar membrane. This process was demonstrated by freeze fracture electron microscopy.[7] The entire scheme of autophagy in yeast is shown in (Fig. 1)

Autophagy is induced not only by nitrogen starvation, but also by the depletion of other nutrients such as carbon, sulfate, phosphate and auxotrophic amino acids, which means that autophagy is a rather general physiological response to adverse nutrient conditions. What is the physiological function of autophagy in yeast? Mutant cell defective in autophagy show normal growth in a nutrient-rich medium.[8,9] This fact means bulk protein degradation is not crucial for vegetative growth. However, autophagy-defective mutants are not able to sporulate and cannot survive for long incubation periods in nitrogen free-medium.[8,9] In the life cycle of *S. cerevisiae*, sporulation and meitotic cell division is induced by nitrogen starvation, which means bulk protein degradation is required for this cell differentiation event. Thus, it is not surprising that autophagy defective mutants cannot complete sporulation. We do not know the reason why autophagy mutants cannot maintain their viability during starvation. One simple explanation is that a minimum essential supply of building blocks from autophagic degradation is necessary for survival during starvation. Another possibility is that a reduced state of cellular activity is critical to endure adverse conditions. In growing yeast cells autophagy does not occur

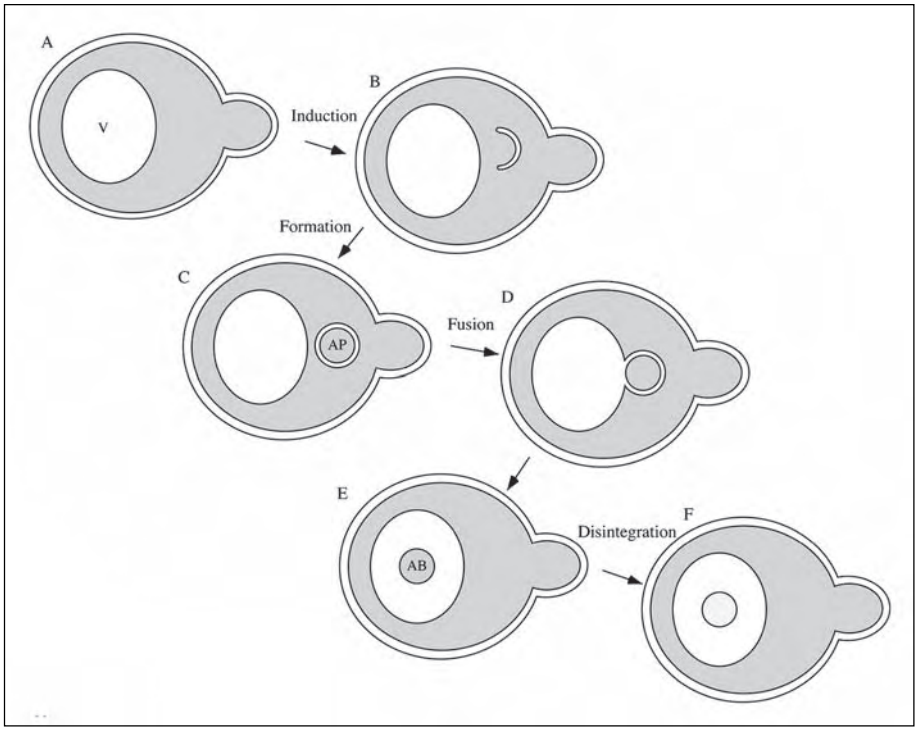

Figure 1. Schematic picture of the macroautophagic process. (A) Induction: When the yeast cell is subjected to starvation, autophagosome formation is induced. V: Vacuole. (B) Formation: A cup-like sac structure appears. It is a precursor of the autophagosome, and the cytoplasmic materials are enwrapped during the vesicle formation. (C) The cup-like structure is sealed to become an autophagosome (AP). (D) Fusion: The outer membrane of the autophagosome fuses with the vacuolar membrane. (E) As a result of the fusion process, the inner membrane structure of the autophagosome is released into the vacuolar lumen. The single membrane structure is called an autophagic body (AB). (F) Disintegration: The autophagic body is disintegrated rapidly in the vacuole, and the contents are degraded.

at a detectable level. A high level of cyclic AMP blocks the induction of autophagy, therefore, it seems likely that autophagy and cell growth are regulated in opposite ways.[10]

Stages of Autophagy

Autophagy consists of many steps and there are many questions to be answered about the molecular mechanism governing each step.

1. How does the cell recognize the environmental conditions and transduce a starvation signal to induce autophagy?
2. How are autophagosomes formed?
3. What is the mechanism of fusion between autophagosomes and the vacuole?
4. How are autophagic bodies disintegrated within vacuoles?
5. How are the degradation products transported out of the vacuole and how are they reutilized?

Genetic approaches have been undertaken to address these problems. The initial screen to identify mutants that were defective in autophagy relied on monitoring the morphological change in the vacuole under starvation conditions. Mutants in which autophagic bodies were not accumulated were selected, and one mutant, *apg1*, was obtained.[8] This strain grows nor-

mally in rich medium, but does not maintain viability under long-term nitrogen starvation. Using this starvation-sensitive phenotype as the basis for a subsequent screen, a total of fourteen autophagy (*apg*) mutants were obtained.[8] Another approach by Thumm et al relied on the immunoscreening of cells which retained a cytosolic enzyme, fatty acid synthase, after starvation. Originally six autophagy (*aut*) mutants were obtained from this analysis.[9] Klionsky's group isolated mutants defective in maturation of precursor Ape1, a resident vacuolar enzyme, that transits through the cytoplasm to vacuole targeting (Cvt) pathway in vegetative conditions (see chapter 7).[11] These *cvt* mutants showed extensive overlap with autophagy defective *apg* and *aut* mutants.[12,13]

The *apg* mutants showed quite similar phenotypes and were indistinguishable from each other. All of these mutants are normal in vegetative growth. Their vacuolar function, secretion and endocytosis are also normal. However, they do not induce bulk protein degradation during starvation and show a loss of viability phenotype under starvation conditions. EM analyses revealed that the *apg* mutants do not accumulate any intermediates of autophagosome formation; therefore, they should have a primary defect in autophagosome formation or at a stage just before the formation process. Because these mutants are nonconditional, it is possible that the *APG* genes are specifically required for autophagy. That is, the *APG* gene products do not have related activities in, and are not common factors for, several other pathways.

Induction of Autophagy

Autophagy was first discovered under nitrogen starvation conditions. Then carbon, sulfate, phosphate, and even single auxotrophic amino acid depletion was also shown to induce the same membrane dynamics, although the extent of the response varied (K, Shirahama and Y. Ohsumi, unpublished results).[5] At stationary phase, autophagic degradation occurs. However, so far any other type of stress including osmotic or salt stress, and heat shock does not induce autophagy in yeast. Under growing conditions the autophagic level is negligible, therefore, autophagy in yeast may be mainly a nutritional response. This does not appear to be the case in mammalian cells where autophagy is induced by various stress conditions and is regulated in part through hormonal control (see chapters 3 and 4).

So far, little is known about the induction of autophagy in yeast other than the involvement of Tor kinase.[10] When the Tor inhibitor rapamycin is added to cells growing in a rich medium, the cells respond in a similar manner to transfer into a starvation medium—that is, autophagy is induced. This implies that Tor negatively regulates autophagy (see chapters 3 and 5).

Autophagosome Formation

What is the origin of the autophagosomal membrane? The answer is mostly unknown, but a novel structure seems to play a pivotal role in autophagosome formation.[14-16] Many of the Apg/Aut/Cvt proteins display a punctate, perivacuolar localization to a compartment termed the pre-autophagosomal structure (PAS). The precise role of the PAS is still obscure, but it bears a protein profile distinct from autophagosomes. Below we describe the current knowledge of each Apg/Aut/Cvt protein involved in autophagosome formation.

VPS34/YLR240w (101 kDa, pI 7.96)—Vps34 is the sole phosphatidylinositol (PtdIns) 3-kinase in *Saccharomyces cerevisiae*.[17] It contains a phosphoinositide 3-kinase family accessory domain (Fig. 2; black box) and PtdIns 3- and 4-kinase domains (Fig. 2; hatched box). The *vps34* mutant shows a wide range of phenotypes, including defects in autophagy, vacuolar protein sorting, endocytosis, and slow growth, meaning that PtdIns(3)P plays a variety of roles, particularly in membrane trafficking pathways.[18] Vps34 associates with some membrane, and its membrane recruitment is dependent on the kinase activity of Vps15.[19,20] Vps34 interacts with Vps15 through a 28-amino acid element near its C terminus (black underline).[21]

VPS15/YBR097w (166 kDa, pI 6.51)—Vps15 is a serine/threonine protein kinase (Fig. 2; kinase domain, black box).[22] The kinase activity is important for the association between Vps15 and Vps34 and membrane recruitment of Vps34.[19,20] Vps15 associates with Vps34 through

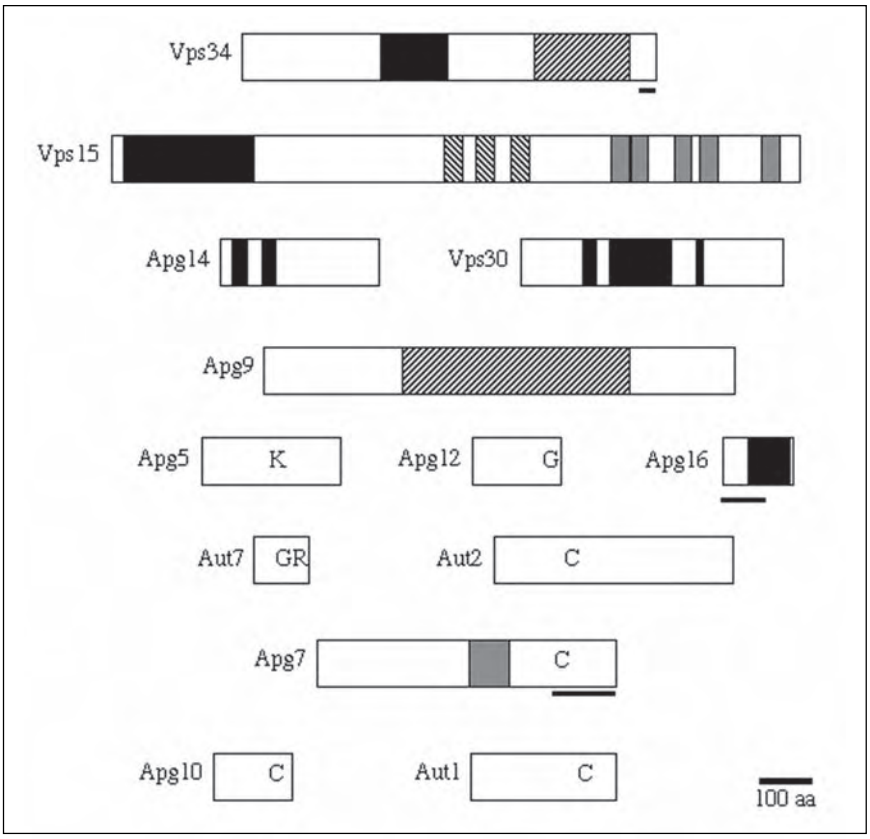

Figure 2. Schematic drawing of proteins involved in autophagosome formation. The bar represents a length of 100 amino acids. Details are in the text. Continued on next page.

three tandem HEAT repeats of about 39 amino acids located in its center region (Fig. 2; hatched box).[21] Additionally, 5 WD repeats exist at its C terminus (Fig. 2; gray box). The null mutant of Vps15 shows mostly the same phenotypes with that of Vps34, therefore, Vps15 may be understood as a regulator of Vps34.[20]

VPS30/APG6/YPL120w (63 kDa, pI 4.94)—Vps30 was originally isolated based on a screen for mutants defective in vacuolar protein sorting of carboxypeptidase Y (Prc1).[23] The *vps30* mutant shows defect in the localization of the sorting receptor for Prc1 (Vps10/Pep1).[23] Later, it was discovered that one of the autophagy defective mutant genes (*APG6*) is isogenic with *VPS30*.[24] Thus, Vps30 is required both for autophagy and vacuolar protein sorting.[24] Vps30 forms part of two distinct protein complexes.[25] One complex consists of Vps30, Apg14, Vps34 and Vps15, and the other is composed of Vps30, Vps38, Vps34 and Vps15.[25] The former complex is responsible for autophagy, and the latter for vacuolar protein sorting. Vps30 is a possible coiled-coil protein (Fig. 2; black box) and is peripherally membrane associated. Lack of Vps34 or Vps15 results in solubilization of Vps30.[25] A *vps34* mutant shows more severe phenotypes than a *vps30* mutant, for example with regard to slow growth; therefore, all the roles of Vps34 may not be regulated by Vps30.[25]

APG14/CVT12/YBR128c (40 kDa, pI 9.44)—Apg14 is a possible coiled-coil protein (Fig. 2; black box) that is associated with Vps30.[24] Overexpression of Apg14 partially suppresses the

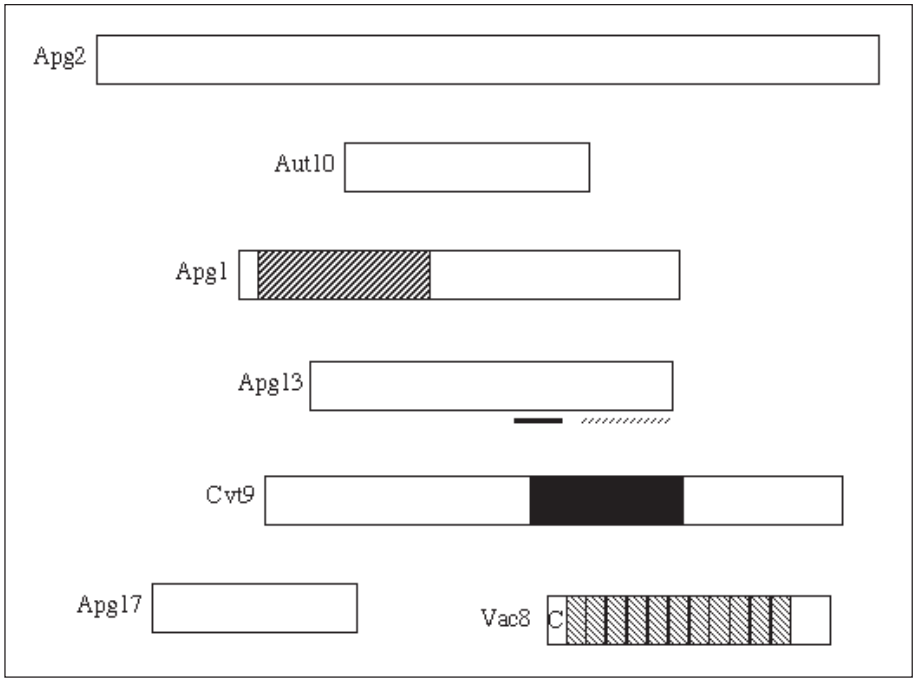

Figure 2 (continued). Schematic drawing of proteins involved in autophagosome formation. Details are in the text.

autophagic defect of a truncated mutant of Vps30 (*apg6-1*), in which Glu269 changes to a Stop codon, but does not suppress that of the deletion allele of Vps30.[24] This suggests that Apg14 interacts with the N-terminal half of Vps30 to exert its function for autophagy. In contrast to *vps30*, the *apg14* mutant does not show a defect in vacuolar protein sorting. Apg14 is the sole specific factor in the autophagy-specific PtdIns 3-kinase complex, therefore it is presumed to play an important role in determining the specificity of the complex, possibly by controlling its localization.[25] Apg14 is peripherally associated with membrane.[24] Overexpressed YFP-Apg14 is localized on vacuolar membrane and at the PAS.[14]

Eight Gln3 (GATA-type zinc finger transcription factor for nitrogen regulation)-binding motifs (GATAA) are found in the 500 base pair upstream untranslated region of the *APG14* gene.[26] The expression of *APG14* mRNA is induced over 20-fold by rapamycin-treatment or nitrogen starvation, and it is reduced in a *gln3* mutant.[26] The *apg14* mutant abolishes the localization of Apg proteins (Aut7, Apg5, Apg1, Apg2) at the PAS.[16] Thus the autophagy specific PtdIns 3-kinase complex is suggested to play an important role in the organization of the PAS.

APG9/AUT9/CVT7/YDL149w (115 kDa, pI 5.79)—Apg9 is an integral membrane protein.[27] In its center segment (Fig. 2; amino acids 295-775, hatched box), Apg9 possesses a region conserved through eukaryotes which contains 5 hydrophobic stretches, long enough for membrane spanning.[27-29] Apg9 localizes to a perivacuolar compartment, possibly the PAS.[14,27] The *apg9* mutant loses the localization of several autophagy proteins (Aut7, Apg5, Apg1, Apg2) at the PAS, and Apg9 is physically associated with Apg2.[16,30,31] Thus Apg9 is suggested to play an important role in the organization of the PAS.

APG5/YPL149w (34 kDa, pI 8.74)—GFP-Apg5 localizes at the PAS at least transiently.[14,16] At Lys149, Apg5 is conjugated with the C-terminal carboxyl group of Apg12 via an isopeptide

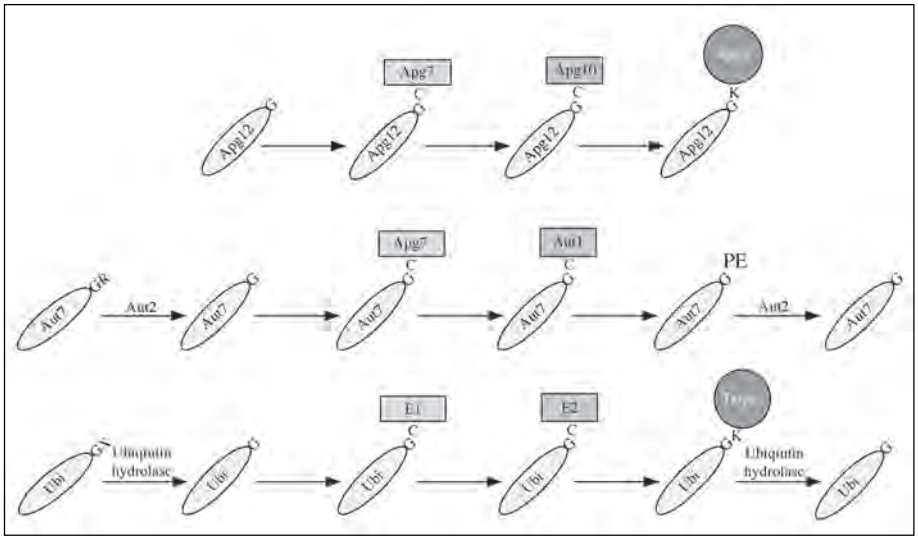

Figure 3. Dual ubiquitin-like reactions involved in autophagosome formation. Apg12 and Aut7 are ubiquitin-like proteins. They are subjected to reactions closely resembling ubiquitination (shown schematically at the bottom). Details are in the text.

bond.[32] This conjugation is essential for autophagy,[32] but not for the localization of Apg5 at the PAS.[16] Apg5 also binds to Apg16,[33] resulting in the formation of a 350 kDa protein cytosolic complex which includes Apg12-Apg5 and Apg16.[34] The localization of Apg5 at the PAS is dependent on Apg16.[16] Apg9 and the autophagy-specific PtdIns 3-kinase complex are also required for Apg5 localization.[14,16] A temperature sensitive mutant of *apg5* exhibits a discontinuous membrane sac-like structure associated with or partly covering the Cvt complex (multiple prApe1 oligomers) at the nonpermissive temperature.[35] A null mutant of *apg5* shows a defect in the localization of Aut7 at the PAS and affects the efficiency of lipidation of Aut7 (Fig. 3).[14,16]

APG12/YBR217w (21 kDa, pI 6.54)—Apg12 is an ubiquitin-like protein.[32] The C-terminal amino acid of Apg12 is a glycine residue.[32] Following translation, the Apg12 protein is activated by Apg7 (an E1 homologue, see below), and then transferred to Apg10 (that functions as an E2 enzyme, see below).[32,36,37] Finally, the C-terminal glycine of Apg12 is conjugated to the lysine residue of Apg5 via an isopeptide bond (Fig. 3).[32] At steady state, most of the Apg12 exist as this conjugate form with Apg5. In the cytosol, a 350 kDa protein complex which includes Apg12-Apg5 and Apg16 is formed.[34] A null mutant of *apg12* does not affect the localization of Apg5 and Apg16 at the PAS, but subsequent autophagosome formation does not occur.[16] An *apg12* null mutant does show a defect in the localization of Aut7 at the PAS and affects the efficiency of Aut7 lipidation.[14,16]

APG16/YMR159c (17 kDa, pI 6.14)—Apg16 binds to Apg5, and more preferentially to the Apg12-Apg5 conjugate, via the N-terminal half of the protein (Fig. 2; black underline).[33] The C-terminal half of Apg16 contains a coiled-coil region (Fig. 2; black box). Multiple Apg16 monomers interact to form an oligomer.[33] As a result, a 350 kDa complex is formed that includes multiple Apg12-Apg5 conjugates and Apg16.[34] Apg16 is localized at least transiently at the PAS.[16] In an *apg16* mutant, the Apg12-Apg5 conjugate is still formed,[33] but the localization of Apg5 at the PAS is abolished.[16] Aut7 localization at the PAS is also impaired, although some Aut7 is still conjugated to phosphatidylethanolamine (PE; see below) in the *apg16* mutant.[16]

AUT7/APG8/CVT5/YBL078c (14 kDa, pI 9.28)—Aut7 is an ubiquitin-like protein.[38] The close homologues of Aut7 in mammalian cells (GATE-16 and GABARAP) have a three-dimensional structure that is very similar to ubiquitin.[39-42] The Aut7 C-terminal sequence is glycine followed by an arginine residue.[43] After translation, this arginine residue is immediately cleaved off by Aut2 (see below), converting the penultimate glycine to the most C-terminal residue.[43] The Aut7 protein is subsequently activated by Apg7 (E1), and then transferred to Aut1 (that acts as an E2 enzyme, see below).[38] Finally, the C-terminal glycine is conjugated with a lipid molecule, PE (Fig. 3).[38] The PE that is conjugated with Aut7 is again cleaved off by Aut2, exposing the free glycine residue.[43] Thus, Aut7 cycles between the glycine-exposed and the PE-conjugated forms.[43] The PE-conjugation is important for autophagic activity of Aut7.[38,43] Aut7 is localized within the autophagic bodies, and a significant amount of Aut7 is transported into the vacuolar lumen to be degraded during autophagy.[44,45] Aut7 is also found on the membrane of autophagosomes during formation, and the amorphous structure located nearby.[44] GFP-Aut7 stains both autophagosomes and the PAS.[14,16] PE conjugation is essential for the localization at the PAS.[14,16,46] Possibly, Aut7 is conjugated with PE at or before the PAS, and detached from PE on the forming autophagosome.

Aut7 is transcriptionally up-regulated under starvation conditions via the Tor signaling pathway.[44] A null mutant of *aut7* is defective in autophagy, while some small or aberrant autophagosomes are still formed and approximately 50% of prApe1 is transported to the vacuole only under starvation conditions.[44,47,48] Similar phenotypes are seen in *aut2* and *aut1* mutants.[47] Therefore, the increased level of Aut7-PE that is synthesized under starvation conditions is proposed to facilitate the formation of autophagosomes that have a normal size and shape.

AUT2/APG4/YNL223w (57 kDa, pI 4.53)—Aut2 is a novel type of cysteine endopeptidase (C54 in MEPRO database classification).[43] Aut2 cleaves the C-terminal arginine residue from newly synthesized Aut7, resulting in a C-terminal glycine (Fig. 3).[43] The glycine residue is essential for the cleavage by Aut2, but the downstream sequence seems not to be recognized by Aut2.[43] Aut2 also cleaves the amide-bond between the C-terminal carboxyl-group of the glycine and the head-group of PE.[43] Thus, Aut2 functions in two steps during the autophagic process. The Aut2 endopeptidase activity is inhibited by N-ethylmaleimide, implicating Cys159 as a possible catalytic site.[43] Aut2 is a soluble protein that is diffused within the cytosol.[46] The cleavage of the arginine residue and exposure of a glycine residue by Aut2 is essential for an ubiquitin-like reaction involving conjugation of Aut7 to PE.[38,43] Expression of the processed form of Aut7 lacking the C-terminal arginine bypasses the initial need for Aut2.[43] In such a strain, however, deletion of Aut2 decreases the autophagic activity, implying that the second role of Aut2 in cleaving the bond between Aut7 and PE is important in autophagy.[43]

APG7/CVT2/YHR171w (71 kDa, pI 6.09)—Apg7 is an ubiquitin activating enzyme-like protein (E1).[37] It activates both Apg12 and Aut7 (Fig. 3).[37,38] Apg7 shows sequence similarity with the other ubiquitin activating enzyme Uba1 around the ATP binding domain (Fig. 2; residues 322-407, gray box).[37] Mutation of the active site cysteine (Cys507) or the ATP binding domain abolished its function.[37,38] Apg7 forms a homodimer, and the C-terminal region (Fig. 2; residues 508-630, black underline) is important for dimerization and activity.[49] Apg7 binds to two E2-like enzymes (Apg10 and Aut3, see below).[36,49] Apg7 also binds to two ubiquitin-like proteins (Apg12 and Aut7) through a thioester bond at the active site.[37,38] Apg7 is mostly a soluble protein, while some portion of GFP-Apg7 is observed as a dot-like structure under starvation conditions.[50]

APG10/YLL042c (20 kDa, pI 4.81)—Apg10 is an ubiquitin conjugation enzyme-like protein (E2) for Apg12 (Fig. 3).[36] A mutation at the active site cysteine (Cys133) abolishes its function, and the Apg12-Apg5 conjugate is not formed.[36] Apg10 physically interacts with Apg7, and forms an intermediate with Apg12 via a thioester bond at its active site in a reaction dependent on previous activation of Apg12 by Apg7.[36] In an *apg10* mutant, Apg5 is localized at the PAS while Aut7 is not.[14,16,46]

AUT1/APG3/YNR007c (36 kDa, pI 4.55)—Aut1 is an ubiquitin conjugation enzyme-like protein (E2) for Aut7 (Fig. 3).[38] A mutation at the active site cysteine (Cys234) abolishes its function, and the Aut7-PE conjugate is not formed.[38] Aut1 interacts with Apg7, and forms a reaction intermediate with Aut7 via a thioester bond at its active site.[38,49] The region around the Aut1 active site shares some similarity to the region around the active site of Apg10.[38] In an *aut1* mutant, Aut7 is not localized at the PAS.[14,16,46]

APG2/AUT8/YNL242Ww (178 kDa, pI 5.78)—Apg2 is a peripheral membrane associated protein that is localized at the PAS.[30,31] A point mutation (G83E) abolishes both the localization and its autophagic activity, so the localization at the PAS seems to be important for Apg2 to execute its function.[30] The localization of Apg2 depends on the presence of Apg1, Apg9, and the autophagy-specific PtdIns 3-kinase complex.[30,31] Apg2 physically interacts with Apg9.[31] In the absence of Apg2, the Aut7, Apg5 and Apg1 proteins localize at the PAS, but subsequent autophagosome formation does not occur.[31]

AUT10/CVT18/YFR021w (55 kDa, pI 5.42)—Aut10 is a member of a gene family consisting of *AUT10*, *MAI1*, and *YGR223c*.[51-53] The *aut10* mutant is defective in both autophagy and the Cvt pathway, while the *mai1* mutant shows a defect only in the Cvt pathway.[51-53] Aut10 is a mostly soluble protein and a small portion is peripherally associated with a membrane.[51,53] GFP-Aut10 shows a cytosolic pattern and a dot structure at a perivacuolar region.[53] The *aut10* mutant shows a defect in the localization of Apg2 and Apg5 at the PAS. Aut10 may also play some role in amino acid signaling.[53]

APG1/AUT3/CVT10/YGL180w (102 kDa, pI 6.40) —Apg1 is a serine/threonine protein kinase.[54,55] Its N terminus consists primarily of a protein kinase domain (Fig. 2; hatched box), and the Apg1 protein shows protein kinase activity in vitro.[56] An Apg1 mutant without kinase activity cannot induce autophagy, implying the kinase activity is essential for Apg1 function.[54,56] The Apg1 kinase activity is increased during induction of autophagy, so the magnitude of the kinase activity seems to be important for the regulation of autophagosome formation.[56] The kinase activity is regulated by Apg13 and possibly by Apg17.[56] Apg1 physically associates with Apg13, Apg17 and Cvt9 respectively.[56] Apg1 is localized at least transiently at the PAS.[16] In an *apg1* mutant, Aut7 and Apg5 can be localized at the PAS, but subsequent autophagosome formation does not occur.[16]

APG13/YPR185w (83 kDa, pI 6.79)—Apg13 is highly phosphorylated under nutrient-rich conditions.[56] Upon induction of autophagy either by starvation or rapamycin treatment, Apg13 is partially dephosphorylated by an unknown phosphatase.[56] The dephosphorylation is reversible and Apg13 is rapidly phosphorylated when cells are transferred from starvation conditions to nutrient-rich media, without requiring de novo protein synthesis.[56] Thus, the phosphorylation state of Apg13 is regulated by the nutrient conditions through the Tor signaling pathway. A genetic interaction exists between Apg1 and Apg13, as evidenced by the suppression of the autophagy defective phenotype of the *apg13* mutant upon overproduction of Apg1.[57] Through its central region (Fig. 2; residues 432-520, black underline), Apg13 physically associates with the Apg1 protein.[56] Under starvation conditions, Apg13 is strongly associated with Apg1, while under nutrient rich conditions the affinity is lowered.[56] In addition, in the a*pg13* deletion mutant, the in vivo kinase activity of Apg1 is lowered.[56] Altogether, these results suggest that Apg13 is a positive regulator for the Apg1 protein kinase. Transport of prApe1 is completely blocked when the *apg13* null mutant is grown in nutrient rich medium, but the block can be partially overcome by incubation in starvation conditions.[48] In an *apg13* mutant that lacks most of the Apg1-binding region (residues 1-448), transport of prApe1 is mostly normal but autophagy is completely defective.[56] Thus, Apg13 regulates autophagy and the Cvt pathway through Apg1 protein kinase. Apg13 also associates with Vac8 via its C-terminal region (Fig. 2; residues 563-718, hatched underline).[58]

APG17/YLR423c (49 kDa, pI 4.97)—Distinct from the other *APG* genes, a null mutation of *apg17* does not affect the Cvt pathway, while autophagy is defective.[56] Apg17 was first identified through a two-hybrid screen with Apg1 as bait.[56] In the null mutant of *apg17*, the in

vitro kinase activity of Apg1 is largely impaired (~20% of wild-type), suggesting that Apg17 is also involved in Apg1 activation.[56] Overexpression of Apg1 suppresses the autophagic defect of the *apg17* deletion mutant.[56] Apg17 also physically associates with Cvt13 and Cvt20, proteins that contain a PtdIns(3)P-binding PX domain, and that are involved specifically in the Cvt pathway (see chapter 7).[59] Apg17-YFP shows a punctuate localization in the cytoplasm.[59,60]

CVT9/YPR049c (135 kDa, pI 5.55)—In contrast to Apg17, a null mutation of Cvt9 does not abolish autophagy, while the Cvt pathway is defective (see chapter 7).[61] Thus, an opposite role to that of Apg17 is postulated for Cvt9. Cvt9 is a coiled-coil protein (Fig. 2; black box), which forms a homo-oligomer.[61] The Cvt9 protein physically interacts with Apg1.[56,61] Cvt9 is localized at least transiently to the PAS.[14]

VAC8/YEL013w (63 kDa, pI 5.01)—Vac8 functions in several divergent pathways, such as vacuole inheritance, nuclear vacuolar junction-formation, homotypic vacuole fusion and the Cvt pathway (see chapters 7 and 9).[58,62-67] Vac8 is localized on the vacuolar membrane and on some other membrane.[62,66] Vac8 is a member of the armadillo repeat family of proteins, which contains 11 armadillo repeats (Fig. 2; hatched boxes).[62,63,66] Its N terminus is myristoylated and this modification is required for vacuolar localization of Vac8 but not for the Cvt pathway.[66] An N-terminal cysteine residue of Vac8 is palmitoylated, and this is essential for vacuolar inheritance and homotypic vacuole fusion but again, not for the Cvt pathway.[65-67] Vac8 is associated with Apg13 under both nutrient-rich and starvation conditions.[58] In a *vac8* mutant, autophagy occurs but less efficiently than in wild type cells.[58]

Fusion of the Autophagosome with the Vacuole

An in vitro system that reconstitutes autophagy has not been established. Therefore, a direct involvement of the fusion machinery in autophagy can be proved only by using conditional mutants to exclude indirect effects resulting from perturbation of other membrane trafficking pathways. In such experiments, Vam3 (t-SNARE), Vam7 (t-SNARE), Vti1 (v-SNARE), Sec18 (NSF), Sec17 (α-SNAP), Vps18 (HOPS complex) and Ypt7 (Rab) have been shown to be directly involved in the autophagosome to vacuole fusion step. These proteins are also involved in other trafficking pathways that deliver proteins to the vacuole.[68-72] To date, no mutant has been found that shows a defect in the fusion between autophagosomes and the vacuole but not in the other membrane trafficking pathways. Therefore, it is assumed that the fusion machinery for autophagy is completely, or at least mostly, shared with the other vacuolar delivery pathways.

Disintegration of Autophagic Bodies in the Vacuoles

In wild type cells autophagic bodies disappear from the vacuole lumen within a minute of their initial appearance. The breakdown of the autophagic body exposes the cytoplasmic cargo to degradation by vacuolar proteases. It is not known why the autophagosomal inner membrane is so quickly disintegrated. This is particularly interesting considering that the outer autophagosomal membrane becomes part of the vacuole and cannot be degraded prematurely without losing the integrity of the vacuole. In the *prb1* mutant defective in proteinase B, a vacuolar resident serine proteinase, the autophagic body is not disintegrated.[3,5,73] A similar result is seen with the *pep4* mutant that is defective for proteinase A, the vacuolar resident serine proteinase that is responsible for activation of proteinase B, and in cells treated with PMSF, an inhibitor of proteinase B.[3,5] Acidification of the vacuolar lumen by the vacuolar proton-translocating ATPase is also necessary for the breakdown of the autophagic body.[74]

Two more specific factors have been shown to be involved in the breakdown of the autophagic body. Cvt17/Aut5 is a possible lipase, which contains a lipase-like domain. In the *cvt17* null mutant, or its possible active site mutant, the autophagic body is not disintegrated.[75,76] Cvt17 is a glycosylated integral membrane protein and is transported to the vacuole via the secretory multivesicular body (Mvb) pathway but not via autophagy.[75,76] Another factor, Aut4, is also involved in the degradation of autophagic bodies. Aut4 is a putative integral membrane

protein with ten to twelve predicted transmembrane domains and shows limited homology to permeases.[77] GFP-Aut4 is visualized at the vacuolar membrane and in punctate structures attached to the vacuole.[77] The detailed mechanism of these proteins in the degradation of the autophagic body remains to be determined, but at least their defects are not due to dysfunction of proteinase B or the vacuolar ATPase.[76,77]

Conclusions

Yeast has proven to be a useful model system for the molecular, genetic and biochemical analysis of autophagy. In the past five years, many of the protein components of the autophagy pathway have been identified and characterized. The identification of autophagy genes in *S. cerevisiae* has allowed for a major advance in the study of this process in other eukaryotes due to the finding that homologues exist in other fungi and in plant and animal cells. Future studies will focus on the interactions among the various autophagy proteins and a determination of their function in autophagy, the cytoplasm to vacuole targeting pathway and pexophagy.

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CHAPTER 7

Cytoplasm to Vacuole Targeting

Per E. Strømhaug and Daniel J. Klionsky

Abstract

The cytoplasm to vacuole targeting (Cvt) pathway is a biosynthetic membrane transport mechanism for the delivery of the resident enzymes aminopeptidase I (Ape1) and α-mannosidase (Ams1) to the vacuole. These hydrolases are synthesized on free ribosomes in the cytosol where they rapidly oligomerize. Precursor Ape1 dodecamers further aggregate into a higher order Ape1 complex that subsequently interacts with the soluble receptor Cvt19. Cvt19 also binds Ams1 oligomers and the resulting structure is called a Cvt complex. The Cvt complex is brought into proximity of a sequestering membrane by the interaction of Cvt19 with Cvt9 and Aut7. A Cvt vesicle is formed in a process that requires the same set of proteins that is used for starvation induced non-selective autophagy. The similarity of the Cvt pathway to autophagy is also demonstrated by the selective uptake of the Cvt complex into autophagosomes during nutrient deprivation. The formation of the Cvt vesicles and autophagosomes requires the membrane protein Apg9, the Apg1 protein kinase complex and the phosphatidylinositol 3-kinase complex I. In addition, the formation of the Cvt vesicles requires phosphatidylinositol(3)phosphate-binding proteins, which are not used by autophagy. Upon completion of the vesicles, the outer membrane of the Cvt vesicle fuses with the vacuolar membrane and a single membrane bound Cvt body is released into the vacuolar lumen. A concerted action of lipases and proteases then liberates α-mannosidase and allows the proteolytic maturation and activation of aminopeptidase I dodecamers.

Introduction

Normal cell growth and homeostasis requires balanced protein synthesis and degradation. Yeast cells harbor two major systems for degradation of proteins, the proteasome and the vacuole. While the proteasome is a large multi-protein particle predominantly breaking down poly-ubiquitin tagged proteins in the cytosol and nucleus, the vacuole is a complex membranous compartment with several functions in addition to protein degradation. Within an acidic lumen favoring hydrolysis is an arsenal of enzymes capable of hydrolyzing all cellular macromolecules, not only proteins. The delimiting membrane of the vacuole contains a number of membrane transporters mediating the recirculation of hydrolyzed monomers such as amino acids and sugars back into the cytosol, a large protein complex that maintains the acidic interior by actively transporting protons across the membrane, as well as permeases facilitating transport of metal and other ions into the vacuole. Through these various transport processes the vacuole also serves a purpose in osmoregulation, in detoxifying the cytosol of elevated levels of harmful compounds, maintaining cytosolic pH and calcium levels, and in storage of metabolites. The vacuolar membrane also contains a number of trans-membrane and membrane-associated proteins that regulate the targeting of vesicles destined for fusion with the vacuole. These vesicles may be endosomes or autophagosomes, and hence carriers of macromolecules and organelles destined to be degraded, or they may be biosynthetic vesicles containing newly synthesized membrane proteins and lumenal vacuolar hydrolases delivered to maintain the vacuolar compartment during homeostasis or for expansion during cell proliferation.

Autophagy, edited by Daniel J. Klionsky.

The vast majority of the biosynthetic vesicles contain proteins that have been co-translationally synthesized and translocated into the endoplasmic reticulum membrane or lumen and transported through the Golgi apparatus to the *trans*-Golgi network. Reaching the *trans*-Golgi network, the proteins are sorted into transport vesicles that traffic to the vacuole by two different pathways. The vesicles of the ALP pathway, named after the cargo protein alkaline phosphatase (Pho8), fuse directly with the vacuole following scission from the Golgi. Other proteins traveling by this pathway include the SNARE proteins Vam3 and Nyv1. The other vesicle-mediated pathway is called the CPY pathway after the cargo protein carboxypeptidase Y (Prc1), and these vesicles are assimilated into the endosomal compartment before the cargo proteins subsequently are transported from the endosomes to the vacuole. Other proteins employing this route of transport include the vacuolar proteinases Pep4 (proteinase A) and Prb1 (proteinase B), and the main component of the vacuolar proton pump, Vph1. Some of the resident hydrolases that are transported to the endosome on their way to the vacuole follow the multi-vesicular body (Mvb) pathway. After transit to the endosome, some membrane proteins invaginate into the endosomal lumen (see chapter 14). Subsequent delivery to the vacuole is presumed to involve direct fusion of the multi-vesicular body with the vacuolar membrane.

Over the last two decades more than sixty *VPS* (Vacuolar Protein Sorting) genes that are involved in the transport of resident proteins to the vacuole have been identified and characterized. Some of these genes encode receptors (e.g., *VPS10* encodes the Prc1 receptor) directly involved in protein targeting, while others may have an indirect function in retrieving receptors from the vacuole and endosome and maintaining the integrity of intermediate compartments. Many of these gene products are shared between the different vacuolar transport pathways as well as with other cellular processes such as protein secretion and uptake of proteins by endocytosis. However, there also exists a fourth route for the import of resident vacuolar hydrolases. Ten years ago our lab discovered that the vacuolar lumenal protein aminopeptidase I does not transit through the secretory pathway en route to the vacuole, but is instead first synthesized on free ribosomes in the cytosol prior to vacuolar import.[1] Genetic analysis identified a set of genes that govern a unique pathway termed the cytoplasm to vacuole targeting (Cvt) pathway.[2] So far, two vacuolar hydrolases have been shown to exploit this mode of transport in *S. cerevisiae*; aminopeptidase I (Ape1/API) and α-mannosidase (Ams1).[3] The four pathways for delivery of resident vacuolar proteins are illustrated in Fig. 1.

Two Proteins Are Transported by the Cvt Pathway; Aminopeptidase I and α-Mannosidase

Aminopeptidase I. Aminopeptidase I, hereafter referred to as Ape1, is a metallopeptidase belonging to the M18 family of the MH clan. It is a leucine aminopeptidase with preference for neutral or hydrophobic amino acids using zinc as a cofactor, and its peptidase activity is also enhanced by chloride ions.[4] Unlike most vacuolar proteins, which pass through the secretory pathway, precursor Ape1 (prApe1) does not contain a signal sequence that would direct the newly synthesized peptide chain to the endoplasmic reticulum, but is instead synthesized free in the cytosol. The first 45 amino acids constitute an inhibitory propeptide, which is cleaved off by proteinase B after the protein enters the vacuole. This cleavage generates an active aminopeptidase and can be monitored as a molecular mass shift from 61 kDa to 50 kDa. This shift can easily be detected by SDS-polyacrylamide gel electrophoresis and western blotting and provides a convenient assay that can be used to monitor the transport of prApe1 into the vacuole. However, the mature protein does not appear to exist as a monomer. By the use of sucrose gradients it was shown that mature Ape1 has a sedimentation coefficient of about 22S, which corresponds to a molecular mass of 640 kDa for a spherical particle, suggesting that functional Ape1 is a dodecamer.[5,6] The assembly of the dodecamer requires the C terminus of Ape1 but at present does not appear to involve any other gene products, and occurs spontaneously in the cytosol shortly after synthesis. The resulting precursor Ape1 dodecamer has a molecular mass of 732 kDa.[6] Several of these protein complexes further aggregate in a process

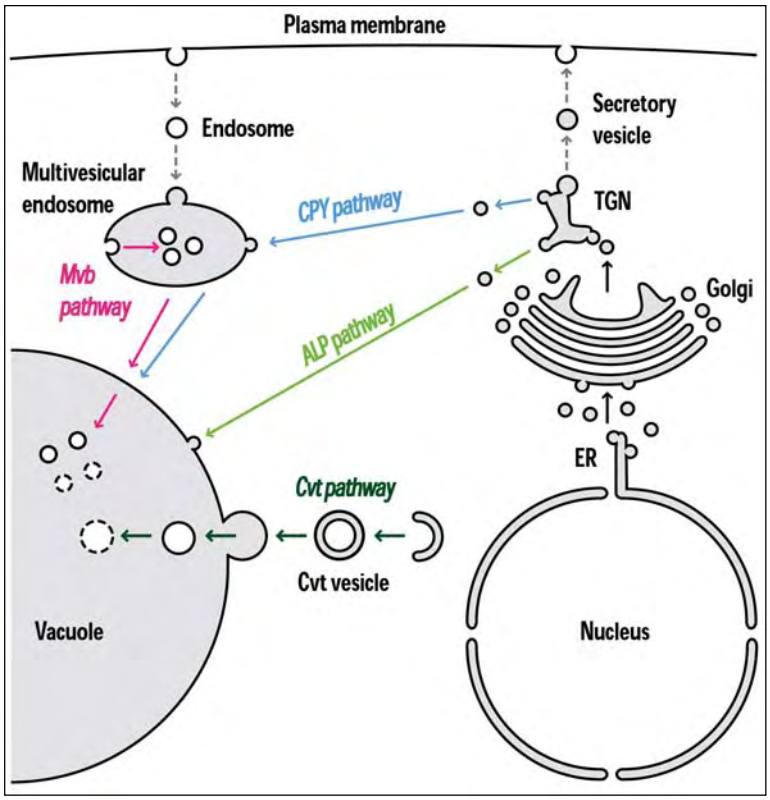

Figure 1. Trafficking of vacuolar proteins. Most vacuolar proteins are synthesized into the endoplasmic reticulum (ER) and transported to the Golgi complex. In the *trans*-Golgi network (TGN), the proteins are either sorted into vesicles traveling directly to the vacuole by the ALP pathway, or into vesicles that are first assimilated into the endosomal compartment (CPY pathway) before the proteins are transferred to the vacuole. Proteins may also in some cases enter the endosomes after being transported to the plasma membrane. At the endosome, membrane proteins may be sorted to the interior of the endosome by the Mvb pathway. These proteins probably enter the vacuole after the multivesicular endosome fuses with the vacuole. The fourth route to the vacuole is employed by aminopeptidase I and α-mannosidase. These hydrolases are synthesized free in the cytosol and thereafter enclosed by a membrane resulting in vesicles that subsequently fuse with the vacuole.

depending on the N-terminal propeptide leading to a higher order structure termed the Ape1 complex.[7] The Ape1 complex can be seen by transmission electron microscopy as a dense protein aggregate in the cytosol, or can be easily seen by live cell fluorescent microscopy with prApe1 fused to different varieties of the green fluorescent protein.[7-9]

While the three-dimensional structure of Ape1 or any other related peptidase within the M18 family of aminopeptidases is not known and the structure of Ape1 therefore cannot be predicted, the three-dimensional organization of the dodecamer was resolved by electron microscopy using negative staining with uranyl formate.[5,10] The electron micrographs of Ape1 purified from yeast together with the observation that the purified fraction contained dimers and hexamers in addition to dodecamers, suggested that the dodecamer consists of two stacked hexamers that are organized according to a pseudo D3 structure (i.e., with three axes of symmetry) where a dimer is the smallest unit. A D3 organization (i.e., two stacks of trimers) has

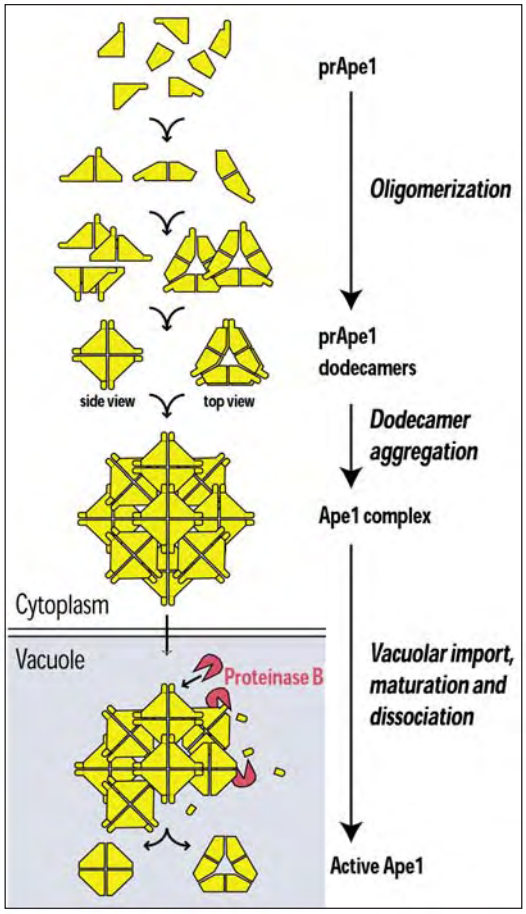

Figure 2. The assembly and structure of aminopeptidase I dodecamers. The three dimensional organization of purified Ape1 dodecamers was resolved by electron microscopy after negative staining with formyl acetate (see text for reference). The scheme for the assembly of the dodecamer is based on these micrographs and on the biochemical characterization of the complex. The dodecamer consists of two stacked hexamers, each made up of three homodimers. The localization of the propeptide is arbitrary and placed in a corner to illustrate that one of the subunits of the dimer has to be rotated 90 degrees. Several prApe1 dodecamers further aggregate into an Ape1 complex in a process that requires the propeptide. After prApe1 is transported to the vacuole, the propeptide is cleaved off by proteinase B, and the Ape1 complex dissociates into proteolytically active Ape1 dodecamers.

also been shown to be the structure of several hexameric aminopeptidases from other peptidase families in the MH clan. A suggested scheme for how the oligomerization may occur is depicted in Figure 2, although the placement of the propeptide is pure speculation as the structural studies were performed only on mature Ape1 dodecamers.

The propeptide of prApe1 functions not only during Ape1 complex assembly, but also plays an additional pivotal role in transport of the protein particle to the vacuole.[7,11] Highly enriched in charged amino acids, the first half of the propeptide is predicted to form an amphipathic α-helix followed by a β-turn defined by a proline, and then another α-helix, resulting in a helix-turn-helix formation or coiled-coil.[12] Mutations or deletions (e.g., deletion of amino acids nine to eleven) in the propeptide that result in disruption of the first amphipathic α-helix lead to the accumulation of Ape1 as a precursor dodecamer in the cytosol.[11] A similar result is seen if the coiled-coil formation is disrupted by mutation of the proline separating the two α-helices (e.g., P22L). A change of the lysine in position twelve to arginine (K12R) results in a temperature sensitive mutant that is thermally reversible.[6] Thus, even conservative changes in the first α-helix of the propeptide have substantial effects on import competency.

α-Mannosidase. α-mannosidase, or Ams1, belongs to family 38 of glycosyl hydrolases and hydrolyzes terminal, non-reducing α-D-mannose residues. Like prApe1, this vacuolar hydrolase is also translated on free cytosolic ribosomes before the protein is transported into the vacuole by a mechanism requiring the same machinery as is used for the transport of prApe1.[3,7]

Earlier studies on Ams1 were ambiguous regarding whether this protein is cleaved upon entry into the vacuole, but in our hands a peptide-generated antiserum recognized mainly a 122 kDa band.[3,13] This is close to the estimated size of Ams1 suggesting that there is no proteolytic activation of Ams1 taking place upon entry into the vacuole. The apparent absence of a Pep4-dependent molecular weight shift upon vacuolar delivery agrees with previous studies indicating that Ams1 activity is Pep4-independent.[13] Study of the vacuolar import of Ams1 is therefore much more tedious than for prApe1, also because the synthesis of Ams1 is low during normal growth conditions. As with prApe1, Ams1 exists as an oligomer. In glycerol velocity gradients Ams1 peaked at 450-669 kDa suggesting that the oligomeric form of Ams1 contains 4 to 6 subunits. Fluorescent tagging suggests that the Ams1 oligomer ultimately localizes to the Ape1 complex.[7]

The Cvt Pathway Resembles Autophagy

Our lab's initial characterization of the transport of prApe1 showed that the protein traffics independently of several gene products essential for transport through the endoplasmic reticulum and Golgi complex.[1] Temperature sensitive mutants of the COPII proteins Sec12 and Sec23, the SNARE priming proteins Sec18 and Sec1, as well as the guanine nucleotide exchange factor Sec7 showed relatively normal maturation of prApe1 at the restrictive temperature. Furthermore, prApe1 transport was normal in the presence of tunicamycin, a glycosylation inhibitor perturbing transport through the Golgi apparatus. Finally, newly synthesized prApe1 was accessible to exogenous pronase in osmotically-lysed yeast spheroplasts in the absence of detergent and hence had not translocated into a membrane compartment (see below). The half-time for processing of prApe1 has been estimated to be in the range of 25-45 minutes and assumed to be essentially equivalent to the half-time for vacuolar import.

Acknowledging the relatively rapid maturation of prApe1 in wild type cells, our lab generated antiserum against the prApe1 propeptide and used this in a colony overlay assay to identify mutant strains retaining the precursor form of the protein.[2] The initial screen identified *cvt* mutants falling into eight complementation groups, and five of these had mutations in novel genes whose products are not required for transport of proteins to the vacuole through the secretory system. Furthermore, these mutants accumulated cytosolic prApe1. Expansion of the screen resulted in nine more complementation groups, and by scrutinizing the diploid and haploid deletion libraries we have identified additional *cvt* mutants bringing the current total up to more than twenty.[14,15]

To determine if the *cvt* mutants were unique we crossed them with various mutants that were known to affect protein delivery to the vacuole, and analyzed the diploids for complementation of the prApe1 accumulation defect. A small number of mutants showed overlap with the *vps* mutants that are defective in the ALP, CPY and Mvb pathways.[2] This result was not surprising because in some cases the *vps* mutants are severely defective in vacuole morphology and lack a normal target for prApe1 delivery. There was no overlap with *end* mutants that are blocked in endocytosis. The major pathway for the transport of cytosolic cargo destined for degradation in the vacuole is by autophagy.[16] Accordingly, we crossed the *cvt* mutants with *aut* mutants and *apg* mutants defective in starvation induced autophagy and found considerable overlap among these mutants.[14,17-19] An electron dense complex containing prApe1 could be seen by immunoelectron microscopy to be enwrapped within a double-membrane vesicle further confirming the autophagic nature of the Cvt pathway.[8,20] This cytosolic vesicle subsequently fuses with the vacuole resulting in an intravacuolar single-membrane bound Cvt body. The membrane of the Cvt body is next degraded and prApe1 released and matured by the vacuolar proteinases.[20] This mode of transport allows prApe1 to retain its dodecameric conformation during the vacuolar import process, which may be essential for the stability of mature Ape1 in the vacuole.[6]

The complementation analysis showed that almost all *APG* and *AUT* genes required for autophagy are also required for the Cvt pathway. However, while autophagy is a degradative

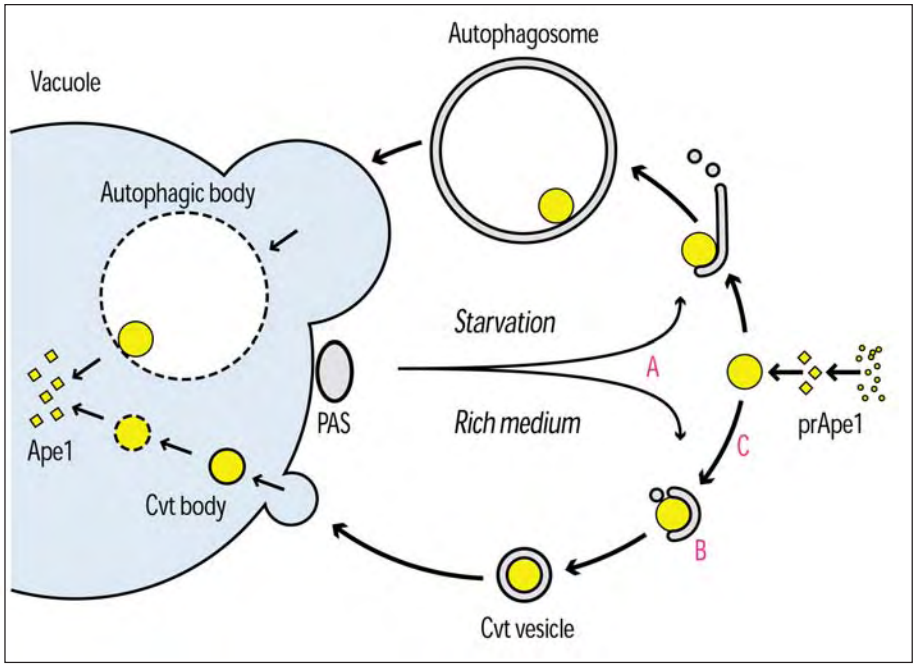

Figure 3. The Cvt pathway and autophagy. A schematic illustration of the two pathways used to transport prApe1 to the vacuole. The Cvt pathway is biosynthetic while autophagy is degradative. Both pathways transport prApe1 to the vacuole, while autophagy also delivers bulk cytoplasm for degradation and recycling. A. The transition between the pathways is regulated by the nutrient availability affecting the activity of Tor kinase complex 1 resulting in alterations in the phosphorylation pattern of Apg1 and Apg13. This in turn leads to changes in the switching complex as depicted in Figure 5. B. The Cvt vesicle is substantially smaller than an autophagosome. This appears to be due in part to the synthesis level of Aut7, which is enhanced during starvation. Aut7 is recruited to the membrane through conjugation to PE as depicted in Figure 6. C. Cargo of the Cvt pathway is localized to and sequestered within the forming vesicle through the action of the Cvt19 receptor, the Cvt9 tether and Aut7, as depicted in Figure 4. In both the Cvt pathway and autophagy, the PAS has been proposed to play a crucial role, either as a source of membrane or as an assembly point for the autophagic machinery. The increasing number of gene products shown to be specific to either the Cvt pathway or autophagy could suggest that there are differences with regard to the origin of the sequestering membrane and/or the mechanism of vesicle formation.

pathway initiated upon starvation, the transport of prApe1 and Ams1 to the vacuole is a constitutive biosynthetic pathway. This suggests that the common gene products constitute the basic vesicle forming machinery. The Cvt pathway is also highly selective while autophagy has been shown to randomly sequester cytosolic enzymes and organelles.[21,22] Electron microscopy revealed that the Cvt vesicles seem to exclude normal cytosol.[8,20] These vesicles have a diameter of approximately 150 nm, significantly smaller than the 300-900 nm sized autophagosomes. However, it is also clear that prApe1 may be transported to the vacuole by autophagy (Fig. 3). Several mutants that are defective in transport of prApe1 in rich media but display normal starvation induced autophagy will mature prApe1 upon starvation.[15,23,24] Electron microscopy has revealed that prApe1 under starvation conditions travels to the vacuole in an autophagosome along with sequestered cytosol.[8]

The vesicular nature of prApe1 transport provides a convenient assay to determine whether a *cvt* mutant is deficient in Cvt vesicle formation or in fusion of a completed vesicle with the vacuole.[20] While the mature form of dodecameric Ape1 is highly resistant to degradation by

proteases, the propeptide of precursor Ape1 is readily accessible to exogenous proteinase K in lysed spheroplasts. Protease treatment of osmotically lysed spheroplasts will therefore result in a molecular weight shift of prApe1 if the sequestration process is inhibited. This shift can easily be detected by Western blotting. Precursor Ape1 that is enclosed within a completed cytosolic vesicle will be protected from the exogenously added protease. It should be noted, however, that care has to be taken to ensure that the vacuole and putative Cvt vesicles/autophagosomes are not lysed in the process, and proper controls therefore have to be included.

Cvt19 Orchestrates the Formation of the Cvt Complex

The fact that more genes are required for specific autophagy of prApe1 and Ams1 compared to starvation-induced autophagy reflects the increased complexity inherent in a selective process. The purpose of autophagy that is initiated upon nutrient limitation is to generate building blocks that the cells can utilize as part of an adaptive response. Autophagy is hence a random bulk process that under normal circumstances will not deplete a particular cellular component. The Cvt pathway faces the opposite challenge, to transport all newly synthesized prApe1 and Ams1 to the vacuole in a timely manner, and this requires several proteins that participate in the recruitment process. Overproduction of prApe1 from a high-copy plasmid results in accumulation of a cytosolic pool of prApe1, showing that there is a rate-limiting step in constitutive prApe1 sequestration.[19] This could be due to a limitation in the vesicle forming machinery during nutrient rich conditions, or due to saturation of a vital component involved in recruitment.

The limitation in prApe1 transport may be overcome by starvation, except in the mutant *cvt19*.[25] Unlike all the other Cvt proteins that mediate the specificity of the Cvt pathway, Cvt19 is also required for sequestration of prApe1 during starvation, even though non-selective autophagy appears to be normal in cells lacking this component. However, upon long-term starvation of *cvt19*Δ cells, a small amount of mature Ape1 can be detected consistent with a non-selective autophagic uptake. Cvt19 therefore has some of the characteristics of a potential receptor, and physical interaction of Cvt19 with prApe1 was demonstrated by two-hybrid interaction and co-immunoprecipitation.[25,26] The direct interaction of Cvt19 with prApe1 does not require other proteins or post-translational modifications, as in vitro translated prApe1 also was co-immunoprecipitated with bacterially expressed Cvt19.[25] Initial reports indicated that Cvt19 interacted either with the propeptide of prApe1, or with the mature protein.[25,26] However, more recent data from our lab suggests that putative two-hybrid interactions of Cvt19 with cytosolic Ape1 lacking the propeptide in wild type cells is indirect; this interaction most likely reflects the formation of hetero-oligomers between endogenous prApe1, which is capable of interacting with Cvt19, and the propeptide-deficient construct.[7] Cvt19 is also required for vacuolar import of Ams1, and we have recently been able to verify that Cvt19 is able to co-immunoprecipitate Ams1 and also interacts with Ams1 using the two-hybrid assay.[7,25] In cells lacking Cvt19, a fluorescent prApe1 construct containing the propeptide will still form an Ape1 complex. In contrast, in the absence of Cvt19 a fluorescent Ams1 construct will display a diffuse cytoplasmic staining.[7] In order to accommodate the transport of two different proteins, it appears that Cvt19 has two different binding sites for its substrates. Precursor Ape1 binds to a coiled-coil domain situated centrally in Cvt19 between amino acids 152-191. The binding site for Ams1 has not been completely defined but requires the amino acids following the coiled-coil domain. Cvt19 may therefore potentially bind the two proteins at the same time. The resulting structure containing prApe1, Ams1 and Cvt19 is termed the Cvt complex (Fig. 4). A consequence of Cvt complex assembly is that prApe1 and Ams1 are transported to the vacuole in a shared Cvt vesicle.

Unlike traditional receptors, Cvt19 does not have a trans-membrane domain, but instead behaves like a peripheral membrane protein.[25] Tagging with GFP reveals that a substantial amount of Cvt19 can be seen within the vacuole in wild type cells while a fainter staining can be detected at a perivacuolar structure. In contrast, the protein co-localizes exclusively with

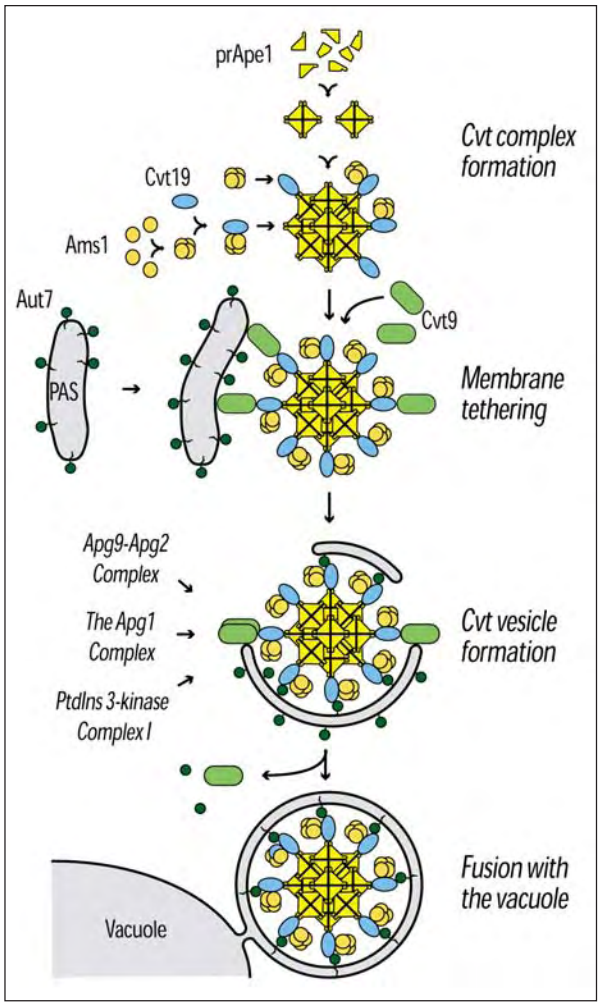

Figure 4. Cvt vesicle formation. Precursor Ape1 dodecamers aggregate into an Ape1 complex, and this complex is bound by the soluble receptor Cvt19. Cvt19 also binds α-mannosidase oligomers and the resulting structure is called a Cvt complex. The Cvt complex next associates with the sequestering membrane by the interaction of Cvt19 with Cvt9 and Aut7. The VFT complex may possibly also play a role in this membrane tethering event. The formation of the Cvt vesicle involves a sorting event whereby Cvt9 is released and Aut7 sorted to the inside of the Cvt vesicle. This sorting and the homotypic fusion event taking place requires the Apg9-Apg2 complex, the Apg1 protein kinase switching complex and PtdIns 3-kinase complex I. These protein complexes are not associated with the completed Cvt vesicle. The outer membrane of the Cvt vesicle finally fuses with the vacuolar membrane. PAS, pre-autophagosomal structure.

prApe1 to a perivacuolar structure in cells defective in transport of prApe1.[7] Pulse-chase experiments furthermore show that in wild type cells Cvt19 is transported to the vacuole and degraded with kinetics similar to that of maturation of prApe1, with an estimated half-time of 25-60 minutes.[25] However, both the punctate localization of Cvt19 as well as its transport to the vacuole is entirely dependent upon prApe1. In cells lacking prApe1, Cvt19 still binds Ams1, but without the prApe1 complex, the Cvt complex is unable to form.[7] As a consequence, cells lacking prApe1 are unable to deliver Ams1 and Cvt19 to the vacuole. A scheme for the assembly of the Cvt complex is depicted in the upper half of Figure 4.

The vacuolar degradation of Cvt19 prohibits receptor recycling and the protein has therefore to be synthesized at a rate matching the production of prApe1. Transcriptional regulation of *APE1* and *CVT19* also appear to be similar, as the synthesis of the corresponding proteins is induced three- to four-fold upon one hour of nitrogen starvation and more than ten-fold after two hours of starvation.[1,25] The increased synthesis of prApe1 upon starvation may reflect the need for increased vacuolar protease activity during starvation due to the high input of proteolytic substrates by autophagy.

The Cvt Complex Localizes to the Pre-Autophagosomal Structure (PAS)

Precursor Ape1, Cvt19 and Ams1 all accumulate in cells deficient in the Cvt pathway at a location proximal to the vacuole, at a structure that has recently been termed the pre-autophagosomal structure, or PAS.[7,27,28] This structure may be of similar character as the phagophore seen in mammalian cells and which has been proposed to represent the sequestering autophagic membrane at an initial stage of autophagosome formation (also called the "isolation membrane" or "cup-shaped structure;" see chapter 2).[29] Alternatively, the PAS could represent a compartment where the autophagic membrane (or phagophore) is originating. In addition to prApe1, Cvt19 and Ams1, most other autophagy components have also been localized to the PAS. This includes the membrane protein Apg9 and components of the protein and lipid conjugation cascade (Apg5, Apg12 and Aut7, see below).[27,28] The serine/threonine protein kinase Apg1, and Apg14, which is a component of the phosphatidylinositol (PtdIns) 3-kinase complex I (see below and chapter 6), have been shown to be present at the PAS.[27,28] Several pathway-specific proteins have also been localized to the PAS. These include Apg17, which is only required for starvation-induced autophagy, and the proteins Cvt9, Cvt13 and Cvt20, which are required for both the Cvt pathway and for the selective autophagy of peroxisomes (pexophagy) but not for bulk autophagy.[15]

With the exception of Aut7 and Cvt9, it seems that only a minor fraction of most autophagy proteins are heavily represented at the PAS during growth in rich medium. The vast majority of Apg1, Apg2, Apg5, Apg12, Cvt13 and Cvt20 display a diffuse cytosolic localization by fluorescent microscopy. The membrane protein Apg9 has only a minor fraction represented at the PAS in rich medium, while the majority is localized to disperse membrane structures. These membranes are of unknown origin and do not seem to be associated with known endomembrane compartments.[27,28,30] An increase in PAS localization can be seen for several of the Cvt and Apg proteins upon starvation or after treatment with the drug rapamaycin. Rapamycin simulates starvation by forming a toxic complex with the protein FKBP12 that binds and inhibits the protein kinase complex TORC1 (see chapters 3 and 5).[31] The increased PAS localization of many autophagy proteins can also be seen in several Cvt pathway-specific mutants and in several autophagy mutants.

Subcellular fractionation using isotonic density gradients separates the autophagic proteins into at least two different populations.[28] It is therefore possible that the PAS is not a traditional membrane compartment but rather an assembly point for the autophagic membrane and the autophagic machinery. Apg1, Apg2 and Cvt9 peak with the membrane protein Apg9 at high density, while other proteins including Cvt19, Apg5 and Apg12 peak at a somewhat lower density that is similar to the density of the completed Cvt vesicle. Completed autophagosomes, on the other hand, have a much lower density.[28,32,33] Subcellular fractionation using OptiPrep and sucrose density gradients separate PAS marker proteins from plasma membrane proteins (Pma1), vacuolar membrane proteins (Pho8), Golgi proteins (Pep12 and Kex2) and ER marker proteins (Dpm1).[28,30,32] Although the PAS is localized close to the vacuole, it does not stain with the dye FM 4-64, which is taken up by endocytosis and labels the endocytic-vacuolar compartment.[27,28] The PAS therefore does not seem to be in equilibrium with the endocytic or vacuolar compartment despite the proximity to these structures. We have also not been able to detect by fluorescent microscopy endocytic cargo proteins (Ste2) or endocytic marker proteins (Vps4, Snf7 and Vps10) at the PAS.

With the exception of Aut7, none of the proteins required for the formation of the Cvt vesicles and autophagosomes seem to be associated with the completed vesicles.[34,35] This is particularly intriguing in the case of the membrane protein Apg9. If Apg9 is supplying the sequestering membrane, there must be a mechanism retaining Apg9 or retrieving it so that it is prevented from ending up on the completed vesicle. Alternatively, Apg9 may be an assembly point for the autophagic vesicle-forming machinery. We have been able to show by affinity isolation experiments that there is an interaction between Apg9 and Apg2 that may confirm the subcellular fractionation data.[32] Localization studies using fluorescent Apg2 and Apg9 sug-

gest that this interaction occurs mainly at the PAS, as the dispersed Apg9 membranes do not seem to stain with Apg2. Apg9 is also required for the localization of Apg2 to the PAS.[36] Apg2 has a molecular mass of 180 kDa, which makes it the largest of proteins known to be required for the Cvt pathway and autophagy. The function of Apg2 is unknown and the protein does not contain any prominent domains. Presumably, Apg2 functions late in the sequestration event, as the localization of Apg2 to the PAS requires Apg1, Apg14 and Cvt18 in addition to Apg9.[32,36,37]

Membrane Tethering Events of the Cvt Pathway Involve Cvt9 and the VFT Complex

Cvt9. Because the prApe1 receptor Cvt19 is not a membrane protein, a mechanism is required for the association of the Cvt complex with the sequestering membrane or PAS. In cells lacking prApe1, neither Cvt19 nor Ams1 is seen at the PAS. This suggests that the receptor does not self-associate with the membrane even though it still associates with one of the cargo molecules. Instead, Cvt9 was shown to be a protein required for the stable membrane association of prApe1.[24] In cells lacking Cvt9, fluorescent prApe1, Cvt19 and Ams1 still colocalize showing that the Cvt complex is able to form. However, in the absence of Cvt9 the Cvt complex is not membrane-associated. Furthermore, the Cvt complex is not contained at a perivacuolar location and does not colocalize with other autophagy proteins such as Aut7, which predominantly localizes to the PAS.[7] Cvt9 therefore exhibits some of the properties of being a tethering factor or a motor protein bringing the Cvt complex to the sequestering membrane, or vice versa. Structurally, Cvt9 also resembles the Golgi tethering factor Uso1 and large contractile proteins like the myosins. Cvt9 has a large centrally placed coiled-coil domain that may function as an assembly point for other proteins. We have recently been able to show that Cvt9 binds Cvt19 by both the two-hybrid assay and by affinity isolation experiments. The binding site was mapped to eight amino acids near the extreme C terminus of Cvt19, well separated from the binding site for prApe1 and Ams1 (Fig. 4).[7] Interestingly, Cvt9 is not only required for the uptake of the Cvt complex, but is also essential for autophagy of peroxisomes after shifting from a medium based on oleic acid as the sole carbon source to glucose-containing starvation medium (see chapters 10 and 11).[24] As Cvt19 is expendable for pexophagy, Cvt9 must be binding to a different receptor-like protein when exerting membrane tethering to peroxisomes. Cvt9 is not well preserved among different species, but in collaboration with the Dunn lab we found Gsa9 as a homologue in the yeast *P. pastoris.* Fluorescent Gsa9 was shown to localize to the vacuolar membrane and to be required for microautophagy of peroxisomes. When cells grown on methanol as the sole source of carbon were shifted to glucose-containing medium, Gsa9 could be seen to mediate tethering of the vacuolar membrane to the peroxisomes (see chapter 10).[24]

While Cvt9 is required for autophagy of peroxisomes during starvation conditions, it is not essential for uptake of prApe1 under these same conditions. Cells lacking Cvt9 accumulate precursor Ape1 in rich media, but a substantial portion of prApe1 is transported to the vacuole and matured within a few hours following nitrogen removal. In contrast, in cells lacking the Cvt19 receptor, only a minor fraction of prApe1 is matured even after 24 hours of starvation, and the amount of mature Ape1 corresponds to a totally random uptake by non-selective autophagy in these cells.[25] There must therefore be an additional factor involved in the membrane tethering that can compensate for the loss of Cvt9 during starvation. This factor is most likely the small ubiquitin-like protein Aut7. We have verified by two-hybrid analysis and affinity isolation experiments that Aut7, in addition to prApe1, Ams1 and Cvt9, binds to Cvt19.[7,28] The binding site is contained in the amino acids following the Cvt9 binding site at the extreme C terminus of Cvt19, and the last six amino acids appear to be particularly important. Cvt19 may therefore be able to bind four proteins at the same time, although it has not yet been shown that Cvt9 and Aut7 are bound simultaneously. There is also a significant difference between these two proteins. Aut7 is transported to the vacuole inside the Cvt vesicle, while

Cvt9 appears not to be associated with the completed vesicle. Unlike Gsa9, the homologue of Cvt9 in *P. pastoris*, Cvt9 does therefore not end up in the vacuole.[24,34,35] Accordingly, there seems to be a sorting mechanism during the sequestration event whereby Cvt9 is kept from entering the inside of the forming vesicle, while Aut7 is either passively allowed to enter or actively sorted to the inside through the interaction with Cvt19. As discussed below, this sorting may be facilitated by other Cvt-specific proteins like Cvt13 and Cvt20, in conjunction with phosphoinositides.[15] The function of Cvt9 and Aut7 in the formation of the Cvt vesicle is illustrated in Fig. 4.

Because cells lacking Cvt9 do not degrade peroxisomes upon shift from oleic acid medium to glucose-containing starvation medium, it is possible that a putative peroxisome receptor does not interact with Aut7 directly and that the Cvt9 tethering step therefore cannot be bypassed in these cells during starvation. So far, however, we have not been able to detect any interaction between the putative pexophagy tag Pex14 (see chapter 11) and Cvt9 (W.-P. Huang and D.J. Klionsky, unpublished results).

The VFT complex. In addition to Cvt9, we have recently been able to show that another tethering complex is also important for the Cvt pathway while being less important for non-selective autophagy.[38] Screening of a yeast deletion library for strains defective in prApe1 maturation revealed a mutant, *cvt22*, lacking an uncharacterized gene encoding a small coiled-coil protein. In addition to accumulating prApe1 during growth in rich media, the *cvt22* mutant has a fragmented vacuole and displays defects both in the CPY pathway and in the recycling of the SNARE Snc1 to the plasma membrane. The phenotype of the *cvt22* mutant therefore appears to be similar to, but somewhat less severe than, the phenotype of mutants lacking components of the VFT (Vps Fifty-Three) complex. This complex has been shown to include the proteins Vps52, Vps53 and Vps54.[39] By affinity isolation experiments we were able to confirm that the protein lacking in the *cvt22* mutant is the fourth subunit of this complex, Vps51.[38,40] Vps52, Vps53 and Vps54 have been shown to form a stable, stoichiometric complex and the absence of any of these components leads to the destabilization and degradation of the other two.[39] Deletion of *VPS51*, however, does not destabilize the other three subunits of the VFT complex and this may account for the slightly less retarded growth phenotype and fragmented vacuole of this mutant.[38] The C terminus of Vps51 contains the coiled-coil domain and this domain is essential for the binding of Vps51 to the other VFT components.[40] All components of the VFT complex hence contain amphipathic coiled-coil domains that function in complex formation. Vps51 is furthermore the subunit that binds the VFT complex to the t-SNARE protein Tlg1 through the conserved 30 N-terminal amino acids, while Vps52 is the subunit that binds the GTP bound form of the small rab protein Ypt6.[40] Although mutants of the VFT complex show rapid maturation of prApe1 upon nitrogen removal, starvation induced autophagy is somewhat reduced in these cells. Electron microscopy revealed that the autophagosomes formed in cells lacking either Vps51 or Vps52 were smaller than autophagosomes formed in wild type cells.[38]

While it is not yet clear if Ypt6 is pivotal for the Cvt pathway, both Tlg1 and its t-SNARE partner Tlg2 are required for the sequestration of prApe1 in rich media, and so is the SM-protein (Sec1/Munc18-like protein) Vps45.[41] Tlg2 complexed with the two light chains Tlg1 and Vti1 functions as a t-SNARE in endocytosis as well as in homotypic fusion within the *trans*-Golgi network.[42,43] It has also been shown that Vti1 is required for the maturation of prApe1.[44] However, it is not clear whether Vti1 is required for the formation of the Cvt vesicle or for the fusion of the Cvt vesicle with the vacuole. Vti1 forms a complex with Vam3 in homotypic vacuole fusion and in fusion of endosomes and autophagosomes with the vacuole, and is not required for the formation of prApe1-containing autophagosomes upon nutrient deprivation.[33,45] These data suggest that Vti1 may play a role in the Cvt pathway in the final fusion event with the vacuole.

Tlg2 was originally shown to co-fractionate with Cvt vesicles on Nycodenz density gradients and therefore was proposed to be a SNARE involved in the fusion step leading to the

formation of the Cvt vesicle.[41] However, using fluorescent tagged proteins, we have not been able to visualize any fraction of Tlg2, Tlg1 and components of the VFT complex at the PAS as judged by co-localization with Aut7, Cvt19 or prApe1. It may therefore be that these proteins are involved in a vesicle transport step leading up to the PAS. Alternatively, there may be a recycling transport step from the PAS or the sequestering membrane to the *trans*-Golgi network. Along these lines, the VFT complex has been shown primarily to function in recycling from endosomes to the *trans*-Golgi network.[39] In VFT mutants, Aut7 does not colocalize with Cvt9, Cv19 and prApe1 to the Cvt complex during growth in nutrient rich medium.[38] This could mean that the VFT complex is required in addition to Cvt9 for the tethering of the Cvt complex to the PAS. Alternatively, there may be a transport step to or from the PAS that is abolished in the VFT mutants or in the associated *tlg1*, *tlg2* and *vps45* mutants, which leads to mislocalization of Aut7. The VFT-sensitive step is bypassed upon starvation and Aut7 gains correct localization at the PAS together with prApe1. The uptake of prApe1 to the vacuole is therefore normal in VFT mutants in nutrient-limiting conditions. The bypass upon starvation may be the reason why the VFT complex is not required for pexophagy upon shift from oleic acid-containing medium to glucose-containing nitrogen starvation medium.[38]

The Components of the Cvt Vesicle Forming Machinery

The Apg1 complex. Exactly how Cvt9 binds to the autophagic membrane or PAS is not yet known, but we have been able to show that overexpression of Cvt9 may increase the localization of Apg9 at the PAS. This suggests that there may be a functional relationship between these two proteins although a direct interaction has not yet been confirmed.[28] However, it has been shown by the two-hybrid assay and affinity isolation experiments that Cvt9 interacts with the serine/threonine protein kinase Apg1.[24] Apg1 further interacts with Apg17 and Apg13, and Apg13 interacts with Vac8, and this putative protein complex has generally been referred to as the "switching complex." The switching complex is believed to regulate the transition from the Cvt pathway to autophagy upon starvation.[23] The transition is regulated by dephosphorylation resulting from inhibition of the protein kinase complex TORC1 by starvation or by rapamycin, which leads to a removal of phosphate groups on Apg1 and Apg13.[46] Dephosphorylated Apg1 and Apg13 have increased affinity for each other and the result may be the assembly of two subcomponents. One of the putative subcomponents consists of Apg1 complexed with Cvt9 and Apg17, the other of Apg13 bound to Vac8. The assembled complex is required for autophagy and may be large, as Cvt9 is also able to interact with itself (Fig. 5).[24] An intriguing feature of this complex is that the only protein component that is entirely required for both the Cvt pathway and autophagy is Apg1. As discussed above, Cvt9 is only required for uptake of prApe1 in rich conditions while bulk autophagy is essentially normal without Cvt9. In contrast, cells lacking Apg17 are entirely blocked in bulk autophagy while the transport of prApe1 is normal. Cells lacking Apg13 are completely blocked in bulk autophagy, but also have a partial block in prApe1 uptake in rich media. Cells lacking Vac8, on the other hand, have a severe, but not complete, reduction in both prApe1 uptake in rich medium as well as in bulk autophagy. Cells lacking Apg13 or Vac8 will still mature prApe1 upon nitrogen starvation.

Apg1 and Apg13 were originally thought to be regulators or initiators of autophagy.[47-49] Lack of Apg13 could be suppressed by overexpression of Apg1, and it has been shown in vitro using the artificial substrate myelin basic protein that Apg13 increases the kinase activity of Apg1.[46,47] It was therefore assumed that high Apg1 kinase activity was required for autophagy but not for the Cvt pathway. However, an endogenous substrate of Apg1 has not yet been found other than that Apg1 is able to autophosphorylate in vitro. This ability to autophosphorylate is reduced when Apg1 is extracted from starved cells.[48] Observations from the yeast *P. pastoris* suggest that the Apg1 homologue Gsa10 is not required for initiation of microautophagy of peroxisomes upon shift from methanol to glucose medium, but rather for an expansion step (see chapter 10).[50] Recent experiments from our lab confirm a non-regulatory

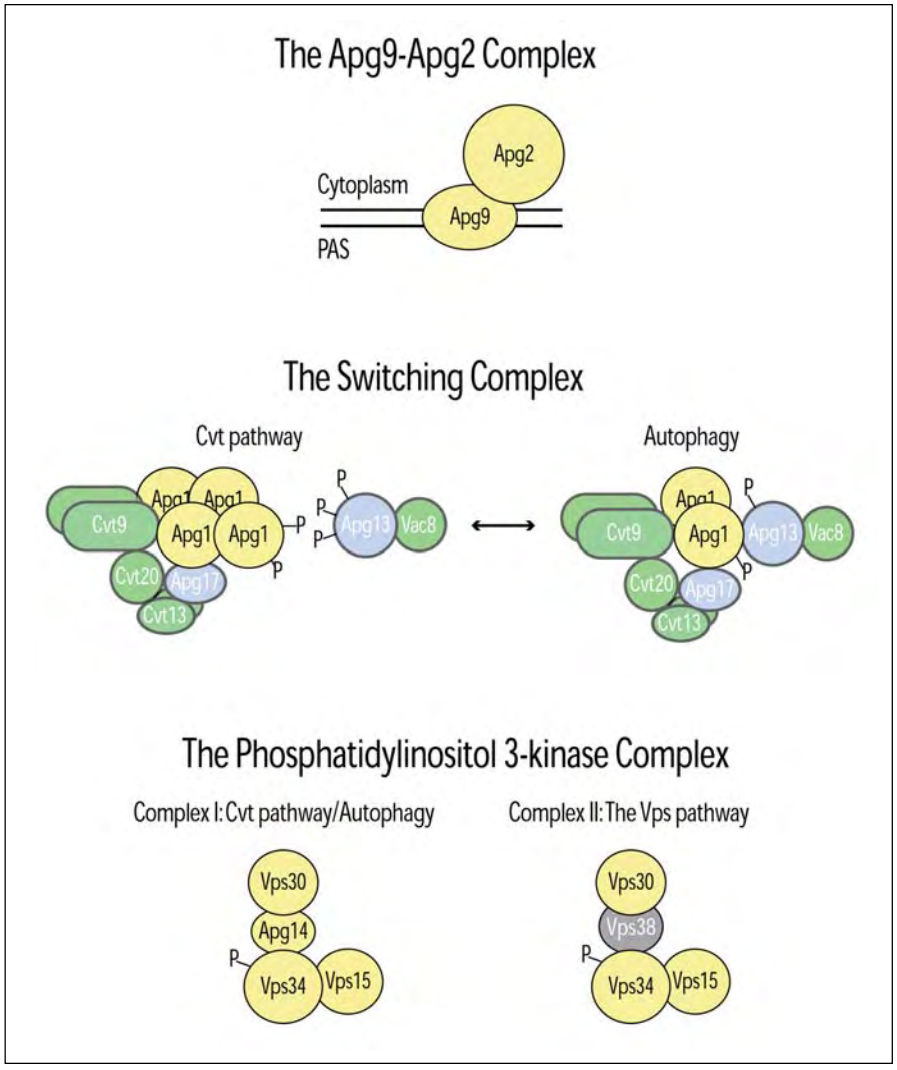

Figure 5. Protein interactions required for the Cvt pathway. Several of the proteins required for the Cvt pathway and autophagy have been shown to interact by affinity isolation experiments and two-hybrid analyses. Apg2 is pulled down with the membrane protein Apg9. Fluorescent microscopy suggests that this interaction predominantly occurs at the PAS, although the function is not clear. The protein kinase Apg1 is the central component of the putative switching complex. This complex was so named because it is believed to be a determinant in the switch from the Cvt pathway to autophagy upon nutrient deprivation. Concomitant with starvation, dephosphorylation of Apg1 and Apg13 results in the assembly of two components along with the dissociation of tetrameric Apg1 into dimeric Apg1. The switching complex contains a number of proteins that are specific either for the Cvt pathway or for autophagy. The PtdIns 3-kinase complex I is required for the association of several proteins with the PAS. The only component of this complex that is specific for the Cvt pathway and autophagy is Apg14. A similar complex where Apg14 is replaced with Vps38 is required for sorting of proteins at the TGN that are going to the vacuole and is referred to as PtdIns 3-kinase complex II. Proteins mainly required for the Cvt pathway are colored green, proteins that are more important for autophagy than the Cvt pathway are colored blue, while proteins that are absolutely required for both pathways are yellow.

role for Apg1 also in autophagy in *S. cerevisiae*. The ATP binding site of the kinase domain of Apg1 was mutated to allow binding and inhibition by 1-NA-PP1.[51] 1-NA-PP1 is a bulkier, cell-permeable analogue of the non-specific protein kinase inhibitor PP1 (4-amino-1-tert-butyl-3-phenylpyrazolo[3,4-*d*] pyrimidine) that is only active at ATP binding sites where a methionine is replaced with a smaller amino acid such as alanine.[52] The inhibitor not only selectively abolished the autophosphorylation of the mutated form of Apg1 in vitro; it also blocked the uptake of prApe1 in vivo in rich medium.[51] However, the block in prApe1 uptake was reversed upon starvation even in the presence of the specific inhibitor, and bulk autophagy was found to be normal. These data suggest that the kinase activity of Apg1 may be more important for the Cvt pathway than for autophagy, and that the role of Apg1 in autophagy may rather be of a structural nature. Concomitant with the shift to starvation, a structural change was seen for Apg1 using sucrose velocity gradient centrifugation that could correspond to a shift from a tetrameric to a dimeric Apg1 complex. A complex corresponding to the putative switching complex could not be detected using velocity centrifugation, nor could components of the switching complex be detected by silver staining following affinity isolation experiments using protein A-tagged Apg1. This suggests that the switching complex may be a series of functional, dynamic protein interactions at the PAS or at the site of vesicle formation rather than a stoichiometric unit. This view is also supported by the fluorescent data showing that only a minor fraction of Apg1 seems to be present at the PAS. The interaction of Apg1 with Cvt9 could be to ensure proximity of Apg1 and the associated switching complex proteins to the Cvt complex during growth in rich media. The composition of the Apg1 switching complex is illustrated in Figure 5.

The function of the other Apg1-associated proteins is even less obvious. Apg17, which does not seem to play a role in the Cvt pathway, has come out in two-hybrid screenings as a protein with a range of possible binding partners. With one exception, as described below, it is unclear how meaningful these putative interactions may be. Vac8, on the other hand, is a palmitoylated and myristoylated armadillo repeat protein with a documented role in vacuole inheritance and in homotypic vacuole fusion.[53,54] In the latter process it has been shown that Vac8 functions after the docking stage and that the lipidation is essential.[32,55] However, lipidation of Vac8 is not required for its role in the Cvt pathway.

Phosphatidylinositol 3-kinase complex I. In addition to the putative Apg1 protein kinase complex, a lipid kinase complex is essential for both the formation of Cvt vesicles and autophagosomes. This complex consists of the PtdIns 3-kinase Vps34 associated with the protein kinase Vps15 in addition to Vps30/Apg6 and Apg14, and is referred to as PtdIns 3-kinase complex I (see Fig. 5 and chapter 6).[56] Of these components, only Apg14 does not play a role in other transport pathways but is specific for the Cvt pathway and autophagy. A similar complex where Apg14 is substituted with Vps38 is referred to as PtdIns 3-kinase complex II. This complex is vital for transport of proteins from the *trans*-Golgi network to the vacuole through the CPY pathway. Complex II is assumed to generate the majority of the PtdIns(3)P in the cells, although some of this lipid is also found in cells containing only the Vps34 and Vps15 core components. The cellular contribution of PtdIns(3)P by the Cvt pathway- and autophagy-specific complex has been shown to be almost undetectable.[56]

PtdIns 3-kinase complex I seems to be important for several steps in the formation of Cvt vesicles and autophagosomes. It has been shown that both the recruitment of Aut7 as well as Apg2 to the PAS is deficient in cells lacking Apg14.[27,36] These recruitment events are independent of each other, as Apg2 localizes to the PAS in cells lacking Aut7, and vice versa.[36] Although it has not been proven that the mislocalization of Apg2 and Aut7 in an *apg14* mutant is due to the absence of PtdIns(3)P at the PAS, we have been able to show that a fraction of Apg14 colocalizes with Cvt9 at this location.[28] Intriguingly, none of these proteins contain recognizable phosphoinositide binding domains, so it is possible that other proteins mediate the targeting. One such candidate is the small trans-membrane protein Etf1.[57] Etf1 contains a basic hexapeptide that has been shown to bind PtdIns(3)P. Mutations in the hexapeptide that

abolishes the binding of Etf1 to PtdIns(3)P result in a defect in the transport of prApe1. However, Etf1 is not required for starvation-induced autophagy. Two other possible candidates that are currently under scrutiny are the two homologous proteins Aut10/Cvt18 and Mai1/Cvt21.[37,58,59] These two proteins display a vacuolar membrane localization that is dependent upon Vps34, although they do not contain traditional lipid binding domains. The function of these proteins is completely unknown except that the localization of Apg2 to the PAS seems to require Cvt18.[37] While Cvt18 is required for the Cvt pathway, bulk autophagy and pexophagy, Cvt21 is only required for the Cvt pathway.

PtdIns(3)P is important for a number of intracellular sorting and targeting events through proteins that have FYVE or PX domains that specifically bind to this lipid. By screening of a deletion library, we found that two mutants lacking putative members of the sorting nexin family were defective in the Cvt pathway. These mutants had wild type capacity for starvation-induced autophagy.[15] One of the mutants was allelic to *cvt13* and the other was named *cvt20*. Both mutants lack proteins containing a PX domain that has a specific but rather moderate to low affinity for PtdIns(3)P.[15,60] The PX domain is essential for the activity of Cvt13 and Cvt20 in the Cvt pathway, and also for the localization of these proteins to the PAS. However, while mutation in the PX domain in one protein results in a predominantly cytosolic localization of the mutated protein, these cells retain a partially active transport of prApe1. Mutation within the PX domain of both proteins appears to be required for the inactivation of the Cvt pathway. These data, combined with affinity isolation experiments showing that Cvt13 and Cvt20 physically interact, led us to propose that a putative heterodimer would increase the affinity of the complex for PtdIns(3)P. Alternatively, this complex may contain more than two components, as we were also able to show by affinity isolation that Cvt13 interacts with itself. PtdIns(3)P at the PAS was further assumed to be important for correct localization, as these proteins did not colocalize with Cvt19 in cells lacking Apg14. Instead, Cvt13 and Cvt20 displayed a localization corresponding to endosomes or a pre-vacuolar compartment (PVC) in these cells. These data, together with the localization of Apg14 suggest that PtdIns(3)P is present at the PAS where it may be essential for several independent events. While the exact function of Cvt13 and Cvt20 is not known, members of the nexin family have been suggested to play a role in protein sorting. At the multivesicular body (MVB) or endosome, PtdIns(3)P binding proteins are responsible for sorting of the PtdIns(3)P itself as well as selected proteins into the lumen of the endosome (see chapter 14). The observation that the localization of Cvt13 and Cvt20 to the PAS also requires Cvt9, and that deletion of Cvt13 and Cvt20 did not result in aberrant localization of other proteins normally present at the PAS, led us to hypothesize that they could be involved in a PtdIns(3)P-dependent sorting step whereby the Cvt complex with Cvt19 and Aut7 is sorted into the Cvt vesicle while the putative tethering factor Cvt9 is retained. Cvt13 and Cvt20 are also required for pexophagy, where Cvt9 presumably performs a similar tethering function and a homologous sorting event would be required. As mentioned above, Cvt13 interacts with Apg17 by the two-hybrid assay, and we were also able to show that these proteins interact by affinity isolation experiments.[15] This puts the Cvt13-Cvt20 complex functionally together with the putative Apg1 complex and could furthermore suggest that this complex is only fully assembled at the PAS or at the site of vesicle formation (see Fig. 5).

The conjugation cascade. Another set of components that is absolutely required for the formation of both Cvt vesicles and autophagosomes, is a cross-linked protein conjugate consisting of Apg5, Apg12 and Apg16 (Fig. 6).[61,62] Western blotting studies on Apg5 and Apg12 showed that these proteins displayed two molecular weight forms, and the higher molecular weight form was found to be a covalent conjugate of the two monomers.[63] Further molecular characterization revealed that the C-terminal glycine of Apg12 is conjugated to the internal Lys149 of Apg5, and this conjugation requires Apg7 and Apg10 (see chapter 6).[64-67] Apg7 is a protein that is relatively conserved across species and a putative human homologue was one of the first human autophagy proteins to be sequenced.[66] Apg7 contains an ATP binding domain with strong similarity to the ubiquitin activating enzyme E1, and probably functions in a

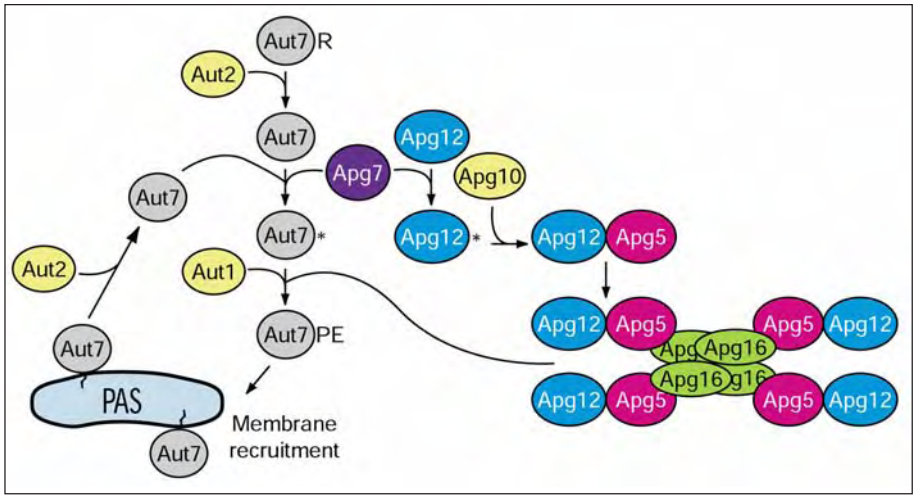

Figure 6. Two conjugation reactions involving ubiquitin-like proteins are required for the Cvt and autophagy pathways. The C-terminal arginine of Aut7 is removed by the Aut2 proteinase and Aut7 is then activated by the E1-like enzyme Apg7. Aut7 is subsequently conjugated to phosphatidylethanolamine (PE) by Aut1 in a reaction that allows its attachment to the membrane. A population of Aut7 is delivered to the vacuole inside of the completed Cvt vesicle or autophagosome while the remainder is removed from the membrane by a second Aut2-dependent cleavage. Apg12 is activated by Apg7 and is subsequently conjugated to Apg5 through the action of Apg10. The Apg12-Apg5 conjugate binds Apg16, which tetramerizes to link four of the conjugates. The Apg12-Apg5·Apg16 complex is needed for stability of the Aut7-PE conjugate. Modified from ref. 98.

homologous manner in the conjugation of Apg12 to Apg5. This involves the activation of the conserved Cys507 of Apg7 by addition of the AMP moiety of ATP, before the AMP is replaced with Apg12 through the carboxyl group of the C-terminal glycine. By replacing the active cysteine with a serine, we were able to detect this intermediate due to the formation of an ester linkage, which is more stable than the normally formed thioester linkage.[66] Apg12 is next transferred to Cys133 of Apg10, which acts as an E2 enzyme, before being conjugated to Apg5. Apg5 further interacts noncovalently with Apg16, which is a small coiled-coil protein that has been shown to dimerize, and this leads to a 350 kDa protein complex.[62]

The formation of the 350 kDa Apg12-Apg5·Apg16 complex is an absolute requirement for Cvt vesicle and autophagosome formation, but it is not yet known in what way. Conjugation of Apg12 to Apg5 appears to occur right after synthesis, and under normal conditions the majority of these proteins exist in a conjugated form.[62] A peptidase that cleaves the conjugate has not been found, suggesting that conjugation is static rather than a dynamic event. The site of conjugation appears to be the cytosol and conjugation is not dependent on the other autophagy proteins such as the membrane protein Apg9, the Apg1 complex, or the PtdIns 3-kinase complex I. In agreement with this localization, Apg7 displays a primarily cytosolic distribution, although localization studies have been somewhat ambiguous. A small fraction of Apg5 and Apg12 is seen at the PAS, however, a similar localization may also be seen for Apg5 in mutants lacking the remaining conjugation machinery.[27] The localization of the 350 kDa complex is probably dependent upon PtdIns(3)P at the PAS as fluorescent protein-tagged Apg5 cannot be seen at this location in mutants lacking Vps30/Apg6. The most intriguing observation on the function of the Apg12-Apg5·Apg16 complex comes from studies on mouse embryonic stem cells, where fluorescent protein-tagged Apg5 can be seen on structures believed to be the forming autophagosomes (see chapter 15).[68] However, upon completion of the vesicle,

GFP-Apg5 is lost from the structure. Although a similar dynamic event has not been observed in *S. cerevisiae*, it places the Apg12-Apg5·Apg16 complex at the site of vesicle formation where a dynamic cascade of events that probably conclude with homotypic vesicle fusion takes place. Electron microscopy of yeast cells that lack Apg5 shows that the Cvt complex is associated with membrane sac structures and small vesicles.[69] A similar membrane association can be seen at the restrictive temperature in cells that harbor a temperature sensitive version of Apg5, suggesting that Apg5 functions at the site of Cvt vesicle formation also in *S. cerevisiae.* Whether the Apg12-Apg5·Apg16 complex binds directly to the sequestering membrane at the site of vesicle formation, or if it first binds to membrane that is subsequently targeted to this site, is not known. Care has to be taken during subcellular fractionation of Apg5, as Apg5 is very "sticky" unless salt is added in the fractionation buffer.[62] Earlier studies therefore concluded that the majority of Apg5 was membrane associated.

The 350 kDa Apg12-Apg5·Apg16 complex is also required for the localization of Aut7 to the PAS.[70] Aut7 is a small protein that has been predicted to contain an ubiquitin fold, as the human homologue GATE-16 was shown by crystallography to bear structural resemblance to ubiquitin.[71] Aut7 is physically conjugated to the lipid phosphatidylethanolamine (PE), but only after the terminal arginine is cleaved off by the peptidase Aut2 exposing a glycine.[70,72,73] Aut7 is next activated by Apg7, which is the same enzyme that also initiates the Apg12-Apg5 conjugation, before it is transferred to Cys234 in Aut1.[73] Aut1 acts as an E2 enzyme in the conjugation reaction transferring Aut7 to the amino group of PE, or vice versa. In cells incapable of forming the Apg12-Apg5·Apg16 complex, the lipidated form of Aut7 is almost undetectable; however, it has been shown by pulse/chase experiments that some lipidation does occur, but that the lipid is rapidly removed by Aut2.[27] In contrast to the conjugation of Apg12 to Apg5, Aut7 lipidation therefore appears to be a dynamic event. It has been proposed that the function of the Apg12-Apg5·Apg16 complex in the lipidation reaction is to stabilize Aut7-PE that is correctly localized to the PAS or the to the site of vesicle formation.[27] Cells that are lacking Aut2 or Aut4 do not contain the lipidated form of Aut7 and have a similar phenotype as cells lacking Aut7 altogether, showing that the localization and activity of Aut7 is entirely dependent upon the protein being conjugated to PE.[74] In the case of Cvt vesicle formation, as discussed previously, Aut7 interacts with the prApe1 and Ams1 receptor Cvt19 and is sorted to the inside of the vesicle together with the Cvt complex (see Fig. 4).[7] A substantial amount of Aut7 also ends up on the inside of autophagosomes formed during starvation, but it is not known whether this is due to the fact that the Cvt complex is transported inside autophagosomes under these conditions.[34,35]

While the 350 kDa Apg12-Apg5·Apg16 complex is essential for Cvt vesicle and autophagosome formation, Aut7 is not.[74] In rich media, cells lacking Aut1, Aut2 or Aut7 do not show any mature Ape1. If the corresponding mutant cells are starved, however, a population of prApe1 is transported to the vacuole and matured. By electron microscopy, we were also able to show that some aberrantly small autophagosomes are formed during these conditions in the *aut1*, *aut2* or *aut7* mutants. A similar result was obtained if a protein synthesis inhibitor was added at the same time that autophagy was initiated in wild type cells by the addition of rapamycin or nutrient deprivation. Aut7 protein levels are normally induced substantially by starvation. We therefore proposed that Aut7 is required for the formation of the Cvt vesicle in rich media, and for the formation of large autophagosomes by membrane expansion during starvation conditions. The mechanism by which this occurs could be by stimulating membrane fusion. In the yeast *P. pastoris*, cells lacking Gsa7, which is the homologue of Apg7, were blocked at a late stage of the vacuolar uptake of peroxisomes upon shift from methanol to glucose based medium (see chapter 10).[66] It was therefore proposed that Gsa7/Apg7 functions at a late step of micropexophagy in *P. pastoris,* which terminates in homotypic vacuolar fusion. A mammalian homologue of Aut7, GATE-16 (see chapter 15), was identified in a screen for proteins that stimulate fusion between Golgi vesicles in an in vitro assay.[75,76] Aut7 from *S. cerevisiae* was shown to be able to substitute for GATE-16 in the assay. In yeast, it has also been

shown that *aut7* interacts genetically with the Golgi to endoplasmic reticulum SNARE genes *BET1* and *SEC22*, and that Aut7 interacts physically with Bet1 and the vacuolar SNARE Nyv1. So far, it does not seem that these interactions are of importance for the Cvt pathway and autophagy. It should also be noted, however, that although some autophagosomes form upon nitrogen removal in cells lacking Aut7, the amount is not adequate to support survival upon starvation. Measurement of the rate of autophagy using PMSF-sensitive protein degradation or the vacuolar uptake of the truncated, cytosolic form of Pho8 (Pho8Δ60), show that bulk autophagy is almost completely abolished in cells lacking Aut7.[77]

Fusion of the Cvt Vesicle with the Vacuole and Disintegration of the Cvt Body

In the previous chapters we have described how the cargo of the Cvt pathway forms a cytosolic Cvt complex together with the receptor Cvt19 and is brought into proximity with the sequestering membrane or PAS by a putative tethering factor. In addition, we have discussed how several protein interactions and protein complexes play essential roles in vesicle formation. Despite all the knowledge presented, we are still relatively ignorant as to how the sequestering Cvt vesicle or autophagosome actually forms. This process may be quite unique. Freeze fracture studies on purified autophagosomes from rat liver cells, and similar studies on whole yeast cells suggest that the formed vesicle is practically devoid of transmembrane proteins (see chapters 2 and 6).[78,79] Our early characterization of the Cvt pathway showed that transport of prApe1 was independent of the cis-SNARE dissociation factor Sec18, and the same has been verified for autophagosome formation in yeast.[1,33] While no SNARE protein has so far been found to be important for autophagosome formation, we have described how we think that Tlg1 and Tlg2 are important for steps leading up to Cvt vesicle formation.[38,41] How priming of these SNAREs occurs in this instance is not known, but it is possible that a Sec18 homologue is involved.

After the Cvt vesicle is formed, it is rapidly targeted to the vacuole for fusion. This generally requires the same machinery as for homotypic vacuole fusion. In the case of autophagosomes, Vti1 has been proposed to be the v-SNARE and this may also be the case for the Cvt vesicle, although some evidence now suggests that Vti1 functions as a t-SNARE.[33,42,44] Other SNAREs involved are Vam3 and Vam7.[80] Tethering to the vacuole is mediated by the class C-Vps/HOPS complex, which include the proteins Vps11, Vps16, Vps18 and Vps33 complexed with Vps39/Cvt4/Vam6 and Vps41/Cvt8/Vam2.[45,81,82] Vps39 acts as a GDP-GTP exchange factor for the small rab GTPase Ypt7, and HOPS together with GTP-bound Ypt7 orchestrates the docking step. In addition to these components, we have recently characterized two new proteins required for fusion of Cvt vesicles and autophagosomes with the vacuole as well as for homotypic vacuole fusion; Ccz1 and Mon1.[83] The *ccz1* mutant has been shown to be allelic with *cvt16*, and Ccz1 forms a complex with Mon1 that is localized to the vacuolar membrane and the PVC.[83,84] This complex is also required for the CPY pathway and endocytosis, but is less important for the ALP pathway. Preliminary data suggest that the Ccz1-Mon1 complex acts before docking and fusion, although we have not been able to detect any interactions with the other membrane tethering components. It has also not been shown that any of the components required for membrane tethering, docking and fusion are present on the completed Cvt vesicles and autophagosomes.

Upon fusion with the vacuole, the outer membrane of the Cvt vesicle and the autophagosome fuses with the vacuolar membrane releasing a Cvt body or autophagic body into the lumen of the vacuole.[79] The subvacuolar body is next degraded in a process requiring Cvt17, Pep4 and the vacuolar proton pump.[85-87] Cvt17 is a putative lipase that is also required for the degradation of the vesicles arising during multivesicular body formation, although it has been proposed that Cvt17 is also transported to the vacuole by the Mvb pathway.[88] The putative active site of Cvt17 has been shown to be required for activity, but it is not yet known what lipid Cvt17 is acting on.[85] The fact that Cvt17 also is involved in the degradation of vesicles formed

by the Mvb pathway, suggests that the inner membrane of the Cvt vesicle and autophagosome may resemble the membrane of the intralumenal MVB vesicles. Accordingly, during vesicle formation, a membrane sorting event must ensure that lipids are segregated correctly between the inner and outer vesicle membrane. While the inner membranes are readily degraded in a Cvt17-dependent manner inside the vacuole, the outer membrane fuses with the vacuolar membrane and must be resistant to vacuolar lipases and proteases. Degradation of autophagic bodies, but not Cvt bodies, have also been shown to be partially blocked in cells lacking Aut4.[89] This could suggest that there may be differences in the composition of the membrane of these two structures. Aut4 is a putative membrane protein with limited homology to permeases and localizes to the vacuolar membrane. It is not known in what way Aut4 may be involved in disintegration of autophagic bodies.

Conclusion and Future Directions

The Cvt pathway is a mechanism for delivery of vacuolar resident hydrolases that has so far only been described to occur in the yeast *S. cerevisiae.* Similarity searches have not been able to identify aminopeptidases in other organisms that contain a propeptide with homology to the propeptide of aminopeptidase I. Mammalian homologues of most of the Cvt pathway-specific proteins have not yet been described, with the exception of the Cvt13 homologue Snx4. Nevertheless, the fact that the molecular machinery required for the formation of the Cvt vesicle is mostly identical to the machinery of starvation-induced autophagy, provides us with a unique assay for the molecular characterization of how these vesicles are formed. The discovery of the Cvt pathway has therefore been of great importance to the field of autophagy, even though the purposes of these pathways are the opposite of each other. Many of the components required for the Cvt pathway that are not used for non-selective autophagy are furthermore required for the selective autophagy of peroxisomes. The selective process of pexophagy has been shown to occur upon nutritional adaptation in other yeast species as well as in mammalian cells after ending treatment with peroxisome proliferators.[90-92] Knowledge about proteins involved in both the Cvt pathway and pexophagy may therefore result in knowledge about the turnover of peroxisomes in mammalian cells. In addition, other components of mammalian cells appear also to be selectively degraded by autophagy. Apoptotic death of sympathetic neurons involves the selective removal of mitochondria, and this degradation has been proposed to occur by selective autophagy. In addition to a function in certain types of programmed cell death, there is increasing information that autophagic dysfunction also may play an important part in a variety of human diseases (see chapters 19-22).

Several important issues remain to be solved about how Cvt vesicles and autophagosomes form. A question that has intrigued scientists in the field of autophagy since the pathway was discovered more than sixty years ago is the origin of the autophagic sequestering membrane. All cellular membranes, including the plasma membrane, have been "shown" by various methods to be able to sequester cytoplasm, although some consensus has been built that the ER is most likely the source of the autophagic membrane (see chapter 2).[93,94] In the yeast *S. cerevisiae,* it is possible that the origin of the sequestering membrane may be different for the Cvt pathway and autophagy. Cvt pathway-specific proteins like the VFT complex and the associated proteins Tlg2, Tlg1 and Vps45 could be involved in a membrane retrieval step.[41] The proteins Cvt13 and Cvt20 have been shown to bind to endosomes or the PVC in addition to the PAS.[15] There are also proteins that have been recently described to be required only for autophagy and not for the Cvt pathway. These include components of the COPII vesicle coat Sec12, Sec16, Sec23 and Sec24.[33] Intriguingly, the COPII components Sec13 and Sec31 are not required for autophagy and it was therefore proposed that the autophagic dependency on the COPII components is not due to transport from the ER to the Golgi. If these pathway specific proteins are involved in membrane generation, it is possible that the PAS represents an assembly point for the autophagic membranes and molecular components rather than a membrane compartment. Finally, the differential role for Aut7 and Apg1 in formation of Cvt vesicles versus

autophagosomes suggests that the mechanism of vesicle formation in addition to the membrane source may be distinct between these two pathways.

The fusion mechanism generating the Cvt vesicles and autophagosomes may also be quite unique from other vesicle formation processes. Intriguingly, some of the yeast autophagy proteins have several possible homologues in humans. The small ubiquitin-like protein Aut7 has three such homologues that are all activated by the human homologue of Apg7 (see chapter 15).[95] Of these three homologues, only LC3 appear to be involved in the formation of autophagosomes.[96] Another homologue, GABARAP, has been shown to bind to the $GABA_A$-receptor and to link this receptor to the cytoskeleton.[97] As discussed above, Aut7 also binds to a receptor, the receptor for prApe1 and Ams1. It is therefore a possibility that molecular mechanisms employed by the Cvt pathway in yeast may have been adapted for other purposes in mammalian cells. Discoveries concerning the Cvt pathway in *S. cerevisiae* could therefore have relevance for processes unrelated to autophagy in human cells.

Acknowledgements

This work was supported by National Institutes of Public Health Servie grant GM53396 to D.J.K.

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CHAPTER 8

Microautophagy

Chao-Wen Wang and Daniel J. Klionsky

Abstract

Micro- and macroautophagy are both processes in which portions of the cytoplasm are non-specifically sequestered, delivered to the lysosome/vacuole, degraded and recycled. The primary morphological difference between these pathways has to do with the site of sequestration and the origin of the sequestering membrane. Macroautophagy involves the formation of a cytosolic double membrane vesicle that fuses with the lysosome/vacuole. This vesicle does not appear to be derived from the lysosomal/vacuolar membrane. In contrast, during microautophagy portions of cytosol or whole organelles are sequestered directly at the surface of the degradative organelle; there is no intermediate transport vesicle. The sequestration process occurs either by invagination of the limiting membrane or by septation or protrusion of arm-like structures. This process has been observed in a wide range of eukaryotic cells, however, the molecular details and functional importance of this pathway are still waiting to be explored.

Introduction

While the majority of our knowledge of autophagy comes from the study of macroautophagy, studies with a wide range of eukaryotic cells have revealed a second mechanism of autophagy termed microautophagy. In microautophagy the sequestration of cytoplasm occurs directly at the limiting membrane of the degradative organelle. Considering the utilization of the lysosome/vacuole membrane for the uptake of cytoplasmic material, two general possibilities could be proposed: Direct transport of proteins across the lysosomal/vacuolar membrane through the action of a specific receptor and a proteinaceous membrane channel, or a lysosome/vacuole membrane-mediated sequestration event. Both processes have been observed. The receptor-mediated transport of specific proteins that occurs via chaperone-mediated autophagy falls into the first category (see chapter 12). This specific pathway appears to be saturable and depends upon a 73-kDa heat-shock protein.[1] Detailed studies suggest that this mechanism has similarities to mitochondrial protein import. In this chapter we will focus on the microautophagic process, uptake via a dynamic rearrangement of lysosomal/vacuolar membrane that results in the sequestration of cytoplasm within a transient membranous vesicle or compartment within the lysosome/vacuole lumen. Although our knowledge of microautophagy is far from complete, several lines of evidence suggest that microautophagy is a mechanistically distinct process from macroautophagy.

Differences between Microautophagy and Macroautophagy

Both microautophagy and macroautophagy are processes by which the cell can engulf cytoplasm and deliver it to the lysosome or vacuole for subsequent degradation and recycling. The primary difference between the two processes concerns the membrane that is used for sequestration. In macroautophagy, sequestration is initiated away from the degradative organelle and involves a double membrane vesicle termed an autophagosome (see chapters 6 and 7). The

Autophagy, edited by Daniel J. Klionsky.

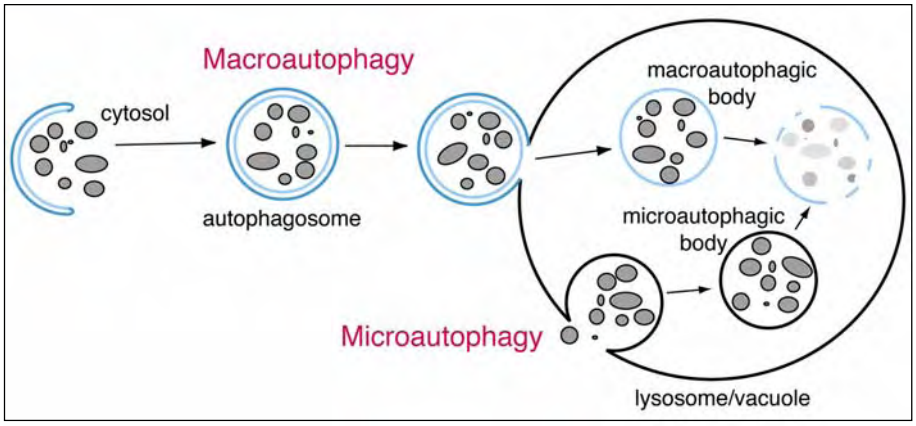

Figure 1. Morphological events for macro- and microautophagy. Macroautophagy involves a sequestration event that takes place separate from the lysosome/vacuole membrane. Membranes of unknown origin, but presumably distinct from the lysosome/vacuole, form a double membrane autophagosome that engulf portions of cytoplasm and, upon completion, fuses with the lysosome/vacuole. In contrast to macroautophagy, microautophagy involves uptake of cytoplasm or whole organelles directly at the lysosome/vacuole membrane. Microautophagy occurs either by protrusion of arm-like structures, by septation or by invagination of the limiting membrane. Both micro- and macroautophagy ultimately generate a single membrane macro- or microautophagic body that is ultimately broken down inside the vacuole lumen.

origin of the vesicle membrane is still not known but is thought to be distinct from the lysosome/vacuole. In contrast, in microautophagy the sequestration event occurs directly at the surface of the degradative compartment—there is no intermediate transport vesicle. The lysosome/vacuole membrane either invaginates or protrudes via arm-like extensions or a septation process to engulf portions of cytoplasm. Following scission of the sequestering membrane from the lysosome/vacuole, or following fusion of the double membrane autophagosome with this organelle, a single membrane vesicle is released into the lumen. The presence of this vesicle in the lysosome/vacuole therefore cannot be used to distinguish between the two processes. One fundamental question that needs to be answered concerns the fate of the sequestering membranes. In both micro- and macroautophagy the single-membrane vesicle that is delivered to the lysosome/vacuole lumen must be degraded to allow access to the contents. In macroatuopahgy, the cytosolic double membrane vesicle is originally not derived from the lysosome/vacuole. Following fusion, the outer membrane becomes continuous with the lysosome/vacuole. This membrane must be rapidly modified so that it is protected from degradation or it must be removed from the limiting membrane so that the integrity of the organelle is maintained. On the other hand, because the sequestering membrane is of lysosomal/vacuolar origin in microautophagy, it is not clear how this membrane is modified so that the cell is now able to distinguish it from the limiting membrane and break it down. A schematic representation of micro- and macroautophagy is shown in Figure 1.

Both macro- and microautophagy have been visualized in eukaryotic cells. It is believed that macroautophagy is regulated by a conserved machinery, whereas our current knowledge on microautophagy is very limited. In most cell types, macroautophagy is carefully regulated by the concentration of amino acid and hormones. Thus, its contribution to proteolysis is mainly controlled by environmental cues. In contrast, microautophagy is insensitive to amino acids based on studies in mammalian cells. There is evidence suggesting that hepatocytic microautophagy declines under conditions of long term starvation, however, the corresponding signals and mechanism are still unknown.[2,3] It is possible that microautophagy is also alterable, but its responses seem to be slower and probably more adaptive depending on the

particular conditions.[4] Macroautophagy is an ATP-dependent process with energy being required for sequestration, vesicle fusion and proteolysis.[5] It had been suggested that microautophagy is ATP-independent,[6] however, further study indicates that this process does require energy. For example, when isolated lysosomes are incubated with ferritin, the protein is delivered to the lysosomes via an ATP-dependent microautophagic-like structure.[7] Furthermore, studies with a cell-free microautophagy system have indicated that ATP is essential for the formation of microautophagic tubes by isolated yeast vacuoles.[8] Similar to macroautophagy, the microautophagy pathway is able to deliver not only soluble proteins but also entire organelles into the vacuole/lysosome for degradation. The later has been most clearly demonstrated with peroxisomes (see chapters 10 and 11) .[9-11] The mechanism that drives the formation and the scission of the microautophagic structures are unknown.

Observation of Microautophagy in Mammalian Cells

The breakdown of cytosolic proteins is mediated through both lysosomal and nonlysosomal (i.e., proteasome) pathways. The first evidence showing that cytosolic proteins could be sequestered and degraded within the lysosome was reported in an isolated rat liver system.[12] Further studies indicate that cytoplasmic sequestration by lysosomes can be divided into two categories, macro- and microautophagy. Macroautophagy accounts for most of the accelerated breakdown of long-lived proteins under amino acid deprivation conditions. Macroautophagy is relatively easy to detect morphologically because it can be monitored through the formation of large cytosolic autophagosomes. In contrast, microautophagy is more difficult to discern because it must be differentiated from non-autophagic perturbations of the lysosome/vacuole membrane. Because of these difficulties, less is known about the role of microautophagy in protein turnover.

Microautophagy Might Regulate Basal Long-Lived Protein Degradation in Rat Liver

Several forms of microautophagy-like sequestration structures have been described in rat liver hepatocytes,[2,3,13,14] however, how these structures are formed is not clear. Mortimore's group suggested that microautophagy might be involved in basal protein turnover. Basal turnover is typically defined experimentally by measuring the release of valine in liver homogenates. This group identified a population of secondary lysosomes termed the type A lysosomes that might have a role in microautophagy and thus employed it as a marker for microautophagy studies.[2,3] Basal proteolytic rates of long-lived protein decreased during 48 hours of starvation. Quantitative electron microscopy data suggested a close correlation between the decline in basal turnover rate and a decline in the volume fraction of microautophagic structures during starvation.[2] Similar results were also observed using the perfused rat liver system.[3] In contrast, macroautophagy is induced during starvation and remained at about the same level over 48 hours in their study.[2]

It should be noted that much of the analysis of microautophagy in mammalian cells is based on morphological analyses of the numbers and types of autophagic structures. However, as indicated in Figure 2, different sectional planes of vacuoles undergoing microautophagy might result in different interpretations. For example, de Waal et al challenged the role of microautophagy in basal turnover during starvation by stressing the importance of other types of putative microautophagic vacuoles.[14] In addition, artifacts might be introduced during sample fixation and sectioning for ultrastructural studies that could generate structures at the lysosome that resemble microautophagic vacuoles. Accordingly, these studies are far from conclusive. More convincing evidence for microautophagy in hepatocytes is seen with isolated lysosomes that are able to internalize particles in vitro. Using ferritin as a marker, these studies found that a microautophagy-like structure is likely to be associated with in vitro transport.[6,7,15] Future studies will be re necessary to confirm the regulation of microautophagy in regard to both morphological and mechanistic aspects.

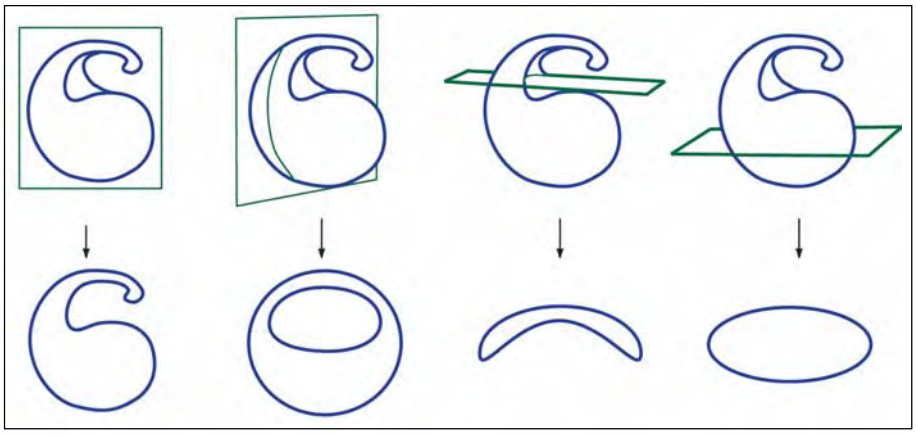

Figure 2. Diagram of an open microautophagic lysosome/vacuole that undergoes different planes of sectioning. After sectioning, at least four different profiles could be observed as shown. These variant appearances might result in arbitrary interpretations in ultrastructural morphometric analyses. This diagram is modified from reference 14.

Can Microautophagy Be Induced?

Many factors have been shown to increase the occurrence of macroautophagy (see chapters 3-5). Thus, it is of interest to understand the factors that might induce this process. Similar to the rat liver system, several autophagic-lysosomal compartments have been observed in cultured cells. Under normal growth conditions, ultrastructural analyses of fibroblasts reveal numerous lysosomes that are usually grouped, whereas autophagosomes and residual bodies (small, very electron-dense structures without acid phosphatase activity) are rare.[16] The induction of microautophagy has been suggested to occur under several conditions based on the increase in the population of multivesiclar body-like structures. For example, incubating fibroblasts in the presence of vinblastine, which causes the breakdown of microtubules, resulted in a ten-fold increase in the volume fraction of active lysosomes.[16] In many circumstances the active lysosomes resembled multivesicular bodies and contained acid phosphatase activity. One explanation is that vinblastine treatment might cause typical round lysosomes to develop into pleomorphic lysosomes. The latter are thought to participate in microautophagy leading to the formation of multivesicular-like structures. Alternatively, pre-existing lysosomes may fuse with nascent autophagosomes or amphisomes (see chapter 2) giving the lysosome a similar multivesicular body appearance. This possibility presents an example of the difficulty of basing these analyses solely on ultrastructural studies.

Another possible example of induced microautophagy is seen when fibroblast cells are treated with protease inhibitors. Early studies suggest that microinjected proteins in fibroblasts quickly assemble into disulfide cross-linked forms that behave as detergent insoluble aggregates and are subject to lysosomal degradation.[17] Lysosomal degradation of protein aggregates has also been observed when pulse-labeled fibroblasts are chased in the presence of protease inhibitors such as leupeptin.[17] Subsequent morphological studies reveal an increased number of electron dense multivesicular bodies following treatment with the cysteine protease inhibitor E-64.[18] Taken together, these results suggest a possible role of microautophagic-like invagination involved in the sequestration of protein aggregates for lysosomal degradation.

Microautophagic-Mediated Degradation Pathways: A Lesson from Fungi

Selective Targeting of Organelles to The Vacuole/Lysosome

The autophagic degradation of organelles has been demonstrated in yeasts such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Candida boidinii* and *Yarrowia lipolytica*.[9,11,19-23] Among these model systems, the macroautophagy-mediated organelle degradation pathway has been extensively studied in *S. cerevisiae* and *H. polymorpha* (see chapters 7, 9 and 11). *P. pastoris* and *H.polymorpha* exhibit both macroautophagic and microautophagic degradation of peroxisomes, a process termed pexophagy, in response to different environmental conditions,[11] thus making it possible to determine proteins that overlap or are distinct for the two types of autophagic processes (see chapters 10 and 11). In brief, a transfer of *P. pastoris* cells from glucose to methanol results in an increase in peroxisome levels in order to metabolize methanol. Once the culture is shifted from methanol to ethanol, selective degradation of peroxisomes by the macroautophagic pathway is activated. In contrast, the microautophagic pathway is triggered upon shifting from methanol to glucose. Micropexophagy in *P. pastoris* is thus far the greatest research model for microautophagy and is covered in detail in chapter 10. It should be noted that both micro- and macroautophagic degradation has also been studied using plant models, a topic covered in chapter 16.

The key enzymes of metabolic pathways are precisely regulated. Some of these enzymes are cytosolic suggesting that they might be inactivated or degraded in the cytosol following a signal transduction event based on some environmental cue. In contrast, other enzymes are localized to membrane structures or organelles such as peroxisomes; the targeting of the corresponding organelles into the vacuole/lysosome for degradation appears to play a critical role in catabolite inactivation. In the filamentous fungus *Aspergillus nidulans*, the peroxisomal enzyme isocitrate lyase is induced in oleate-containing medium. Once subjected to a medium using glucose as sole carbon source, the catabolic inactivation of isocitrate lyase is induced.[24] Such glucose-dependent inactivation of isocitrate lyase has been reported in several fungi including *S. cerevisiae*.[25] In *S. cerevisiae*, however, isocitrate lyase is localized in the cytosol and is dependent upon phosphorylation/dephosphorylation to control its enzyme activity.[25] In *A. nidulans*, isocitrate lyase is localized to the peroxisome, and its inactivation requires proteolysis of the whole organelle. Ultrastructural studies demonstrate that peroxisomes are first associated with one or various vacuoles at 1-2 hours following a shift from oleate to glucose. Complete sequestration within a single vacuole is seen after 2-6 hours.[24] Accordingly, these data suggest that the vacuole is directly involved in degradation of peroxisomes, which further suggests that microautophagy is the autophagic process that is involved in the sequestration event. In *S. cerevisiae*, excess peroxisomes can be delivered to the vacuole for degradation when cells are replenished with glucose.[26] Similarly, ultrastructural studies indicate that engulfment at the vacuole membrane might account for the uptake of peroxisomes indicating that microautophagy is probably involved, although these studies are not conclusive.[26] While our current understanding of the extent to which autophagy is involved in catabolite inactivation is still very limited, we expect that additional examples will be uncovered where this mechanism is used in microorganisms for the precise control of cellular physiology.

Another example of microautophagic degradation of organelles is seen with piecemeal microautophagy of the nucleus. In this process, portions of the nucleus are targeted to and degraded within the vacuole. This process is described in detail in chapter 9.

Specific Targeting of Cytosolic Proteins into the Yeast Vacuole

Direct import of cytosolic proteins into the yeast vacuole has been described for few proteins. These include the resident vacuolar hydrolases aminopeptidase I and α-mannosidase, the gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase), and glyceraldehyde-3-phosphate dehydrogenase. Some resident vacuolar hydrolases are delivered to the yeast vacuole by the cytoplasm to vacuole targeting (Cvt) pathway that is mechanistically similar to macroautophagy but occurs under nutrient rich conditions (Chapter 7).[19,20] FBPase is the key regulatory enzyme in the gluconeogenesis pathway that is activated in response to glucose starvation.[1] The degradation of FBPase has been the subject of debate. It has been shown that the enzyme can be ubiquitinated and degraded in the cytosol by the proteasome.[27,28] However, other evidence suggests that FBPase is selectively targeted from the cytosol to the vacuole for degradation when glucose starved cells are replenished with glucose (chapter 13).[1,26] In the latter process, FBPase enters 30 nm cytosolic vesicles that subsequently fuse with the vacuole or are taken into the vacuole through microautophagy.[26] The method of sequestration of FBPase into the cytosolic vesicles is not fully understood but may involve a direct translocation mechanism similar to chaperone-mediated autophagy (chapter 12). Thus, the sequestration of FBPase might itself represent a type of specific microautophagy, albeit one that uses a set of protein components distinct from those used in macroautophagy or pexophagy. In vitro studies indicate that glyceraldehyde-3-phosphate dehydrogenase is imported into and degraded by vacuoles.[29] Comparing vacuoles from cells grown in rich-medium or following starvation, the authors found a five-fold increase in protein uptake by the vacuoles derived from starved cells. In addition, this vacuole delivery process is Hsp70-dependent and requires protease-sensitive components on the outer vacuole surface.[29] Accordingly, this transport process shares many features with the direct import of proteins across the mammalian lysosomal membrane.

Microautophagic Vacuole Invagination

The formation of a specialized microautophagic-like structure termed autophagic tubes has been observed in *S. cerevisiae*. Based on studies of both intact and purified vacuoles, Mayer and colleagues found that the vacuole membrane could form a very mobile tubular, often branched, invagination, and then bud off into the lumen of the organelle within less than 40 seconds.[30] Formation of these vacuolar invaginations is constitutive but increased by 63% under starvation conditions. In contrast to the wild type vacuoles, those from several autophagy-defective mutants could not increase the frequency of autophagic tubes upon starvation. Freeze-fracture analysis revealed a striking heterogeneity in the membrane structure. A smooth zone devoid of transmembrane particles was seen at the tip of the bubble-like structure where invagination/budding occurs. In contrast, the base of the autophagic tube has the normal content of transmembrane proteins.[30] The smooth membrane structure of the autophagic tube is similar to that of the autophagic bodies that have been described previously.[31] Accordingly, it was proposed that one role of this microautophagic event may be to help regulate vacuole membrane homeostasis. In particular, microautophagy may allow the removal of the autophagosome outer membrane from the limiting membrane of the vacuole following fusion.[8,30] A second study further reconstituted microautophagic tube formation in vitro. In this in vitro system, it was observed that the uptake of dextran-coupled FITC inside the isolated vacuoles as well as the formation of autophagic tubes occurs in an ATP-dependent manner.[8] Unlike macroautophagy, the in vitro microautophagy system is independent of components involved in homotypic vacuole fusion such as Sec17 (α-SNAP), Sec18 (NSF), and SNAREs. In vitro microautophagy is cytosol-dependent. Adding cytosol from mutants defective in macroautophagy only partially supports microautophagic uptake activity, suggesting that this specific microautophagy pathway might participate in some amount of cross-talk with or depend upon some of the identified macroautophagy machinery.[8] However, recent work has suggested that the *APG* gene products are actually required for the final stage of sequestration during microautophagy of peroxisomes in yeast (Y. Sakai, personal communication). These data indicate that

microautophagy and macroautophagy are mechanistically different processes in *S. cerevisiae*. However, it should also be noted that the precise mechanism of microautopahgy of peroxisomes, micropexophagy, may not involve invagination of the vacuole membrane. A recent study of micropexophagy in *P. pastoris* suggests that the vacuole typically septates into multiple compartments that engulf peroxisomes, as opposed to sequestration by a single invaginating vacuole.[32] This means that micropexophagy may involve yet a different mechanism than the type of microautophagy described above. Thus, unlike the conserved macroautophagy pathway, microautophagy seems to operate at different levels and by different mechanisms from one situation to another. Further study is required to prove this speculation.

Summary and Concluding Remarks

Microautophagy is defined as the uptake of cytoplasm by a direct involvement of lysosomal/vacuolar membrane. It results in vesicles budding into the lumen of the lysosome/vacuole and their subsequent degradation by lysosomal hydrolases. It appears that microautophagy might be a widespread phenomenon in many cell types. While the macroautophagy pathway that regulates protein degradation in response to environmental change is physiologically important, the functional role and regulatory control of microautophagy is largely unknown (except in the case of micropexophagy). Future studies will be important to understand these aspects of microautophagy. In particular, is microautophagy generally involved in basal protein turnover? Is there any particular modification that is required for this degradation pathway? Under certain circumstances, microautophagy seems to be up-regulated for selective targeting. How are the appropriate signals transduced to the lysosomal/vacuolar membrane? In addition to the corresponding signaling pathway(s), it will be important to determine the molecular mechanism and the protein components that are required for the membrane rearrangements that are involved in this pathway.

Acknowledgements

The authors would like to thank Dr. Yasuyoshi Sakai for helpful comments. This work was supported by National Institutes of Health Public Health Service grant GM53396 (to D. J. K.), and the Lewis E. and Elaine Prince Wehmeyer Trust (to C.-W. W.).

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CHAPTER 9

Microautophagy of the *Saccharomyces cerevisiae* Nucleus

David S. Goldfarb

Abstract

Portions of the *Saccharomyces cerevisiae* nucleus are targeted to the vacuole and degraded by "piecemeal microautophagy of the nucleus" (Pmn). During Pmn small teardrop-like nuclear envelope blebs are engulfed by invaginations of the vacuole membrane, pinched into the vacuole lumen, and degraded by lumenal hydrolases. Pmn occurs in the context of nucleus-vacuole (NV) junctions, which are Velcro-like patches formed through specific interactions between Vac8 on the vacuole membrane and Nvj1 on the outer nuclear membrane. Pmn occurs at low levels in early log phase cells and is gradually induced as nutrients become limiting. Degradation of nonessential nuclear components such as nucleolar preribosomes appears to be a normal physiological process because cells undergoing Pmn survive and continue to divide. Like other autophagic processes, Pmn is induced by rapamycin, an inhibitor of Tor kinase function. Pmn occurs at normal levels in *apg7*Δ cells, which are defective in macroautophagy. Because yeast has a closed mitosis, Pmn may have evolved to deliver damaged or excess nuclear contents to the vacuole for degradation and recycling.

The Yeast Nucleus As a Substrate for Autophagy

Nuclear Structure and Composition

The purpose of this article is to review what is known about "piecemeal microautophagy of the nucleus" (Pmn) in *Saccharomyces cerevisiae* (Fig. 1A). Pmn begins with the formation of nucleus-vacuole (NV) junctions, which position the vacuole over an area of the nucleus that then becomes a potential substrate for degradation. During Pmn the vacuole membrane engulfs and pinches off nuclear envelope vesicles, called "blebs," that enclose a portion of the nucleoplasm. Because it is important to consider the contents of Pmn vesicles, and the implications of their degradation, we will begin with a brief overview of nuclear structure.

Eukaryotic cell growth, metabolism, and reproduction are directed by the chromosomes, which are enclosed within the nuclear envelope (NE). The NE is comprised of concentric inner and outer nuclear membranes that are fused at various points creating pores occupied by nuclear pore complexes (NPCs). Whereas a typical mammalian nucleus contains approximately 2000 NPCs, the nuclear envelope of a *S. cerevisiae* cell in early G1 phase contains, on average, about 86 NPCs.[1] The outer nuclear membrane is continuous with the rough endoplasmic reticulum (ER), which is studded with ribosomes in the act of cotranslational secretion. In plants and animals the nuclear envelope is supported from the inside by a rigid meshwork of intermediate filaments called the nuclear lamina. Yeasts lack a nuclear lamina and the various inner nuclear membrane proteins such as "LAPs" that link the inner nuclear membrane of higher cells to the lamina.[2] If cells are to survive Pmn, which they do, it follows that bulk

Autophagy, edited by Daniel J. Klionsky.

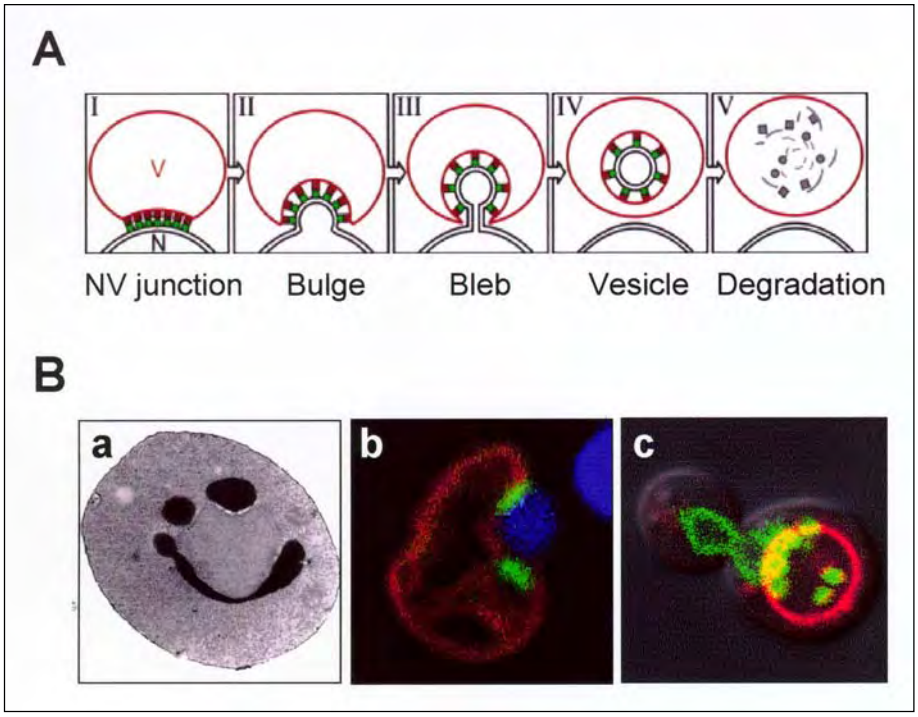

Figure 1. A. Pmn in five acts. (Stage I) NV junctions are formed by the interaction and clustering of Nvj1 (green circles) in the nuclear envelope with Vac8 (red squares) in the vacuolar membrane. (N, nucleus; V, vacuole). Nuclear bulges (Stage II) develop into tethered blebs (Stage III). Intra-vacuolar vesicles are formed by scission of the vacuolar and two nuclear envelope membranes of the blebs and the release of intra-vacuolar vesicles into the vacuole lumen (Stage IV). Finally the Pmn vesicle and its contents are degraded by vacuolar hydrolases (Stage V). B. Transmission electron micrograph showing abnormally large NV junctions between black-stained vacuoles and the nucleus in *NVJ1* overexpressing cells (panel a); confocal micrograph showing sandwiching of Nvj1-GFP (green) between FM 4-64-stained vacuole (red) and Hoechst-stained chromatin (blue) (panel b); and confocal micrograph showing Pmn blebs and vesicles emerging from an NV junction in a mitotic cell (panel c). Panel A and panels B.b and B.c were reprinted from *Molecular Biology of the Cell* (2003, vol. 14, pgs. 129-141) with permission by the American Society for Cell Biology.

chromosomal DNA must be excluded from Pmn vesicles. This is a potentially delicate matter, since the chromosomes are attached to the NE. In higher cells, chromosomes are linked to the NE through the nuclear lamina. The nature of the association of yeast chromosomes with the NE is less well understood, but may involve tethering telomeres to NPCs.[3]

Approximately 10% of cellular proteins are nuclear, including large numbers of factors committed to the assembly and maintenance of chromatin and its transcription. The nucleus is also the site of ribosome biogenesis. The nucleolus is a prominent feature of nuclei, and in yeast occupies about one third of the nucleus. The primary function of the nucleolus is the production of 40S and 60S ribosomal subunits.[4] Nucleoli are organized around nascent ribosomal RNA (rRNA) gene transcripts, and contain precursor and mature rRNAs, rRNA processing enzymes, snoRNPs (small nucleolar ribonucleoprotein particles), ribosomal and nonribosomal proteins, and partially assembled subunits. The bulk of the nucleolus in dividing cells is com-

posed of preribosomes, which appear as dense granular material in electron micrographs. *S. cerevisiae* contains a single crescent or cone-shaped nucleolus positioned directly underneath the NE. Perhaps owing to its great density, the nucleolus is prone to stress-induced aggregation and often requires chaperone-mediated remodeling.[5,6,7]

The NE breaks down during mitosis in most higher eukaryotes and the kinetichores attach to the centrosomes located in the cytoplasm. Later during telophase the NE reassembles on the surface of the chromosomes and then expands as the chromosomes decondense and nuclear proteins, which had mixed with the cytoplasm, are reimported through NPCs to generate the two daughter cell nucleoplasms. Nucleoli, which disassemble during prophase, begin to reform and accumulate new preribosomes during telophase.[8] In contrast, the *S. cerevisiae* NE does not break down during mitosis. Instead mitotic spindles form within the "closed" nucleus using spindle pole bodies permanently embedded in the NE. During mitosis the yeast nucleolus remain intact, RNA polymerase I transcription of rDNA (chromosomal DNA encoding rRNA) continues (it ceases during mitosis in higher cells), and the soluble contents of the nucleoplasm do not mix with the cytoplasm.[9]

Degradation of Nuclear Proteins and RNAs

The lysosome/vacuole plays an important role in the degradation of the cytosol and its numerous organelles, especially in response to nutritional cues. Historically, the nucleus has been ignored as a possible substrate for autophagy, probably because there is usually only one and it is essential (for an exception to this rule see ref. 10). Instead, the bulk of nuclear proteolysis has been assumed to be catalyzed by nuclear enzymes such as the proteasome. The proteasome catalyzes the selective degradation of polyubiquitin-tagged proteins in both the cytoplasm and nucleus.[11,12] An important role of the proteasome in the nucleus is the timely degradation of cell cycle factors such as G1/S phase cyclins. However, the proteasome may also play a role in stress responses, since oxidatively damaged histones are degraded in the nucleus by the proteasome,[13] and the proteasome appears to be recruited by PA200 to the DNA of gamma-irradiated tissue culture cells.[14] Of course, eukaryotic cells contain many other proteases that remain candidates for the degradation of nuclear proteins. Finally, nuclear proteins such as the lamins, NPC subunits, and inner nuclear membrane proteins are cleaved by caspases during apoptosis in mammalian cells.[15] Recently, a metacaspase that localizes to the nucleus was shown to be required for apoptotic-like cell death in *S. cerevisiae.*[16,17]

Because many proteins shuttle between the nucleus and cytoplasm, either continuously or in a regulated fashion, it is possible that some might be degraded while they are transiently exposed to the cytoplasm. It is also possible that components of the interphase nucleus are targeted for degradation during open mitoses when they are mixed with the cytoplasm. For example, vertebrate M phase cyclins and anaphase inhibitor are imported into the nucleus during interphase and then degraded by the proteasome during mitosis—although these are specialized cases. This latter option is not available to nuclear proteins in nondividing cells such as circulating lymphocytes and neurons, or to organisms such as *S. cerevisiae* with closed mitoses.

Recent progress has been made toward elucidating mechanisms that degrade RNA in the nucleus. Studies of rRNA processing identified a complex of ten riboexonucleases called the exosome that plays a central role in the precise formation of the 3' ends of several types of RNAs.[18] In the nucleus, the exosome also appears to function in a regulated mRNA surveillance system that degrades transcripts in response to defects in the mRNA processing and export pathways. Nonsense-mediated premRNA degradation is a fascinating but poorly understood process that degrades vertebrate premRNAs containing premature termination codons before they reach the cytoplasm.[19] Finally, yeast mRNAs that are retained in the nucleus can be degraded by a pathway that requires the nuclear mRNA cap-binding protein Cbc1p.[20]

Nucleus-Vacuole Junctions

Composition and Structure of NV Junctions

The selective microautophagy of organelles is almost certainly directed by specific protein-protein interactions between the lysosome/vacuole membrane and target organelles; however, most of these factors remain undiscovered. An exception is the role of Vac8 and Nvj1 in Pmn. Here, multiple tightly clustered copies of Vac8 in the vacuole membrane form Velcro-like junctions with clusters of Nvj1 in the outer nuclear membrane (Fig. 1A).[21]

Because the nucleus and vacuole(s) in yeast are large and, by TEM, obvious features of the cytoplasm, NV junctions are easy to find where they occur in published micrographs. Usually left unmentioned, they may have been dismissed in the minds of many, ourselves included, simply as places where the nucleus and vacuole happened to be pressed together. However, an early report confirmed by us noted that NPCs are excluded from areas of close association between the nucleus and vacuole.[21,22,23] More recently, vacuole membrane patches were found to copurify in association with preparations of yeast nuclear envelopes.[24] Perhaps because there was no obvious reason for their existence, the discovery of NV junctions had to await serendipity.

We identified Vac8 as an uncharacterized ORF in a BLAST search for new importin α genes in yeast. Importin α proteins are conserved cytosolic receptors for classical nuclear localization signals. Metazoan animals contain three types of conventional importin α genes, designated α1, α2, and α3.[25] Of these, yeasts and plants contain only importin α1 genes. Unbeknownst to us, Vac8 had been previously identified in a mutant screen for vacuole inheritance genes.[26] Our first indication that Vac8 was not a nuclear transport factor was the localization of a Vac8-GFP reporter to the vacuole membrane.[21]

Importin α, β-catenin (*Drosophila* "Armadillo"), and Vac8 are similar proteins that each contain a central domain composed of 10-12 tandem Armadillo (Arm) repeats bracketed by short N- and C-terminal domains. Arm repeats are degenerate 40-45 amino acid structural elements that fold into units of three α-helical segments. Arm repeats associate in tandem to form superhelical rods whose crystal structures in association with binding partners look like thick twisted slugs.[27,28] The third α-helices of each Arm repeat are lined-up in staggered parallel along the length of the rod to create a binding groove. The R-groups projecting from these helices specify each Arm domain's panel of unique binding partners.

The N-terminal domains of Vac8, β-catenin, and the importin α proteins direct their localization to the nuclear envelope, plasma membrane, and vacuole membrane, respectively. The N-terminus of β-catenin binds α-catenin, which recruits the cortical actin cytoskeleton to the plasma membrane and adherens junctions.[28] The N-terminus of importin α binds importin β, which targets the complex to the NE.[29] The N-terminus of Vac8 is also key to its membrane localization, but, instead of protein-protein interactions, acylation sites modified with myristate and palmitate residues anchor the protein in the vacuole membrane.[30-32] Protein-protein interactions probably play a role in specifying the exclusive targeting of Vac8 to the vacuole membrane, because a reporter protein containing only the first 69 amino acids localized both to the vacuole and plasma membranes.[21]

Mutational analysis has implicated Vac8 in multiple processes, including vacuole inheritance, cytoplasm to vacuole targeting (Cvt; see chapter 7), vacuole fusion and, as described below, Pmn. The observation that Vac8-GFP accumulated between vacuole-vacuole and vacuole-nucleus contact sites foreshadowed the discovery of its roles in homotypic fusion and Pmn.[21] Interestingly, *vac8* mutants that preclude palmitoylation inhibit vacuole inheritance and vacuole fusion, but not Cvt transport.[30,32] Consistent with its accumulation between clustered vacuoles,[21] Vac8 is required on both membranes during homotypic fusion, probably in association with a SNARE complex.[33,34] Since the palmitoylation of some signaling proteins is known to be reversible and regulated, it was of some interest when it was found that [^{3}H]palmitate is incorporated into Vac8 during the initial priming stage of in vitro fusion assays.[33,34] These studies raise the possibility that palmitoylation dynamics could play a role in Pmn.

Yeast two-hybrid screens have identified three Vac8 binding partners: Apg13, Vab2, and Nvj1.[21,35] The cosedimentation of Vac8 with F-actin, which has been implicated in vacuole inheritance[36] and fusion,[37] suggests that there may be additional binding partners that link Vac8 to actin.[30] It is not known if the apportionment of Vac8 among its different binding partners is regulated. This would only be necessary if the available pool of Vac8 was limiting, which can occur under artificial conditions by overexpressing *NVJ1*.[21] There is ample precedent for the regulation of partner binding by Arm domain proteins like Vac8. The distribution of β-catenin between adherens junctions and the Wnt/wingless signaling pathway is tightly regulated,[38] and the small GTPase Ran determines whether importin α binds importin β or CAS (the cellular apoptosis susceptibility gene product) in the nucleus.[29]

Because Vac8 is basically an adapter protein, it can be incorporated into complexes that serve either structural or regulatory roles. The role of Vac8 in Cvt targeting appears to be regulatory. Here, Vac8 is recruited by Apg13 into a multi-subunit complex that includes Apg1 (a protein kinase) and its associated polypeptides Cvt9 and Apg17[35,39] (see chapters 6 and 7). This complex may control switching between the Cvt pathway and macroautophagy.[35] Interestingly, Vac8 is required for the Cvt pathway but not macroautophagy.[35] This and other data suggest that autophagosomes may not arise through the simple expansion of preformed Cvt vesicles.

The binding of Vac8 to Vab2 is consistent with their having subtle roles in the control of vacuolar acidification, because Vab2 also interacts by two-hybrid with the vacuolar ATPase subunit Vma8.[21] The importance of these interactions is unclear, because neither the synthesis nor the assembly of the vacuolar ATPase, nor the acidification of the vacuole, requires *VAC8* or *VAB2* (P. Roberts, unpublished results).

Vac8-Nvj1 complexes play a striking structural role in the formation of NV junctions. Vac8-Nvj1 interactions are remarkable because they create discrete junctions between dissimilar organelles (Fig. 1B, panel a). The novelty of this phenomenon compelled us to persevere even after it became obvious that Vac8 was not an importin α-like nuclear transport receptor. Nvj1 is an integral outer nuclear membrane protein with a putative signal peptide and a single membrane-spanning domain. *NVJ1* homologues are currently restricted to *Saccharomyces* species. Vac8 binds the C-terminal 30-50 amino acids of Nvj1, which must therefore be exposed to the cytosol. In most cells Nvj1-GFP localizes to short stripes or patches sandwiched between FM 4-64-stained vacuoles and Hoechst-stained nuclei (Fig. 1B, panel b). The highly restricted localization of Nvj1-GFP to small patches of the nuclear envelope requires its interaction with Vac8, because it spreads over the entire surface of the nucleus in *vac8*Δ cells. It is likely that Nvj1 contains some kind of perinuclear membrane sorting signal, because Nvj1-GFP does not spread to the peripheral ER in *vac8*Δ cells or when overexpressed in wild type cells.

Although Vac8-Nvj1 complexes may be sufficient to physically link vacuole and nuclear membranes, other proteins probably associate with the junction to regulate and facilitate Pmn. Two proteins that accumulate in NV junctions include Tsc13, an ER membrane protein that is a putative enoyl reductase required for very long chain fatty acid synthesis,[40] and Osh1,[41] a putative oxysterol binding protein. An ankyrin repeat domain is required for the NV junction localization of Osh1.[41] The role of these proteins in lipid metabolism is potentially important because cells control the lipid composition of membranes at specific locations to facilitate deformation, fusion, and fission, and to recruit specific proteins into lipid domains.[42,43] Very long chain fatty acids are thought to facilitate membrane bending around nuclear pores and possibly during NE fission in yeast.[32,44] It is intriguing to note that NE fission in *S. cerevisiae* occurs both during mitosis and Pmn. Thus it is possible that Tsc13 and Osh1 participate in the modification of NV junction lipids. Alternatively, or in addition, the localization of these proteins to NV junctions may serve a more general role in lipid biosynthesis and/or transport such as occurs at regions of close contact between the ER and other organelles.[45,46,47] Finally, it is possible that the GFP-tagged versions of Osh1 and Tsc13 used to establish their localizations are recognized as misfolded proteins and targeted to NV junctions for degradation by Pmn.

Physiological Control of NV Junctions

NV junctions appear to be induced by nutrient depletion, as their frequency increases from ~50% during early log phase to greater than 90% by late log.[23] The total surface area of NV junctions per cell also increases at higher cell densities. *NVJ1* expression levels rise concomitant with the expansion of NV junctions. Also, because the ectopic over-expression of *NVJ1* is sufficient to radically increase the surface area of NV junctions in rapidly dividing cells (Fig. 1B, panel a), it is likely that cellular levels of Nvj1 are limiting for the size of NV junctions.[21] As *VAC8* expression levels do not significantly change in response to nutrient depletion, during which time NV junctions are on the increase, a larger proportion of the cellular Vac8 pool must be recruited into complexes with Nvj1 from elsewhere on the vacuole membrane. In fact, the high over-expression of *NVJ1* causes defects in vacuolar inheritance and fusion. [21]

Finally, Nvj1 binds Yih1, and the morphology of NV junctions and Pmn structures appear altered in starved *yih1*Δ cells (E. Kvam, unpublished results). Yih1 is a member of the conserved "Impact" family of proteins and, in *S. cerevisiae*, competes with Gcn1 for binding to the GI domain of the eIF2α kinase Gcn2.[48] Gcn1 and Gcn2 play important roles in general amino acid control by regulating the translation of the Gcn4 transcription factor. Regarding the possible role of Yih1 in the metabolic control of both processes, it is interesting that nitrogen starvation represses Gcn4 translation[49] but induces Pmn.[23]

Microautophagy of the Nucleus

Morphological, Genetic and Biochemical Evidence for Pmn

Pmn occurs in the context of NV junctions and involves several morphologically distinct intermediates (Fig. 1). The bulging of NV junctions is the first morphologically distinguishable Pmn intermediate. Those bulges that continue along the pathway become increasingly engulfed by the vacuole until they are attached to the nucleus by only the narrowest of tethers. Pmn blebs detach by scission of the vacuolar and two nuclear membranes, and are released into the vacuole lumen as Pmn vesicles. During the blebbing process, the separation between the inner and outer nuclear membranes remains constant, as if they were attached (or under pressure from inside). This is not always the case, because the two nuclear membranes separate to form blisters in certain NPC mutants.[50] Finally, Pmn vesicles are degraded by soluble vacuolar hydrolases, and the products are presumably either stored in the vacuole or recycled to the cytoplasm.

The existence of Pmn is supported by morphological, genetic, physiological, and biochemical evidence. The striking appearance of Pmn intermediates lends itself especially well to microscopic analysis. Transmission electron microscopy images of NE blebs protruding into adjacent vacuoles first suggested the existence of Pmn. These structures are absent in *vac8* and *nvj1* mutant cells. A direct role of Nvj1 in the formation of Pmn blebs and vesicles was confirmed using functional Nvj1-GFP reporters, which, when expressed at physiological levels, localize exclusively between Hoechst-stained nuclei and FM 4-64-stained vacuole membranes (Fig. 1B, panel b). Pmn vesicles are distinguished from autophagic bodies and other intra-vacuolar vesicles by their fluorescent costaining with Nvj1-GFP and FM 4-64 (Fig. 1B, panel c). Like autophagic bodies, free-floating Pmn vesicles bounce around like pinballs in the vacuole lumen. Importantly, Hoechst-stained chromatin is absent from the vast majority of Pmn blebs and vesicles.

Three lines of evidence support the conclusion that NE vesicles and their contents are actually degraded in the vacuole.[23] First, like other autophagic bodies, Pmn vesicles accumulate in *pep4*Δ cells, indicating that they are normally degraded by *PEP4*-dependent vacuolar hydrolases. Second, Nvj1-GFP/FM 4-64-stained Pmn vesicles are sometimes (infrequently) observed by confocal microscopy to "pop" and then dissipate. Third, the half-life of Nvj1-GFP as assessed by immunoblot is significantly longer than normal in *vac8*Δ and *pep4*Δ cells.

Pmn occurs independent of the Cvt pathway and macroautophagy, both of which are absent in *apg7*Δ cells (see chapters 6 and 7). In *apg7*Δ cells, Pmn structures are present at wild type levels and the half-life of Nvj1-GFP is normal. This is curious because the formation of microautophagic tubes in *S. cerevisiae*[51] and micropexophagy in *Pichia pastoris*[52] are both strongly affected by mutations in genes such as *APG7* (*GSA7 in P. pastoris*) that are required for macroautophagy (see chapters 8 and 10). It is not obvious why *AGP7/GSA7* should be required for these microautophagic processes but not for Pmn. It has been suggested that the fusion of autophagosomes with the vacuole is required to replenish vacuole membrane depleted by microautophagic invagination and scission.[51] It is possible that the rate of vacuole membrane degraded by Pmn is low enough not to require the contribution of new membrane from Cvt vesicles or autophagosomes. Instead, the net delivery of membrane derived from ER-Golgi vesicle trafficking pathways normally employed in the biogenesis and growth of vacuoles may suffice.[53] Many questions remain regarding the physiological, regulatory, and mechanistic relationship between "macroautophagic" and "microautophagic" processes in yeast and higher cells.

Physiological Control of Pmn

Pmn probably acts during log growth to degrade unneeded or damaged nuclear components, and in starving cells to recycle excess materials such as preribosomes (see below). Unlike macroautophagy, which is repressed in cells growing in rich medium, Pmn occurs at measurable levels in rapidly dividing cells. At higher cell densities in dividing cultures, the numbers of Pmn structures increase in parallel with rises in the frequency of NV junctions. Specifically, the frequency of cells containing at least one Pmn bleb or vesicle increased from ~5-10% in very early log phase cells to ~35% by early stationary phase.

Thus it appears that Pmn is controlled by signaling pathways, which respond to the gradual depletion of available carbon and nitrogen in the medium. In contrast, macroautophagy is induced by acute starvation and is virtually undetectable in dividing cultures.[35] Given these differences, both Pmn and macroautophagy are induced by rapamycin, an inhibitor of the Tor kinase nutrient signaling pathway.[23,54] The induction of Pmn at high cell densities might also be explained by the accumulation of secreted factors or metabolic side-products within the cells or in the culture medium. For example, Pmn is induced when hydrogen peroxide is added to the medium of early log phase cells (S. Moshkovitz, unpublished results). Nutrient depletion is sufficient, however, because Pmn is induced when dividing cells are transferred to fresh nitrogen or glucose starvation media.[23] Possible cross-talk between Pmn and macroautophagy, which could be mediated by the regulatory kinase Apg1,[39] has not been investigated.

Pmn Cargo

The nucleus plays absolutely critical roles in gene expression, metabolism, ribosome biogenesis, growth, and division, and is continuously undergoing structural remodeling. Efforts to elucidate the role(s) of Pmn in cell physiology will definitely profit from knowing more about which nuclear components are targeted for degradation by Pmn. Defining the contents of Pmn vesicles is also interesting because it is possible, if not likely, that proteins, RNAs, and possibly even DNAs may be selectively partitioned into Pmn blebs. For example, nitrogen limitation may trigger the degradation of different nuclear components than heat shock. Alternatively, there may be no positive selectivity to what is degraded. The formation of NV junctions on the surface of the nucleus could be haphazard, and Pmn blebs that develop from within NV junctions might just take random 'bites' out of the nucleoplasm in the same fashion as autophagosomes nonselectively incorporate bulk cytosol.

Because Vac8 and Nvj1 are defining components of every Pmn vesicle, they are, in a sense, selectively targeted for degradation by Pmn. By analogy, Aut7/Apg8 is required for the formation of Cvt vesicles and autophagosomes and is degraded in the vacuole along with autophagic bodies.[55] The degradation of Tsc13 and Osh1 might also be considered to be selective, because

they concentrate in NV junctions.[40,41] Still, these membrane proteins may be special cases. More interesting will be the fate of nucleoplasmic components.

There does appear to be a mechanism(s) that prevents the inclusion of bulk chromatin into Pmn blebs and vesicles. Obviously, the targeting of chromosomes to the vacuole would kill the cell. The evidence for the exclusion of chromatin from Pmn vesicles is twofold. First, cells generally do not die of Pmn, because greater than 90% of late log phase cells are viable even though 25-30% of this population contains Pmn blebs and/or vesicles.[23] Second, by confocal microscopy Hoechst-stained chromatin is absent from the lumen of Pmn blebs and vesicles in healthy cultures (unpublished results).

NPCs are also absent from NV junctions and Pmn blebs in dividing cells.[21-23] Because NPCs protrude at least 20 nm into the cytoplasm,[56] their sheer bulk may be sufficient to exclude them from NV junctions, where the separation between nuclear and vacuole membranes is only ~12.5 nm. The exclusion of chromatin and NPCs from Pmn blebs in dividing cells could be related because it has been suggested that chromosomes in yeast are tethered to the NE via NPCs.[3]

The study of Pmn is made difficult by its built-in inefficiency. Pmn mediates the partial degradation of the nucleus and, as such, the turnover of most abundant nuclear proteins can be expected to be incomplete and slow. In contrast, the targeting of precursor Ape1 by the Cvt pathway[35] and fructose-1,6-bisphosphatase by the vacuolar import and degradation (Vid) pathway[57] are both highly efficient and go to near completion (see chapters 7 and 13). The turnover of Nvj1 by Pmn can, of course, be studied, but its quantitation is hampered by the fact that the same conditions (nutrient depletion or starvation) which induce its degradation also induce its expression.[23] To overcome this difficulty a pulse of Nvj1-EYFP was generated by transiently inducing P_{GAL1}-*NVJ1*-EYFP expression with galactose for 3 hrs. The cells were then switched to glucose-containing SD-N nitrogen starvation medium to shut-off expression, stop division, and induce Pmn. Under these conditions, the turnover of the Nvj1-EYFP 'pulse' could be quantified by immunoblot in various genetic backgrounds.[23]

Some Pmn blebs contain material that is clearly derived from the granular nucleolus. The granular nucleolus owes its characteristic morphology to the punctate appearance of densely packed preribosomes.[58] Figure 2 shows a starved cell containing two Pmn blebs, one of which clearly contains a portion of the granular nucleolus. In addition, preliminary results indicate that specific nonribosomal nucleolar proteins such as Nop8, which is required for 60S subunit biogenesis,[59] are partitioned into Pmn vesicles (S. Moshkovitz, unpublished results). In lieu of a careful morphometric analysis, we can not say whether NV junctions and Pmn blebs occur at a greater or lesser frequency over the nucleolus. However, preribosomes are a logical substrate for autophagic recycling in starving cells because most of them are no longer needed in the cytoplasm.

Prospects

Many basic questions remain about the molecular mechanism of Pmn and its physiological role(s) in the cell. Virtually nothing is known about the molecular mechanism of bleb formation, nor is it known what factors facilitate the coordinated scission of vacuole and nuclear membranes. Although NV junctions specify the nucleus as a target of microautophagy, it remains to be determined if mechanisms exist which selectively target specific nuclear components into Pmn blebs, or if there are differences in the composition of Pmn vesicles in dividing versus starving cells. Issues regarding the positioning of NV junctions on the nuclear surface, especially during mitosis, may contribute to the polarity of the yeast cytoplasm. The degradation of the nucleolus by Pmn is especially interesting because of its diverse roles in gene silencing, cell cycle control, and aging.[4,60,61] There is an intriguing possibility that the study of Pmn in apoptotic *S. cerevisae* cells may provide insight into the conserved role that autophagy is thought to play in programmed cell death.[62] *S. cerevisiae* contains a metacaspase and undergoes a form of apoptosis that shares many features with programmed cell death in higher cells.[16]

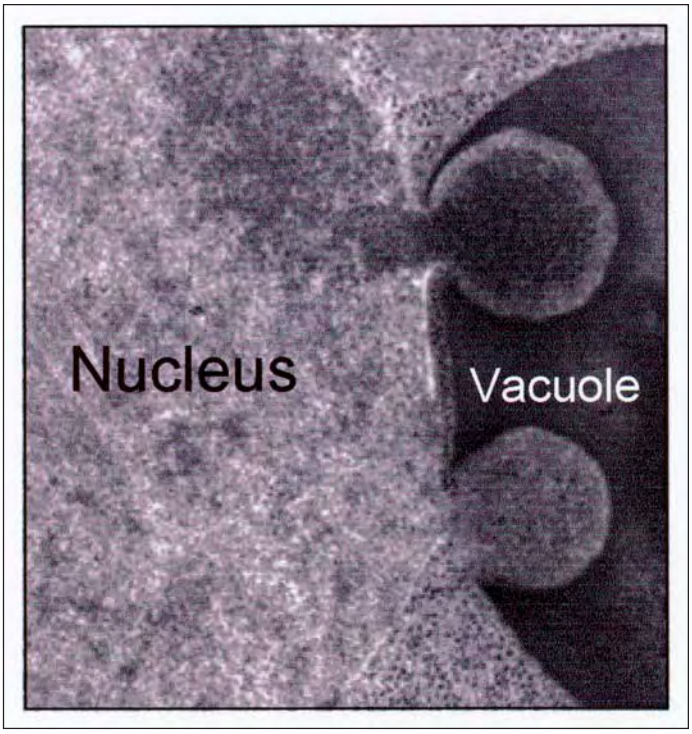

Figure 2. Transmission electron micrograph of a cell containing two Pmn blebs. The upper bleb contains material from the granular nucleolus. Reprinted from Molecular Biology of the Cell (2003, vol. 14, pgs. 129-141) with permission by the American Society for Cell Biology.

Preliminary results suggest that Pmn and apoptosis in yeast may be coordinated (S. Moshkovitz, unpublished results). Finally, even though *VAC8* and *NVJ1* are not conserved, and the possibility remains that Pmn evolved strictly to service nuclei that do not break down during mitosis, we are eager to investigate whether Pmn-like processes occur in plants and animals.

Acknowledgements

The author wishes to acknowledge past and present contributors to this project, including Xioazhou Pan, Paul Roberts, Erik Kvam, Nataliya Shulga, and Sharon Moshkovitz, and Mark Winey and Eileen O'Toole (University of Colorado) for their exceptional electron microscopy. This work was supported by grants from the NIH (5RO1 GM362-11) and NSF (MCB-0110972).

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CHAPTER 10

Glucose-Induced Pexophagy in *Pichia pastoris*

Pouran Habibzadegah-Tari and William A. Dunn, Jr.

Abstract

Pexophagy is the selective degradation of peroxisomes by the yeast vacuole. In *Pichia pastoris*, pexophagy occurs when cells adapt from utilizing methanol as the sole carbon source to metabolizing glucose. Upon glucose adaptation from methanol, the peroxisomes are engulfed within the vacuole by an invagination at the vacuole surface followed by the extension of arm-like projections of the vacuole, which sequester the peroxisomes. Once inside the vacuole the peroxisomes are rapidly degraded by the hydrolytic enzymes present. At least 21 genes have been identified that are known to be essential for glucose-induced pexophagy. A few of these genes appear to be required for the regulation of pexophagy while many of them are essential for the sequestration of peroxisomes and others are necessary for the degradation of peroxisomes. In this chapter, we will discuss our current understanding of the functional roles of these genes in the molecular events of pexophagy.

Introduction

Cell survival depends upon the ability to adapt to changing environmental conditions by synthesizing required proteins and degrading those that are no longer required or have become nonfunctional. Protein synthesis can be regulated at both transcriptional and translational levels. The selective and nonselective removal of proteins is mediated by both nonlysosomal and lysosomal events. Nonlysosomal proteolysis can be performed by the ubiquitin-mediated proteasome pathway and cytosolic proteinases such as calpains and caspases. Lysosomal proteolysis requires the prior sequestration of cellular components by autophagic events. There exist three classifications of autophagy: chaperone-mediate autophagy, microautophagy, and macroautophagy. Chaperone-mediated autophagy is the receptor-mediated selective insertion of soluble proteins directly into the lysosome (see chapter 12).[1] Microautophagy occurs at the lysosome surface whereby the membrane invaginates and extends arm-like projections to sequester proteins and organelles into the lysosome (see chapter 8). Macroautophagy involves the sequestration of cellular components including organelles within an autophagosome that then delivers its contents to the lysosome by fusing with it (see chapters 2-7). These pathways of protein degradation have been documented in many eukaryotic systems including plants and yeast.[2-4]

Both microautophagy and macroautophagy have been characterized in a number of yeast models. We have shown that *Pichia pastoris* is capable of selectively degrading peroxisomes by both microautophagy and macroautophagy mechanisms. In addition, depriving the cells of nitrogen and amino acids can induce nonselective autophagy. Methylotrophic yeasts such as *Pichia pastoris* and *Hansenula polymorpha* (*Pichia augusta*) are able to utilize methanol as the sole carbon source by synthesizing peroxisomal and cytosolic enzymes required for methanol assimilation. Upon adapting these cells to a different carbon source such as glucose or ethanol, the now superfluous peroxisomes are rapidly and selectively degraded within the vacuole. When *P. pastoris* adapts from methanol to glucose, the peroxisomes are sequestered for degradation at

Autophagy, edited by Daniel J. Klionsky.

the vacuole surface by a microautophagic process. In contrast, adaptation from methanol to ethanol results in the sequestration of peroxisomes into autophagosomes and their degradation by macroautophagy. The selective degradation of peroxisomes referred to as pexophagy has also been documented in *S. cerevisiae*, *Yarrowia lipolytica*, *Aspergillus nidulans*, and mammalian cells.[2,5-8] In this review, we will discuss our current knowledge of pexophagy in *P. pastoris*.

Autophagic Pathways in *Pichia pastoris*

Cells respond to stress caused by intracellular pathogens or environmental changes by nonselectively and selectively degrading proteins and organelles. What makes *Pichia pastoris* an excellent model for studying these degradative events is that this yeast is capable of nonselectively degrading organelles when nitrogen and amino acid sources are in short supply and selectively degrading superfluous peroxisomes when nutritional substrates are changed. *P. pastoris* is a methylotrophic yeast capable of growing on methanol as the sole carbon source. Methanol is oxidized to formaldehyde by alcohol oxidase within peroxisomes. The formaldehyde is then degraded to carbon dioxide by cytosolic enzymes with the production of energy in the form of $NADH_2$. The generation of biomass occurs upon entry of formaldehyde into the xylulose 5-phosphate cycle yielding three-carbon compounds, glyceraldehyde 3-phosphate and dihydroxyacetone. When the carbon source is changed from methanol to glucose or ethanol, the peroxisomes are rapidly and selectively degraded by micro- and macropexophagy, respectively.

Autophagy

Autophagy is a cellular response to amino acid deprivation observed in virtually all eukaryotic cells. When mammalian and yeast cells are deprived of essential amino acids, cellular components and organelles are non-selectively delivered to the lysosome and the proteins degraded into their monomeric subunits—amino acids, fatty acids and sugars—by the acid hydrolases present.[9] These sequestration events occur by both microautophagy and macroautophagy. Microautophagy proceeds by invaginations of the vacuole (lysosome) membrane that bud and form intravacuole vesicles. Invaginations at the vacuole surface are observed when *P. pastoris* are starved for nitrogen and amino acids (Fig. 1A). Similar observations were reported in *S. cerevisiae*.[10] During macroautophagy, double-membrane-bound autophagosomes are formed within minutes to hours of nitrogen deprivation (Fig. 1B). In mammalian cells, these vesicles mature in a step-wise fashion and ultimately fuse with lysosomes.[11,12] The autophagosome may first acquire lysosomal membrane proteins including the acidifying H^+-ATPase prior to the delivery of acid hydrolases. This maturation model of autophagy in mammalian cells has not yet been defined in the yeast system.

Both micro- and macroautophagy are enhanced in starved cells. However, which pathway predominates during starvation has not been evaluated. This analysis requires a means to selectively inhibit one of these pathways, but no way has been determined to accomplish this in *P. pastoris*. However, it has been shown in *S. cerevisiae* that macroautophagy requires Vam3 while microautophagy does not (see chapter 8).[13,14] Further studies are needed to assess the importance of these pathways in starvation-induced protein degradation.

Pexophagy

The selective degradation of peroxisomes, pexophagy, has been documented in both yeast and mammalian cells. In *P. pastoris*, glucose-induced peroxisome degradation occurs by micropexophagy while ethanol-induced degradation occurs by macropexophagy.[15] Micropexophagy occurs when the vacuole invaginates and sequesters the peroxisomes for degradation (Fig. 1C). Results from our laboratory and those of Drs. S. Subramani and Y. Sakai have suggested that micropexophagy proceeds through five morphologically and genetically defined events that include: 1) glucose signaling; 2) peroxisome recognition and early sequestration events whereby the vacuole begins to invaginate and/or send out arm-like projections (or alternatively undergo septation); 3) intermediate sequestration events whereby the arm-like

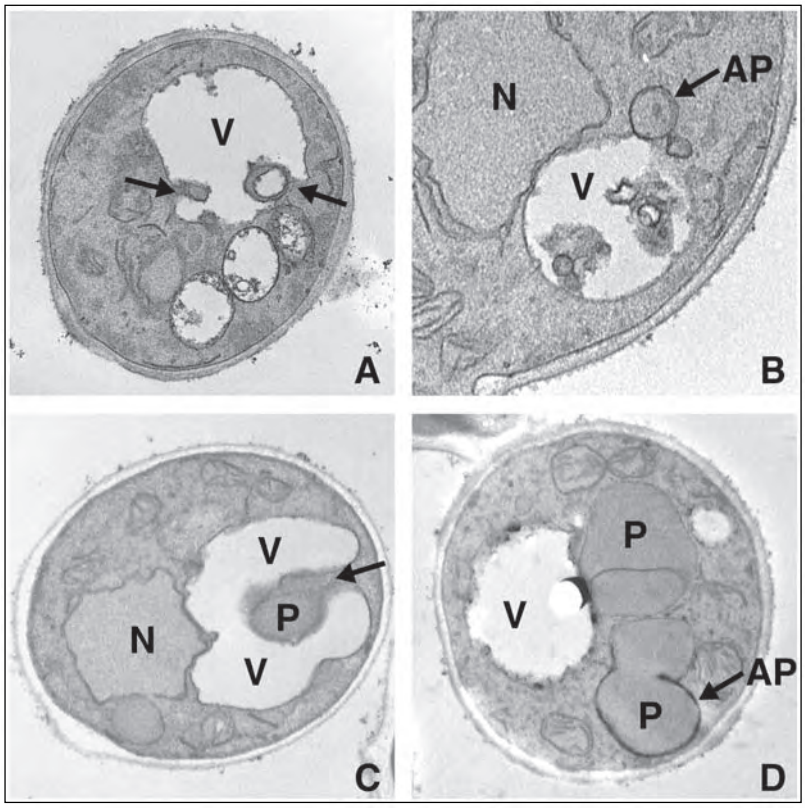

Figure 1. Microautophagy and macroautophagy in *Pichia pastoris*. Autophagy or pexophagy were induced in GS115 cells by starving the cells for amino acids and nitrogen for 3 hours (panels A and B) or by adapting the cells from methanol media to glucose (panel C) or ethanol (panel D) for 3 hours. The cells were then fixed in potassium permanganate and prepared for viewing by transmission electron microscopy.[15,41] In starved cells, autophagsosomes (AP) can be observed as well as profiles of vacuole invaginations suggestive of ongoing microautophagy (arrows in panel A). Micropexophagy can be observed when cells adapt from methanol to glucose (arrow in panel C). During ethanol adaptation, individual peroxisomes are sequestered within autophagosomes (AP) by macropexophagy (panel D). N, nucleus; V, vacuole; P, peroxisome.

projections extend from the vacuole to almost completely engulf the peroxisomes; 4) late sequestration events that include homotypic fusion of the juxtaposed vacuole membranes and formation of a microautophagic body; and 5) degradation of the microautophagic body and peroxisomes by vacuolar hydrolases (Fig. 2). The events of macropexophagy in *P. pastoris* have not been as well characterized. Layers of sequestering membranes, which arise from the rough endoplasmic reticulum or some unknown source (see chapter 2), engulf individual peroxisomes resulting in the formation of an autophagosome (Fig. 1D). The autophagosome then fuses with the vacuole and an autophagic body within the vacuole is formed. After destruction of the autophagic body membrane, the peroxisome is exposed to the vacuolar hydrolases and degraded. These hydrolases include proteinases, glycosidases, phosphatases, nucleases, and lipases that can degrade all cellular components.

Glucose-induced micropexophagy is selective and requires the synthesis of one or more proteins. We have shown that the cellular amounts of two peroxisomal enzymes, alcohol oxidase and dihydroxyacetone synthase, were significantly diminished, but that the amounts of

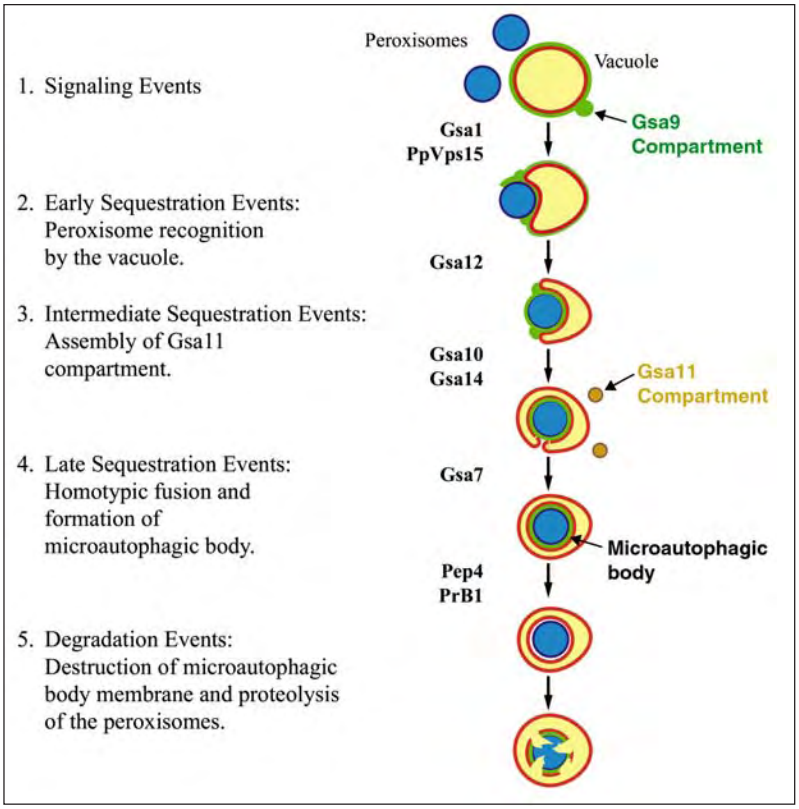

Figure 2. Micropexophagy in *Pichia pastoris*. Glucose-induced micropexophagy proceeds through a series of events that include: 1) glucose signaling; 2) early sequestration events that include the Gsa9 compartment and the invagination of the vacuole; 3) intermediate sequestration events that include the assembly of the Gsa11 compartment and the extension of arm-like projections from the vacuole to engulf the peroxisomes; 4) late sequestration events that include homotypic fusion of the juxtaposed vacuole membranes and formation of a microautophagic body; and 5) degradation of the peroxisomes by vacuolar hydrolases.

other cellular proteins identified by Coomassie staining were not altered during glucose adaptation.[16] The loss of these peroxisomal proteins was due to enhanced degradation that required proteinases A and B.[15,16] In addition, the β-subunit of the mitochondrial F_1 ATPase was not degraded during glucose adaptation.[16] Micropexophagy requires protein synthesis. In the presence of cycloheximide, the peroxisomes are not sequestered and the vacuole remains rounded with no evidence of invagination.[15] The data suggest that protein synthesis is required for either glucose signaling or an early sequestration event.

The selective removal of peroxisomes has also been reported to occur in plants and mammalian cells. When cells of *Aspergillus nidulans* are adapted from oleate medium to glucose, the peroxisomes are sequestered into vacuoles by events similar to micropexophagy.[2] The removal of excess peroxisomes by pexophagy has been described in hepatocytes that have been previously treated with either clofibrate or dioctyl phthalate.[7,17,18] These drugs interact with peroxisome proliferator-activated receptors to induce the production of peroxisomes in mammalian cells. These drugs are used clinically to treat dyslipidemia and hypertriglyceridemia.[19] Upon removal of these drugs, the peroxisomes are selectively degraded by mechanisms analogous to macropexophagy. Pexophagy is also responsible for the selective degradation of abnormal per-

oxisomes present in cells isolated from Zellweger patients.[20] 3-methyladenine, an inhibitor of autophagy, suppresses the degradation of these peroxisomes. The ability of the cell to modulate peroxisome levels by synthesis and degradation is essential in the regulation of lipid metabolism, which is central to energy production.

Glucose-Induced Selective Autophagy *(GSA)* Genes

P. pastoris is a proven genetic model which has been useful to identify genes and characterize proteins required for peroxisome synthesis and degradation. A number of laboratories have utilized this yeast to identify many unique *PEX* genes required for peroxisome biogenesis.[21] Utilizing a novel mutagenesis technique, twenty *GSA* and *PAZ* genes have been identified and shown to be required for the degradation of peroxisomes by micropexophagy (Table 1). Mutagenesis was done by restriction enzyme mediated integration (REMI) that involved the random genomic insertion of a vector containing a Zeocin-resistance gene (Fig. 3). Afterwards, pexophagy mutants were identified by direct colony assays by screening for colonies that could not degrade alcohol oxidase following a shift from methanol to glucose. We found that pREMI-Z inserted into a single locus in most of the mutants. The *GSA* gene disrupted by this insertion is then sequenced. Many of the Gsa and Paz proteins are structurally homologous to either Cvt proteins required for the transport of cytosolic aminopeptidase I to the vacuole, Vps proteins required for the transport of proteins from the Golgi to the vacuole, or Apg and Aut proteins required for autophagy in *S. cerevisiae* (see chapters 6 and 7). We have found that many of these genes are also required for the formation of the autophagosome in *P. pastoris*. The data suggest that the membrane events responsible for the delivery of proteins and organelles to the vacuole (e.g., microautophagy and macroautophagy) share a number of common proteins.

We have tentatively classified the functional roles of the Gsa proteins based on their requirements for signaling, sequestration (early, intermediate, and late), and degradation events. These classifications were done initially by comparing the vacuole morphology of the mutants during glucose-induced pexophagy. However, such comparisons are problematic because many of the *gsa* mutants are "leaky" and a complete blockage is not observed. Therefore, we have based our classifications not only on vacuole morphology but also on an examination of the cellular distribution of GFP-Gsa protein constructs in normal and *gsa* mutant cells. We have found that virtually all the *GSA* genes identified so far are required for the sequestration of peroxisomes.

Sequestration Signaling Events

GSA1 was the first pexophagy gene to be identified.[22] *GSA1* encodes the alpha subunit called Pfk1 of the phosphofructokinase enzyme complex. Pfk1 is a highly regulated protein with conserved domains for binding the activators AMP and fructose 2,6-bisphosphate and the inhibitors ATP and fructose 6-phosphate. Pfk1 associates with Pfk2 and an unknown protein to form a 975 kDa hetero-oligomeric enzyme complex.[23] This complex catalyzes the rate-limiting event in the glycolytic pathway, allowing the cell to respond to changes in glucose concentration. The evidence suggests that Gsa1 is likely required for a glucose-signaling event. First, Gsa1 is required for glucose-induced micropexophagy, but not ethanol-induced macropexophagy nor starvation-induced autophagy. Second, the vacuole remains rounded when *gsa1* mutants are adapted to glucose suggesting that Gsa1 is required for an early event in micropexophagy. Third, the primary function of phosphofructokinase is to modulate glucose flux through glycolysis. However, we have shown that the phosphofructokinase activity is not required for pexophagy suggesting that in this process Pfk may act independent of its glycolytic function.[22] Nevertheless, it is likely that Gsa1 is required for a glucose-signaling event.

The *P. pastoris* (Pp) Vps15 (Gsa19/Paz13) protein is a serine/threonine protein kinase required for both pexophagy and autophagy in *P. pastoris*.[24,25] The initial events of vacuole invagination were not observed in *paz13* mutants suggesting that this protein is required for glucose signaling or an early sequestration event. In *S. cerevisiae*, this protein is essential for

Table 1. GSA and PAZ genes required for glucose-induced pexophagy

GSA	PAZ	Aliases	Yeast Homologues	Comments	Reference
1			PFK1	Phosphofructokinase	22
7	12		APG7, CVT2	E1-like enzyme responsible for the conjugation of Apg12 to Apg5 and Aut7 to lipids	25, 36, 35
9	6		CVT9	Coiled-coil protein found at the vacuolar surface	25, 30
10	1		APG1, CVT10	Serine/threonine protein kinase that complexes with Cvt9, and Vac8	25, 43, 44
11	7		APG2	Soluble protein that associates with an organelle juxtaposed to the vacuole	25, 31, 45
12			AUT10, CVT18	WD40 protein associated with the vacuolar surface	33
13				Putative transmembrane protein	
14	9		APG9, AUT9, CVT7	Transmembrane protein associated with an organelle juxtaposed to the vacuole	25, 46
15	14	PEP4	PEP4	Endopeptidase	25
17				Zinc-finger protein	
18				WD40 protein associated with the vacuolar surface	
19	13	PpVPS15	VPS15	Serine/threonine protein kinase anchored to the membrane via myristoylation	24, 25
20			AUT1, APG3	E2-like enzyme responsible for the conjugation of Aut7 to lipids	36
21		PpVAC8	VAC8	Armadillo-repeat protein anchored to the vacuole surface via myristoylation and palmitoylation	39, 47
	2		AUT7, APG8, CVT5	Soluble protein conjugated to lipids at the surface of the autophagosome	25, 36
	3		APG16	Coiled-coil protein that interacts with the Apg12-Apg5 conjugate	25, 36
	4	UGT51		UDP-glucose:sterol glucosyltransferase	25
	5		GCN3	alpha subunit of eIF2B	25, 48
	8		AUT2, APG4	Cysteine peptidase required to expose the C-terminal glycine moiety of Aut7 to allow its conjugation to lipids	25, 36
	10		GCN1	Starvation-induced activation of Gcn2 and translational activation of Gcn4	25, 49
	11		GCN2	eIF2α kinase	25, 49
	16		CVT13, SNX4	Phox protein that interacts with Cvt20 and Apg17	50 (Y. Sakai, personal comm.)
	19		GCN4	Leucine-zipper protein that positively regulates gene transcription during amino acid starvation	51 (Y. Sakai, personal comm.)

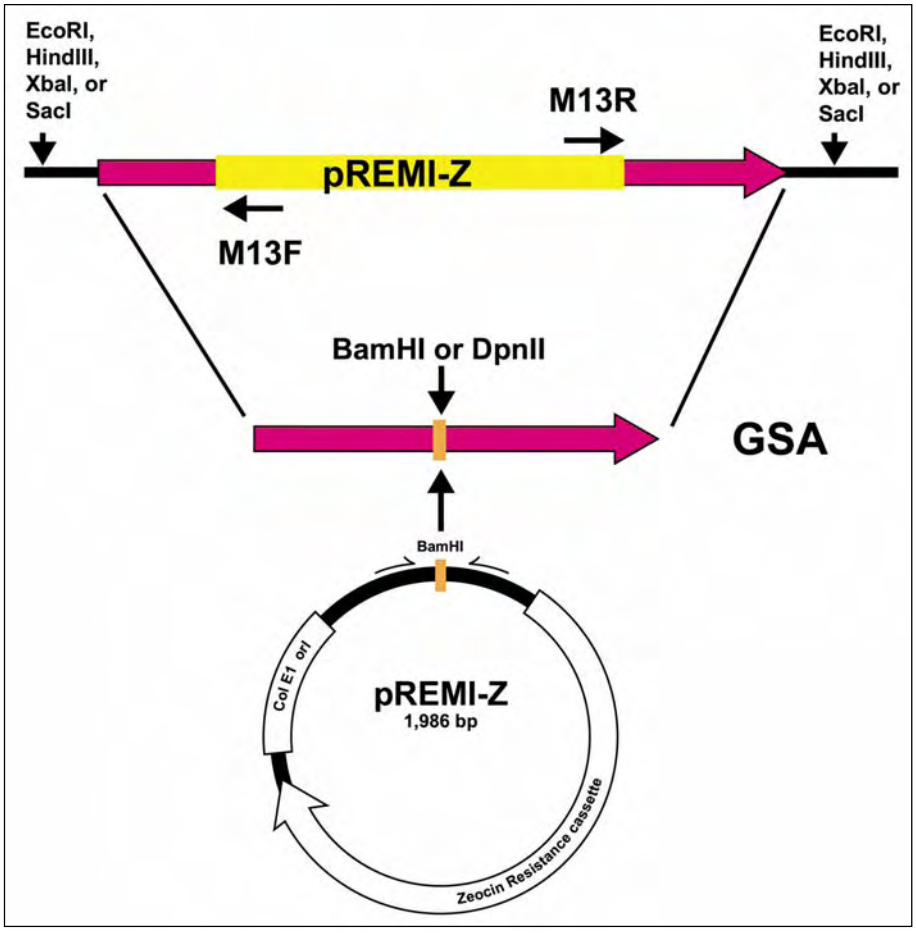

Figure 3. Restriction enzyme-mediated integration (REMI) mutagenesis. GS115 cells were transformed in the presence of BamHI or DpnII enzyme with pREMI-Z (AF282723) that had been linearized with BamHI. The *gsa* mutants were then identified and isolated. The genomic DNA from these mutants was recovered, digested with EcoRI, HindIII, XbaI or SacI, and the DNA circularized by ligation. The resulting vector containing the Zeocin resistance gene flanked by yeast genomic DNA was then amplified and the disrupted *GSA* gene sequenced using M13 primers.

sorting proteins from the Golgi apparatus to the vacuole by activating Vps34. Vps34 belongs to a family of class III phosphatidylinositol (PtdIns) 3-kinases. In addition, Vps34 has been shown to be required for glucose-induced pexophagy in *H. polymorpha*.[26] Apg6/Vps30 and Apg14 are peripheral membrane proteins that interact to form a stable complex with Vps15 and Vps34 (see chapters 6 and 7).[27] This complex also appears to be required for autophagy in mammalian cells (see chapter 3). The formation of the autophagosome in mammalian cells requires a class III PtdIns 3-kinase that can be inhibited by wortmannin and 3-methyladenine, a Bcl2-binding protein called Beclin, which is homologous to Apg6 (see chapters 15 and 20), and a protein kinase p150, which is homologous to Vps15.[28,29] The protein substrate for this kinase has not been determined. In addition to its protein kinase signature at the N-terminus, Vps15 from both *P. pastoris* and *S. cerevisiae* has a myristoylation membrane anchoring site and four WD40 domains at its C terminus that can encourage protein-protein interactions.

Early Sequestration Events

The early sequestration events include peroxisome recognition and invagination of the vacuole surface. The specific degradation of peroxisomes during glucose adaptation requires their recognition and sequestration by the vacuole itself. Therefore, we expect one or more Gsa proteins to localize to or interact with the vacuole surface and possibly act as a recognition protein.

A likely peroxisomal recognition protein at the vacuole surface is Gsa9.[30] Gsa9 is a 150 kDa protein that is structurally homologous to Cvt9 in *S. cerevisiae* (see chapter 7). In *gsa9* cells, the vacuole was found to have arm-like projections partially extending around the peroxisome within two hours of glucose adaptation. This would suggest that Gsa9 is required for an intermediate sequestration event. Gsa9 localizes to a region where the vacuolar membrane appears to make contact with the peroxisomes. As predicted for a protein that may function in the vacuolar recognition of peroxisomes, Gsa9 is not required for starvation-induced nonselective autophagy. Similarly, the constitutive selective autophagy of aminopeptidase I, but not starvation-induced nonselective autophagy is impaired in *cvt9* mutants. In addition, we have shown that Gsa9 localized around the peroxisomes independent of vacuole sequestration. In fact, our data suggest that Gsa9 localizes to the peroxisomes prior to vacuole sequestration and independent of Gsa7, Gsa11, or Gsa12.[31] The data suggest that Gsa9 may function in the vacuolar recognition of peroxisomes. A potential target for Gsa9 is Pex14. Pex14 has been shown to be required for peroxisome degradation in *H. polymorpha* and both Gsa9 and Pex14 contain protein interacting coiled-coil domains.[32] Although the role of the coiled-coil domain in peroxisome biosynthesis and degradation has not been examined, it appears that the N terminus of Pex14 that also contains the Pex5 binding site is required for pexophagy. We project that Gsa9 is required for directional movements of the vacuolar arms around the peroxisomes. In the absence of Gsa9 the initial events of micropexophagy proceed, but the engulfment process is not completed.

Another member of the family of pexophagy genes found at the vacuole membrane is Gsa12.[33] *GSA12* encodes a 60 kDa protein that is homologous to Aut10/Cvt18 in *S. cerevisiae*. Both proteins contain two WD40 repeats, which have been shown in other proteins to mediate protein-protein interactions.[34] Mutants lacking Gsa12 are blocked at an early sequestration stage with the vacuole being indented but lacking arm-like extensions that are characteristic of the intermediate sequestration stage. The exact function of Gsa12 in pexophagy and autophagy is not known, but it is required for the assembly of the Gsa11 compartment that occurs during micropexophagy (see below). Gsa12 is localized in the cytosol and at the vacuolar membrane. It is possible that the equilibrium between these two pools may have an essential role in the regulation of pexophagy. The absence of any transmembrane domains suggests that Gsa12 is not an integral membrane protein but instead interacts with one or more membrane proteins at the vacuole surface. The WD40 domains within Gsa12 likely mediate this interaction. However, Gsa12 has only two repeats, and a minimum of four WD40 domains is needed to form the β-propeller platform that has been shown to interact with proteins.[34] Gsa18 and Gsa19 (PpVps15) also contain WD40 domains. Like Gsa12, Gsa18, which has three WD40 motifs, is found at the vacuole surface (unpublished observations). In principle, the dimerization of Gsa12 or its association with Gsa18 will form a four or five bladed propeller.

Intermediate Sequestration Events

The intermediate sequestration events include the extension of arm-like projections from the vacuole partially surrounding the peroxisome cluster. The assembly of a unique *Gsa11* compartment is coincident with these vacuolar movements.

GSA11 encodes a 208 kDa protein required for selective micro- and macropexophagy, as well as for starvation-induced autophagy.[31] Gsa11 is structurally similar to *S. cerevisiae* Apg2 (see chapters 6 and 7). Apg2 is required for starvation-induced autophagy and for sorting of aminopeptidase I through the Cvt pathway. In *gsa11* mutants, the movements of the vacuole arm-like protrusions around the peroxisomes were limited. However, Gsa9 was shown to dis-

tribute around the peroxisome cluster in the absence of the vacuole arms.[31] These results indicate that Gsa11 is not required for Gsa9 to interact with the peroxisomes, but is required for vacuolar engulfment of peroxisomes. Gsa11 is a cytosolic protein that relocates to 1-3 structures juxtaposed to the vacuole during glucose adaptation.[31] This relocation requires PpVps15, Gsa12, Gsa10, and Gsa14, but not Gsa7 or Gsa9. This is consistent with PpVps15 and Gsa12 being essential for an upstream signaling and an early sequestration event, respectively. Gsa7 is required for a late sequestration event (see below). Meanwhile, Gsa10 and Gsa14 appear to be essential for an intermediate sequestration event.

Gsa10 is a serine/threonine protein kinase homologous to Apg1 (see chapters 6 and 7). Gsa10 is an 85 kDa protein with a well-conserved N-terminal "kinase domain" and a C-terminal "C-domain" which are separated by a variable "PS domain" which is rich in prolines and serines. Cells lacking Gsa10 are defective in glucose induced pexophagy and starvation-induced autophagy. Based on vacuole morphology, mutants lacking Paz1/Gsa10 are blocked at a late stage in pexophagy.[25] However, Gsa10 is required for Gsa11 to associate with perivacuolar structures.[31] At this time, the protein substrate for Gsa10 has not been identified nor is it known if Gsa11 is phosphorylated. Therefore, it is unclear if Gsa10 directly or indirectly affects Gsa11 function.

GSA14 encodes a 115 kDa membrane protein required for both selective pexophagy and nonselective autophagy. Gsa14 is structurally similar to Apg9, which is required for aminopeptidase I targeting to the vacuole and starvation induced autophagy (see chapters 6 and 7). Gsa14 contains five to six putative transmembrane domains with no recognized motifs to suggest function. In cells grown in methanol or glucose, Gsa14 localizes to unknown organelles juxtaposed to the vacuole. These structures are similar in appearance and number to those containing Gsa11 when cells adapt to glucose. We propose that Gsa14 resides in these perivacuole vesicles and recruits Gsa11 upon the onset of pexophagy.

Late Sequestration Events

A key protein in micropexophagy and non-selective autophagy in *P. pastoris* is an E1-like enzyme encoded by *GSA7*.[35] This 71 kDa protein is analogous to Apg7 from *S. cerevisiae* (see chapters 6 and 7). Gsa7 belong to a family of E1 enzymes, which has been shown to activate ubiquitin and ubiquitin-like proteins. Gsa7 contains an ATP binding motif as well as the catalytic cysteine site characteristic of E1 enzymes. Site directed mutagenesis of these sites showed that they are required for Gsa7 function in peroxisome degradation.[35] Our studies reveal that Gsa7 acts at a late step in the sequestration of peroxisomes. The blockage is virtually complete in the *gsa7* mutants and occurs at the late sequestration stage whereby the vacuole almost completely surrounds the peroxisomes. In addition, Gsa7 is not required for the association of Gsa11 with the perivacuolar structures. These results suggest that Gsa7 is necessary for either bringing the opposing vacuolar membranes together or assisting in the fusion of the opposing vacuolar membranes.

In *S. cerevisiae*, Apg7 catalyzes two protein conjugation events that are essential for the formation of the autophagosome.[36] The first event is the conjugation of the C terminus of Apg12 to a lysine moiety of Apg5. This reaction requires an E2-like enzyme called Apg10. The Apg12-Apg5 conjugate then interacts with Apg16 to somehow initiate the formation of the autophagosome. The second conjugation event is the linkage of the C terminus of Aut7/Apg8 to phosphatidylethanolamine at the autophagosome membrane. This event requires the prior removal of the C-terminal arginine from Aut7 by Aut2/Apg4 and an E2-like enzyme called Aut1/Apg3. In addition, the membrane recruitment of Aut7 requires Apg7, Apg12, and Apg5.[37]

The many screens of pexophagy mutants done by our laboratory and that of Dr. Y. Sakai have not found the *P. pastoris* homologues of Apg10, Apg12 and Apg5 indicating that this pathway may not be essential for pexophagy. However, the *P. pastoris* homologues of Aut7, Aut2 and Aut1 have been identified as Paz2, Paz8, and Gsa20, respectively (Table 1). Mukaiyama et al., have shown that Paz8 removes 9 amino acids from the C terminus of Paz2 to expose a

glycine moiety.[25] Furthermore, when this glycine is mutated to an alanine, Paz8 cannot process Paz2. Based on comparisons of vacuole morphology, Mukaiyama et al., have indicated that Paz8 and Gsa7/Paz12 are required for a late sequestration event.[25] Conversely, *paz2* null mutants appear to be blocked at an early sequestration stage. These results suggest that Paz2 may be required for more than one sequestration event.

Degradation Events

The yeast vacuole is analogous to the mammalian lysosome containing numerous hydrolytic enzymes that include proteinases, glycosidases, nucleases and phosphatases. In *S. cerevisiae*, many of these hydrolases are synthesized as inactive precursors, which are then transported to the vacuole and activated by proteinases A and B (Pep4 and Prb1).[38] Therefore in the absence of these proteinases, vacuolar hydrolase activities are reduced and overall protein degradation is suppressed. We have found that carboxypeptidase Y activities are not detected in *P. pastoris* mutants lacking Pep4.[15]

The degradation of peroxisomes by glucose-induced or ethanol-induced pexophagy is almost completely inhibited in *pep4 prb1* double mutants resulting in the accumulation of peroxisomes within the vacuole.[15] In addition, protein degradation induced by amino acid starvation is efficiently suppressed in *pep4*, *prb1* and *pep4 prb1* mutants.[15] We have also shown that autophagic bodies accumulate in *pep4 prb1* cells starved for amino acids. These observations suggest that in *P. pastoris*, proteinases A and B are essential for protein degradation during pexophagy and autophagy.

The *paz5*, *paz10*, and *paz11* mutants are blocked late in pexophagy.[25] In these mutants, the peroxisomes are within the vacuole but the limiting membranes of the microautophagic body and the peroxisome are not disrupted. These genes encode proteins homologous to Gcn3, Gcn1, and Gcn2, respectively, which are involved in the cellular response to starvation. However, the role of these proteins in vacuolar degradation is unknown.

Compartments of Micropexophagy

We have identified two unique compartments that house some of the essential elements of pexophagy. These compartments reside at or near the vacuole and appear to be distinct from each other. The Gsa9 compartment appears as a single dot at the vacuole surface, while the Gsa11 compartment is observed as 2-3 structures adjacent to the vacuole (Fig. 4). The Gsa9 compartment is visible regardless of environmental conditions. Meanwhile, the assembly of Gsa11 into the perivacuolar structures occurs only when pexophagy is stimulated.

Gsa9 Compartment

When cells are grown in YPD or YNM, Gsa9 associates with the vacuole membrane and a single structure located at the vacuole surface, which we call the Gsa9 compartment. During glucose-induced pexophagy, Gsa9 is positioned at the concave surface of the invaginating vacuole between the vacuole and those peroxisomes being sequestered (Fig. 4A,B). Gsa9 at the convex surface of the vacuole is virtually nondetectable. The dynamics of this compartment are best evident in the *gsa11* mutants. In these mutants, the vacuole arms only partially surround the peroxisomes. However, Gsa9 surrounds the peroxisomes and localizes to two structures situated at the vacuolar arms on opposite sides of the peroxisome cluster. These structures appear to be organizing centers for the movements of Gsa9 around the peroxisomes, which occur independent of the vacuole. In *S. cerevisiae*, there is evidence to suggest this compartment contains in addition to Cvt9 (Gsa9), Apg1, Aut7, Apg5, Apg12, Apg1, Apg13, Vac8, Cvt19, and Apg9 (Gsa14).[39,40] In addition, the recruitment of Aut7 to this compartment requires Apg5 and Apg12, but not Apg9.[37] However, our data show that the Gsa9 compartment resides at the concave surface of the involuting vacuole while the Gsa11 and Gsa14 organelles are juxtaposed to the convex surface (Fig. 4).

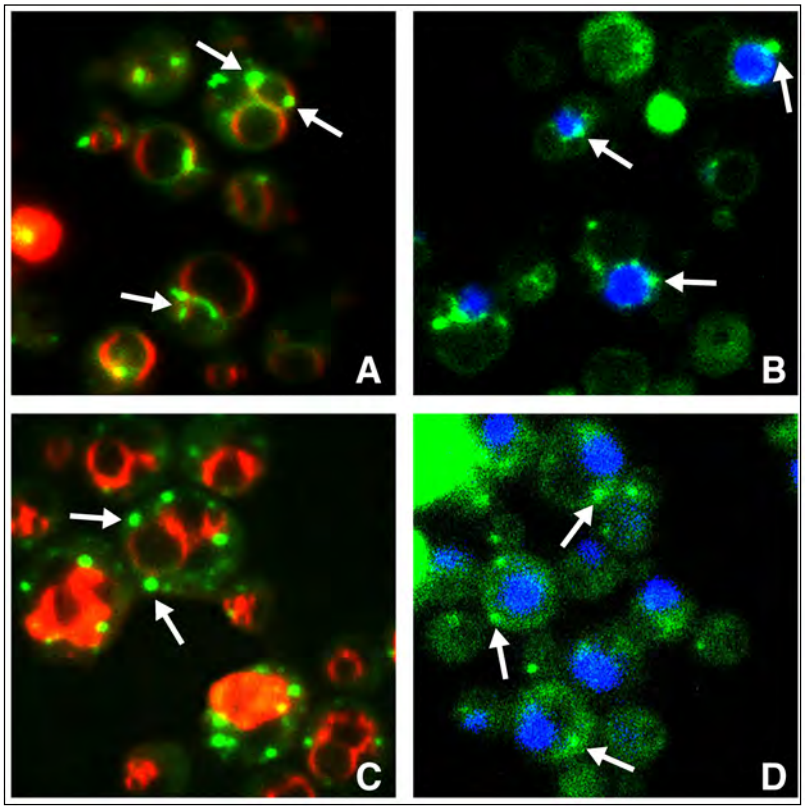

Figure 4. Cellular localization of Gsa9 and Gsa11 during glucose-induced pexophagy. Cells expressing GFP-Gsa9 (panels A and B) or GFP-Gsa11 (panels C and D) were grown in methanol and then adapted to glucose for two hours. The GFP conjugated proteins, the vacuoles labeled with FM 4-64 (panels A and C), and the peroxisomes containing BFP-SKL (panels B and D) were visualized in situ by fluorescence microscopy. GFP-Gsa9 localized to the vacuolar arms in close association with the peroxisomes (arrows in panels A and B). GFP-Gsa11 was found in discrete dots adjacent to the vacuole, but clearly distant from the peroxisomes (arrows in panels C and D).

Gsa9/Cvt9 is required for selective autophagy (e.g., pexophagy and the Cvt pathway), but is not necessary for nonselective starvation-induced autophagy.[30] In *S. cerevisiae*, this compartment may act as a signaling complex to switch between Cvt and autophagic transport. In addition, this compartment contains Cvt19 suggesting it may function in the formation of the Cvt vesicles that transport aminopeptidase I to the vacuole (see chapter 7).[40] In *P. pastoris*, our data are consistent with Gsa9 interacting with the peroxisomes, which then directs the movements of the vacuole membrane around the peroxisomes. These events will require proteins at the peroxisome and vacuole surfaces that will interact with Gsa9. Two such proteins include a peroxisomal membrane protein, PpPex14, and a vacuole membrane protein, PpVac8. Pex14 has been shown to be required for pexophagy in *H. polymorpha*.[32] We have found that *vac8* mutants are defective in pexophagy in *P. pastoris* (unpublished observations). Nevertheless, further studies are needed to verify these interactions and to examine the regulation of these interactions.

Gsa11 Compartment

Unlike Gsa9, Gsa11 is required for selective pexophagy and non-selective autophagy. In growing cells, Gsa11 remains cytosolic. However, once cells are starved for nutrients and autophagy is stimulated, Gsa11 becomes associated with 2-3 structures adjacent to the vacuole. These structures are also observed in cells undergoing glucose-induced pexophagy.[31] During pexophagy the Gsa11 compartment is composed of one or more cytoplasmic structures juxtaposed to the vacuole at the surface opposite where sequestration of peroxisomes is occurring. The assembly of Gsa11 into this compartment appears to be a requirement for pexophagy to proceed beyond the intermediate sequestration stage. When the C-terminal region of Gsa11 is deleted, Gsa11 fails to localize to these structures and appears to be nonfunctional. In addition, the assembly of the Gsa11 compartment appears to be highly regulated requiring Gsa12 and Gsa14, and two serine/threonine protein kinases Gsa10 and PpVps15. Although we have not yet localized the cellular distribution of Gsa10 and PpVps15, Gsa12 is found in the cytosol and at the vacuole surface, while Gsa14 resides in structures similar to those containing Gsa11. The role of protein phosphorylation in the assembly of the Gsa11 compartment will need to be investigated.

Although the Gsa11 compartment functions in a similar manner in *P. pastoris* and *S. cerevisiae*, there appear to be some differences in the characteristics of this compartment between yeasts. In *S. cerevisiae*, Apg2 (Gsa11) colocalizes with Aut7, Apg5, Apg16, and Apg1.[41,42] This "pre-autophagosomal" structure normally appears as a single dot near the vacuole in both fed and starved cells. However, in *P. pastoris*, the Gsa11 compartment is only observed when autophagy or pexophagy is stimulated. In addition, this compartment appears as multiple structures near the vacuole (Fig. 4C,D). Despite these differences, this unique Gsa11 compartment appears to be a conserved functional unit required for autophagy and pexophagy in yeasts.

Summary

Eukaryotic cells are able to respond to environmental changes and stress by selectively and nonselectively degrading cellular organelles by a process called autophagy. *S. cerevisiae* and *P. pastoris* have proven to be excellent genetic models to characterize the molecular events of the autophagic pathway. The studies on the selective degradation of peroxisomes have rapidly advanced in the last few years with the identification of many proteins required for pexophagy. The goal now is to better characterize these proteins and define their functional roles.

Of the pexophagy genes, only a few are not required for autophagy. These include Gsa9, Pex14, and Vac8 that likely promote interactions between the vacuole and the peroxisomes. The majority of the *GSA* and *PAZ* genes are required for pexophagy and autophagy suggesting that these pathways share common features or that the corresponding proteins are multifunctional. A common element for both pexophagy and autophagy is the Gsa11 compartment (pre-autophagosome) and those proteins required to "assemble" this compartment. A better understanding of the function and regulation of this compartment as well as the proteins associated with it will be critical in defining the molecular events that regulate pexophagy and autophagy in eukaryotic cells.

Acknowledgements

We wish to acknowledge Dr. Ben Glick for providing us with the pREMIz vector and to Dr. Per E. Strømhaug for identifying and cloning many of our *GSA* genes. This work was supported by grants from NSF (MCB-9817002) and NIH (1R01CA095552).

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CHAPTER 11

Selective Degradation of Peroxisomes in the Methylotrophic Yeast *Hansenula polymorpha*

Jan A.K.W. Kiel and Marten Veenhuis

Abstract

Peroxisomes are ubiquitous organelles, morphologically characterized by a single membrane that encloses a proteinaceous matrix. These organelles are inducible in nature, and their functional diversity is unprecedented. Their importance is probably best illustrated by the existence of peroxisomal diseases like Zellweger syndrome in man, which are associated with major neurological abnormalities sometimes resulting in an early death.

Until recently, most molecular studies on peroxisomes have focused on the biogenesis of the organelles and the function of their matrix enzymes, and much progress has been made in this field. However, the highly selective process of peroxisome degradation (designated pexophagy) via the major lytic compartment in the cell - the vacuole - has been largely neglected. Only recently, molecular data have become available regarding this process, especially using methylotrophic yeast species, like *Hansenula polymorpha*, as model organisms. The latter is not surprising as the morphological events accompanying peroxisome biogenesis and selective degradation are much more pronounced in these organisms relative to other fungi, including baker's yeast.

A recent finding is that pexophagy and other transport pathways to the vacuole (vacuolar protein sorting, autophagy, cytoplasm to vacuole targeting and endocytosis) seem to require common but also unique genes. Since some of the transport processes to the vacuole are either nonselective or constitutive in nature, it has become of utmost importance to understand what makes peroxisome degradation the highly selective process that it is and what signals trigger it.

It has been demonstrated that the prime determinant of selective peroxisome degradation in *H. polymorpha* is located at the peroxisomal membrane. Thus, to understand the selectivity of the process it is essential to study the role of peroxisomal membrane proteins in pexophagy in detail. Recent data suggest that at least two membrane-bound proteins required for the biogenesis of peroxisomes (so-called peroxins) also have specific roles in the destruction of peroxisomes via macropexophagy.

This chapter highlights the main achievements in understanding the process of pexophagy in methylotrophic yeast species, in particular *H. polymorpha*.

Homeostasis of Peroxisomes in *H. polymorpha*

When cells of *H. polymorpha* are grown in liquid media containing glucose and ammonium sulphate as sole carbon and nitrogen sources they contain a single, small peroxisome with a largely unknown function (Fig. 1). However, when such cells are shifted to peroxisome-inducing conditions (e.g., methanol), this original organelle increases in size as a result of the import of peroxisomal enzymes involved in methanol metabolism. When the organelle has reached a certain size, proliferation initiates. The level of this proliferation is largely determined by the growth conditions. In methanol-containing batch cultures that are in mid-logarithmic growth

Autophagy, edited by Daniel J. Klionsky.

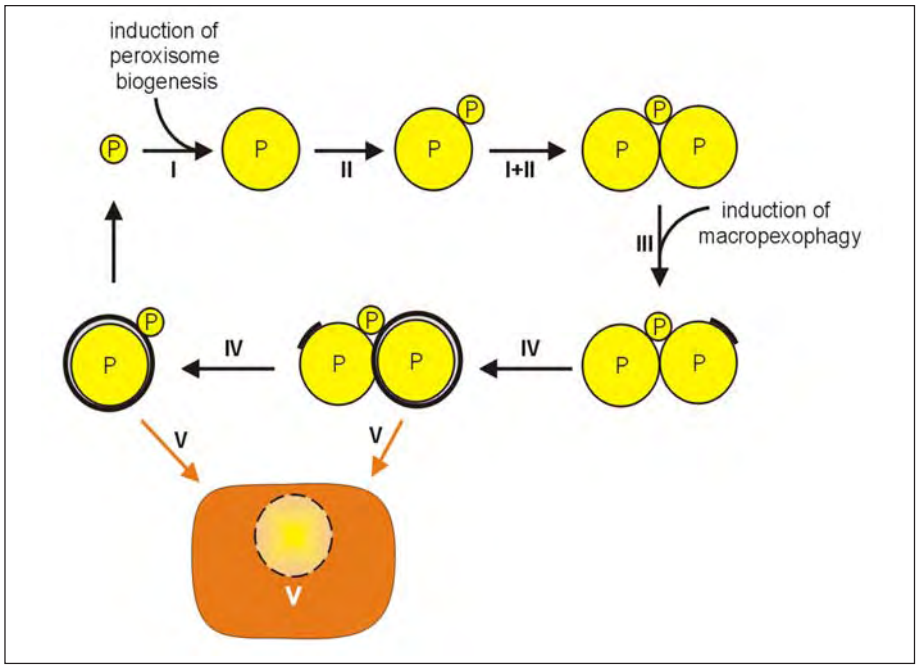

Figure 1. Homeostasis of peroxisomes in *H. polymorpha*. Schematic representation of peroxisome biogenesis and selective peroxisome degradation (macropexophagy) in *H. polymorpha*. During cultivation on glucose-containing medium *H. polymorpha* cells contain a single small peroxisome. When the cells are shifted to methanol as sole carbon and energy source, this peroxisome grows as a result of matrix protein import (step I) and proliferates (step II) until the cell contains many large peroxisomes. When macropexophagy is induced by the addition of excess glucose (step III) degradation starts on a single import-incompetent peroxisome, that is sequestered by additional membranes (step IV). After complete sequestration, the organelle fuses with the vacuole (step V). Subsequently, the next import-incompetent peroxisome is being sequestered and degraded by the vacuole. Finally, only the single import-competent peroxisome remains, and can function as the progenitor of newly formed organelles upon peroxisome induction. P, peroxisome; V, vacuole. A color version of this figure can be viewed at http://www.eurekah.com/abstract.php?chapid=1120&bookid=98&catid=69.

stage, cells contain several large peroxisomes that harbor the key enzymes of methanol metabolism (alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT)). It is thought that these large organelles function solely as "enzyme bags" that allow the cell to utilize methanol for energy and biomass production, but are no longer capable of importing matrix proteins. Additionally, the cells contain one, or a few, small organelles that display biosynthetic functions. In summary, import of matrix enzymes, organelle proliferation and inheritance seem to be restricted to the few small organelles in the cells.[1,2]

When methanol-grown *H. polymorpha* cells are shifted to a new environment not requiring the methanol-utilizing enzymes, the large peroxisomes are quickly degraded by a highly selective process known as macropexophagy. Morphological analysis suggested that the small organelle(s) escape the selective degradation process thus enabling the cell to rapidly respond to changes in the environment that require new peroxisome functions.[1,3] Thus, in *H. polymorpha* peroxisome biogenesis and degradation are tightly regulated by the growth conditions of the cells, which render this yeast species an ideal model organism to study these topics.

Recently, we obtained data that were in line with our earlier morphological observations. We isolated a *H. polymorpha* mutant that lacks a fungal $Zn(II)_2$-Cys_6 type transcription factor

required for methylotrophic growth. In cells of this mutant, a single peroxisome is present even when they are grown under conditions that induce peroxisome proliferation. Biochemical and ultrastructural analysis (using among others fluorescence microscopy and GFP tagged peroxisomes) revealed that upon exposure of these cells to excess glucose the single peroxisome remained stably in the cytoplasm, whereas in wild type control cells massive peroxisome degradation occurred (Fig. 2; A.N. Leão et al, submitted). Additional evidence came from *H. polymorpha* wild type cells briefly induced on methanol. As denoted above, during early growth on methanol, *H. polymorpha* cells contain a single peroxisome that increases in size until proliferation occurs (Fig. 1). When cells grown to this stage are subjected to excess glucose, macropexophagy is exclusively observed in those cells where peroxisomes have started to proliferate. In cells with a single peroxisome, the organelle is invariably retained in the cytoplasm (A.N. Leão et al, submitted). Additionally, it was observed in wild type *H. polymorpha* cells that at the final stage of macropexophagy generally only a single fluorescent spot (corresponding to a GFP-tagged peroxisome) is present in the cells, reflecting the import-competent peroxisome that escapes degradation (A.N. Leão et al, submitted). Thus, a clear difference seems to exist between import-competent peroxisomes and the import-incompetent ones with respect to their sensitivity to selective degradation.

Macropexophagy in *H. polymorpha*

Selective degradation of peroxisomes in *H. polymorpha* has been observed under conditions of (i) carbon catabolite inactivation and (ii) damage to peroxisomes. The latter process includes both damage to peroxisomal matrix components (e.g., by KCN)[4] or to membrane components (by the toxin peroxysomicine A-1).[5] It must be noted that this process may have an important physiological function, since occasionally we observe in methanol-grown *H. polymorpha* cells peroxisomes inside the vacuole undergoing degradation. It is thought that during methylotrophic growth peroxisomes undergo a sort of aging process, e.g., because the organelles become damaged by reactive oxygen species like hydrogen peroxide or oxygen radicals. It is likely that in case of irreparable damage to peroxisomes, the organelles are removed from the cytoplasm in a selective manner. So far this process has not been studied at the molecular level.

Initial experiments on selective peroxisome degradation during carbon catabolite inactivation in *H. polymorpha* demonstrated that the rate of inactivation of the peroxisomal enzymes AO and CAT was dependent on the carbon source used in the new environment.[1,3] Maximal degradation rates were obtained when compounds were used that fully repress the synthesis of AO (glucose, ethanol), while moderate rates were observed under partially repressing conditions (glycerol, dihydroxyacetone).[3] Notably, peroxisome degradation is not induced in *H. polymorpha* after a shift to another nitrogen source. When *H. polymorpha* cells are grown on methanol as carbon source and methylamine as nitrogen source they contain peroxisomal enzymes that are involved in the oxidation of both the carbon (AO, DHAS and CAT) and nitrogen source (amine oxidase (AMO) and CAT). A shift to glucose/ammonium sulphate resulted in the degradation of peroxisomes (containing both AO and AMO protein). A similar result was obtained when the cells were placed in glucose/methylamine medium, conditions still requiring AMO and CAT enzyme activity. Under these conditions the original organelles that contained both AO and AMO were degraded. Concomitantly with this degradation new peroxisomes were synthesized that harbor exclusively newly synthesized AMO and CAT. Thus in these cells the opposite processes of peroxisome destruction and assembly proceed simultaneously. Peroxisome degradation was not observed when methanol/methylamine-grown cells were transferred to fresh methanol medium supplemented with an AMO-repressing nitrogen source, namely ammonium sulphate.[6] Similar results were obtained using the nonmethylotrophic yeast *Trichosporon cutaneum.* When cells of this yeast species are grown on ethylamine (requiring peroxisomal AMO) as sole carbon and nitrogen source, only a transfer to medium containing a new carbon source (ethanol) resulted in selective degradation of peroxisomes.[7]

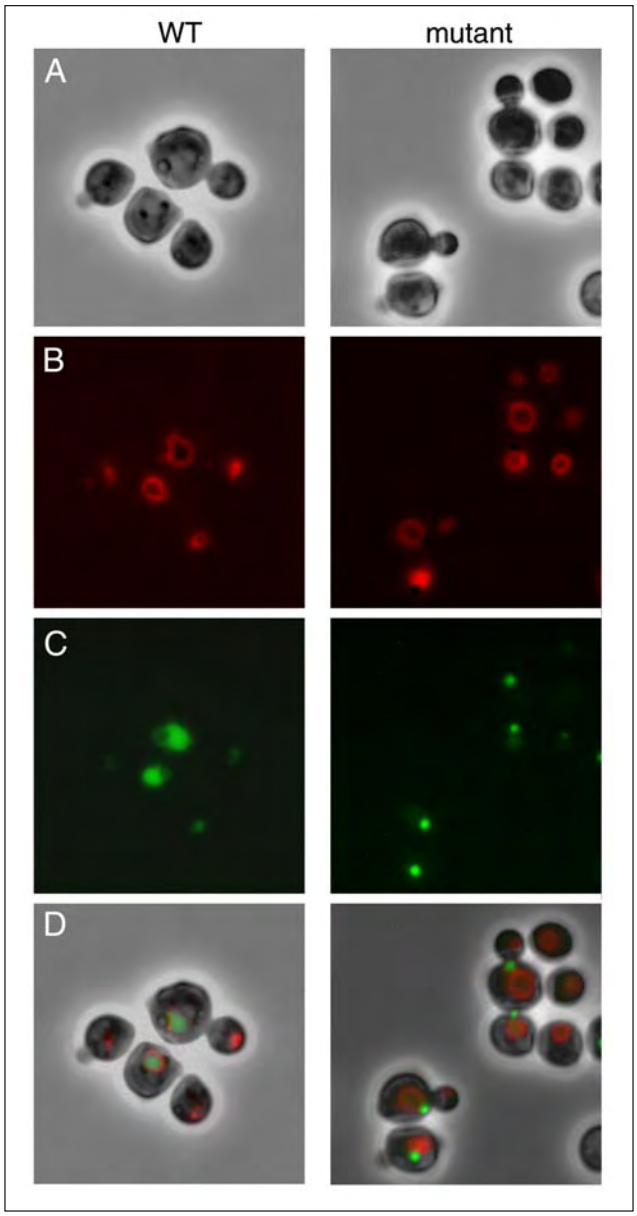

Figure 2. Analysis of pexophagy by fluorescence microscopy. Methanol-grown wild type (WT) *H. polymorpha* cells and cells of a mutant lacking a $Zn(II)_2$-Cys_6 type transcription factor required for methanol metabolism, both harboring peroxisomes with eGFP-SKL, were treated with the red fluorecent dye FM 4-64 to label the vacuolar membrane. Subsequently, the cells were subjected to glucose-excess conditions to induce macropexophagy. The pictures show the cells after 1 h of incubation with glucose. Under these conditions peroxisomes in wild type cells become degraded since the eGFP fluorescence is observed in the FM 4-64 fluorescent vacuoles. In contrast, in cells of the mutant lacking the $Zn(II)_2$-Cys_6 type transcription factor the single eGFP containing peroxisome remains stably in the cytoplasm. A, bright field images; B, FM 4-64 fluorescence; C, eGFP fluorescence; D, merged pictures.

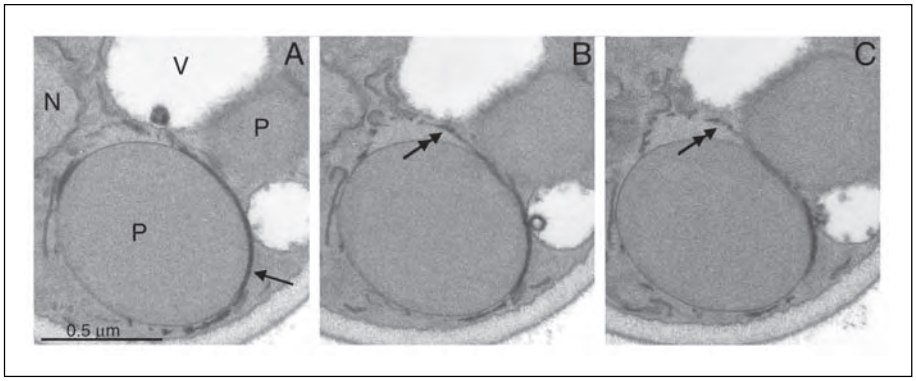

Figure 3. Initiation of peroxisome sequestration during macropexophagy. A,B,C: Three consecutive sections from a set of serial sections of methanol-grown wild type *H. polymorpha* to show the initial peroxisome sequestration events during macropexophagy. The figure shows the formation of the sequestration membranes around the peroxisome (arrow); also other building blocks of these membranes en route to the organelle can be seen (double arrow). N, nucleus; P, peroxisome; V, vacuole.

The process of selective peroxisome degradation in *H. polymorpha* (macropexophagy) has been unraveled at the morphological level in detail (for schematic model see Fig. 1).[1,3] As already denoted above, predominantly large, mature peroxisomes are sensitive to macropexophagy. At the final stage one, or a few, small peroxisomes, which harbour AO protein, escape the degradation process (Fig. 1).[1] In *H. polymorpha*, the first visible sign of macropexophagy is the sequestration of a peroxisome tagged for degradation by several membranous layers (Fig. 3). The number of membrane layers can vary considerably, ranging from 2 up to 12 (thickness of approximately 60-70 Å). Freeze etch data suggest that these membranes do not contain abundant integral membrane proteins.[1,3] Although it is not known where these additional membranes originate from, morphological data occasionally show close contacts between these membranes and mitochondria (Fig. 4). This may suggest that mitochondria play an important role in the formation of the sequestering membranes. However, so far this has not been studied in detail. It must be emphasized that sequestration of peroxisomes during macropexophagy is a highly selective process; other cell components (cytosol, ribosomes, mitochondria, endoplasmic reticulum, etc.) are not taken up in the sequestered compartment.[8]

After complete sequestration of the organelle (Fig. 5), hydrolytic enzymes are donated by the vacuole. This may be accomplished by fragmentation of the central vacuole into a number of vacuolar vesicles, which fuse with the sequestered organelle. Alternatively, a direct fusion can occur between the sequestered peroxisome and the central vacuole. In both cases the outer membrane of the sequestered organelle fuses with the vacuolar membrane (Fig. 6A). Finally, vacuolar hydrolases lyse the membranes surrounding the organelle and degrade its contents (Fig. 6B).[3] Immunocytochemical analysis using antibodies against peroxisomal AO protein demonstrated the presence of this enzyme in the vacuole after uptake of the peroxisome.

So far, no data are available implying a direct role for any of the vacuolar proteases in macropexophagy. Unfortunately, mutants in the main vacuolar proteinases A and B are not available for *H. polymorpha*. However, our data indicate that the vacuolar hydrolase carboxypeptidase Y does not play a significant role during macropexophagy.[9]

Remarkably, macropexophagy in *H. polymorpha* is not dependent on the synthesis of new proteins, since peroxisome degradation during glucose-adaptation is not blocked in the presence of cycloheximide (M. Veenhuis and A.R. Bellu, unpublished data). Nevertheless, macropexophagy in *H. polymorpha* is an energy-requiring process, because pexophagy is inhibited by cyanide or under anaerobic conditions.[3]

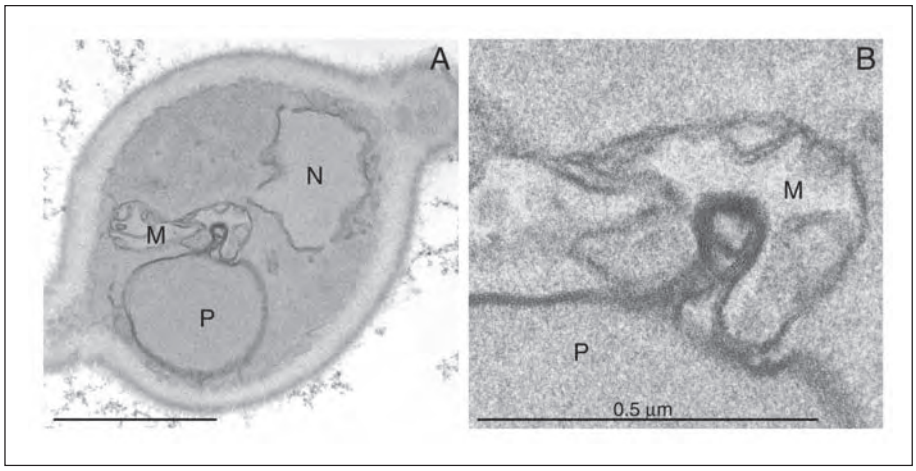

Figure 4. Close association of sequestering membranes with mitochondria. A. Wild type *H. polymorpha* cell in the initial stage of macropexophagy demonstrating a characteristic example of the intimate relationship that is frequently observed between the sequestering membranes and the mitochondria. A detailed view is shown in B. M, mitochondrion; N, nucleus; P, peroxisome. The bar represents 1 µm unless otherwise indicated.

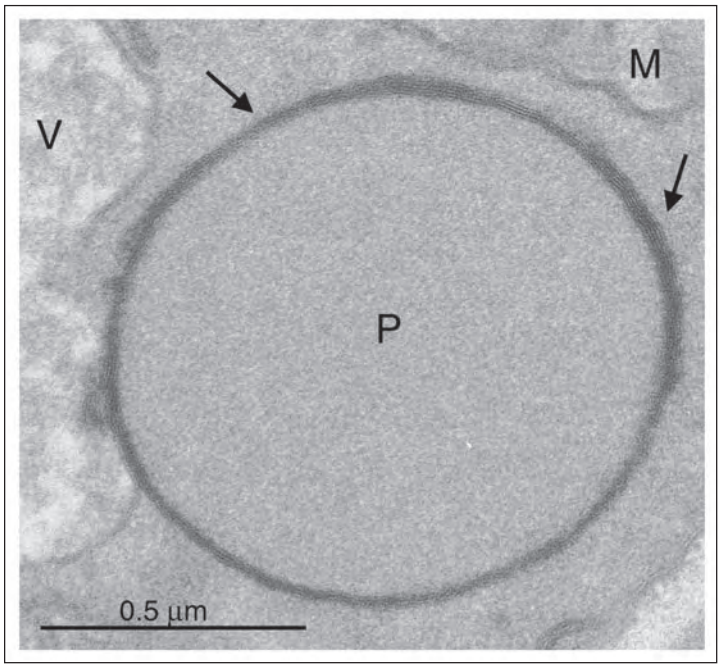

Figure 5. Complete sequestration of peroxisome during macropexophagy. Detail of a wild type *H. polymorpha* cell during pexophagy to demonstrate a typical example of the sequestration of a cytosolic peroxisome tagged for degradation by various membrane layers (arrows). M, mitochondrion; P, peroxisome; V, vacuole.

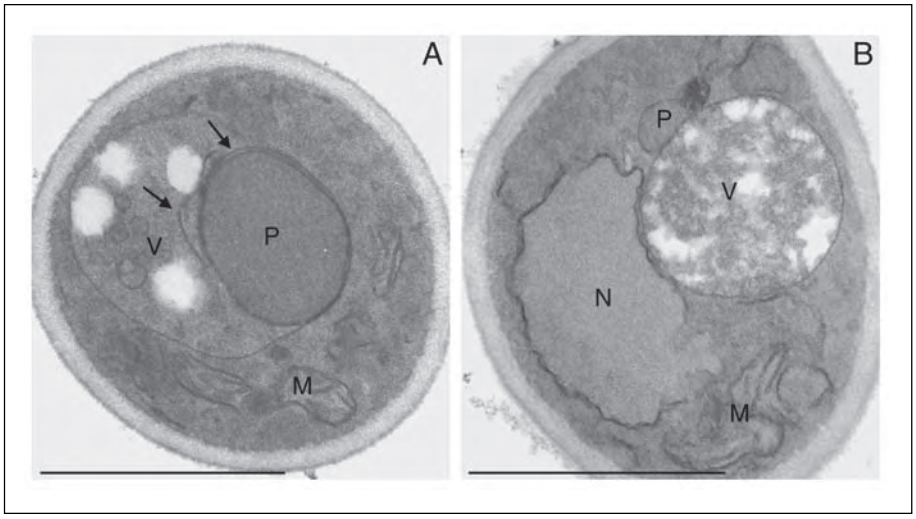

Figure 6. Final stages of macropexophagy in *H. polymorpha.* A. Methanol-grown wild type *H. polymorpha* cell shifted to glucose in the presence of PMSF for 1 h. Typically, an intact peroxisome is included in the vacuole. The original surrounding membranes are also still partly visible (arrow). B. Final stage of glucose-induced peroxisome degradation in methanol-grown wild type *H. polymorpha* cells. Typically one small organelle remains after degradation of the mature peroxisomes. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole; the bar represents 1μm.

Alternative Modes of Peroxisome Degradation in *H. polymorpha*

In the methylotrophic yeast *Pichia pastoris* pexophagy may proceed through two morphologically quite distinct processes, macropexophagy and micropexophagy, dependent on the carbon source used to induce the degradation process (see chapter 10).

Also in *H. polymorpha* selective degradation of peroxisomes does not always occur via macropexophagy. We have recently isolated a *H. polymorpha* mutant, designated *pdd4*, which is disturbed in macropexophagy. Nevertheless, during glucose-adaptation, but also during methylotrophic growth, peroxisomes are constitutively degraded in *pdd4* cells via a slow process. Surprisingly, in contrast to macropexophagy, this process is dependent on new protein synthesis (I. Monastyrska et al, in preparation). This type of degradation is normally not observed in wild type *H. polymorpha* cells when these are grown on methanol or under conditions inducing macropexophagy.

In addition, we have observed that peroxisomes in methanol-grown *H. polymorpha* cells can be degraded via a process resembling micropexophagy in *P. pastoris* (see chapter 10) when they are shifted to nitrogen-starvation conditions in the presence of methanol.[10] Under these conditions, peroxisomes are enclosed by long, interconnected tubular vacuolar structures, resembling those observed during micropexophagy in *P. pastoris,*[11] followed by their degradation in the vacuole. As mentioned above, this type of peroxisome degradation appears to be dependent on new protein synthesis (I. Monastyrska et al, in preparation). Notably, nitrogen starvation conditions also induce autophagy of portions of the cytosol and mitochondria, which are taken up separately via invaginations of the vacuolar membrane.[10] The occurrence of this nonselective degradation process makes it hard to assess whether the degradation of peroxisomes taking place during nitrogen starvation is truly a selective process.

Signaling of Pexophagy

In methylotrophic yeast species glucose is a major inducer required to signal the onset of peroxisome degradation. However, peroxisomes in methanol-grown *H. polymorpha* cells are not degraded when they are shifted to 2-deoxyglucose, a glucose analogue that can be taken up by the cell, phosphorylated by glucokinase/hexokinase, but not further metabolized. However, under these conditions AO synthesis is fully repressed. Morphological analysis indicated that peroxisomes in 2-deoxyglucose adapted cells do become sequestered from the cytoplasm, but that the fusion event with the vacuole is blocked.[3] This suggests that the initial signal that triggers sequestration proceeds in the presence of the glucose analogue. One possibility to explain this phenomenom is that during 2-deoxyglucose adaptation the cells do not have sufficient energy to complete the degradation process. Alternatively, glucose (or a metabolite) could provide a second signal that is required to fully allow peroxisome degradation in *H. polymorpha*. Recently, Parpinello et al described a regulatory mutant (*glr2-1*) that is capable of synthesizing peroxisomal enzymes involved in methanol metabolism (AO, DHAS, CAT) even in the presence of glucose.[12] When methanol-grown cells of this mutant were adapted to glucose, massive peroxisome degradation occurred. Concomitantly with this degradation, new peroxisomes were synthesized that harboured newly synthesized AO, DHAS and CAT protein. Notably, the newly formed organelles were not degraded. These data clearly indicate that the signal to initiate macropexophagy in *H. polymorpha* is not directly related to glucose repression. How the glucose signal is actually transmitted to the degradation machinery remains to be established.

Turnover of peroxisomes has also been observed in the methylotrophic yeast *Pichia methanolica* (also known as *Pichia pinus*) during glucose and ethanol adaptation. Ethanol adaptation of methanol-grown *P. methanolica* cells seems to induce degradation of peroxisomes by a process similar to macropexophagy.[13] For *P. methanolica* an attempt has been made to dissect the signalling pathway induced by ethanol. It was observed that certain mutants affected in enzymes of the glyoxylate pathway, required for ethanol-growth, were unable to degrade peroxisomal enzymes by macropexophagy during ethanol-adaptation.[13,14] Nevertheless, under these conditions the mutants were still capable of carbon-catabolite repression. Based on the phenotypes observed with these glyoxylate pathway mutants the authors concluded that glyoxylate itself was probably the effector that triggered macropexophagy. Notably, in *P. methanolica* the signaling pathways of glucose- and ethanol-induced pexophagy appear to be two distinct processes, because in all mutants analyzed glucose-induced pexophagy remained unaffected.

It has been suggested that also in *H. polymorpha* glyoxylate might function as the effector during ethanol adaptation since a mutant with reduced acetyl CoA synthetase activity was affected in macropexophagy under these conditions.[15] This result should, however, be treated with care because a *H. polymorpha alg2* mutant, disrupted in the gene encoding isocitrate lyase, still showed a decrease in AO activity during ethanol adaptation, suggesting no defects in macropexophagy (E. Berardi et al, in preparation).[16] In contrast, a *P. methanolica* isocitrate lyase mutant (obtained by chemical mutagenesis) was affected in ethanol-induced macropexophagy.[13] Clearly, much is still to be learned concerning the signaling process by ethanol.

Isolation of Mutants Affected in Macropexophagy

In order to elucidate the mechanisms governing selective degradation of peroxisomes, mutants impaired in macropexophagy were isolated for *H. polymorpha*. Initially, generation of pexophagy mutants occurred via classical means (i.e., chemical treatment).[8,17] However, we have recently adapted the gene tagging technique of Restriction Enzyme Mediated Integration (REMI) so that it results in a completely random integration of the tag into the *H. polymorpha* genome. This modified technique was designated Random integration of Linear DNA Fragments (RALF) and allows a genome wide mutagenesis.[18] After selection of mutants affected in pexophagy, the integrated tag is isolated from the genome together with flanking genomic

regions, thus providing the possibility to obtain direct sequence information of the gene mutagenized by the tag.

Selection of pexophagy mutants in *H. polymorpha* is based on the increased retention of peroxisomal AO activity in these mutants after induction of the pexophagy process, which allows their identification by a simple plate assay.[8] To provide an additional tool to isolate pexophagy mutants (and their complementing genes), we recently started to utilize the increased sensitivity of mutants affected in peroxisome degradation towards allyl alcohol as a first screen in the isolation procedure.[19]

Using both classical mutagenesis and RALF, twenty *H. polymorpha* mutants affected in macropexophagy (designated *pdd* mutants, peroxisome degradation-deficient) have been isolated (our unpublished data).[8,18] So far, all mutants studied were found to be affected in both glucose- and ethanol-induced peroxisome degradation. However, recently Stasyk et al[17] isolated a *H. polymorpha* mutant (designated *gcr1*) specifically defective in glucose-induced macropexophagy as a result of a defect in glucose transport. Morphological analysis of the *pdd* mutants demonstrated that they showed defects in either of the two phenotypically distinct steps of the macropexophagy process: (i) signaling/sequestration of peroxisomes (disturbed in e.g., *pdd7*),[20] or (ii) the uptake of the sequestered peroxisome into the vacuole (disturbed in e.g., *pdd2*).[8] Notably, mutants impaired exclusively in the degradation of incorporated peroxisomes in the vacuole, were not isolated. The reason for this may be related to the screening procedure, namely to assay inactivation of AO protein. Because mutant isolation is crucially dependent on AO activity, mutant colonies affected in the actual degradation of the protein cannot be distinguished from wild type colonies.

Genes Involved in Pexophagy

The first genes involved in selective peroxisome degradation in methylotrophic yeasts were isolated by functional complementation. However, it has been rather laborious to isolate genes by a conventional plasmid library screen. In fact the *H. polymorpha PDD1* gene was isolated making use of the temperature sensitivity of the *pdd1* mutant.[21] It must be noted that pexophagy is not an essential process. Moreover, many pexophagy mutants appear to be defective in sporulation. This has so far precluded the isolation of many of the genes involved in selective peroxisome degradation by functional complementation in both *H. polymorpha* (our unpublished results) and *P. pastoris*.[11] Currently, many genes are being isolated by gene tagging techniques.[11,18] Only recently have we succeeded in isolating plasmids complementing a number of the original *pdd* mutants via a positive selection method (enhanced resistance towards allyl alcohol).[19]

Surprisingly, many of the isolated genes involved in the selective degradation of peroxisomes appeared to play essential roles in other cellular processes as well. This is exemplified by the finding that the first isolated gene involved in micropexophagy in *P. pastoris* (*GSA1*) appeared to encode the α-subunit of phosphofructokinase (encoded in *S. cerevisiae* by the *PFK1* gene), part of the key regulatory enzyme complex in glycolysis (see chapter 10).[22] However, it must be noted that the enzymatic activity of Gsa1 was not required during micropexophagy, suggesting a dual function for the protein.

In many cases, the isolated genes required for pexophagy encoded homologues of *S. cerevisiae* proteins that are required for processes involving transport of proteins/organelles towards the vacuole. These include vacuolar protein sorting (Vps), autophagy (Apg), cytoplasm to vacuole targeting (Cvt) and endocytosis (End) proteins (for reviews see refs. 23-25; see also Table 1 and chapters 6 and 7).

The first isolated *H. polymorpha* gene involved in macropexophagy (*PDD1*) was found to encode the functional homologue of *S. cerevisiae* Vps34, a phoshatidylinositol 3-kinase required for vacuolar protein sorting and endocytosis.[21] The *pdd1* mutants are affected in an early stage of macropexophagy, sequestration of peroxisomes from the cytoplasm. Thus the peroxisomes never reach the interior of the vacuole. This made us conclude that the pexophagy defect in *pdd1* mutants cannot be attributed to a lack of vacuolar proteases that normally travel

Table 1. Overlap between pexophagy and other transport processes to the vacuole

Gene [reference]	Mutant Phenotype *H. polymorpha*	Mutant Phenotype *P. pastoris*	Mutant Phenotype *S. cerevisiae*
HpPDD1 [10, 21,*] *ScVPS34* [44,45,46,47]	macro, vps, apg, end	nd	vps, end, apg,cvt
HpPDD7 [20] *ScAPG1* [42,48] *ScAUT3* [49] *ScCVT10* [50] *PpGSA10* [50] *PpPAZ1* [11]	macro, apg	micro, apg	pxg, apg, cvt
HpPDD18 [*] *PpGSA9* [51] *PpPAZ6* [11] *ScCVT9* [51]	macro	micro (NOT apg)	pxg, cvt (NOT apg)
HpPDD19 [*] *PpVPS15* [28] *PpGSA19* [50] *PpPAZ13* [11] *ScVPS15* [46,52]	macro	macro, micro	vps, apg, cvt
PpGSA7 [53,54] *PpPAZ12* [11] *ScAPG7* [42,54] *ScCVT2* [42,54]	nd	micro, apg	pxg, apg, cvt
PpGSA11 [50] *PpPAZ7* [11] *ScAPG2* [55]	nd	micro, apg	apg, cvt
PpGSA12 [56] *ScCVT18* [56] *ScAUT10* [57]	nd	micro	apg, cvt
PpGSA14 [50] *PpPAZ9* [11] *ScAUT9* [58] *ScCVT7* [59] *ScAPG9* [59]	nd	micro	apg, cvt
PpGSA20 [50] *ScAUT1* [60] *ScAPG3* [50]	nd	micro	apg, cvt
PpPAZ2 [11] *ScAPG8* [42] *ScAUT7* [61] *ScCVT5* [62]	nd	macro, micro	pxg, apg, cvt

continued on next page

Table 1. Continued

Gene [reference]	Mutant Phenotype *H. polymorpha*	Mutant Phenotype *P. pastoris*	Mutant Phenotype *S. cerevisiae*
PpPAZ3 [11] *ScAPG16* [63]	nd	micro	apg, cvt
PpPAZ8 [11] *ScAUT2* [61] *ScAPG4* [50]	nd	micro	apg, cvt
ScAPG5 [42,64]	nd	nd	pxg, apg, cvt
ScAPG10 [42,65]	nd	nd	pxg, apg, cvt
ScAPG14 [42,66] *ScCVT12* [50]	nd	nd	pxg, apg, cvt

Key: Hp, *H. polymorpha*; Pp, *P. pastoris*; Sc, *S. cerevisiae*; apg, autophagy; cvt, cytoplasm to vacuole targeting; end, endocytosis; macro, macropexophagy; micro, micropexophagy; nd, not determined; pxg, pexophagy (uncertain whether micro- or macropexophagy was affected); vps, vacuolar protein sorting. The asterisk indicates our unpublished results.
A number of *H. polymorpha* and *P. pastoris* genes involved in pexophagy do not show similarity to *S. cerevisiae APG/AUT/CVT/VPS* genes. These genes are not listed here.

via the Vps pathway, but must affect a specific function of the protein. We found that, similar to *S. cerevisiae* Vps34, *H. polymorpha* Pdd1 is also required for the Vps, Apg and End pathways (Table 1).[21,26] In *S. cerevisiae*, Vps34 is bound to membranes via the membrane-attached protein ScVps15, a protein kinase essential for activation of ScVps34.[27] Recently it was found that the *P. pastoris VPS15/PAZ13* and *H. polymorpha PDD19* genes, required for selective degradation of peroxisomes, encode homologues of *S. cerevisiae* Vps15 (refs. 11,28 and our unpublished data).

It was observed that many genes essential for micropexophagy in *P. pastoris* encode homologues of *S. cerevisiae* proteins involved in the highly related Apg and Cvt pathways (see chapters 6 and 7). Also in *H. polymorpha* some homologues of *APG/CVT* genes have been found to be essential for macropexophagy (Table 1). Thus *H. polymorpha PDD7* and *PDD18* encode the putative homologues of *S. cerevisiae* Apg1/Cvt10 and Cvt9, respectively (our unpublished data).[20] However, many of the *pdd* mutants that we have isolated are not affected in autophagy (e.g., *pdd2* and *pdd4*).[10,26] Furthermore, initial sequence analysis of the genomic regions of *H. polymorpha pdd* mutants obtained by the RALF technique indicated that these mutants are not affected in homologues of *ScAPG/CVT* genes. Also novel genes with no homologues in the available databases have been found (e.g., *PDD4* and *PDD6*; our unpublished data).

So far the common denominator for macropexophagy, micropexophagy and other transport processes to the vacuole seems to be in the early stages of these processes, namely signaling/initiation of sequestration. Thus, in all organisms homologues of *ScAPG1*, *ScVPS34, ScVPS15* and *ScCVT9* are required to initiate the transport process during pexophagy. In the next step, sequestration of the material to be transported towards the vacuole, the macro- and micropexophagy processes tend to diverge. Clearly, in *P. pastoris* sequestration of peroxisomes during micropexophagy requires many homologues of *ScAPG/CVT* genes.[11] In *H. polymorpha* sequestration of single peroxisomes during macropexophagy may require a different set of genes, although it can still not be excluded that *APG/CVT* homologues also play a role in this process.

Upon uptake into the vacuole, more general proteins (e.g., lipases, proteases, etc.) are expected to play a role in the final degradation of peroxisomes and their contents. Thus, although at first sight all transport processes to the vacuole seem to require the same set of genes, each process definitely also requires (some) unique genes. Clearly, we should focus on identifying these genes to understand macro- and micropexophagy at the molecular level.

A comparison of the nomenclature of the genes found to be involved in the different transport processes to the vacuole in *H. polymorpha*, *P. pastoris* and *S. cerevisiae* (see Table 1) demonstrates what can become a major problem in this field. For one and the same gene many acronyms have been chosen: e.g., the *H. polymorpha PDD7* gene is the functional homologue of *P. pastoris GSA10* and *PAZ1* and *S. cerevisiae APG1*, *AUT3* and *CVT10* (Table 1). Since most if not all of these proteins have a role in either the biogenesis of the vacuole or the degradation of substrates (proteins, organelles) by the vacuole, it may be preferable to come to a unified nomenclature in the near future based on these two roles (i.e., biogenesis of the vacuole versus degradation of substrates by the vacuole). A unified nomenclature was recently adopted in the peroxisome biogenesis field.[29]

The Role of Peroxins in Macropexophagy

Proteins involved in peroxisome biogenesis are collectively known as peroxins and are encoded by *PEX* genes.[29] *Pex* mutants of methylotrophic yeasts have lost the capacity to grow on methanol as the sole carbon and energy source because they lack intact peroxisomes. Upon induction of peroxisome biogenesis, most *pex* mutants harbor peroxisomal structures (known as "remnants" or "ghosts") in conjunction with matrix proteins, which are mislocalized to the cytosol. This mislocalization is actually the cause of the failure of these mutants to grow on methanol as sole carbon and energy source. Under these conditions mislocalized AO enzyme converts methanol into formaldehyde and hydrogen peroxide in the cytosol. Normally, the latter product is decomposed by catalase in the peroxisome. However, in *pex* mutants the hydrogen peroxide formed in the cytosol is removed by energy-requiring processes that are detrimental for growth.[30] It must be noted that peroxisomal membrane proteins are normally incorporated into the membrane remnants in *pex* mutants (for review see ref. 31).

To understand the prime target of macropexophagy, van der Klei et al studied a *H. polymorpha pex* mutant that harboured very small peroxisomes and had most of the peroxisomal matrix proteins mislocalized to the cytosol.[32] They observed that exclusively the very small peroxisomes were degraded, suggesting that peroxisomal membrane remnants contain the determinants that are recognized by the macropexophagy machinery. In contrast, mislocalized matrix proteins remained stably present in the cytosol. Subsequently, degradation of peroxisomal remnants was studied in a series of *H. polymorpha* Δ*pex* mutants.[33] It appeared that in most *H. polymorpha* Δ*pex* mutants, peroxisomal remnants are normally susceptible to glucose-induced selective degradation, a property that has in fact been used as an additional criterion for their peroxisomal nature. However, in *pex14*Δ cells these membrane remnants were not sensitive to degradation, suggesting an involvement of Pex14 in selective peroxisome degradation. Pex14 is a protein tightly associated with the cytoplasmic side of the peroxisomal membrane. In peroxisome biogenesis Pex14 plays an essential role in matrix protein import as a component of the docking site for receptor/cargo protein complexes.[31] *H. polymorpha pex14*Δ peroxisomes contain little matrix protein. However, a suppression of this phenotype can be achieved when the receptor protein for PTS1 matrix proteins, Pex5, is overproduced in this strain, and virtually normal peroxisomes are formed.[34] Recent data indicate that the peroxisomes formed under these conditions are not susceptible to degradation during glucose adaptation, again implying a Pex14 requirement during macropexophagy.[35] Using mutants producing truncated forms of HpPex14, Bellu et al obtained evidence that the N-terminus of HpPex14 is essential for macropexophagy.[35] Notably, this N-terminus is also required for efficient matrix protein import. Since sequestration of peroxisomes was not observed in these cells, it seems that Pex14 is involved in a step prior to the sequestration process. Apparently, HpPex14 acts as a molecular switch in which peroxisome biogenesis and degradation converge.

However, Pex14 is not the only peroxin that has a role during macropexophagy in *H. polymorpha*. Biochemical analysis of two strains affected in macropexophagy, i.e., the *pdd2* mutant (disturbed in the fusion of sequestered peroxisomes with the vacuole) and the *pex14*Δ strain overproducing Pex5 (disturbed in the sequestration process, see above) indicated that the levels of the peroxin Pex3 decreased during glucose-adaptation, whereas the peroxisomes remained stably in the cytoplasm.[36] This decrease was not observed in *pdd1* cells, which are presumed to be affected in a very early step of macropexophagy.[21] In *H. polymorpha,* Pex3 is tightly associated with the cytoplasmic side of the peroxisomal membrane.[37] We recently showed that upon exposure of methanol-grown wild type *H. polymorpha* cells to glucose-excess conditions, Pex3 levels drop rapidly in a proteasome dependent manner.[36] This suggest that Pex3 is removed from the peroxisomal membrane prior to degradation of the organelle in the vacuole. Three additional lines of evidence confirm that Pex3 plays an essential role during selective peroxisome degradation in *H. polymorpha*: (i) it has been observed that overexpression of *PEX3* prevents degradation of peroxisomes during glucose adaptation;[38] (ii) addition of eGFP to the C-terminus of Pex3 interferes with macropexophagy[36] and (iii) introduction of the *S. cerevisiae PEX3* ortholog succesfully restored peroxisome biogenesis in a *H. polymorpha pex3*Δ mutant,[39] but not pexophagy.[36] In these cells, ScPex3 remained completely stable and peroxisomes were not degraded, suggesting that *H. polymorpha* Pex3 apparently has a role in peroxisome degradation that ScPex3 cannot fulfill. Our interpretation of these data is that *H. polymorpha* Pex3 plays a role in protecting peroxisomes from degradation during growth on methanol. Upon induction of macropexophagy, Pex3 is removed from the peroxisomal membrane and degraded by the proteasome. Subsequently, in a Pex14 dependent manner peroxisomes are sequestered from the cytosol and incorporated into the vacuole for degradation. Recently it was observed that Pex3 and Pex14 are in fact part of a protein complex,[31,40] suggesting that Pex3 may in some way shield Pex14 from recognition by the macropexophagy machinery. Removal of Pex3 would then initiate the degradation process. Apparently, when Pex3 is not sufficiently removed from the peroxisomal membrane (overproduced Pex3, presence of eGFP-tagged Pex3 or a heterologous Pex3), sequestration of peroxisomes is blocked. A hypothetical model depicting the role of Pex3 and Pex14 in macropexophagy is shown in Figure 7.

Concluding Remarks

So far, our knowledge of pexophagy is mainly based on data gathered using the methylotrophic yeasts *H. polymorpha* and *P. pastoris*. Unfortunately, *S. cerevisiae* has not been used extensively for studies of pexophagy, thus precluding a genome-wide analysis. So far, it is unclear whether in *S. cerevisiae* peroxisomes are degraded via micro- or macropexophagy.[41,42] Nevertheless, the role of *APG/CVT* genes in pexophagy in *S. cerevisiae* has provided a clear indication of how sequestration of peroxisomes could occur.[42] Also in higher eukaryotes degradation of peroxisomes has been observed (reviewed in ref. 43). However, it remains unknown whether the processes of macro- and micropexophagy also take place in higher eukaryotes. Possibly, these processes are typical methylotrophic traits, designed to rapidly remove the many large peroxisomes that occupy a significant portion of the cytoplasmic volume in methylotrophic yeasts during growth on methanol.

Still, many questions await an answer. To name but a few:

1. What are the principles of the differentiation between matrix protein import-incompetent peroxisomes that are susceptible to pexophagy and matrix protein import-competent peroxisomes that escape the degradation process?
2. How is the glucose signal translated into the first visible sign of macropexophagy: removal of Pex3 from the peroxisomal membrane?
3. Are other peroxisomal membrane proteins involved in macropexophagy?
4. What is the nature of the sequestering membrane in macropexophagy?

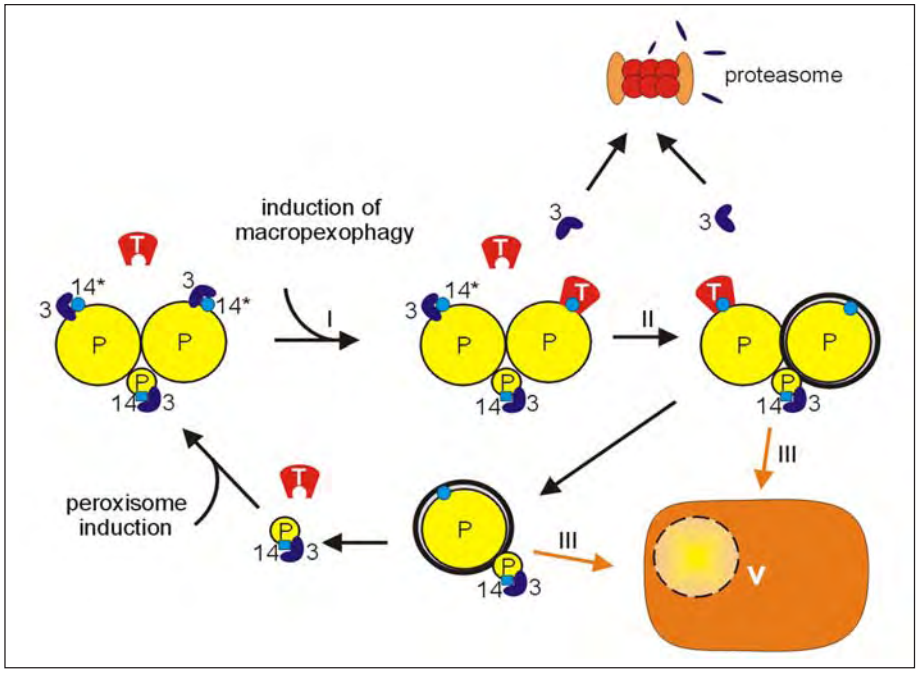

Figure 7. Hypothetical role of *H. polymorpha* Pex3 and Pex14 in macropexophagy. Schematic representation of the role that *H. polymorpha* Pex3 and Pex14 could play during macropexophagy. Methanol-grown *H. polymorpha* cells contain a few large, mature peroxisomes (P) that can no longer import matrix proteins. In these peroxisomes Pex14 is inactive (indicated by the asterisk), while Pex3 is thought to shield these Pex14 molecules from recognition by a preexisting protein, designated Terminator (T). In addition, the cells contain a small peroxisome that is still import-competent and requires both Pex3 and Pex14 to import matrix proteins. It is hypothesized that upon induction of macropexophagy (step I) Pex3 on mature peroxisomes undergoes a conformational change, allowing it to be removed from the peroxisomal membrane in a proteasome-dependent manner. Pex3 is subsequently degraded by the proteasome. The removal of Pex3 allows the terminator protein to recognize the inactive Pex14 molecules at the peroxisomal membrane, thus triggering the sequestration of the organelle (step II). Subsequently, the sequestered organelle is targeted to the vacuole (V) for degradation (step III). The small import-competent peroxisome containing Pex3 and Pex14 molecules active in matrix protein import escapes the degradation process and provides the cell with the opportunity to quickly respond to changes in the environment. A color version of this figure can be viewed at http://www.eurekah.com/abstract.php?chapid=1120&bookid=98&catid=69.

Acknowledgements

JAKWK is supported by a grant from ALW, which is subsidized by the Dutch Organization for the Advancement of pure Research (NWO). We thank the members of our group for providing data prior to publication.

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CHAPTER 12

Chaperone-Mediated Autophagy

J. Fred Dice, Patrick F. Finn, Amy E. Majeski, Nicholas T. Mesires and Ana Maria Cuervo

Abstract

Chaperone-mediated autophagy (Cma) is responsible for the degradation of 30% of cytosolic proteins from fibroblasts, hepatocytes and many other cell types during prolonged starvation. All substrate proteins for this pathway of proteolysis contain a compositional peptide motif related to KFERQ. Isolated lysosomes carry out Cma, and these preparations demonstrate a requirement for a molecular chaperone, the heat shock cognate protein of 73 kDa (hsc73) and ATP for optimal activity. Substrate proteins bind to a receptor in the lysosomal membrane, the lysosome-associated membrane protein type 2a (lamp2a). After binding to lamp2a, the substrate protein is unfolded probably by a group of molecular chaperones still associated with the substrate-lamp2a complex. An intralysosomal form of hsc73 (ly-hsc73) is required for entry of the substrate protein into the lysosomal matrix. Lamp2a in the lysosomal membrane and ly-hsc73 are rate-limiting components of Cma. The amount of lamp2a in the lysosomal membrane can be increased in two ways; its degradation rate can be decreased and a fraction of the protein in the lysosomal matrix can be reinserted into the lysosomal membrane. Lamp2a is found in the lysosomal membrane as a homomultimer, but whether or not this multimerization is regulated is unclear. The intracellular signals that activate Cma are under investigation, and they seem distinct from activators of macroautophagy. Ketone bodies that accumulate in prolonged starvation activate Cma. Aging is accompanied by reduced activity of Cma, and the resulting reduced protein degradation may lead to the accumulation of damaged proteins. Three specific examples of substrates for Cma are discussed.

Initial Discovery

Chaperone-mediated autophagy (Cma) refers to a process by which particular cytosolic proteins are targeted to lysosomes for degradation.[1,2] We chose this name because it is a form of autophagy or "self eating" by lysosomes, as are macroautophagy and microautophagy (see chapters 2-8), but in this proteolytic pathway there are multiple, critical roles for molecular chaperones. In addition, while most transport pathways to the lysosome require vesicle traffic or dynamic membrane rearrangements, Cma involves neither.

In the mid-1970s one of us (JFD) began studying the increased protein degradation in liver of rats made acutely diabetic with the drug streptozotocin.[3,4] These animals were hyperglycemic and ketotic, and they died in 3-4 days unless repeatedly injected with insulin. This information is important because less severe insulin deficiency may elicit an opposite response in protein degradation; animals may adapt by decreasing protein degradation in many tissues.[5] The increased protein degradation in severe insulin deficiency applied most dramatically to those proteins that were normally very long-lived suggesting that the enhanced degradation was selective for particular substrate proteins to some degree.[6] The longer-lived cytosolic proteins tended to be small,[7] basic,[8] nonglycosylated[9] and/or hydrophilic.[10] These classes of proteins are synthesized at the same rates during insulin deficiency, so their increased degradation leads to a decline in their intracellular concentration in response to insulin withdrawal.[6]

Autophagy, edited by Daniel J. Klionsky.

Such protein degradation studies involved injecting a radioactive amino acid into rats and following the incorporation and loss of radioactivity from different protein fractions. This experimental approach was instrumental in establishing that different cellular proteins had different half-lives but was of limited value for understanding mechanisms of proteolysis. Thousands of proteins and an unknown number of proteolytic pathways were being followed simultaneously in such studies.

We realized that much more detailed information could be gained if a single radiolabeled protein could be introduced into cells followed by quantification of its degradation. Okada,[11] Loyter,[12] and Rechsteiner[13] and their colleagues used red blood cells (rbcs) to microinject proteins into cultured cells (Fig. 1A). In this process rbcs are first lysed to remove most of the hemoglobin. A radiolabeled protein is introduced into these resulting ghosts by a second round of hypotonic lysis. Isosmotic conditions are restored, and the captured radioactive protein is separated from the protein outside the ghosts by centrifugation. The loaded ghosts are then added to confluent monolayers of fibroblasts, and fusion between the rbc membrane and the fibroblast membrane is induced using chemicals such as polyethylene glycol or viral fusogenic proteins. Under the right conditions, rbc membranes will fuse with fibroblast membranes without significant fibroblast-fibroblast fusion (Fig. 1A).

We also used a second method for introducing proteins into the cytosol of cultured cells called osmotic lysis of pinosomes.[14] Cells are briefly exposed to a radiolabeled protein in the presence of 0.5 M sucrose and 10% polyethylene glycol followed by osmotic lysis of the pinocytic vesicles with a hypotonic medium (Fig. 1B).

These two procedures for introducing radiolabeled proteins into the cytosol of cultured cells have distinct strengths and limitations. The amount of protein that can be successfully transferred to the cytosol is small; less than 1% of the protein for rbc-mediated microinjection, and less than 0.1% for osmotic lysis of pinosomes. Protein metabolism is disturbed for 2 hours after rbc-mediated microinjection and for up to 12 hours after osmotic lysis of pinosomes.[15,16] Therefore, protein degradation measurements must be postponed until after these times.

Other laboratories, as well as our own, radiolabeled and introduced into the cytosol a wide variety of different proteins. Most of the different purified proteins had characteristic and reproducible half-lives.[17] We focused our attention on proteins whose degradation rates increased in response to the withdrawal of serum growth factors.[18]

Cells in culture respond to serum withdrawal by increasing rates of proteolysis. Based on partial inhibition of the increased proteolysis by lysosomotropic agents such as ammonium chloride or chloroquine or by inhibitors of lysosomal proteases such as leupeptin, the increased proteolysis was widely accepted as being lysosomal.[19,20] The increased proteolysis in liver and other tissues during starvation and in diabetes was also known to be lysosomal.[21] Our hope was to use fibroblasts in culture along with serum deprivation to study the increased proteolysis associated with starvation. As it turned out, there were two different lysosomal pathways of proteolysis activated during starvation, macroautophagy followed by Cma. All of our studies used confluent cultures, and fibroblasts activate macroautophagy in response to contact inhibition of growth.[19] Serum withdrawal from confluent cultures causes increased proteolysis due to Cma.[22]

The substrate selectivity of Cma was evident very early in our microinjection studies; bovine pancreatic ribonuclease A (RNase A) and lysozyme are both small, basic proteins. [^{3}H]RNase A was consistently degraded more rapidly in response to serum withdrawal while [^{3}H]lysozyme was not (Fig. 2).[17,18] More definitive results came from analysis of the degradation characteristics of derivatives of RNase A. This protein can be cleaved by subtilisin into residues 1-20 (RNase S-peptide) and residues 21-124 (RNase S-protein). A pivotal finding was that RNase S-protein was degraded at the same rate in the presence and absence of serum (Fig. 3).[18] However, RNase S-peptide radiolabeled and microinjected alone was degraded more rapidly in response to serum withdrawal (Fig. 3). We expected from this result that the targeting information for enhanced degradation during serum withdrawal would be contained within the primary sequence of the first 20 amino acids of RNase A.

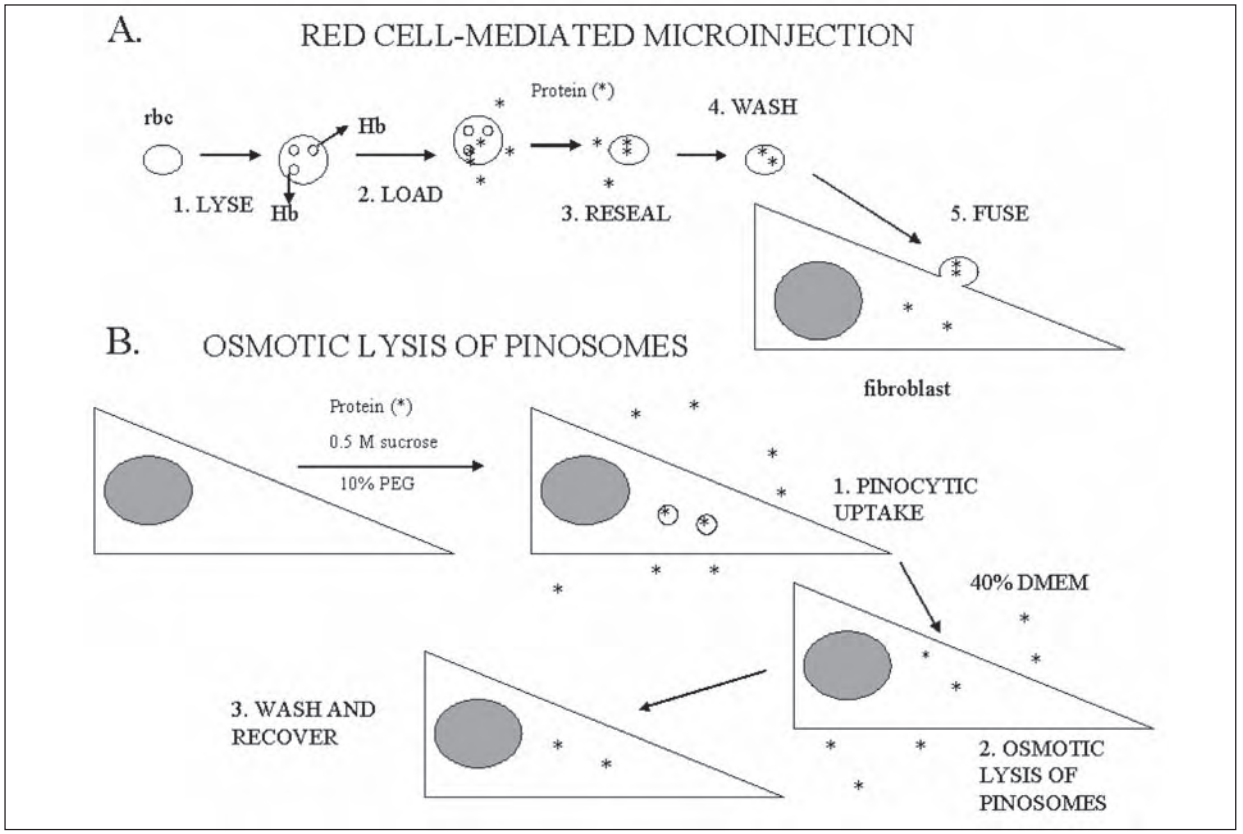

Figure 1. Two methods to introduce radiolabeled proteins into living cultured cells. A. The 5 steps in red blood cell-mediated microinjection are shown. rbc, red blood cell; Hb, hemoglobin B. The 3 steps in osmotic lysis of pinosomes are shown. PEG, polyethylene glycol; DMEM, Dulbecco's Modified Eagle's Medium.

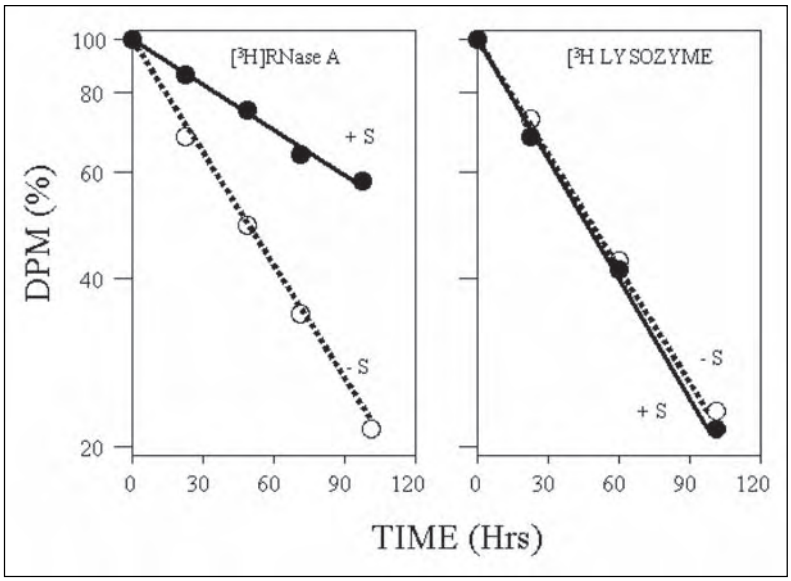

Figure 2. Degradation of microinjected [^{3}H]RNase A and [^{3}H]lysozyme. Both proteins were labeled by reductive methylation using NaB_3-H_4 and introduced into fibroblasts by red blood cell-mediated microinjection. [3-H]RNase A is degraded with a half-life of 100 hours in the presence of serum (+S), but its half-life decreases to 50 hours in the absence of serum (-S). [^{3}H]Lysozyme is degraded with a half-life of 40 hours both in the presence and absence of serum.

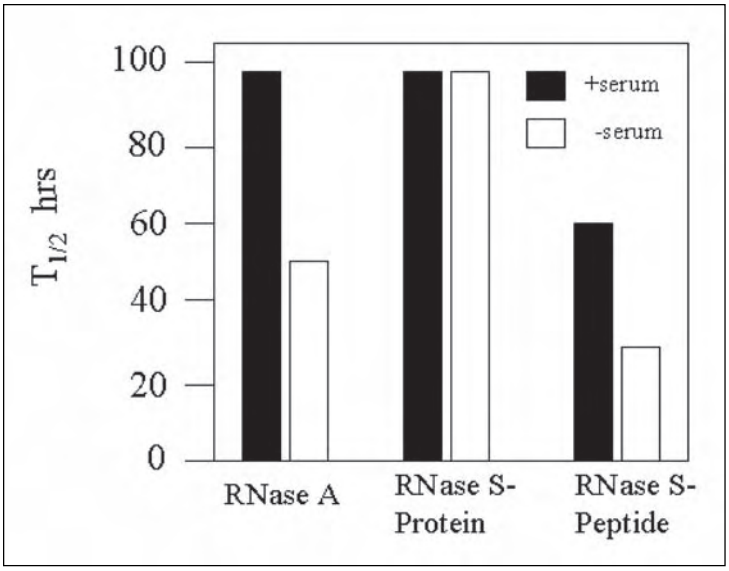

Figure 3. Degradation properties of microinjected [^{3}H]RNase A, [^{3}H]RNase S-protein, and [^{3}H]RNase S-peptide. RNase S-protein (amino acids 21-124 of RNase A) shows no increase in degradation rate in the absence of serum (-S). RNase S-peptide (amino acids 1-20 of RNase A) is degraded more rapidly than RNase A in the presence of serum (+S), but its degradation rate doubles in the absence of serum (-S).

To confirm that RNase S-peptide contained information for the increased degradation of proteins in response to serum withdrawal, we covalently linked RNase S-peptide to other proteins whose degradation rates were not regulated by serum.[23] An average of one RNase S-peptide molecule linked to one molecule of [^{3}H]lysozyme, or one molecule of [^{3}H]insulin A chain, caused those heterologous proteins to be degraded more rapidly in the absence of serum.[23]

To test whether the degradation of RNase A after microinjection into fibroblasts occurred within lysosomes, we covalently linked an inert carbohydrate to RNase A.[16] The carbohydrate [^{3}H]raffinose did not alter the degradation properties of RNase A. The degradation products consisted of [^{3}H]raffinose-lysine and [^{3}H]raffinose-dipeptides which cannot be further metabolized or transported across membranes. The [^{3}H]raffinose degradation products were localized to lysosomes when attached to RNase A but were localized to the cytosol when attached to denatured albumin, a substrate for the ubiquitin/proteasome proteolytic pathway.[16] These studies showed that RNase A is degraded entirely by lysosomes both in the presence and in the absence of serum. In confluent cultures maintained in serum, the lysosomal pathways responsible for the degradation of RNase A are presumably macroautophagy or microautophagy while in the absence of serum it is Cma. Despite the fact that RNase A is degraded entirely by lysosomes, ammonium chloride treatment of cells inhibited the degradation of RNase A by only 40-50%.[16] Therefore, the effect of lysosomotropic agents on proteolysis provides a minimum estimate for the role of lysosomes in a proteolytic pathway.

Identifying KFERQ As the Targeting Peptide in RNase A

To determine whether or not the entire RNase S-peptide was required for enhanced protein degradation in the absence of serum, we synthesized a variety of smaller peptides and comicroinjected them along with [^{3}H]RNase A to determine which ones specifically blocked the increased degradation in response to serum withdrawal.[2,24] This approach lead to the identification of residues 7-11 (KFERQ) as the critical pentapeptide within RNase S-peptide.

We also used recombinant DNA techniques to produce a fusion protein between RNase S-peptide and *E. coli* β-galactosidase. β-galactosidase by itself is degraded at the same rate in the presence and absence of serum after introduction into human fibroblasts. The direct fusion of RNase S-peptide to β-galactosidase resulted in a protein in which the KFERQ sequence was not accessible to antibodies against KFERQ. This product was also degraded at the same rate in the presence and absence of serum. However, when we introduced a 24 amino acid linker sequence between RNase S-peptide and β-galactosidase, this allowed the KFERQ region to become accessible, and the fusion protein was degraded more rapidly in the absence of serum (LJ Terlecky, SA Goff, and JF Dice, unpublished results). This construct is currently being used to mutate the KFERQ region to define changes in the KFERQ motif that can act as a targeting sequence for Cma.

The increased degradation of RNase A and RNase S-peptide in response to serum withdrawal were not unique to these proteins. Cytosolic aspartate aminotransferase could also be radiolabeled and microinjected into fibroblasts, and its degradation was also increased two-fold in response to serum withdrawal (EA Beard and JF Dice, unpublished results). We and others have so far identified 14 different protein substrates and have shown that 10 proteins are not substrates for Cma (Table 1).

The KFERQ-Like Targeting Sequence

KFERQ is an example of a targeting peptide, but this exact sequence is not required.[2,22,25] Sequence analysis of 4 substrate proteins suggested that the motif was a glutamine (Q) at one end and in any order a very hydrophobic (F), an acidic (E), a basic (R), and a second very hydrophobic or basic (K) amino acid.[22,25] We have experimentally shown that, at least in some cases, the Q can be replaced by the biochemically related N (AE Majeski, AM Cuervo, and JF Dice, unpublished results). Thus, the tentative KFERQ-like motif can be described as: (+,-,[],+/[]) Q/N or Q/N (+,-,[],+/[]) where the parentheses indicate that the order is unimportant and

Table 1. Substrates and nonsubstrates for Cma

Protein Substrates	Sequence	Representation	Reference
RNase A	KFERQ	+[]-+Q	18
aspartate aminotransferase	RKVEQ	++[]-Q	*
pyruvate kinase	QDLKF	Q-[]+[]	77
hemoglobin (β-chain)	QRFFE	Q+[][]-	76
GAPDH	NRVVD	N+[][]-	31
aldolase B	QKKEL, QFREL, IKLDQ	Q++-[], Q[]+-[], []+[]-Q	26
hsc73	QRDKV, QKILD	Q+-+[], Q+[][]-	**
IκB	VKELQ	[]+-[]Q	73
annexin II	QKVFD	Q+[][]-	75
annexin IV	QELLR	Q-[][]+	75
α_2-microglobulin	VDKLN, RIKEN	[]-+[]N, +[]+-N	41
glutathione transferase	NKKFE	N++[]-	***
c-fos	NLLKE	N[][]+-	78
Pax 2	DVVRQ, QRIVE	-[][]+Q, Q+[][]-	79
Nonsubstrates			
RNase S-protein			18
ovalbumin	RDILN, RELIN, NFEKL	+-[][]N, +-[][]N, N[]-+[]	31
lysozyme			17
ubiquitin			28
hexokinase			31
β-galactosidase	IRELN	[]+-[]N	***
annexin V	NIRKE, EFRKN	N[]++-, -[]++N	75
annexin XI			75
insulin A chain			28
green fluorescent protein	NRIEL	N+[]-[]	****

The putative KFERQ-like sequences are shown where + = K, R; - = D, E; and [] = F, I, L, V. References refer to evidence that the proteins are or are not substrates for Cma. *EA Beard and JF Dice, unpublished **AM Cuervo and JF Dice, unpublished ***AE Majeski and JF Dice, unpublished ****M Han and JF Dice, unpublished

+ = K, R; - = D, E; and [] = F, I, L, V. This motif currently identifies all fourteen known substrate proteins (Table 1). However, 4 of the 10 proteins that are not substrates also have putative N-based targeting motifs (Table 1). Probability calculations show that KFERQ-motifs should appear in 55% of proteins based on the frequencies of the amino acids and the average size of cytosolic proteins (JF Dice, unpublished results). Taken together these considerations suggest that the motif must be more restricted than shown above since we find experimentally that only 30% of cytosolic proteins contain KFERQ-like motifs that can be recognized by antibodies.[22] We believe that N can replace Q only in certain cases, and are currently defining

these N-based sequences using site-directed mutagenesis of the RNaseS-peptide-linker -β-galactosidase construct.

Some proteins such as aldolase B contain multiple KFERQ-like sequences. Experimentally, only one of these three motifs is necessary for aldolase B degradation by Cma.[26] The other KFERQ motifs may not be accessible to hsc73 binding, but this conjecture remains to be proven.

KFERQ-like sequences are found in a variety of secondary structures in proteins, and this has led some researchers to question how such targeting sequences could possibly be recognized by cellular proteins.[27] The answer appears to be that protein structures in cells are much more dynamic than they appear in X-ray crystallography. As an example, the KFERQ sequence in RNase A is within an α-helix, but intact RNase A can be immunoprecipitated with an antibody raised against linear KFERQ.[22] Certain molecular chaperones also recognize linear protein sequences yet hsc73 binds to the KFERQ region of RNase A.[28] Clearly, this pentapeptide motif folds and unfolds in solution.

Isolated Lysosomes Can Carry Out Chaperone-Mediated Autophagy

Highly purified lysosomes from cultured human fibroblasts isolated over two consecutive density gradients showed little degradation activity when incubated with protein substrates. However, addition of the constitutively expressed form of the heat shock cognate protein of 73 kDa (hsc73) together with ATP and an ATP-regenerating system selectively stimulated degradation of substrates for Cma (Fig. 4).[28,29] No other heat shock proteins of 70 kDa (hsp70s) tested were active in this assay.[28] Cma was temperature dependent and required an acidic lysosomal lumen (Fig. 4).

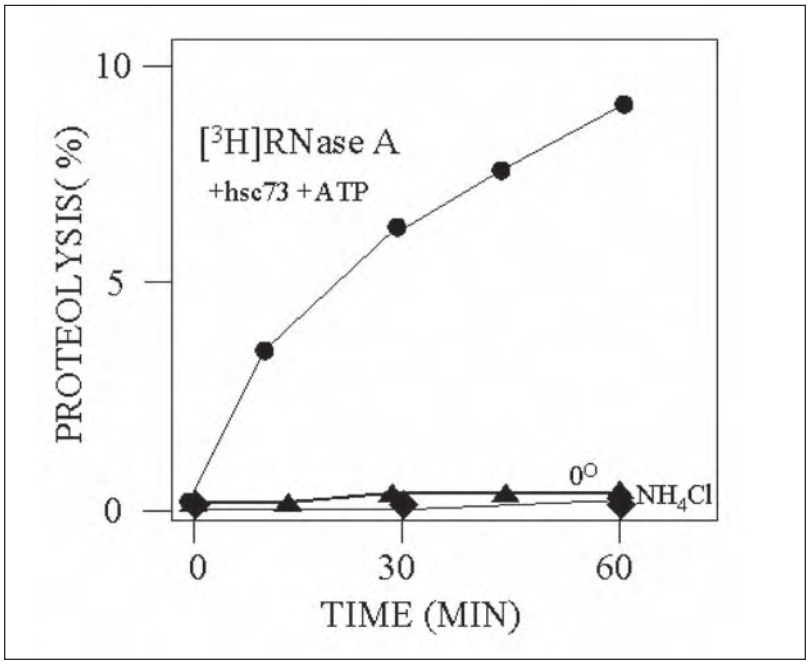

Figure 4. Degradation of [^{3}H]RNase A by lysosomes isolated from cultured human fibroblasts. Highly purified lysosomes are able to degrade [^{3}H]RNase A if hsc73 and an ATP regenerating system are included. Degradation is inhibited at 0°C and in the presence of a lysosomotropic agent, ammonium chloride (NH_4Cl).

The degradation of substrate proteins was saturable (K_m = 5 μM for [^{3}H]RNase S-peptide), and different substrate proteins competed with each other for degradation. For example, degradation of [^{3}H]RNase A could be competed by addition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) but not by an equal concentration of ovalbumin.[30,31] At 4°C substrate proteins bound to the lysosome surface but did not translocate into the lysosome. This binding was saturable and could be inhibited by prior mild trypsinization of the lysosomes.[30] These results suggested that a receptor for substrate proteins might exist in the lysosomal membrane (see below).

All vesicular traffic and presumably both macro- and microautophagy can be inhibited by GTPγS.[32,33] We found no effect of GTP or GTPγS on degradation of substrate proteins (SR Terlecky and JF Dice, unpublished results) and concluded that vesicular traffic was not involved in Cma.

Rat Liver Lysosomes

Our early studies using an antibody against KFERQ suggested that Cma is active in many but not all mammalian tissues. We found that starvation induces the loss of KFERQ-containing proteins in liver, kidney, spleen and heart, but not in skeletal muscle.[22,34] Even for the same tissue, Cma rates vary in different cell types. For example, astrocytes in culture display high rates of Cma (AM Cuervo and A Martin, unpublished results), but Cma is inactive in brain, which contains mainly neurons.[34]

One advantage of liver compared to other organs is its homogenous cellular composition with 90% of liver cells being hepatocytes. In addition, protein degradation is important in the regulation of the total protein content in liver.[35] The large size and easy dissection of this organ facilitate the isolation of large amounts of highly purified lysosomes by conventional procedures.[36] This fact has been critical for the isolation and characterization of lysosomal components that participate in Cma.

Isolated lysosomes from rat liver also carry out Cma. Uptake of GAPDH and RNase A was stimulated by hsc73 and ATP, and these two substrates competed with each other for binding to the lysosomal membrane.[31] RNase S-peptide was another substrate for Cma by rat liver lysosomes, and nonsubstrates included RNase S-protein, ovalbumin, and hexokinase.[31]

Using rat liver lysosomes, Knecht and colleagues were able to separately measure substrate binding to lysosomal membranes, import into the lysosomal lumen and degradation within the lysosome.[31,39] Figure 5 shows that mixing a radiolabeled substrate with lysosomes results in binding of some of the substrate to the lysosomal membrane. Any substrate that was transported into the lysosome must be rapidly degraded because little could be found inside at steady state. On the other hand, when the same incubation contained chymostatin to inhibit lysosomal proteases, the isolated lysosomes contained both bound and transported populations of substrate. Treatment with proteinase K removed the bound form and left only that transported. Unexpectedly, a kinetic intermediate in the transport of RNase A was evident in which 2 kDa of the protein remained susceptible to attack by proteinase K. The 2 kDa fragment corresponded to the C terminus of RNase A indicating that the transport of this protein into lysosomes was with the N terminus leading.[31]

A Receptor in the Lysosomal Membrane

The saturable binding of substrate proteins to lysosomes suggested the existence of a receptor associated with the lysosomal membrane. We found that substrate proteins could bind to a known integral membrane protein of lysosomes called lysosome-associated membrane protein type 2a (lamp2a).[40] Most of this protein resides in the lysosomal lumen and is heavily glycosylated. There is a single transmembrane segment and a 12 amino acid tail in the cytosol (Fig. 6).

Substrate proteins bind to the cytosolic tail of lamp2a.[40] Substrate binding could be blocked by incubation in the presence of a soluble 12 amino acid peptide corresponding to the cytosolic

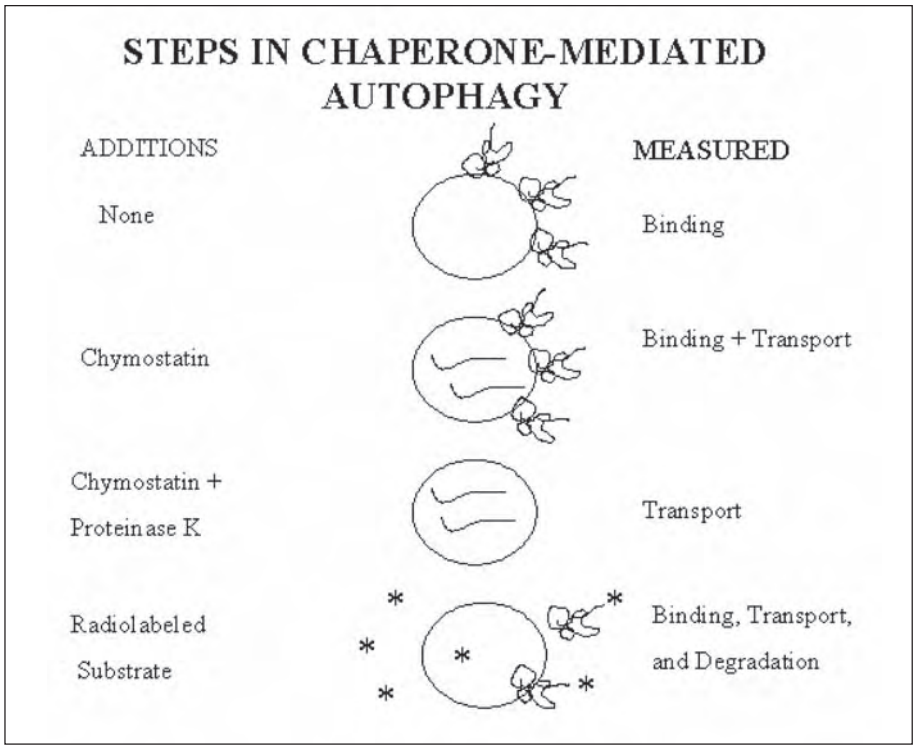

Figure 5. Different steps in Cma can be distinguished using lysosomes isolated from rat liver or cultured cells. Without additions, substrate proteins associated with lysosomes are bound to the surface; any transported molecules are quickly degraded. When degradation is blocked with chymostatin, lysosome associated substrates represent both bound and transported molecules. Bound substrate can be digested with proteinase K. Degradation of a radiolabeled substrate provides a measure of binding, transport, and degradation.

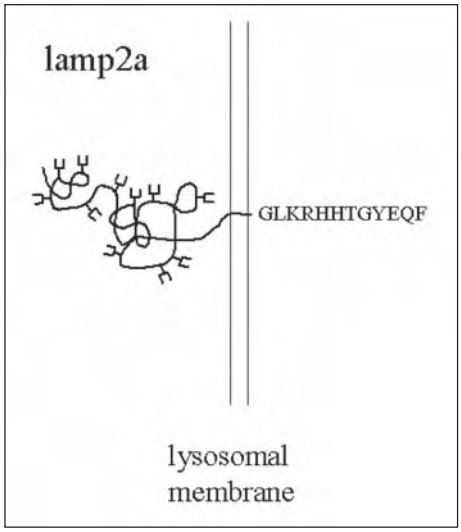

Figure 6. Lamp2a in the lysosomal membrane. The 12 amino acid cytosolic tail sequence is shown. The line represents the polypeptide backbone, and the branched structures represent sugar groups.

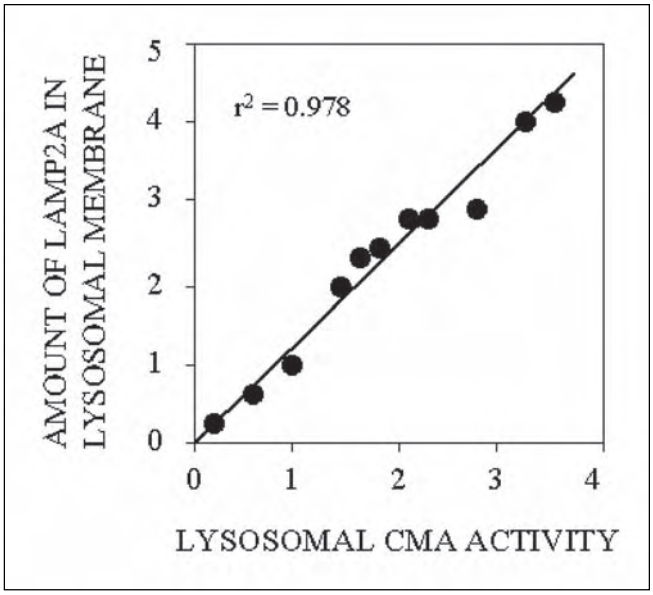

Figure 7. Correlation between lysosomal Cma activity and levels of lamp2a in the lysosomal membrane. Lysosomal Cma activity was measured using isolated lysosomes and radiolabeled RNase A or GAPDH. Lysosomal membrane lamp2a was measured by separating lysosomal membranes by SDS-PAGE, transferring the proteins to nitrocellulose filters, and immunoblotting the membranes using an antibody specific to lamp2a. The conditions in order of increasing Cma activity are: liver lysosome population less active in Cma, lysosomes from 22-month old rats, lysosomes from fed rat liver or cultured fibroblasts in the presence of serum, liver lysosomes from rats starved for 20 hours, liver lysosome population more active in Cma, lysosomes from cultured fibroblasts in the absence of serum, liver lysosomes from rats with $\alpha 2\mu$ nephropathy, liver lysosomes from rats starved 64 hours, lysosomes from cultured cells stably overexpressing human lamp2a, liver lysosomes from rats starved for 88 hours, lysosomes from cultured cells stably overexpressing human lamp2a at a higher level.

tail of lamp2a but not by a peptide with the same amino acid composition but a random sequence. Furthermore, an antibody to the cytosolic tail also blocked substrate binding.[40]

Overexpression of lamp2a in cultured cells revealed that binding to the receptor is a rate-limiting step for Cma.[40] Cell clones expressing higher lysosomal levels of lamp2a also displayed higher Cma activity. This correlation between levels of lamp2a and Cma activity was also later corroborated in vivo. We have documented higher content of lamp2a at the lysosomal membrane in all conditions that activate Cma,[37] such as prolonged starvation in rat liver, serum deprivation in cultured cells, or exposure to gasoline derivatives in rat kidneys.[41] Conversely, rates of Cma decrease with age and lysosomal membrane levels of lamp2a are lower in old animals when compared to young animals (Fig. 7).[42]

Changes in the lysosomal content of lamp2a are, at least in part, a consequence of the tightly regulated degradation of this receptor in lysosomes.[43] The combined action of a serine protease and a metalloprotease in the lysosomal membrane cleaves the cytosolic/transmembrane region of lamp2a resulting in the release of a truncated lamp2a that is rapidly degraded in the lysosomal lumen.[43] This process decreases when Cma is activated, contributing to the increase in the lysosomal levels of lamp2a under those conditions. We are currently trying to identify the proteases involved in lamp2a cleavage and to understand their regulation.

A unique characteristic of lamp2a compared to receptor proteins in other intracellular transport systems is its dual location at the lysosomal membrane and in the lysosomal lumen.[43-45]

The lumenal lamp2a associates with other lumenal proteins and lipids in a pH-dependent manner.[44,45] Though it is not clear how lamp2a reaches the lysosomal lumen, some of it is internalized together with the substrates for Cma during their translocation.[43] The distribution of lamp2a between the lysosomal membrane and lumen is dynamic. Under conditions resulting in maximal activation of Cma, part of the lumenal lamp2a can be recruited into the lysosomal membrane becoming a functional receptor.[43] Further studies are required to identify components of the lysosomal membrane and lumen involved in the insertion/deinsertion process.

Lamp2a is one of 3 different lamp2s that originate from alternative splicing of a single gene.[46-48] These variants differ in the amino acid composition of their membrane and cytosolic region, but all have identical lumenal domains.[47] A complete knock-out of the lamp2 gene in mice leads to abnormal accumulation of autophagosomes in different tissues.[49,50] Lamp2b is the isoform involved in macroautophagy; a patient with accumulation of autophagosomes (Danon's disease) carries a single mutation in an exon specific to lamp2b (see chapter 22).[50]

In contrast, lamp2a is the only variant involved in Cma.[37] The four positive residues in the cytosolic tail (KRHH) (Fig. 6) that are essential for substrate binding are only conserved in lamp2a, and substrate proteins do not bind to other lamp2 isoforms.[37] Lysosomal levels of lamp2a, but not of other forms of lamp2, correlate with Cma activity (Fig. 7). Furthermore, only lamp2a exists as a multimer which is possibly required for the translocation of the substrates. We presume that Cma was also defective in the lamp2 knock-out mice, and the absence of this pathway may contribute to the animals' phenotype such as their reduced ability to withstand starvation.[49]

Two Different Populations of Rat Liver Lysosomes

Cuervo and colleagues[51] separated subpopulations of lysosomes with different activity for Cma based on slight density differences. The main difference between these groups of lysosomes, otherwise identical in their morphological and biochemical properties, is that lysosomes more active for Cma have higher levels of lamp2a in the lysosomal membrane[34] as well as higher levels of hsc73 in their lumen (ly-hsc73).[51] The elevated amount of lamp2a in the lysosomal membrane results from a decrease in its degradation rate combined with increased reinsertion of lumenal lamp2a into the lysosomal membrane.[37,43] Interestingly, low activity lysosomes become highly active for Cma if ly-hsc73 is increased by prior incubation with hsc73.[51] Our interpretation is that cells normally contain a population of lysosomes displaying low Cma activity that can be quickly committed to Cma when required by changes in cellular conditions. The starvation-induced enrichment of ly-hsc73 in this group of lysosomes results from a decrease in the degradation rate of the chaperone in the lysosomal lumen.[51] Further investigations are required to determine whether activation of this group of lysosomes for Cma is a final step in their maturation process or if they could undergo dynamic activation/inactivation cycles depending on the cellular requirements for Cma.

Role of Lysosomal Lumen Hsc73

Multiple lines of evidence suggest that a distinct isoform of hsc73 exists in the lumen of lysosomes. Immunostaining of fibroblasts for hsc73 results in colocalization with vesicular structures that are also positive for the lysosomal membrane glycoprotein of 120 kDa, a major glycoprotein in the lysosomal membrane,[52,53] indicating that hsc73 is associated with either the lysosomal membrane and/or matrix. Two-dimensional gel electrophoresis demonstrated that the lysosomal matrix hsc73 had a pI = 5.3 whereas most cytoplasmic hsc73 and hsc73 associated with the lysosomal membrane had pIs of 5.5, indicating that ly-hsc73 is a distinct isoform of hsc73.[53] It is unknown how ly-hsc73 becomes localized to the lysosomal matrix; microautophagy and macroautophagy are two mechanisms that have been proposed previously.[51] It is also possible that hsc73 is incorporated into lysosomes by Cma because it contains two KFERQ motifs.[28] However, the significance of the more acidic pI of ly-hsc73 remains to be elucidated.

Ly-hsc73 appears to facilitate the transport of substrates into the lysosome for degradation, although direct evidence for this remains inconclusive. As described above, populations of lysosomes containing higher levels of ly-hsc73 exhibit greater rates of Cma compared to populations of lysosomes with less ly-hsc73.[51]

Further in vivo studies using neutralizing concentrations of endocytosed anti-hsc73 antibodies demonstrated a role for ly-hsc73 in Cma. Pretreatment of fibroblasts with anti-hsc73 antibodies abolished the increase in protein degradation by Cma in response to serum deprivation.[22,53] Endocytosed anti-hsc73 antibody pretreated with hsc73 did not abolish the increase in protein degradation, indicating that this response could be attributed to the neutralizing properties of anti-hsc73. Furthermore, the degradation of endocytosed [^{3}H]RNase A was not affected by pretreatment with anti-hsc73 antibodies suggesting that overall lysosomal function was not changed.[53] Taken together, these results suggest that ly-hsc73 is necessary for efficient Cma. Hsp70s in the lumen of the endoplasmic reticulum and in the mitochondrial matrix are required to pull substrate proteins into those organelles,[54] and ly-hsc73 may play a similar role.

More recently, anti-hsc73 antibodies have been shown to decrease the reinsertion of lamp2a from the lumen of resealed lysosomal membranes.[43] Therefore, it is possible that the effect of endocytosed anti-hsc73 antibodies on inhibition of Cma may be due to indirect effects of ly-hsc73 on lamp2a levels in the lysosomal membrane. Further experiments are required to clarify the role(s) of lumenal ly-hsc73 in Cma.

Unfolding of Protein Substrates Is Required for Protein Translocation

Recent evidence suggests that unfolding of substrate proteins is required for efficient transport into lysosomes by Cma.[55] An experimental approach originally developed by Eilers and Schatz[56] to study the requirement for protein unfolding prior to mitochondrial import was used. Dihydrofolate reductase (DHFR) can be locked into a folded, protease-resistant conformation through high affinity interactions with the substrate analog methotrexate. In the presence of methotrexate, binding of DHFR (which contains a KFERQ motif) to lysosomes was unchanged but uptake was decreased by 80%.[55] Similarly, uptake of a fusion protein comprising full length DHFR with the presequence and first three amino acids of subunit 9 of the F_o-ATP synthase (Su9-DHFR) by isolated lysosomes was abolished in the presence of methotrexate. Partial removal of methotrexate from Su9-DHFR bound to lysosomal membranes resulted in increased uptake of Su9-DHFR protein into lysosomes. Furthermore, competition of methotrexate binding with its substrate dihydrofolate also increased the uptake of Su9-DHFR presumably by allowing DHFR to adopt a more relaxed conformation. DHFR and Su9-DHFR were also shown to be specifically transported into lysosomes for degradation via Cma as demonstrated by increased uptake into liver lysosomes from rats starved for 88 hours as well as the ability of RNase A to compete for uptake of these substrates into the lysosome in vitro.[24,38] Taken together, these data demonstrate that unfolding is necessary for the efficient transport of substrate proteins by Cma.

Hsc73 at the Lysosomal Membrane

In lysosomes from rat liver and from cultured cells, most (80%) lysosome-associated hsc73 is in the lumen (ly-hsc73). The remaining 20% is associated with the cytosolic face of the lysosomal membrane (lym-hsc73).[53] Agarraberes and Dice[57] characterized the lym-hsc73 associated with the lysosomal membrane by extracting hsc73 from purified lysosomal membranes using a variety of treatments. They discovered that high salt concentrations and alkali treatment could not extract the lym-hsc73; only urea was able to extract lym-hsc73 from the lysosomal membrane, while none of these treatments could extract lamp2a. To determine whether or not hsc73 was associated with lipids, lysosomal membranes were treated with [^{125}I]-labeled 3-trifluoromethyl-3-(m-iodophenyl) diazirine (TID), a compound that labels molecules exposed to hydrophobic environments. Lym-hsc73 remained unlabeled by treatment with

[^{125}I]TID, while lamp2a was readily labeled. Taken together, these results indicated that lym-hsc73 was not an integral membrane protein itself but localized to the lysosomal membrane through strong interactions with other membrane proteins.[57]

Hsc73 is known to exist in complexes with other chaperones in the cytosol.[58-60] Therefore, the possibility that lym-hsc73 also existed in a complex was examined. To evaluate this possibility, solubilized lysosomal membranes were immunoprecipitated with anti-hsc73 antibodies or anti-hsp70 interacting protein (hip) antibodies. Western blot analysis identified several chaperones associated with hsc73; the heat shock protein of 90 kDa (hsp90), hsp70-hsp90 organizing protein (hop), hip, the Bcl2-associated anthogene 1 protein (Bag-1) and hsp40. GAPDH was also in these complexes indicating that at least some of these complexes were functionally transporting substrates. A further interesting discovery was that both hip and Bag-1 coimmunoprecipitated with hsc73.[57] Hip and Bag-1 have overlapping binding sites on hsc73 and cannot bind to the same molecule of hsc73.[59] Therefore, multiple complexes must be present at the lysosomal membrane.[57]

To directly assess the requirement for some of the chaperones/cochaperones in the binding and transport of substrates across the lysosomal membrane, blocking antibodies against hsc73, hip, hop, and hsp40 were used. Purified lysosomes were incubated with antibodies against these molecular chaperones along with a substrate for Cma (RNase A or GAPDH). These antibodies could inhibit substrate transport across the membrane of the lysosome.[57] These additional chaperones and cochaperones have been incorporated into a more detailed working model of Cma (Fig. 8).

In contrast to these results with lym-hsc73, lumenal ly-hsc73 was not complexed with any other molecular chaperones. It may be that the chaperone complex dissociates and only hsc73 is transferred to the lysosomal lumen. Alternatively, the entire molecular chaperone complex might enter the lysosome lumen, but chaperones other than ly-hsc73 might be rapidly degraded.

Physiology of Cma

An important role for Cma in prolonged starvation is undoubtedly to provide amino acids to support ongoing protein synthesis and to act as an energy source. Mechanisms by which Cma is activated are under investigation. The causes and effects of reduced Cma in aging are also being studied.

Activation of Cma in Starvation

The mechanism of activation of Cma is unknown. Cma is activated when confluent fibroblasts are switched to serum-free media and in rat tissues during prolonged starvation.[22,38] It is possible that starvation and the acute withdrawal of serum growth factors reduces amino acid transport into cells and that intracellular amino acid depletion may signal the activation of Cma just as it activates macroautophagy (see chapter 3).[2]

However, activation of macroautophagy and Cma are clearly through separate mechanisms. For example, phosphatidylinositol (PtdIns) 3-kinase plays an important role in the activation of macroautophagy because this process can be inhibited by PtdIns 3-kinase inhibitors (see chapters 3 and 4).[61,62] However, these PtdIns 3-kinase inhibitors had no effect on the activation of Cma (PF Finn and JF Dice, unpublished results).

Another pathway that is required for activation of macroautophagy utilizes the mammalian target of rapamycin (mTor; see chapters 3 and 5). The mTor signaling pathway is a nutrient sensing pathway that controls cell growth in organisms ranging from yeast to mammals.[63] Treatment with rapamycin inhibits mTor and results in stimulation of macroautophagy.[63] We are currently testing whether or not the mTor pathway also participates in the activation of Cma.

One small molecule that does activate Cma is the ketone body, β-hydroxybutyrate (BOH) (PF Finn and JF Dice, unpublished results). Ketone bodies accumulate in the circulation of mammals during prolonged fasting similar to the timing of activation of Cma. Interestingly, BOH stimulated Cma equally in the presence and absence of serum. We are currently defining the mechanisms by which BOH activates Cma.

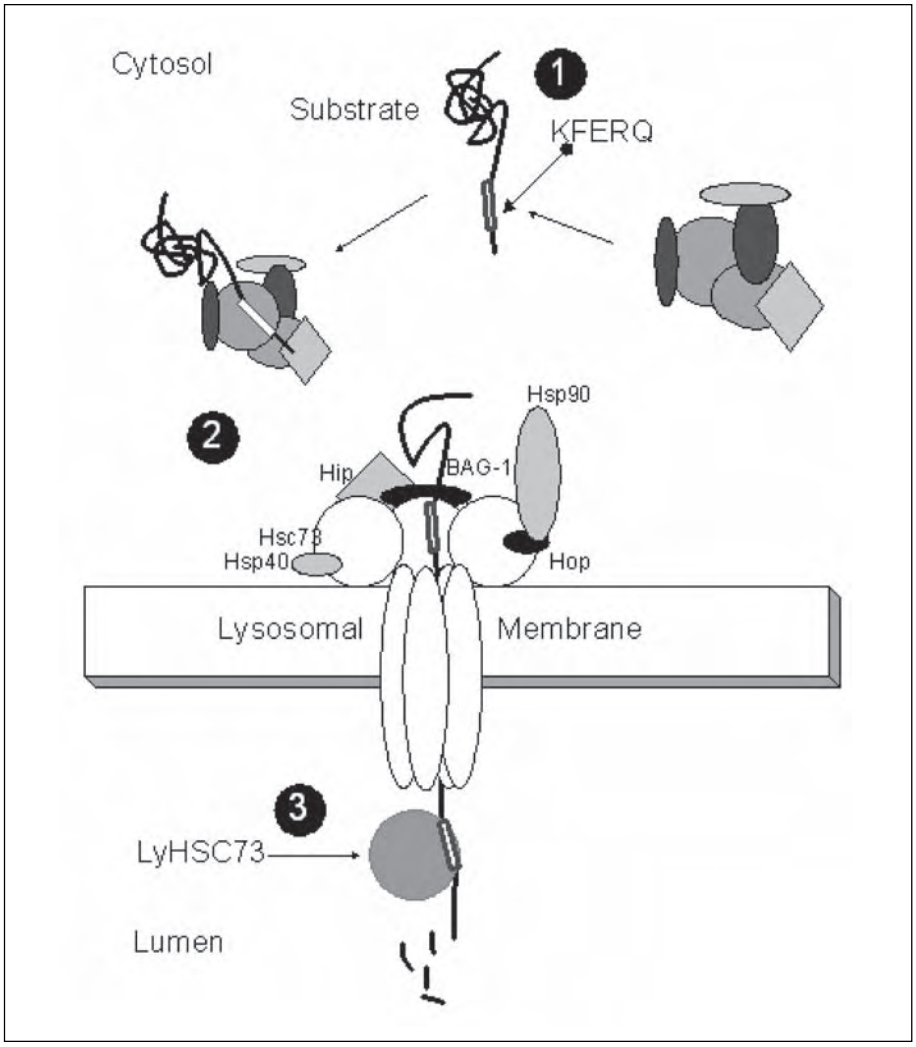

Figure 8. The current working model of Cma. 1. Hsc73 chaperone/cochaperones complex binds to a peptide region containing the pentapeptide motif, KFERQ. 2. The protein complex then is delivered to the lysosome where the chaperone/cochaperones complex docks the substrate to lamp2a, the multimeric lysosomal membrane protein. 3. The substrate is then translocated across the lysosomal membrane with the help of ly-hsc73 into the lumen of the lysosome. The substrate is then degraded by the lysosomal hydrolases. Adapted from Agarraberes F.A., Dice J.F. Journal of Cell Science. 2001; 114:2491-2499.

Decline of Cma in Aging

As mentioned earlier in this chapter, activated Cma is associated with starvation and also with exposure to certain toxins. In aging there is a marked decline in Cma.[42] One characteristic of aging cells and tissues is an accumulation of damaged proteins. One reason for their accumulation could be a reduced rate of degradation. Overall rates of proteolysis do decline with age. Macroautophagy is reduced in aged liver due to reduced formation of autophagosomes coupled with reduced fusion with lysosomes (reviewed in reference 64).

The age-related decline in Cma was first shown in human fibroblasts by studying the degradation rates of microinjected proteins.[65] Senescent cells broke down these proteins more slowly than young cells. Some of these proteins were subsequently identified as substrates for Cma.

Later studies utilizing lysosomes isolated from rat liver and fibroblasts showed similar results as the earlier whole cell analyses.[42] Substrate degradation rates by isolated lysosomes of starved old rats or serum-deprived senescent fibroblasts were found to be lower than for young rats and cells. Separating lysosomal binding and uptake, each was found to be lower in old rats. This can be explained by the lower levels of lamp2a found in old rats and late population doubling level fibroblasts.

The amount of ly-hsc73 is higher in old rats, but only in the oldest rats. The increase in ly-hsc73 may be a compensatory mechanism due to the decrease in lamp2a levels. Ly-hsc73 levels are modulated under conditions such as fasting, so the increased ly-hsc73 in old cells may be an attempt to activate Cma.[42]

Normal cells rid themselves of damaged components such as oxidized proteins. The changes in protein degradation may participate in the accumulation of these damaged proteins in "old" cells. Increasing the level of lamp2a in young cells makes chaperone-mediated autophagy more active.[37,40] Future studies will investigate whether or not increasing lamp2a levels in aged cells will result in an increased pathway activity and decreased amount of damaged proteins.

Interesting Substrates for Cma

There are 14 known substrates for Cma (Table 1), and here we review 3 of particular interest.

α_2-Microglobulin

α_2-microglobulin ($\alpha 2\mu$) is a secreted protein that is synthesized in the liver.[66] Among its proposed functions are the transport of fatty acids to renal epithelial cells[67] and the transport of odorants to male rodent urine.[68] In the syndrome hyaline droplet nephropathy or $\alpha 2\mu$ nephropathy, hyaline droplets with lysosomal $\alpha 2\mu$ as the principal component are found in the proximal tubules of affected kidneys.[66,67,69] This syndrome is caused from exposure to environmental toxins including gasoline derivatives of aliphatic, alicyclic, and aromatic nature. The lysosomal overload of $\alpha 2\mu$ results in severe cellular damage and proximal tubule necrosis. The lysosomal $\alpha 2\mu$ accumulation is not due to a change in transcription of the $\alpha 2\mu$ gene, in translation of the $\alpha 2\mu$ mRNA, or in the transport of $\alpha 2\mu$ from liver to kidney.[70] Also, there is no decrease in its lysosomal degradation following endocytosis by proximal tubule cells.[41]

Besides being a secreted protein, $\alpha 2\mu$ is also localized in the cytosol in both liver and kidney.[41] How this protein escapes the secretory and endocytic pathways to enter the cytosol is not known. This protein has characteristics that are common to substrates of Cma including two KFERQ-like sequences (see Table 1). $\alpha 2\mu$ is taken up and degraded by lysosomes isolated from liver and kidney, and $\alpha 2\mu$ can compete with other known Cma substrates. $\alpha 2\mu$ also binds to lysosomal membranes, presumably to lamp2a.[41] The chemicals that cause hyaline droplet nephropathy stimulate Cma and make $\alpha 2\mu$ an especially good substrate so that $\alpha 2\mu$ is preferentially taken up under these conditions. The increased uptake combined with reduced degradation leads to the accumulation of $\alpha 2\mu$ in lysosomes eventually forming the hyaline droplets.[41]

Inhibitor of Nuclear Factor κB

The well-studied transcription factor nuclear factor κB (NF-κB) is ubiquitously expressed and regulates transcription of many genes important for immune and inflammation responses as well as cell cycle regulation and development. NF-κB moves from the cytosol to the nucleus upon stimulation.[71] Otherwise it is kept in the cytosol through its binding to proteins called inhibitors of NF-κB (IκB).[72]

Several nonlysosomal degradative systems can degrade IκB depending on the type of cell and circumstance; these include the 26S proteasome, calpains and caspases.[71] Both short-lived and long-lived pools of IκB exist within cells, and the long-lived pool is a substrate for Cma.[73] IκB is found in the lumen of rat liver lysosomes active for Cma if cathepsin activity is inhibited by leupeptin. IκB is degraded with a half-life of 4.4 days in Chinese hamster ovary (CHO) cells in the presence of serum, but is degraded with a half-life of 0.9 days in the absence of serum.[73] IκB binds to lamp2a, and cells overexpressing lamp2a show increased rates of degradation of IκB. Targeting of IκB for Cma does not require phosphorylation but does depend on reactive oxygen species.[73] As the IκB levels decreased, NF-κB activity increased under conditions of increased Cma. Therefore, the relative lack of IκB due to its degradation by Cma is associated with an increase in NF-κB sensitivity to activating stimuli.[73]

Annexins

Annexins are a group of proteins that can bind calcium and phospholipids and have diverse roles in the cell including the regulation of endocytosis, exocytosis, and apoptosis.[74] Annexins are found in the cytosol, but can traffic to parts of the vesicular system, mitochondria, and the nucleus.[74] Some of this traffic is regulated by calcium binding.[70] How annexin protein levels are regulated is not known. Most annexins have KFERQ motif sequences but some do not.[75]

The KFERQ motif-containing annexins tested (II and VI) are substrates for Cma and those without the motif sequence (V and XI) are not. The degradation rates of the different annexins were measured in serum-supplemented and serum-deprived cells following immunoprecipitation. Isolated lysosomes were utilized to follow transport and degradation of the different purified annexins. The amount of each protein in lysosomes more active in Cma was also measured. These results showed that annexins with a KFERQ motif can be degraded through Cma.[75] It is possible that their selective degradation has a regulatory role, which would allow certain annexin functions to be depleted while others are maintained.[75] For example, endocytosis and exocytosis are maintained during starvation, and annexins critical for these processes must be retained. Annexins that associate with polypeptide growth factor receptors may be degraded and contribute to receptor internalization under these conditions.[2]

Future Directions of Research

Additional experiments required to understand various aspects of Cma are mentioned throughout the text. A major component of the protein import machinery for Cma not yet identified is the translocon. Attempts to identify these proteins by their expected interaction with lamp2a have yielded no candidates. The protein import machinery of chloroplasts uses molecules that act as combination receptors and translocons,[54] and it seems possible that lamp2a in a multimeric form may also be a dual receptor and translocon. We are attempting to reconstitute binding and import into liposomes using defined molecular chaperones and lamp2a.

It is also very important to complete the definition of a KFERQ-like targeting sequence in substrates. It seems certain that N will be able to replace Q only within limited sequences such that the putative N-based sequences listed in nonsubstrates (Table 1) will be excluded. Once the exact targeting sequences are known, protein sequence databases may be searched to identify classes of proteins that are enriched for substrates of Cma. This kind of analysis should expand our understanding of the physiological importance of Cma. Proteins degraded by Cma should be dispensable or maybe even harmful during prolonged starvation.

The reduced Cma in aging appears to be due to a reduction in lamp2a levels in the lysosomal membrane, but other age-related defects may also exist. We plan to overexpress lamp2a in senescent cells using adenoviral vectors. If Cma activity is returned to values in young cells by expression of lamp2a at the levels found in young cells, the reduction in level of this protein will be shown to be the only cause of reduced Cma in aging. We must then pursue why the levels of lamp2a decline in aging. Another important question regards the effects of the reduced Cma in senescent cells. For example, the accumulation of certain abnormal proteins in

aged tissues may be caused by their reduced degradation. A subset of these proteins may be substrates of Cma.

Acknowledgements

Research from the authors' laboratories was supported by grants from the National Institutes of Health (AG06116 to JFD and AG21904 to AMC) and the Howard Hughes Medical Institute (AMC).

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CHAPTER 13

Vacuolar Import and Degradation

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Abstract

The gluconeogenic enzyme fructose-1,6-bisphosphatase is rapidly degraded in yeast cells following a shift from low glucose conditions to high glucose conditions. Although the site of degradation has been controversial, research from our lab and others indicates that a significant portion of FBPase is degraded in the vacuole. A number of mutants have been identified that block FBPase degradation, and these mutants affect various steps in the FBPase degradation pathway. Based on these mutant studies, a model for the FBPase degradation pathway has been developed in which FBPase trafficks from the cytosol to intermediate vacuole import and degradation (Vid) vesicles following a glucose shift. These Vid vesicles then deliver FBPase to the vacuole where it is degraded. The *VID22* gene is required for FBPase import into Vid vesicles, while the *UBC1* gene appears to play a role in vesicle formation. In contrast, the *VID24* gene is required for the trafficking of Vid vesicles to the vacuole. Vid vesicles have been purified to near homogeneity, and purified vesicles have been utilized in an in vitro assay that recapitulates the first FBPase trafficking step. The use of the in vitro assay has assisted in the identification of other proteins that participate in the FBPase degradation pathway. These include the molecular chaperone Ssa2 and the cyclophilin Cpr1, both of which are required for FBPase import into Vid vesicles.

FBPase—A Model Protein for Protein Degradation Studies

Regulation of FBPase Levels

In yeast, there is a coordinate regulation of enzymes involved in pathways such as the TCA cycle, the glyoxylate cycle, and gluconeogenesis.[1-4] Accordingly, a number of enzymes in these processes are induced when yeast cells are grown in media containing poor carbon sources, and as such, this provides more substrates for the production of glucose. When cells are supplied with glucose, however, these enzymes are no longer needed and they are rapidly inactivated. Phosphoenolpyruvate kinase is an example of a key gluconeogenic enzyme that is catabolically inactivated following the addition of glucose.[2] Likewise malate dehydrogenase, an enzyme in the TCA cycle,[3] isocitrate lyase, an enzyme involved in the glyoxylate cycle[4] and sugar permeases, such as galactose permease and maltose permease, have all been reported to be inactivated by glucose.[5, 6]

Fructose-1,6-bisphosphatase (FBPase) is another key gluconeogenic enzyme that is inactivated by glucose.[1] A number of studies have attempted to identify the mechanisms of the catabolic inactivation of FBPase. Although there are many contributing factors, protein degradation is the primary mechanism by which FBPase is inactivated. Protein degradation also plays a role in the inactivation of a number of other enzymes. For example, when cells are transferred to glucose, the plasma membrane galactose transporter is selectively delivered to the vacuole for degradation.[6] Mutants defective in endocytosis block the degradation of the galac-

Autophagy, edited by Daniel J. Klionsky.

tose transporter, suggesting that it is delivered to the vacuole for degradation through the endocytic pathway.[6] In a similar manner, the maltose transporter is delivered to the vacuole via endocytosis following a shift of cells to glucose.[5] The inactivating enzyme for phosphoenolpyruvate kinase has been identified as proteinase B (Prb1),[7] whereas the inactivating enzyme for uridine nucleosidase is proteinase A (Pep4).[8] These proteinases are found in the vacuole and have broad substrate activities. However, whether phosphoenolpyruvate kinase or uridine nucleosidase are targeted to the vacuole for degradation has not been examined.

Because protein degradation can regulate the activity of a number of key enzymes, the study of the degradative process can provide valuable information regarding the regulation of metabolic pathways. A number of characteristics make FBPase an ideal candidate for protein degradation studies. First of all, FBPase expression can be induced under very specific and controlled circumstances. Second, the process of FBPase degradation can also be initiated in a controlled manner via the addition of glucose. Finally, the degradation of FBPase is relatively rapid, as the protein exhibits a half-life of approximately 30 minutes following a glucose shift. Thus the process of protein degradation can be followed over a relatively short period of time.

Protein modification may be a contributing factor for the targeted degradation of FBPase. For example, the phosphorylation of FBPase has been implicated as a possible regulatory factor in the degradation of this protein. However, whether phosphorylation actually plays a role in the degradation of FBPase is the subject of debate. FBPase can be phosphorylated at Ser11,[9] and the level of phosphorylation increases during glucose replenishment. Phosphorylation of FBPase is mediated by the Ras2 signaling pathway.[10] Interestingly, this phosphorylation does not appear to play a direct role in the inactivation of FBPase, because the mutation of the phosphorylation site does not alter the degradation of FBPase.[9,11] Ubiquitination of FBPase has also been cited as a means of targeting this protein for degradation in the proteasome.[12,13]

The Site of FBPase Degradation

The site of FBPase degradation has been the subject of debate.[12-15] There is evidence suggesting that FBPase is ubiquitinated and that the N-terminal proline residue is required for polyubiquitination of FBPase in response to glucose. The ubiquitinated FBPase is then degraded in the proteasome, because proteasome mutants retard the degradation of FBPase.[12, 13] In contrast to these results, other studies have provided evidence that FBPase is degraded in the vacuole. For example, FBPase degradation is retarded in *pep4*Δ mutant strains,[10,16] indicating that FBPase degradation is dependent upon the presence of the vacuolar *PEP4* gene. The redistribution of FBPase from the cytosol to the vacuole following a glucose shift has also been confirmed by immunofluorescence and immunoelectron microscopy studies.[6, 16] Therefore, these studies provide direct evidence that FBPase is targeted to the vacuole in response to glucose. At present, there is no clear explanation for the differences in these studies, although differences in strains or experimental conditions may be a factor. Also, it remains possible that FBPase may be degraded at both of these locations. This type of situation has been described previously for the degradation of the fatty acid synthase subunit β , which can be degraded in either the vacuole or the proteasome, depending upon growth conditions.[17]

Trafficking of FBPase to Vid Vesicles and the Vacuole

Identification of Intermediate Vesicles

One model for the FBPase degradation pathway suggests that FBPase is targeted from the cytosol to intermediate vesicles and then to the vacuole for degradation.[18] Support for the intermediate vesicle hypothesis was obtained following the isolation and purification of FBPase-associated vesicles to near homogeneity.[19] Vesicles were purified from wild type cells that had been shifted to glucose for 30 minutes at 22°C. The cells were homogenized and centrifuged to obtain a 100,000 x g high-speed pellet that was fractionated on a Sephacryl S-1000 sizing column to separate intracellular organelles. The purity of the FBPase-associated

vesicles was assayed by immunoblotting with antibodies specific to FBPase and organelle markers. Following S-1000 fractionation, FBPase appears in two distinct peaks. The first peak overlaps with the plasma membrane marker Pma1 and also the vacuole marker Prc1 (carboxypeptidase Y). In contrast, the second FBPase peak overlaps with a number of cellular organelle markers. These include markers for the vacuole (Prc1), ER (Sec62),[20, 21] Golgi (Mnn1; a-1,3-mannosyltransferase, a membrane protein that marks the medial Golgi compartment),[22] peroxisomes (3-oxoacyl Co A thiolase),[6] mitochondria (cytochrome C), COPI vesicles (Sec21) that are involved in the retrograde and anterograde transport between the ER and Golgi in yeast,[23, 24] and the ER-derived COPII vesicles (Sec22).[24, 25]

Because the second FBPase peak overlaps with a number of organelle markers, this peak was pooled and re-fractionated on density equilibrium gradients. On a sucrose gradient, FBPase is present in fractions 10 and 11, which corresponds to a density of 1.18 —1.22 g/ml. These fractions do not contain any of the above organelle markers, suggesting that FBPase is contained in distinct organelles. The FBPase peak fractions were also examined by electron microscopy.[19] A homogenous population of vesicles 35-45 nm in diameter is seen by electron microscopy. These vesicles appear to be surrounded by a layer of smooth membrane.

Kinetics of FBPase Transport

If Vid vesicles serve as intermediates in the FBPase targeting pathway, then FBPase should be associated with vesicles before entry into the vacuole. The kinetics of FBPase association with Vid vesicles has been examined.[19] FBPase distribution was monitored at 22°C in wild type cells, because FBPase targeting to the vacuole is delayed at this temperature. Wild type cells were shifted to glucose for various times at 22°C. FBPase is found in the Vid vesicle fraction at t=30 min, and is distributed in both the Vid vesicle and vacuole fractions by 60 min. At t=90 min, a small amount of FBPase is associated with vacuoles, but not with Vid vesicles. Thus, FBPase distribution to Vid vesicles is induced by glucose, occurs only transiently, and precedes the association with the vacuole. In contrast, Prc1, which is sorted from the Golgi to the vacuole, is not affected by glucose under these same conditions.

To determine whether FBPase is transported into the lumen of Vid vesicles, vesicles were isolated and incubated in the presence or absence of increasing amounts of proteinase K.[19] FBPase that is sequestered inside vesicles should be resistant to proteinase K digestion, while FBPase that is peripherally associated with the vesicles should be sensitive to proteinase K digestion. FBPase is stable when incubated with proteinase K, in the absence but not the presence of the detergent Triton X-100, indicating that this protein is present within the lumen of Vid vesicles. Therefore, at least a portion of FBPase is sequestered inside Vid vesicles, although these results do not rule out the possibility that a small fraction of FBPase associates with the vesicles peripherally.

Identification of *VID* Genes

Protein trafficking events have been characterized in large part through the use of mutants that block various steps in these processes. Mutants have been identified that have defects in the sorting of vacuolar proteins,[26, 27] proteolysis in the vacuole,[28,29] protein secretion,[30] ubiquitin conjugation [31] and endocytosis.[32] Likewise, there are mutants that exhibit a wide range of FBPase degradation defects. Various mutagenesis techniques have been utilized in order to identify genes that play a role in the FBPase degradation pathway. For example, cells have been subjected to UV mutagenesis and the mutants then examined for their ability to degrade FBPase.[18] Mutants defective in the glucose-induced degradation of FBPase were identified via the use of a colony blotting procedure. Pulse/chase experiments confirmed the degree to which these mutants were defective in FBPase degradation. In wild type cells, FBPase is degraded with a half-life of 30 minutes. In contrast, mutants were isolated that displayed an FBPase degradation half-life ranging from 110-400 minutes. All vacuole import and degradation *(vid)* mutants are complemented for the FBPase degradation defect by crossing with wild type cells and therefore the corresponding mutations are recessive.

A transposon mutagenesis strategy has also been utilized to identify *VID* genes involved in FBPase degradation.[33] To obtain mutants, wild type cells were transformed with a transposon-lacZ/LEU2 library and screened for FBPase degradation defects using the same colony blotting procedure that was used for the original UV mutagenesis. To identify the genes that were mutated, genomic DNA was isolated from the mutants and the nucleotide sequences flanking the transposon insertion site were amplified by PCR and sequenced. The mutated genes were then identified with a BLAST search of the *Saccharomyces* Genome Database. The defects of these mutants were confirmed using yeast strains in which the genes were deleted. All the null mutants examined were defective in FBPase degradation in response to glucose. Furthermore, when the corresponding *VID* genes are transformed into these mutants, FBPase degradation is restored to that seen in wild type cells.

The *vid* mutants have been further characterized by immunolocalization experiments,[18] in order to determine which step in the FBPase degradation pathway is blocked. Based upon these studies, *vid* mutants have been classified into two major groups. Class A mutants accumulate FBPase in the cytosol, while class B mutants accumulate FBPase in Vid vesicles.

VID24 *and Vesicle Trafficking to the Vacuole*

A number of important questions remain to be resolved regarding the nature of Vid vesicles. These include the identification of the site of origin of these vesicles, the mechanism by which FBPase is imported, and the identity of proteins that are required for the proper function of these organelles. If Vid vesicles are a true intermediate in the FBPase degradation pathway, then they should contain proteins that function in FBPase import into the vesicles and also in the delivery of FBPase from the vesicles to the vacuole for degradation. Vid24 has been identified as one such protein that plays an important role in the transport of FBPase to the vacuole. The *VID24* gene was cloned and identified by chromosomal walking.[34] *VID24* encodes a protein of 362 amino acids with a calculated molecular weight of 41 kDa, and an isoelectric point of 6.5.

Characterization of Vid24 has indicated that it is a peripheral protein that localizes to Vid vesicles.[34] Vid24 is undetectable in cells that are grown under glucose starvation conditions. However, Vid24 is transiently induced when cells are glucose-starved and then shifted to glucose-containing media. This induction is blocked when cycloheximide is added with glucose, suggesting that glucose induces de novo synthesis of Vid24. When Vid24 localization is studied by immunofluoresence microscopy, there is no detectable fluorescence during glucose starvation. However, after a glucose shift of 30 to 60 min, there is a strong fluorescence signal. A significant amount of the Vid24 staining appears in punctate structures within cells, suggesting that Vid24 is associated with intracellular organelles. Further experiments have indicated that Vid24 is indeed associated with Vid vesicles. Therefore, Vid24 is a specific marker for Vid vesicles. The *vid24-1* mutant belongs to the Class B family of mutants that accumulate FBPase in Vid vesicles, suggesting that Vid24 is required for the delivery of FBPase from Vid vesicles to the vacuole.

UBC1 *and Vesicle Formation*

Vid vesicles are distinct from most, if not all, known organelles. However, it is not known whether they are derived from existing structures or whether they are present in cells before a glucose shift. Studies on vesicle biogenesis have been limited, due to the fact that Vid24 is the only known Vid vesicle marker. As mentioned above, Vid24 is induced following the shift from glucose starvation to glucose rich conditions. Following a 20-30 minute glucose shift, Vid24 associates with Vid vesicles, but prior to this time, there is no marker available to detect Vid vesicles. Therefore, the events that occur during the first 20-30 minutes following a glucose shift have not been analyzed in detail. As an alternative method, an attempt was made to screen for mutants that fail to form Vid vesicles. Via this approach, the function of specific molecules in Vid vesicle formation can be assigned.

During the screening of various mutants, the *UBC1* gene was identified as playing a role in Vid vesicle biogenesis.[35] In the *ubc1*Δ mutants, a decrease in the rate of FBPase degradation was found. Furthermore, the *ubc1*Δ mutant showed a reduced sequestration of FBPase into Vid vesicle fractions. These results appear to be due to a decrease in the levels of Vid vesicles in *ubc1*Δ cells. In wild type cells, high levels of Vid24 are found in the high-speed pellet, which is representative of Vid vesicles. By contrast, the *ubc1*Δ mutant exhibits a significantly decreased level of Vid24 in the pellet fraction, suggesting that Vid vesicle production is impaired. Although the mechanism of action has not been identified, the formation of multi-ubiquitin chains appears to play a role in the FBPase degradation. This was demonstrated via the use of the R48K/R63K ubiquitin mutant, which inhibits the formation of multi-ubiquitin chains. FBPase degradation is impaired in cells that express this mutant protein. Likewise, there is a decrease in the amount of FBPase associated with the Vid vesicle fraction. Taken together, these results are consistent with the hypothesis that the *ubc1*Δ and R48K/R63K mutations result in a decreased production of Vid vesicles.

In Vitro Reconstitution of FBPase Import into Vid Vesicles

The Semi-Intact Cell FBPase Import Assay

In order to gain a better understanding of the FBPase degradation pathway, a number of in vitro assays have been developed. The targeting of FBPase into the vacuole has been reconstituted using semi-intact yeast cells in which the endogenous *PEP4* and *FBP1* genes were deleted.[36] The *FBP1* gene was deleted so that radiolabeled FBPase could be added exogenously. The *pep4*Δ deletion served to reduce the vacuolar proteolytic activity to ~30% of the wild type level. This allowed FBPase to accumulate within the vacuole, and hence facilitated the detection of the import process. To obtain import competent material, cells were glucose-starved and then shifted to glucose-containing medium for 20 minutes. Semi-intact cells were then prepared from the double deletion strain by converting the cells to spheroplasts, followed by a slow freeze and thaw, and finally treating with hypotonic buffer. This treatment released soluble proteins and small molecules into the medium but still preserved the integrity of most organelles in a functional state.[37, 38] Using this approach, purified, radiolabeled FBPase could be introduced into semi-intact cells and the fate of this protein could be followed. The import of FBPase was measured by incubating semi-intact cells with ^{35}S-FBPase in the presence of an ATP regenerating system and cytosol. At the end of reactions, samples were treated with proteinase K to digest free FBPase that was not imported into membrane-sealed compartments. Following SDS-PAGE, the FBPase radioactivity was quantitated by a phosphorimager.

Using this in vitro assay, about 20-40% of the total FBPase is sequestered inside a membrane protected compartment. When Triton X-100 is added to solubilize the membranes, FBPase sequestration is abolished, indicating that intact membranes are required for the sequestration process. FBPase sequestration reaches 20-40% after 10 or 20 minutes of import but decreases with longer incubation times. Under the same conditions, the amounts of Prc1 are not significantly altered within 60 min of the in vitro reactions, suggesting that the vacuole is stable during the FBPase import process. Similar results are obtained when immunofluorescence studies are performed. A *pep4*Δ strain was used for these studies in order to slow the degradative processes and to allow for the accumulation of FBPase in the vacuole. Indeed, FBPase is found in the vacuole following a 40 min glucose shift.[36]

The preceding experiments indicate that a portion of FBPase can be targeted to the vacuole under in vitro conditions. However, these experiments could not distinguish whether FBPase was targeted directly from the cytosol to the vacuole, or whether it first transited through Vid vesicles. To address this question, an assay was developed to study the targeting of FBPase to Vid vesicles.[35] Vid24 plays an important role in the trafficking of FBPase from Vid vesicles to the vacuole. In the absence of this gene, however, FBPase is still targeted to Vid vesicles. Because trafficking from Vid vesicles to the vacuole is blocked in the *vid24*Δ strain, this mutant

accumulates high levels of FBPase in Vid vesicles. For this reason, FBPase sequestration into Vid vesicles was reconstituted using *vid24*Δ cells. Purified FBPase was incubated with semi-intact *vid24*Δ cells in the absence or presence of ATP, an ATP regenerating system and cytosol. At selected times, proteinase K was added to digest any FBPase that was not protected in a membrane-sealed compartment. In the absence of both ATP and cytosol, FBPase import in the *vid24*Δ semi-intact cells is minimal. In the presence of ATP and cytosol, however, FBPase import increases in a time-dependent manner.[35] When quantitated, approximately 25-35% of the total added FBPase is proteinase K-protected after 30 minutes of import. Therefore, this in vitro system appears to faithfully reproduce the import of FBPase into Vid vesicles.

FBPase Import into Isolated Vid Vesicles

The semi-intact cell in vitro system has been used to define the defective component in mutants that block FBPase import into Vid vesicles. However, the import assay has its limitations, in that it relies on the use of mutants that block parts of the FBPase trafficking pathway. In order to examine FBPase import directly in the presence of wild type components, a more refined in vitro system was developed utilizing isolated Vid vesicles.[39] In this in vitro system, Vid vesicles are isolated by differential centrifugation from a wild type strain in which the endogenous *FBP1* gene is deleted. Consequently, a known quantity of purified FBPase can be added to the reaction mixture. For this in vitro assay, Vid vesicles are incubated with FBPase in the presence of an import cocktail containing ATP, an ATP regenerating system, and wild type cytosol. Following a 20 min incubation period, proteinase K is added to degrade unprotected FBPase. Under the standard in vitro conditions, approximately 20-40% of the exogenously added FBPase is protected from proteinase K digestion in vitro. However, when 2% Triton X-100 is added to solubilize the membranes, FBPase is degraded following the addition of proteinase K. These results suggest that FBPase is sequestered inside Vid vesicles. In the absence of ATP and cytosol, FBPase sequestration is abolished, suggesting that the sequestration of FBPase into Vid vesicles requires ATP and cytosol.[39]

A Role for Molecular Chaperones in FBPase Import

Ssa2

The mechanisms by which FBPase is sequestered inside of Vid vesicles have not been established. However, the successful development of the in vitro assays suggests that FBPase can be imported into intact Vid vesicles. The import of FBPase into Vid vesicles most likely requires the participation of a number of accessory proteins such as receptors, channel forming proteins and molecular chaperones. In mammalian cells, specific receptors, and the heat shock cognate protein hsc73 are required for the degradation of cytosolic proteins containing the KFERQ sequence (see chapter 12).[40] In a similar manner, FBPase import into Vid vesicles is stimulated by cytosolic ATP binding proteins, suggesting that ATPases are required for import. Molecular chaperones of the hsp70 family are ATPases that are known to play an important role in the import of proteins into the ER,[41] the mitochondria [42] and the lysosome.[43-45] The yeast hsp70 family member Ssa2 was found to be required for FBPase degradation, because the *ssa2*Δ strain is defective in FBPase degradation.[39] In contrast, the *ssa1*Δ, *ssa3*Δ and *ssa4*Δ strains degrade FBPase with kinetics similar to wild type strains. When FBPase import was tested using components derived from these mutants, the *ssa1*Δ, *ssa3*Δ o*r ssa4*Δ strains were competent for in vitro FBPase import. However, the *ssa2*Δ strain is defective in FBPase import in vitro. Therefore, Ssa2 is involved in the import of FBPase into Vid vesicles, whereas the other Ssa proteins are dispensable for this process. Consistent with these results, the *ssa1*Δ *ssa2*Δ double mutant was defective in FBPase degradation and in vitro FBPase import. By contrast, the *ssa1*Δ *ssa3*Δ mutant was normal in FBPase degradation and in vitro FBPase import.

The in vitro assay was also used to determine whether the *ssa2*Δ strain contained defective cytosol or defective vesicles. When wild type cytosol is combined with *ssa2*Δ Vid vesicles, FBPase

import is observed. However, when cytosol from the *ssa2*Δ strain is combined with wild type vesicles, FBPase import is impaired. Thus, it appears that Ssa2 must be present in the cytosol in order for FBPase import to occur.[39] These results were verified by adding increasing amounts of purified wild type Ssa2 to the cytosol and vesicle fractions isolated from the *ssa2*Δ strain. Ssa2 stimulates FBPase import in a dose-dependent manner. In contrast, the addition of purified Ssa1 has no effect on import, indicating that Ssa2 has a specific role in FBPase import. The nature of the role that molecular chaperones play in the import of FBPase into Vid vesicles has not been established. However, these chaperones may play some part in stabilizing FBPase or in maintaining it in an unfolded conformation during the import process.

Cpr1

Cyclophilin A (Cpr1) has also been identified as a required component for the import of FBPase into Vid vesicles.[46] Cpr1 is an abundant cytosolic protein and is the major receptor for the immunosuppressant drug cyclosporin A.[47] Interestingly, this protein was initially identified owing to its role as a mediator for the Vid protein Vid22. The *VID22* (YLR373c) gene was identified using a transposon-based mutagenesis strategy. The *VID22* gene encodes a 102 kDa protein that is localized to the plasma membrane.[33] Vid22 is synthesized in the cytosol and is targeted to the plasma membrane independent of the classical ER-Golgi transport pathway. When the *VID22* gene is deleted, FBPase degradation is retarded. In the *vid22*Δ mutant, FBPase accumulates in the cytosol in vivo, suggesting that *VID22* plays a role in FBPase targeting to Vid vesicles.[33] The *vid22* null mutant also reduces the import of FBPase into Vid vesicles in vitro.[46] When *vid22*Δ mutant cytosol is combined with wild type Vid vesicles, FBPase import is defective. In contrast, when *vid22*Δ Vid vesicles are incubated with wild type cytosol, FBPase is imported at a normal rate. Therefore, the *vid22*Δ mutant contains defective cytosol, but competent Vid vesicles.[46]

Vid22 is a plasma membrane protein, but it was found to regulate FBPase import indirectly through the action of a cytosolic protein. For example, the addition of purified Vid22 to the *vid22*Δ in vitro reaction does not rescue the FBPase import defect. Cytosol from the *vid22*Δ mutant fails to support import when mixed with wild type vesicles, whereas cytosol from wild type cells fully complements FBPase import when mixed with *vid22*Δ vesicles. These results indicate that a required cytosolic protein is present in wild type cells. However, it is absent or present at low levels in the *vid22*Δ mutant. In order to identify the cytosolic factor(s) that mediate Vid22 function, wild type cytosol was fractionated by sequential purification using ammonium sulfate precipitation, Superose 6 and G75 sizing chromatography, and DEAE ion exchange chromatography. Fractions from each of these purification steps were added to the *vid22*Δ mutant cytosol in the in vitro reactions to identify the proteins that complemented the *vid22*Δ mutant cytosol. A major protein band migrating at 17 kDa was found in the stimulatory fraction from the final DEAE step. This 17 kDa protein is identical to Cpr1 as determined by MALDI.[46]

The role that Cpr1 plays in FBPase degradation was confirmed via the use of *cpr1* null mutants.[46] FBPase degradation and in vitro FBPase import were found to be defective in the *cpr1*Δ mutant. In addition, cytosol from the *cpr1* null mutant is unable to support FBPase import into wild type Vid vesicles. However, Vid vesicles from *cpr1*Δ mutants are competent in importing FBPase when supplied with wild type cytosol. To test whether Cpr1 is directly involved in FBPase import, increasing amounts of purified Cpr1 were added to the in vitro reaction mixtures containing *cpr1*Δ mutant cytosol and Vid vesicles. The import defect is rescued via the addition of purified Cpr1 in a dose dependent manner. By contrast, the control protein BSA does not stimulate FBPase import. Therefore, Cpr1 plays a direct role in the import process.

Vid22 has been found to regulate the levels of the Cpr1 protein, and thus it affects the degradation of FBPase. The amount of Cpr1 in total lysates is significantly reduced in the *vid22*Δ mutant as compared to that seen in wild type cells, suggesting that the *VID22* gene

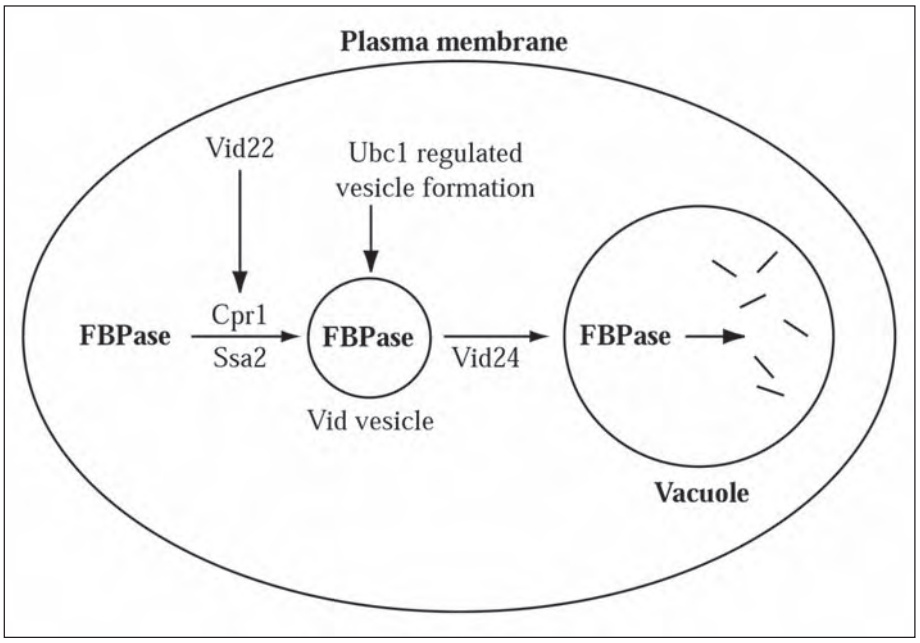

Figure 1. The FBPase degradation pathway. When glucose starved cells are shifted to medium containing fresh glucose, FBPase is imported into Vid vesicles and then to the vacuole for degradation. In the initial step, FBPase is imported into Vid vesicles through a process that is dependent on Ssa2, Cpr1 and Vid22. Although the site of origin for Vid vesicles is unknown, their formation appears to be regulated by the cytosolic ubiquitin conjugating enzyme Ubc1. Following FBPase sequestration, the loaded vesicles traffic to the vacuole via a process that is dependent upon Vid24. FBPase is released into the lumen of the vacuole and subsequently degraded by proteinases. (Reprinted with permission from J Biol Chem 2001; 276:48017-48026.)

product is required to control the Cpr1 protein levels. The *vid22*Δ defect is reversible, however, via the overexpression of the Cpr1 protein in vivo, or by the addition of purified Cpr1 protein to the in vitro assay. Increasing amounts of Cpr1 complement *vid22*Δ mutant cytosol and stimulate FBPase import in a dose-dependent manner.[46] Taken together, these results suggest that Vid22 regulates the cytosolic protein Cpr1, which in turn stimulates FBPase import directly. It is not known how Vid22 may regulate Cpr1 levels. Vid22 may participate in a signal transduction cascade and thereby regulate the expression of Cpr1. Alternatively, Vid22 may control the stability of Cpr1. In this case, the absence of Vid22 may lead to the rapid turnover of Cpr1. Vid22 appears to regulate Cpr1 in an indirect manner, because no direct interaction of Vid22 and Cpr1 was detected.

A Model for the FBPase Degradation Pathway

Based upon the above studies, we have proposed a model for the FBPase degradation pathway (Fig. 1). FBPase is synthesized and remains in the cytosol of cells that are subjected to glucose starvation. However, when starved cells are shifted to glucose-containing medium, FBPase is rapidly imported into Vid vesicles. Vid vesicles then traffic to the vacuole, and thus deliver FBPase for degradation. The site of origin for Vid vesicles is unknown, and the mechanisms of Vid vesicle biogenesis remain largely uncharacterized. However, the ubiquitin-conjugating enzyme Ubc1 does appear to be required for Vid vesicle formation. Levels of Vid vesicles are reduced in the absence of Ubc1, and FBPase degradation is compro-

mised. FBPase import into Vid vesicles is dependent upon the presence of the cytosolic proteins Ssa2 and Cpr1. Vid22 is also required for the import of FBPase, although this protein acts indirectly. In the absence of Vid22, Cpr1 levels are reduced and FBPase import is inhibited. Following FBPase sequestration into Vid vesicles, the loaded vesicles then traffic to the vacuole via a process that is dependent upon Vid24. FBPase is released into the lumen of the vacuole and the protein is then degraded by proteinases.

Future Studies

A number of questions remain to be answered regarding the mechanisms by which FBPase is degraded. An important question concerns the site of formation of Vid vesicles, and the identification of proteins involved in this process. The identification of additional Vid vesicle marker proteins will be instrumental in helping to address this question, as well as determining the mechanisms by which FBPase is imported into Vid vesicles. Although FBPase appears to be directly imported into Vid vesicles, we have not identified any of the machinery that is necessary for this process, with the exception of molecular chaperones. Likewise, FBPase-loaded Vid vesicles must be delivered to the vacuole. It will be informative to determine whether this step is mediated by cytoskeletal components. Finally, additional studies are needed to further characterize the final step in Vid vesicle trafficking. In particular, does Vid vesicle to vacuole trafficking involve the binding and fusion of Vid vesicles with the vacuole? Alternatively, might Vid vesicles be taken into the vacuole via a microautophagy-like process? It will be interesting to determine whether SNAREs play a required role in the trafficking of Vid vesicles to the vacuole.

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CHAPTER 14

Ubiquitin-Mediated Vacuolar Sorting and Degradation

David J. Katzmann

Abstract

Protein sorting within the endosomal system can yield several outcomes. One outcome is sorting into the intralumenal vesicles of a multivesicular body (MVB). MVB formation is required for a number of important cellular functions. It has been appreciated for some time that some cell surface receptors are sorted into MVBs en route to the lysosome/vacuole for degradation. Proper spatial and temporal degradation of signaling receptors is important in the context of growth control, and dysfunction of these processes can lead to developmental defects or even tumorigenesis. More recently, it has come to be appreciated that the Mvb sorting pathway is required for certain non-degradative functions as well, including proper immune response and the budding of viral particles. Advances in our understanding of the *cis*-acting signals for selection by this pathway and the *trans*-acting components that play a role in recognition of cargoes are the focus of this chapter.

Introduction

Vesicle-mediated transport is the major mechanism by which proteins transit the secretory and endocytic pathways in eukaryotic cells. The endosomal system represents a crossroads within the cell, where protein cargoes from both the biosynthetic and endocytic pathways meet. Cargoes within the endosomal system can be directed to the Golgi, plasma membrane or lysosome/vacuole, resulting in recycling, delivery to their site of action or degradation. For example, soluble lysosomal hydrolases can be delivered to the endosomal system by the action of sorting receptors that interact with cargoes within the *trans* Golgi network (TGN).[1] Once the sorting receptors, together with their cargoes, have reached the endosome they are recycled to the TGN to perform additional rounds of sorting, while the hydrolases continue on to their site of action.[1-3] Cell surface proteins that have been internalized and delivered to the endosomal system can either be recycled to the plasma membrane or targeted to the lysosome/vacuole for degradation.

A cell's surface has a large number of receptors that are responsible for recognizing extracellular signals and initiating the appropriate intracellular response. For example, upon binding its agonist, the epidermal growth factor receptor (EGFR) initiates an intracellular signal transduction cascade that stimulates cellular growth. Cell signaling pathways that are initiated by cell surface receptors are, in part, modulated by the steady state pool of activated receptors.[4,5] A number of cell surface receptors have been shown to associate with downstream signaling components subsequent to internalization from the plasma membrane.[6-11] This suggests that signal propagation continues until the activated receptor complex is dissociated or sequestered away from cytosolic effectors. Sorting into a multivesicular body (MVB) can potentially terminate signaling by sequestering activated receptor complexes away from cytosolic effectors, and ultimately by degradation within the lysosome/vacuole. A cell can, therefore,

Autophagy, edited by Daniel J. Klionsky.

fine-tune its response to extracellular agonist by the downregulation of these receptors. Receptor downregulation is a highly regulated, multistep process that is the result of internalization from the plasma membrane, delivery to an endosomal compartment and subsequent delivery to the hydrolytic lumen of the lysosome/vacuole for degradation. In this regard, receptor downregulation can be considered as one type of autophagic process.

The delivery of a transmembrane receptor in its entirety to the lumen of the lysosome/vacuole is the result of a sorting step that takes place at the endosome, during the formation of MVBs. An MVB forms when the limiting membrane of the endosome invaginates and buds into its lumen, thereby creating its characteristic "multivesicular" appearance. During the process of MVB formation, proteins that are destined for delivery to the lumen of the lysosome/vacuole are actively sorted into vesicles as they invaginate and bud from the limiting membrane. Heterotypic fusion of the MVB with the lysosome/vacuole results in the delivery of these intralumenal vesicles, and the protein cargoes contained therein, to the lysosomal/vacuolar lumen where they are degraded by resident hydrolases. Morphologically, the formation of multivesicular bodies is similar to the formation of the phagophore that sequesters cytoplasm during autophagy (see chapter 2). There is no clear evidence at present that the endosome participates in biogenesis of the autophagosome. It is interesting to note, however, that the putative lipase Cvt17/Aut5 that is required for degradation of subvacuolar lumenal vesicles[12,13] transits to the vacuole through the Mvb pathway (see chapter 7).[13]

The sorting of proteins into the inner vesicles of multivesicular bodies is required for a number of important cellular processes, including not only the downregulation of activated signaling receptors, but also non-degradative functions, such as proper stimulation of the immune response and even the budding of certain viruses from the host cell. Recent advances in our understanding of the Mvb-sorting pathway have resulted from the identification of ubiquitin as a signal for efficient sorting into this transport route and from the discovery of components of the sorting and regulatory machinery that directs this complex process.

Background

The endosomal system is highly plastic and has historically been broken down into "early" and "late" endosomes. The distinction between the two is based in part on the kinetics with which endocytosed material is delivered to each compartment (for review, see ref. 14). However, there are also morphological and biochemical distinctions that can be made between the two. Early endosomes tend to be located towards the cell's periphery and display a more tubular morphology, while late endosomes tend to be more spherical and located peri-nuclear (in mammalian cells) or peri-vacuolar (in yeast) (for review, see ref. 15). Late endosomes exhibit additional distinguishing characteristics from both earlier endosomes and the later vacuole or lysosome, such as differing protein/lipid composition and a lower lumenal pH as compared to early endosomes. In addition to being generally more spherical, late endosomes display the characteristic morphology of possessing multiple vesicles within the lumen, leading to their definition as "multivesicular bodies." MVBs have been found in all eukaryotic cell types examined and are characterized by having intralumenal membranes and vesicles that are approximately 50-80 nanometers. The unique morphology of MVBs has been documented by electron microscopy studies dating back to the 1950s.[16,17] In the late 1960s, electron microscopy captured the limiting membrane of the MVB invaginating into its lumen, leading the authors to conclude that the intralumenal vesicles were derived from the limiting membrane.[18]

The physiological significance of MVBs was ascribed by Stanley Cohen and colleagues who analyzed the uptake of Epidermal Growth Factor conjugated to ferretin over a time course to show that its localization shifted from the limiting membrane of the endosome to the intralumenal vesicles of the MVB and eventually the lysosome.[19,20] From such studies, the authors concluded that this represented a potential mechanism by which the cell could mediate the degradation of EGF ligand and potentially even EGF receptors (EGFRs). These elegant morphologically based studies established the conceptual framework for the function of MVBs, and these insightful predictions have subsequently been proven correct.

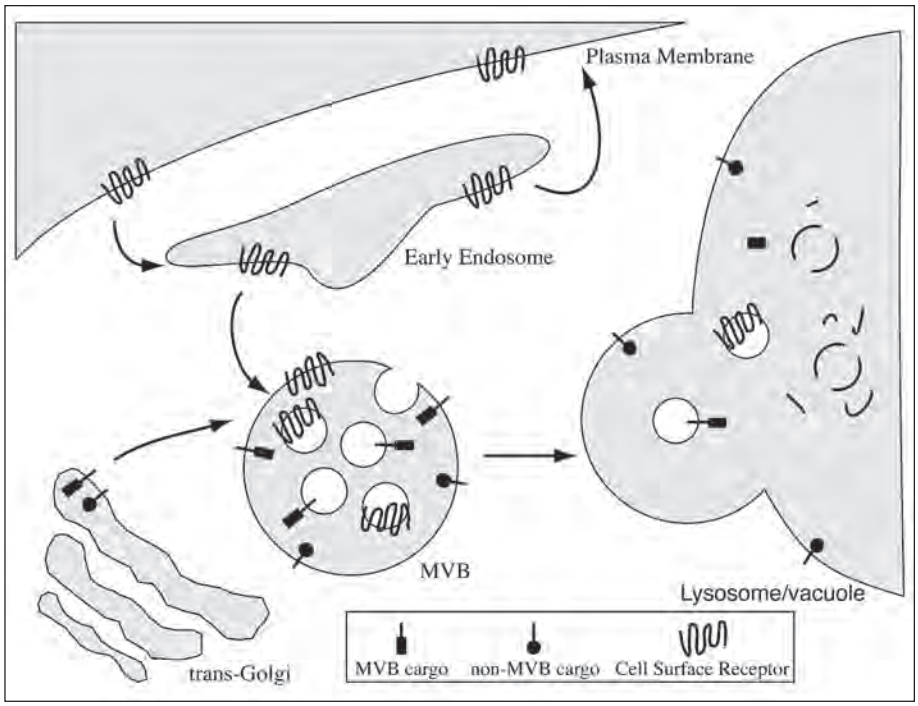

Figure 1. Protein sorting in the endosomal pathway. The endosome serves as a site where biosynthetic traffic from the Golgi and endocytic traffic from the plasma membrane merge. Proteins that have been delivered to the endosomal system can either be recycled to their compartment of origin or can continue on to the lysosome/vacuole. Terminal localization within the lysosome/vacuole is the consequence of a sorting event that occurs during the formation of MVBs. During MVB formation, the limiting membrane of the endosome invaginates and buds into its own lumen. Cargoes that are destined for delivery to the lumen of the vacuole/lysosome are actively sorted into these vesicles, which are delivered to the lumen of the lysosome/vacuole following heterotypic fusion. Resident hydrolases within the lysosome/vacuole degrade vesicles delivered through the Mvb pathway, as well as cargo contained therein. Proteins that are neither recycled from the endosome nor sorted into intralumenal vesicles during MVB formation will be delivered to the limiting membrane of the lysosome/vacuole following heterotypic fusion of the MVB. Ubiquitin has been shown to serve as a positive sorting signal for selection by the Mvb pathway in the context of a number of Mvb cargoes.

Endocytosed material that has been delivered to the endosomal system can be selectively sorted into these invaginating vesicles during the formation of an MVB. Multiple lines of evidence indicate that the MVB matures as such prior to fusing with the lysosome/vacuole in a single heterotypic fusion event. Studies in mammalian cells have demonstrated that, like EGF, EGFR is delivered from the limiting membrane of late endosomes to the intralumenal vesicles and eventually to the lumen of the lysosome.[21] In the yeast *Saccharomyces cerevisiae*, mutants that are defective for the fusion of transport intermediates to the vacuole (the functional equivalent of the lysosome) have been used to demonstrate the existence of mature MVBs.[22-24] Genetic data from yeast, combined with the development of an in vitro mammalian-based heterotypic fusion assay, has led to an extensive list of machinery that is required for MVB fusion with the lysosome/vacuole (for review, see ref. 25).

Heterotypic fusion of the limiting membranes of the MVB and lysosome/vacuole results in the delivery of these internal vesicles to the hydrolytic lumen of the lysosome/vacuole, followed by their degradation (Fig. 1) Endosomal cargo proteins that are not sorted into the intralumenal vesicles of an MVB can either be recycled to their subcellular compartment of origin, or will be

delivered to the limiting membrane of the lysosome/vacuole following heterotypic fusion. A distinction can therefore be made between sorting at the MVB and sorting into an MVB, with the latter indicating that a cargo has entered an intralumenal vesicle while the former can have several outcomes.

Selection of Mvb Pathway Cargo

Clearly, it would be disadvantageous for a cell to non-specifically sort proteins into the Mvb pathway, as this would result in their non-specific degradation. Even in the context of a cell surface receptor that will enter this pathway after becoming activated, it is critical that entry not occur prematurely or the ability to perceive extracellular stimuli would be lost. Conversely, failure to degrade activated receptors involved in stimulating growth will cause prolonged signaling that can result in developmental defects or tumorigenicity. Not surprisingly, the entry of cargo into this pathway is highly regulated, requiring both cis- and trans-acting factors.[26-29] In the case of EGFR, agonist binding activates its own tyrosine kinase activity, resulting in trans-phosphorylation of dimerized receptors at the plasma membrane, as well as phosphorylation of downstream signaling molecules. Phosphotyrosine residues within the cytoplasmic tail of EGFR serve to recruit additional factors that positively affect its rate of degradation, such as the SH2 domain-containing protein Cbl. Cbl marks EGFRs for degration by covalently modifying them with ubiquitin.[26,30-32]

It is impossible to discuss the downregulation of cell surface receptors without a brief introduction to ubiquitin. Ubiquitin is a 76 kDa protein that is covalently added to the sigma-amino group of a lysine residue within substrate proteins. Ubiquitin modification is catalyzed by the action of a cascade of enzymes: an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme and an E3 ubiquitin ligase.[33] It is the E3 that confers substrate specificity onto this reaction. Ubiquitin modification is also reversible by the action of de-ubiquitinating enzymes. Ubiquitin itself contains several lysine residues, through which additional ubiquitin molecules can be added to form branched "polyubiquitin" chains. Perhaps the best-characterized consequence of a substrate's polyubiquitination is targeting to the 26S proteasome for degradation. More recently, it has come to be appreciated that ubiquitin can serve in other regulatory capacities following monoubiquitination (the addition of a single ubiquitin) or in some cases diubiquitination (for review, see ref. 34). In the context of protein trafficking, monoubiquitination of transmembrane cargo proteins results in their degradation, but this degradation appears to be via the lysosome/vacuole, rather than the proteasome. In addition, there have also been a number of examples wherein ubiquitin has been shown to directly modulate the activity of a protein (as opposed to modulating its steady-state levels). These include transcriptional activation,[35] histone function,[36,37] and regulation of kinase activity.[38,39] In addition, certain components of the endocytic machinery have been shown to be ubiquitinated,[40-42] but the significance of this is presently unclear. Ubiquitin can, therefore, serve as a signal for degradation by either the proteasome or the lysosome/vacuole, but can also function as a non-degradative modulator of protein activity. Parallels to phosphorylation are striking, in that there are enzymes that add or remove ubiquitin (as there are kinases and phosphatases) and the addition of ubiquitin can result in a number of outcomes.

One of the first examples of ubiquitin as a signal for intracellular protein targeting came from the G-protein coupled receptor (GPCR) Ste2 in the yeast *S. cerevisiae*.[43] *S. cerevisiae* cells exist in two haploid mating types, a and alpha, and Ste2 is expressed on the surface of mating type a cells. Its function is to bind the mating pheromone (alpha factor) secreted by cells of mating type alpha. The interaction of Ste2 and alpha factor induces a MAPK signaling cascade that initiates the mating response.[44] In addition, binding of alpha factor by Ste2 induces the phosphorylation, ubiquitination and downregulation of Ste2 via its internalization from the cell surface and delivery to the vacuole.[43,45] Mutation of the ubiquitin-accepting lysine residues in the C-terminal cytoplasmic tail of Ste2 results in its stabilization after treatment with alpha factor and translational fusion of ubiquitin to Ste2 is sufficient to drive its internalization.[45]

Additionally, Ste2 accumulates in an ubiquitinated form in mutants that are defective for internalization from the plasma membrane[43] and translational fusion of ubiquitin to a protein that is normally stable at the cell surface is sufficient to drive its downregulation in the vacuole.[46] Studies such as these have shown that ubiquitin plays an important role in the downregulation of cell surface proteins and indicate that in some contexts it can function at the earliest step of this phenomenon, internalization from the cell surface.

Ubiquitin has been shown to play a crucial role in the downregulation of a variety of cell surface proteins, however its precise role does not seem to be the same in all cases. In the context of EGFR, the Cbl ubiquitin ligase is required for its efficient ubiquitination and downregulation. However, ubiquitin modification appears to play a role after internalization of EGFR from the cell surface. Overexpression of Cbl increases both the ubiquitination of EGFR and its degradation rate without affecting its rate of internalization from the plasma membrane.[27,47] As EGFR is a cargo of the Mvb pathway, one idea would be that ubiquitin serves as a sorting signal for entry into this pathway. In this context, certain cell surface proteins would normally be in a recycling loop between an endosomal compartment and the cell surface, and ubiquitin serves as a signal to remove them from this recycling loop and target them for degradation via the Mvb pathway. The IL-2 receptor also receives ubiquitin modification and mutation of its acceptor lysine residues results in mislocalization to intracellular structures, rather than delivery into the Mvb pathway or accumulation at the cell surface.[48] Studies in *S. cerevisiae* have also suggested a post-internalization role for several cell surface proteins, including the ABC transporter Ste6 and the uracil permease Fur4.[49,50] Ste3 (the GPCR that binds a-factor on the surface of mating type alpha cells) has been shown to transit two routes after internalization from the PM, one of which is to recycle between the cell surface from an early endosomal compartment, the second is degradation via the Mvb pathway. Ubiquitin seems to play a critical role in removing Ste3 from the recycling loop and, instead, targeting it into the Mvb pathway.[51] Another yeast protein, the general amino acid permease Gap1, is subject to a sorting decision in a late secretory compartment such that when the external nitrogen source is rich, it is ubiquitinated and targeted directly to the vacuole, without transiting to the cell surface.[52,53] In addition, Gap1 at the cell surface is targeted for vacuolar degradation as the result of ubiquitin modification.[53] Together, these data suggest a role for ubiquitin as a signal for entry into the Mvb pathway. However, given that ubiquitin can serve as an internalization signal, it is difficult to ascribe an Mvb sorting function to ubiquitin-utilizing proteins that transit to the cell surface. Regardless, these studies clearly demonstrated that ubiquitin plays a role in downregulation of cell surface proteins.

In addition to serving as a tag that signals the degradation of proteins, ubiquitin appears to play an additional role during the downregulation of certain cell surface proteins. In mammalian cells, the growth hormone receptor (GHR) has also been shown to display an ubiquitin-dependent step in its downregulation, however, it does not require covalent ubiquitin modification itself.[54] In yeast, it has been demonstrated that fusions between Ste2 and ubiquitin are internalized with near wild type kinetics, unless this fusion is analyzed in the context of a mutant that is defective for the ubiquitin ligase Rsp5.[55] Rsp5 has been implicated as the ubiquitin ligase that tags a number of cell surface proteins with ubiquitin, marking them for degradation. But the delayed internalization of a Ste2-Ub chimera in an *rsp5* mutant, and the ubiquitin requirement in the downregulation of GHR, suggest that ubiquitin ligases play an essential role in MVB sorting in addition to the identification of cargoes. An obvious interpretation would be that various components of the endocytic sorting machinery are themselves receiving ubiquitin modification and that this is a requisite for their function. Indeed, recent reports have described the ubiquitination of several components of the sorting machinery, including Eps15, Hrs and β-arrestin.[41,42,56] While the physiological significance of ubiquitin modification of these proteins remains to be demonstrated, they are clearly candidates as recipients of ubiquitin modification that is required for endocytosis.

Ubiquitin plays an essential role in the downregulation of cell surface receptors, both at the level of cargo identification and the regulation of transport machinery. To address whether ubiquitin serves as a positive sorting signal for entry into the Mvb pathway, irrespective of internalization from the plasma membrane, several groups utilized cargoes that do not transit to the cell surface en route from the Golgi to the vacuole. One of these was carboxypeptidase S (Cps1), a vacuolar hydrolase that is synthesized as a type II integral membrane protein. Cps1 is delivered from the Golgi to the endosome, where it is selected as an Mvb cargo.[57,58] Prior to reaching the Mvb sorting step, Cps1 receives ubiquitin modification and this is a requisite for entry into the Mvb pathway.[59] Furthermore, the fusion of a small peptide from Cps1 (normally the recipient of ubiquitination) to a non-Mvb cargo was sufficient to drive its entry into the Mvb pathway.[59] Another study utilized translational fusions between ubiquitin and cargoes that transit the endosomal system without normally being selected by the Mvb sorting machinery. Likewise, this study revealed that the addition of monoubiquitin to several proteins that would normally not receive ubiquitin modification, nor be selected as Mvb cargoes, was sufficient to drive their incorporation into the Mvb pathway.[60]

While these data would seem to indicate that ubiquitination of endosomal cargoes is both necessary and sufficient to drive their incorporation into the Mvb pathway, it should be noted that another study, while consistent on most levels, identified an Mvb cargo that does not appear to be ubiquitinated (Sna3).[61] It seems that GHR and Sna3 may represent Mvb cargoes that do not require ubiquitin modification to be selected by this pathway. Multiple signals would, therefore, appear to be capable of targeting a protein for entry into the Mvb pathway. The nature of the non-ubiquitin based signal is presently not understood, but one possibility would be a contribution from transmembrane domains within the cargo proteins.[62] Alternatively, ubiquitin itself has a di-leucine-like motif that appears to be responsible for the internalization of cell surface receptors[46] and it may be that certain Mvb cargoes possess such a signal, thereby obviating the need to receive ubiquitin modification. The presence of ubiquitin on an endosomal cargo appears to be sufficient to direct it into the Mvb pathway, regardless of whether this modification occurred at the cell surface or an intracellular compartment.

It is interesting to note that, while ubiquitin serves as a positive sorting signal for entry into the Mvb pathway, it would appear that this tag is removed from cargoes prior to their degradation. This was first suggested by studies involving the deubiquitinating enzyme Doa4. It was noted that yeast cells deleted for this enzymatic activity had lower levels of free ubiquitin, as it was now being rapidly turned over in the vacuole.[63] A genetic screen for mutants that suppressed this phenotype yielded a number of mutants in endosomal trafficking, establishing a link between Doa4 activity and the endosome.[64] Additional analyses revealed that Doa4 is an endosomally-associated deubiquitinase that is responsible for removing ubiquitin from endosomal cargoes destined for entry into the Mvb pathway, thereby allowing ubiquitin to be reused rather than degraded in the vacuole lumen.[64] Direct analyses of several Mvb cargoes (Cps1, Fur4 and Ste6) during their transit into this pathway is consistent with this role for Doa4.[49,50,59] It is not presently understood how recognition of ubiquitinated cargo is coordinated with its sorting and removal of the ubiquitin tag.

Sorting Factors of the Mvb Pathway

Studies in yeast have identified a group of sixteen genes whose products are required for the proper sorting of Mvb cargoes. These genes are referred to as the Class E *VPS* (vacuolar protein sorting) group and represent likely candidates as integral players in the Mvb sorting pathway. When viewed by electron microscopy, Class E *vps* mutants accumulate an aberrant endosomal compartment that consists of multi-lamellar membrane accumulations near the vacuole.[65,66] This aberrant structure has been termed the "class E compartment." Both biosynthetic and endocytic cargoes can be found to accumulate in this structure.[65-68] Perhaps most strikingly, the class E mutants block the delivery of Mvb cargoes to the vacuole lumen without blocking delivery to the vacuole. Therefore, membrane-bound cargoes normally sorted into the vacuole

lumen can be found in the limiting membrane of the vacuole, as well as the class E compartment.[58] As mentioned above, localization of cargo to the limiting membrane of the endosome is the result of a failure to be delivered to the intralumenal vesicles of this organelle during MVB formation. Retention in the limiting membrane of the endosome leads to delivery to the limiting membrane of the vacuole after endosome-vacuole fusion.

It has been well documented that cargoes of the various transport pathways arrive at their appropriate location through the combined effect of sorting determinants within those cargo proteins and their interactions with transport machinery. Ubiquitin as a sorting determinant does not appear to be an exception to this rule. A number of motifs that interact with ubiquitin have been described recently, largely by the use of sequence or structural comparisons. For example, one of the first proteins demonstrated to bind ubiquitin was the proteasomal subunit S5A, and its ubiquitin interacting motif (UIM) was used to describe a whole family of proteins, many of which play a role in endosomal sorting.[69] One of these is Vps27, a class E Vps protein. Both Vps27 and its mammalian homolog Hrs bind ubiquitin in a manner that requires UIM function.[70,71] Point mutations in the UIM domains of Vps27 that confer a defect in ubiquitin binding in vitro confer Mvb sorting defects on Mvb cargoes from both the biosynthetic route (Cps1) and endocytic route (Ste2).[70] However, it is presently unclear whether this defect is the result of an inability to bind ubiquitinated Mvb cargo or another component of the sorting machinery that is itself ubiquitinated. Another class E protein that contains a UIM is Hse1, a homolog of the mammalian STAM protein.[72] In both yeast and mammalian cells Vps27/Hrs has been shown to interact with Hse1/STAM.[72,73] Deletion of Hse1 has been shown to confer a class E phenotype in some strain backgrounds, but not others[70,72] suggesting a role in Mvb sorting that is presently unclear.

The UIM plays another interesting role that may confer a level of regulation onto UIM-containing proteins. Hrs has been shown to be ubiquitinated in a manner that is dependent upon its UIM, as has Eps15, which functions at the internalization step of receptor downregulation.[41] The UIM of Eps15 has been shown to specifically interact with the Nedd4 ubiquitin ligase complex to mediate its own ubiquitination.[41] While the physiological significance of ubiquitination of these transport components remains to be elucidated, an attractive possibility is that these proteins serve as adapter molecules between cargo and other transport components and their own ubiquitination may contribute to this complex network of interactions.

In addition to Vps27, a second class E Vps protein has been shown to bind ubiquitin, although via a different domain. Vps23 and its mammalian homolog Tsg101 contain a domain that has homology to E2 ubiquitin conjugating enzymes (UBCs), yet it lacks a critical active site cysteine required for the function of bona fide UBCs.[74-76] This domain has alternatively been referred to as an UBC-like domain, or a ubiquitin E2 variant (UEV).[77] The UBC-like domain of Vps23 is required for its interaction with ubiquitin in vitro and in vivo.[59] Vps23 can be co-purified with ubiquitinated Cps1 (an Mvb cargo), but not a lysine mutant form of Cps1 that cannot receive this modification (and is not an Mvb cargo).[59] Vps23 assembles into a 350 kDa complex together with two other class E Vps proteins, Vps28 and Vps37.[59,76] This complex was the first of three complexes comprised of Class E Vps proteins described, and given the role of the class E proteins in Mvb sorting, these have been named endosomal sorting complexes required for transport (ESCRT). The Vps23-containing ESCRT-I is transiently recruited from the cytoplasm to endosomal membranes, where it appears to play a role in the recognition of ubiquitinated cargoes destined for entry into the Mvb pathway.[59]

The mammalian homolog of Vps23, Tsg101, also assembles into a 350 kDa complex together with the homolog of Vps28 (hVps28) and this complex transiently associates with endosomal membranes.[76,78] The mammalian homolog of Vps37 has not yet been identified. Like Vps23, Tsg101 binds ubiquitin in a manner dependent upon its UBC-like domain.[79] Inactivation of Tsg101 in fibroblasts results in prolonged EGFR recycling and a consequence of this is prolonged EGF signaling, as measured by increased phosphorylation of the down-

stream signaling components Erk1 and 2.[76] Increased numbers of activated EGFRs at the cell surface and the increased signaling that results provides an attractive mechanism to explain the ability of *tsg101* mutant fibroblasts to induce metastatic tumors in nude mice.[80] If the machinery responsible for recognizing the activated, ubiquitinated EGFR cannot perform its function, the receptor will not enter the Mvb pathway. This is similar to the oncogenic mechanism of Cbl mutants that have lost their ability to add ubiquitin to activated receptors, thereby leading to prolonged recycling and signaling through downstream pathways. The distinction is that in the case of Cbl, the receptors are not identified as Mvb cargo, through a failure to be ubiquitinated, and therefore are not recognized by the Mvb sorting machinery.

In addition to ESCRT-I, two additional ESCRT complexes are also required for the function of the Mvb pathway. ESCRT-II is a 155 kDa complex comprised of the class E Vps proteins Vps22, Vps25 and Vps36.[81] This complex exists in the cytosol and is transiently recruited to the endosomal membrane in a manner that does not rely on ESCRT-I function. However, genetic studies suggest that ESCRT-II functions downstream of ESCRT-I, as overproduction of ESCRT-II can obviate the need for ESCRT-I function, whereas the opposite is not true.[81] This suggests a role for ESCRT-I in the activation of ESCRT-II, rather than in directing its localization. What does seem to be clear is that ESCRT-II is required for the appropriate formation of ESCRT-III.[82] ESCRT-III only forms on endosomal membranes and is actually composed of two functional subunits; a membrane proximal subcomplex made of Vps20 and Snf7/Vps32, as well as a peripherally associated subcomplex made of Vps2 and Vps24.[82] These are defined as functionally distinct because Vps20/Snf7 can still associate with endosomal membranes in the absence of Vps2 or Vps24, whereas Vps2/Vps24 cannot do so without Vps20/Snf7. Association of ESCRT-III with the endosomal membrane would appear to be stabilized in part by the myristoylation of Vps20. Loss of ESCRT-II function results in a mislocalization of ESCRT-III subunits, suggesting that ESCRT-II drives the proper formation of ESCRT-III by an interaction with the Vps20/Snf7 complex. It is possible to co-purify ESCRT-II and ESCRT-III from endosomal membranes, but not cytosol, which would seem to confirm the formation of a higher order complex.[81] However, it would appear that ESCRT-III has a defined stoichiometry once formed on the endosomal membrane, although the precise size of this complex has been elusive.[76,82,83] The specific function of ESCRT-III in Mvb sorting is not clear, however it has been suggested that it plays a role in the concentration of Mvb cargoes.

ESCRT-III appears to serve the additional function of recruiting at least two enzymatic activities that function in Mvb sorting, the AAA-ATPase Vps4 and the deubiquitinating enzyme Doa4. Mutants defective for Vps4/SKD1 (see chapter 15) activity have been shown to accumulate class E proteins on endosomal membranes, indicating a role in the dissociation/disassembly of class E proteins from the endosomal membrane in both yeast and mammalian cells.[59,68,78,81-84] Vps4 forms a homo-oligomeric complex in its ATP-loaded form and mutations that render the protein unable to bind ATP fail to associate with endosomal membranes, suggesting a cycle of ATP binding, assembly into a higher order complex, association with the endosomal membrane and hydrolysis of ATP, leading to dissociation of ESCRT complexes.[83] The activity of Doa4 appears to be required for the removal of ubiquitin from Mvb cargoes prior to their degradation, thereby allowing ubiquitin to be recycled rather than degraded.[63,64] The *doa4* mutant itself is not a class E mutant. Given that ubiquitin is rapidly degraded via the Mvb pathway in a *doa4*Δ mutant, this mutant clearly does not display a block in Mvb sorting. Vps4 and Doa4 specifically require the outer ESCRT-III subcomplex comprised of Vps2/Vps24 for endosomal recruitment.[64] It is intriguing to speculate that the two functional subcomplexes of ESCRT-III are responsible first for concentrating and/or sequestering Mvb cargoes, and second for recruiting enzymes to execute the removal of ubiquitin and the release of the sorting machinery to allow the cycle to start again.

Clathrin, a coat protein involved in the formation of transport vesicles from a number of subcellular compartments, has been found to form two distinct types of structures on endosomes.

Clathrin-coated buds at the tips of endosomal tubules contain transferrin receptors,[85,86] suggesting that one function of the endosomal pool of clathrin is in the recycling of non-resident proteins to their compartment of origin. In some cell types, such as melanosomes, clathrin has been visualized as a planar lattice that is devoid of clathrin coated buds.[87] While the function of these planar lattices is presently unclear, possible insight comes from studies that indicated a possible link to Mvb sorting through HRS (the mammalian homolog of the class E protein Vps27). HRS contains a clathrin binding domain within its C terminus that is required for interaction with clathrin in vitro and recruitment of clathrin to endosomes in vivo.[88] The use of a dominant negative Rab5, which confers an aberrant (enlarged) endosomal morphology, revealed that HRS, planar clathrin and Mvb cargo colocalized to subdomains of the endosome.[71] It is therefore possible that these subdomains represent sites within the endosome that are actively sorting Mvb cargoes. Whether clathrin is involved directly in the sorting of Mvb cargo or if this represents a sorting event that results in either recycling or entry into the Mvb pathway is not clear.

Lipids and Mvb Sorting

Late endosomes also display a distinct lipid composition relative to early endosomes (reviewed in ref. 15). The internal membranes of MVBs have been shown to accumulate two lipid species in distinct populations: Lysobisphosphatidic acid (LBPA)[89] and phosphatidylinositol (PtdIns)(3)phosphate.[90] It has been suggested that the biophysical characteristics of these lipids may facilitate the deformation of the membrane during invagination from the limiting membrane. For example, LBPA has an unusual head group structure that may promote membrane curvature. Additionally, the limiting membrane is enriched for PtdIns(3)P and this lipid species serves to recruit a number of endosomal proteins containing PtdIns(3)P-binding PH or FYVE domains (for reviews, see refs. 91,92). Lipids such as PtdIns(3)P may, therefore, exert some or all of their influence by recruiting effector proteins that are more directly involved in MVB formation or Mvb sorting. A role for PtdIns(3)P in MVB formation has been suggested by both yeast and mammalian studies. Deletion of Vps34, the sole PtdIns 3-kinase in yeast likewise blocks Golgi-to-vacuole transport.[93] In mammalian cells, treatment of cells with the PtdIns 3-kinase inhibitor wortmanin or microinjection of anti-hVps34 antibodies was demonstrated to block MVB formation.[94,95] Furthermore, deletion of the PtdIns(3)P binding protein (and class E component) Vps27 likewise blocks MVB formation, suggesting that this lipid species is exerting at least a portion of its effects in MVB function through recruited proteins.[58] Perturbation of LBPA levels in mammalian cells results in missorting of the mannose-6-phosphate receptor and increased levels of cholesterol accumulation in late endosomes, a phenotype that mimics the cholesterol storage disorder Niemann-Pick Type C.[96] This lead to the idea that it is possible for proteins and lipids to be retrieved from the lumenal vesicles of an MVB; specifically that the LBPA vesicles serve as a storage site for low-density lipoprotein-derived cholesterol.[96] While LBPA does not appear to be delivered to the lysosome, it has been shown that the major site for the turnover of PtdIns(3)P is the yeast vacuole.[97] This raises the interesting possibility that LBPA-positive vesicles and PtdIns(3)P-positive vesicles serve distinctly different functions, with PtdIns(3)P vesicles being destined for delivery to the lumen of the lysosome/vacuole for degradation and LBPA vesicles serving more of a storage or recycling function for both lipids and proteins. Support for this notion comes from immature dendritic cells, which package major histocompatibility complex class II molecules into the intralumenal vesicles of MVB-like compartments called MIICs (major histocompatibility complex class II compartments). Upon cellular stimulation by antigen, the intralumenal vesicles fuse back with the limiting MIIC membrane, the class II molecules are loaded with antigenic peptide, and the class II-peptide complexes are transported to the plasma membrane for presentation to naive T cells.[98]

PtdIns(3)P appears to play an additional role in Mvb sorting that is presently less clear. PtdIns(3)P is the substrate for the PtdIns(3) 5-kinase Fab1, which converts PtdIns(3)P to

PtdIns(3,5)P_2.[99] Fab1 function has been shown to play a role in the sorting of a subset of Mvb cargoes. Sorting of the biosynthetic cargo Cps1 was found to be defective in *fab1* mutants, while the endocytic cargo Ste2 was still delivered to the vacuole lumen.[58] More recent evidence clearly demonstrates that the Mvb pathway is still functional for the delivery of biosynthetic proteins in *fab1* mutants in specific contexts. In these studies it was revealed that biosynthetic Mvb cargoes that do not require ubiquitination or that were made as translational fusions to ubiquitin were properly sorted into the lumen of the vacuole in a *fab1* mutant.[60,100] Both the endocytic cargo Ste2, which would appear to be ubiquitinated at the plasma membrane by the action of the Rsp5 ubiquitin ligase, and translational fusions between ubiquitin and biosynthetic cargoes are sorted normally into the Mvb pathway.[58,60,100] This would seem to indicate that Fab1 function is not required for the function of the Mvb pathway, but rather in some aspect of cargo selection.

Conclusion

The Mvb pathway plays an important role in several aspects of cellular physiology. Downregulation of activated cell surface receptors such as EGFR, is important not only in the immediate context of modulating growth control signaling, but also in the broader context of development. A recent study of HRS function in Drosophila revealed that *hrs* mutant larvae accumulate aberrant endosomal compartments that are unable to invaginate and form MVBs.[101] This defect is manifested in an inability to downregulate EGFR and subsequent enhanced signaling through the Torso signaling pathway, resulting in multiple morphological defects during development.[101] Another interesting developmental connection to the Mvb pathway would appear to be the Notch signaling pathway. Notch receptors act in a variety of developmental steps including lateral inhibition during vulval development in *C. elegans* , inhibition of neuronal differentiation in *Drosophila* embryonic epidermal cells and neurite outgrowth.[102,103] Notch transmembrane receptors are activated by an interaction with transmembrane DSL proteins (Delta, Serrate and Lag-2) in adjacent cells.[104,105] Activation of Notch by Delta results in several proteolytic events, including the release of the intracellular domain of Notch from the plasma membrane, resulting in its translocation to the nucleus where it regulates the expression of a variety of target genes.[104,105] One of the ways that this signaling pathway is modulated is by the internalization of Notch's ligand, Delta. Mutations in the ubiquitin ligase *neuralized* result in increased plasma membrane localization of Delta whereas increased expression results in increased turnover of Delta.[106-108] Interestingly, Delta appears to be monoubiquitinated, making it a likely substrate for the Mvb pathway. Further studies on the post-internalization trafficking of Delta are needed to determine if this is indeed the case. However, these studies underline the importance of proper modulation of receptor signaling as a growth control mechanism.

The Mvb pathway has also been shown to function in non-degradative capacities as well. In addition to the storage of certain lipids and even proteins (see above), recent studies have demonstrated that budding of certain retroviral particles (including HIV-1) requires the function of the class E Vps proteins. Topologically, the budding events of an invaginating vesicle during Mvb formation and the release of a viral particle from the cell surface are equivalent in that cytosolically oriented machinery is pushing a vesicle away from the cytosol (as compared to the majority of vesicle formation events wherein the machinery pulls a vesicle into the cytosol). The efficient packaging of the viral protein Gag into viral particles correlates with its ubiquitination,[109-111] much as has been shown to be the case for Mvb cargoes. Furthermore, the efficient packaging and release of viral particles from the cell surface requires the function of mammalian homologs of a variety of the class E Vps proteins. Perhaps most interestingly, Tsg101, the homolog of Vps23, was shown to not only be required for the budding of HIV-1 particles, but its affinity for the Gag protein was dramatically increased when Gag was ubiquitinated.[112] Together, this suggests a model in which the virus is usurping the Mvb machinery to sort ubiquitinated cargoes into nascent viral particles. In support of this model,

Tsg101 has recently been shown to re-localize to the plasma membrane in response to specific peptide (PTAP) sequences within the Gag protein.[113] It will be interesting to learn whether the virus is utilizing the entire Mvb machinery, or merely a subset thereof.

There have been a number of recent advances in our understanding of the Mvb sorting pathway. These have revealed exciting connections to a number of interesting cellular phenomenon beyond the obvious connections to function of the vacuole/lysosome. However, many questions remain to be answered. How are proteins that are not ubiquitinated recognized as cargoes for the Mvb pathway? How is it that some proteins are able to escape from the degradative fate of cell surface receptors? What is the machinery that is responsible for tagging Mvb cargoes with ubiquitin? Are viruses, such as HIV-1, utilizing all the class E Vps proteins, or merely a subset thereof? As is usually the case, answers to previous questions have only yielded additional questions. It will be exciting to follow these developments as they unfold.

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CHAPTER 15

Mammalian Homologues of Yeast Autophagy Proteins

Tamotsu Yoshimori and Noboru Mizushima

Abstract

Most of the yeast proteins reported to be involved in autophagy have homologues in mammals. Analyses of some of these homologues have provided new insights into formation of the autophagosome, the double-membrane cytosolic sequestering vesicle, and the relationship between autophagy and organelles in other pathways. LC3 and the Apg12-Apg5 complex, proteins that bind to the forming autophagosomes, will be useful probes to understand the roles of autophagy in mammals.

Introduction

Autophagy was first observed in mammalian cells by electron microscopy in the early 1960s. However, the molecular basis underlying mammalian autophagy had remained cryptic until quite recently because of the difficulty in addressing this problem through a biochemical approach. As has been the case in other fields of membrane trafficking, a breakthrough came from the application of yeast genetics; more than 15 genes essential for yeast autophagy, *AUT* and *APG* were identified from genetic screens in yeast (see chapters 6 and 7). Database searches revealed that for most of the *APG* and *AUT* gene products there are related proteins in mammals, suggesting that the molecular machinery of autophagy may be conserved through evolution. The homologues are good candidates for functional counterparts of the yeast proteins and recent investigation of some of them revealed that they are actually involved in mammalian autophagy. The identification of the homologues has begun a great expansion of our knowledge about the molecular mechanism of autophagy, in particular as it relates to topics that are specific to mammalian cells.

LC3 and its Family

The hallmark of autophagy is the formation of a double-membrane cytosolic vesicle, the autophagosome, which sequesters cytoplasm and delivers it to the lysosome/vacuole. Despite ongoing interest in the protein components that comprise the autophagosome membrane, they remain largely unknown. The absence of a useful marker protein has hindered progress in the study of autophagy. The microtubule-associated protein 1 (MAP1) light chain 3 (LC3),[1] a rat homologue of yeast Aut7/Apg8 (see chapters 6 and 7), is the first identified mammalian protein localizing in autophagosomal membranes.[2]

Two forms of LC3, the 18 kDa and16 kDa forms (named LC3-I and II, respectively), are detected in various cells and show distinct subcellular localization; LC3-I is cytosolic, whereas LC3-II is recovered in a pellet after ultracentrifugation. LC3-II is specifically enriched in the autophagic membrane fraction among various organelle fractions. Furthermore, immunogold

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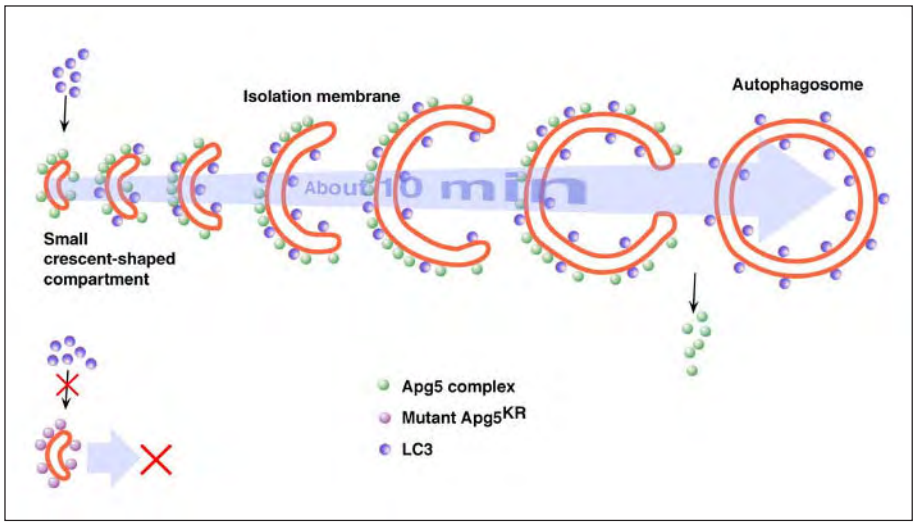

Figure 1. Schematic diagram of autophagosome formation in mammalian cells. The protein complex including Apg5, Apg12, and Apg16L (Apg5 complex) is associated with the membranes of small crescent-shaped compartments (phagophores), which are precursors to autophagosomes. The membranes elongate and grow into an isolation membrane, then mature to autophagosomes in about 10 minutes. The Apg5 complex leaves from the membrane just before or after membrane fusion, while LC3 remains. The mutant $Apg5^{KR}$ that cannot be conjugated with Apg12 targets to the membranes but neither elongates them nor recruits LC3.

electron microscopy confirmed binding of LC3 to the autophagosome membranes (Fig. 1). Ring-shaped autophagosomes can be detected by fluorescent microscopy of GFP-LC3.

The C-terminal 22 amino acids are immediately cleaved from newly synthesized LC3 to form LC3-I. Then, the following post-translational modifications convert part of the cytoplasmic LC3-I to LC3-II, which is associated with autophagosomes. The first cleavage is performed by novel proteases, Apg4A and Apg4B, homologues of yeast Aut2/Apg4 (our unpublished result; see chapters 6 and 7). It is still unclear how LC3-II is produced from LC3-I. LC3-II may possibly receive lipidation at the C terminus, similar to the manner in which yeast Aut7 is conjugated with phosphatidylethanolamine by a ubiquitin-like system (see chapters 6 and 7).[3] In yeast, phosphatidylethanolamine-conjugated Aut7 is membrane bound. Similar to yeast Aut7, LC3-I is activated by mammalian Apg7 whose yeast homologue acts as an E1-like enzyme of the ubiquitin-like conjugation system.[4]

Culturing cells under the serum and amino acids-depleted (starvation) condition that induces autophagy causes enhancement of the conversion from LC3-I to II as well as up-regulation of LC3 expression.[2] The amount of LC3-II is well correlated with the number of autophagosomes. The inhibitors of autophagosome formation (such as 3-methyladenine and wortmannin) suppress the increase in LC3-II levels. On the other hand, treatment with drugs that cause the accumulation of autophagosomes (such as bafilomycin A1 and vinblastine) results in an additional increase in LC3-II. Thus, LC3 is not only a morphological marker for autophagosomes but also an indicator of autophagic progression.

There are no Aut7 homologues in yeast. In contrast, in addition to LC3, there are at least two other Aut7 homologues in mammalian cells. These additional homologues are the Golgi-associated ATPase Enhancer of 16 kDa (GATE-16)[5] and γ-aminobutyric acid $(GABA)_A$-receptor-associated protein (GABARAP).[6] The former has been suggested to modulate intra-Golgi transport and the latter is believed to be involved in clustering and/or transport of

$GABA_A$-receptor. Interestingly, both of these proteins are also substrates of mammalian Aut2/Apg4 proteins (our unpublished observation), Apg7[4] and Aut1/Apg3.[7] If GATE-16 and GABARAP are expressed in cultured cells, both form I and II are produced and the latter co-localizes with LC3-II on the ring-shaped autophagosomes (our unpublished result). Further analyses are required to elucidate the meaning of these results.

Apg12-Apg5 Complex

Another ubiquitin-like conjugation system is essential for autophagosome formation in yeast.[8] It is the Apg12 conjugation system, in which the carboxy-terminal glycine of Apg12 is covalently attached to a lysine at the center of Apg5 (see chapters 6 and 7).[9] This conjugating reaction is catalyzed by Apg7 and Apg10.[10-12]

The Apg12-Apg5 conjugation system is also well conserved in mammals.[13] Mammalian Apg12, a 140 amino acid protein (46 amino acid shorter than yeast Apg12), is first activated by mammalian Apg7,[4] then transferred to mammalian Apg10 (our unpublished data). Finally Apg12 is conjugated with mammalian Apg5.[13] Human Apg5 was also identified during the purification of apoptosis specific protein (ASP).[14] However, the size of ASP (45 kDa) is different from either unconjugated Apg5 or the Apg12-Apg5 conjugate. Furthermore, expression of ASP is induced during (or after) apoptosis whereas the expression of Apg5 is not induced in an apoptotic-dependent manner. In fact recent studies show that ASP and human Apg5 are distinct proteins.[15]

In yeast, the Apg12-Apg5 conjugate further interacts with a small coiled-coil protein, Apg16.[16] Subsequent oligomerization of Apg16 results in the formation of a ~350 kDa protein complex, which is proposed to contain four sets of Apg12-Apg5 and Apg16.[17] In mammalian cells, Apg12-Apg5 forms an ~800 kDa protein complex with a newly identified WD repeat protein designated as Apg16L.[17a] Although the N terminus of Apg16L (63 kDa) shows homology with yeast Apg16 (17 kDa), the C-terminal WD repeat domain is a unique feature of Apg16L. Because the WD repeat domain is considered to be involved in protein-protein interactions, Apg16L may further interact with an as yet unidentified protein(s), which could be specific for mammalian cells. It should be noted that most of the cellular Apg5 is conjugated with Apg12, and that most of the Apg12-Apg5 conjugate forms a ~800 kDa complex with Apg16L irrespective of whether or not autophagy is induced. Thus, the conjugation and complex formation do not appear to be a trigger of autophagy, but rather provide part of the machinery involved in autophagosome formation.

The Apg12 conjugation system has been directly shown to be required for mammalian macroautophagy by generating *APG5*$^{-/-}$ mouse embryonic stem (ES) cells.[18] The localization and role of Apg12-Apg5 was also studied in detail using GFP-fused Apg5 expressed in ES cells.[18] Although most of the Apg12-Apg5·Apg16L complex exists in the cytosol, a small fraction of the complex localizes to the isolation membranes throughout its elongation process (Fig. 1). Apg12-Apg5 dissociates from the membrane when autophagosome formation is completed. LC3 is also present on the isolation membrane together with Apg12-Apg5, and remains on the autophagosomal membrane even after Apg12-Apg5 dissociates. Analysis of an Apg5 lysine to arginine mutant ($Apg5^{KR}$) revealed that the covalent modification of Apg5 with Apg12 is not required for membrane targeting of Apg5 but is essential for its involvement in elongation of the isolation membranes. Apg5 and its modification with Apg12 are also required for targeting of LC3 to the isolation membranes. Thus the Apg12-Apg5 conjugate plays an essential role in isolation membrane development, together with LC3. Studies on these molecules also revealed that autophagosomes are generated from a small crescent-shaped compartment, not directly derived from pre-existing membranes such as ER cisternae. In yeast, a quite unique structure termed the pre-autophagosomal structure (PAS), to which several Apg proteins are targeted, has been found (see chapters 6 and 7).[19] The PAS is probably distinct from the isolation membrane, even at a very early stage. It is not known whether a structure equivalent to the yeast PAS exists in mammalian cells.

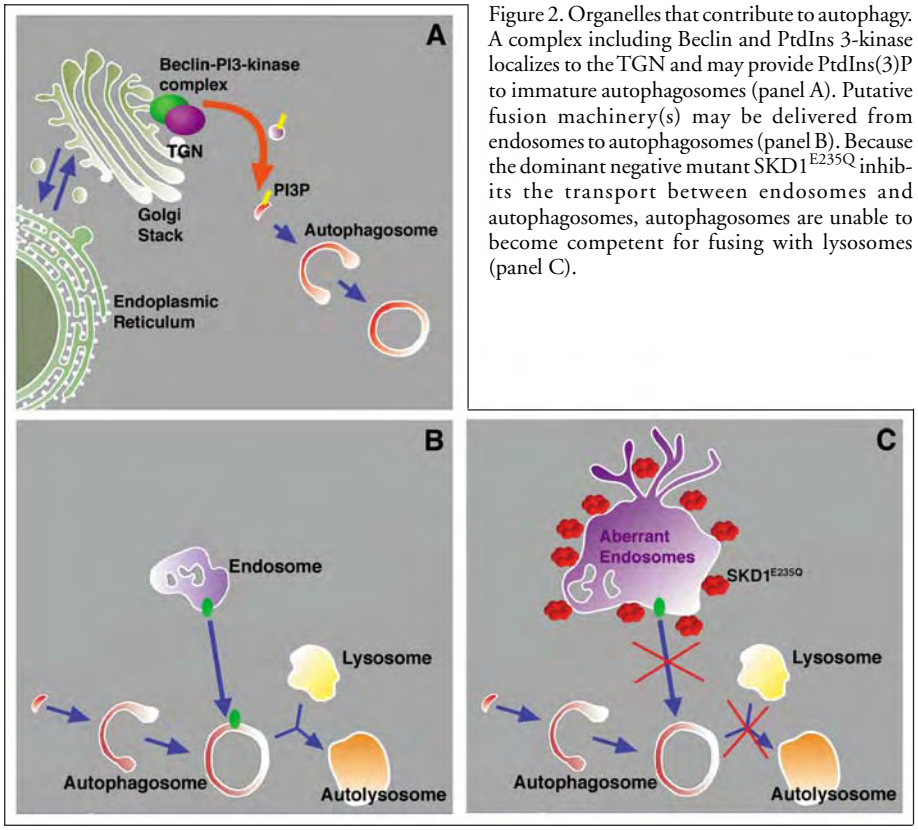

Figure 2. Organelles that contribute to autophagy. A complex including Beclin and PtdIns 3-kinase localizes to the TGN and may provide PtdIns(3)P to immature autophagosomes (panel A). Putative fusion machinery(s) may be delivered from endosomes to autophagosomes (panel B). Because the dominant negative mutant SKD1^{E235Q} inhibits the transport between endosomes and autophagosomes, autophagosomes are unable to become competent for fusing with lysosomes (panel C).

Beclin and SKD1

Studies of other homologues revealed that organelles other than autophagosomes are involved in autophagy (Fig. 2). Beclin, a human homologue of Vps30/Apg6, is required for autophagy (see chapters 6, 7 and 20).[20] The *vps* mutants were isolated due to defects in vacuolar protein sorting of Prc1 (carboxypeptidase Y) from the TGN to the vacuole, the yeast counterpart of the lysosome. The Vps30/Apg6 protein was shown to be co-immunoprecipitated with phosphatydylinositol (PtdIns) 3-kinase (class III phosphoinositide 3-kinase), which is also involved in autophagy.[21] Quantitative analyses using cross-linker indicated that all Beclin binds to PtdIns 3-kinase, while about half of the kinase remains free from Beclin. Thus, Beclin is a component of the PtdIns 3-kinase complex. Interestingly, the complex does not localize to the autophagic membranes, but rather is found in the *trans*-Golgi Network (TGN). In yeast, Vps34 PtdIns 3-kinase forms at least two multi-subunit complexes; one consisting of Vps15, Vps30/Apg6, Apg14, and Vps34 and the other consisting of Vps15, Vps30/Apg6, Vps38, and Vps34.[22] The former works in autophagy and the Cvt pathway (see chapters 6 and 7), whereas the latter is involved in endosomal transport. The Beclin-PtdIns 3-kinase complex at the TGN presumably plays an important role in both autophagy and the endocytic pathway through production of PtdIns(3)P. Because PtdIns(3)P seems to be a lipid component of the autophagosomal membranes, the complex may provide this lipid to immature autophagosomes. Based on the analogy to its role in early endosomes, PtdIns(3)P may function as an anchor for a putative protein essential for autophagosome formation. It is also possible that the PtdIns 3-kinase complex is involved in sorting putative proteinaceous components of autophagosomes

at the TGN. Along these lines, some proteins that must have been glycosylated in the ER and Golgi complex were found in autophagosomes.[23]

Mouse SKD1[24] is not homologous to any of the Apg proteins but rather to Vps4/Csc1.[25,26] The *csc1* mutant is a gain of function allele of *VPS4/CSC1* that was obtained in a screen for mutants that result in the induction of autophagy even in the presence of nutrients. Because disruption of *CSC1* causes a severe defect in autophagy, the gene product apparently plays a role in autophagy as well as in endosomal transport. SKD1 is a member of the AAA-ATPase family and overexpression of the dominant-negative mutant, SKD1^{E235Q}, which is expected to be defective in ATP hydrolysis, induces abnormal endosomes and inhibits transport from endosomes to the plasma membrane and to lysosomes.[27] Whereas most of the wild type SKD1 is distributed in the cytoplasm, the mutant SKD1 binds to the aberrant endosomes. Therefore, SKD1, like Vps4, must be involved in sorting and/or transport from the endosomes probably through transient association with the endosomal membrane. Moreover, in the cells overexpressing the mutant SKD1^{E235Q} autophagic protein degradation under starvation conditions is significantly inhibited.[28]

An obvious question is what is the relationship of the SKD1 ATPase to autophagy. As opposed to Apg5-deficient cells that are unable to form autophagosomes, the SKD1 mutant accumulates large numbers of autophagosomes, even under nutrient-rich conditions. However, the increased level of autophagosomes does not result in fusion with lysosomes; there are only a few lysosomes in the cells that have fused with autophagosomes, suggesting that mutant SKD1 overexpression affects this fusion process. Inhibition of autophagic degradation is thus attributable to a defect in fusion. SKD1 seems to function in autophagy indirectly, because the mutant SKD1 does not localize to autophagosomes. It is noteworthy that transport from late endosomes to autophagosomes (resulting in the production of amphisomes; see chapter 2) is also defective in cells overexpressing the mutant SKD1. Although the presence of such transport has been known for some time, its meaning is still unclear. Endosomes may provide autophagosomes with putative component(s) required for fusion with lysosomes.

Conclusion

The history of autophagy is now entering upon a new phase due to the identification of mammalian proteins involved in autophagy, especially, the autophagic membrane-binding proteins. Various physiological and pathological processes have been correlated with autophagy in mammals. The mammalian autophagy proteins should be good tools for probing these developmental and pathological conditions. It is quite likely that in the near future we will reach a further understanding about the roles of autophagy in higher multicellular systems, as well as an elucidation of the molecular machinery that drives the membrane rearrangements that are the hallmark of this essential process.

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CHAPTER 16

Autophagy in Plants

Yuji Moriyasu and Daniel J. Klionsky

Abstract

There is substantial morphological evidence that plants carry out autophagy. Different types of vacuoles such as the vegetative vacuole and protein storage vacuole are present in plant cells. Morphological studies suggest that these two types of vacuoles function as lytic compartments of autophagy in plant cells. Moreover, autophagy is likely to contribute to the genesis of these vacuoles. Since the total volume of vacuoles accounts for more than 90% of the total cell volume in matured plant cells, the plant vacuole plays an important role in the morphology of whole plants as well as plant cells. Thus, autophagy may contribute to the morphogenesis of plants. This is one of the most conspicuous roles of autophagy in plants, a role that has not been considered in animal and yeast cells.

Although the difficulty of applying genetic approaches in plant systems has made it problematic to identify the molecular components of the autophagic machinery, the sequencing of the *Arabidopsis* genome has allowed the identification of homologues of the yeast autophagy genes. Many of the *APG* genes appear to be in EST collections from various other plants suggesting that the same autophagic machinery is conserved across most plant species. Mutations in these genes result in several developmental abnormalities supporting the concept that autophagy in plants is involved in the normal physiology of plant development as well as in response to starvation.

Introduction

Autophagy occurs in all eukaryotic cells, including fungi, animals and plants. In fungi, autophagy appears to be primarily a response to nutrient depletion and defects in autophagy result in cell death during starvation conditions. Autophagy is induced upon nutrient depletion in animal cells, but is also triggered by a range of other stimuli including hormones and cellular injury (see chapters 3, 4 and 23). In mammalian cells, autophagic dysfunction is associated with a number of genetic diseases (see chapters 20-22).

Autophagy in animal cells plays a developmental role (reviewed in ref. 1). In plants as well, autophagy has been observed at various stages of development. Recent molecular genetic studies show that orthologs of most of the yeast autophagy genes exist in *Arabidopsis thaliana*. Mutations in these genes result in several developmental abnormalities suggesting that autophagy in plants is involved in the normal physiology of plant development as well as in response to starvation. This chapter provides an overview on the role of autophagy in plants.

Vacuole Biogenesis and Development

There are different types of vacuoles present in plant cells and these compartments carry out distinct functions.[2-5] For example, the protein storage vacuole is involved in storing proteins for use in later stages of development such as during seed germination. In contrast, the vegetative or lytic vacuole functions primarily as a degradative compartment similar to the mammalian lysosome. Accordingly, these compartments contain different enzymes and metabolites.

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Both types of vacuoles seem to play a central role in autophagy in plant cells. Moreover, as discussed below, autophagy is thought to participate in the genesis of these two types of vacuoles.

Meristematic cells existing in shoot and root apices do not have large vacuoles. As these cells mature, large vegetative vacuoles are formed. Autophagy may play a critical role in the biogenesis of vegetative vacuoles.[6-8] Vesicles budding from the *trans*-Golgi network fuse together to form an autophagosome-like structure surrounding a portion of cytoplasm. As the inner membrane of an autophagosome and enclosed cytoplasm are degraded, the compartment matures into a small vegetative vacuole. The fusion of small vacuoles formed in this way results in the formation of a large vacuole. In another case, membrane sacs that are differentiated from the ER have been shown to align at the site where new vacuolar membrane is to be formed.[9,10] These membrane sacs enclose cytoplasm, fuse with each other and dilate inward and as a result a large vacuole is formed. In both cases, autophagy or autophagy-like processes contribute to the genesis of these vegetative vacuoles.

During the maturation of seeds and cereal grains, the formation of protein storage vacuoles occurs. It has been reported that autophagy contributes to the formation of protein storage vacuoles in some species.[8,11-14] In wheat, prolamins, storage proteins synthesized in the ER, are ultimately localized in protein storage vacuoles.[15] Some portion of prolamins is transported to vegetative vacuoles via the Golgi apparatus in a manner similar to the transport of vacuolar/lysosomal lumenal proteins found in yeast and mammalian cells. In contrast, the other portion of prolamins is transported directly to vacuoles without passing through the Golgi apparatus resulting in the formation of protein storage vacuoles. In this case, some prolamins initially aggregate within the ER lumen and the aggregates bud off from the ER into the cytoplasm. These aggregates are subsequently internalized into vacuoles by a process analogous to autophagy.[15] This process is initiated by the attachment of electron-translucent vesicles around the aggregates. These vesicles then fuse together and form vacuoles containing protein aggregates, protein storage vacuoles. The selectivity of this autophagic process has not been known, and some observations suggest that this type of autophagic sequestration is not specific to the aggregate of storage proteins.[16]

In the formation of protein storage vacuoles in cotyledon cells during the maturation of dicot seeds, preexisting vegetative vacuoles have been thought to transform to protein storage vacuoles by the deposition of storage proteins into vegetative vacuoles. This concept on the transformation of vegetative vacuoles to protein storage vacuoles, however, is not uniformly accepted and it has been reported that during the development of pea seeds, preexisting vacuoles disappear completely and concomitantly protein storage vacuoles are formed de novo.[2,17] In this case, the newly formed precursor of protein storage vacuoles, which originate from the ER, encloses and engulfs—in an autophagic process—preexisting lytic vacuoles.[2,17] This process is similar to the genesis of vegetative vacuoles.[9]

When seeds germinate, storage proteins stored in protein storage vacuoles are degraded and the organelles are transformed again to vegetative vacuoles. During this transition these vacuoles perform autophagy. In the cotyledon cells of bean, starch granules existing in the cytoplasm appear to be selectively enclosed by electron-translucent membrane sacs and transported to the vacuole.[18] Thereafter, starch granules are degraded by amylase that has been synthesized in the ER and transported through the Golgi apparatus into the same vacuole. Other cellular components are also taken up into the vacuoles by macroautophagy or microautophagy in a nonselective manner.[18-20] Because senescence of cotyledons occurs during seedling growth, this result means that vacuolar autophagy contributes to the degradation of cellular components at some stages during the senescence of cotyledon cells. Autophagy has been known to occur in many other cases of senescence.[21] The degradation of cellular components at the final stage during senescence, however, appears to be done through the rupture of a vacuolar membrane and the release of vacuolar hydrolytic enzymes into the cytosol.[21-23]

Starvation and Autophagy

There is substantial morphological evidence for both micro- and macroautophagy in plants.[7,8,13,22,24] In most cases, structures of cytoplasmic origin were found in vegetative and protein storage vacuoles. In many instances, however, it is unclear whether these structures are transported into vacuoles in a macro- or microautophagic manner (see chapter 8). One of the most well characterized autophagic processes in plant cells is that performed in cultured cells under starvation conditions. When tobacco, rice or sycamore cells cultured in suspension are starved for sucrose, they perform autophagy[25-27] and show a loss of total cellular protein.[28] In sycamore cells, a part of the cytoplasm appears to be transported into the central vacuole through macroautophagy because autophagosome-like structures are detected in the cytoplasm.[8,26] In tobacco cells, autolysosomes (formed from the fusion of autophagosomes and lysosomes) that are distinguished from the vacuole are detected in the cytoplasm, and accordingly this autophagy can be also regarded as macroautophagy.[27]

In sycamore cells under sucrose starvation conditions, the amount of phosphorylcholine increases.[26] Phosphorylcholine is a degradation product of phosphatidylcholine, a major component of cellular membranes, and thus its accumulation is thought to be a marker of autophagy. Glycerol and pyruvate added to culture media block the accumulation of phosphorylcholine. Because the concentrations of hexose phosphates remain low even with the introduction of glycerol and pyruvate, which have the possibility of being converted to hexose phosphates through the gluconeogenesis pathway, it follows that the autophagic response appears to be controlled by the level of mitochondrial respiratory substrates rather than by the level of hexose phosphates in the cytoplasm.[26]

It has not been examined morphologically whether or not autophagy is really induced under nutrient starvation conditions in whole multicellular plants. But maize plants subjected to prolonged darkness display increased protein degradation that is likely due to autophagy.[29]

Other Topics in Autophagy of Plant Cells

Plant Morphogenesis and Autophagy

Matured plant cells usually have one or a few large vacuoles and the total volume of vacuoles accounts for more than 90% of the total cell volume. Thus the plant vacuole plays an important role in the morphology of whole plants as well as plant cells. If autophagy contributes to vacuole genesis, it follows that autophagy is involved in the morphogenesis of plants. This is one of the most conspicuous roles of autophagy in plants that has not been considered in animal and yeast cells. On the other hand, *Arabidopsis* mutants in which the homologues to yeast *APG* genes are disrupted can accomplish a complete life cycle without conspicuous morphological changes under nutrient-rich conditions (See *Molecular Genetic Analysis* in this chapter). This result suggests that autophagy contributes little to vacuole genesis under such conditions, although it is thus far unclear whether or not autophagy is really blocked in the mutant plants. Alternatively, autophagic processes observed during vacuole genesis[6,9] may be different from autophagy that is dependent on the *APG* homologues.

It has been thought that large vacuoles in plant cells are formed by the fusion of many small vacuoles. During this process, the adjustment of vacuolar volume to vacuolar membrane area should occur. In other words, there is a need to reduce the amount of vacuolar membrane. Such adjustment may be done by a kind of autophagy.[30]

The Selectivity of Autophagy

The selectivity of autophagy has not been scrutinized in plants. Autophagy performed by tobacco cells under sucrose starvation conditions is nonselective.[27] In contrast, in the endosperm cells of developing wheat grains, the aggregates of storage proteins appear to be selectively enclosed by membrane sacs that are electron-translucent.[15] Similarly, in cotyledon cells of ger-

minating bean, starch granules existing in the cytoplasm appear to be selectively enclosed by electron-translucent membrane sacs and transported to the vacuole.[18] Another interesting example of selective autophagy in plant cells is a specific transfer of materials in plastids to vacuoles.[31,32]

The Membrane of Autophagic Vacuoles

Plant cells have different kinds of vacuoles. It has been hypothesized that the different vacuoles are characterized by specific membrane proteins, in particular the different tonoplast intrinsic proteins (TIPs).[5] According to this hypothesis, an autophagic vacuole is defined to be a vacuole whose membrane has α-TIP but not γ- or δ–TIP. We do not have much information about autophagic vacuoles in plant cells, but in the aleurone and root tip cells of barley, there is an autophagic vacuole-like organelle whose membrane has only α-TIP.[33,33a] In order to test the hypothesis, more information about autophagic vacuoles is needed.

Phosphatidylinositol 3-Kinase and Autophagy

The activity of phosphatidylinositol (PtdIns) 3-kinase is necessary for autophagy in both mammalian and yeast cells[34,35] (see chapters 3,4,6,7). Well-characterized inhibitors of PtdIns 3-kinase such as wortmannin and LY294002 block autophagy in tobacco cells under sucrose starvation conditions (C. Takatsuka, Y. Inoue, and Y. Moriyasu, unpublished data). Similarly, the mammalian autophagy inhibitor 3-methyladenine, the target of which is now thought to be PtdIns 3-kinase,[34] inhibits autophagy in tobacco cells. Together, these data suggest that PtdIns 3-kinase also plays a central role in autophagy in plant cells.

Molecular Genetic Analysis

Homologues of Yeast Autophagy Genes

Until recently, most of the analyses concerning autophagy in plants (and also in mammalian cells) were morphological in nature. The identification of autophagy genes in yeast has expanded the analysis of autophagy in animal cells (see chapter 15). Similarly, the recent sequencing of the *Arabidopsis* genome has allowed a genome-wide search for plant homologues to the yeast *APG* genes. Hanaoka et al[36] and Doelling et al[37] identified 25 plant genes that are homologous to 12 of the yeast *APG* genes. Many of the essential residues in the yeast Apg proteins are conserved in these *Arabidopsis* homologues.[36,37] The potential orthologs include gene products corresponding to *APG1* to *APG10*, *APG12* and *APG13* (see chapters 6 and 7). Among these genes, the *Arabidopsis AtAPG4* gene has been shown to complement a yeast *aut2/apg4* mutant.[36] In addition, many of the *APG* genes appear to be in EST collections from various other plants suggesting that the same autophagic machinery is conserved across most plant species.

Yeast Apg1 is a protein kinase that is regulated by Apg13, and that may be involved in regulating the conversion between the Apg and Cvt pathways.[1,38] A function for Apg2 has not been established. However, it has been known that the localization of Apg2 is dependent on the transmembrane protein Apg9.[39,40] The Aut1/Apg3, Aut2/Apg4, Apg5, Apg7, Apg10 and Apg16 proteins comprise two conjugation systems of the ubiquitin-like proteins Aut7/Apg8 and Apg12 (reviewed in ref. 41). All of these proteins with the exception of Apg16 have homologues in *Arabidopsis*. It seems likely that a protein(s) carrying out analogous functions to Apg16 exists in *Arabidopsis*. Apg6/Vps30 is a common component of two kinase complexes composed of the PtdIns 3-kinase Vps34 and the protein kinase Vps15.[35] These two kinase complexes may interact through proteins that bind PtdIns 3-phosphate.[42] Apg14 is a component of one of the two kinase complexes and is thought to provide functional specificity for these complexes.[35] As with Apg16, an *Arabidopsis* homologue of Apg14 apparently does not exist, but it seems likely that there will be a similar protein that carries out an analogous function.

Other proteins that are required for autophagy including Apg17 and Aut10/Cvt18 have not yet been identified in plants. In addition, proteins that are specific for the Cvt pathway such as the receptor/adaptor for precursor aminopeptidase I, Cvt19,[43] and proteins that may be part of the Apg1 kinase complex, Cvt9, Cvt13 and Cvt20,[42,44] may not have homologues in all systems (see chapters 6 and 7). At present, there is no evidence for the existence of the Cvt pathway in any organism other than *S. cerevisiae*.

Of 12 *Arabidopsis* Apg proteins, the AtApg1, 4, 8, 12 and 13 proteins are encoded by multiple loci.[36,37] For example, there are three loci for *AtAPG1* and nine for *AtAPG8*. Some of the corresponding proteins are expressed in an organ-specific manner.[36]

The Ohsumi and Vierstra labs examined the effect of autophagy mutations in plants by examining T-DNA insertion lines for *AtAPG* genes that exist as single copies in the genome. An *atapg9-1* and *atapg7-1* mutant were chosen for detailed analysis, respectively.[36,37] In both cases, the plants apparently undergo normal embryogenesis, germination, cotyledon development, both root and shoot elongation, and seed production under standard nutrient-rich conditions. These results suggest that autophagy is not essential for the completion of a life cycle in *Arabidopsis*. A close examination, however, shows that even in nutrient-rich conditions the *atapg9-1* plants exhibit an early flowering phenotype, resulting in a reduction in rosette leaf number, and earlier senescence of rosette leaves.[36] Earlier chlorosis of the mature rosette leaves following bolting (transition from a rosette form to one with an elongated stem) is also observed in the *atapg7-1* plants.[37] These results show that autophagy does play some role in normal plant development even when nutrient conditions are not specifically limiting.

When *atapg7-1* and *atapg9-1* plants are grown in media depleted for or limiting in nitrogen, they display a range of more severe defects. Both *atapg7-1* and *atapg9-1* plants show accelerated senescence of cotyledons and rosette leaves more dramatically than that seen under nutrient-rich conditions. Moreover, both the mutant plants develop fewer flowers and siliques (seed pods or fruits), resulting in fewer seeds. In addition, in *atapg7-1* mutants, the growth of roots is slower than that in wild type plants. The mRNA levels of the senescence markers *SEN1* and *YSL4* increase at earlier times in the *atapg9-1* mutant.[36]

There are no obvious morphological defects in the vacuoles in both the mutant plants suggesting that the mutant phenotypes are due to blocks in autophagic delivery rather than vacuolar function per se. The RNA encoding *AtAPG7* and *AtAPG8* accumulates along with the senescence-associated *SEN1* transcript during dark-induced senescence in detached leaves,[37] showing the expression of these autophagy genes can be used as other markers of senescence.

Under the conditions that senescence is induced, *atapg7-1* and *atapg9-1* plants exhibit more rapid chlorosis and loss of chloroplast enzymes, indicating that the degradation of chloroplast proteins, one of the most conspicuous symptoms of senescence, is accelerated in these plants. This result is consistent with the concept that the degradation of chloroplast proteins happening at the initial stage of leaf senescence is not performed by vacuolar autophagy. It has been thought that the degradation of chloroplast proteins may be done by a proteolytic system in chloroplasts themselves.[22,45,46] Furthermore, in *atapg7-1* mutant plants, the degradation of rRNA is not blocked, but instead is performed more rapidly than that in wild type plants.[37] This result may suggest that rRNA, in addition to chloroplast proteins, is not a substrate of autophagy during senescence.

Early senescence in the autophagy mutants can be explained by the deficiency of nutrients in the plants. Autophagy may in general contribute to the recycling of cellular constituents and such contribution may be enhanced under conditions where a nutrient supply from outside is limited and/or photosynthesis is blocked. As a result of the less efficient supply of nutrients, the autophagy mutants may be forced into premature senescence with a resulting decrease in seed production; plants undergo senescence to recover nutrients, especially from old leaves, for the production of seeds. Accelerated chlorosis may be due to a need to produce seeds under conditions that are perceived as nutrient limiting.

Homologues of SNARE Proteins

In macroautophagy, autophagosomes that are formed in the cytoplasm must fuse with the vacuole or lysosome to deliver their contents. The fusion process requires various proteins that have been characterized in both yeast and animal cells including SNARE, Rab and Sec1 proteins (reviewed in ref. 47). A number of proteins that are orthologs to the yeast and mammalian fusion components have been identified in plants.[47] These include the SNARE proteins AtTlg2,[48] AtVti1[49] and AtVam3.[50] AtVam3 likely plays a role in membrane fusion in a manner similar to the yeast Vam3 although the site of action of AtVam3 may be the prevacuolar compartment[51] in addition to the vacuole.[50] AtVam3 is more similar to *Arabidopsis* and yeast Pep12 indicating that it may function on multiple organelles or at different locations in different cell types.[47] AtPep12 is known to be located at prevacuolar compartments (late endosomes) in *Arabidopsis*.[52] How the fusion of autophagosomes or autophagic vacuoles with various types of vacuoles is controlled will become one of the immediate issues concerning autophagy in plant cells.

Conclusion

For many years morphological evidence has indicated that plants carry out autophagy. The difficulty of applying genetic approaches in plant systems has made it difficult to identify the molecular components of the autophagic machinery. Two recent advances, however, have resulted in a major breakthrough. First, many of the proteins required for autophagy in yeast have been identified. Second, the sequencing of the *Arabidopsis* genome has allowed the identification of homologues of the yeast autophagy proteins. The majority of the yeast *APG* genes have homologues in *Arabidopsis* and the conservation of key residues suggests that the corresponding proteins are orthologs. The analysis of *atapg* mutants indicates that autophagy plays a role in both normal plant development and response to nutrient-limited conditions. Future experiments will provide more specific information on the function of autophagy in plants, whether this process occurs in an organ-specific manner, and the role of the different isoforms of the Apg proteins.

Acknowledgements

The authors would like to thank Drs. Laura Olsen, John Schiefelbein and Charles Yocum for helpful comments. This work was supported by National Institutes of Health Public Health Service grant GM53396 (to D. J. K.).

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CHAPTER 17

Autophagy in *Caenorhabditis elegans*

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Abstract

The first detailed morphological description and quantitative data on autophagy in *C. elegans* show the appearance of autophagic vacuoles in various stages of development in most cell types of wild type and certain mutant animals. The preliminary results concerning some autophagy-related genes and the genomic search for the *C. elegans* orthologs of the *Saccharomyces cerevisiae* autophagy genes forecast the existence of differences in the autophagic machinery in the two model organisms. The initial results of an effort to start the characterization of autophagy in *C. elegans* are presented in this chapter.

Introduction

The fact that the Medline search for "autophagy" gave 1036 hits near the end of the year 2002, while entering the double query of "autophagy and elegans" resulted in two articles only, convincingly shows that autophagy research has not really started in *Caenorhabditis elegans*. How could *C. elegans*, one of the most promising and exciting multicellular model organisms, avoid the interest of researchers of autophagy for so long?

A partial answer to this question may be related to the fact that autophagy (used as the synonym of macroautophagy in this article) has been discovered[1] and described by electron microscopy (see refs. 2-4 for a review of the early literature). Electron microscopy is still an indispensible method for studying this process. Starting in the 1990s, the unicellular yeast with its relatively big vacuole proved to be uniquely suited to study autophagy by light microscopy (with supporting studies by the electron microscope). A similar approach for multicellular animals, however, seemed and still seems to be unworkable. However, the new era had already begun with the discovery of autophagy genes in yeast (see chapters 6 and 7) and the challenge was there to look for a genetically tractable metazoan organism.

In principle (and a priori) *C. elegans* is an obvious choice. However, the first sight of this organism makes the electron microscopist at least somewhat discouraged. As one of the few masters of *C. elegans* electron microscopy David H. Hall sums it up: "Your quarry is a small slippery worm wrapped in an impenetrable cuticle."[5] Our own work has proved that these qualities make *C. elegans* a rather special object for autophagy studies. Even with the background of 30 years vertebrate electron microscopy and the support of the excellent methodological review of Hall,[5] handling and preparation of the samples needed a lot of inventiveness, practice, and most of all patience to achieve the necessary speed and precision for routine work. The summary below is based on more than three thousand electron micrographs taken from nearly three hundred worms from different stages of development.

Autophagy, edited by Daniel J. Klionsky.

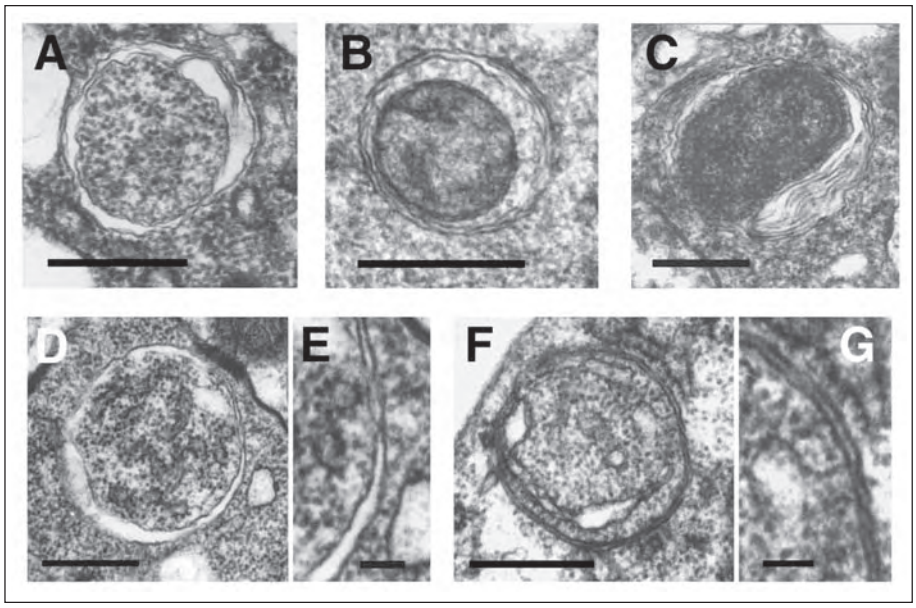

Figure 1. Autophagosomes contain well recognizable cytoplasmic material (A-D), their double membrane is the thin type (E is enlarged from D), is often myelinated (C), and its course is finely tortuous having a wider or narrower empty looking cleft between the two layers (A-D). Sections of cellular interdigitations may look like autophagosomes (F), but their membranes are the thick type (G is enlarged from F) that keep in parallel without the characteristic cleft between them. Scale bars are 0.5 μm in A-D and F, and 0.1 μm in E and G).

Identification of Autophagosomes in *C. elegans*

Without preceding results it is necessary to present here the basic electron microscopic description of autophagosomes in *C. elegans*. This is all the more important because there are subcellular stuctures in *C. elegans* cells which may lead to false identification of autophagosomes that must be avoided in order to get reliable results.

As there are still no specific markers for routine morphological work, autophagosomes are identified by the qualities of their bordering membrane and isolated content. To be able to follow the dynamics of the autophagic process, autophagosomes of different age are usually discerned. The initial form is delineated by a ribosome-free double-membrane and the content is morphologically unchanged. Typical examples of early autophagosomes from various cell types of *C. elegans* are shown in Figure 1. Characteristic features of the sequestering membrane (following the most used aldehyde-OsO_4 double fixation) are that it is of the thin type having the same thickness as the membrane of the rough ER or the mitochondrion,[6] usually has a finely tortuous course, often has a narrower or wider empty-looking cleft between the two phospholipid bilayers and is liable to split up into myelinated structures (Fig. 1A-E). With the help of serial sections we have demonstrated in several cases that such structures are indeed closed compartments (Fig. 2).

The most frequently encountered structure that can be incorrectly identified as an autophagosome is sections of cellular interdigitations that are quite common in *C. elegans*. Their exclusion can be based on their membrane being the thick type, the space between the two membrane layers having a constant width and containing some low-density intercellular material. The course of the membranes is smooth (not tortuous), and their splitting into myelin structures is much less frequent (Fig. 1F,G).

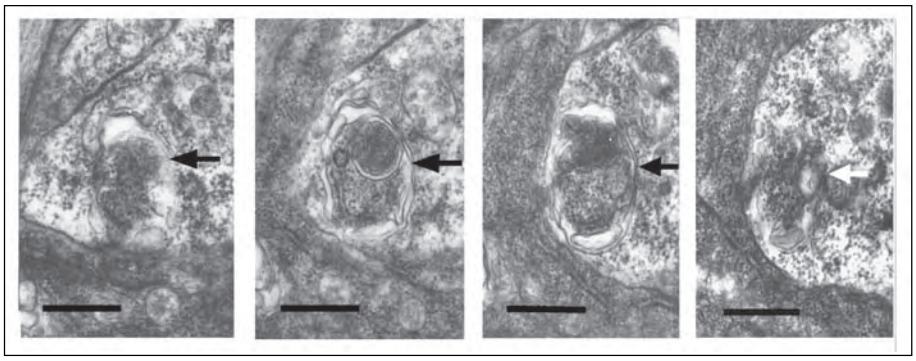

Figure 2. Serial section of an autophagosome in a body wall muscle cell. The autophagosome (arrows) appears and disappears from left to right. Scale bar 0.5 μm.

The subsequent digestive form that results from fusion with a lysosome and/or endosome has a content that shows morphological signs of weaker or stronger structural deterioration. The inner vesicle membrane may persist for some time but eventually falls apart. Most frequently in *C. elegans* active lysosomes initially become denser as a whole with each segregated component following this trend. In later stages the content of active lysosomes gradually becomes more and more electron lucent leaving only clumps of the dense material (Fig. 3A-D) and frequent accumulation of myelinated structures (Fig. 3E and Fig. 4). It is possible, however, to reconstruct a succession of images that connect later forms to earlier ones thereby identifying their origin. The parallel presence of transitional forms of all types of autophagic vacuoles is one of the most important signs of a genuine autophagic process in the cell under investigation.

Secretory vacuoles with a content of heterogeneous density may be incorrectly identified as autophagosomes that have fused with lysosomes (Fig. 3F). In most cases transitional forms can be found nearby with features that link them to clearly recognizable secretory vacuoles rather than to autophagosomes or active lysosomes. A similar pattern of identifying the transitional forms can be followed to avoid the false categorization of swollen or damaged mitochondria that are demarcated by a thin type of double membrane.

We have found that in *C. elegans* typical autophagosomes and single lysosomes have approximately 0.5 μm average diameter that is smaller than those in several mammalian cell types.[4,6,7] In a few encountered cases of strongly enhanced autophagy (see below) the diameter of single autophagosomes may increase to more than 1 μm. We have also found that the usual autophagosome in *C. elegans* contains cytoplasm with ribosomes and rough ER. Other organelles are uncommon (e.g., smaller sized mitochondria in Fig. 1B) or rare (secretory vacuoles, not shown) in autophagosomes.

Under conditions of strong stimulation (see below) complex multifocal structures emerge by multiple fusions of autophagosomes and lysosomes resulting in large vacuoles where the focuses can be in different stages of degradation (Fig. 4). Such complexes provide excellent help to identify transitional forms for the morphological reconstruction of the degradation process. In most cases their further advance seems to lead to more or less myelinated multifocal lysosomes with mostly unidentifiable content as a possible morphological representation of the late stage of the degradation process (Fig. 4).

Autophagy during Postembryonic Development

Our first attempts with randomly taken samples of all larval stages (including the special dauer larvae;[9] see chapter 20) from growing or starved cultures and sectioned perpendicularly to the long axis of the animals revealed that satisfactory results can only be obtained by quan-

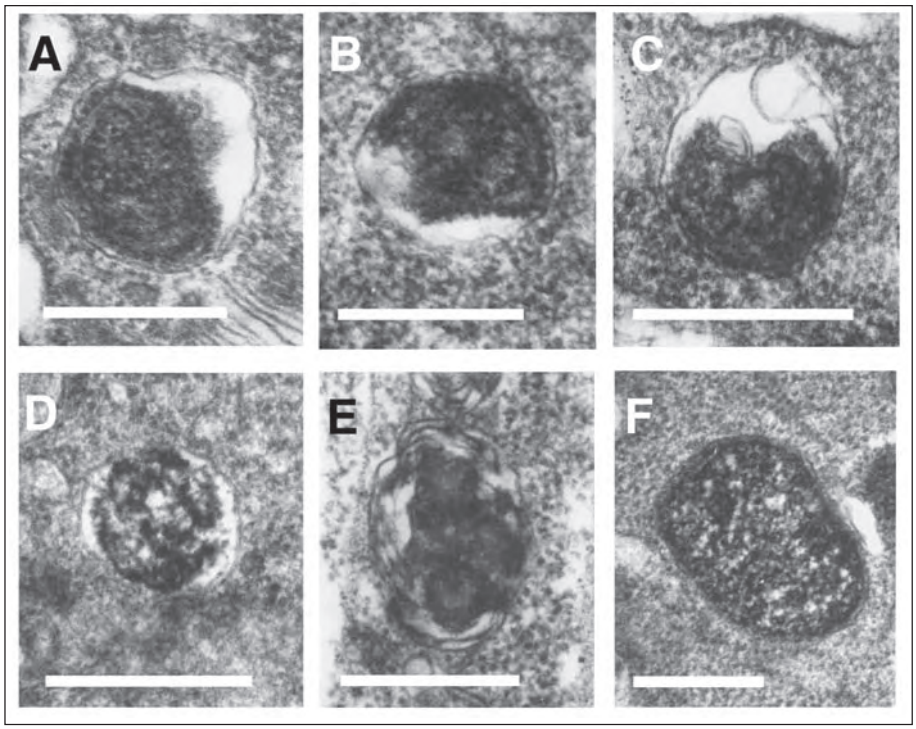

Figure 3. A series of active lysosomes (A-E) showing the gradual deterioration of the content leading to highly heterogeneous irregular density of the clumps of ribosomes from the rough endoplasmic reticulum and myelinated remnants (E). Some types of secretory vacuoles (F) which can be incorrectly identified as autophagosomes are also heterogeneous, but the pattern is more regular and dense secretory material is attached to the membrane all around. Scale bars are 0.5 μm.

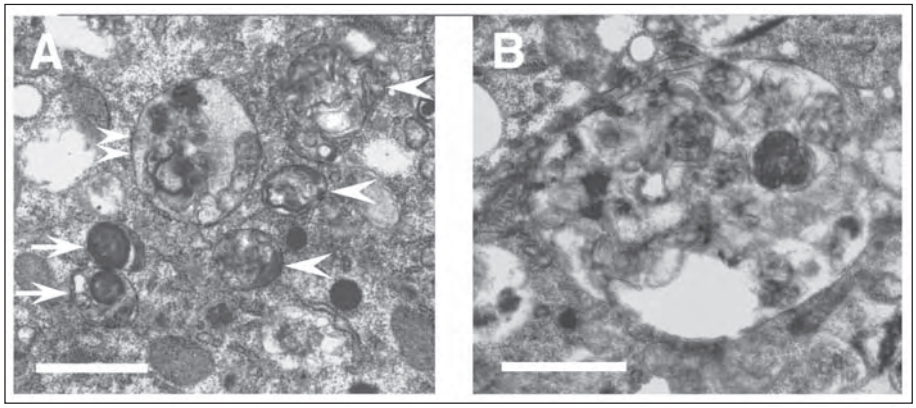

Figure 4. Various types of autophagic elements in the gut epithelial cells of *C. elegans*. A. Autophagosomes in an adult worm with the *apg6* ortholog inactivated by RNAi showing autophagosomes (arrows), myelinated forms (arrowheads), multifocal complex form (double arrowhead). B. A large complex autophagosome with several (mostly myelinated) foci from previous fusion of autophagosomes in a rare lethargus with extreme autophagic response from starved culture. Scale bars are 1 μm.

titative evaluation methods. Regional differences along the body and the need to obtain the maximum test area for morphometry necessitated the sectioning of the animals along their longitudinal axis.

By this approach we measured the quantity of autophagic vacuoles in three main body tissues: The hypodermis, the body wall muscle and the gut epithelial cells. Our data show that the total autophagic compartment in the cells of continuously feeding animals of well fed cultures in all larval stages has an average of less than 0.001% of the cytoplasmic volume, being actually zero in most animals (unpublished results). However, a significant elevation occurs in these cell types at the end of all four molting periods (lethargus phases) giving a value of 0.2-0.4%. This is in the range of the so called "physiological autophagy" in some tissues of mammals in vivo,[8,10] but still an order of magnitude lower than the stimulated autophagy in several mammalian systems.[8,10]

Our preliminary observations show that animals in the predauer stage that have already accumulated substantial amount of glycogen and lipid do not yet display autophagy, while definitive dauer larvae usually contain regional accumulations of autophagosomes in their gut epithelial cells which persist for several hours even in the postdauer period indicating a possible delay in degradation.

We have found autophagosomes in most cell types of *C. elegans*. The two notable exceptions have been so far the neurons and gonadal reproductive cells. Distribution of autophagosomes seems to be rather uneven along the longitudinal axis of the animal in the three main body tissues. They tend to appear in groups in certain cells without any apparent system of regional distribution. Differential reaction of cells can also be seen in mammalian systems where cells with lots of autophagic vacuoles frequently border others with no autophagy at all. Differences, however, become smaller when autophagy is strongly stimulated (unpublished observations).

Our systematic starvation experiments in the L2 larval phase show that individual variation is rather big among the animals, but the average quantity of autophagic vacuoles do not seem to be higher than those in the lethargus phase. We have found so far two wild type animals (one lethargus and one starved for 24 h) that displayed extremely high autophagic activity in their gut epithelial cells (Fig. 4B) while their parallels did not. The reason for this variation is unclear at the moment but indicates the existence of substantial reserve in autophagic capacity.

The *C. elegans* Orthologs of the Yeast Autophagy Genes

A direct approach for identifying autophagy-related genes in *C. elegans* is to use standard BLAST searches of genetic mediators of yeast autophagy against the *C. elegans* genome (Table 1). Interestingly, many of the yeast autophagy genes have no significant *C. elegans* counterpart. In contrast, some of the *C. elegans* genes display high evolutionary conservation with the yeast ortholog. This suggests that, although a conserved core machinery of autophagy exists throughout the eukaryotic kingdom, autophagy may be regulated in a different way in the two organisms. The characteristics of those *C. elegans* orthologs that have already been under investigation also support this assumption. For example, the genes *let-363* and *let-512* being orthologous to yeast *TOR2* and *VPS34* respectively,[11,12] have been shown to be essential for viability. Our preliminary data suggest that the *C. elegans apg6* ortholog, whose product interacts in vitro with LET-512/VPS34, is also an essential component for *C. elegans* development. In contrast, the *apg/vps/cvt* loss-of-function mutant yeast strains, although defective in autophagy and vacuolar protein sorting, are viable under standard laboratory conditions.

Another intriguing example is the gene *unc-51* encoding a novel serine/threonine protein kinase that is the ortholog of yeast *APG1*.[13] UNC-51 is a neuron specific protein required for axonal elongation and guidance. According to our observations, however, *C. elegans* neurons have not shown detectable autophagic activity. In addition, autophagic vacuoles can be seen in the lethargus phase worms in *unc-51(e369)* loss-of-function mutants just like in wild type animals.

Table 1.* C. elegans *orthologs of yeast genes required for the autophagy and Cvt pathways

Yeast Gene	*C. elegans* Ortholog ORF	Gene	Probability of Blast Hit	Predicted Function
APG1/AUT3/CVT10	Y60A3A.1	*unc-51*	7.7e-38*	Serine/threonine kinase
APG2/AUT8	M03A8.2		0.00013	?
	Y87G2A.3		1.4e-10*	Microtubule-associated Protein
APG5	Y71G12B.12		0.0025	?
APG6/VPS30	T19E7.3		7.4e-14*	Coiled-coil protein
APG7/CVT2	M7.5		4.5e-95*	Yeast YHX1-like Protein
APG9/AUT9/CVT7	T22H9.2		1.2e-41*	?
APG10	D20.85.2		0.00018	?
APG12	B0336.8	*lgg-3*	0.0022	?
APG13	C01F6.6a		0.12	PDZ domain
APG14/CVT12	D1014.4		0.4	?
APG16	C06G3.2		0.0024	Kinesin-like protein
APG17	Y11D7A.14		1.3e-0.5	Myosin head (motor domain)
AUT1/APG3	Y55F3AM.4		5.2e-26*	?
AUT2/APG4	ZK792.8		9.8e-19*	?
AUT7APG8/CVT5	C32D5.9	*lgg-1*	5e-32*	?
	ZK593.6	*lgg-2*	2.6e-19*	Microtubule-associated Protein
AUT10/CVT18	F41E6.13		1.4e-36*	?
CVT9	F59A2.6		5.9e-11*	?
CVT17	F23F12.9		0.37	?
PRB1	C51E3.7	*egl-3*	0.034	?
TOR1	B0261.2	*let-363*	2.2e-252*	Inositol/ phosphatidylinositol kinase
VAC8	F26B1.3	*ima-2*	2.7e-12*	Nuclear-membrane associated protein
VAM3	ZC155.7		2e-06	?
VAM7	C15C7.1		0.0053	?
VPS11	R06F6.2		1.8e-15*	Vacuolar biogenesis Protein
VPS15	ZK930.1		8.9e-69*	Serine/threonine kinase
VPS16	C05D11.2		4.6e-15*	?
VPS33	B0303.9	*slp-1*	1.3e-10*	?
VPS34/CSC1/ END13	B0025.1	*let-512*	6.7e-106*	Phosphatidylinositol 3-kinase
VPS38	T22C1.6		0.00042	?
VPS39	T08G5.5		1.3e-11*	?
VPS41	F32A6.3		6e-16*	?

C. elegans orthologs showing a significant sequence homology are indicated by an asterisk.

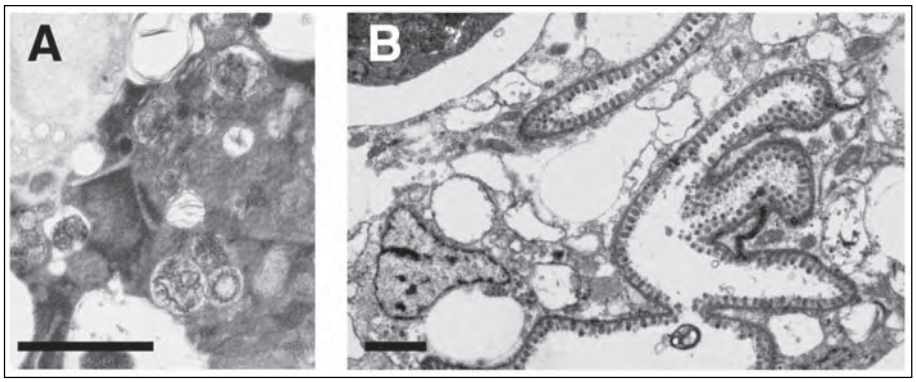

Figure 5. A. Several active lysosomes in an embryo following inactivation of the *apg6* ortholog by RNAi. B. A portion of the gut epithelial cell from the TOR mutant with a lot of glycogen, lipid and little functional cytoplasm without autophagic vacuoles. Scale bars are 1 μm.

Surprisingly, our further characterization of *let-512/vps34* null mutants using the allele *h510* revealed a strongly significant increase in autophagic vacuole formation in the gut epithelial cells of the arrested L4 stage larvae to a level of over 1% of the cytoplasmic volume.[12] This strongly suggests that the absence of LET-512/VPS34, which is required for vesicular transport from the nucleus toward the cell periphery is compatible with intense autophagy in *C. elegans* intestinal cells. Consistently, inactivation of the *C. elegans apg6* ortholog by RNAi results in a strong autophagic response in the adults (Fig. 4A) as well as in the embryonic phase of development (Fig. 5A). Whereas yeast *APG6* and *VPS34* are known to be essential for autophagy,[14] their *C. elegans* counterparts T19E7.3 ORF and LET-512, respectively, appear to be dispensable, raising the requirement of important stimulatory contribution by another factor(s).

One of the most compelling lines of evidence showing differences in regulation of autophagy between yeast and *C. elegans* comes from the study of the TOR proteins. In yeast, TOR is a key regulator of autophagy: Starvation conditions generate an inhibitory signal that blocks the TOR kinase leading eventually to the activation of the autophagic cascade.[15] In *C. elegans*, however, the *CeTor* null mutant animals, *let-363(h111)*,[12] which arrest growth and development at the L3 larval stage are completely devoid of autophagic vacuoles (Fig. 5B).

Future Perspectives

Our results show that *C. elegans* is a tractable model system to study the mechanism and regulation of autophagy in a metazoan organism. Its genome contains a highly conserved set of the yeast autophagy genes. Possibly many of the *C. elegans* autophagy genes cannot be revealed by simple BLAST searches using yeast sequences, thus genetic screens should be performed to isolate new components of the autophagy machinery operating in *C. elegans*. This multicellular model organism appears to be suitable to find those genetic factors involved in autophagy that make it different from the unicellular yeast system.

Acknowledgements

This work was supported by an OTKA grant (T033047) to A.L.K. and T.V. who also want to express their gratitude to A. Fodor for his initial help and encouragement in working with *C. elegans* and to M. Saródy and L. Kovács for excellent technical assistance.

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CHAPTER 18

Role of Autophagy in Developmental Cell Growth and Death: Insights from *Drosophila*

Thomas P. Neufeld

Abstract

During development in *Drosophila*, larvae increase in mass by 1,000-fold over the course of a few days. This high rate of growth is controlled by TOR, a potent regulator of both protein synthesis and autophagy. At metamorphosis, most larval tissues are histolyzed through autophagy-mediated cell death in response to high levels of the steroid hormone 20-hydroxyecdysone. Other tissues are dramatically remodeled into adult structures at this time, and autophagy again plays a role. This chapter describes the occurrence and function of autophagy in *Drosophila* development, emphasizing the regulatory roles of TOR and ecdysone signaling.

Introduction

Division of development into distinct embryonic, larval and pupal stages has been an enormously successful strategy for a large number of insect species. In the fruit fly *Drosophila melanogaster*, embryogenesis is characterized by rapid cell proliferation and differentiation; the larval stage is specialized for feeding and exponential growth; and during the pupal stage the organism undergoes metamorphosis into the adult. This division of labor requires substantial reorganization and elimination of cells and tissues during transitions from one stage to the next. Furthermore, dependence of organismal growth on resources obtained by feeding during the larval stage requires mechanisms for surviving periods of nutrient shortage. In this chapter I describe the role of autophagy in these processes, and the potential use of *Drosophila* as a model system to learn more about the regulation of autophagy during development.

Steroid Hormone-Mediated Autophagic Cell Death and Remodeling

Insects are capable of resculpturing their bodies on a massive scale, and many of these remodeling projects occur through autophagy. For example, in worker honeybees not destined to become queens, the obsolete ovaries degenerate through autophagic cell death in response to pheromones emitted by the queen.[1] In many insect species, flight muscles of adult females are autolyzed to allocate more resources toward egg production.[2] However, the most extreme examples of autophagic recycling and cell turnover occur during metamorphosis. In holometabolous insects (those that undergo complete metamorphosis), larvae are comprised of two fundamentally distinct cell types: Diploid imaginal cells, which are destined to give rise to adult structures, and large polyploid larval cells, whose role is to support and nourish the imaginal tissues. Metamorphosis occurs during the pupal stage, at which time most of the larval cells are histolyzed to make way for and provide nourishment to the differentiating cells of the adult.

Autophagy, edited by Daniel J. Klionsky.

Ultrastructural studies in the 1960s and 1970s revealed that an early step in the destruction of most larval tissues is the accumulation of large numbers of autophagic vacuoles (presumably autophagosomes; see chapter 2);[3-5] hence this process was classified as type II (autophagic) cell death, as distinguished from type I (apoptotic).[6] More recent analyses have blurred this distinction somewhat, as aspects of both autophagic and apoptotic deaths have been shown to occur in the same cell (see chapter 23). For example, cells in the degenerating prothoracic and salivary glands of the tobacco hornworm *Manduca sexta* display clear signs of autophagy, yet also become TUNEL-positive with highly condensed chromatin.[7,8] Histolysis of salivary gland and midgut cells in *Drosophila* is also autophagic in appearance, yet recent studies have found that these cells upregulate expression of the apoptotic activators *grim*, *reaper*, and *hid*, and the caspase *dronc*.[9] Furthermore, overexpression of the caspase inhibitor P35 was shown to prevent degradation of these tissues.[9,10] Caspase inhibition was found to completely prevent DNA fragmentation but had only a limited, though significant, effect on autophagy. Interestingly, the mammalian apoptotic regulator DAPK was recently shown to induce autophagic cell death.[11] More study is needed to clarify the relationship between these types of cell death.

The signal that triggers entry into metamorphosis is a high titer pulse of the steroid hormone 20-hydroxyecdysone (ecdysone), which also controls molting and feeding behavior earlier in larval development.[12] Ecdysone is capable of inducing autophagic death in isolated tissues in vitro,[4,9] indicating that its degradative effects are direct. Interestingly, different tissues appear to become competent to respond to ecdysone at different times in development. For example, the midgut initiates histolysis almost immediately after pupation, whereas salivary gland degeneration begins 12-14 hours later.[9] The fat body, which serves as a storage source of lipid, glycogen and protein akin to the vertebrate liver, initiates autophagy prior to pupation in response to increasing levels of circulating ecdysone,[13] which also induce a "wandering" behavior as the larva prepares to pupate. Fat body cells also generate numerous protein storage granules at this time, through endocytosis of hemolymph protein. These protein granules appear to eventually fuse with autophagic granules, indicative of the close link between the endocytic and autophagic pathways (see chapter 2).[13] Degeneration and death of the fat body occurs gradually and continuously throughout the pupal phase, over which time cell volume is greatly reduced (fourfold decrease in cross-sectional area).[14] Starvation during the larval phase can also induce autophagy and massive cell shrinkage in the fat body.[15]

Transcriptional responses to ecdysone are mediated through induction of a set of "early" genes including *BR-C*, *E74A* and *E93,* which encode transcription factors that in turn regulate expression of "late" or effector genes (e.g., *reaper* etc.). A fourth gene, *bFTZ-F1*, appears to act as a competence factor for the early genes, and its expression governs the timing of various responses to ecdysone.[16] Mutations in any of these four genes impair salivary gland degeneration during metamorphosis. *E93* appears to play an especially important role, as mutations in this gene cause an early block in autophagy induction, and ectopic *E93* expression can induce cell death in other tissues.[10] Although transcription of cell death regulators is altered in these mutants, the target genes regulating autophagy are not known. Post-transcriptional responses to ecdysone may also influence autophagy, as phosphorylation of lysosomal acid phosphatases is induced by ecdysone.[17]

Finally, it should be noted that not all ecdysone-induced autophagy leads to cell death. Malpighian tubules, a form of nephric system in many insects, are extensively remodeled by autophagy during metamorphosis to support differing physiological needs of the adult.[18]

Regulation of Autophagy by TOR and Nutrient Signaling

TOR Mediates Cell Growth and Metabolism in Response to Nutrient Levels

Rapamycin is a potent inducer of autophagy in a number of cell types including mammalian hepatocytes, kidney cells and myotubes, as well as yeast.[19-22] Rapamycin acts by binding to and inhibiting the large serine-threonine kinase TOR, whose inactivation leads to a number of

cellular phenotypes that mimic the effects of starvation, including cell cycle arrest in the G_0/G_1 phase, reduced protein synthesis, a specific transcriptional profile, and autophagy (see chapters 3 and 5).[23] Inhibition of Tor in yeast also leads to specific degradation of a number of proteins including the tryptophan permease Tat2 and the translation initiation factor eIF4G.[24,25] In higher eukaryotes, TOR is required for phosphorylation of the translational regulators p70S6 kinase and 4EBP in response to insulin and other growth factors. Thus TOR proteins regulate cell growth and metabolism at multiple levels. The similar effects of TOR inactivation and starvation strongly suggest a role for TOR in nutrient or energy sensing, although the mechanisms by which TOR activity is regulated are not fully understood.

Mutations in *Drosophila* TOR also phenocopy the effects of nutrient withdrawal, including a cell-type specific cell cycle arrest, changes in fat body morphology, and reduced nucleolar size.[26,27] TOR mutants become arrested in development during the larval phase just prior to the period of exponential growth period, and like wild type larvae deprived of amino acids, will survive as small larvae for several weeks. Cell size in TOR mutant animals is severely reduced, a phenotype also caused by mutations in components of the insulin/phosphatidylinositol 3-kinase pathway. Biochemical and genetic interaction studies confirm the requirement for TOR in insulin signaling. In addition, they demonstrate that p70S6 kinase is a physiologically relevant target of TOR, since overexpression of p70S6 kinase can partially alleviate TOR mutant phenotypes.[26] Finally, TOR mutants also display a striking upregulation of the lysosomal content of tissues such as the fat body and gut during larval development (Fig. 1). These results are consistent with a conserved role for TOR in inhibiting autophagy in *Drosophila*, although this has not yet been confirmed by ultrastructural or biochemical studies.

Mechanism of Autophagy Regulation by TOR

Induction of autophagy in response to rapamycin or starvation is rapid and does not require protein synthesis,[28,29] indicating that modification of existing factors rather than de novo synthesis is key. In yeast, Tor signaling has been shown to regulate autophagy through interactions with a complex comprised of Apg1, Apg13, and Apg17 (see chapters 6 and 7).[30] Tor causes hyperphosphorylation of Apg13 during nutrient-rich conditions, which prevents its association with Apg1. Starvation or rapamycin treatment results in rapid dephosphorylation of Apg13, increasing its affinity for Apg1, whose protein kinase activity is increased upon Apg13 binding. Interestingly, this pathway from Tor to autophagy is independent of Tap42, an essential mediator of most other Tor functions.

Although Apg1 has readily identifiable orthologs in *Drosophila* and other higher eukaryotes,[31,32] Apg13 and Apg17 in most cases do not, suggesting that regulation of autophagy by TOR may occur through other mechanisms in multicellular organisms (see chapter 17). Signaling through the p70S6 kinase-S6 branch of the TOR pathway has been shown to be tightly correlated with inhibition of autophagy in cultured hepatocytes, leading to the proposal that phosphorylation of ribosomal protein S6 is directly inhibitory to autophagy, perhaps through a mechanism of competition between protein synthesis and autophagy for free endoplasmic reticulum.[20] Although compelling, these experiments did not demonstrate a causative role for S6 phosphorylation in autophagy, and it is possible that the correlation between S6 phosphorylation and autophagy merely reflects the fact that both are regulated by TOR, perhaps independently.

In *Drosophila*, mutations in p70S6 kinase delay development and lead to reduced body size,[33] but metamorphosis occurs normally, indicating that induction of autophagic cell death and remodeling do not require modulations in p70S6 kinase signaling. Furthermore, transgenic expression of constitutively active p70S6 kinase also does not disrupt metamorphosis.[26] Thus as in yeast, TOR may use an independent pathway to regulate autophagy. This can be directly tested through measurements of autophagy rates in *Drosophila* p70S6 kinase and ribosomal protein S6 mutants.

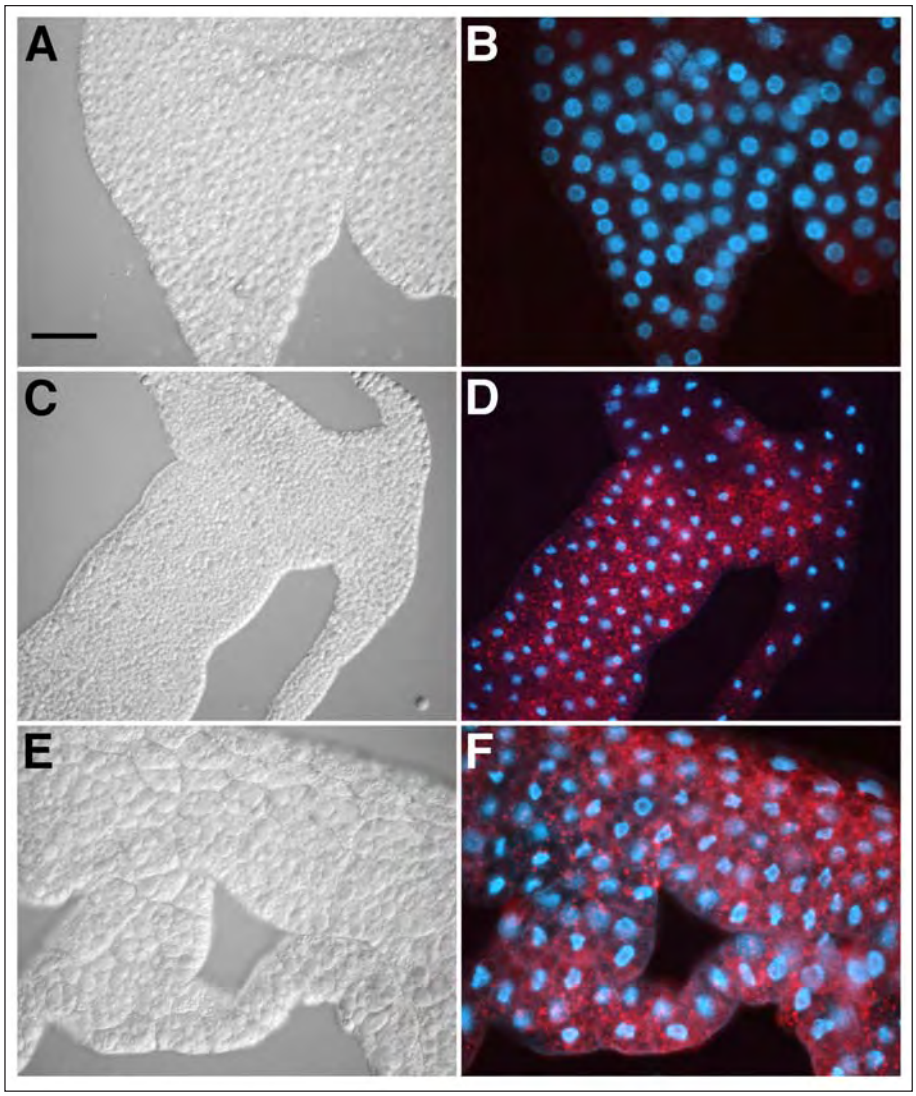

Figure 1. Lysosomal activity increases in response to TOR loss of function and starvation. Nomarski (A, C, E) and corresponding fluorescent (B, D, F) images of larval fat body from fed wild type (A, B), fed TOR mutant (C, D) and 24 hr starved wild type (E, F). Nuclei (blue) and acidic lysosomes labeled with LysoTracker (red) are shown. Note the increase in lysosomal staining in TOR mutant and starved wild type fat bodies. Note also the reduced nuclear size of *Drosophila* TOR fat body cells, resulting from a block in endoreplication. Scale bar in (A) represents 50 micrometers for all panels.

Role of Autophagy in TOR Signaling and Cell Growth

Inactivation of TOR signaling, either genetically or via rapamycin treatment, causes a profound inhibition of cell growth in a variety of cell types. To what degree does induction of autophagy account for this effect? Much of the effort in addressing the mechanism of TOR-mediated growth control in higher eukaryotes has focused on the well-known anabolic

effects of TOR signaling, particularly stimulation of protein synthesis through activation of p70S6 kinase and eIF4E. However, measurements of protein synthesis in rapamycin-arrested cells have shown that bulk translation is reduced by only ~10-30% following mTOR inactivation in mammalian cells.[20,34] Thus, suppression of autophagy may be a predominant means be which TOR promotes cell growth. Experiments in *Drosophila* in which autophagy is blocked genetically in a TOR mutant background may provide a productive avenue to pursue this question.

More generally, the role of autophagy in controlling cell growth during development is largely unknown. Is autophagic degradation critical only to allow survival during starvation, or could it also function to regulate rates of mass accumulation in normally developing organisms? Treatments that stimulate tissue growth, such as partial hepatectomy,[35] refeeding following starvation,[36] and growth factor addition,[37] cause a reduction in the rate of autophagy below that of untreated cells, whereas growth-suppressive signals such as contact inhibition[38] and substrate detachment[39] can induce autophagy. A comparative study of protein synthesis, retention, and degradation rates in the livers of adult and neonatal mice found that reduced protein turnover accounted for the majority of the difference between growing and non-growing liver.[40] The identification of a human Apg6 homolog, Beclin 1, as a tumor suppressor also provides evidence for a role for autophagy in growth control (see chapters 15 and 20).[41] Together, these results suggest that modulation of autophagy rates may be an important mechanism in regulating cell growth. Systematic clonal inactivation of a number of autophagy genes in *Drosophila* (see below), followed by careful analysis of the effects on cellular growth rates, will provide a rigorous test of this thesis.

Relationships between TOR and Ecdysone in Control of Autophagy

Since autophagy can be regulated by either ecdysone or nutrition/TOR signaling at different stages of fly development, it will be interesting to dissect the connections between these pathways. For example, one can postulate a simple mechanism in which ecdysone induces autophagy during metamorphosis by downregulating TOR activity. While direct evidence for such a mechanism is lacking, it is worth noting that steroid hormone signaling has been shown to inhibit TOR in rat L6 myoblasts.[42] In addition, since pupae are encased and thus not feeding during metamorphosis, reduced nutrient levels may play a role in ecdysone-mediated autophagy during metamorphosis. Alternatively, TOR and ecdysone could signal independently to the core autophagic machinery, at either the same or different entry points.

Although a role for TOR in ecdysone-induced autophagy is speculative, TOR signaling has been shown to play a critical role in ecdysone synthesis. Ecdysone is produced in the prothoracic gland in response to a brain neuropeptide, prothoracicotropic hormone (PTTH). In the prothoracic gland of *Manduca*, PTTH strongly induces phosphorylation of ribosomal protein S6, and stimulates synthesis of a small number of specific proteins.[43] Addition of rapamycin was found to prevent both the phosphorylation and protein synthesis responses to PTTH, and to strongly inhibit ecdysteroidogenesis. In *Drosophila*, certain hypomorphic mutant alleles of *Drosophila* TOR arrest development just prior to metamorphosis, despite reaching a size normally sufficient for pupation.[26,27] This unusual arrest point may reflect a requirement for high levels of TOR signaling for ecdysone synthesis in the prothoracic gland.

Conservation of Core Autophagic Machinery

Orthologs of many of the genes required for autophagy in yeast have been identified in *Drosophila* and other higher eukaryotes (see ref. 44 and Table 1), and recent studies indicate that a role in autophagy has been conserved for at least some of these genes. For example, Apg5-deficient mouse embryonic stem cells (see chapter 15) are unable to form autolysosomes (autophagosomes fused with lysosomes; see chapter 2) in response to amino acid starvation, and bulk protein degradation is greatly reduced.[45] Likewise, expression of Beclin 1, the human homolog of Apg6, can restore normal levels of autophagy to *APG6*-disrupted yeast, as well as to

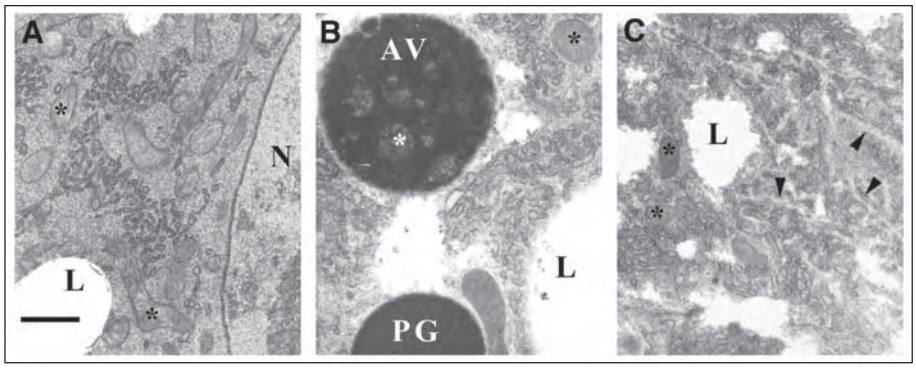

Figure 2. Aut1 expression is required for autophagy in larval fat body. Electron micrographs showing fat body cells from third instar (L3) feeding larva (A), L3 larva in the wandering stage (B), and L3 wandering larva lacking Aut1 (C). Loss of Aut1 expression prevents the appearance of autophagic granules normally induced during the wandering stage. Note the accumulation of microtubules (arrowheads in C) in fat bodies lacking Aut1. L, lipid droplet; N, nucleus; AV, autophagic vacuole (autophagosome); PG, protein granule; asterisks, mitochondria. Scale bar in (A) represents one micrometer for all panels. Figure courtesy of G. Juhasz.

human breast carcinoma cells lacking Beclin 1 expression (see chapters 15 and 20).[41] Moreover, mammalian Aut7, Apg5, and Apg12 localize to autophagosomes, and display biochemical interactions similar to those described in yeast.[45-47] In *Drosophila*, a transgenic RNA interference approach was used to show that Aut1 (see chapters 6 and 7) is required for autophagy in fat body cells of late stage wandering larvae (Fig. 2), and its absence causes lethality during the late larval and pupal stages.[47a] Using microarray analysis, it was also found that expression of *Drosophila* Aut7 (see chapters 6 and 7) homologs was strongly induced in larval fat body undergoing autophagy (G. Juhasz, O. Komonyi, P. Maroy, L. Puskas, M. Sass, personal communication).

Although the basic cell biological functions of the core autophagic machinery thus appear to have been conserved in higher eukaryotes, regulation of these factors is likely to be more complex than in yeast, given the multiple modes of autophagy induction. It will be of interest to determine whether autophagy caused by starvation, hormones, or death-inducing signals use the same, overlapping, or completely different sets of cellular regulators. As discussed above, regulation of autophagy by TOR is likely to differ between yeasts and metazoans as well, since genes related to yeast Apg13 and Apg17 have not been identified in most higher eukaryotes.

Mutations in Apg homologs in *Drosophila* will provide useful tools to address these issues. P element insertions in a number of such genes have been identified (Table 1). Analysis of the lethal phases of these mutants finds that they span the range from viable to larval or pupal lethal (R. Scott and T.N., unpublished). These differences may be explained in a number of ways. Trivially, the weak phenotype (viability) may reflect the action of weak alleles; P elements insertions commonly produce hypomorphic mutant alleles. However, at least one of the viable mutants, $Apg4^{P0997}$, has been shown to be a null allele.[48] Disruption of autophagy in *Drosophila* may not result in obvious phenotypic consequences under normal growth conditions, as in yeast; if this is indeed the case, the observed lethal phenotypes may indicate that some of these genes have pleiotropic functions beyond regulation of autophagy. In this regard, it is notable that *Drosophila* Apg4 was identified through its genetic interactions with the Notch signaling pathway,[48] and human Beclin/Apg6 associates with the apoptosis regulator Bcl-2.[41] Alternatively, the phenotypic range of these mutants may reflect the roles of these genes in different stages of the autophagic process, or in different forms of autophagy (i.e., autophagic cell death versus starvation-induced autophagy). Apg4, Aut7 and Aut10 each have two

Table 1. Yeast genes involved in autophagy and related genes in flies

Yeast Gene	Fly Gene	P Element	Lethal Phase
Apg1/Aut3	CG10967	ep3348	pupal lethal
Apg2	CG1241		
Apg5	CG1643		
Apg6/Vps30	CG5429	l(3)00096	pupal lethal
Apg7/Cvt2	CG5489		
Apg9/Aut9/Cvt7	CG3615		
Apg10	CG12821		
Apg12	CG10861		
Aut1/Apg3	CG6877		
Aut2/Apg4	CG4428	P0997	viable (ref. 48)
	CG6194		
Aut7/Apg8	CG1534	ep362	viable
	CG12334		
Aut10/Cvt18	CG7986	KG03090	larval lethal
	CG8678	KG02403	viable

closely-related orthologs in flies (Table 1), consistent with the development of specialized functions following duplication of these genes. Interestingly, the non-lethal P element insertions listed in Table 1 all map to these genes, suggesting the possibility of redundant functions.

Summary

Lysosome- and endosome-mediated functions, whose study has long been the domain of cell biologists, have recently begun to capture increased attention from the developmental biology community. From the effects of transcytosis on morphogen gradient formation to the internalization and activation of ligand-receptor complexes, the importance of these cell biological functions to metazoan development is gaining recognition.[49,50] The vast collection of eye color mutants in *Drosophila*, a number of which have recently been found to result from defects in lysosomal delivery,[51] are likely to provide additional tools with which to analyze these processes. Studies in model organisms such as *Drosophila* should increase our understanding of the developmental roles and mechanisms of autophagy and other lysosomal processes.

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CHAPTER 19

Trafficking of Bacterial Pathogens to Autophagosomes

William A. Dunn, Jr., Brian R. Dorn and Ann Progulske-Fox

Abstract

Bacteria have evolved a variety of mechanisms to subvert the eukaryotic defenses and survive intracellularly. Many bacterial pathogens have been shown to establish an intracellular niche for survival and replication by lysing the phagosome and entering the cytosol, by suppressing the maturation of the phagosome into a phagolysosome, or by trafficking to autophagosomes that fail to mature into autolysosomes. A subset of intracellular pathogens, including *Brucella abortus*, *Legionella pneumophila*, and *Porphyromonas gingivalis*, are diverted from phagosomes to autophagosomes where the pathogen presumably modifies this compartment to establish an environment necessary for its replication. In this chapter, we will examine the biochemical and morphological evidence for the survival of bacterial pathogens within an autophagosome-like compartment.

Introduction

Many bacteria that invade eukaryotic cells have evolved mechanisms to evade degradation and survive intracellularly.[1,2] Bacterial pathogens enter the host cells by phagocytosis, being incorporated into a phagosome. Some pathogens such as *Actinobacillus actinomycetemcomitans*, *Listeria monocytogenes*, *Rickettsia*, and *Shigella* lyse the phagosome and enter the cytoplasm to replicate.[3,4] Other intracellular pathogens such as *Mycobacterium tuberculosis* and *Salmonella typhimurium* reside within the phagosome that has been modified by the bacterium to prevent fusion with the lysosome and acquisition of hydrolytic enzymes.[1] A third group of bacteria that include *Brucella abortus*, *Legionella pneumophila*, and *Porphyromonas gingivalis* leave the phagocytic pathway to enter the autophagic pathway where they replicate within vacuoles that resemble autophagosomes.[5-7] In this chapter, we will examine the relationship between these intracellular pathogens and autophagy.

Autophagy

Recent data from a number of laboratories suggest a convergence between phagocytic and autophagic pathways (Fig. 1). Autophagy is a cellular pathway for the degradation of organelles and cellular components. This lysosomal process is responsible for the selective removal of "nonfunctional" or "damaged" cellular components that occurs when cells are stressed or are remodeling.[8] In addition, autophagy is essential for the nonselective degradation of cellular proteins to maintain cellular amino acid levels for protein synthesis (reviewed in references 9 and 10). Autophagy is constitutively active, but is dramatically enhanced when cells are starved for amino acids or growth factors.[10,11] In fact, under conditions whereby protein synthesis is suppressed, autophagy is enhanced, suggesting that these pathways are inversely regulated.

Autophagy, edited by Daniel J. Klionsky.

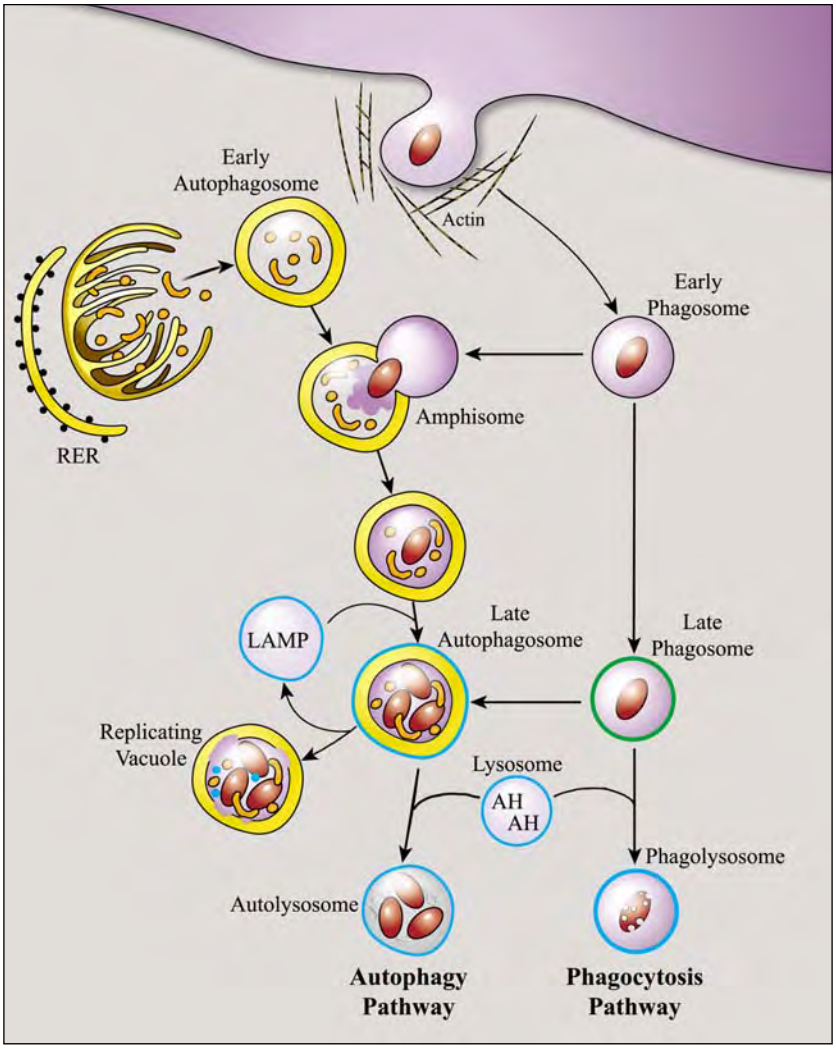

Figure 1. Model for bacterial trafficking in eukaryotic cells. Upon cellular invasion, the bacterium is contained within an early phagosome formed by an invagination of the plasma membrane that is mediated by actin polymerization events. The vacuole quickly acquires early endosome markers such as Rab5. The early phagosome then matures into a late phagosome having late endosome markers such as Rab7 and Rap1. The late phagosome subsequently acquires acid hydrolases (AH) by fusing with a lysosome. The bacterium is then destroyed within the resulting phagolysosome. In the case of *P. gingivalis*, *B. abortus*, and *L. pneumophila*, the phagosome may fuse with autophagosomes instead of maturing into the phagolysosome. The early autophagosome may form from cisternae of the rough endoplasmic reticulum (RER). This vacuole can fuse with an endosome or an endosome-like vacuole such as the phagosome resulting in a vacuole referred to as an amphisome. The early autophagosome or amphisome then matures into a late autophagosome by acquiring the vacuolar H^+-ATPase and lysosomal associated membrane proteins (LAMP). Finally, the late autophagosome fuses with a lysosome thereby becoming a degradative organelle known as an autolysosome. Based on marker proteins, it appears that *B. abortus* and *P. gingivalis* replicate within a replicating vacuole derived from a late autophagosome that has lost the V-ATPase and LAMP-1, while *L. pneumophila* appears to replicate within an autolysosome. If autophagy is suppressed, these bacteria will remain within the phagocytic pathway whereby they are destroyed and degraded within phagolysosomes.

Furthermore, FRAP/mTOR, a phosphatidlyinositol kinase-like protein kinase that stimulates the phosphorylation of the ribosomal protein S6, has been shown to enhance protein synthesis while suppressing autophagy (see chapter 5).[12,13] In addition to starvation stress, autophagy is also stimulated in cells stressed by heat, toxins (e.g., sulindac sulfide and ricin), or intracellular pathogens, suggesting a protective role in cell survival.[14-18]

The events of autophagy are depicted in Figure 1. The nascent or early autophagosome is a vacuole bound by two or more membranes that may be derived from cisternae of the rough endoplasmic reticulum and/or a unique structure referred to as a phagophore (see chapter 2).[19-22] This vacuole containing sequestered organelles (e.g., ribosomes, mitochondria, peroxisomes, and other vesicles) and cytosol acquires vacuolar H^+-ATPase (V-ATPase) and lysosomal associated membrane protein-1 (LAMP-1) and matures into an acidic late autophagosome.[23] The late autophagosome acquires hydrolytic enzymes by docking and fusing with a lysosome thereby becoming an autolysosome.[23,24] The sequestered cytoplasmic material is then degraded, and the resulting breakdown products reutilized for the synthesis of new cellular components. The characterization of the molecular events required for the formation and maturation of the autophagosome is essential for our understanding of the regulation of autophagy. A number of yeast proteins required for autophagy have been characterized and their mammalian homologues identified (see chapters 6, 7 and 15).

Trafficking of Bacteria within Eukaryotic Cells

Bacteria are initially internalized into phagosomes, which mature by dynamic interactions with endosomal compartments through a series of steps that include budding and fusion events controlled by Rab GTPases, and ultimately fuse with a lysosome becoming a phagolysosome.[25-27] The harsh acidic environment maintained within the phagolysosome promotes death and degradation of the microbe thereby providing the host a defense against such intracellular pathogens. Therefore, in order to survive within the host cell, the bacterial pathogen must either interfere with the normal maturation of the phagosome to the phagolysosome or exit this pathway. Some intracellular pathogens survive and replicate within the phagosome or a phagosome-like vacuole by preventing the fusion events required for the acquisition of hydrolytic enzymes and maturation into a phagolysosome. Such is the case for *Salmonella typhimurium* which traffics to a late endosome-like vacuole that does not mature into a phagolysosome.[28] *Mycobacterium tuberculosis* survives in non-acidic phagosomes that lack the V-ATPase proton pump.[29] Meanwhile, some bacteria exit the phagocytic pathway to be incorporated into vacuoles that resemble autophagosomes. These bacteria include: *Porphyromonas gingivalis*, *Brucella abortus*, and *Legionella pneumophila*.

As noted above, bacteria are initially internalized into phagosomes, which interact with endosomes, thereby maturing into vacuoles having the characteristic endosomal markers.[26] We refer to these vacuoles as either early or late phagosomes, containing early or late endosome markers, respectively (Fig. 1). The late phagosome acquires lysosomal proteinases, thus becoming a phagolysosome wherein the pathogen is destroyed. *P. gingivalis*, *B. abortus*, and *L. pneumophila* are rapidly incorporated into early endosome-like vacuoles containing Rab5.[7,30,31] However, these vacuoles do not acquire late endosome markers such as Rap1 and mannose 6-phosphate receptor or phagolysosomal markers such as cathepsin L. Instead, these bacteria are found in vacuoles that contain the autophagosomal marker, monodansylcadaverine, or proteins that are associated with autophagosomes (e.g., BiP, protein disulphide isomerase, calnexin, and LAMP-1) or HsGsa7, a protein that is required for the formation and expansion of autophagosomes (see chapters 6, 7 and 15).[14] Like the autophagosome, some of these vacuoles are bound by two or more membranes (Fig. 2C). In addition, these vacuoles contain organelles and cytosol that presumably had been sequestered by the autophagosome (Fig. 2).

The presence of these bacteria in vacuoles that resemble autophagosomes suggests that the phagocytic and autophagic pathways converge. Indeed, the convergence between endocytic and autophagic pathways has been documented.[20,32-34] Although endosomes may be sequestered into autophagosomes, it is likely this convergence is due to the fusion of endosomes with

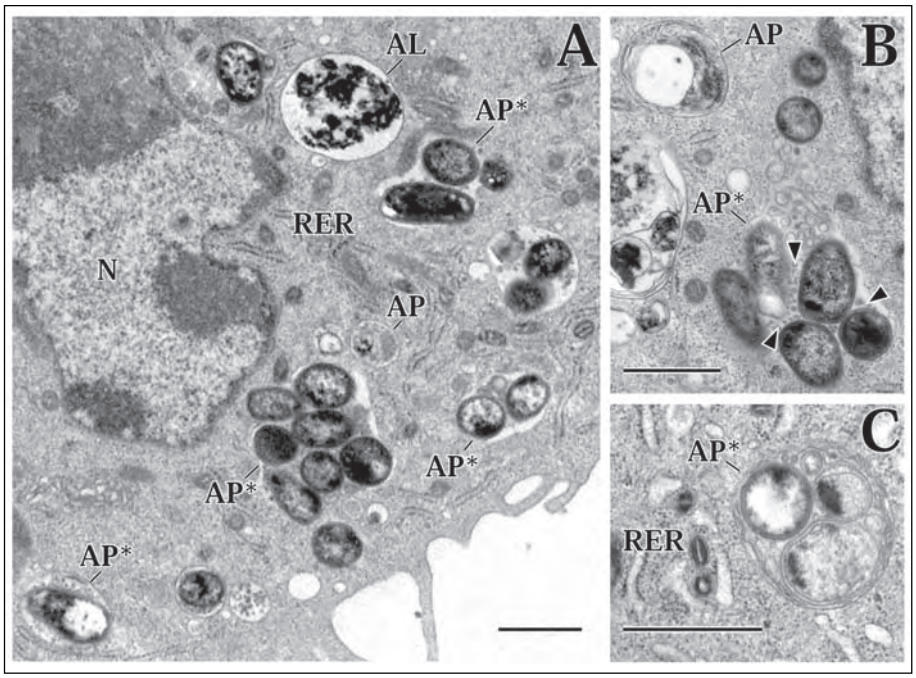

Figure 2. *P. gingivalis* infection of human coronary artery endothelial cells. Human coronary artery endothelial cells were cultured in the presence of *P. gingivalis*. Profiles of autophagosomes with (AP*) and without (AP) *P. gingivalis* can be observed within infected cells. These vacuoles contain vesicular organelles and undegraded cytosol. Some of the vacuoles that contain *P. gingivalis* are bound by two or more membranes, which are characteristic of early autophagosomes (panel C). Secretory blebs presumably containing gingipains (endopeptidases) can be observed associated with bacteria within the autophagosomes (arrowheads in panel B). AP, autophagosome; AL, autolysosome; M, mitochondria; N, nucleus; RER, rough endoplasmic reticulum. Bar = 1.0 μm.

autophagosomes. Data suggest that endosomes fuse primarily with early autophagosomes and to a lesser extent with late autophagosomes and autolysosomes.[32,33] These autophagosomes that contain endocytosed proteins have been referred to as amphisomes (see chapter 2).[32] The fusion of endosomes with autophagosomes and mixing of their contents provides an avenue for the transport of bacteria to the autophagosome.

L. pneumophila appears to have the same trafficking characteristics in a variety of cell types.[35-37] However, the intracellular trafficking for *B. abortus* and *P. gingivalis* appears to be cell specific. For example, *B. abortus* traffics to the autophagosomes in epithelial cells but not in macrophages.[38] *P. gingivalis* invades the autophagosomes of endothelial and smooth muscle cells, but not of oral epithelial cells.[7,39]

Autophagic Response to Bacterial Pathogens

It is not known if the bacteria promote autophagy to make available a replicative niche or if autophagy is a cellular response to the stress of the bacterial invasion. Because both *P. gingivalis* and *L. pneumophila* utilize short peptides as carbon and energy sources, it would be advantageous for these bacteria to activate the autophagic pathway thereby forming a vacuole containing endogenous proteins that can be utilized by these bacteria for growth. *L. pneumophila* utilizes a type IV secretion system that is encoded by the *icm*/*dot* (intracellular multiplication/defect in organelle trafficking) complex to transduce a cis-acting signal to establish the replica-

tive vacuole.[40-43] The type IV secretion system in *B. abortus* is also essential for its survival within an autophagosome-like vacuole.[44,45] *dot* and *icm* mutants fail to establish residence in an autophagosome-like compartment and instead enter the phagocytic pathway.[46] Current models postulate that proteins are delivered from the bacteria via this secretion system directly to the host cell cytoplasm.[47] It is possible that one or more secreted proteins could initiate the autophagic response thereby providing the permissive environment for bacterial replication.[36] An alternative scenario is that the secreted proteins suppress the trafficking of the bacterium to the phagolysosome. In this case, the infected cell would respond by activating an alternative pathway to deliver the intracellular pathogen to the lysosome (i.e., autophagy). Indeed, cells are capable of activating autophagy in response to many forms of stress such as radiation, drugs, and viral pathogens.[16,48-51] Therefore, a cell compromised with an intracellular pathogen would likely respond by activating autophagy to destroy the foreign entity.

Our data indicate that *P. gingivalis* may promote its entry into the autophagic pathway by enhancing the autophagic response in these cells. We have observed an increase in the occurrence of autophagosomes coincident with infection (unpublished observations) and a change in the cellular distribution of HsGsa7 in infected cells that is comparable to that seen in starved cells undergoing autophagy.[14] When autophagy is suppressed in fed cells, HsGsa7 localizes to the Golgi apparatus. Upon removal of amino acids and serum to stimulate autophagy, HsGsa7 is located to vesicles throughout the cell. In cells infected with *P. gingivalis*, HsGsa7 is also localized to vesicles suggesting autophagy is enhanced. However, when the infected cells were treated with wortmannin, an autophagy inhibitor, HsGsa7 was found in the Golgi compartment. Interestingly, neither *P. gingivalis* nor *B. abortus* survive intracellularly when autophagy is suppressed by wortmannin or 3-methyladenine.[7,52-55] This is likely due to the bacterium being trafficked to phagolysosomes and destroyed. To support this, we have shown that in the presence of wortmannin, *P. gingivalis* resides in vacuoles that contain the lysosomal proteinase cathepsin L.[7]

Bacterial Replication within an Autophagosome-Like Vacuole

During autophagy, the early autophagosome is likely formed from the rough endoplasmic reticulum and acquires the lysosomal associated membrane protein-1 (LAMP-1, also known as lysosomal glycoprotein 120 (lgp120)) and the V-ATPase while retaining rough endoplasmic reticulum proteins. The resulting late autophagosome then matures to an autolysosome by acquiring acid hydrolases that degrade the inner membrane and the constituents of the compartment.[23] *L. pneumophila* appears to transit from the phagosome to the autophagosome and eventually to the autolysosome, which is acidic and contains LAMP-1 and cathepsin D.[56] It is apparent that this pathogen is capable of surviving not only the acid environment but also the hydrolytic enzymes present in this lysosomal compartment. Meanwhile, *B. abortus* and *P. gingivalis* appear to replicate in vacuoles that are distinct from autophagosomes and autolysosomes. *B. abortus* replicates in a unique vacuole (termed a replicating vacuole) bound by a single membrane that lacks the lysosomal proteins, LAMP-1 and cathepsin D, but maintains endoplasmic reticulum proteins, sec61b and calnexin.[30] We have characterized a similar compartment for the replication of *P. gingivalis* and propose that it originated from the late autophagosome.[7] The data suggest that *B. abortus* and *P. gingivalis* somehow prevent the late autophagosome from acquiring lysosomal hydrolases but promote the recycling or loss of LAMP-1 (and V-ATPase). Indeed, we have suggested that the mannose 6-phosphate receptor cycles between the autophagosome and the Golgi apparatus.[57]

Bacterial Subversion of Autophagy

Bacterial trafficking to the autophagic pathway may provide not only a protective niche from the host's defense mechanisms but also a means to supply peptide substrates for their metabolic pathways. The availability of peptides and amino acids would be especially beneficial to *P. gingivalis* and *L. pneumophila*, which utilize these compounds as carbon and energy

sources.[58-60] Therefore, these bacteria may be subverting a normal eukaryotic process to derive their energy needs for reproduction.

Because the vacuoles containing *P. gingivalis* do not appear to contain host cell proteinases, this bacterium would rely on its own proteinases (e.g., gingipains) to break down the host proteins that had been sequestered within the autophagosome. The delivery of these proteinases occurs by vesicle secretion.[61,62] Such vesicles can be seen associated with the surface of *P. gingivalis* in autophagosomes (arrowheads in Fig. 2). These endopeptidases may provide the means by which the bacteria can degrade host cell proteins, thereby providing themselves with the peptides necessary for their survival. In addition, the membrane structure of the late autophagosome or replicating vacuole will provide protection for the host cell. In contrast, *L. pneumophila*, which lacks endogenous proteinases, appears to rely on the proteinases of the host cell to provide the necessary peptides and amino acids for growth. These scenarios are consistent with *P. gingivalis* and *L. pneumophila* residing in compartments that lack or contain host proteinases, respectively (Fig. 1). By utilizing the autophagic pathway, these pathogens are exposed to nutrients in the form of host cell proteins that are essential for growth of these pathogens.

Summary

Some pathogenic bacteria are able to invade cells and escape the destructive environment of the lysosome. Many of these bacteria survive within vacuoles derived from phagosomes while others including *P. gingivalis*, *B. abortus*, and *L. pneumophila* survive in vacuoles that appear to be derived from autophagosomes. The autophagosome-like compartment provides an environment for the survival and proliferation of *P. gingivalis* and *B. abortus*. This compartment lacks hydrolytic enzymes but contains host cell proteins that had been sequestered within the autophagosome, which will provide carbon and energy sources needed by *P. gingivalis*. The data suggest that these bacteria may secrete proteins that promote an autophagic response within the host cell. If the bacterial secretion system is rendered inactive by mutations or if autophagy is suppressed by drugs, these bacteria are ultimately destroyed within phagolysosomes.

It is possible that other intracellular pathogens may also utilize the convergence of the phagocytic and autophagic pathways for survival. The bacterial and mammalian signals that stimulate autophagy and promote residence of the pathogen within the permissive organelle have yet to be identified. A better understanding of these intracellular pathogens and their ability to traffic to autophagosomes will not only lead to their use as biological tools to study the regulation and events of autophagy but also to further our knowledge of the mechanisms of pathogenesis thereby advancing better therapeutics.

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CHAPTER 20

Autophagy and Cancer

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Abstract

Cancer is a complex multigenic disorder involving the perturbation of several different pathways that regulate cell differentiation, cell proliferation and cell survival. In theory, the process of macroautophagy (herein referred to as autophagy) may protect against cancer by sequestering damaged organelles, permitting cellular differentiation, increasing protein catabolism, and promoting autophagic cell death. Alternatively, autophagy may contribute to cancer by promoting the survival of nutrient-starved tumor cells. While there is some experimental evidence to support each of these possibilities, recent advances in understanding the molecular mechanisms of autophagy favor a model in which autophagy contributes to tumor suppression and in which defects in autophagy contribute to oncogenesis. Biochemical evidence in mammalian cells and genetic evidence in *C. elegans* suggests that autophagy is positively regulated by the *PTEN* tumor suppressor gene and negatively regulated by the oncogenic Class I phosphatidylinositol 3-kinase signaling pathway. Furthermore, the mammalian *APG* gene, *beclin 1*, has tumor suppressor activity and maps to a tumor susceptibility locus that is commonly deleted in human breast and ovarian cancers. The genetic disruption of autophagy control in human cancer provides new insights into understanding molecular mechanisms of oncogenesis. Such insights may foster the development of novel approaches to restore autophagy in the chemoprevention or treatment of human malignancies.

Introduction

Recent advances in elucidating the genetics, biochemistry, and cell biology of autophagy in yeast (see chapters 6 and 7) are expected to open new frontiers in research on the role of mammalian autophagy in cancer biology. Since several of the yeast autophagy genes (e.g., *APG1*, *APG3*, *APG4*, *APG5*, *APG6*, *APG7*, *APG8*, *APG12*) have mammalian orthologs, genetic approaches can now be used to directly assess the role of autophagy genes in the regulation of tumorigenesis. If bi-allelic mutations in autophagy genes are found in human cancers and/or if autophagy gene disruption in mice increases susceptibility to tumorigenesis, such data will provide important evidence that autophagy is a tumor suppressor pathway. As-of-yet, however, such studies have not been completed, and there is no definitive genetic proof for a role of autophagy genes in cancer biology.

While genetic proof for a role of autophagy in tumor suppression is lacking, there are, however, several other lines of evidence that support the hypotheses that autophagy plays a role in negative growth control and that autophagy deregulation contributes to tumorigenesis. These include: (1) inverse associations between levels of autophagic activity and malignant transformation; (2) inhibitory effects of oncogenic signaling pathways on autophagy; (3) tumor suppressor activity of the mammalian autophagy gene, *beclin 1*; and (4) death-promoting activity of autophagy in response to ionizing radiation and certain chemotherapeutic agents. On the other hand, there is also evidence that autophagic activity may increase in tumor cells, perhaps as a survival adaptation to high-density or low nutrient conditions. This chapter will summa-

Autophagy, edited by Daniel J. Klionsky.

rize the relevant data for each of these areas and will highlight the important questions that need to be addressed with further research.

Autophagic Activity in Normal and Malignant Cells

Autophagy is an evolutionarily conserved pathway that is triggered in both yeast and mammalian cells by nutrient deprivation and by high-density conditions (see chapters 3-7). In response to these stimuli, cell growth becomes arrested concurrently with increased proteolysis of long-lived proteins and cellular organelles by the autophagolysosomal pathway. While multiple cellular biochemical events have been identified that regulate starvation-induced cell growth arrest and contact-dependent growth inhibition, several investigators have postulated that increased autophagic protein degradation also plays a contributory role.[1-3] The role of autophagy in negative control of cell growth in these settings is thought to relate directly to its effects on the balance between protein synthesis and protein degradation. A fundamental aspect of cell growth is the increase in cell mass, which is a representation of the quantity of cellular protein. Because the accumulation of cellular protein represents a balance between the rates of cellular protein synthesis and protein degradation, increased long-lived protein degradation through autophagy is a potential mechanism of negative control of cell growth. Conversely, decreased autophagic protein degradation is a potential mechanism of deregulated growth in cancer cells.

In this context, it is noteworthy that starvation or high-density-induced autophagy is diminished in some cell lines transformed in vitro as well as in some malignant cells derived from primary tumors. Reduced rates of long-lived protein degradation following amino acid deprivation or confluent culture conditions have been observed in chemically transformed rat hepatocytes and SV40-transformed mouse fibroblasts as compared to rates observed in nontransformed control cells.[1,4,5] In addition, MCF7 human breast carcinoma cells do not increase long-lived protein degradation following amino acid starvation,[6] and rat hepatocytes derived from primary rat hepatomas or ascites hepatoma have diminished rates of long-lived protein degradation as compared to normal hepatocytes.[3,7] Furthermore, in a rat chemical heptaocellular carcinogenesis model, a linear relationship was observed between stage of malignancy and reduced autophagic activity.[3]

While these data are consistent with the concept that decreased autophagy in malignant cells contributes to deregulated cell growth, there are two major limitations of this interpretation. First, these data are only correlative and do not address whether there is a direct cause and effect relationship between decreased autophagy and tumorigenesis. It is possible that decreased autophagy is an epiphenomenon that arises as a result of malignant transformation rather than a fundamental cellular process that plays a primary role in tumorigenesis. Second, other studies have failed to find decreases in nutrient deprivation-induced autophagy in transformed human fibroblasts,[8] in transformed human bronchial epithelial cells,[9] in colon carcinoma cells,[10] in HeLa cell lines,[11] in murine teratocarcinoma cell lines,[12] and in lymphocytes from leukemic patients.[13] Thus, decreased starvation-induced autophagy is not a universal property of malignant cells.

Considering these limitations, the associations between decreased autophagy and malignant transformation are of uncertain significance in the pathogenesis of cancer. In fact, a contrasting model has also been proposed in which increases in autophagy may accompany malignant transformation, and promote the survival of tumor cells that are subjected to low oxygen and nutrient-limited growth conditions.[14] Rez et al found that cellular autophagic capacity is highly increased in azaserine-induced premalignant atypical acinar nodule cells.[15] Houri et al have found that human colon cancer cells, which can survive for a long duration in the absence of nutrients, are characterized by a high rate of autophagy,[10] and we have found that the expression of the Beclin 1 autophagy protein is increased in a subpopulation of human hepatocellular carcinomas associated with hepatitis B and hepatitis C virus infections (W. Noguery-Irizary and B. Levine, unpublished data). It is not possible, however, to conclude from these studies whether increased autophagy in certain malignant cells merely represents an adaptive response

to a nutrient-poor environment or whether it plays a biologically important role in the survival and outgrowth of neoplastic cells.

In summary, there is evidence that autophagic activity may either be decreased, unchanged, or increased in malignant cells. To the extent that autophagy may be involved in negative growth control and in cell death, decreases in autophagy could contribute to tumor development or progression. To the extent that autophagy may allow tumor cells to adapt to nutrient poor environmental conditions, increases in autophagy could also contribute to tumor progression. These two possibilities are not mutually exclusive, and increases or decreases in autophagy may contribute differentially to tumors that arise in different tissues or to different stages of tumorigenesis. For example, as noted above, in a chemical liver carcinogenesis rat model, hepatocyte autophagic activity decreases with malignant progression,[3] but the reverse is observed in a chemical carcinogenesis pancreatic carcinoma rat model.[16] The precise roles of autophagy deregulation in different stages of tumorigenesis and in different types of tumors will need to be elucidated through genetic studies involving loss-of-function mutations in autophagy-regulatory and autophagy-execution genes.

Signaling Pathways that Regulate Oncogenesis and Autophagy

The dual regulation of autophagy and oncogenesis by similar upstream signaling pathways provides further evidence for functional links between autophagy deregulation and cancer. Not only have there been important recent advances in identifying genes required for autophagy, there has also been significant progress in the past decade in identifying signaling molecules that either positively or negatively regulate autophagy in yeast and in mammalian cells (see chapters 3-7). Many of the molecules that negatively regulate mammalian autophagy are involved in mitogenic signaling and oncogenesis (e.g., phosphatidylinositol (PtdIns) 3-kinase, Akt, mTOR, p70S6 kinase). Conversely, molecules that positively regulate mammalian autophagy have established (e.g., PTEN) or possible links (e.g., eIF2α kinases) to tumor suppression.

Class I PtdIns 3-kinase is a plasma-membrane associated-kinase that converts PtdIns(4)P and PtdIns(4,5)P_2 to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, respectively, which bind to pleckstrin homology (PH) domains of Akt (also known as protein kinase B) and its activator PDK-1 (phosphoinositide-dependent kinase-1). Akt and PDK-1 phosphorylate several other protein kinases including p70S6 kinase and mTOR (reviewed in refs. 17-19). PTEN is a dual protein/lipid phosphatase that dephosphorylates the 3' position of the Class I PtdIns 3-kinase products and consequently, downregulates the PtdIns 3-kinase/Akt pathway (see Fig. 1 for schematic of pathway; reviewed in ref. 20).

Both genetic and functional data indicate that the Class I PtdIns 3-kinase signaling pathway is involved in oncogenesis, and that PTEN is involved in tumor suppression.[20] These data include evidence that: (1) the catalytic subunit of Class I PtdIns 3-kinase is overexpressed in ovarian cancers;[21] (2) Akt genes are amplified or overexpressed in several human cancers, including gastric, ovarian, breast, pancreatic, and prostate cancer;[22] (3) mammary carcinogenesis is enhanced in certain mouse models by constitutively active Akt;[23] (4) Akt is upregulated in many tumors with mutations in *PTEN* (reviewed in ref. 24); (5) biallelic genetic alterations in *PTEN* are frequently detected in glioblastomas (23-44%), endometrial cancers (35-50%), metastatic protstate cancers (35%), malignant melanomas (43%) and less commonly in breast cancers (4-6%) (reviewed in ref. 24); and (6) *PTEN*$^{+/-}$ heterozygous mice develop neoplasms in multiple organs including the endometrium, liver, prostate, gastrointestinal tract, thyroid, and thymus.[24,25]

It has been commonly assumed that the mechanisms by which the Class I PtdIns 3-kinase signaling pathway promotes oncogenesis involves the induction of cell growth in response to mitogenic signals, the inhibition of apoptosis, or the promotion of angiogenesis. Likewise, it has been commonly assumed that PTEN exerts its tumor suppressor effects by antagonizing these functions of Class I PtdIns 3-kinase signaling. However, new evidence is emerging indi-

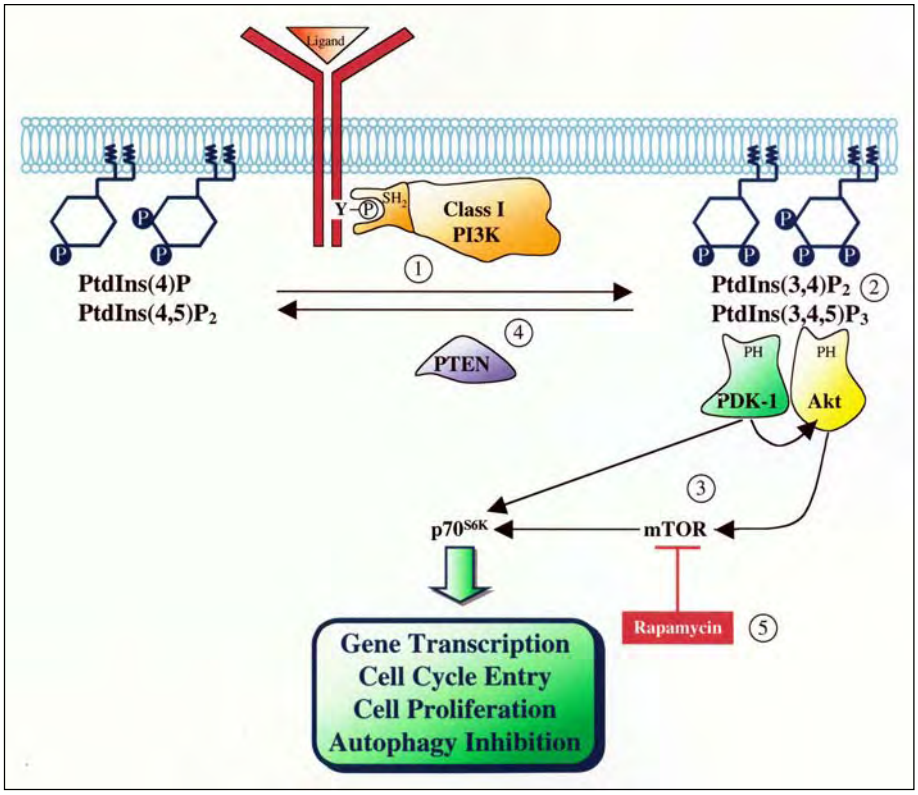

Figure 1. The Class I PtdIns 3-kinase signaling pathway and autophagy. (1) Class I PtdIns 3-kinase activated by receptor protein-tyrosine kinase, converts PtdIns(4)P and PtdIns(4,5)P_2 to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, respecitively. (2) Akt and its activator PDK-1 bind to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 via pleckstrin homology (PH) domains. (3) Phosphorylation of p70S6 kinase by PDK-1 (or mTOR) and of mTOR by Akt stimulate oncogenesis and inhibit autophagy. (4) PTEN dephosphorylates the 3' position of class I PtdIns 3-kinase products and consequently downregulates the PtdIns 3-kinase/Akt pathway. (5) Rapamycin, a macrolide drug that inhibits mTOR kinase, is a potent inducer of autophagy and exerts antitumor effects in cells with aberrant activation of Akt or inactivation of PTEN.

cating that negative regulation of autophagy may also be an important function of the oncogenic PtdIns 3-kinase signaling pathway that is inhibited by the PTEN tumor suppressor. Petiot et al found that an increase in Class I PtdIns 3-kinase products caused by either feeding cells with synthetic lipids or by IL-14 activation of PtdIns 3-kinase enzymatic activity results in decreased rates of autophagy in human colon cancer HT-29 cells.[26] Overexpression of wild type PTEN, but not a phosphinositide phosphatase mutant of PTEN, counteracts the IL-13 down regulation of autophagy in these cells,[27] indicating that PTEN counteracts the autophagy inhibitory effects of Class I PtdIns 3-kinase. In addition, a dominant negative mutant of Akt increases autophagy rates and a constitutively active form of Akt decreases autophagy rates in HT-29 cells,[27] suggesting a critical role for the Akt oncogene in the negative regulation of autophagy. Together, these data demonstrate that, in addition to stimulating other pathways involved in oncogenesis, the Class I PtdIns 3-kinase signaling plays an inhibitory role in mammalian autophagy that is counteracted by the PTEN tumor suppressor gene, at least in HT-29 cells. Further studies are needed to evaluate the role of Class I PtdIns 3-kinase signaling and PTEN in autophagy regulation in other cell types.

Transformation mediated by Class I PtdIns 3-kinase and Akt is thought to be mediated by mTOR and ribosomal p70S6 kinase (reviewed in ref. 20), which are two protein kinases that play well-established roles in both translational control and autophagy. The activating phosphorylations of p70S6 kinase by PDK-1 (or mTOR) and of mTOR by Akt are involved both in the stimulation of translation as well as in the inhibition of autophagy (see chapters 3 and 4). A mutant of Akt that retains kinase activity but does not induce phosphorylation of p70S6 kinase or activation of 4E-BP-1 by mTOR fails to transform chick embryo fibroblasts, suggesting a correlation between the oncogenicity of Akt and the activation of mTOR-p70S6 kinase signaling.[22] The inhibitor of mTOR kinase, rapamycin, effectively blocks transformation induced by either Class I PtdIns 3-kinase or Akt but does not reduce the transforming activity of 11 other oncoproteins.[22] Furthermore, mTOR inhibition with rapamycin reduces neoplasia in tumors that arise in *PTEN*-deficient mice,[28,29] and correlations have been observed between tumor cell sensitivity to rapamycin and Akt activation.[30]

Although oncogenic versions of mTOR and p70S6 kinase have yet to be identified in human malignancies, both molecules are likely to play a role in oncogenesis. The role of mTOR in oncogenesis is suggested by the links between mTOR and upstream oncogenic signals such as PtdIns 3-kinase and Akt as well as the anti-tumor effects of the mTOR inhibitor, rapamycin. The role of p70S6 kinase in oncogenesis is suggested by the amplification and overexpression of p70S6 kinase in breast cancers,[31,32] the increased activation of p70S6 kinase in *PTEN*-deficient cells,[28,29] and the role of constitutive activation of mTOR-p70S6 kinase signaling in the transformation of human pancreatic cancer cells.[33] Interestingly, studies of loss-of-function mutants in *Drosophila* indicate that both dTOR and dp70S6 kinase play roles in determining organ and organism size,[34,35] suggesting an evolutionarily conserved role of these molecules in cell growth control.

Given the dual role of the Class I PtdIns 3-kinase signaling pathway (including PtdIns 3-kinase and the downstream molecules Akt, mTOR, and p70S6 kinase) in both oncogenesis and autophagy inhibition, the possibility arises that activation of this signaling pathway may promote oncogenesis through a mechanism that involves reduction of autophagy. However, in addition to mTOR, Class I PtdIns 3-kinase signaling has many different downstream targets that play known or potential roles in oncogenesis (reviewed in refs. 18, 20). mTOR also regulates many different cellular functions and aspects of cell growth besides its inhibitory effects on autophagy (reviewed in ref. 36). At present, there are no data that directly address whether autophagy inhibition by Class I PtdIns 3-kinase, Akt, mTOR, or p70S6 kinase plays a role in the oncogenicity of any of these molecules. Similarly, there are no data that address whether autophagy stimulation by PTEN plays a role in its tumor suppressor action. Further studies to dissect the role of autophagy regulation by the Class I PtdIns 3-kinase signaling pathway in oncogenesis are therefore a high research priority.

To address whether autophagy genes are required for growth arrest phenotypes that are negatively regulated by the Class I oncogenic PtdIns 3-kinase signaling pathway, we used a genetic approach involving *C. elegans*. In *C. elegans*, the Class I PtdIns 3-kinase signaling pathway is similar to that found in mammalian cells, and loss-of-function mutations in this pathway promote a developmental arrest phenotype known as dauer (see chapter 17).[37-39] Of note, dauer is stimulated physiologically in *C. elegans* by nutrient starvation and overcrowding,[40] two conditions that promote autophagy in yeast and mammalian cells. The dauer phenotype induced by loss-of-function mutations in the Class I PtdIns 3-kinase signaling pathway can be blocked by a loss-of-function mutation in the *C. elegans* ortholog of PTEN.[41,42] Thus, the *C. elegans* Class I PtdIns 3-kinase pathway negatively regulates dauer entry and the *C. elegans* PTEN positively regulates dauer entry, suggesting that dauer entry in *C. elegans* is regulated in a parallel manner to growth inhibition in mammalian cells.

We recently found that dauer formation in *C. elegans* with a loss-of-function mutation in the insulin-like tyrosine kinase receptor, *daf-2*, (which is upstream of the PtdIns 3-kinase, *age-1*) requires the *C. elegans* orthologs of the yeast autophagy genes, *APG1* (also known as *C.*

elegans unc-51), *APG6* (also known as *Ce beclin-1*) and *APG8* (A. Melendez and B. Levine, unpublished data). These findings provide genetic evidence that functions regulated by autophagy genes are downstream inhibitory targets of the evolutionarily conserved oncogenic PtdIns 3-kinase signaling pathway. Furthermore, they demonstrate that, at least in the *C. elegans* model, autophagy genes are required for a growth arrest phenotype negatively regulated by a signaling pathway that is also involved in mammalian oncogenesis. Such findings further highlight the need to investigate the role of autophagy deregulation in mammalian oncogenesis that results from aberrant activation of the Class I PtdIns 3-kinase signaling pathway.

In addition to the Class I PtdIns 3-kinase signaling pathway, there are other autophagy-regulatory signaling pathways that may have relevance to cell growth control and cancer biology. In contrast to mTOR-p70S6 kinase signaling which positively regulates translation and inhibits autophagy in response to mitogenic stimuli, the eIF2α kinase signaling pathway plays an evolutionarily conserved role in translational arrest (reviewed in refs. 43-45) and autophagy induction in response to starvation and other forms of cellular stress.[46] The relationship between eIF2α kinase signaling and cancer is far less established than that discussed above for the Class I PtdIns 3-kinase signaling pathway and cancer. Nonetheless, certain observations raise the possibility that eIF2α kinase signaling may play a role in negative growth control. These include the observations that (1) overexpression of catalytically inactive mutants of the interferon-inducible eIF2α kinase, PKR, transform NIH3T3 cells;[47] (2) overexpression of a nonphosphorylatable mutant of eIF2α transforms NIH3T3 cells;[48] (3) PKR promotes apoptosis under certain stress conditions (reviewed in refs. 49, 50); and (4) some correlations exist between higher levels of PKR expression in tumors and favorable prognosis.[51] Arguing against a role for this pathway in cancer, tumors have not been observed in *PKR*$^{-/-}$ mice[52] and there is no genetic evidence thus far of alterations in *PKR* or other eIF2α kinases in human malignancies. Nonetheless, in view of the translational inhibitory and autophagy stimulatory effects of eIF2α kinase signaling, coupled with the transforming effects of in vitro blockade of this pathway, more extensive studies should be performed to evaluate whether the eIF2α kinase signaling pathway plays a role in tumor suppression.

GTP-binding proteins of the Ras superfamily provide another possible link between autophagic control and cellular events involved in oncogenesis. Both the trimeric $G_{\alpha i3}$ protein and the regulator of trimeric G proteins, GAIP (G_{α}-interacting protein), are important in the control of autophagy in human colon cancer cells (see chapter 3). $G_{\alpha i3}$ inhibits autophagy when it is associated with GTP and stimulates autophagy when it is bound to GDP.[53] GAIP promotes autophagy by accelerating the rate of GTP hydrolysis by $G_{\alpha i3}$.[54] In contrast to trimeric GTPases, less is known about the role of the Rab and Rho families of monomeric GTPases in autophagy control. However, the Rho family of GTPases are involved in cell cycle progression and tumorigenesis (reviewed in refs. 55, 56) and a recent observation in yeast indicates a possible role in autophagy regulation. A large-scale yeast two-hybrid experiment demonstrated that the yeast autophagy gene product, Apg17, binds to Rho1 and Rho2 GTPases,[57] although the significance of this interaction is not yet known.

Furthermore, the small GTPase, Ras, an important oncogene that is activated by mutation in approximately 25% of all tumors, is thought to promote cellular transformation, at least in part, through activation of the PtdIns 3-kinase/Akt pathway (reviewed in ref. 18). Several studies suggest that PtdIns 3-kinases can be activated by direct interaction with Ras.[58-60] Furthermore, PtdIns 3-kinase/Akt activation is essential for anchorage-independent growth and cytoskeletal reorganization associated with Ras-dependent transformation,[61] suppression of c-Myc-induced apoptosis by Ras,[62] and the aberrant survival of Ras-transformed detached epithelial cells.[63] Given the role of the PtdIns 3-kinase/Akt signaling pathway in autophagy inhibition and the importance of Ras activating mutations in human cancers, it will be of interest to determine whether autophagy is another downstream effector function that is regulated by Ras through the PtdIns 3-kinase/Akt signaling pathway.

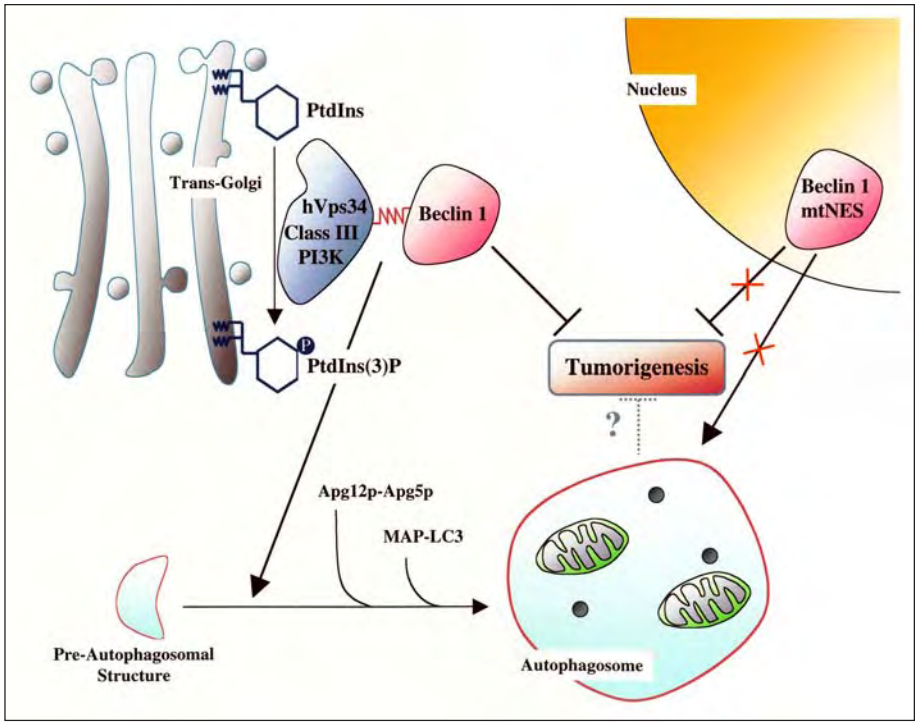

Figure 2. The Class III PtdIns 3-kinase complex, hVPS34 and Beclin 1, and autophagy. Beclin 1, a mammalian homologue of yeast Vps30/Apg6, forms a complex with the class III PtdIns 3-kinase, hVPS34, and localizes in the trans-Golgi network. This complex is thought to be essential for the localization of other Apg proteins to the pre-autophagosomal structure, and consequently, for autophagosome formation. Beclin 1 also has tumor suppressor function, which may be due to its effects on autophagy or alternative mechanisms. Mutation of the leucine-rich nuclear export signal of Beclin 1 (Beclin 1 mtNES) blocks its nucleocytoplasmic transport, and its autophagy, and tumor suppressor function.

Beclin 1 a Genetic Link between Tumor Suppressor and Autophagy Pathways

As noted above, the negative regulation of autophagy by the oncogenic Class I PtdIns 3-kinase signaling pathway and the positive regulation of autophagy by the PTEN tumor suppressor raise the possibility that genetic disruption of autophagy control may contribute to oncogenesis. This concept is further supported by observations linking functional inactivation of one of the downstream autophagy effector genes, *beclin 1*, to mammary tumorigenesis.

Beclin 1, the mammalian homologue of yeast Apg6/Vps30 (see chapter 6, 7 and 15), is a 60 kDa coiled-coil protein that was originally identified in a yeast two-hybrid screen for novel Bcl-2-interacting proteins.[64] Beclin 1 has been shown to complement autophagy in yeast disrupted in the *beclin 1* ortholog, *APG6/VPS30*, and to promote starvation-induced autophagy in MCF7 human breast carcinoma cells.[6,65] Beclin 1 localizes to the trans-Golgi network,[66] and in both yeast and mammalian cells, is part of a Class III PtdIns 3-kinase complex thought to be important in mediating the localization of other Apg proteins to the pre-autophagosomal structure (see Fig. 2 for schematic of pathway).[66-68]

The *beclin 1* gene maps to a tumor susceptibility locus on chromosome 17q21 that is monoallelically deleted in 40-75% of the cases of sporadic breast and ovarian cancer (reviewed

in ref. 69), raising the possibility that it may be a tumor suppressor. However, biallelic inactivations of *beclin 1* have not been demonstrated in human cancers and therefore as-of-yet, the gene does not fulfill genetic criteria for classification as a tumor suppressor. In a mutational analysis of breast cancer cell lines with net *beclin 1* deletions, no mutations were found in the coding regions or splice junctions of remaining alleles.[69] Additionally, Northern blot analysis did not identify abnormalities in *beclin 1* transcripts.[69]

Although biallelic mutations of *beclin 1* have not been demonstrated in human cancer, three lines of evidence suggest that functional inactivation of *beclin 1* may contribute to mammary tumorigenesis (Fig. 3). First, Beclin 1 protein is expressed ubiquitously in all normal breast epithelial cells, but frequently has low or undetectable expression in malignant breast epithelial cells.[6] Second, the autophagy-promoting effects of *beclin 1* gene transfer in MCF7 human breast carcinoma cells are associated with tumor suppressor function. Inducible expression of wild type Beclin 1, but not an autophagy-defective nuclear export signal mutant of Beclin 1, in MCF7 cells results in the loss of malignant morphologic properties, decreased rates of cell proliferation, impaired clonigenicity in vitro, and impaired ability to form tumors in nude mice.[6,65] Third, tamoxifen, an anti-estrogen compound that is widely used in the chemoprevention and treatment of breast cancer, induces Beclin 1 expression and autophagy in MCF7 cells (Fig. 3F-G).

Together, these findings suggest that decreased expression of the Beclin 1 autophagy protein may contribute to the development and/or progression of breast carcinoma and that pharmacologic induction of Beclin 1 expression in breast carcinoma cells may be clinically beneficial. Further studies are required to determine whether mutations are found in both alleles of *beclin 1* in human tumors; whether *beclin 1* demonstrates haploinsufficiency for tumor suppression; what mechanisms are responsible for decreased Beclin 1 expression in malignant breast epithelial cells; whether the mechanism by which *beclin 1* acts as a tumor suppressor is through its effects on autophagy; whether *beclin 1* inactivation contributes to tumorigenesis of tissues other than breast and ovary; and whether *beclin 1* represents a useful drug target for cancer therapy. In addition, it will be important to investigate whether other mammalian orthologs of yeast autophagy genes are functionally inactivated in human malignancies.

Autophagic Cell Death and Cancer

Although autophagy represents a mechanism by which cells adapt and survive in nutrient-limiting conditions, autophagy also may directly contribute to programmed cell death, and in this context, have additional relevance to cancer biology. Based upon morphologic criteria, programmed cell death has been traditionally classified either as Type I which is "classical" apoptosis or Type II which is autophagic death (reviewed in refs. 70,71; see chapter 23). In classical apoptotic cell death, there is cell rounding, membrane blebbing, cytoskeletal collapse, cytoplasmic condensation and fragmentation, chromatin condensation and fragmentation, and formation of membrane-bound apoptotic bodies. A characteristic feature of apoptotic death is the preservation of cytoplasmic organelles until late in the process. In contrast, in autophagic cell death, there is an early accumulation of cytosolic autophagic vacuoles that is accompanied by degradation of organelles, including Golgi complex, polyribosomes, endoplasmic reticulum, and mitochondria. Unlike apoptotic death, cytoskeletal elements are largely preserved, presumably because of their role in autophagocytosis.

Despite these distinctions, there may also be considerable morphologic, biochemical, and/or genetic overlap between apoptosis and autophagy in programmed cell death. In some cases, apoptotic and autophagic cell death morphologies coincide in vivo in certain tissues[72] and less commonly, in the same cell.[73-75] Using genetic approaches to studying programmed cell death in insect metamorphosis, Lee and Baehrecke demonstrated that the steroid-inducible gene E93 is both required for the autophagic destruction of larval salivary glands during metamorphosis and sufficient to trigger hallmark features of apoptosis, indicating that autophagic and apoptotic death share common regulatory mechanisms.[76] In mammalian tissue culture studies of trophic

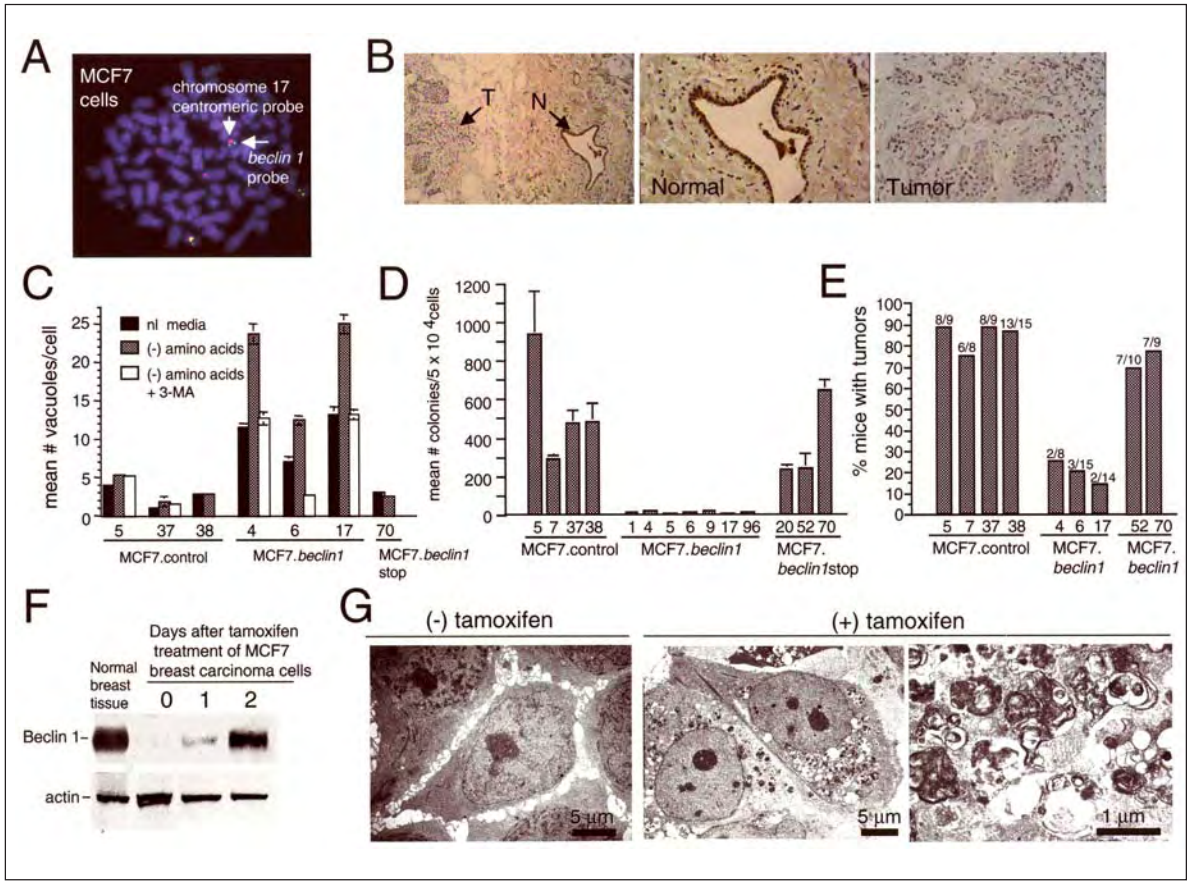

Figure 3. Links between the *beclin 1* autophagy gene and the negative regulation of mammary tumorigenesis. (A) FISH analysis of MCF7 breast carcinoma cells, demonstrating net deletion of the *beclin 1* allele (adapted with permission from ref. 6). (B) Immunohistochemical analysis of Beclin 1 expression in normal and malignant breast epithelial cells in a case of sporadic invasive breast carcinom. Loss of Beclin 1 protein expression is observed in the malignant breast epithelial cells (right panel) (adapted with permission from ref. 6). (C, D, E) Effects of *beclin 1* gene transfer on MCF7 starvation-induced autophagy (C), in vitro clonigenicity (D), and in vivo tumorigenicity in nude mice (E) (MCF7.*beclin1*stop clones are MCF7 cells transfected with a *beclin 1* mutant containing a stop codon at nucleotide position 270) (adapted with permission from ref. 6). (F) Induction of Beclin 1 expression in MCF7 cells by treatment with tamoxifen (1 μM). (G) Induction of autophagy in MCF7 cells by treatment with tamoxifen (1 μM). Images shown are for cells treated for 48 hours.

factor deprivation of sympathetic neurons and tumor necrois factor treatment of T-lymphoblastic leukemic cells, features of autophagy are observed in apoptotic cells and treatment with the autophagy inhibitor, 3-methyladenine, inhibits or delays apoptosis.[73,74]

One candidate cellular signal for the overlap between apoptosis and autophagy is the mitochondrial membrane permeability transition, since it both regulates cytochrome C release and caspase activation during apoptosis (reviewed in ref. 77) and also has been shown to initiate autophagy in rat hepatocytes.[78,79] In addition, the Ca^{2+}/calmodulin-regulated Ser/Thr death kinases, DAPk and DRP-1, are necessary for both bleb formation in apoptotic cells and for the formation of autophagic vesicles during autophagic cell death,[80] indicating that certain morphologic features of apoptotic and autophagic death are regulated by common upstream signaling molecules. Conversely, the inhibition of apoptosis and autophagy can be regulated by common upstream signaling pathways, as the autophagy inhibitory PtdIns 3-kinase/Akt signaling pathway discussed above also inhibits apoptosis (for reviews, see refs. 19,81,82). Furthermore, it is possible that the Apg proteins themselves are involved in both autophagy and apoptosis, since the mammalian homolog of Apg6, Beclin 1, was originally isolated as a Bcl-2-interacting protein.[64] However, no studies have yet been published that evaluate the functional role of mammalian Apg proteins in apoptosis.

The involvement of autophagy in programmed cell death, and potentially, in apoptosis, suggests that the death-promoting effects of this process (like those of apoptosis) may play a role in tumor suppression and in the death response of tumor cells to ionizing radiation and chemotherapy. A role for autophagic cell death in tumor suppression is only speculative at present, although at the morphologic level, autophagic cell death has been shown to predominate in spontaneous regression of neuroblastomas[83] and in hormone-dependent mammary tumor regression.[84]

While the role of autophagic cell death in the prevention of tumorigenesis is unknown, there are some data demonstrating that ionizing radiation and chemotherapeutic agents induce autophagic death of tumor cells. Ionizing radiation induces caspase-independent autophagic death of human tumor cell lines of epithelial cell origin, including breast, colon, and prostate,[85] as well as autophagic death of human glioblastoma cell lines.[86] Autophagic cell death has also been observed in human prostate cancer cell lines treated with ligands of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ),[87] in malignant human glioma cells treated with arsenic (I. Germano, personal communication) or lymphokine-activated killer cells,[88] in rat ascites tumor cells treated with vinblastine,[89] and in human breast carcinoma cell lines treated with tamoxifen alone[90,91] or combinations of epirubicin, medroxyprogesterone acetate, and tamoxifen.[92] Besides ionizing radation and chemotherapy, there is one report of a gene therapy approach that induces autophagic tumor cell death; expression of an oncogenically mutated *ras* gene triggers caspase-independent autophagic death of human glioma and gastric cancer cell lines.[93]

These data suggest that autophagy induction may be a mechanism by which, at least in part, certain known cancer therapies exert their anti-tumor effects. Since many cancer cells have evolved mechanisms to evade apoptosis (for review, see ref. 94), an alternative, apoptosis-independent, death autophagic pathway may be a particularly useful target in such settings. For example, MCF7 cells lack a functional caspase 3[95] and are resistant to certain triggers of apoptotic death,[96] but, as noted above, are sensitive to radiation and tamoxifen-induced autophagic death.[85,90] Thus, in the treatment of cancers that have specific alterations in apoptosis regulation, it may be essential to induce autophagic death for effective elimination of tumor cells.

Conversely, it is possible that some cancer cells have evolved mechanisms to evade autophagic cell death, and in such cases, it will be necessary to either target apoptotic death pathways (if they are intact) or develop new approaches to restore autophagic cell death pathways. For example, if autophagy induction proves to be involved in the anti-tumor effects of rapamycin, the efficacy of rapamycin would be expected to be reduced in tumors with functional inactivation of Beclin 1 or other autophagy execution genes that are downstream of TOR. This limita-

tion could be bypassed by therapeutic manipulations that restore autophagy gene expression, such as tamoxifen treatment in the case of breast carcinoma. Therefore, in the clinical trials that are presently in progress to determine molecular markers of rapamycin-responsiveness of different patients' tumors, it will be important to assess the status of autophagy genes downstream of TOR.

The participation of autophagy in programmed cell death of cancer cells is in seeming contradiction to the fundamental role of autophagy as a cellular adaptive and survival response. Further studies need to be done to elucidate the factors that determine whether autophagy promotes the survival or the death of the host cell and such factors will need to be exploited in cancer therapy. With our present limited understanding of such factors, the contradiction is perhaps best understood if cellular adaptation/survival and cellular death are viewed as continuous, rather than dichotomous, functions of autophagy. A model is emerging in which autophagy is initially triggered as an adaptive response to stress stimuli, but if unsuccessful in controlling the stress stimuli or too excessive, either apoptotic or autophagic death may ensue.

With respect to the outcome of apoptotic death, the selective targeting of mitochondria for autophagic sequestration by the mitochondrial membrane permeability transition prevents the cytoplasmic diffusion of mitochondrial pro-apoptotic factors and protects the cells.[78,79] However, when the autophagic capacities of the cells are overloaded, mitochondrial pro-apoptotic factors are free to activate the death program. This concept is supported by studies demonstrating that autophagy reduction in human colon cancer cells, either by treatment with 3-methyladenine or overexpression of a GTPase-deficient mutant of the $G_{\alpha i3}$ protein, both increases the rate of mitochondrial cytochrome C release and accelerates sulindac sulfide-induced apoptosis.[97] With respect to the outcome of autophagic death, moderate amounts of autophagy are protective in cellular responses to ionizing radiation, since autophagy inhibition increases radiation sensitivity, but larger amounts of autophagy result in lysosomal destruction of the cell.[85] Therefore, if autophagy induction is to be used as a strategy for cancer therapy, it will be important to ensure that it promotes, rather than protects, against cancer cell death.

In addition to the death-promoting consequences of excessive autophagy induction, it is possible that autophagy induction by certain chemotherapeutic agents also acts through other mechanisms to negatively regulate tumorigenesis. These other mechanisms may be particularly important in the chemoprophylactic effects of the autophagy-inducing compound tamoxifen for breast cancer and in the chemoprophylactic effects of rapamycin in *PTEN*-deficient mice, (since chemoprophylaxis involves the prevention of normal or preneoplastic cells from progressing to a malignant state rather than the killing of established tumors). As discussed elsewhere in this book (see chapters 10, 11, 22 and 23), autophagy is a method by which cells rid themselves of damaged organelles and toxic metabolites, presumably including those which can act as carcinogens. Autophagy is also thought to be involved in cellular differentiation (reviewed in ref. 98) and in inhibiting cell proliferation through its catabolic effects (reviewed in ref. 3). All of these functions of autophagy could help retard the development or progression of human malignancies and potentially contribute to the beneficial clinical effects of autophagy-inducing, anti-tumor compounds.

In conclusion, there is emerging evidence that autophagy induction plays a role in programmed cell death and in cancer therapy. Based on recent advances in understanding the molecular mechanisms of autophagy and its regulation, the tools are now available to dissect the role of autophagy in normal and deregulated cell growth as well as in cell death. This chapter has reviewed numerous clues that suggest that autophagy deregulation plays a role in tumorigenesis, a possibility that has far-reaching implications for cancer biology and cancer therapy. The time is long overdue for rigorous scientific proof of this concept.

Acknowledgements

Work done in the authors' laboratory was supported by an American Cancer Society Grant RPG-98-339-CCG, an NIH Grant RO1 CA84254, and a Mallinckrodt Scholar Award to B.L. We thank Alicia Melendez for helpful discussions.

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CHAPTER 21

Autophagy in Neural Function and Neuronal Death

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Abstract

Autophagic activity in the nervous system has long been noted. Autophagy is activated in neurons during development, after injury, and in a range of genetic disorders. At times autophagy coincides with regeneration, while at other times it appears to lead to neurodegeneration. Accordingly, arguments have arisen about the functions of autophagy in neural cells; for example, does autophagy enhance repair (perhaps by removing molecules and organelles that malfunction), or does it participate in cell death? Proper evaluation of the roles and potential benefits of autophagy are becoming possible in light of our deeper understanding of the molecular mechanisms that govern this process.

Introduction

The idea that autophagy may participate (or be deficient) in cell death in neurodegenerative diseases has re-emerged in the past few years and is becoming a hot topic of debate (see chapter 22). However, the study of autophagy in relation to the nervous system is not a new topic. The first studies go back to the late 1960s, around the time that de Duve first summarized his ideas about autophagosomes/lysosomes, their origins and possible functions (reviewed in ref. 1). An explosion of ultrastructural studies using electron microscopy reported the presence of increased numbers of autophagic/autolysosomal vacuoles in several types of neurons after axonal injury,[2-4] toxin exposure,[5] in genetic models of degeneration,[6-8] and disease.[9] This review explores the possible functions of autophagy in the nervous system with special regard to neural regeneration and developmental cell death and suggests that both these topics require re-examination in light of our recent understanding of the autophagic process.

Autophagy and Regeneration in the Nervous System

Autophagy and Chromatolysis

The emergence of autophagy as an important process in neurons began with histological studies of the reaction of neurons to injury. When the connection of a neuron to its axon or dendrite is severed, a retrograde reaction known as "chromatolysis" occurs in a neuronal cell body. The term chromatolysis was first coined by Nissl,[10] who showed that stacks of ribosomes (the "Nissl substance") are scattered, fragmented and their staining is reduced after axotomy. Using acid phosphatase histochemistry as an indicator of lysosomal activity, subsequent electron microscopy (EM) studies of chromatolytic neurons[11] suggested that the decrease in Nissl staining occurs through autophagic degradation in lysosomal-like structures.[2,3,12-14] In addition to the proposed removal of ribosomes, EM pictures also showed evidence for removal of mitochondria and ER/Golgi constituents (collectively termed GERL[15]) as well as a host of poorly identified constituents. Identification of the vesicular structures as being autophagic

Autophagy, edited by Daniel J. Klionsky.

was based on structural similarities with the autophagic vacuoles that were described to engulf mitochondria in glucagon-treated liver.[16] These observations raised three main questions: What are the origins of the membranes that form the engulfing vesicles, what are the signals that activate the autophagic process, and what is its purpose?

Origins of Autophagic Vacuoles

The origins of the vacuoles are still not entirely clear. It is thought that the vacuoles not only appear to derive from de novo structures produced in the cell body but also from tubular-vesicular structures that are transported back into the cell body from the stump of the severed axon in a retrograde process. These conclusions are mainly based on results from Matthew's lab,[17,18] who conducted a systematic study of the changes that occur in sympathetic ganglia after axotomy up to 143 days, and performed what is probably one of the best quantitative studies of autophagy in neurons. They reported that autophagic activity begins in the cell body within two hours after axotomy and peaks within two days, concomitant with the very beginnings of a regenerative response. Activity continues for several weeks at lower levels. The sheer increase in the number of vacuoles in the cell body, the rapidity of this response, and the fact that many of the vacuoles contain granular ER, which is absent in the axon, supports the idea that many of the vacuoles arise locally de novo. However, the huge surge in vesicle formation observed in the stub of the axon that remains connected to the cell body after axotomy—from whence the regenerating axon will arise—gave rise to the suggestion that some vacuoles arise from the axon. In support of this possibility, Hollenbeck,[19] who studied the trafficking of organelles between axons and cell bodies, showed that in cultured superior cervical ganglion (SCG) neurons, mitochondria in axons that are engulfed in autophagic vacuoles are transported in a retrograde process to the cell body.

It should be noted that not all the degradation in chromatolytic neurons is necessarily mediated by autophagy. Diagnosis of the process is extremely difficult. First, depending on the type of neuron and locus of injury, a myriad of different vesicle shapes and sizes have been reported, only some of which have the appearance of "typical" autophagosomes and autolysosomes (see chapter 2).[20] Many vesicles share similarities with multivesicular bodies (MVB) and multilamaellar bodies (MLB). Though MLB are consistent with maturation of autophagosomes into lysosomes,[1] MLB may also arise directly from endosomes or from other processes involving the lysosomes themselves (see chapters 2 and 14). For example, MLB are commonly found in neurons from patients with glycosphingolipid lysosomal storage diseases like Tay-Sachs disease[21-23] without concomitant increases in autophagic intermediates having been observed. As for MVB, it is now recognized that the same class of phosphatidylinositol 3-kinase (Vps34) that initiates the formation of the autophagic vacuole (see chapters 3,4,6,7), is also involved in the formation of MVB and endosomal/lysosomal trafficking.[24] Hence, inhibitors of phosphatidylinositol 3-kinases that are used as diagnostic markers for autophagy will also arrest MVB and MLB formation. Lastly, in many of these studies it is difficult to assess the true prevalence of autophagy because publications contain no more than one or two images and there is no quantitative analysis.

The Nature of the Inductive Signals

Although it is clear that axotomy provides a strong pro-autophagic signal, no work has been published that identifies the mediators of this effect. Interestingly, it appears that physical cutting of the axon is not necessary and that nerve paralysis is sufficient for the chromatolytic response to develop. For example, a chromatolytic response has been reported to occur in hypoglossal neurons (which are motoneurons that innervate the tongue) after its neurotransmitter releasing activity was paralyzed with Botulinum toxin, at times that coincide with sprouting of collateral branches.[25] However, the response after cutting was much faster and stronger than that observed after Botulinum treatment in the same type of neurons. Since increased neuronal autophagy is induced by severing neuronal connections to their target, it was initially

speculated that the cut-off of supply of factors from the targets is the signal that gives rise to autophagy. Indeed, the further away the neuron is cut from the cell body, the lesser the intensity of the chromatolytic/autophagic response, consistent with the idea that a retrograde supply of factors in the axon suppresses autophagy. SCG neurons depend on nerve growth factor (NGF) for their survival during development. However, the target-derived factor that suppresses autophagy in mature neurons is unlikely to be NGF as these neurons, which showed such robust autophagy after axotomy,[17] did not show significant chromatolysis (or cell death) when they were chronically deprived of NGF over 12 months.[26] However, it is well established that SCG neurons lose their dependence on NGF as they mature. Thus, there may be other target-derived factors that are responsible for suppressing autophagy in the adult rat. Identification of these factors is of major interest.

The Function of Autophagy

It was suggested that autophagy participates in nerve regeneration, based on the fact that autophagic activity peaks at the beginning of the regenerative response regardless of whether it was induced by cutting or by paralysis. At the time that many of these studies were performed, there were two notions about the function of autophagy: Ashford and Porter[16] suggested that autophagy is a protective device used to isolate potentially damaging reactions in the cell, while Napolitano[27] suggested that production of autophagosomes is a means of producing cytolysosomes that are metabolic switches "set aside in order to produce breakdown products that serve to re-orient physiological functions in new directions."[27] Matthews[17,18] proposed that in the cell body, the autophagic activity is used to clear away those parts of the neuronal synthetic machinery, such as the production of secretory vesicles containing neurotransmitter, and receptors on the plasma membrane, that are not required until later on in the regenerative process ("lysis may be related preferentially to processes subserving transmitter and receptive functions rather than to those which concern the production of material for regrowth of the axon"[17]). An analogy is given to the autophagic process that clears prolactin-containing vesicles from prolactin-producing neurons in the pituitary (which control milk production) after cessation of suckling, when prolactin is no longer required.[28] Remarkably however, there were much higher numbers of autophagic vesicles in the SCG neuron cell bodies compared to the pituitary neurons, though the latter are almost completely resorbed. In the axon, autophagic activity was suggested to serve as a repository of membranous constituents that can be redistributed locally into the axon once regeneration and axonal transport resume.

A particular emphasis was put on the autophagic engulfment of mitochondria, in light of Ashford and Porter's influential study.[16] Engulfment of a subset of mitochondria in the cell body was suggested by Matthews[17] to be due to the fact that these mitochondria express monoamine oxidase, an enzyme involved in noradrenaline catabolism, hence not important at this time for neuron function, and even potentially toxic. This is an interesting idea, given the recent observation that dopaminergic midbrain neurons (the neurons that die in Parkinson's disease) show a huge accumulation of numerous MLB, interpreted to be the result of a surge in autophagic activity, when dopamine (which is a catecholaminergic neurotransmitter and substrate of monoamine oxidase, like noradrenaline) is forced to accumulate in the cytoplasm by methamphetamine. Unlike the case of regeneration, however, Larsen et al[29] correlate increased autophagy in these neurons with the accumulation of toxic dopamine products, loss of mitochondria, and axonal degeneration. In Matthew's study,[18] the highest location of exuberant autophagy in the axon stump occurred just behind the sites where mitochondria were clustered. It was suggested that these mitochondria must be cleared before regeneration can take place but no direct evidence for such a clearance is provided. Some of these mitochondria looked slightly swollen, indicating perhaps an "abnormal" mitochondrial physiology. However, mitochondria are not the only organelles that are engulfed after axotomy and many mitochondria remain intact both in the cell body and the axon stump. Thus, it appears that mitochondria are not a major focus of autophagy during chromatolysis and regeneration.

In summary, it is probable that autophagy during chromatolysis is fulfilling nutritive, regenerative, and protective roles at different times and only in rare cases does it mediate cell death. It is interesting that chromatolysis is much reduced or even absent in central nervous system-projecting neurons after they are axotomized, and these neurons do not regenerate. Even in sensory neurons that project one branch into the periphery and one branch into the central nervous system, axotomy of the peripheral branch induces a chromatolytic/autophagic response commensurate with regeneration, but axotomy of the central branch produces no chromatolysis or regeneration.[25] The study of the role of autophagy in neuronal injury is ripe for reassessment in light of recent advances in understanding the molecular basis of autophagy and the molecular markers available for identification of different organelles.

Role of Autophagy in Neuronal Cell Death

Evidence for Autophagic Neuronal Cell Death

Although axotomy is followed by regeneration in the adult peripheral nervous system, it results in rapid and severe neurodegeneration when conducted in young animals (rodent and chick). Some adult neurons may also die after axotomy but this phenomenon has been less studied, perhaps because death can occur with remarkable delays of up to two years after injury.[25] Given that axotomy can result in neuronal cell death of less mature neurons, and with the discovery that programmed cell death during development is governed by factors supplied by neuronal targets,[30] it was not long before investigations began into the roles of autophagy in programmed cell death during development of the nervous system. Pilar and Landmesser[31] and Chu-Wang and Oppenheimer[32] examined carefully the mechanisms of cell death in two different neuronal types, and reported increased incidences of autophagy both in the cell body and especially in the proximal axon. Both groups concluded, however, that most natural cell death was not autophagic but rather showed hallmarks of a Type I process, which we would now classify as apoptotic (see chapter 23). Axotomy, however, was still suggested to result in a higher incidence of death by autophagy. Pilar and Landmesser[41] proposed that "immature" neurons that have no time to develop their protein synthetic machinery died by apoptosis but more mature neurons (e.g., those that reached their targets) die showing "cytoplasmic manifestations" of two types, cytoplasmic vacuolization and autophagic vacuolization. A study by Clarke's group[33] further investigated whether lysosomal activity participated in cell death, using colchicine (which prevents axonal transport) to induce a kind of chemical axotomy. Using immunohistochemical methods as well as EM, they concluded that both autophagy and endocytosis participate in this kind of cell death. The arguments that support the notion that autophagy participates in neuronal cell death are covered in great detail in a review by Clarke,[34] who also explores the question of whether autophagy is a mechanism of cell destruction, or whether it is a reflection of signals that trigger the neurons to die. He argues in favor of the former though he cannot exclude the latter. We now know that apoptotic signals can also induce autophagy in neurons.

Autophagy and Removal of Mitochondria

Recently, we reported that cultured SCG neurons induced to undergo apoptosis by NGF-deprivation display a dramatic increase in autophagic activity.[37] If apoptotic death is prevented by caspase inhibitors, the neurons go on to remove all their mitochondria from the cell bodies and die subsequently over several days by what appears to be a process of starvation.[35] Removal of mitochondria seems to be mediated by a very specific process since there were no signs of degradation of the Golgi or the ER.[36] The autophagic profiles induced in cultured SCG neurons resemble the autophagic profiles reported by Matthews and Raisman,[20] although autophagy was apparently induced by pro-apoptotic signals and not by axotomy (e.g., by NGF-deprivation or treatment with cytosine arabinoside in the presence of NGF).[37] We also showed that 3-methyladenine (3-MA) inhibited autophagic activity as well as the

removal of mitochondria. However, it must be stressed that 3-MA also inhibited the activity of JNK and p38, two kinases implicated in pro-apoptotic signalling in SCG neurons.[37] Hence it is not possible to separate the anti-autophagic and anti-apoptotic effects of 3-MA (for further discussion see ref. 38).

One of the recurrent findings is the apparently specific clearance of mitochondria by autophagy in various types of cells. Despite many years of research, it is still not clear which signals specify the particular autophagy of mitochondria. Lemasters[39] has suggested that autophagy of liver mitochondria is induced by the opening of the mitochondrial permeability transition pore (mPTP) and that it occurs as a protective device that prevents mitochondria from releasing toxic proteins. Lemasters lab has further demonstrated a correlation between mPTP opening and autophagic sequestration of mitochondria in live cultured hepatocytes using a combination of dyes, though the frequency of such incidences relative to the total number of mitochondria was very small.[40] Interestingly, 3-MA can inhibit mPTP opening in isolated liver and heart mitochondria at the same concentrations that it prevents autophagy in cells.[41] Since the mPTP is exclusively located in the mitochondrial membrane, this could be a highly accurate marker for mitochondrial recognition, but it is difficult to envisage how a change between the open and closed conformation of a channel would be recognized by the autophagic machinery as a signal and promote autophagic arousal. In neurons, Bax activation at the mitochondrial membrane could be a signal for their autophagy; Bax is recruited to the mitochondria whereupon it changes its conformation markedly (a change which is recognized by the antibody 1D1), and it is responsible for initiating cytochrome c release and subsequent opening of the mPTP.[42] Moreover, Bcl-2, which prevents activation of Bax and release of cytochrome c in this experimental paradigm, also prevented the activation of autophagy and the loss of mitochondria.[36] Another potential connection between autophagy and mitochondria is the Bcl-2-binding, autophagic protein Beclin1 (the human homologue of Apg6; see chapters 6, 7, 15 and 20).[43,44] However, we have been unable to find Beclin1, or Beclin1 lacking the Bcl-2-binding domain, localized to mitochondria when expressed in cell lines (unpublished data; see also ref. 45 and below). Since mitochondria can release several proteins in conjunction with opening of the mPTP,[46] it is possible that some of these could also provide a localized signaling mechanism for autophagic induction. Thus, more work is required to understand the intriguing connection between autophagy and mitochondrial removal.

Molecular Events Associated with Autophagic Cell Death

Though neuronal death by autophagy during development may be rare, it has assumed renewed prominence in a range of neurodegenerative models (see chapter 22). One model already discussed is the accumulation of free dopamine, which is followed by its oxidation into toxic products and neuron degeneration (see also the recent review by Larsen and Sulzer on autophagy in neurons[47]).[29] A second model is that of cell death after treatment of telencephalic neurons with the lysosomotropic agent chloroquine.[48] In a third model, chick embryos chronically treated with sublethal doses of the glutamate receptor agonist NMDA showed a massive upregulation of vesiculo-tubular structures mainly in a Nissl-free area of the cell body. Though previously the authors suggested that the neurons were spared from programmed (apoptotic) death, they suggest here that these pathological alterations may lead to a slow form of death prevalent in neurodegenerative disorders.[49] Using a range of markers to trace the origins of the vacuoles, it is suggested that they arise as a result of the derangement of Golgi/endocytic interactions, in keeping with some views as to the origins of autophagic vacuoles (see chapter 2). Interestingly, the demarcated area they outline that contains an immense jumble of different types of vesicles looks very similar to the structure that is contained in a membrane-bound organelle observed previously in a cortical neuron from the "brindled" heterozygous mouse (MobrY).[8] A fourth model relates to the mechanism of neuronal death of cerebellar Purkinje neurons in the lurcher mouse, a long-standing conundrum. Here, evidence for a possible connection between Beclin1 (Apg6), autophagy and neuronal death has been recently uncovered.[50]

Briefly, lurcher is a gain-of-function mutation in the δ2 glutamate receptor gene (Grid2) that turns the receptor into a leaky ion channel. The lurcher (LC) mouse shows a massive degeneration of the entire cerebellum, a part of the brain structure that controls movement and balance. The main output from the cerebellum is carried by Purkinje cells, upon which two main fiber types form synapses; the climbing fibers and the parallel fibers from granule cells. When the Purkinje cells die, the granule cells that depend on them die by target-dependent, Bax-mediated apoptosis. However, Bax elimination does not protect the Purkinje cells from death.[51,52] In a yeast two-hybrid screen set up to identify proteins that mediate the toxicity of the GluRδ2^{LC}, Yue et al[50] identified a protein named nPIST, which binds via its C terminus and PDZ domains to the C terminus of GluRδ2. A second yeast two-hybrid screen with the two coiled-coiled domains of nPIST as bait isolated fragments of Beclin1 (also containing a coiled-coiled domain). Confirming their interaction in cell lines overexpressing these two proteins, and noting their expression in Purkinje cells in the cerebellum, they analyzed whether nPIST/Beclin1 interactions promote/regulate autophagy. They found that Beclin1 alone did not increase autophagy, and that nPIST does not increase localization of Beclin1 to vacuolar structures (detected in Beclin1 overexpressing cells by its translocation to punctate structures, which appear to be autophagosomal by EM as they are largely incomplete membrane crescents). However, when a PDZ-deficient form of nPIST was expressed, Beclin1 relocalized to vacuolar structures in about 40% of cells compared to 6% of control cells. Further, expression of GluRδ2^{LC} (in the absence of nPIST) was sufficient to promote co-expressed Beclin1 localization to vesicles. Indeed dying lurcher Purkinje mouse neurons show what look like enhanced autophagic profiles though whether the entire cascade operates in the neurons has not yet been demonstrated.

Finally, evidence for lysosomal involvement in neuronal cell death has been provided by work from Driscoll's lab. In *C. elegans*, mutations that persistently activate the touch-sensitive mechanically gated ion channels of the ENaC family (Mec/DEG) are said to result in necrosis as cells typically swell at the end stages of their degeneration. In 1997, an EM study showed that one of the earliest events in the neurons was the accumulation of membrane whorl structures close to the plasma membrane.[53] Subsequently, these whorls grew into MLB-like structures although single-membraned vacuoles are also observed. The axon also fragments into whorls. Subsequent genetic screens for factors that mediate the death identified calcium released from the ER as being a necessary component of this death pathway,[54] and showed that death is mediated by sequential activation of at least two calcium-activated aspartyl (calpain-like?) proteases and two cysteine (cathepsin-like?) proteases.[55] Consistent with this massive proteolysis, the cytoplasm becomes slightly more translucent as degeneration proceeds. However, no autophagic gene homologs were picked up in this screen. Altogether, it is clear that caution is required in interpreting processes within cells as being autophagic based on the shape of vacuoles. For example, in some neurons, extensive vacuolization is reported during non-apoptotic degeneration but these vesicles do not resemble autophagic vacuoles.[56]

Conclusions

There are tantalizing bits of evidence that autophagy participates in, and may be utilized to direct, important aspects of neuronal physiology. What is required now is a proper molecular definition of the processes concerned. As more concrete evidence is gained as to its roles, perhaps it will be possible to manipulate autophagic function, for example, to enhance regeneration or clear unwanted/damaged proteins such as those that form toxic inclusions in neurodegenerative diseases.

Acknowledgements

Work from my lab reported in this review was supported by grants from the Wellcome Trust, the BBSRC, and the MRC. I am grateful to Ed Bampton for his patience whilst listening to the arguments put forward in this review and his comments on the manuscript.

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CHAPTER 22

Autophagy and Neuromuscular Diseases

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Abstract

Autophagy, a process by which bulk cellular proteins are turned over via the lysosomal/vacuolar system, substantially contributes to the quality control of cytoplasmic components by removing aged or injured cell constituents that are formed in cells exposed to various stimuli and stresses. Once accumulated, these damaged proteins vitiate normal cellular functions and eventually cause cell death. The effects of damaged proteins are more serious in non-proliferative tissues such as muscles and neuronal tissues, because they are unable to regenerate normal healthy cells.

In a number of inherited neuromuscular diseases, aberrant autophagic protein turnover has been shown to play a substantial role in the progression of disease. One disease (Danon's disease) has been demonstrated to be due to mutation of the gene encoding lysosome-associated membrane protein 2b (lamp2b) necessary for the fusion of autophagosomes with the lysosome. In most other neuromuscular diseases, the responsible genes are not directly relevant to autophagy. However, abnormal accumulation of autophagic vacuoles or the appearance of irregular-shaped autophagic vacuoles is frequently observed as a common symptom. One common mechanism underlying these diseases is that mutations cause conformational disorders of some particular proteins. The altered proteins have a strong tendency to form insoluble aggregates. These aggregates can be sequestered into autophagic vacuoles as the substrates of autophagy, but they are resistant to lysosomal proteinases. The accumulation of these aggregates results in a block in autophagy.

Introduction

As all living creatures have their own lifespan, cellular proteins have their own lifespan, too. Every protein exists in a dynamic equilibrium between continuous synthesis and degradation (turnover). The average duration of protein life is frequently expressed as a half-life. As the representative examples show (Table 1), diverse proteins have diverse half-lives. The half-lives of proteins vary in the range spanning from thirty minutes to over ten days. The proteins listed in Table 1 can be divided into two groups, short-lived and long-lived proteins. Usually, proteins having half-lives within a couple of hours are defined as short-lived, whereas proteins having longer half-lives are defined as long-lived. Many proteins and enzymes involved in progression of the cell cycle, cell signaling, transcriptional regulation, cell proliferation, and key metabolisms belong to the short-lived group (Table 1). It is well known that some short-lived proteins are degraded by calpain and that many other short-lived proteins are selectively degraded by the ubiquitin-proteasome system. As these proteins function under limited or some particular conditions, it is conceivable that they must be destined for a rapid degradation immediately after their roles are accomplished. Long-lived proteins account for a large majority of total cellular proteins. It has been demonstrated that most long-lived proteins are degraded through a common pathway, autophagy. Autophagy is a universal process by which bulky cellular components are degraded collectively and non-selectively by a lysosomal/vacuolar sys-

Autophagy, edited by Daniel J. Klionsky.

Table 1. Half-lives of cellular proteins

Half-Life	Protein	Localization
Less than	Ornithine decarboxylase	Cytosol
30 minuites	5-aminolevulinic acid synthase	Mitochondria
1~5 hours	Phosphoenolpyruvate carboxykinase	Cytosol
	HMG-CoA reductase	Cytosol
	Tyrosine aminotransferase	Cytosol
	Tryptophan oxigenase	ER
	Serine dehydratase	Cytosol
	RNA polymerase	Nucleus
	Deoxythimidine kinase	Nucleus
	HSP70	Cytosol
5~50 hours	Glucokinase	Cytosol
	Glucose 6-phosphate dehydrogenase	Cytosol
	3-phosphoglycerate dehydrogenase	Cytosol
	AcetylCoA carboxylase	Cytosol
	Alanine aminotransferase	Cytosol
	Cytochrome P450 reductase	ER
	Catalase	Peroxisome
	Insulin receptor	Plasma membrane
50~200 hours	Lactate dehydrogenase	Cytosol
	Carbamoylphosphate synthase	Mitochondria
	Argininase	Cytosol
	Cytochrome c	Mitochondria
	Cytochrome P450	ER
>200 hours	Phosphoglycerate kinase	Cytosol
	Fumarase	Mitochondria

tem. There are two categories of autophagy: Macroautophagy and microautophagy (see chapters 2 and 8). In microautophagy, relatively small portions of cytoplasm are directly surrounded by invaginating lysosomal membranes for subsequent sequestration and degradation. Degradation of organelles and high levels of cytosolic proteins occurs via macroautophagy. Quantitatively, the contribution of macroautophagy to total protein turnover is much greater than that of microautophagy. Therefore, we have focused our review on macroautophagy and for simplicity use the term autophagy.

One unequivocal characteristic of autophagy is its dependence on the nutrient conditions of the extracellular milieu (see chapter 3). In the rat liver, the rate of autophagic protein degradation accounts for about 1.5% of total proteins per hour when serum amino acids are at a maximal level.[1] The rate is accelerated to 4.5% of total proteins per hour under fasting conditions when serum amino acids are at the lowest level.[1] The mean half-life of bulk proteins calculated from these rates is 15 hours during fasting and 46 hours after feeding. Thus, the mean half-life of long-lived proteins fluctuates greatly between feeding and fasting cycles. It has been well documented that 8 out of 20 amino acids are sufficient for suppressing autophagy in the liver.[2] Likewise, the level of extra-cellular nitrogen source controls autophagy in lower eukaryotes (see chapters 6 and 7).

As the major system for bulk protein degradation, autophagy plays a pivotal role in cell survival. First, autophagy greatly contributes toward supplying amino acids that are necessary for keeping cells alive under nutrient-deprived conditions. A significant part of these amino acids must be used for the synthesis of proteins that are essential for adapting cells to starvation

conditions. Another population of the recycled amino acids must be metabolized for the production of energy. Second, autophagy has a house-cleaning role. It participates in removing aged or injured cell constituents that are inevitably produced in the cells' exposure to various stimuli and stresses. Autophagic activity of rat liver declines with increasing age[3] and this decline conversely correlates with an increase in the accumulation of oxidized proteins.[4] Functionally inactive or damaged proteins remaining in the cytoplasm hinder normal cell functions, which eventually causes disease or cell death. In yeast, *Saccharomyces cerevisiae*, autophagy-defective mutants such as *aut* and *apg* manifest a common phenotype: They have a tendency to die earlier than wild type cells under starvation conditions.[5]

Although autophagy is a daily occurrence depending on the feeding-fasting cycle, it is profoundly accelerated under particular circumstances. Dramatic cellular remodeling that accompanies bulk degradation of cell constituents occurs during development, metamorphosis, and cell differentiation. Because not only soluble cytosolic proteins, but also large cell organelles such as the endoplasmic reticulum, peroxisomes, and mitochondria, are the substrates of autophagy, this process is a useful mechanism for removing a mass of cellular protein in a short time. In apoptotic programmed cell death (PCD), autophagy is activated by apoptotic stimuli.[6] Elevated levels of autophagosomes and cathepsin D-positive granules are observed in the early stages of apoptosis in PC12 cells.[7] It has also been shown recently that autophagy plays an important role in type II PCD. Autophagic or type II PCD is characterized by bleb formation and the appearance of numerous autophagic vacuoles in the cytoplasm, but contrary to type I PCD or apoptosis, neither DNA fragmentation, chromatin condensation, cytoplasmic shrinkage, nor formation of apoptotic bodies occurs.[8-10] In type II PCD, the entire process occurs independent of caspase activity, and autophagy instead of heterophagy participates in disposing of dying cell constituents. Thus, autophagy seemingly functions as a protective mechanism that can eliminate dying cells, which would otherwise be harmful to the organisms.

In view of the importance of autophagy in cell life, it is important to understand what kind of pathological conditions or diseases can be attributable to the dysfunction of autophagy. The theme of this chapter is autophagy and neuromuscular diseases. There are a large number of inherited neuromuscular diseases. In a substantial number of these diseases, dysfunction of autophagy or aberrant autophagic proteolysis has been suspected as an underlying mechanism. Analyses of autophagy-related genes that have been identified during the last decade enable us not only to understand elementary steps of autophagy at the molecular level (see Chapters 6, 7 and 15), but also to locate genetic defects, which would interfere with, or in some cases enhance, autophagy. First, more details about the genetic basis underlying the dysfunction of autophagy will be considered in the next section.

Mechanistic Considerations of Autophagy Dysfunction

Genes Involved in Elementary Steps of Autophagy

The entire process of autophagy can be divided into four steps (Fig. 1):

1. Nutrition-deprivation signaling to induce autophagy.
2. Formation of double-membraned autophagosome coupled with sequestration of cytoplasmic components.
3. Fusion of the autophagosome with the endosome/lysosome to form an autolysosome.
4. Degradation of sequestered components in the autolysosomal lumen.

Recently, genes involved in each of the four steps have been identified. Before considering the relationship between genetic disorders and dysfunction of autophagy, we briefly outline the functions of these genes.

It has been postulated that there is a sensor or receptor that recognizes decreased levels of serum amino acids.[11] Release of autophagy-suppressive amino acids from presumptive receptors elicits a series of intracellular signaling cascades (step 1). Genes encoding signaling molecules involved in these cascades operate prior to autophagosome formation. Representative

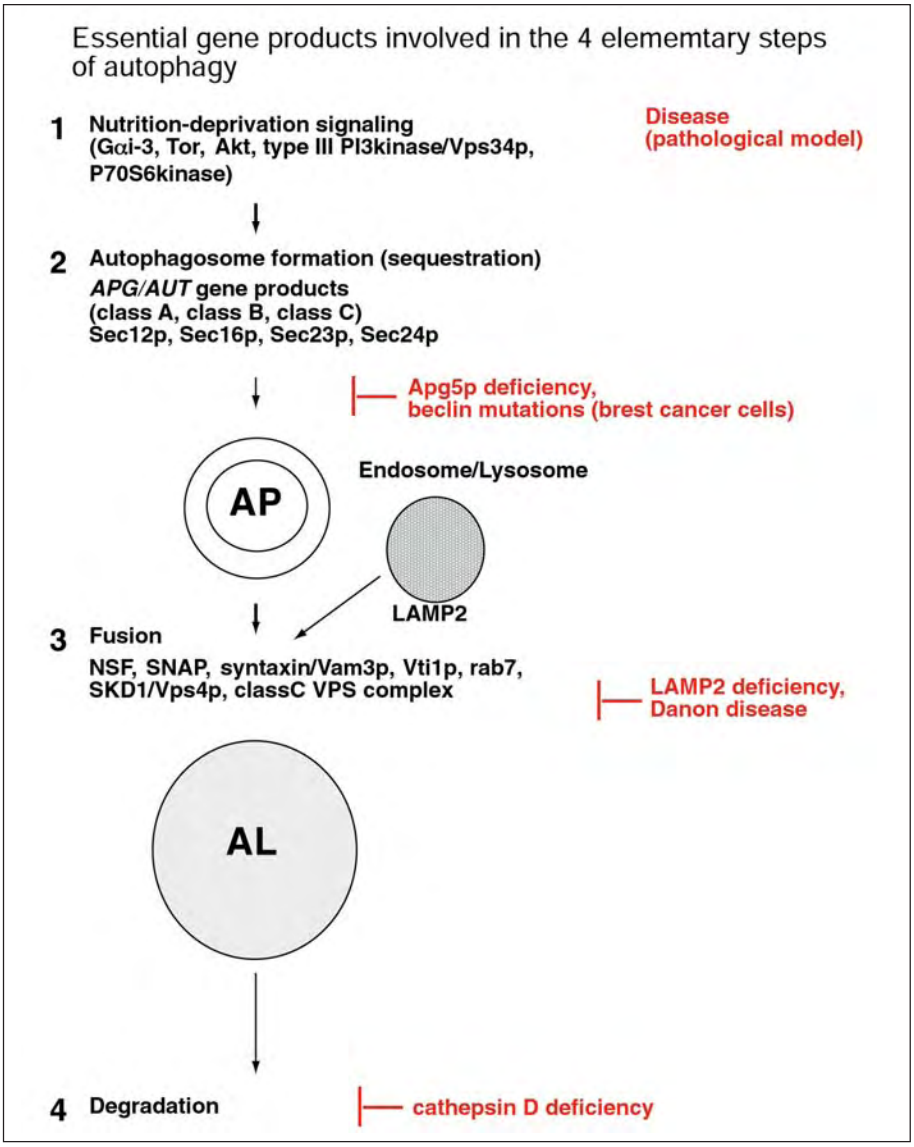

Figure 1. Essential gene products involved in the 4 elementary steps of autophagy. The entire process of autophagy, which begins with non-selective sequestration of cytoplasmic components into autophagosomes and ends up with degradation of these components, comprises four elementary steps. In step 1, a decrease in the serum amino acid level elicits a series of intracelluar signaling cascades. Tor, Vps34/PI3-kinase, and p70S6 kinase are closely linked with an amino acid starvation signal. Following this cascade, a double-membraned autophagosome (AP), which sequesters cytosolic proteins and cell organelles, is formed (step 2). Most of the *APG/AUT* gene products are required for the formation of AP. The AP then fuses with either an endosome or lysosome to mature into an autolysosome (AL) (step 3). As autophagy-specific membrane fusion components, syntaxin 7/Vam3, Vti1, and rab7 play a pivotal role. Acquisition of lysosomal proteinases through step 3 enables subsequent degradation of sequestered proteins in the acidic lumen of the AL (step 4). Some diseases and animal models that are known to be caused by the defects of the genes involved in the four elementary steps are shown in red letters on the right.

members involved in this cascade are $G\alpha_{i3}$, Akt, class III phosphatidylinositol (PtdIns) 3-kinase/Vps34, Tor kinase, and p70S6 kinase (see chapters 3-5). The p70S6 kinase phosphorylates ribosomal subunit S6 and phosphorylation of S6 strongly correlates with the inhibition of autophagy. The activity of p70S6 kinase is regulated by Tor kinase that is positioned downstream of the amino-acid starvation signal. Inactivation of Tor by rapamycin causes dephosphorylation of p70S6 kinase, which enhances autophagy.

The induction of autophagy by the signaling cascade is followed by the action of the machinery that is involved in the formation of the autophagosome (step 2). It has been demonstrated that almost all of the *APG* and *AUT* genes are directly required for autophagosome formation and thus included in the members of the machinery. Most closely linked with the starvation signaling are the class C *APG* genes consisting of *APG6/VPS30/beclin*, *APG9*, *APG14* and *APG16*. Vps30/beclin and Apg14 are part of a PtdIns 3-kinase complex specific for autophagy.[12] There are two other groups; class A *APG* genes (*APG1*, *APG2*, *APG13*, *APG17*) and class B *APG* genes (*AUT1*, *AUT2*, *APG5*, *APG7*, *APG10*, *APG12*) (see chapters 6 and 7).[12] Class A gene products consist of Apg1 protein kinase and its regulators, Apg13 and Apg17. ULK1, a mammalian homologue of Apg1 is necessary for the development of granular neurons in the cerebellar cortex.[13] Class B gene products are required for two protein conjugation systems that have a critical role in forming the pre-autophagosomal compartment. Furthermore, some additional proteins involved in ER-to-Golgi transport have been shown to be necessary for autophagosome formation in the yeast, *Saccharomyces cerevisiae*.[14] These proteins are Sec12, Sec16, Sec23, and Sec24. Therefore, mammalian homologues of these gene products should also participate in the formation of the autophagosome.

As autophagosomes do not possess protein degradative enzymes, cytoplasmic components sequestered in the lumen of the autophagosome remain intact. After fusion of the autophagosome with an endosome or lysosome the compartment matures into an autolysosome; the sequestered components are degraded by lysosomal hydrolases in this compartment. Various factors are required for the autophagosomal fusion (step 3). In general, a set of proteins including NSF, SNAP, v- and t-SNAREs, and tethering factors are necessary for membrane docking and fusion, and this is also the case with autophagy. Syntaxin 7/Vam3 and Vti1 have been established as the t- and v-SNARE, respectively,[14,15] that are needed for membrane fusion between the autophagosome and endosome or lysosome. Also, rab7/Ypt7, a low molecular weight GTP-binding protein, is necessary for fusion.[16] Both Syntaxin 7 and rab7 are localized on late endosomal membranes. In addition to the factors directly involved in membrane fusion, some components necessary for the endosomal-lysosomal pathway are also required. These include SKD1/Vps4, a factor necessary for membrane transport between the early endosome and the plasma membrane[17] and the class C Vps complex, which is indispensable for the transport from the early endosome to the late endososme.[18,19] The fact that the genes required for endosomal trafficking are also essential for the maturation of autophagosomes to autolysosomes may indicate that the endosome rather than the lysosome is more important as a fusion partner for the autophagosome. It has been shown more recently that the gene encoding lamp2a, a lysosomal membrane glycoprotein, that is essential for hsc73-dependent lysosomal transport (see chapter 12), is also necessary for fusion with the autophagosome.[20] In addition, lumenal acidification driven by a vacuolar H^+-ATPase is necessary for the fusion between autophagosomes and the endosome/lysosome, as evidenced by the inhibition of autophagosomal fusion in bafilomycin A-treated cells.[21]

Degradation of sequestered cytoplasmic components in the autolysosomal lumen is dependent on proper functioning of lysosomal hydrolytic enzymes in an acidic milieu. Hence, the genes encoding lysosomal hydrolytic enzymes as well as structural components of lysosomal membranes are critical for degradation (step 4). Moreover, a previous study reported that normal activity of an ubiquitin-activating enzyme (E1) is needed for maturation of the autolysosome into a residual body, though the precise role of E1 has not been clarified.[22] It is plausible that new genes that have yet to be identified will be added to the list in Table 2 in the future.

Table 2. Autophagy-related diseases

Name of Disease	Mode of Inheritance	Principal Part Affected	Histopathology	Responsible Gene or Gene Locus	Age of Onset	OMIM Number
Muscle Disease						
Muscle disease with autosomal mutations						
Distal myopathy with rimmed vacuoles (DMRV)	autosomal recessive	distal leg muscles, anterior compartment	rimmed vacuoles, LAMP1, LAMP2, acid phosphatase, cathepsins B, H, L, α- and γ-adaptin, ubiquitin, amyloid β, MAP-LC3 positive	gene encoding UDP-N-acetylglucos-amine 2-epimerase/ N-acetylmannosamine kinase	20~30 years	605820
Tibial muscular dystrophy (TMD)	autosomal dominant	distal leg muscles	rimmed vacuoles	2q31	>35 years	600334
Welander distal myopathy(WDM)	autosomal dominant	hand muscles	rimmed vacuoles	2p13	> 40 years	604454
Inclusion body myositis	autosomal dominant	proximal parts of the limbs	line vacuoles, sarcoplasmin inclusions	unknown	20~30years	147421
Marinesco-Sjorgen syndrome	autosomal recessive	brachial muscles	disarranged myofibrils, rimmed vacuole with numerous myeloid bodies	unknown	2~40 years	248800
Oculopharyngeal muscular dystrophy	autosomal dominant	proximal limb, eylid	nuclear aggregation of PABP2, rimmed vacuoles	PABP2 encoding polyadenine binding protein 2	50~60 years	164300
Hypertrophic cardiomyopathy (HCM)	autosomal dominant	heart muscles	vacuoles with sarcomic inclusions	PRKAG2 encoding γ2 regulatory subunit of AMP-activated protein kinase	40 years	600858
Limb girdle muscular dystrophy 1A (LGMD1A)	autosomal dominant	proximal leg muscles, Achilles tendon	rimmed vacuoles	gene encoding myotilin	18~35 years	159000

Continued on next page

Table 2. Continued

Name of Disease	Mode of Inheritance	Principal Part Affected	Histopathology	Responsible Gene or Gene Locus	Age of Onset	OMIM Number
Muscle disease with X-chromosomal mutations						
Duchenne muscular dystrophy	X-linked recessive	proximal leg muscles, gastrocnemius	muscle necrosis, hyper contracted fibers	gene encoding dystrophin (deletion)	2~4 years	310200
Becker muscular dystrophy	X-linked recessive	proximal leg muscles	muscle necrosis, hyper contracted fibers	gene encoding dystrophin	5~25 years	300376
X-linked myopathy with excessive autophagy	X-linked recessive	proximal and distal muscles (thigh, shoulder, lower limbs,etc)	autophagic vacuoles	Xq28	10s	310440
Danon's disease	X-linked recessive	skeletal and cardiac muscles	autophagic vacuoles	gene coding for LAMP2	2~16 years	309060
Neuronal Disease						
Huntington's disease	autosomal dominant	striatal neurons in basal ganglia	cytoplasmic insoluble aggregates, autophagic vacuoles with sequestsered huntingtin, accumulation of endosomes and lysosomes	huntingtin	30~40 years	143100
Parkinson's disease	autosomal dominant	dopaminergic neurons of substantia nigra	Lewy bodies (deposition of α-synuclein, synphilin, parkin, ubiquitin,etc)	gene encoding ubiquitin C-terminal hydrolase	59 years (mean)	168600
	autosomal recessive		endoplasmic stress	gene encoding parkin	<40 years	
	autosomal dominant		Lewy bodies, α-synuclein fibrils	gene encoding α-synuclein	46 years (mean)	
Alzheimer's disease	autosomal dominant	hippocampal and cortical neurons	deposition of Aβ42 and neurofibrillar tangles, elevation of cathepsins, enlarged endosomes, granulovacuoles, amyloid	gene encoding amyloid protein precursor	30~40s	104300
	autosomal dominant	presenilins 1and 2	plaques		40s	

Predicted Outcome Caused by Mutations of Autophagy-Related Genes

Autophagy-related genes described in the previous section are divided into two groups. One group includes the genes encoding signaling molecules, most of which operate in step 1. Additionally, some genes such as *APG13* and *APG1* operating in step 2 are also included in this group. As gene products of this group are involved in the switching mechanism of autophagy, mutations of these genes cause positive or negative effects on autophagic induction. For example, $G_{\alpha i3}$ must be in the GDP-bound form for autophagy to occur (see chapter 3).[23] Mutations that fix $G_{\alpha i3}$ in a GDP bound form, result in constitutive autophagy. In contrast, mutations that stabilize the GTP bound form result in constitutively suppressed autophagy.

The majority of other autophagy-related genes that operate in steps 2, 3, and 4 comprise the second group. Mutations in these genes cause "loss of function" and hence negatively affect autophagy. In the context of neuromuscular diseases, impairment of normal protein turnover is frequently recognized as a common defect, so dysfunction of autophagy due to "loss of function" type mutations appears to correlate more closely with pathological symptoms. Consequently, a greater emphasis should be placed on analyses of the latter type of mutations.

Morphological studies using rat livers showed that a relatively small number of autophagic vacuoles (autophagosomes plus autolysosomes) can be detected in the steady state of normal autophagy under starvation conditions.[24] The number is profoundly increased when lysosomal proteolysis is inhibited with lysosomal proteinase inhibitors.[24-26] This means that autophagic vacuoles themselves are rapidly turned over and that the rate-determining step in autophagosome biogenesis is autophagosome formation (step 2), not fusion or degradation (step 3 or 4).

Lysosomal proteinases degrade autophagosomal membrane-specific components remaining in autolysosomes to regenerate lysosomes.[27] Thus, inhibition of lysosomal proteolysis prevents the turnover of autophagic vacuoles. Accordingly, it is possible to determine if a particular mutant is defective in degradation by examining the level of autophagic vacuoles. Similarly, other mutation-induced defects in autophagy can be analyzed with regard to the intermediates that accumulate. That is, if a defect caused by mutation of an autophagy-related gene occurs at a certain point in the above steps, reaction intermediates formed prior to this point would accumulate, while those produced in subsequent steps would be markedly diminished. Therefore, depending on the step that is affected by the mutation, different pathological outcomes would emerge (Fig. 1).

Defects of the genes involved in step 2 cause impairment or loss of autophagosome formation under starvation conditions. As a typical symptom, the cytoplasm would fill with accumulated proteins and organelles that would otherwise be sequestered into autophagosomes for degradation. As a prolonged effect, an abnormal increase in cell mass may also be predicted. There is an excellent experimental model for studying this type of mutation. *APG5*-deficient mouse embryonic stem cells lack autophagosomes completely and bulk protein degradation is severely hindered.[28] Using this model system, it is possible to understand the process and mechanism of deterioration caused by accumulated cellular proteins. The absence of autophagosomes under starvation conditions is reported in certain breast cancer cell lines in which Apg6/beclin/Vps30, a key component of the class C Apg complex is mutated (see chapters 6, 7, 15 and 20). There is another pathological example, which appears to mimic the step 2 defects. Phalloidin, a fungal toxin, which inhibits bile secretion through its effect on hepatocyte microfilaments, also hinders autophagosome formation.[29] There is an increase in the number of cytosolic and organellar enzymes that accumulate in the cytoplasm of phalloidin-administered rat livers.[29]

Defects in the genes involved in steps 3 and 4 would elicit a common phenotype, accumulation of autophagic vacuoles. In the case of defects in step 3, the accumulated vesicles would comprise autophagosomes almost exclusively. In contrast, in the case of defects in step 4, both autolysosomes (active lysosomes resulting from fusion with autophagosomes; see chapter 2) and autophagosomes would accumulate. Many autophagic vacuoles (presumably autophagosomes) accumulate in the cytoplasm of lamp2-deficient mice[20] and in the muscles of patients with Danon's disease (human lamp2-deficiency disease),[30] in which step 3 and/or step

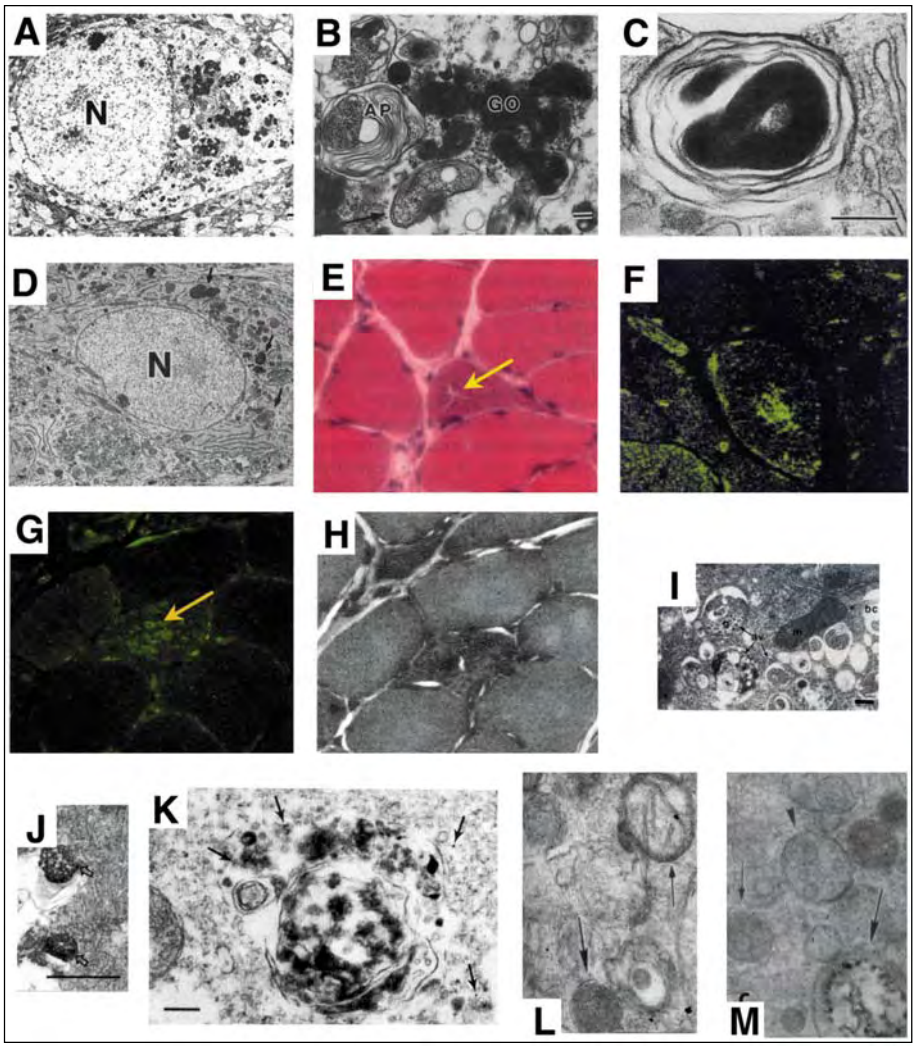

Figure 2. Legend Next Page

4 are impaired. Accumulation of both autophagosomes and autolysosomes can be expected in some typical lysosomal diseases in which step 4 is hindered due to mutations of lysosomal proteinases. One well-known model is cathepsin D-deficient mice. Cathepsin D is one of the major and most active lysosomal proteinases. The deficiency of this enzyme causes remarkable accumulation of autophagosomes and autolysosomes with ceroid lipofuscin in neuronal and epithelial tissues (Fig. 2A-D).[31] Many autophagsomes and autolysosomes containing osmiophilic deposits and myelin-like structures appear in the neuronal perikarya. This pathological feature resembles those of typical neurodegenerative disorders.

Criteria for Diagnosing Dysfunction of Autophagy

To clarify whether defects in autophagy play a role in the etiology of particular diseases, it is important to obtain evidence showing that an aberration occurs at the level of autophagic vacuoles. For diagnosis of diseases, usually only trace amounts of patient tissues are available;

Figure 2. Histopathological characteristics of representative neuromuscular diseases and some pathological models showing accumulated autophagic vacuoles and lysosomes (shown on previous page). A-D: Electron micrographs of the brain of cathepsin D-deficient mice (Reprinted by permission from Journal of Neuroscience 2000; 20: 6898-6906. Copyright 2000 by the Society for Neuroscience). A cerebral cortex neuron obtained from a cathepsin D-deficient mouse. Numerous dense lysosomes and autophagic vacuoles are seen at low (A) and high (B) magnifications. Note that autophagic vacuoles (AP) with or without fingerprint-like myelin figures accumulate along with granular osmiophilic deposits (GO). A fingerprint membranous structure similar to that in B is also found in the neuronal perikaryon of Purkinje cell (C). In D, the dense granular bodies (arrow) appear in the neuronal perikaryon of CA3 pyramidal neurons. E-H: Morphological characteristics of skeletal muscles in hereditary and experimental myopathies (Reprinted by permission from Journal of Biochemistry 2002; 131: 647-651. Copyright 2002 by the Japanese Biochemical Society). HE-staining pattern of rimmed vacuoles (arrow) in experimental chloroquine myopathy (E). Immunofluorescence microscopy showing MAP-LC3-positive autophagic vacuoles in DMRV muscle biopsy specimen (F) and chloroquine-treated rat soleus muscle (G). Immunofluorescence on limiting membranes of autophagic vacuoles is shown by the arrow. Serial section of G stained with HE (H). I: Autophagic vacuoles accumulating in the liver of a lamp2-deficient mouse as revealed by electron microscopy (Reprinted by permission from Nature 2000; 406: 902-906. Copyright 2000 by Macmillan Publishers Ltd.). Autophagic vacuoles with sequestered glycogen and cell constituents are seen. J: Electron micrograph of patient brain with Huntington's disease (Reprinted by permission from Sapp et al., "Huntingtin localization in brains of normal and Huntington's disease patients," Annals of Neurology 1997; 42: 604-612. Copyright 1997 by John Wiley and Sons, Inc.). Huntingtin-labeled granules (arrow) appear in the cytoplasm of pyramidal neurons. K: Immunogold labeling of FLAG-huntingtin with expanded polyQ tract expressed in X57 clonal striatal cells (Reprinted by permission from Journal of Neuroscience 2000; 20: 7268-7278. Copyright 2000 by the Society for Neuroscience). Gold deposits appear within double-membraned autophagosomes and surrounding cytoplasm. L and M: Electron micrographs of two PC12 cell lines expressing mutant (A53T) α-synuclein (Reprinted by permission from Journal of Neuroscience 2001; 21: 9549-9560. Copyright 2001 by the Society for Neuroscience). Autophagic vacuoles with sequestered organelles appear in the cytoplasm of two PC12 clones (M1 (L) and M15 (M)).

therefore, a morphological examination is optimal. Using electron microscopy, autophagosomes and autolysosomes can be distinguished from each other. Autophagosomes have double membranes and clear structures of sequestered proteins, and organelles can be seen in the lumen. In contrast, autolysosomes have single membranes and dimmed or blurred lumenal structures due to protein degradation. Acid phosphatase staining also has been used to distinguish autophagic vacuoles. Acid phosphatase, a lysosomal marker protein, is present in autolysosomes but absent from autophagosomes. However, as described in the next section, there are many neuromuscular diseases that display the accumulation of vacuoles and vesicles of varied types. In such cases, it is often very difficult to distinguish subtle differences in membrane structures by ordinary transmission electron microscopy. In order to circumvent this difficulty, detection of a definitive marker of the autophagic vacuolar membranes is recommended.

Staining autophagic vacuoles with monodansylcadaverine (MDC) has been used for light microscopic analysis.[32,33] MDC is a hydrophobic probe whose fluorescence is strongly enhanced in a hydrophobic environment with a concomitant shift of its emission wavelength maximum to the blue spectrum.[34] When MDC is incorporated into cells, it is believed to concentrate and diffuse in lipid-rich autophagic vacuolar membranes. As an organic amine, MDC can be significantly incorporated in the acidic lumen of lysosomes and endosomes. Fluorescence cytochemistry using MDC is a simple and convenient procedure to survey autophagic vacuoles in primary cell cultures derived from patient tissues or animal models.

It has been shown that detection of the membrane-bound form of MAP-LC3 is more promising and compelling for diagnosis. Originally, MAP-LC3 was identified as a small subunit of MAP-IA and MAP-IB, microtubule-associated proteins. Both MAP-IA and MAP-IB can bind to microtubules and also exist in free form dispersed in the cytosol. More recently, MAP-LC3 has been identified to be one of the mammalian homologues of Aut7/Apg8 (see chapters 6, 7 and 15) and found to localize on autophagic vacuolar membranes.[35] During starvation-induced autophagy, MAP-LC3 is processed to a lipidated form (LC3-II) that has a greater mobility than

its soluble precursor form in SDS polyacrylamide gel electrophoresis. LC3-II is specifically localized on autophagic vacuoles. Hence, an increase in the cellular LC3-II content is a good indicator for accumulation of autophagic vacuoles. Also, immunofluorescence or immunoelectron microscopy to localize LC3-II on punctate or vesicular structures is useful for distinguishing autophagic vacuoles from other vesicles. As described later, this is seen in a recent study of experimental chloroquine myopathy.[36]

Dysfunction of Autophagy in Neuromuscular Disorders

Living cells are exposed to various environmental stresses such as reactive oxygen species, UV irradiation, chemical substances, bacterial and viral infections, etc. These stresses frequently cause various types of protein injuries that vitiate normal cellular functions and may eventually cause cell death. As autophagy substantially contributes to quality control of cytoplasmic components by removing these injured proteins or organelles, dysfunction of autophagy elicits severe effects on cell activities. These effects are more serious in non-proliferative tissues such as muscles and nerves, because it is difficult to regenerate normal healthy cells to replace damaged ones. As a result, the compromised cells would last or accumulate for a long time and whole tissues including the injured cells would be affected. It is well known that in many neuromuscular diseases, the onset of disease is age-related and the symptoms proceed chronically.

This section deals with inherited neuromuscular disorders, in which aberrant autophagic protein turnover has been thought to play some roles in the progression of diseases. In some diseases an abnormal number and/or appearance of autophagic vacuoles have been observed. In others, aggregates of cytoplasmic remnants, lipofuscin deposits, and accumulated autophagic vacuoles and lysosomes are the primary hallmarks. These morphological features have been reported to be attributable to abnormal elevation or suppression of autophagy, directly or indirectly linked to the etiology of the diseases. Here, we aim at evaluating the role of autophagy dysfunction in these neuromuscular diseases from genetic viewpoints mentioned in the previous section. For details of each disease, see also the online web site [Online Mendelian Inheritance in Man (OMIM); http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM].

Muscular Disorders

Myopathies Caused by Autosomal Mutations

The primary myopathies have been classified into two categories. In one group, as represented by Duchenne muscular dystrophy, proximal muscles of the extremities and trunk are predominantly affected. In the other group, distal muscles are preferentially affected. The latter group includes Welander distal myopathy (OMIM #604454), tibial muscular dystrophy (OMIM #600334), oculopharyngeal muscular dystrophy (OMIM #164300), Marinesco-Sjogren syndrome (OMIM #248800), distal myopathy with rimmed vacuoles (DMRV or Nonaka myopathy, OMIM #605820), and inclusion body myositis (IBM, OMIM #147421) (Table 2). Among these diseases, Walander distal myopathy, tibial muscular dystrophy, and IBM are autosomal dominantly inherited diseases, whereas Marinesco-Sjogren syndrome and DMRV are autosomal recessively inherited diseases. Responsible genes or gene loci have been determined as summarized in Table 2.

In spite of the different modes of inheritance and diversity in the responsible genes affected, all of them are characterized by peculiar rimmed vacuoles accumulating beneath the sarcolemma. Characteristics of these rimmed vacuoles have been best investigated with biopsied specimens of DMRV patients, which are summarized below.

Distal myopathy with rimmed vacuoles (DMRV)—In most cases, rimmed vacuoles are present in atrophic muscle fibers that occasionally pack into small groups (Fig. 2E). Considerable amounts of necrotic and regenerating muscle fibers are observed, but they are not enough to account for muscle atrophy.[37] Electron microscopy has shown that vesicles with single- or double-limiting membranes are present in the area of rimmed vacuoles.[38] Immunocytochemi-

cal analyses have revealed that the vacuoles possess various lysosomal markers including lysosomal acid phosphatase, lamp1, lamp2, and cathepsins B, D, H, and L.[39,40] Only faint signals of these lysosomal markers are detected in healthy muscles. It has been also reported that α and γ-adaptin,[41] ubiquitin, β-amyloid, and microtubule-associated protein τ[42] are present in the vacuoles. Based on these data, it has been assumed that the rimmed vacuoles are autophagic vacuoles abundantly formed in atrophic fibers.

Marked enhancement of lysosomal enzyme activities and lysosomal membrane markers as well as the abundance of the rimmed vacuoles in atrophic fibers may indicate that autophagy is participating in ongoing muscle degradation that is characteristic of DMRV, and that muscle atrophy can be attributable to enhanced autophagy. However, accumulation of autophagic vacuoles itself may be also considered indicative of stagnation of autophagy (step3 and/or step4 deficit). Which of these two possibilities is the case? Interestingly, this issue may be resolved by studies carried out using an experimental disease model system. Formation and accumulation of vesicles that resemble the rimmed vacuoles in DMRV and IBM have been demonstrated in chloroquine myopathy. Chloroquine, an antimalarial drug that is also used in the treatment of rheumatic diseases, has been known to cause neuropathy and myopathy.[43] This effect is interpreted in terms of the neutralizing action of chloroquine on the acidic milieu of the lysosomal lumen; deacidification suppresses lysosomal protein degradation by inhibiting fusion of the autophagosome with the lysosome. Administration of chloroquine into rats causes myopathy in the soleus and other skeletal muscles, and many rimmed vacuoles are formed in these muscle fibers. Suzuki et al has recently demonstrated that MAP-LC3, an autophagosomal membrane marker, is localized in the region of rimmed vacuoles formed in chloroquine-administered rat muscles and human DMRV patient specimens (Fig. 2E-H).[36] The authors speculate that lysosomal function is compromised in the chloroquine-treated rat model system. As a result, autophagosome maturation through fusion with lysosomes is hampered as in the case with bafilomycin-treated cells. Autophagosomes with sequestered muscle proteins in the lumen then aggregate with lysosomes to form rimmed vacuoles. As the morphological appearance of the rimmed vacuoles of DMRV patients is quite similar to that of chloroquine-affected muscles, stagnation of autophagy at step 3 and/or step 4 is likely to be involved in DMRV.

The responsible gene for DMRV has been recently identified as the UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine gene.[44] This gene is necessary for sialic acid biosynthesis, so it is still a mystery as to why this type of mutation causes the accumulation of rimmed vacuoles. The identical gene is also responsible for hereditary inclusion body myopathy (OMIM #600737),[45] which used to be considered as a distinct hereditary disease.

Oculopharyngeal muscular dystrophy (OPMD)—OPMD (OMIM #164300) is an adult-onset disease with autosomal dominant inheritance. The disease is characterized by progressive eyelid drooping, swallowing difficulties and proximal limb weakness. Pathological analysis revealed that tubular intranuclear filamentous inclusions exist in patient skeletal muscles.[46] The gene responsible for OPMD was identified as PABP2, which encodes the poly (A) binding protein 2.[47] The normal PABP2 gene has a $(GCG)_6$ trinucleotide repeat at its 5' end, whereas this $(GCG)_6$ repeat is expanded to $(GCG)_{8\text{-}13}$ in the PABP2 gene of patients with OPMD. The $(GCG)_6$ trinucleotide repeat codes for a polyalanine stretch. Due to the presence of a GCA GCA GCA GCG coding sequence adjacent to the $(GCG)_6$ repeat, the wild-type PABP2 protein has a 10 alanine stretch at its N terminus. In contrast, the mutant proteins have 12-17 alanines. Pathogenesis of OPMD is very similar to that of Huntington's disease, which is described later. The mutant PABP2 protein with an expanded polyA repeat accumulates and forms misfolded aggregates in nuclei, which causes cell death. For the aggregate formation, oligomerization of mutant PABP2 protein plays a key role.[48] Rimmed vacuoles are occasionally found in a substantial number of OPMD patient's muscles. It has been shown that overexpressed green fluorescent protein (GFP) fused with 19 polyA repeats tends to form misfolded aggregates that are resistant to autophagic protein degradation.[49]

Myopathies Caused by Mutations in the X-Chromosome

Duchenne muscular dystrophy—In proximal muscular dystrophies including Duchenne muscular dystrophy (OMIM #310200), and Becker muscular dystrophy (OMIM #300376), massive protein degradation is accompanied by progressive necrosis of muscle fibers. Duchenne muscular dystrophy is caused by mutations in dystrophin, a giant fibrous protein localized beneath the sarcolemma. Baker muscular dystrophy is caused by mutations in the dystroglycan complex. In both cases, gross degeneration or collapse of sarcolemmal membrane skeleton is a primary event, followed by myofibrillar disorganization. The contribution of both the intramyofibral autophagic-lysosomal system and phagocytosis by infiltrating macrophages to the muscle degradation has been evaluated in Duchenne dystrophy and its animal models. Immunocytochemical investigations revealed that at early stages of muscle breakdown, strong reactivity of anti-cathepsin B, anti-cathepsin L, and anti-cathepsin H was seen in atrophic myofibers before infiltration of macrophages.[50,51] Autolysosomes possibly involved in ongoing muscle protein degradation were also detected. For complete degradation of muscle fibers, however, phagocytosis by infiltrating macrophages is required and this latter step contributes profoundly to the massive muscle breakdown.

X-linked myopathy with excessive autophagy—X-linked myopathy with excessive autophagy (XMEA) is a slowly progressive inherited myopathy characterized by membrane bound sarcoplasmic vacuoles (OMIM #310440) (for a review see ref. 52). It was first described in 1988 by Kalimo et al,[53] and was characterized as displaying excessive autophagic activity. Sarcoplasmic vacuoles in myofibers of XMEA male patients is positive for dystrophin, laminin, esterase activity, and lamp2.[54,55] The vacuoles contain lysosomal enzymes and cell debris including degenerating organelles, basophilic granules, and membrane whorls.[54] These characteristics resemble those found in the myofibers of patients with Danon's disease, another type of X-linked vacuolar myopathy. There is a definitive difference, however, between the myofiber of XMEA patients and that of Danon's disease patients. The surface of the XMEA myofibers and some vacuoles in the XMEA myofibers are decorated by deposited calcium and complement C5b-9 membrane attack complex (MAC) in addition to lamp2-positive vacuoles.[54-57] The muscle weakness of XMEA patients is mainly observed in proximal muscles of the lower limbs. Creatine kinase activities in the patients' sera are significantly elevated as is the case with those of Danon's disease patients. However, while patients of Danon's disease have cardiomyopathy and mental retardation, XMEA patients do not display these symptoms. These differences suggest that a defect of XMEA is specific for skeletal muscle, but not for cardiac muscle nor the central nervous system. XMEA patients have a normal life expectancy. XMEA-carrier females are almost normal or display a minimal defect, and have normal activity of creatine kinase in their serum. The XMEA gene maps to the region between DXS8103 and PAR (Xq pseudoautosomal region) on Xq28, the most telomeric band of the long arm of chromosome X.[58,59] Because the gene encoding lamp2 maps to Xq24,[30] it is different from the XMEA gene. Thus, XMEA is genetically different from Danon's disease, even though some characteristics of the vacuoles of XMEA patents are similar to those of patients with Danon's disease. Two pathogenic hypotheses were proposed for the XMEA defect. The first is that sublethal injury to the myofibers may result in an induction of intracellular autophagy to degrade sarcoplasmic debris, and the debris may be further extruded from the myofibers.[53] Another hypothesis is that deposition of complement MAC on the damaged cell surface membrane myofibers may mediate an injury to the cells resulting in muscle-disorder and the formation of autophagosomes, and that the membrane-bounded vacuoles may be a consequence of sarcolemmal invagination.[54]

Danon's disease—Danon's disease was first described as a lysosomal glycogen storage disease with normal acid maltase in 1981 by Danon et al (OMIM #309060).[60] The muscular pathology of Danon's disease can be distinguished from that of Pompe disease in that the former has normal acid maltase (acid α-glucosidase) activity, whereas the latter is caused by acid maltase deficiency.[60-63] Danon's disease is also called X-linked vacuolar cardiomyopathy, myopathy and mental retardation (XVCM-MR). The vacuoles of Danon's disease are similar to those of X-linked

myopathy with excessive autophagy (XMEA), and are positive for dystrophin, laminin, acid phosphatase and esterase staining.[60,64] The vacuoles are autophagosomes containing lysosomal enzymes. Unlike the vacuoles of XMEA patients, those of Danon's disease do not contain deposited calcium, complement C5b-9 MAC, or lamp2.[56,57] Danon's disease vacuoles were observed in both skeletal and cardiac muscles, while those of XMEA patients were observed only in skeletal muscle. Clinically, patients of Danon's disease show cardiomyopathy (100%) and mental retardation (60-80%). Creatine kinase activities of patient sera were elevated. Muscle weakness is often mild, so all patients remain ambulatory. However, they died of cardiac failure at 30-60 years of age probably because of a vacuolation of cardiac myofibers. Effective treatment for the disease involves cardiac transplantation.

Recent genetic analyses of independent patients of Danon's disease and lamp2-deficient mice revealed that lamp2 deficiency definitively causes Danon's disease (for a review see ref. 65).[20,30] Lamp2 is one of the lysosomal membrane proteins that is highly glycosylated.[66] All Danon disease patients have a mutation in the LAMP2 gene. The expression of two alternative splicing isoforms of LAMP2, LAMP2a and LAMP2b in human tissues is different: LAMP2b is mainly expressed in skeletal muscle and at a low but significant level in heart, while LAMP2a mRNA is expressed in human placenta, lung, and liver.[67] Examination of patients having a mutation in the LAMP2b-specific region leads to the conclusion that deficient levels of lamp2b causes cardiomyopathy, myopathy, and cardiac failure.[30] Analyses of lamp2-deficient mice provide further insight into the precise defects associated with Danon's disease.[20] Autophagic vacuoles accumulate extensively in many tissues including liver, pancreas, spleen, kidney, and skeletal and heart muscle of lamp2-deficient mice (Fig. 2I). Mortality of lamp2-deficient mice increases and the survivors display reduced weight compared with their wild type littermates. Cultured hepatocytes from lamp2-deficient mice display the accumulation of early and late autophagic vacuoles. The early autophagic vacuoles in lamp2-deficient hepatocytes contain cathepsin D and lamp1 at four-fold lower levels than that in wild type hepatocytes, and show slightly higher pH (pH 6.7) compared with the wild type autophagic vacuoles. Furthermore, degradation of long-lived proteins, which is mediated by autophagy, in lamp2-deficient mice is significantly inhibited. It is reported that lamp2a is essential for selective protein uptake by the lysosome (see chapter 12), but the molecular function of lamp2 in autophagy is still unknown. However, lamp2 plays indispensable roles in the maturation of autophagic vacuoles, and a defect in their maturation causes cardiomyopathy, myopathy and a defect in the central nervous system.

Neurodegenerative Disorders

There are some unique characteristics in inherited neurodegenerative disorders. First, cell death in certain populations of neurons is strongly correlated with the symptoms of the disease. Death of hippocampal and cortical neurons is responsible for the symptoms of Alzheimer's disease. Death of substantia nigra in the midbrain that uses dopamine as the neurotransmitter underlies Parkinson's disease. In Huntington's disease, neurons in the striatum, which controls body movement, are degenerated. Second, in many neuronal diseases, the etiology has revealed the role of aggregation or misfolding of particular proteins, which is the key to the onset or progression of the disease. Thus, the word "protein conformational disorder" is often used to refer to these diseases. Third, accumulation of endosomes, lysosomes, and autophagosomes is frequently associated with the symptoms. This accumulation may reflect a block rather than enhancement of autophagic protein degradation. Here, we mainly focus on three major diseases, i.e., Huntington's disease, Alzheimer's disease, and Parkinson's disease, which apparently involve severe disorders of autophagic-lysosomal proteolysis.

Huntington's disease—Huntington's disease is an autosomal dominant disorder caused by mutations of huntingtin, a cytosolic protein whose function is unknown and that is enriched in striatal and cortical neurons.[68-70] Huntingtin has a polyglutamine (polyQ) tract in its N-terminus. In Huntington's disease, abnormal expansion of the polyQ caused by codon (CAG)

reiterations in exon 1 of the Huntingtin gene produces mutated huntingtin with an expanded polyQ repeat (more than 37 tandem glutamines). The mechanism as to how the abnormal expansion is elicited is not yet elucidated. The mutant huntingtin or N-terminal polyQ fragments cleaved from it by caspases accumulate in the cell to form insoluble aggregates, which cause toxicity and cell death.

The severity of the disease closely correlates with the length of the polyQ tract. Mutant huntingtin with a longer polyQ repeat has a stronger tendency to form aggregates, and both accelerates the onset and strengthens the severity of the disease. This is confirmed in animal models. Mice transgenic for exon 1 of the human Huntingtin gene carrying CAG repeat expansions develop neuronal intranuclear inclusions containing huntingtin and ubiquitin and subsequently develop a neurological phenotype.[71] Transgenic expression of cDNA encoding a short N-terminal fragment with more than 82 polyQ repeats is sufficient to cause neurite aggregates and nuclear inclusions, and behavioral abnormalities of animals.[72] Thus, the formation of insoluble polyQ aggregates is an early event leading to manifestation of the disease.

The mutant huntingtin is associated with endosomes of patient fibroblasts and accumulates aberrantly in punctate structures in the cytoplasm. This morphology resembles that seen in samples from the brains of patients with Huntington's disease (Fig. 2J).[73,74] As the progressive aggregate formation of polyQ repeats in patient brains is a long-term process, other approaches using animal models and cultured cells have also been attempted to clarify the role of polyQ aggregation in cell toxicity. Using a clonal mouse striatal cell line (X57) transiently transfected with human huntingtin, it has been shown that mutant huntingtin formed nuclear and cytoplasmic inclusions and vacuoles, which increased with polyQ expansion and with time after transfection.[75] N-terminal fragments from mutant huntingtin, possibly cleaved at the presumptive caspase sites were localized to the vacuoles that are also cathepsin D positive. Ultrastructural analysis of huntingtin-transfected cells[76] has revealed that autophagic vacuoles with or without sequestered components, dense lysosomes, and multilamellar and tubulovesicular structures accumulate abundantly. Autophagic vacuoles and lysosome-like dense vacuoles could be also stained with anti-huntingtin antibody (Fig. 2K). However, most of the huntingtin molecules reside on the outer surface of these vacuoles. These morphological data suggest that aggregates of mutant huntingtin stimulate the autophagic, endosomal-lysosomal system but the entire process of autophagy is somehow hampered due to huntingtin sequestered in the lumen or associated with the outer surfaces of these vacuoles.

Ravikumar et al investigated whether mutant huntingtin with expanded polyQ repeats can be degraded by autophagy.[49] Seventy-four polyQ repeats fused to the N terminus of green fluoresecent protein (polyQ74-GFP) was transfected into COS7 cells. Degradation of polyQ74-GFP is inhibited by 3-methyladenine, a specific inhibitor of autophagy, and enhanced by rapamycin. Bafilomycin also inhibited the degradation of polyQ74-GFP. Similarly, in stable transfectants of PC12 cells expressing polyQ74-GFP, 3-methyadenine and bafilomycin cause accumulation of polyQ74-GFP, whereas rapamycin inhibits the accumulation. Interestingly, inhibitors of autophagy enhance cell death whereas rapamycin reverses the effects. It should also be noted that the overexpressed polyQ74-GFP form insoluble aggregates in a time-dependent manner. Once formed, the insoluble aggregates become resistant to rapamycin-induced autophagy. The data again confirm that the inability to degrade polyQ expansions by autophagy accelerates the progression of Huntington's disease.

It is difficult to explain why striatal neurons are primarily affected in Huntington's disease. As the striatum receives dense dopaminergic innervation in the brain, dopamine toxicity in relation to mitochondrial dysfunction and production of reactive oxygen species has been proposed as a promoting factor for cell death.[77] Petersen et al showed that both accumulation of autophagic vacuoles and cell death are promoted by dopamine in a cultured transgenic mouse cell line (Bates R6) expressing mutant huntingtin.[78] Interestingly, ubiquitin and DCF fluorescence, a dopamine-induced oxidation stress marker, colocalize on autophagic vacuoles. The results may indicate that dopamine-induced oxidative stress produces oxidized proteins, which are sequestered into autophagosomes.

In addition to Huntington's disease, seven inherited neurodegenerative diseases have been known to be caused by the expansion of polygulutamine repeats.[79] These are spinobulbar atrophy, dentatorubural pallidoluysian atrophy, and spinocerebellar ataxia types 1, 2, 3, 6 and 7.

Parkinson's disease—Parkinson's disease (PD) is a neurodegenerative disorder characterized by rigidity, hypokinesia, and postural instability accompanying trembling extremities.[80] The progressive loss of dopaminergic neurons of the substantia nigra is a major symptom. Pathological examination shows prominent accumulation of cytoplasmic inclusions of proteinaceous material with lipids called Lewy bodies. Lewy bodies consist of lipids, ubiquitin, enzymes involved in ubiquitin-related pathways, neurofilament proteins, α-synuclein, synphilin-1, etc. So far, three genetic causes of PD have been identified.[81-84] The gene encoding ubiquitin C-terminal hydrolase L1 (UCH-L1) has been identified in a German family.[85] Mutations in the gene encoding parkin, a ubiqutin E3 ligase, are identified in another familial juvenile parkinsonism with autosomal recessive inheritance.[86] Third, mutations in the gene encoding α-synuclein that localizes in presynaptic terminals and is abundantly contained in Lewy bodies are identified in certain cases of familial PD. The involvement of two genes responsible for PD in the ubiquitin-proteasome system has drawn intense attention to the possible role of ubiquitin-proteasome dysfunction in the progression of PD.

UCHL-1 is a deubiquitinating enzyme responsible for degrading polyubiquitin chains into ubiquitin monomers to regenerate the monoubiquitin pool. A mutation in UCHL-1 decreasing its enzyme activity was identified in a familial PD. Inability to degrade the polyubiquitin chain must retard the overall reaction of the ubiquitin-proteasome system, cause accumulation of ubiquitinated proteins, and ultimately menace the survival of neuronal cells. There is another disease named gracial axonal dystrophy, in which an in-frame deletion of UCHL-1 exons is elicited.[87] Accumulation of β-amyloid and ubiquitin was found in the neurons of mice with gracile axonal dystrophy.

Parkin is an ubiqutin E3 ligase, possessing consensus ubiquitin-like and RING finger motifs. Mutations in parkin cause autosomal recessive PD. As an E3, it utilizes ER-associated Ubc6 and Ubc7 as well as UbcH7 and UbcH8 as its E2.[88] Recently, it has been shown that parkin suppresses unfolded protein-stress-induced cell death.[88] Unfolded proteins formed in the ER are translocated across the ER membrane into the cytosol and degraded via the ubiquitin-proteasome system (ER-associated protein degradation; ERAD). Parkin participates in ubiquitination of unfolded proteins in ERAD.[88] Parkin-associated endocelin-receptor-like receptor (Pael-R) has been identified as an unfolded protein substrate recognized by parkin. When overexpressed in cells, this receptor tends to become unfolded, insoluble, and ubiquitinated in vivo. The insoluble Pael-R leads to unfolded protein-induced cell death. Parkin specifically ubiquitinates this receptor in the presence of Ubc6 and Ubc7, and promotes the degradation of insoluble Pael-R, resulting in the suppression of cell death. Moreover, the insoluble form of Pael-R accumulates in the brains of PD patients.

α-Synuclein is a protein of unknown function and a major component in Lewy bodies. Two point mutations in α-Synuclein (A53T and A30P) have been found in an autosomal dominant form of familial PD.[89,90] In vitro conformation analyses have shed light on a causative role of fibril formation by α-synuclein in PD. α-Synuclein existing in an unfolded state without any particular conformation in solution turns into β-sheet structures typical of amyloid fibrils in a concentration-dependent manner.[91,92] The conformation transition is reminiscent of that of prion protein in bovine spongeform encephalopathy. Mutant α-synuclein has a stronger tendency to form fibrils than wild type synuclein. It has been shown recently that synphilin-1, an α-synuclein interacting protein, which is enriched in Lewy bodies,[93] is recognized by parkin for ubiquitination.[94] Co-transfection of α-synuclein, synphilin-1, and parkin results in the formation of ubiquitin-positive protein inclusions. Hence, similar to huntingtin with abnormal polyQ expansions, misfolded or aggregated α-synuclein is believed to cause cell toxicity or stress and inhibits the ubiquitin-proteasome system. Lewy bodies may contribute to segregate aggregated α-synuclein into inclusions to moderate its toxicity.[95,96]

Dysfunction of the ubiqutin-proteasome system is directly or indirectly associated with hereditary PD and this is likely the case with other sporadic PD. It has been reported recently that autophagic-lysosomal dysfunction may be also involved in PD. Using stable PC12 transfectants expressing wild type and A53T mutant α-synuclein, Stefanis et al showed that marked accumulation of autophagic vacuoles and impairment of lysosomal and ubiquitin-proteasome functions are principal phenotypes in the cells expressing mutant α-synuclein.[97] Many vacuoles with double-limiting membranes and sequestered organelles in their lumen are observed, suggesting abundant accumulation of autophagosomes (Fig. 2L,M). These morphological features are similar to those predicted when a defect of autophagy at step 3 (see Fig. 1) is elicited. Because the cells expressing mutant α-synuclein exhibit no obvious features of apoptosis, the authors interpret these observations in terms of autophagic cell death. However, other studies reported that apotosis is induced by α-synuclein overexpression.[98,99] The selective loss of nigral neurons has been related with dopamine metabolism producing reactive oxygen species, which stimulate signaling cascades inducing apoptosis.[100,101] Coexistence of apoptotic features and characteristics of autophagic degeneration was reported in the brains of PD patients by an ultrastructural analysis.[102] For a better understanding of the cell death mechanism in PD, further investigations into the role of autophagy are necessary (see chapter 23).

Alzheimer's disease—Alzheimer's disease (AD) is a progressive neurodegenerative disease accompanying impairments in cognition and memory. The pathological hallmark of Alzheimer's disease is the accumulation of fibrillogenic amyloid peptides (Aβ40 and Aβ42) in brain hippocampal and cortical neurons as senile or amyloid (Aβ) plaques.[103] Progressive deposition of Aβ peptides is thought to be a causative event in early phases of AD, and the deposition increases with the progression of the disease, leading in turn to the formation of neurofibrillar tangles (NFTs). Aβ and phosphorylated tau protein together with lipids are the major constituents of NFTs. The NFTs cause cell death, resulting in the loss of affected parts of the neurons. Two important genes are known in familial Alzheimer's disease with autosomal dominant inheritance.[104] One gene encodes amyloid precursor protein (APP), a type I membrane protein. Newly synthesized APP is targeted to the plasma membrane where APP undergoes proteolytic processing to form a secreted form of APP (sAPP-α) and Aβ. Presumptive α-, β-, and γ-secretases are involved in the processing. Cleavage of APP by α-secretase produces sAPP-α. Cleavages by β- and γ-secretases produce 40 and 42 amino acid fragments (Aβ40 and Aβ42), respectively. Mutations of APP in familial AD facilitates production of more Aβ42 and Aβ40 than soluble APP, whereas processing of normal APP produces more soluble APP than Aβ40 and Aβ42. It has been shown that Aβ42 is much more exacerbating than Aβ40 for the formation of senile plaques. Presenilins (presenilin-1 and presenilin-2) are polytopic transmembrane proteins that are proteolytically processed to N- and C-terminal fragments.[105,106] Presenilin plays a critical role in the generation of Aβ from APP. It is believed to exert γ-secretase activity by itself, or control the trafficking of γ-secretase.[107]

It has been known for some time that cellular levels of lysosomal cathepsins, such as cathepsin B, cathepsin D, and cathepsin L are remarkably elevated in AD brains.[108,109] Immunocytochemical analyses showed that in normal control brains, these cathepsins are localized exclusively in intracellular lysosomes of neurons. In AD brains, immunoreactivities to these enzymes are associated also with extracellular senile plaques in addition to lysosomes of neurons, implying that these lysosomal cathepsins colocalize on amyloid plaques together with β-amyloid and lipofuscin granules.[108] More recently, elevated levels of cathepsin D and cathepsin D mRNA were shown by immunoblotting and Northern blotting, respectively, of patients' brains.[110,111] It is assumed that activation of the lysosomal system occurs as an early and distinctive response of neurons in AD, and after cell death, released lysosomal hydrolases accumulate in the neuronal parenchyma in association with lipofuscin granules and Aβ to form amyloid plaques. The upregulation of cathepsins in AD may be somehow linked with the cell death process. A role of cathepsin D in cytokine-incuced apoptosis and active recruitment of cathepsin D to the

death process has been reported.[112,113] Cathepsin B released from destabilized lysosomes into the cytoplasm of neuronal cells exerts principal injury effects on neuronal cells in experimental brain ischemia using primates.[114]

In parallel with elevated levels of cathepsins, a three-fold enlargement of endosomes as compared with the control was found to occur in sporadic AD brains. These endosomes are enriched with cathepsins B and D, and cation-independent mannose 6-phosphate receptor. To better characterize enlarged endosomes, an extensive ultrastructural examination was performed.[115] Enlarged endosomes were found in brain specimens of early-stage sporadic AD patients as well as patients with Down's syndrome. These endosomes possess rab 5, rabaptin 5, EEA1, and rab4, early and recycling endosome markers. These abnormally developed endosomes were observed in early stage AD and Down's syndrome but not in other neurodegenerative diseases such as Huntington's disease, encephalitis, and Lewy body dementia. These large endosomes may contribute to the endocytosis of Aβ and apoE, another risk factor of AD.

Granulovacuolar degeneration (GVD) is another unique pathological hallmark of AD together with NFTs and amyloid plaques. Usually GVD occurs in hippocampal pyramidal neurons of AD patients and is recognized as cytoplasmic inclusions of 3 to 5 μm in diameter accumulating in the cytoplasm. Using electron microscopy, the inclusions appear as membrane-bound inclusions (granulovacuoles) comprising irregular clusters of electron-dense granules.[116] Often, granulovacuoles with double membranes containing neuronal cytoplasm are seen but seldom do these vacuoles contain NFTs and lipofuscin granules. The number of granulovacuoles increases with age and severity of AD. Morphologically, granulovacuoles resemble autophagic vacuoles and recent findings that activated caspase 3 is present in granulovacuoles[117] may indicate that granulovacuoles are the autolysosome involved in caspase 3 maturation. The functional or pathological relevance to an aberrantly elevated endosomal and lysosomal system has not been investigated.

Conclusion and Perspective

The identification of many autophagy-related genes during the last decade has allowed us to adopt genetic approaches to understand the mechanism of autophagy dysfunction in various neuromuscular disorders. Growing numbers of studies dealing with this topic have been published recently. In many studies, however, descriptions of "aberrant autophagy" are mainly based on morphological data and are largely phenomenological. Information on what kind of autophagy dysfunction occurs and how it correlates with the etiology is still premature.

Close examination of the literature has picked up one inherited disease (Danon's disease) that has been shown to be due to the mutation of an autophagy-related gene. There are still many diseases for which the responsible genes have yet to be identified (Table 2). Now that a complete human genome sequence has been determined, responsible genes for these diseases will soon be elucidated: Some diseases may be identified as caused by the defects of other autophagy-related genes shown in Table 2. It is also possible that some diseases caused by the mutations of autophagy-related genes will be newly discovered in the near future.

There are many neuromuscular disorders whose responsible genes are not directly relevant to autophagy, yet they manifest abnormal accumulation of autophagic vacuoles or the appearance of irregular-shaped autophagic vacuoles in common with autophagy defects. This pathological sign implies that autophagy is a normal cell-survival response to counteract cellular disorganization elicited by diverse mutations. One on hand, autophagy is markedly upregulated in response to many diseases, but on the other hand, the entire process is simultaneously retarded at some step. In cases of DMRV and chloroquine myopathies, rimmed vacuoles, a prominent hallmark of the diseases, have been found positive for MAP-LC3-II, an autophagosomal membrane marker, demonstrating that rimmed vacuoles include autophagic vacuoles.[36] In view of chloroquine action and the similarity in the phenotypes among different myopathies, it is suggested that inhibition of autophagy at the fusion step (step 3) is likely to occur. As for neurodegenerative disorders, the histopathological appearance of patient brains

and experimental models of Huntington's and Parkinson's diseases is very similar to that of cathepsin D-deficiency. Hence, the accumulation of autophagic vacuoles in these disorders appears to reflect a block in autophagy at the degradation step (step 4). One common characteristic of these disorders is that mutations cause conformational disorders of particular proteins. Mutant proteins have a strong tendency to form insoluble aggregates, which can be sequestered into autophagic vacuoles as the substrates of autophagy, but are resistant to lysosomal proteinases. Importantly, insoluble protein aggregates formed in the cytoplasm exert a strong inhibitory effect on proteasome activity.[118] In Parkinson's disease, mutations are directly related with the ubiquitin-proteasome system. Thus, the two major systems for protein turnover are suppressed in neurodegenerative disorders. In addition to the effects on cellular protein degradative machineries, insoluble protein aggregates in the cytoplasm cause various types of cell toxicity, including oxidative stress, disturbance of Ca^{2+} homeostasis, etc. These toxic effects are known to induce apoptosis and other types of cell death, which are described elsewhere in this book (see chapter 23).

Recently, more attention has been attracted to the effects of diet restriction on age-related neurodegenerative disorders.[119] Dietary restriction significantly extends the lifespan. One hypothesis for this mechanism is that dietary restriction increases the resistance of the nervous system to age-related neurodegenerative disorders. Increases in the level of antioxidant enzymes, neurotrophic factor, and enzymes involved in DNA replication, may contribute to this mechanism. Obviously, dietary restriction prolongs starvation, under which autophagy is maximally enhanced. Therefore, enhancement of autophagy by dietary restriction may considerably contribute toward removing aggregate-prone proteins before they elicit irreversible damage to neurons.

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CHAPTER 23

Autophagocytosis and Programmed Cell Death

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Abstract

In the last decade tremendous progress has been achieved in understanding the control of apoptosis by the cytokine/growth factor network of organisms as well as the molecular mechanisms of signal-transduction in preparation and final execution of the cell's suicide. Accumulating evidence suggests that apoptosis is not the only type of programmed cell death (PCD) but that cells use different pathways for active self-destruction as reflected by different morphologies: While in apoptosis (or "type I") condensation events are predominant, autophagy is a prominent characteristic of a second type of PCD ("type II"). Autophagic PCD appears to be a phylogenetically old phenomenon, it may occur in various organisms including humans in physiological and disease states. Recently, distinct biochemical and molecular features have been assigned to this type of PCD. However, autophagic and apoptotic PCD do not appear as mutually exclusive phenomena. Rather, they may occur simlutaneously in tissues or a cell may even switch from apoptotic to autophagic degradation of cytoplasmic structures. This chapter reviews morphological, functional and biochemical/molecular data on the role of the autophagosomal and lysosomal compartments in programmed cell death.

Historical Perspectives: Programmed Cell Death—Apoptosis—Autophagic Programmed Cell Death—Necrosis

The occurrence of cell death under a variety of physiological and pathological conditions in multicellular organisms has been documented many times during the past 150 years.[1-3] In 1871, Virchow described the diversity of cell death as "necrosis" and "necrobiosis."[4] Subsequently, cell death was reported to occur during metamorphosis of invertebrates and lower vertebrates and the development of mammals; in adults, cell loss may occur according to physiological demands as well as after various kinds of damage by chemicals, injury, radiation, hypoxia etc (for review see refs. 5-10). In developmental biology cell death essentially was considered as a "programmed" event.[10-13] On the other hand, in toxicology and pathology cell death mainly was viewed as a passive, degenerative phenomenon occuring after severe damage of tissues.[14] It was not before the early 1970s when Farber et al—based upon a characteristic morphology of cell death and its requirement for protein synthesis—suggested the occurrence of a "suicide" type of cell death in liver, intestine, and other organs after treatment with cytotoxic anti-cancer drugs.[15] The widespread occurrence and biological relevance of programmed cell death was also advocated by Kerr, Wyllie and Currie, who in 1972 proposed a morphologically based classification of cell death into two broad categories. According to this proposal the term "necrosis," which usually was used for all types of cell death, was redefined and restricted to events caused by violent environmental perturbation leading to collapse of internal homeostasis. The new term "apoptosis" (now often and in a broader sense called programmed cell

Autophagy, edited by Daniel J. Klionsky.

death) was coined to describe an orchestrated self-destruction of a cell.[5,6] Apoptosis gained considerable credit when it became clear that it constitutes an essential part of life for many multicellular organisms and modern techniques provided insights into its molecular pathways; these were revealed to be conserved from worms to mammals. Thus, in the last decade, a tremendous gain in knowledge concerning the molecular events of signaling, preparation and execution of apoptosis has been achieved (for review see refs. 9,13, 16-25).

However, morphological and biochemical evidence suggests that programmed cell death is not confined to apoptosis but rather cells may use different pathways for active self-destruction: Condensation prominent, type I or apoptosis; autophagy prominent, type II PCD, etc.[26-31] Thus, Schweichel and Merker[26] and Clarke[28] noted that under certain conditions programmed cell death may exhibit morphological features clearly different from apoptosis. In the developing embryo, three morphologically distinct types of cell death were described: Type I is most likely identical to apoptosis. Type II is characterized by a prominent formation of autophagic vacuoles ("autophagic cell death"). Type III is described as occurring through disintegration of cells into fragments without involvement of the lysosomal system and without marked condensation.[28] The present chapter reviews the role of autophagy in programmed cell death. In addition to a comparative view on morphological and functional features of apoptosis and those of autophagic PCD, an attempt is made to identify links between the control of autophagocytosis in general and those molecular events that specifically may affect the life-death decision of cells.

Programmed Cell Death (PCD): Morphological and Functional Diversity

Apoptosis

Apoptosis originally was defined on the basis of a specific pattern of morphological changes in the dying cell: Condensation of cytoplasm, in solid tissues separation from neighboring cells, condensation of chromatin at the nuclear membrane to sharply delineated masses and cell fragmentation into apoptotic bodies (Fig. 1a,b; the sequence of histological stages of apoptosis is schematically depicted in Fig. 2).[5,6] In highly condensed dead cells or cell fragments organelles are still intact as shown by electron microscopy; cellular membranes are well preserved and, consequently, cell contents are not liberated. In vivo, apoptotic bodies are rapidly phagocytosed and degraded by neighboring cells. In fact, apoptotic cells display "eat me signals" at their surface serving for recognition and rapid uptake by adjacent vital cells and thus prevent inflammation and secondary tissue damage.[5,6,19] No evidence for lysosomal or autophagic events in apoptotic cells in vivo was noted in morphological and histochemical studies.[5,6,32] Rather, degradation of apoptotic bodies ensues as the final step of phagocytosis by vital cells (heterophagy).[5,6,19,32]

The occurrence of apoptosis cannot be narrowed down to certain biological conditions, rather apoptosis is a widespread phenomenon in the living world and plays important roles in states of health and disease.[5,6] Nevertheless, it seems that cells consisting of relatively small amounts of cytoplasm such as non-secretory cells enter the apoptotic pathway more readily than large, secretory cells (see below).[29]

Autophagic Cell Death

Definition and Occurrence

Autophagic cell death, at the level of electron microscopy, is characterized by the degradation of cytoplasmic components resulting in progressive loss of electron density; the descriptions of autophagic cell death consistently include that degradation of cytoplasmic components precedes nuclear collapse (see below and Fig. 1; the sequence of histological stages of autophagic PCD is schematically depicted in Fig. 2). However, denoting cell death as "autoph-

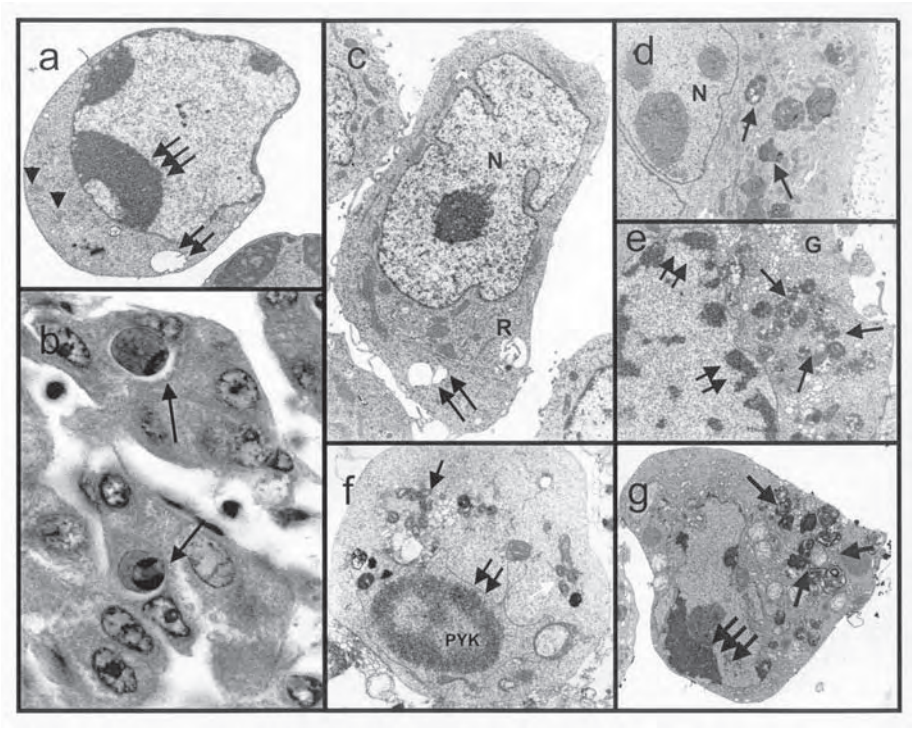

Figure 1. Morphological features of programmed cell death. Apoptosis (a,b). (a) Ultrastrastructure of spontaneous apoptosis in hepatoma cell cultures (Hep3B). Apoptotic body exhibiting a highly condensed cytoplasm containing nuclear fragments (↑↑↑), electron translucent vacuoles (↑↑), intact mitochondria (↑). Note the absence of autophagic vacuoles (see Fig. 1g), x14,400 (b) Hematoxylin and eosin stained human liver tumor section, intracellular apoptotic bodies (↑). Note dense homogenous and eosinophilic cytoplasm with highly condensed chromatin. Autophagic PCD (c-g). Ultrastrastructure of cell death in MCF-7 cultures after tamoxifen treatment. (c) Control, day 7; electron translucent vacuoles (↑↑), intact nulceus (N). The plasma membrane exhibits extended areas with villi (v), the cytoplasm typically shows multiple polyribosomes (R). (d) 10^{-6} M tamoxifen, day 7; the nucleus appears normal (N), in the cytoplasm numerous AVs (↑) are visible. (e) Tamoxifen 10^{-6} M, day 7; ribbons of condensed chromatin are detached from the nuclear envelope (↑↑). Numerous AVs (↑) and prominent Golgi regions (G) of the cytoplasm. (f) Tamoxifen 10^{-6} M, day 7; rounded cell, surface characterized by loss of microvilli. The condensed chromatin is detached from the nuclear envelope and concentrated in the center of the nucleus (PYK); the nuclear envelope appears intact (↑↑). In the amorphous cytoplasm polyribosomes are not visible; mitochondria and AVs are clustered at the cell poles (↑). (g) Tamoxifen 10^{-6} M, day 7; the chromatin is condensed to crescent masses that abut the nuclear envelope (↑↑↑), providing an example of apoptotic elements in autophagic PCD. Numerous vacuoles of different contents mark the cytoplasm (↑). Bars 2 μm.

agic/type II PCD" needs a cautionary note. Reviewing the literature reveals a non-consistent use of terms to describe cell death associated with autophagocytosis as it includes necrosis, non-apoptotic types of cell death, apoptosis/type I PCD, autophagic cell death/type II PCD and others (for review see ref. 30). Relatively little is known of the molecular events underlying the initiation and execution of autophagic cell death. Therefore, in the present chapter a demonstration of autophagic vacuoles (AVs) in dying cells by electron microscopy is taken as conditio sine qua non (i.e., an absolute prerequisite) to denote cell death as autophagic/type II PCD; AVs in the first steps of the pathway (autophagic sequestration) are separated from the cytoplasm by a double-membrane followed by vacuolation of the intercisternal space and loss

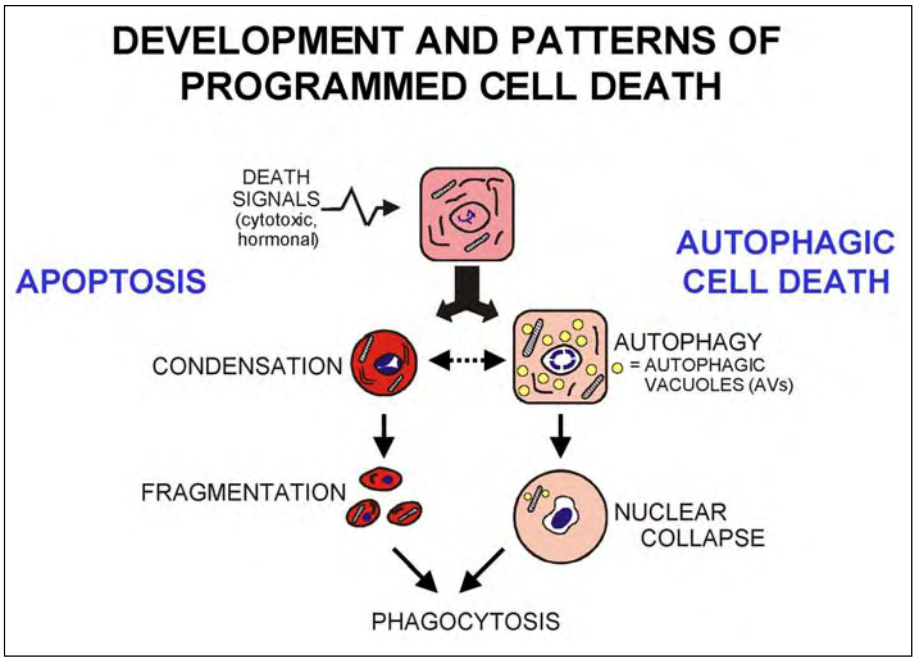

Figure 2. Development and patterns of cell death. Apoptosis: Condensation of cytoplasm and of chromatin at the nuclear membrane to sharply delineated masses (often like crescents) followed by cell fragmentation into apoptotic bodies. Phagocytosis (in vivo) and heterophagic degradation. Note: According to original description autophagy/lysosomes do not play a distinct role early in apoptosis. Autophagic cell death: Autophagy; formation of autophagic vacuoles (AVs) and degradation of cytoplasmic constituents. Nuclear collapse (as observed in MCF-7 cells); pyknosis, single pyknotic mass in the center of the nucleus, nuclear envelope still intact, cytoplasm amorphous with few clusters of AVs and mitochondria. Note: Autophagocytosis with apoptotic-like DNA condensation/fragmenation may also occur; Phagocytosis (in vivo) and final degradation. See text for details and references.

of inside membrane. Typical inclusions of the enveloped cytoplasmic portions comprise mixed contents like curled membranes and organelles gradually undergoing degradation (Fig. 1d-f).[33] In addition, histo- and biochemical criteria indicating a role of the autophagosomal-lysosomal compartment can be taken into account as reviewed in detail elsewhere.[30] It should be emphasized, that referring to the morphological/histochemical features does not imply a causative relationship between autophagcoytosis and eventual manifestation of a cell's suicide; this will require either an established functional link between these phenomena and/or elucidation of specifically related molecular events. Finally, in cultured cells cytoplasmic vacuolization—which might be mistaken as autophagic vacuoles—is widely observed; however based upon their electron translucent appearance this type of vacuoles can be descriminated from autophagic vacuoles (for review see ref. 34).

Autophagic cell death appears to be a phylogenetically old phenomenon as it has been observed in the slime mold *Dictyostelium discoideum* and in the nematode *C. elegans*.[35-37] It is important during insect metamorphosis which is one of the most extreme biological conditions of tissue remodeling; here cells of ecto-, endo- and mesodermal origin are affected (for review see refs. 7, 28-30). Likewise, in vertebrate development, autophagic cell death appears to be a prominent feature. It is associated with organ morphogenesis as exemplified by shaping of extremities, cavity formation in intestine and regression of sexual anlagen (for review see

refs. 7, 26, 28, 30). Autophagic cell death also is reported to occur in adult insects and vertebrates including humans; it is often associated with the elimination of (large secretory) cells during adjustment of sexual organs and ancillary tissues to seasonal reproduction. As to pathophysiology, autophagic cell death has been associated with experimental and human neurological diseases, after cytotoxic drug treatment, but also during spontaneous regression of human tumors (for review see ref. 30).[38]

Taken together, autophagic PCD predominantly appears to be activated when the developmental program or (in adulthood) homeostatic mechanisms demand massive cell elimination; in all cases, the bulk of cytoplasm is degraded by autophagy before nuclear collapse ensues. In instances of cell injury, damaged organelles or membranes may be transferred into the autophagic pathway serving as a protective response at the subcellular scale and in case becoming overwhelmed, elimination of the whole cell may result. Thus, these functional features of autophagic cell death are in line with the general function of autophagy, namely being the major inducible pathway for degradation of cytoplasmic components including whole organelles.[39-41] It should be noted, however, that autophagic cell death and apoptosis are not mutually exclusive phenomena. Thus, both types of cell death can occur simultaneously in tissues, but also subsequently as governed by the developmental program. Moreover, individual dying cells may exhibit both, apoptotic and autophagic features ("mixed type") (for review see refs. 7, 29, 30).

Remarkably, the mode of cell death does not necessarily affect the efficient clearance of cell residues from the body through phagocytosis. Thus, in vivo autophagic PCD has been found to be completed by heterophagy (for review see refs. 7, 28, 30). Obviously, dying cells in general display surface signals to facilitate their phagocytosis, the expression of which constitutes an integral part of the overall PCD-signaling.[19]

Human Mammary Carcinoma (MCF-7) Cells As a Model for Autophagic PCD

For almost 3 decades, human mammary carcinoma (MCF-7) cells have been used as a biological test system in drug development, namely to select drugs with a strong anti-proliferative potency for treatment of human mammary tumors (for review see refs. 42, 43). More recently, we and others have used MCF-7 cells as a model to study the anti-survival effect of anti-estrogens such as tamoxifen, ICI 164384 and toremifene.[44-49] Thus, tamoxifen at high doses (10^{-5} M) causes lysis (necrosis) of almost all cells within 24 hours that cannot be prevented by estradiol.[48] The cytotoxic action of tamoxifen may result from perturbations in membrane fluidity,[50] formation of reactive oxygen species,[51] DNA damage by DNA adducts or chromosomal aberrations, which have been found to occur in kidney and liver.[52,53] On the other hand, lower concentrations of tamoxifen (10^{-6} M and below) induced a gradual appearance of cell death starting to occur 3 days after treatment. This type of cell death is considered to be a receptor-mediated, active cell suicide because it can be inhibited by estradiol.[48] This "mitogen rescue" is considered to be characteristic for an active or programmed mode of death and is often used to functionally discriminate PCD from necrosis, which—according to this view—would only be prevented by removal of the noxious agent (for review see refs. 23, 54). Closer electron microscopy studies revealed that the active self-destruction of MCF-7 cells after tamoxifen treatment belongs to the autophagic type of PCD; respresentative morphological features are shown in Fig. 1c-g. In MCF-7 cells upon tamoxifen treatment, the first changes visible at the electron microscope level comprise formation of autophagic vacuoles (AV; see chapter 2), which gradually degrade cytoplasmic structures (Fig. 1c-f). Notably, in cells exhibiting a highly condensed nucleus, structures required for protein synthesis such as polyribosomes, ER, and Golgi have disappeared, whereas a few clusters of intact mitochondria persist in close vicinity to AVs and the nuclear envelope (Fig. 1f). The electron microscopy studies were confirmed and extended by histochemical studies with monodansylcadaverine (MDC), which has been described to selectively accumulate in autophagic vacuoles.[55,56] MDC was used to visualize AVs in MCF-7 cells and to compare the kinetics of AV formation with those of nuclear condensation at the light (fluorescence) microscopy level. Clearly, AV formation pre-

ceded nuclear collapse which reflects the irreversible stage of cell death.[48,49] As will be outlined subsequently, this experimental model has been successfully used to elucidate some of the functional and molecluar features of autophagic PCD.

Programmed Cell Death: From Morphology to Molecular Mechanisms

Apoptosis

Many of the morphological features typical of apoptosis can be traced to the activation of cysteine proteases, which belong to the large protein familiy of caspases (for review see refs. 23, 57-59). Caspases are highly conserved through evolution and have been found in insects, nematodes, and hydra.[57,61] A set of sequentially acting "initiator" and "executioner" caspases have been identified as central players in apoptosis (for review see refs. 23, 57-60). Two major processes are active upstream of the caspase cascades: 1. Receptor-mediated death signaling ("death receptor") that ultimately triggers caspase-8 as exemplified by the interaction of CD95 with its ligand ("extrinsic" pathway); 2. Release of a set of molecules (cytochrome c, apoptosis-protease-activating factor-1 (APAF1), apoptosis-inducing factor (AIF)) from mitochondria as central targets for mediating intracellular death signals as exemplified by oxidative stress or DNA damage; cytochrome c and APAF1 form a complex responsible for the activation of caspase-9 ("intrinsic" pathway).[25] This pathway is subjected to the control of pro- and anti-apoptotic members of the Bcl-2 family (for review see refs. 23,25,55-60). Both pathways are considered to join at the level of caspase-3, which is the common substrate for caspase-8 and -9, and thereby mediates the final execution of apoptosis and its characteristic morphological manifestation. In addition, the extrinsic and intrinsic pathways may communicate upstream of caspase-3 through caspase-8-mediated Bcl-2 interacting domain (BID) cleavage at Arg59; truncated BID targets to mitochondria and stimulates release of mitochondrial pro-apoptotic molecules (for review see refs. 23, 25, 55-60).

Recently, BID has been also found to link the lysosomal compartment to the intrinsic pathway of apoptosis (for review see refs. 22, 62, 63). Lysosomal cathepsins cleave BID at Arg65 and thereby, a diversity of relatively unspecific signals such as photodamage or lysosomotropic agents may be transduced to the specific enzyme cascades that trigger the coordinated PCD program.[22] Thus, the lysosomal compartment may play specific roles in different PCD pathways: 1. In caspase-dependent apoptosis; 2. By providing the cell's hydrolytic machinery required for autophagocytosis (for review see refs. 22, 62-64). Furthermore, in the endoplasmic reticulum caspase-12 appears to be activated by stress stimuli,[65] the Golgi membranes and the nucleus contain caspase-2,[66] and discrete subnuclear structures may also carry caspase-9 all of which may provide the initial death signal.[67] Taken together, it appears that these organelles play specific roles in channeling a wide variety of different pertubations to the caspase-driven self-destruction of cells.[68]

Caspases: A Molecular Switch for Different PCD Morphologies?

The model of "caspase-dependent" apoptosis is an extremely important paradigm, however, apparently not universal. Thus, caspase-coding sequences are absent from many non-mammalian species but nevertheless, these organisms may undergo PCD under conditions of stress (for review see refs. 23, 29, 68). For instance, PCD in yeast is associated with DNA fragmentation, membrane blebbing, and phosphatidylserine exposure at the outer cell membrane and can be selectively triggered or blocked by Bax-like or CED-9-related genes (for review see ref. 68). Several authors have recently pointed out that the introduction of caspases during evolution may reflect a decisive refinement of the ancient caspase-independent death programs including autophagic PCD (for review see refs. 23, 31, 39).

Nevertheless, in mammalian systems caspase-independent modes of cell death have been reported manyfold (for review see refs. 13, 21, 23, 31, 69). Early reports on caspase-indpendent

cell death, for instance, include bax-expressing thymocytes dying in the presence of caspase inhibitors,[70] neuronal cells (PC12) developing a necrosis-like phenotype in the presence of caspase inhibitors,[71] and cell death resulting from transfection with BNIP3 (a death protein of the Bcl-2 familiy member associated with mitochondria).[72] Recently, AIF was found to play an important role in the regulation of caspase-independent cell death.[69] Notably, some studies provided evidence that caspase-independent mode(s) of cell death might belong to the autophagic type PCD as exemplified by neuronal cell death resulting from oncogenic RAS overexpression,[38,73,74] death of sympathetic cells[74] and death of MCF-7 cells after tamoxifen treatment,[48] or consequent to DAP-kinase expression.[76] However, autophagic PCD in salivary glands during development of *Drosophila* has been reported to require caspase activity; the role of caspases at distinct stages of autophagy is not clear.[77] In conclusion, autophagic PCD cannot be specifically attributed to caspase-independent pathway(s) of PCD.

It is of interest to note, that unoccupied insulin-like growth factor receptor I has been found to mediate death of fibroblasts.[78] The dying cells lack chromatin condensation, DNA cleavage and any evidence for caspase activation. The cytoplasm exhibited strong vacuolization, however, not of the autophagic type.[78] Notably, caspase-9, but none of the other caspases, are causative for this non-apoptotic manifestion of cell death. Moreover, this PCD type is not simply a result of the lack of the apoptosis machinery, as Bax can induce "classical" apoptosis. Sperandio et al denoted this type of cell "paraptosis"; it is tempting to speculate that paraptosis might correspond to the non-lysosomal or type III cell death as reviewed by Clarke.[28]

Autophagic PCD

Prior to discussing the relation of autophagy to PCD in detail, it should be noted that autophagocytosis constitutes the major inducible pathway for degradation of cytoplasmic components including whole organelles, which does not necessarily result in the death of cells. By autophagocytosis cells may adapt to environmental changes such as nutrient deprivation, damage of subcelullar structures including membranes, and whole organelles.[39-41] Thus, the question arises whether autophagy might be just a "side-effect" of the stress imposed on the cells. Or does a functional link exist between autophagocytosis and execution of the (final) death program? If so, what are the underlying molecular mechanisms? Which are the key pathways mediating external signals to autophagocytosis and/or cell death?

To approach some of these questions, current knowledge on autophagy was applied in studies on non-apoptotic programmed cell death/PCD in MCF-7 cells. Autophagy ensues through a sequence of events which are highly conserved from yeast to humans including: Sequestration of cytoplasmic constitutents, formation and maturation of autophagosomes, their fusion with lysosomes to give rise to autolysosomes and the final degradation of cytoplasmic material.[39,41] A functional link between autophagocytosis and cell suicide was suggested by inhibition experiments with 3-methyladenine (3-MA), wortmannin and LY294002 (see chapters 3 and 4).[48,73,74,79,80] 3-MA, an inhibitor of the sequestration of cytoplasmic components,[81,82] has been found to prevent both the formation of autophagic vacuoles and the eventual cell death (indicated by nuclear destruction) in a variety of different cell types including tamoxifen-treated human mammary carcinoma cells (MCF-7),[48] gastric and glioma cells overexpressing Ras,[38,73,74] TNF–α treated human T-lymphoblastic leukemic cells,[79] neuronal cells upon serum withdrawal or treatment with arabinoside,[75] and kidney cell lines treated with bacterial toxins such as ricin, abrin, Shiga toxin and diphtheria toxin.[82] Likewise, our studies on "mitogen rescue" of MCF-7 cells with estradiol revealed that estradiol not only prevents nuclear destruction, but also inhibits the preceding formation of autophagic vacuoles.

At the molecular level, recent studies suggested the involvement specifically of the class III phosphatidylinositol (PtdIns) 3-kinase product PtdIns(3)P in sequestration: Formation of PtdIns(3)P as well as of autophagosomes was found to be inhibited not only by 3-MA, but also by wortmannin and LY294002.[80] Importantly, it was pointed out by Tolkovsky et al that although 3-MA has been described to specifically block the sequestration step, its effects are not

exclusively limited to the autophagic process.[83] Thus, 3-MA was found to inhibit the phosphorylation of JNK and p38 kinases in NGF-deprived neurons (both may be involved in apoptosis signalling[75]) and to attenuate mitochondrial permeability transition pore opening. Nevertheless, although 3-MA may affect additional pathways, studies with autophagy inhibitors other than 3-MA, namely estradiol, wortmannin and LY294002, strongly suggest a functional link between formation of autophagic vacuoles and PCD pathway(s).

In further studies we addressed the fate of the cytoskeleton during autophagic cell death as well as apoptosis. In apoptosis, the cell's preparatory as well as executional steps include depolymerization or caspase-driven cleavage of actin, cytokeratins, lamins and other cytoskeletal proteins, most probably resulting in the typical final shape of apoptotic cells; representative examples are shown in Fig. 3a (for review see refs. 13, 16-21, 23, 59). On the other hand, all steps of autophagocytosis are known to depend on certain cytoskeletal elements (for review see ref. 40). For instance, intermediate filaments (cytokeratin, vimentin) are necessary for sequestration of cytoplasmic structures.[84,85] Furthermore, all stages including the final degradation of cytoplasmic material in AVs are ATP-dependent.[86-88] Therefore, we investigated whether cleavage of the cytoskeleton as described for apoptosis would, or would not occur during autophagic cell death. The fate of cytoskeletal elements was closely followed during autophagic cell death in individual MCF-7 cells by immunocytochemistry after tamoxifen treatment; in addition, the protein pattern was analysed by biochemical means.[48,49] Indeed, the cytoskeleton was found to be redistributed but largely preserved, even in cells exhibiting nuclear condensation/fragmentation, i.e. the irreversible stage of cell death (Fig. 3b).[48] A pronounced fragmentation of the cytokeratin was not detected before MCF-7 cells detached from the substrate which is a very late stage of cell death in MCF-7 cells and probably reflects secondary necrosis.[49] Remarkably, the vast majority (about 85%) of MCF-7 cells still contained F-actin when the nucleus was already condensed.[49] Polymerization of G- to F-actin is an ATP-dependent process and, therefore, F-actin is a sensitive indicator of the metabolic state of a cell. In support of this notion, electron micrcoscopy and rhodamine 123 staining revealed that even at late stages of the death process autophagic vacuoles were associated with clusters of structurally and functionally intact mitochondria although most of the cytoplasm appeared amorphous (Fig. 1f).[48,49] It appears likely that ATP synthesis is maintained during the progression of autophagic cell death at a level required for the completion of autophagocytosis. Moreover, transglutaminase, an enzyme cross-linking proteins and subcellular structures, is activated in apoptotic hepatocytes,[14] but not in tamoxifen-induced PCD of MCF-7 cells. Thus, the preservation of the cytoskeleton during autophagic death of MCF-7 cells matches with current concepts of the cytoskeleton's function in autophagy.

What about the molecular control and signal transduction for autophagic PCD? One of the first molecular links between autophagy and programmed cell death in mammalian cells has been provided by studies on the RAS-signaling pathway: Expression of an oncogenically mutated Ras gene in human glioma and gastric cancer cell lines induced cell death associated with autophagocytosis.[38,73,74] The nuclei remained relatively well preserved and were negative for TUNEL staining thus matching with the features of autophagic PCD (Table 1).[38,73,74] Furthermore, Ras-induced cell death occurred in the absence of caspase activation, it did not require wild type p53 activity and was not inhibited by the anti-apoptotic Bcl-2 protein.[38,73,74] These features of Ras-induced cell death as demonstrated in experimental systems were recently confirmed and extended by clinical observations on spontaneous regressing neuroblastoma in humans: Cell death was found to be associated with increased Ras expression, but lack of capase-3 activation and DNA fragmentation.[38] Notably, the functional effector machinery for the execution of apoptosis could be activated in the Ras-transformed cells by TNF-α demonstrating that the manifestation of autophagic cell death does not simply reflect defective apoptosis.[73] Rather, cells apparently switch between different suicide pathways depending on the external death signal. Likewise, MCF-7 cells may enter the autophagic, caspase-independent PCD pathway upon antiestrogen treatment whereas TNF-α/TNF-related apoptosis-inducing ligand (TRAIL)-induced cell death of MCF-7 cells was found to involve activation of the

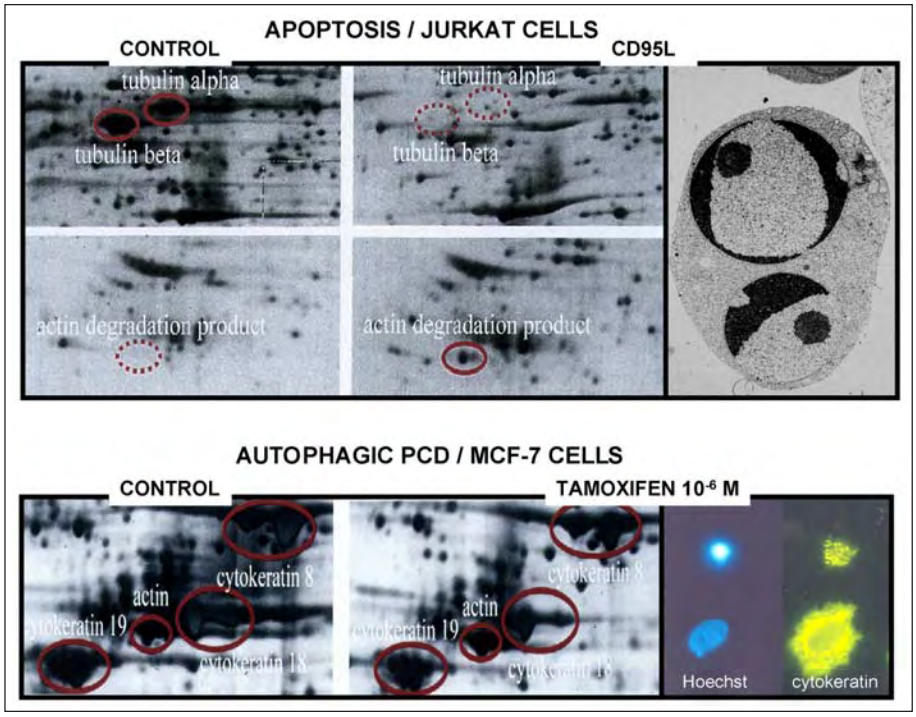

Figure 3. Different fates of the cytoskeleton in apoptosis and autophagic PCD as demonstrated by immunocytochemical and biochemical means. (a) Apoptosis. Jurkat cell were treated with CD95L for 4 hours as described in detail elsewhere.[112] (b) Autophagic PCD. MCF-7 cells were treated with tamoxifen (10^{-6}M) as described in detail elsewhere.[48,49] Proteome analysis: Subcellular fractions (cytosol, crude membranes, nuclear matrix) were isolated from normal and dying cells and analyzed by high-resolution two-dimensional (2D) gel electrophoresis; thereby, more than 2,000 proteins from the cell fractions can be separated. Labeling of proteins by ^{35}S-methionine and subsequent autoradiography of 2D gels allowed the differentiation of newly synthesized proteins, while silver staining of these gels revealed the whole protein pool of the respective fraction. Proteins were identified based upon their immunoreactivity or by mass spectrometry of tryptic digests.[112] A color version of this figure can be seen at http://www.eurekah.com/abstract.php?chapid=1322&bookid=98&catid=69.

initiator caspase–8 at the apex of a caspase cascade including cleavage of cytoskeletal proteins (Table 1).[89]

Of note, cell death induced by the oncogenic Ras pathway was dependent on the activity of PtdIns 3-kinases, a physiological downstream effector of Ras.[73] In turn, the PtdIns 3-kinases downstream effectors comprise the mTOR/p70S6 kinase pathway, which is considered to be the master switch between catabolism and anabolism of cells (see chapters 3 and 4).[39-41,90] mTOR may also exert a plethora of functions in various pathways of programmed cell death.[91] The hypophosphorylated p70S6 kinase promotes detachment of ribosomes from endoplasmic reticulum, presumably one of the initial molecular events in the sequestration step of autophagy (for review see refs. 39-41; see chapter 2). mTOR also might help to link recent observations on some of the yeast *APG* genes (required for autophagy) to cell death. To date fourteen *APG* genes are known to act in a conjugation cascade controlling initiation and execution of autophagy (see chapters 6 and 7); notably, these molecular processes have been found to be highly conserved from yeast to humans as reviewed elsewhere (see chapter 15).[39-41,92] In the context of programmed cell death, two members of this gene family will be annotated briefly. First, human Apg5 (hApg5) was considered to be homologous to "apoptosis specific protein"

Table 1. Differences and commonalities between apoptotic (type I) and autophagic (type II) PCD

	AUTOPHAGIC PCD	APOPTOTIC PCD	
	MCF-7 / TAM	MCF-7 / TNFα	Jurkat Cells/CD95L Hepatocytes/TGFβ1
Autophagic vacuoles	+	--	(+)*
Stress response (hsp 90 translocation)	+	n.d.	+
Protein synthesis			
(auto)phagosomes: annexin VI, grp78,	+		-
lysosomes: cathepsin B, D	-	n.d.	n.d.
Cytoskeletal protein breakdown			
intermediate filaments, fodrin[1,2], actin[2], myosin light chain	-	+	+
Caspase involvement	-	+	+
Transglutaminase involvement	-	n.d.	+
Nuclear protein breakdown			
SAF-A[2], SATB1[2], p40, lamin B [2,3]	-	n.d.	+
SUPT6H, HA95, PWP-1	+		+
DNA fragmentation			
HMW	+	+	+
LMW	- (±)	- (±)	+
TUNEL (in situ)	- (±)	- (±)	+

HMW = high molecular weight (50-300 kbp) and LMW = low molecular weight (20 kbp and below) DNA fragmentation as revealed by PULSE-field and conventional agarose gel electrophoresis.[48] (1): calpain target; (2) caspase 3 target; (3) caspase 6 target; SAF, scaffold attachment factor A; STAB1, Special AT-rich sequence binding protein 1; p40, laminin–binding receptor precursor p40; (4-6) chromatin-associated proteins: SUPT6H, putative chromatin structure regulator; HA95, neighbor of A-kinase anchoring protein95; PWP-1, nuclear phosphoprotein similar to *S.cerevisiae* PWP-1. n.d., not determined. (+)* under cell culture conditions we occasionally have observed the occurrence of autophagic vacuoles in intact as well as apoptotic cells; although autophagy in these cases appears not to be related specifically to the induction of cell death, this phenomenon should not be concealed.[49] See text for further references.

(ASP)[93] and this work has been repeatedly cited as providing evidence for a molecular link between programmed cell death and autophagy (for review see ref. 94). However, this hypothesis can no longer be maintained. As most recently demonstrated by Tolkovsky and co-workers, the apoptosis-specifc protein (ASP, 45kDa) and hApg5 are unrelated proteins that share the property (along with other proteins) of interacting with c-jun polyclonal antibodies used in the earlier studies.[94] Secondly, beclin 1 (Apg6/Vps30) has been described to induce autophagocytosis in mammalian cells; beclin 1 is a Bcl-2-interacting protein with structural similarity to the yeast autophagy gene APG6/VPS30.[95] In MCF-7 cells, the autophagocytosis-promoting activity of beclin 1 was associated with inhibition of MCF-7 cell proliferation, in vitro clonogenicity and tumorigenesis in nude mice, but no evidence for an induction of cell death was observed (see chapter 20).[95] However, most recent studies on neurodegeneration in Lurcher mice provided evidence for an interaction of beclin 1 with two other proteins, namely the mutated glutamate GluRδ2(Lc) receptor and nPIST, a novel isoform of a PDZ domain-containing protein that binds to this receptor, and that the interaction of these proteins results in autophagic death of cerebellar Purkinje cells.[96]

Of note, most recent studies by Kimchi and co-workers revealed the death-associated protein (DAP)-kinases as important regulators for both, apoptosis and autophagic PCD.[76] DAP-Kinases (DAPk) are a group of Ca^{2+} calmodulin-regulated serine/threonine kinases, known since a few years to be involved in a wide array of apoptotic pathways initiated by interferon–γ, TNF-α, CD95-L and detachment from extracellular matrix.[97] Recently, DAPk-related protein kinase (DRP-1) was isolated as a novel member of the DAP-kinase family of proteins.[98] DRP-1 and DAPk have been found to possess rate-limiting functions in two distinct cytoplasmic events, namely membrane blebbing (characteristic of apoptotic cell death) as well as extensive autophagy (typical of autophagic PCD). These two different cellular enzyme activation outcomes were independent of caspase activity.[76] Furthermore, expression of a dominant negative mutant of DRP-1 or of DAPk antisense mRNA reduced autophagy induced by antiestrogens, amino acid starvation, or administration of interferon-gamma.[76] Notably, these mutants did not prevent nuclear fragmentation, suggesting that DRP-1 and DAPk specifically act in signal transduction for cytoplasmic rather than nuclear degradation.[76] In line with this, immunogold staining showed that DRP-1 is localized inside the autophagic vesicles.[76] Taken together, these findings strongly suggest a direct involvement of DRP-1 in the process of autophagy.

As outlined above, there is cumulative evidence that a set of molecules closely associated with the initial steps of autophagocytosis also appears to affect the life-death decision of cells. What about interactions between the control of biogenesis of lysosomes[99] and their subsequent fusion with autophagosomes with that of the cell's suicide? In regressing endocrine-dependent tumors de novo synthesis and an increased activity of lysosomal enzymes were described.[100] More recently, TNF-α was found to induce an autophagic type of cell death in T-lymphoblastic leukemic cells; 3-MA inhibited both the formation of autophagosomes and cell death.[79] However, asparagine, which inhibits the fusion of lysosomes with autophagosomes, did not prevent TNF-α induced cell death.[79] Thus, inhibition of an event downstream of sequestration did not affect the execution of autophagic cell death, suggesting that at least in T lymphocytes the supply of lysosomes might not be a check point for initiation of this type of programmed cell death. Furthermore, increasing the lysosomal pH by monensin or NH_4Cl did not protect kidney cells against ricin-induced lysis, a type of cell death exhibiting characteristic features of PCD.[82] Notably, 3-MA has been reported to slightly increase lysosomal pH.[83] However, as an increase in lysosomal pH seems not to protect from cell death, an increase in lysosomal pH as a possible cause for the protective action of 3-MA in the MCF-7 model of autophagic PCD appears unlikely.[82] In support of this hypothesis, tamoxifen-induced autophagic cell death in MCF-7 cells was found not to be associated with an expansion of the lysosomal compartment. Thus, based upon histochemical and biochemical studies we found neither evidence for an increased rate of synthesis nor activity of lysosomal proteases (Table 1; L. Török, U. Fröhwein, C. Gerner and W. Bursch, unpublished observations). In summary, at present the interactions of lysosome biogenesis with the pathway(s) leading to autophagic cell death remain elusive. However, the current functional and molecular data suggest that the events controlling the formation of autophagosomes rather than the biogenesis of lysosomes might provide a superior regulatory link(s) between autophagocytosis and cell suicide.

The most engimatic part of the autophagic cell death sequence is how the cytoplasmic events are linked to the final nuclear collapse. In apoptosis, many of the essential players have been identified. Briefly, the typical morphology of apoptotic nuclei, namely condensation of chromatin to crescent masses abutting to the nuclear membrane, results from a specific sequence of cleavage events. Thus, the genomic DNA is cleaved into large chromatin domains of 50-300 kbp, and then in many, but not all, cell types to oligonucleosomes (for review see refs. 16-23, 57-60, 101). All but the earliest large-domain cleavage events depend on caspases: The caspase activated DNAse (CAD/DFF-40), normally sequestered in the cytoplasm by the chaperone ICAD/DFF-45, is released and translocated to the nucleus once the chaperone has been degraded by caspase-3, finally resulting in the oligonucleosomal cleavage pattern. Furthermore, the nuclear envelope becomes discontinuous, the lamin polymer that normally underlies

the nuclear membrane is broken down by proteolysis.[102] What about the nuclear destruction during autophagic PCD? Cell death induced in caspase-3-deficient MCF-7 cells by TNF-α as well as staurosporine was not associated with low molecular weight, i.e. (oligo)nucleosomal DNA fragmentation, cell shrinkage and blebbing.[103] We have confirmed and extended these observations by showing that caspase-3-deficient MCF-7 cells exhibit high molecular weight DNA fragmentation into 50-300 kbp.[48] These observations meet well with others showing that high molecular weight DNA fragmentation is brought about by caspase-independent mechanism(s). Alternative non-caspase proteases conceivably involved in completion of cell suicide may include cathepsins,[104] calpains,[105] serine proteases,[106] granzymes[109] and/or the proteasome complex.[108,109] A non-apoptotic type of cell death was identified in insect tissues characterized by strong expression of the polyubiquitin gene and of the multicatalytic proteinase (proteasome).[27] Ubiquitin binds to cellular proteins to label them for proteolytic degradation by the proteasome protease. In our studies, MCF-7 cells treated with tamoxifen showed neither induction of ubiquitin mRNA expression nor synthesis of proteasome protein above the control level. However, preliminary results of our own obtained with high throughput two-dimensional gel electropheresis of subcelluar fractions suggest translocation of proteasome subunits (α, δ, ζ, τ) from the cytoplasm to the nucleus. Interestingly, Arnoult et al reported that cytoplasmic extracts from dying *Dictyostelium* cells were found to trigger the breakdown of isolated mammalian and *Dictyostelium* nuclei in a cell-free system.[110] DNA fragmentation was prevented by a polyclonal antibody specific for *Dictyostelium discoideum* apoptosis-inducing factor (DdAIF), and therefore the authors suggested that DdAIF is involved in DNA degradation during *Dictyostelium* cell death.

Exciting new insights into molecular events regulating cytoplasmic and nuclear destruction has been provided by studies on developmental cell death in *Drosophila*.[77] Thus, salivary gland cell death during development of *Drosophila* has been found to include autophagocytosis. At the molecular level, the gene E93 was reported to be sufficient to trigger cell death and, based upopn gain-of-function studies, E93 is considered to be necessary for autophagy.[77] A downstream effector of E93 is *crq* (croquemort) for pro-autophagic signaling, a gene that is required for phagocytosis during normal embryonic development.[111] Importantly, other downstream E93 effectors, namely *rpr, hid* and *grim* are considered to be essential for the nuclear apoptotic responses such as DNA fragmentation.[77] Taken together, these data suggest that in *Drosophila* the E93 gene may constitute a key regulator driving a concerted cytoplasmic and nuclear breakdown during autophagic PCD.

Differences and Commonalities of PCD Pathways

As outlined above, apoptosis and autophagic PCD are not mutually exclusive phenomena; they may occur simultaneously in tissues. Cells also may respond to death signals by entering either the apoptotic (type I) or autophagic (type II) PCD-pathway as demonstrated by Kuchino and coworkers.[38,73,74] Furthermore, cells may even switch from apoptosis (cytoplasmic condensation) to autophagic degradation of cytoplasmic components. Thus, studies on isolated neurons revealed that the manifestation of autophagic cell death may be controlled upstream of caspase cascades, but downstream of JNK/p38 (after NGF-withdrawal) and p53 (after cytosine arabinoside).[75] These studies also suggested that the same apoptotic signals that target mitochondria also activate autophagy. Once activated, autophagy may mediate caspase-independent neuronal cell death.[75] From a teleogical point of view, it is tempting to speculate that caspase-driven apoptosis with its precise and selective proteolyis of relatively few but crucial proteins may be completed much more rapidly than a caspase-independent autophagic PCD. The advantage of the rapid completion of an apoptotic pathway might be facilitating the preservation of the tissue structure. On the other hand, the advantage of an autophagic pathway would be to contribute to the removal of large masses of cytoplasm/cells and thereby, to cope with demands on tissue remodeling. At the morphological level, in studies with MCF-7 cell cultures a subfraction of dying cells showed autophagic cell death with an apoptotic nuclear morphology.[48] At the biochemical level, some nuclear proteins are cleaved during apoptosis

and autophagic cell death such as SUPT6H, HA95, PWP-1; other nuclear proteins are cleaved exclusively during apoptosis (of Jurkat cells) but not during autophagic PCD, for example, lamin B and scaffold attachment factor (Table 1).[112] Likewise, autophagic and apoptotic PCD seem to share the cell's "stress response" as indicated by translocation of heat shock protein-90 (Table 1). The recent studies on the role of DAP-kinases also revealed some commonalities between the apoptotic and autophagic death pathway.[76] Thus, the expression of both the death-associated protein kinase (DAPk) and DRP-1 were found to trigger two major cytoplasmic events: 1. Membrane blebbing, which is characteristic of "classical" apoptosis; and 2. Autophagy as typical of type II PCD.

In conclusion, to date the formation of autophagic vacuoles along with de novo synthesis of their components and its requirement for the cytoskeleton appears to provide the most clear indicator of autophagic PCD. Although a few molecular patterns such as the DAP-kinases and RAS signaling pathway in mammals as well as the E93 pathway in *Drosophila* have emerged recently, specific molecular pattern(s) of autophagic cell death remain to be proven.

Conclusions

Programmed cell death (PCD) is an essential phenomenon in normal development and adulthood of multicellular organisms. Cells use different pathways for active self-destruction, with the morphology ranging from apoptosis to autophagic cell death. Autophagic cell death appears to be activated when massive removal of cells or cytoplasm is demanded, for instance as part of the developmental program. Autophagy preceding cell death may also reflect a cell's adaptive response to sublethal (non-necrotic) conditions such as nutrient/growth factor deprivation or cell damage by cytotoxic drugs, hypoxia, etc. A functional link is suggested by a number of studies showing that 3-methyladenine inhibits both formation of autophagosomes and the manifestation of cell death (nuclear collapse). In mammalian systems, molecular links between autophagocytosis and eventual cell death have been provided by recent findings on DAP-kinases and Ras signaling (including PtdIns 3-kinases). Additional, but less clear, evidence for molecular events that might be associated with a cell's decision for autophagic PCD include mTOR/p70S6 kinase signaling and the autophagocytosis gene *APG6/VPS30* (Beclin 1). In *Drosophila*, the E93 gene appears to constitute a key regulator driving a concerted cytoplasmic and nuclear breakdown during autophagic PCD. However, apoptosis and autophagic cell death are not mutually exclusive phenomena, they may occur simultaneously in tissues or even, conjointly in the same cell; in vivo, cell residues resulting from both processes may be cleared by heterophagy. It should be emphasized, that autophagic and apoptotic PCD appear to be highly conserved during evolution as they occur in unicellular organisms,[114] in the green algae Volvox regulating the germ-soma dichotomy,[114] the slime mold *Dictyostelium discoideum*[31] and, last but not least, in plants.[115] Golstein and coworkers raised the hypothesis that a single core mechanism of PCD may have developed before the postulated multiple emergences of multicellularity.[35,36] According to this hypothesis, the phenotypic variations of PCD would result from differences in a cell's enzymatic equipment and mechanical constraints. Probably, the "older" autophagic cell death pathway has been improved by caspases rendering possible a more precise and more rapid cutting down of molecules that is essential for survival of cells.

Acknowledgements

Our studies reviewed herein comprise research over many years during which excellent technical assistance was provided by E. Bayer, B. Bublava, A. Dutter, M. Eisenbauer, J. Stockinger E. Scherzer and C. Unger and is gratefully acknowledged. These studies were supported by grants from the "Anton-Dreher-Gedächtnisschenkung für medizinische Forschung," the Austrian "Herzfelder'sche Familienstiftung," the Austrian Ministry of Traffic and Science, by Hafslund-Nycomed Austria, the Österreichische Nationalbank project 8093 as well as the Institute of Cancer Research, University of Vienna. Due to space limits, we referred to review articles whenever possible. Therefore, we apologize to the many authors whose original publications were not cited directly.

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