

Autophagy in the Eukaryotic Cell

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The major cellular pathways for protein and organelle turnover are autophagy and proteasome-mediated degradation. These processes are important to maintain a well-controlled balance between anabolism and catabolism in order to have normal cell growth and development. They play an essential role during starvation, cellular differentiation, cell death, and aging but also in preventing some types of cancer (59). These degradation pathways permit the cell to eliminate unwanted or unnecessary organelles and to recycle the components for reuse (54, 59).

The lysosome or vacuole is the major catabolic factory in eukaryotic cells and contains a range of hydrolases capable of degrading all cellular constituents. Organelle turnover is accomplished exclusively at this location through a process of autophagy that is conserved among yeast, plant, and animal cells. Microautophagy involves the uptake of cytoplasm at the lysosome or vacuole surface but has not been well characterized. In contrast, degradation by macroautophagy involves membrane engulfment at an initial site that is separate from this organelle. In mammalian cells, this process has been known for a long time, but the early studies were primarily phenomenological. Molecular components have been identified in the last decade by genetic screening of the yeast *Saccharomyces cerevisiae* (32, 80, 117, 121) and in recent years by two-hybrid screening of the same organism with predetermined baits or by genome-wide approaches (20, 42, 45, 71, 123). Surprisingly, molecular genetic studies with yeast have shown the overlap of the macroautophagy machinery with that used for peroxisome degradation (pexophagy) and the cytoplasm-to-vacuole targeting (Cvt) pathway (31, 39, 99), which ensures the delivery of the resident vacuolar protease aminopeptidase I (Ape1) (58, 97). These processes operate under different nutritional conditions, and the Cvt pathway in particular is biosynthetic. However, biochemical and morphological analyses have shown that the basic mechanism in all three processes is the sequestration of the cargo material (precursor Ape1 [prApe1], bulk cytoplasm, or specific organelle) within double-membrane structures (5–7, 39, 113).

The biogenesis and consumption of these vesicles can be divided into four discrete steps: induction and cargo packaging, formation and completion, docking and fusion, and breakdown. Figure 1 shows schematically these events for macroautophagy, the Cvt pathway, and pexophagy. The induction of vesicle formation during macroautophagy is stimulated by cellular signals such as starvation (113), whereas during Cvt trans-

port the binding of prApe1 to its receptor may be the signal that triggers induction (98). Upon completion, the sequestering vesicle (called an autophagosome or Cvt vesicle, respectively) docks with the lysosome or vacuole and then fuses with it. In this way, the inner vesicle is liberated inside the lysosome or vacuole, where it is finally consumed by hydrolases. In addition to induction, another major difference between these pathways appears to be the regulation of the size of the vesicle. Autophagosomes that form during starvation have anywhere from 8- to 200-fold more volume than Cvt vesicles that are induced under nutrient-rich conditions (300 to 900 nm versus 140 to 160 nm in diameter, respectively) (6). Finally, several lines of evidence suggest that the source of the sequestering vesicles for macroautophagy and the Cvt pathway differ at least in part. For example, macroautophagy but not the Cvt pathway requires the Sec12, Sec16, Sec23, and Sec24 proteins for formation of the membrane coat, COPII, that drives the formation of vesicles from the endoplasmic reticulum (41). Conversely, only the Cvt pathway utilizes the tSNARE protein Tlg2 and the Sec1 homologue Vps45 (1).

This review focuses on the yeast *S. cerevisiae* because of the recent advances in understanding of the molecular mechanism of autophagy, pexophagy, and Cvt transport in this organism. However, this compendium will also point out the high degree of conservation of these processes among eukaryotes, emphasizing the relevance of the studies with yeast.

INDUCTION OR CARGO PACKAGING

With macroautophagy, pexophagy, and the Cvt pathway, cells use largely the same machinery to accomplish a similar goal, delivery of cytoplasmic components into the interior of the vacuole. Growth conditions dictate which components must be targeted for delivery.

Under conditions of nutrient stress, it becomes necessary for the cell to eliminate nonutilized energy-consuming cytosolic proteins and organelles. These components are delivered by macroautophagy to the vacuole, where they are degraded in order to generate an internal supply of nutrients (113). Tor is a serine/threonine kinase that, in response to amino acids and growth factors, coordinates different aspects of cell growth, such as transcription, translation, tRNA and ribosome biogenesis, actin organization, and protein kinase C signaling. Starvation inhibits Tor activity, provoking various cellular responses, including cell arrest in the early G₁ phase, inhibition of protein synthesis, nutrient transporter turnover, transcriptional changes, and autophagy (81, 87, 90, 96). An identical cell reaction can also be triggered by treatment with rapamycin, a specific Tor inhibitor (34, 63).

Tor inactivation induces autophagy at two different levels: transcription and autophagosome formation (2). Gln3 is a

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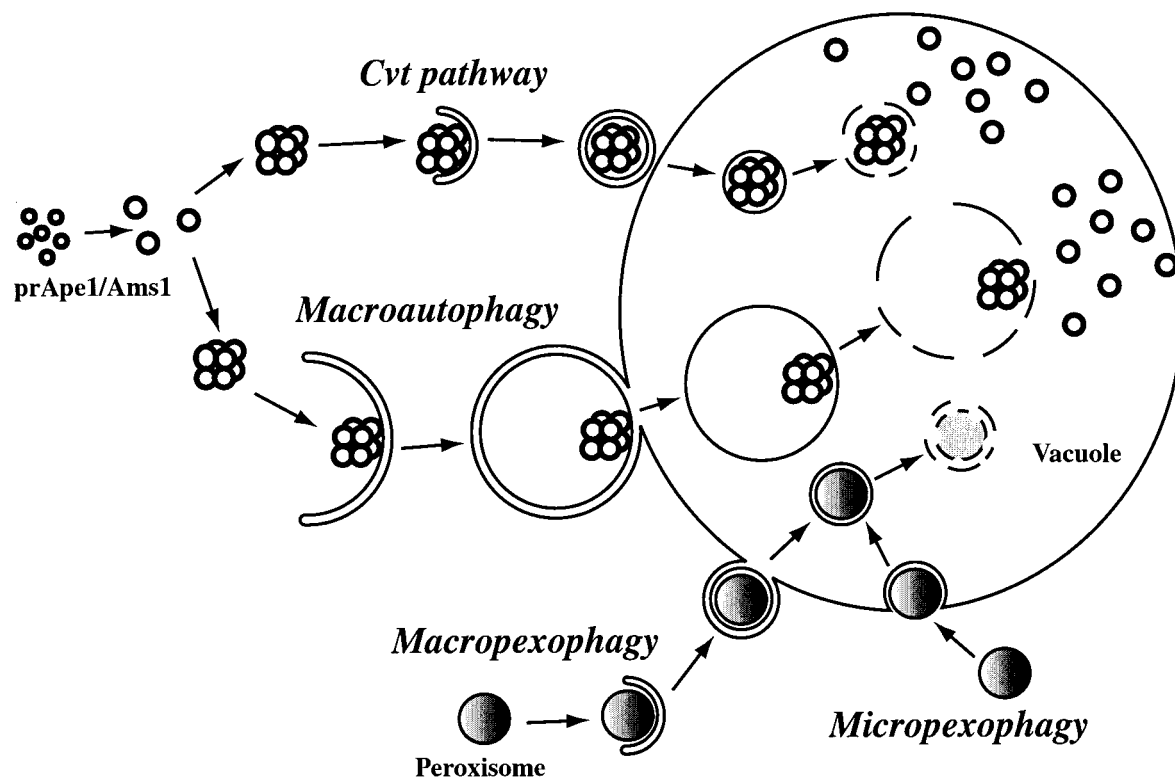


FIG. 1. Models for macroautophagy, pexophagy, and the Cvt pathway in the yeast *S. cerevisiae*. The basic mechanism in macroautophagy, macropexophagy, and the Cvt pathway is the sequestration of cargo material (bulk cytoplasm, peroxisomes, or prApe1 and Ams1, respectively) by a cytosolic double-membrane vesicle. Upon completion, the sequestering vesicle docks with the vacuole and then fuses with it. In this way, the inner vesicle is liberated inside the vacuole, where it is finally consumed by hydrolases. In micropexophagy, sequestration occurs directly at the vacuole surface, again resulting in the release of a vesicle within the vacuolar lumen. The small circles representing prApe1 or Ams1 are monomeric forms, and the medium circles indicate the oligomeric forms of these hydrolases.

transcriptional regulator of nitrogen source utilization genes, and it normally resides in the cytoplasm. Its localization is due to Tor-dependent phosphorylation that promotes its binding to the cytoplasmic repressor Ure2 (9). Tor inactivation leads to the dephosphorylation and dissociation of the inhibitory subunits of protein phosphatase 2A (PP2A), a phosphatase that acts on several Tor substrates, including Gln3 (90, 96). Dephosphorylation of Gln3 by PP2A promotes its dissociation from Ure2 and its successive translocation into the nucleus, where it activates the transcription of several genes (9, 14). Among those genes, some are part of the macroautophagy machinery (see below; genes shown in bold type have been shown to be induced only through microarray studies): *APG1/AUT3*, *AUT1/APG3*, *AUT2/APG4*, *APG5*, *AUT7/APG8*, *APG7/CVT2*, *APG12*, *APG13*, and *APG14* (15, 33, 37, 56, 78). After treatment with rapamycin, macroautophagy is not blocked even if the de novo biosynthesis of induced proteins is inhibited by cycloheximide, suggesting that up-regulation of those factors is not essential for this process (2). Autophagosomes formed under those conditions are significantly smaller than normal, however, indicating a role for de novo protein synthesis in the regulation of autophagosome expansion (2).

An important role in switching from the Cvt pathway to macroautophagy in response to nutrient conditions seems to be played by the cytosolic Apg1-Apg13 complex. Apg1/Aut3 is a serine/threonine kinase required for both the Cvt pathway

and macroautophagy (70, 99, 109), and its activity is modulated by Apg13 (27, 45). Tor signaling negatively regulates the association between Apg1 and Apg13. Under nutrient-rich conditions, active Tor causes hyperphosphorylation of Apg13, preventing or moderating its association with Apg1 (45). It is not known whether Tor directly phosphorylates Apg13. Tor inactivation by starvation or rapamycin treatment promotes the rapid dephosphorylation of Apg13, a process that seems to be independent of PP2A (45, 100). Dephosphorylated Apg13 binds to Apg1; this association promotes autophosphorylation and activation of Apg1, leading to the induction of macroautophagy (45, 70).

In addition to Apg13, Apg1 also interacts with three other proteins whose function is specific either for macroautophagy or the Cvt pathway: Apg17, Cvt9, and Vac8 (45, 53, 100). *APG17* is one of the small group of known genes with a function exclusively restricted to macroautophagy, and its gene product seems to participate in the formation or stabilization of the Apg1-Apg13 complex (45). It is possible that Apg17 has a role in cellular physiology other than autophagy because it seems to be a general factor required for the coordination of several cellular processes (20). Vac8 and Cvt9 are phosphoproteins specific for the Cvt pathway (53, 100, 134). It is possible that Apg1 and Apg13, both essential for macroautophagy and Cvt transport (27, 45, 70, 99, 100, 109), are the core of a regulatory system that controls conversion between those two

pathways. Modulation of this system is accomplished in part through phosphorylation or dephosphorylation reactions and through interactions with factors specific for macroautophagy or for the Cvt pathway (45, 53, 100). However, this general scenario is complicated by the fact that other protein kinases, such as Snf1 and Pho85, recently were shown to have a role in the regulation of autophagy, probably via Apg1 and Apg13 (135). It remains to be determined whether all these components are assembled into a single large protein conglomerate or whether they form separate complexes depending on nutritional conditions.

Autophagy is a nonspecific degradative process and is used to deliver bulk cytoplasmic components to the vacuole. In contrast, the Cvt pathway is biosynthetic and selects specific cargo components for transport. Two hydrolases are known to use this route: Ape1 (58) and α -mannosidase (Ams1) (38). After translation, Ams1 and prApe1 rapidly assemble into large oligomers (38, 55) that cannot be translocated to the endoplasmic reticulum. Thus, these proteins are unable to follow the normal route through a portion of the secretory pathway that most vacuolar proteins use to reach their final localization. The presence of an alternative route to the vacuole, such as the Cvt pathway, may have developed to allow the transport of large resident protein complexes.

Cvt19 is the receptor required for the transport of prApe1 and Ams1 to the vacuole via the Cvt pathway (65, 98). Cvt19 is not required for macroautophagy, but in its absence, prApe1 is not efficiently delivered to the vacuole under starvation conditions (98). Cvt19 specifically binds the propeptide of prApe1 and travels along with this protein to the vacuole, where it is finally degraded by vacuolar proteases. The association of prApe1 with Cvt19 promotes the inclusion of both proteins into the forming Cvt vesicles (98), but it is not clear yet if the formation of this complex is the event that triggers Cvt vesicle formation. The absence of Cvt19 destabilizes but does not prevent the association of prApe1 with membranes, indicating that prApe1 itself is able to bind a specific lipid or another protein (98). The lack of Cvt9 interferes with the proper association of prApe1 with membranes (53), but it is not known if this protein binds prApe1 or Cvt19. Interestingly, both Cvt9 and Cvt19 are peripheral membrane proteins that localize in a single punctate structure near the vacuole, possibly the site of Cvt vesicle formation (53, 98). It is tempting to speculate that an interaction of prApe1, Cvt19, and Cvt9 modulates the activity of the Apg1-Apg13 complex and consequently also the induction of Cvt vesicle formation.

Pexophagy, like autophagy, is a degradative process (Fig. 1). However, it is not induced by starvation conditions per se. When yeast cells are grown in the presence of oleic acid as the sole carbon source, peroxisome biogenesis is induced in order to carry out fatty acid β oxidation (128). After shifting of cells to a glucose-containing medium, the excess peroxisomes are degraded in the vacuole (17, 24, 39). The degradation of superfluous peroxisomes by micropexophagy has been shown to require most of the Apg and Cvt proteins, including the Cvt transport-specific component Cvt9/Gsa9 (39, 53). This requirement suggests that pexophagy may be induced in a manner similar to the Cvt pathway. It is not surprising that both the Cvt pathway and micropexophagy require Cvt9, because these two processes are active under the same growth conditions. Perox-

isome degradation is rapid and highly specific (39), but it does not use the Cvt19 receptor (98). It is not known how peroxisomes are selected by the enwrapping membranes, but Cvt9/Gsa9 probably plays an important role (53).

VESICLE FORMATION AND COMPLETION

Vesicle formation starts with the enwrapping of the selected cargo, organelle, or cytosol by a membrane. This process ends with the fusion of the extremities of the surrounding membrane, leading to the completion of a double-membrane vesicle, an autophagosome or a Cvt vesicle, depending on size and on nutritional conditions (Fig. 1).

Several components are necessary for this process and are shared by macroautophagy, pexophagy, and the Cvt pathway. In particular, some of the factors are parts of two different ubiquitin-like (UBL) systems that are essential for vesicle biogenesis (84). Ubiquitin is first activated by binding to a ubiquitin-activating enzyme (also called E1). Then it is transferred to a ubiquitin-conjugating enzyme (also called E2). Then a ubiquitin protein ligase enzyme (also called E3) catalyzes its covalent binding to the target substrate (136). The specificity of substrate recognition is modulated by the E2 and E3 enzymes, but other factors are also implicated.

The first UBL protein shown to be involved in macroautophagy was Apg12 (72). As in the ubiquitin system, Apg12 is first activated by the E1 enzyme Apg7/Cvt2 in an ATP-dependent reaction that leads to the formation of a high-energy thioester bond between the carboxy-terminal glycine of Apg12 and cysteine 507 of Apg7 (50, 61, 72, 114). Subsequently, Apg12 is transferred to the E2 enzyme Apg10, forming a new thioester bond with its cysteine 133 (72, 105). The last step of this sequence of reactions is the covalent linkage of Apg12 to lysine 149 of Apg5 (46, 72). This conjugation system is constitutive and does not possess the counterpart of an E3 enzyme (84); this function is probably accomplished by Apg10. The Apg12-Apg5 conjugate then associates with Apg16, forming a trimeric complex that is able to multimerize (71). The function of this large complex is unknown, but the proteins seem to be localized at the site of autophagosome formation (28).

The second UBL protein involved in macroautophagy is Aut7/Apg8/Cvt5. Aut7 is a soluble protein, and its carboxy-terminal arginine is removed by the Aut2/Apg4 cysteine protease, leaving a glycine residue at the carboxy terminus (51, 57). As with Apg12, Aut7 is activated by the E1 enzyme Apg7/Cvt2 through a thioester bond between its carboxy-terminal glycine and cysteine 507 of Apg7 (40, 57, 61). Aut7 is subsequently transferred to the E2 enzyme Aut1/Apg3 via a new thioester bond between those two proteins (40, 51, 95). Aut7 is finally covalently conjugated to a phosphatidylethanolamine (PE) molecule, becoming tightly membrane associated (40). Until the step when they form their respective E2 conjugates, the Aut7 and Apg12 conjugation systems proceed independently but may be coordinated through the dual requirement for Apg7. The Apg12-Apg5 complex forms in the absence of the Aut7 conjugation system (28, 72), whereas the linkage between Aut7 and PE is blocked in the absence of the Apg12 system (51).

The Aut2 protease, in addition to removing the carboxy-terminal arginine of newly synthesized Aut7, is also able to

cleave the amide bond linking Aut7 to PE, changing it back to a soluble form (57). This second cleavage event is part of the dynamic utilization of Aut7 during autophagosome and Cvt vesicle formation and is required as part of the normal itinerary of this protein. Under nutrient-rich growth conditions, Aut7 localizes to unidentified dot structures dispersed in the cytoplasm (51, 56). After shifting of cells to a minimal medium that induces starvation, Aut7 becomes concentrated to punctate perivacuolar structures (56). These structures appear to be autophagosomes in the process of formation. Aut7 localizes along the entire preautophagosomal membrane during expansion (56). Upon completion of the autophagosome, the Aut7 pool on the surface dissociates from the membrane, becoming soluble, an event probably catalyzed by the action of Aut2 (56). The Aut7 pool trapped inside the autophagosome is successively degraded in the vacuolar lumen together with the autophagic body (37, 56). The behavior of Aut7 suggests that it is a structural element necessary for autophagosome and Cvt vesicle formation. This idea is also supported by the fact that Aut7 is induced by starvation (37, 56) and is required for expansion of the membrane that forms the autophagosome (2).

Aut7, like the coat proteins involved in other vesicular trafficking events, may have a double role in coordinating autophagosome biogenesis. As discussed above, Aut7 plays a structural role in vesicle formation. In addition, the presence of Aut7 during this event may have the function of preventing the premature fusion of an unfinished autophagosome with the vacuole. This activity can be achieved through the binding of Aut7 in an inhibitory manner to one of the SNARE proteins required for the fusion of the autophagosome with the vacuole (66). The result is that only complete, Aut7-uncoated autophagosomes can fuse with the vacuole. The signal that triggers Aut7 release upon completion of the autophagosome is not yet known.

PE is an abundant lipid present in all cellular membranes, but Aut7 is specifically conjugated only to PE present in the forming autophagosome. It is unclear how the Aut7-Aut1 activated complex is recruited to the right place. It is known that the binding of Aut7 to PE depends on the Apg12 conjugation system (51). It is possible that one of the events stimulated by this system is the supply of a docking point for the Aut7-Aut1 complex. In addition, an E3 enzyme implicated in the transfer of Aut7 to PE has not yet been identified. It is conceivable that this enzyme provides such a landmark.

Apg9/Cvt7/Aut9 is the only characterized multispinning transmembrane protein essential for autophagosome and Cvt vesicle formation (64, 79). It localizes to perivacuolar punctate structures but appears to be excluded from the membranes composing autophagosomes and Cvt vesicles (79). Several components involved in the induction and formation of Cvt and macroautophagy transport vesicles are peripheral membrane proteins, but the nature of their targeting to membranes is unknown. Apg9 shares a localization pattern similar to that of those proteins, and it is likely that it is used as a docking point by some of them. This seems to be the case at least for Apg2, a protein colocalizing with Apg9 and implicated in the process of autophagosome and Cvt vesicle formation as well as in pexophagy (106, 132). Apg2 directly binds Apg9 (132) and, like Apg9, is excluded from autophagosomes (106). In the absence of Apg9, Apg2 becomes cytosolic (106, 132). The

association between Apg9 and Apg2 requires the presence of Apg1 but not its kinase activity (106). Apg2 also colocalizes with Aut7, but its distribution does not depend on Aut7 and vice versa, suggesting that these two proteins are independently recruited to the same perivacuolar structures (51, 106).

Cvt18/Gsa12 is a cytosolic protein that contains two WD40 domains and that is also essential for double-membrane vesicle formation in macroautophagy, the Cvt pathway, and pexophagy (29). Cvt18 localizes to perivacuolar punctate structures and to the vacuolar rim, but its association with membranes does not appear to depend on the characterized *APG/CVT/AUT* genes. The function of Cvt18 is not known, but it is required to target Apg2 to punctate structures adjacent to the vacuole (29).

DOCKING AND FUSION

Vacuoles can fuse with late endosomes (multivesicular body pathway) or possibly with vesicles derived from endosomes (carboxypeptidase Y pathway), with Golgi complex-derived vesicles (alkaline phosphatase pathway), and with themselves (homotypic fusion). In all of these scenarios, cells use an identical fusion machinery, which consists of SNARE proteins, a rab-GTPase, and the class C vps protein complex, also known as the HOPS (homotypic fusion and vacuole protein sorting) complex (18, 67, 94, 101, 137, 139).

It is not surprising that the same components are exploited for the fusion of autophagosomes and Cvt vesicles. Precursor Ape1 maturation was shown to be blocked in strains where the two vacuolar SNARE proteins, Vam7 and Vti1, and the subunits of the HOPS complex, Vps39 (the product of a gene allelic to *CVT4*) and Vps41 (the product of a gene allelic to *CVT8*), were nonfunctional (26, 32, 93). These results should be considered with caution because these proteins are essential for protease delivery to the vacuole, and the observed defect in prApe1 processing could be indirect (18, 26, 67, 76, 93). However, the possibility of their direct role has been highlighted because strains with defects in other components of this fusion machinery, such as the vacuolar SNARE protein Vam3, the small rab-GTPase Ypt7, and two other subunits of the HOPS complex, Vps16 (the product of a gene allelic to *CVT15*) and Vps18, have been shown to accumulate autophagosomes or Cvt vesicles (2, 7, 19, 28, 50, 89, 97).

VESICLE BREAKDOWN

Once the autophagosomes and Cvt vesicles have fused with the vacuolar membrane, the interior single-membrane vesicles are released into the vacuolar lumen. These vesicles, now termed autophagic and Cvt bodies, are subsequently consumed by hydrolases (Fig. 1). This breakdown requires normal acidification of the vacuole (77), presumably to maintain the optimal pH of the degradative enzymes but also because it is essential for the autocatalytic cleavage and subsequent activation of proteinase A (Pep4) and proteinase B (the product of the *PRB1* gene, allelic to *CVT1*) (43, 124). These proteases are at the apex of a proteolytic cascade that leads to the activation of most vacuolar hydrolases. Mutations in the *PEP4* or *PRB1* genes stabilize autophagic and Cvt bodies as well as peroxi-

somes that have been delivered to the vacuole (6, 31, 39, 58, 113, 117).

In addition to proteases, the complete degradation of luminal vesicles requires the action of lipases. The *cvt17/aut5* mutant exhibits an accumulation of both Cvt and autophagic bodies inside the vacuole (32, 97). The *CVT17* gene was recently cloned and shown to code for an integral membrane protein with an essential domain conserved among lipases (116). One model is that Cvt17 enters the inner lipid bilayer of Cvt and autophagic bodies, probably at the place of their formation, and is successively degraded with this membrane in the vacuolar lumen (116). It is not clear if Cvt17 is required for the direct lysis of subvacuolar vesicles. It is also possible that its lipase activity is necessary to alter the lipid composition of the subvacuolar vesicles as they form, a modification that may be essential to target other hydrolases inside the bodies or to render the lipid bilayer more susceptible to other vacuolar lipases.

Another protein that has also been shown to play a role in the degradation of subvacuolar vesicles is Aut4, a multispansing transmembrane protein (112). The fact that Aut4 is completely immersed in membranes suggests a function linked to the lipid composition of subvacuolar vesicles (lipid synthase, lipase, or flipase); however, a role in targeting hydrolases to autophagic bodies cannot be excluded. Aut4 function seems to be distinct from that of Cvt17 for two reasons. First, Aut4 is autophagy specific, and *aut4Δ* cells grown under nutrient-rich conditions show normal prApe1 processing (112). Second, Aut4 localizes to perivacuolar punctate structures and to the vacuolar membrane but does not enter autophagic bodies (112).

Both Cvt17 and Aut4 have homologues only in bacteria or other fungi (112, 116) (Table 1), reflecting a possible involvement with a particular lipid. In order for lipases to recognize membranes destined for degradation, the lipid bilayer composition of autophagic and Cvt bodies should be different from that of the delimiting vacuolar membrane. Multivesicular bodies are another population of subvacuolar vesicles that are degraded within the vacuolar lumen (67, 83). In mammalian cells, they are reported to have a unique lipid composition, being enriched in the unusual lipid *lyso*-bis-phosphatidic acid (60). Cvt17 and Aut4 may play a role in modifying transport membranes to differentiate them from the vacuolar membrane and allow for their specific degradation.

CONSERVED MACHINERY

Macroautophagy is a very well known process in mammalian cells, and it is mechanistically identical to the process that occurs in yeast cells (21, 22, 25, 59, 75, 103, 104, 119). Macroautophagy has also been demonstrated in plants (4, 16, 120).

Several molecular components of the mammalian machinery have been cloned because of their homology to genes identified in *S. cerevisiae* (Table 1). In particular, the two UBL systems are conserved. Aut7 is the core of one of these two systems, and its rat homologue, LC3, has identical functional characteristics. LC3 is cytosolic, but after processing it becomes membrane bound and localizes to both the inside and the outside of forming autophagosomes (44, 74). In addition, the human LC3 homologue is also activated by the Apg7 ho-

mologue (hApg7) (115). The human homologue of the second UBL, Apg12 (hApg12), is similarly activated by hApg7 and finally forms a complex with the Apg5 homologue (hApg5) (73, 115). The mouse Apg12-Apg5 conjugate is essential for macroautophagy and localizes to the forming autophagosomes (74). As in yeast cells, the Apg12-Apg5 complex is necessary for the last step of the LC3/Aut7 conjugation system that leads to the tight association of LC3 with the nascent autophagosome (51, 74). LC3/Aut7 is probably one of the principal structural elements of the autophagosome, and the absence of its targeting to membranes in Apg5-deficient mouse embryonic stem cells can explain why those cells are impaired in autophagosome formation (2, 37, 56, 74).

The human Bcl-2-interacting protein, Beclin 1, is the functional homologue of yeast Vps30/Apg6 (see below) (68). Beclin 1 is able to complement the autophagy deficiency of yeast cells lacking Vps30/Apg6, but it also restores autophagy in human MCF7 breast carcinoma cells and reduces the tumorigenicity of various other malignant cell lines (68). In yeast cells, Vps30/Apg6 is associated with another protein required for macroautophagy: the phosphatidylinositol (PI) 3-kinase Vps34 (see below) (49). PI 3-phosphate also has been shown to be essential for autophagy in mammalian cells (11, 86).

Even if the basic machinery for autophagy is identical between yeast and mammalian cells, the possibility that this phenomenon is more complex in higher eukaryotes cannot be excluded. For example, in addition to LC3, there are two more human Aut7 counterparts that play a role in other cellular processes (44, 66, 85, 91, 115, 133). Interestingly, all three human Aut7 homologues are activated by hApg7, suggesting that various mammalian pathways are coordinated during the induction of autophagy (115). This variegation of Aut7-like proteins is also present in other higher eukaryotes (Table 1). In yeast cells these multiple functions are probably accomplished by a single protein (66). Aut2 is the protease involved in the processing of Aut7 and then in its subsequent release from membranes (see above) (56). It seems that there are also several Aut2 homologues in every higher eukaryotic organism (Table 1). This finding may reflect the necessity to process different Aut7-like factors that are ultimately linked to different substrates. In yeast cells, Aut7 is covalently bound to PE (see above) (40); further investigations are required to demonstrate if the same is true for all the mammalian Aut7 homologues.

In addition to *S. cerevisiae*, several other yeasts can modulate their peroxisome population to better exploit nutrient conditions. When methylotrophic yeasts such as *Pichia pastoris*, *Pichia methanolica*, *Hansenula polymorpha*, and *Candida boidinii* are grown in media containing methanol, peroxisome biogenesis is induced in order to optimize the utilization of this carbon source (125, 127). Upon adaptation to an alternative carbon source, such as glucose or ethanol, peroxisomes are rapidly degraded in the vacuole (13, 35, 62, 92, 122, 126, 130). An identical fate is also reserved for the peroxisomes of *Yarrowia lipolytica* and *Aspergillus nidulans* after shifting of those fungi from a medium containing oleic acid to one containing glucose (3, 30). Peroxophagy occurs by two mechanisms. Macroperoxophagy is morphologically identical to macroautophagy in *S. cerevisiae*, while micropexophagy involves uptake of the

TABLE 1. Yeast genes involved in autophagy, the Cvt pathway, and pexophagy

| Gene | Step(s) | Role or Interactions | Homologues ^a (% identity, % similarity) |
|-----------------------|--|---|--|
| <i>APG1/AUT3</i> | Induction | Serine/threonine kinase | <i>Homo sapiens</i> (32, 48); <i>Mus musculus</i> (32, 48); <i>Caenorhabditis elegans</i> (24, 41); <i>S. pombe</i> (42, 60) |
| <i>APG2</i> | Formation and completion | Associated with Apg9 | <i>H. sapiens</i> (24, 39); <i>C. elegans</i> (35, 50); <i>S. pombe</i> (22, 41); <i>P. pastoris</i> (28, 45); <i>Drosophila melanogaster</i> (32, 48) |
| <i>APG5</i> | Formation and completion | Apg12 conjugation system | <i>H. sapiens</i> (23, 41); <i>M. musculus</i> (24, 41); <i>C. elegans</i> (23, 41); <i>S. pombe</i> (29, 50); <i>D. melanogaster</i> (20, 42); <i>Arabidopsis thaliana</i> (24, 42) |
| <i>APG6/VPS30</i> | Formation and completion | Forms a complex with Apg14, Vps34, and Vps15 | <i>H. sapiens</i> (29, 48); <i>M. musculus</i> (30, 47); <i>Rattus norvegicus</i> (27, 44); <i>C. elegans</i> (28, 49); <i>S. pombe</i> (27, 45); <i>D. melanogaster</i> (29, 45); <i>A. thaliana</i> (25, 43); <i>Triticum aestivum</i> (25, 43) |
| <i>APG7/CVT2</i> | Formation and completion | Apg12 and Aut7 conjugation systems (E1) | <i>H. sapiens</i> (37, 54); <i>M. musculus</i> (38, 54); <i>C. elegans</i> (37, 56); <i>S. pombe</i> (39, 58); <i>P. pastoris</i> (44, 61); <i>D. melanogaster</i> (37, 54); <i>A. thaliana</i> (39, 56) |
| <i>APG9/CVT7/AUT9</i> | Formation and completion | Transmembrane protein associated with Apg2 | <i>H. sapiens</i> (30, 52); <i>C. elegans</i> (26, 45); <i>S. pombe</i> (38, 57); <i>D. melanogaster</i> (25, 49); <i>A. thaliana</i> (27, 53) |
| <i>APG10</i> | Formation and completion | Apg12 conjugation system (E2) | <i>C. elegans</i> (22, 44) |
| <i>APG12</i> | Formation and completion | Apg12 conjugation system (UBL) | <i>H. sapiens</i> (32, 59); <i>M. musculus</i> (31, 58); <i>C. elegans</i> (23, 46); <i>S. pombe</i> (33, 57); <i>D. melanogaster</i> (28, 56); <i>A. thaliana</i> (36, 64) |
| <i>APG13</i> | Induction | Modulates Apg1 activity | <i>S. pombe</i> (21, 40) |
| <i>APG14</i> | Formation and completion | Forms a complex with Ap6, Vps34, and Vps15 | <i>Methanococcus jannaschii</i> (28, 51) |
| <i>APG16</i> | Formation and completion | Associates with the Apg12-Apg5 complex | |
| <i>APG17</i> | Induction (autophagy specific) | Probably involved in the Apg1-Apg13 interaction | <i>S. pombe</i> (21, 45); <i>Candida albicans</i> (20, 39) |
| <i>AUT1/APG3</i> | Formation and completion | Aut7 conjugation system (E2) | <i>H. sapiens</i> (32, 45); <i>M. musculus</i> (32, 44); <i>R. norvegicus</i> (32, 44); <i>C. elegans</i> (35, 50); <i>S. pombe</i> (34, 54); <i>D. melanogaster</i> (32, 48); <i>A. thaliana</i> (32, 44) |
| <i>AUT2/APG4</i> | Formation and completion | Aut7 conjugation system | <i>H. sapiens</i> (28, 44), (26, 40), (27, 42); <i>C. elegans</i> (26, 40); <i>S. pombe</i> (29, 48); <i>D. melanogaster</i> (28, 42), (29, 45); <i>A. thaliana</i> (29, 44) |
| <i>AUT4</i> | Vesicle breakdown (autophagy specific) | Transmembrane protein | <i>S. pombe</i> (23, 46); <i>S. coelicolor</i> (25, 41); <i>Bacillus subtilis</i> (20, 40); <i>Clostridium acetobutylicum</i> (20, 40) |
| <i>AUT7/APG8/CVT5</i> | Formation and completion | Aut7 conjugation system (UBL) | <i>H. sapiens</i> (55, 78), (55, 77), (54, 77); <i>Bos taurus</i> (55, 78); <i>M. musculus</i> (55, 78), (55, 77), (54, 77); <i>R. norvegicus</i> (55, 78), (55, 77); <i>Cavia porcellus</i> (54, 77); <i>C. elegans</i> (53, 74), (34, 64); <i>S. pombe</i> (73, 88); <i>D. melanogaster</i> (56, 78), (54, 76); <i>A. thaliana</i> (73, 90), (71, 84), (71, 86), (65, 80), (70, 85), (76, 90), (54, 75), (49, 73); <i>Laccaria bicolor</i> (76, 90); <i>Gillichthys mirabilis</i> (55, 75) |
| <i>CVT9</i> | Induction (Cvt pathway and pexophagy specific) | Associated with Apg1 | <i>S. pombe</i> (26, 44); <i>P. pastoris</i> (24, 42) |
| <i>CVT17/AUT5</i> | Vesicle breakdown | Putative transmembrane lipase | <i>S. pombe</i> (45, 63); <i>C. albicans</i> (48, 62); <i>Cladosporium fulvum</i> (38, 54) |
| <i>CVT18</i> | Formation and completion | | <i>H. sapiens</i> (27, 43), (30, 45), (26, 45), (28, 44); <i>C. elegans</i> (27, 44), (26, 43), (25, 43); <i>S. cerevisiae</i> (28, 44), (26, 45); <i>S. pombe</i> (33, 50), (26, 46), (26, 44); <i>Neurospora crassa</i> (34, 52); <i>D. melanogaster</i> (25, 42), (25, 42), (23, 40); <i>A. thaliana</i> (30, 51), (41, 60); <i>Oryza sativa</i> (39, 59) |
| <i>CVT19</i> | Induction (?) and cargo packaging (Cvt pathway specific) | Ape1 and Ams1 receptor | <i>S. cerevisiae</i> (28, 44) |
| <i>VAC8</i> | Induction (Cvt pathway and pexophagy specific) | Associated with Apg13 | <i>S. pombe</i> (64, 80) |

^a Homologues were identified by a BLAST search of the encoded protein (<http://www.ncbi.nlm.nih.gov/BLAST/>).

peroxisomes directly at the vacuole surface (reviewed in references 54 and 111) (Fig. 1).

Genetic screens to identify mutants defective in pexophagy have been performed with *H. polymorpha*, *P. pastoris*, and *Y. lipolytica* (30, 92, 118, 122). The identified genes, *PDD1*, *PpVPS15/GSA19*, *PDD7*, *GSA7*, *GSA9*, *GSA10*, *GSA11*, *GSA12*, and *GSA14*, are functional homologues of the *S. cerevisiae* genes *VPS34*, *VPS15*, *APG1*, *APG7*, *CVT9*, *APG1*, *APG2*, *CVT18*, and *APG9*, respectively, showing that pexophagy in yeasts employs the same machinery as that required for macroautophagy and the Cvt pathway (29, 48, 53, 108, 110, 129, 140). The *P. pastoris* *GSA1* gene is also essential for pexophagy and encodes the regulatory subunit of phosphofructokinase, an enzyme that itself is not required for pexophagy (141). The role of *Gsa1* is not clear, and it is not known if mutations in the phosphofructokinases of other yeasts have the same effect. Pexophagy has also been observed in mammalian cells (12, 69), but there are no reports characterizing the machinery used for this process.

The existence of the Cvt pathway has only been demonstrated in the yeast *S. cerevisiae*. Even if the autophagy machinery is present in all eukaryotic cells, homologues of the factors specific for the Cvt pathway, such as *Cvt9* or *Vac8*, seem to be restricted only to other yeasts (Table 1). The major difficulty in the identification of this transport route in other organisms is that no putative cargo molecules are known, even if it seems that *Ape1* and *Ams1* have counterparts. *Ape1* has clear homologues in other eukaryotic cells, but it is difficult to predict if they follow a Cvt pathway because alignments show that those proteins have divergent N termini. This region contains the information to target *prApe1* to the Cvt pathway (82). These divergences may reflect differences in the targeting determinant but may also be symptomatic of a diverse localization. For example, the closest mammalian homologue of *Ape1* localizes to the cytosol (138). *Ams1* also has homologues in *Schizosaccharomyces pombe*, *A. nidulans*, humans, and rats. None of these homologues has a classical signal sequence or transmembrane domains, suggesting they may not travel through the secretory pathway (23, 38). Confusingly, the rat homologue localizes to the endoplasmic reticulum (10). More detailed studies are required to show where and how *Ape1* and *Ams1* homologues are localized in other organisms. The absence of *Cvt9* or *Vac8* but also of the *Cvt19* receptor in higher eukaryotes may reflect only the fact that the Cvt pathway in these organisms selects other cargo molecules.

FUTURE DIRECTIONS

Several genes essential for macroautophagy, the Cvt pathway, and pexophagy have now been identified and ordered in functional groups. This classification is based on the step in the import pathway in which they act and on their interactions with other proteins. A future task will be to try to find the connections among these clusters of genes in order to obtain the complete picture of these processes. For example, it has been determined that *Apg1* kinase activity is essential for the induction of autophagosomes and Cvt vesicles. Many proteins interacting with *Apg1* are also known, but the target of this cluster of proteins remains unidentified. The binding of cargo molecules to the *Cvt19* receptor also seems to be important for the

induction step, but at the moment, no connection between such complexes and other known elements has been made. Similarly, three groups of genes are involved in the biogenesis of double-membrane vesicles: the *Apg12* and *Aut7* conjugation systems and the *Apg2-Apg9* complex. The interactions among these sets of proteins remain largely unknown. Identification of the connections among these groups of genes will help to order the events that lead to the formation and completion of these vesicles.

The origin(s) of the sequestering membranes in the Cvt, macroautophagy, and macropexophagy pathways is not known. In mammalian cells, it is generally believed that autophagosomes are derived from the endoplasmic reticulum (22). It is now evident that in yeast cells, double-membrane vesicles are formed in a perivacuolar punctate structure. The only known structure with this characteristic location is the late endosome or prevacuolar compartment (88, 131). Several observations argue against the participation of this organelle in the formation of double-membrane vesicles. For example, *Pep12* is a SNARE protein that localizes to the late endosome (8, 36). Gradient analyses have shown that *Pep12* does not completely cofractionate with markers of the perivacuolar structure involved in the macroautophagy and Cvt pathways (53, 79, 106, 132). Moreover, nonfunctional *PEP12* alleles that block the vacuolar delivery of proteins passing through the late endosome do not affect the transport of *prApe1* (1, 8). Finally, mutations that affect the morphology of the late endosome do not interfere with that of the macroautophagy and Cvt pathway perivacuolar structure (79). Conversely, other lines of evidence implicate the endosome or a domain of the endosome in these pathways. The *csc1* mutant is constitutive for macroautophagy. *CSC1* is allelic with the gene encoding endosomal protein *Vps4* (107). Another protein that has been implicated in endosomal function, *Vps30/Apg6*, is part of two similar complexes, one required for sorting of carboxypeptidase Y and the other required for the macroautophagy and Cvt pathways (47, 49, 102). In addition to *Apg6*, these complexes contain two other common subunits: the PI 3-kinase *Vps34* and the protein kinase *Vps15* (49). The specificity for the pathway is given by the fourth subunit: *Apg14* is essential for the macroautophagy and Cvt pathways, whereas *Vps38* is necessary for the Vps pathway (49). Recent studies showed that *Apg14* colocalizes with many of the other *Apg/Cvt* proteins (52). It will now be interesting to determine where the mammalian homologues of the yeast proteins in this newly discovered perivacuolar structure are localized.

The presence of a newly discovered organelle in the yeast endosomal system or the involvement of a subdomain of the endosome raises new questions connected with subcellular protein trafficking. Three of the components localizing to this structure are integral membrane proteins (64, 79, 112, 116). One of those, *Cvt17*, is glycosylated, indicating passage through the Golgi complex (116). After translation, all the transmembrane proteins of the endosomal system are translocated to the endoplasmic reticulum and follow the secretory route until the late Golgi compartment (18, 67). In this compartment, they are recognized by coat proteins and specifically packaged into vesicles directed to their final destinations (18, 67). Which class of vesicles is involved in the transport of *Cvt17*, *Apg9*, and *Aut4* to the perivacuolar structure involved

in the macroautophagy and Cvt pathways? How are these proteins recognized and segregated from other proteins present in the endoplasmic reticulum? The involvement in macroautophagy of a subset of components required for vesicle coat formation at the endoplasmic reticulum (41) may provide some insight into these questions.

Finally, vesicle fusion with the target compartment is catalyzed by the interaction of SNARE proteins present on the two approaching membranes. The best candidate for a SNARE protein on the perivacuolar organelle is Tlg2. This tSNARE protein localizes to punctate structures (36) and is required for the normal processing of prApe1 (1). *TLG2* deletion strains block the formation and completion of Cvt vesicles (1). However, Tlg2 is specific for the Cvt pathway and is unnecessary for macroautophagy (1). These findings leave open the question of which SNARE proteins are essential for autophagosome formation. Are there different perivacuolar structures, some necessary for Cvt vesicle formation and others necessary for autophagosome biogenesis? Analysis of the trafficking of integral membrane proteins under different growth conditions will help to answer this question.

Autophagy, the Cvt pathway, and pexophagy are complex processes involving dynamic rearrangements of membranes to deliver proteins and organelles from the cytoplasm to the vacuole. These processes are conserved in eukaryotes. Future studies will continue to provide molecular details about these alternative vacuolar targeting pathways.

ACKNOWLEDGMENTS

F.R. is supported by a long-term fellowship from the European Molecular Biology Organization, and D.J.K. is supported by Public Health Service grant GM53396 from the National Institutes of Health.

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