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Transport of proteins to the yeast vacuole: autophagy, cytoplasm-to-vacuole targeting, and role of the vacuole in degradation

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The vacuole/lysosome performs a central role in degradation. Proteins and organelles are transported to the vacuole by selective and non-selective pathways. Transport to the vacuole by autophagy is the primary mode for degradation of cytoplasmic constituents under starvation conditions. Autophagy overlaps mechanistically and genetically with a biosynthetic pathway termed Cvt (Cytoplasm-to-vacuole targeting) that operates under vegetative conditions to transport the resident vacuolar hydrolase aminopeptidase I (API). API import has been dissected to reveal the action of a novel mechanism that transports cargo within double-membrane vesicles. Recent work has uncovered molecular components involved in autophagy and the Cvt pathway.

Key words: autophagy / Cvt pathway / lysosome / protein targeting / vacuole

The yeast vacuole is analogous to the mammalian lysosome and is the main cellular site of protein and organelle turnover. Sequestered within this organelle are numerous degradative enzymes that allow the cell to regulate levels of cellular constituents. Transport pathways control the extent and selectivity of vacuole-mediated turnover, as degradation can only occur upon delivery to the vacuole lumen. In addition, biosynthetic transport pathways deliver resident lytic enzymes to the organelle; most vacuole proteins are transported through a portion of the secretory pathway.[1]

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Several pathways mobilize proteins to the vacuole for subsequent degradation. For example, endocytic transport delivers extracellular material and plasma membrane proteins to this organelle.[2] In animal cells subjected to prolonged periods of starvation, a mechanism for direct translocation across the lysosomal membrane accomplishes uptake of specific cytosolic proteins. This pathway entails recognition of a specific sequence of the target protein by a receptor on the lysosomal membrane, and requires the action of cytosolic and vacuolar isoforms of the heat-shock protein Hsc73.[3,4] An hsp73-mediated pathway has also been described for import into the yeast vacuole.[5] Another specific vacuolar uptake pathway characterized in yeast transports the gluconeogenic enzyme fructose-1,6-bisphosphatase, and occurs when yeast cells are shifted from respiratory to fermentative conditions.[6–8]

Autophagy is the primary delivery pathway used to transport proteins and organelles for degradation. During nutrient stress, massive protein turnover occurs in the vacuole, resulting in the destruction of nearly half the total cellular protein in a 24 h period.[9] This dramatic turnover of cellular material cannot be accommodated by the proteasome, and in general, the vacuole mediates bulk turnover of components, while the cytoplasmic ubiquitin/proteasome system is responsible for selective turnover of proteins.

Autophagy was first described by electron microscopic studies. At a morphological level, autophagy can be differentiated into two modes. Macroautophagy [Figure 1(a)] involves the formation of double-membrane vesicles, which are distinct from the vacuole. In contrast, microautophagy proceeds by vacuolar membrane-mediated engulfing or enwrapping of cargo [Figure 1(b)]. In yeast, autophagy appears to occur primarily by macroautophagy, based on electron microscopic studies of nitrogen-starved cells.[10]

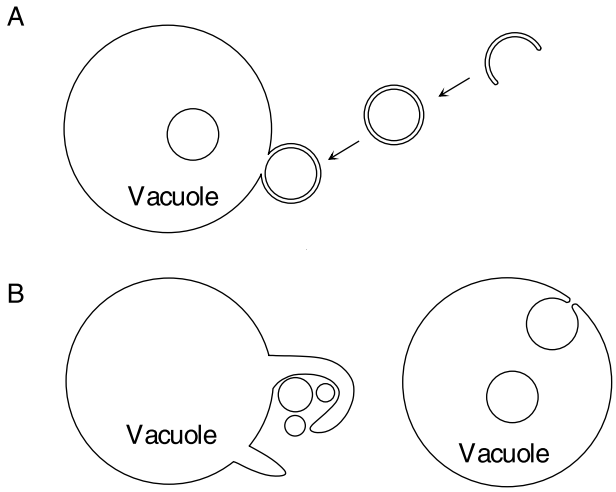

Figure 1. Autophagy may occur by macroautophagic (a) or microautophagic (b) modes of uptake. Macroautophagy occurs through formation of vesicles separate from the vacuole that are composed of two membranes. Fusion of the outer membrane with the vacuole delivers membrane-enclosed cargo to the organelle for subsequent degradation. In microautophagy, uptake of cytosolic material occurs by an enwrapping mechanism that utilizes finger-like projections of the vacuolar membrane (left panel), or invaginations of the vacuolar membrane (right panel).

A yeast model for study of autophagy and cytoplasm-to-vacuole transport

Genetic screens for yeast mutants defective in autophagy (*apg, aut*) have been performed by screening for an inability to degrade cytoplasmic proteins during starvation, or by selecting for an increased sensitivity to starvation.[11,12] Yeast treated with the serine protease inhibitor PMSF, or strains lacking vacuolar proteinase B activity accumulate intravacuolar vesicles when they are starved.[13] Presumably, the inhibition of vacuolar proteolysis leads to an inability to break down vesicles within the lumen that are derived from autophagic transport (Figure 2, Stage 4). In a secondary screen, putative mutants were assessed at a microscopic level to determine if these subvacuolar vesicles, the 'autophagic bodies', were lacking in PMSF-treated, starved cells, as an indicator of defective autophagic transport.[11,12]

An independent genetic screen in yeast led to the discovery of a genetic overlap between degradative autophagy and the biosynthetic transport of a vacuolar hydrolase, aminopeptidase I (API).[14–16] Unlike most other resident vacuolar proteins, API is known to be transported independently of the secretory pathway, by a cytoplasm-to-vacuole targeting (Cvt) pathway. Many mutants defective in autophagy are unable to transport API. Most of the *cvt* mutants show typical *apg* phenotypes, such as decreased viability under starvation, inability to accomplish degradation of cytoplasmic marker proteins during nutrient limitation, and in most cases, the lack of accumulation of autophagic bodies in the vacuole in the presence of PMSF and nutrient stress. More recently, it has become clear that the vacuole-mediated specific degradation of peroxisomes is also mediated by many of the same molecular components that are active in autophagy and Cvt.[17] This organelle degradation pathway, termed pexophagy, is probably not unique; it is likely that the same basic autophagic machinery can also specifically target other organelles for disposal when they become damaged, or when they are no longer required for efficient cellular function. Thus, the vesicular machinery involved in these pathways accomplishes specific uptake as well as non-selective transport.

Biochemical and genetic dissection of starvation-induced autophagy in yeast, coupled with microscopic evaluation, has elucidated the stages of membrane dynamics in the transport process, leading to the model presented in Figure 2. The basic features of macroautophagy and Cvt transport are formation of a double-membrane vesicle (Stage 2), successive fusion of the outer bilayer with the membrane (Stage 3), and finally, lumenal degradation of internalized autophagic bodies/Cvt bodies (Stage 4). Using a molecular genetic approach, a number of groups are starting to identify protein components involved in modulating these complex membrane dynamics. Analyzing the subcellular localization of precursor API (prAPI) in *cvt* and *apg* mutants has been useful in classifying the basic stages

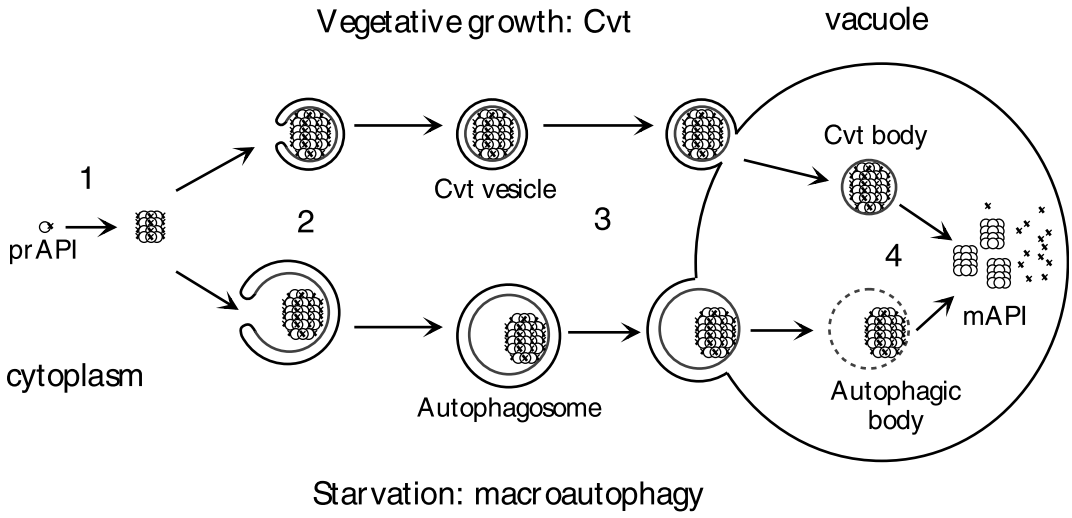

Figure 2. Model of autophagy and Cvt transport to the yeast vacuole. Stage 1: Cvt complex formation. Precursor API (prAPI) oligomerizes in the cytosol into a dodecamer that is then assembled into a higher-order structure, the Cvt complex. Stage 2: Formation of the double-membrane vesicles. The Cvt complex is enclosed by two membranes, to form a Cvt vesicle under vegetative growth conditions, or rather, forming an autophagosome under nutrient limitation. Stage 3: Fusion with vacuolar membrane. The outer membranes of Cvt vesicles / autophagosomes fuse with the vacuolar membrane, releasing vesicles into the organelle lumen. The subvacuolar vesicles are called Cvt bodies (during nutritive conditions) or autophagic bodies (when autophagy has been induced). Stage 4: Vesicle breakdown. The Cvt and autophagic bodies are targeted for degradation to release cargo into the vacuolar lumen.

at which various proteins act. As part of its biosynthetic delivery process, prAPI monomers oligomerize into homododecamers, which then associate into a higher-order protein assembly called a Cvt complex. The N-terminal propeptide of prAPI is necessary for the formation of the Cvt complex and for its specific delivery to the vacuole.[18] Because of the unique nature of this transport event, the *APG*, *CVT*, and *AUT* genes are likely to encode proteins that have novel functions.

Unique mechanistic questions

The membrane dynamics involved in autophagic and Cvt transport are different from other characterized membrane-trafficking systems. The standard process invoked in classical endomembrane trafficking is budding of vesicles from a source membrane. These vesicles are enclosed by a single membrane, and are complete upon scission from the donor membrane. The cargo that becomes enclosed in these vesicles is initially in contact with the lumenal face of the donor membrane, and remains associated within the lumen of budded vesicles. In contrast, Cvt and autophagy proceed by recruitment of membranes into sacs that ultimately form a fully enclosed double-membrane vesicle (Figure 2, stage 2). This mechanism encloses cargo which is in contact with the cytoplasmic face of the vesicle membrane.

One of the most glaring omissions in our mechanistic understanding of autophagy and related transport pathways is the identification of a source for the sequestering membrane. Related to this central question is the effort to elucidate mechanistically how membranes physically enwrap cytoplasmic proteins and organelles. Intense focus in the last couple of years, facilitated by the application of yeast genetics, has uncovered several proteins required for this sequestration step.

Characterization of four *apg* mutants reveals that a novel protein conjugation system is employed during autophagy and Cvt. Apg5p is conjugated via an isopeptide bond to a specific lysine residue of Apg12p,[19] reminiscent of the type of conjugation utilized in ubiquitination of proteins. In fact, the *APG7/CVT2* gene, which is required for Apg12p-Apg5p conjugate formation, encodes an E1-like ubiquitin-activating enzyme.[20,21] Biochemical analysis of another protein required for prAPI import and autophagy, Apg10p, suggests that it is an Apg12-conjugating enzyme, although it has no homology to the E2 ubiquitin carrier proteins that have analogous function.[22] In addition, Apg12p itself is not homologous to ubiquitin, nor is

it related in structure to ubiquitin-like molecules recently implicated in various other cellular regulatory checkpoints.[23] Another component, Apg16p, was shown to interact specifically with Apg5p, with a preference for the Apg12p-conjugated form of Apg5p.[19] Both free Apg5p, as well as the Apg12p–Apg5p conjugate partially localize with a membrane, even though these proteins are hydrophilic.[19, 24] Analysis of an Apg7pGFP fusion protein demonstrates that Apg7p is primarily cytosolic but shows a partially punctate distribution upon starvation. The punctate staining pattern is dependent on Apg12p, suggesting that membrane binding of Apg7p requires the presence of its substrate.[21]

It is likely that the conjugation machinery performs its function in association with the autophagosome/Cvt vesicle membrane. Biochemical studies carried out in null mutants lacking any of the conjugation machinery components indicate that prAPI transport is arrested at a protease-accessible and membrane-associated stage; the Cvt complex of prAPI dodecamers is still formed, thus the conjugation machinery is involved in the formation/completion of Cvt vesicles and autophagosomes. Recently, analysis of a conditional allele of *apg5* also demonstrated the role of this conjugation machinery in sequestering prAPI into double-membrane vesicles.[24] Finally, localization of API by immunoelectron microscopy in *apg5* mutant cells prepared under conditions non-permissive for Cvt and autophagic transport shows that prAPI is associated with membranes, but is not enclosed into vesicles, supporting the biochemical studies.[24]

Analysis of other *cvt*, *apg* and *aut* mutants for localization of prAPI indicates that a number of other proteins in addition to the conjugation machinery act to accomplish the formation of autophagosomes and Cvt vesicles. Recently, two groups independently analyzed the localization of prAPI in the *aut7/cvt5/apg8* mutant and concluded that the defective gene encodes another component involved in forming the double-membrane structure. *AUT7* encodes a small, hydrophilic protein that is membrane associated. Aut7p is normally enclosed within the autophagosome or Cvt vesicle as demonstrated by immunofluorescence, protease protection experiments, and biochemical localization in mutants defective in various stages of the import process.[25, 26] Earlier work had speculated that Aut7p may act as an adapter for linking completed vesicles to the cytoskeletal network;[27] however, the more recent localization of this protein is incompatible with this hypothesis. Interestingly, Aut7p is upregulated when cells are starved for nitrogen. The increased membrane mobilization into autophagosomes which occurs upon induction of autophagy may require the increased abundance of components required for the formation or maintenance of the double-membrane vesicles; Aut7p is a likely candidate for such a component. Another protein, Aut2p, has been implicated genetically and biochemically as an interacting parter with Aut7p.[27] The nature of Aut2p association with Aut7p *in vivo* is still not known.

One reasonable expectation is that a protein required for autophagosome/Cvt vesicle completion should be localized at least partially to the forming double-membrane vesicles. Apg9p is an integral membrane protein required for the acquisition of a double membrane surrounding Cvt complexes; in cells lacking functional Apg9p, prAPI is protease accessible and membrane associated, the hallmark characteristics for a block during sequestration.[28] However, the localization of Apg9p *in vivo* as evidenced by fluorescence of a GFP fusion protein is somewhat surprising given this prAPI phenotype; Apg9p is seen in discrete punctate structures that are frequently close to the vacuolar membrane.[28] Furthermore, in cells that accumulate completed autophagosomes and Cvt vesicles due to a defect in the vacuolar fusion step, Apg9p does not co-localize with prAPI. Finally, immunoelectron microscopy indicates that Apg9p is excluded from cytosolic autophagosomes, indicating that Apg9p is not a resident membrane protein of these double-membrane vesicles. By biochemical analysis, Apg9p does not co-localize with typical endomembrane marker proteins. Thus, Apg9p is a membrane protein whose function is directly required for the complete formation of autophagosomes/Cvt vesicles that is not itself found in these structures. Apg9p may act at the membrane source for autophagosomes or Cvt vesicles; however, other explanations for its function, including a role in a non-source compartment cannot be excluded.

Several components recently implicated in the formation of Cvt vesicles may also play a role in other vesicle-trafficking events. These include Tlg2p and Vps45p, which are syntaxin and Sec1p homologues, respectively. These proteins may function specifically in membrane fusion during the double-membrane vesicle formation step.[29] The *vac8* mutant blocks prAPI transport and causes a similar phenotype. Vac8p is an armadillo-repeat-containing protein that has a genetically-separable function in vacuole inheritance.[30, 31]

Components acting subsequent to autophagosome/Cvt vesicle formation have also been charac-

terized. Fusion of vesicles with the membrane of the vacuole occurs in a number of cellular-trafficking pathways; the vacuole is the 'terminal destination' for numerous vesicular transport pathways; Vam3p is a vacuolar membrane protein of the syntaxin family of t-SNAREs. A temperature sensitive mutant of *vam3* is defective in biosynthetic delivery of proteins through the secretory pathway and is also blocked in autophagy and the Cvt pathway.[32] Vps18p and Vps16p are components of a larger complex that are active in a late step of protein transport to the vacuole, functioning in biosynthetic transport as well as endocytic transport to this organelle; these proteins have also been implicated in Cvt delivery.[10, 33, 34] *YPT7* encodes a small GTP-binding protein of the *rab* family that is required for homotypic fusion of vacuoles as well as fusion of endosomal vesicles with the vacuole.[35, 36] Ypt7p is also required in Cvt transport and autophagy, acting after formation of the Cvt vesicle or autophagosome.[21] Thus, the docking and fusion machinery operative in the Cvt pathway and in autophagy is at least partly shared with other pathways, which involve fusion of single-membrane vesicles with the vacuole.

Following fusion of the outer membrane of the Cvt vesicle or autophagosome with the vacuole membrane, the internalized inner vesicles are then degraded in the vacuole lumen. This suborganellar recognition of a vesicle destined for lysis is another aspect of Cvt and autophagic transport that is unique among other known vacuolar membrane transport systems. The *cvt17* mutant accumulates prAPI inside the vacuole[34] (Figure 2, Stage 4), thus the *CVT17* gene product is required for intravacuolar vesicle breakdown. The mechanism by which Cvt17p action results in Cvt vesicle breakdown is currently unknown.

What determines the mode of transport: Cvt transport versus autophagy?

Despite the fact that Cvt transport and autophagy use essentially the same molecular machinery, a number of differences distinguish the two modes of transport. First, prAPI import is rapid, occurring with a half time of 30–45 min[37] while autophagy is slow, occurring maximally at a rate of 4% per hour.[16] Autophagy occurs at a low basal level, and is induced by starvation. Precursor API uptake is constitutive, occurring under both starvation and nutrient-rich conditions, but is upregulated by starvation.[16] Morphologically, double-membrane vesicles seen in rapidly growing cells are smaller than their autophagosome counterparts. Cvt vesicles are approximately 150 nm in diameter while autophagosomes are approximately 300–900 nm.[13, 38] Finally, the Cvt pathway is specific (excluding bulk cytosol) and saturable, while autophagy is non-specific and is not saturable. The ramification of these observed differences is that there must be some molecular components that make the process rapid and specific for prAPI delivery, and that allow autophagic transport to accommodate more cargo. One possibility is the use of a specific receptor that can recognize the pro region of prAPI; however, such a receptor has not been identified thus far. A similar issue arises in addressing the specificity of organelle uptake: do individual receptors act to recognize cognate proteins localized to individual organelles, such as peroxisomes?

The switch from specific Cvt uptake to the upregulated, non-specific autophagic mode of transport is known to depend on a Tor kinase signaling cascade. Tor kinases in yeast and other eukaryotes are operative in sensing nutritional status. *Saccharomyces cerevisiae* Tor kinase activity has been implicated by pharmacological and genetic studies as a regulating component that suppresses autophagy under nutrient-rich conditions.[39]

Candidate components for controlling the specificity of uptake by Cvt transport versus autophagic transport include proteins encoded by genes that demonstrate pathway-specific defects when they are inactivated. *cvt9* mutants accumulate prAPI during vegetative growth conditions, but can transport the protein to the vacuole when autophagy is induced by a shift to nitrogen-poor conditions (J. Kim, J. Guan, Y. Kamada, Y. Ohsumi and D. J. Klionsky, unpublished data). The inactivation of Cvt9p in a conditional mutant results in an arrest in prAPI transport at a protease-sensitive, membrane-associated stage, indicating that it is active prior to final enclosure of cargo into Cvt vesicles. It will be interesting to determine what biochemical function Cvt9p performs in the process of Cvt vesicle formation; presumably, this activity is not essential for autophagosome formation.

CVT3 is another gene required for Cvt transport that is not essential for autophagy.[16] Analyzing the morphology of a *cvt3* strain under vegetative conditions revealed that while prAPI can form the Cvt complex (Figure 2, Stage 1) in this strain, the subsequent formation of Cvt vesicles is defective.[38] The *CVT3* gene has yet to be cloned.

Tlg2p, another component mentioned earlier in regard to Cvt vesicle formation, also displays a Cvt pathway-specific phenotype.[29] Deletion in this gene

blocks prAPI targeting during nutritive growth, but not when autophagy is induced. The autophagic bypass of the block in prAPI transport suggests that Tlg2p might not be needed for the formation of autophagosomes. Likewise, the prAPI accumulation phenotype exhibited by the *vac8* mutant, also blocked in vesicle formation, can be reversed under nitrogen starvation conditions (D.C. Nice, S.V. Scott, L.S. Weisman and D.J. Klionsky, unpublished data). This suggests that the Cvt pathway, but not autophagy, is defective in the *vac8* mutant. The fact that several mutants demonstrate Cvt-specific function at the stage of vesicle formation / sequestration indicates that this is a crucial step in regulating Cvt transport / autophagy at a mechanistic level.

In contrast to the above Cvt-specific mutants, the *aut4* mutant does not accumulate prAPI under vegetative conditions.[15] This mutant accumulates autophagic bodies in the vacuole under nitrogen-limiting conditions, suggesting that Aut4p may function specifically for autophagic body breakdown.

The proteins discussed above are a subset of a number of components that have been implicated by genetics as proteins that play a role in Cvt transport and autophagy. Importantly, the set of *apg*, *aut*, and *cvt* mutants is likely incomplete; it is probable that new mutants will be identified. For the existing mutants, a number have not yet been examined to establish the stage at which vesicular transport has been blocked. Finally, several mutants defective in pexophagy in various fungi have been obtained that promise to shed some light on the process of autophagic uptake to the vacuole. Depending on the nutritive conditions, methylotrophic yeasts such as *Pichia pastoris* can take up peroxisomes by macro- or micropexophagy (Figure 1).[40] Mutants in this system should allow a better characterization of these two aspects of vacuolar transport, a subject that is currently poorly understood. As the characterization of molecular components continues, we should come to a more complete understanding of the mechanisms involved in autophagy and related vacuolar transport pathways.

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