Delivery of proteins and organelles to the vacuole from the cytoplasm

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The vacuole/lysosome is a primary catabolic site in the eukaryotic cell. One implication of its cellular role is that delivery systems must exist to target both hydrolytic enzymes and substrates destined for degradation to this organelle. A number of nonclassical vacuolar targeting pathways that deliver degradative substrates and at least one resident enzyme from the cytosol to the vacuole have recently been described. The pathways identified so far include cytoplasm to vacuole targeting, macroautophagy, pexophagy and vacuolar import and degradation. Cytological, genetic and molecular genetic approaches have begun to provide insight into the molecular basis of these processes.

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Current Opinion in Cell Biology 1998, **10**:523–529

http://biomednet.com/elecref/0955067401000523

Abbreviations

API	aminopeptidase I
csc	constitutive sequestration of cytosol by autophagy
Cvt	cytoplasm-to-vacuole targeting
ER	endoplasmic reticulum
FBPase	fructose-1,6-bisphosphatase
gsa	glucose-induced selective autophagy
pag	peroxisome degradation via autophagy
pdd	peroxisome degradation deficient
PFK1	α subunit of phosphofructokinase
vid	vacuolar import and degradation
vps	vacuolar protein sorting

Introduction

Compartmentalization is a key feature of eukaryotic cells, allowing enzymes to operate more efficiently and separating competing reactions. Because of these advantages, cells have developed specific processes to ensure the correct targeting and delivery of both resident enzymes and substrates to the proper organelle. The vacuole is the main degradative compartment in the yeast cell [1]. At least three pathways, two that divert secretory traffic from the late Golgi to the vacuole, and cytoplasm-to-vacuole targeting (Cvt), are utilized to supply hydrolases to this organelle. Each of these processes requires a number of sorting/targeting components, some of which play a role in all three pathways. A more complete picture of the complexity of the vacuole is obtained, however, when one considers the diversity of processes used for substrate delivery (Figure 1). The delivery of both bulk and specific components from the cell surface occurs by endocytosis (reviewed in [2]). In addition, there is a basal level of constitutive transport to the vacuole of proteins that are destined for degradation. Most of the flow to the vacuole, however, occurs in response to specific environmental stimuli. Different cues lead to the uptake of organelles such as peroxisomes, particular cytosolic enzymes including those used in gluconeogenesis, or bulk cytosol [3,4]. Each of these sequestration events occurs at particular times, is limited to distinct cytoplasmic components and is restricted to subsets of the target populations; hence, there must be subcellular machinery that senses the environmental signals, activates the appropriate machinery and designates the correct targets. The variety of vacuolar import pathways has been mirrored in the array of screens used by researchers to investigate their function. In this review, we have focused on the delivery of proteins and organelles to the yeast vacuole from the cytoplasm.

Pexophagy and mitophagy are examples of regulated organellar degradation

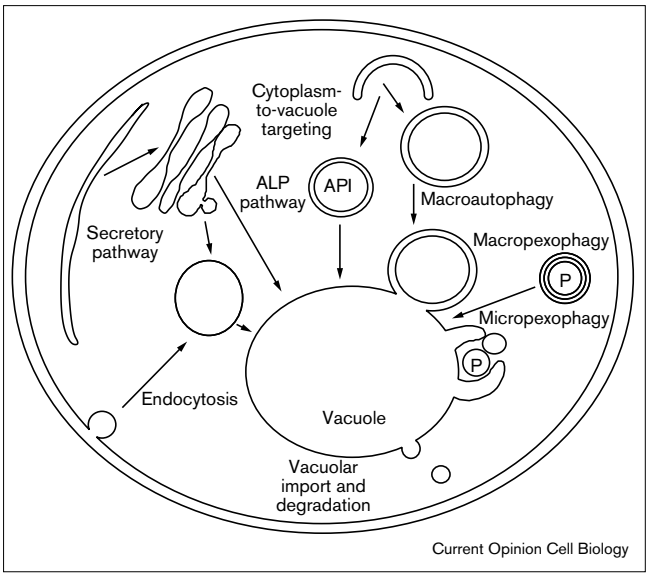
Like the biosynthesis of proteins and organelles, the ultimate destruction of these cellular components is an important regulatory process. Whereas considerable attention has focused on the biogenesis of peroxisomes [5] and mitochondria, much less is known about the processes by which these organelles are degraded.

The turnover of peroxisomes, or pexophagy, has been examined primarily in the methylotrophic yeasts *Pichia pastoris*, *Candida boidinii* and *Hansenula polymorpha* [4]. In these organisms, peroxisomes are synthesized when methanol or oleic acid is the sole carbon source. Peroxisomes are selectively degraded when glucose or ethanol is subsequently added to the growth medium [6,7]. Degradation involves the sequestration of the organelle and subsequent delivery to the vacuole lumen, but the specific mechanism differs depending on the organism and environmental stimulus.

In *H. polymorpha*, uptake occurs through macropexophagy [7]. Peroxisomes are enwrapped by a sequestering membrane(s) that subsequently fuses with a vacuolar compartment. A similar process occurs in *P. pastoris* when cells are exposed to ethanol [8,9••]. Macropexophagy in *P. pastoris*, *C. boidinii* [8,9••,10], and *H. polymorpha* (M Veenhuis, personal communication) is insensitive to cyclohexamide, suggesting the involvement of pre-existing components; in *H. polymorpha*, the sequestering membrane is thought to derive from the endoplasmic reticulum (M Veenhuis, personal communication).

In contrast to ethanol adaptation, a micropexophagic mechanism operates in *P. pastoris* in the presence of glucose [8,9••]. In this case, clusters of peroxisomes rather than individual organelles are engulfed directly by protrusions of membrane from the vacuole surface. This uptake

Figure 1



A variety of pathways are used for the delivery of both resident hydrolases and degradative substrates to the vacuole. The secretory and alkaline phosphatase (ALP) pathways transport resident hydrolases through the endoplasmic reticulum and Golgi complex and, for the secretory pathway, through the endosome en route to the vacuole. The vacuolar import and degradation and pexophagy pathways are used for the specific degradation of cytosolic proteins or peroxisomes, respectively, under particular nutrient conditions. Macroautophagy is a non-specific process involved in the vacuolar degradation of bulk cytosol. The cytoplasm-to-vacuole targeting pathway delivers the resident hydrolase aminopeptidase I (API) into the vacuole from the cytosol. ALP, alkaline phosphatase; API, aminopeptidase I; ER, endoplasmic reticulum; P, peroxisome.

is dependent on new protein synthesis. In addition, micropexophagy is blocked by the phosphatidylinositol-3 (PI3)-kinase inhibitor wortmannin, suggesting a role for phosphatidylinositides in the signaling process [9••]. Micropexophagy can also be distinguished from macropexophagy based on the kinetics of inactivation of peroxisomal enzymes [8] or delivery of peroxisomes to the vacuole [9••]; the loss of activity is slower during glucose adaptation and there is a lag that is not observed in the presence of ethanol. Finally, during glucose but not ethanol adaptation in *P. pastoris*, cytosolic enzymes such as formate dehydrogenase are also degraded [8]. It is not known, however, if the degradation of cytosolic enzymes occurs by micropexophagy or the vacuolar import and degradation pathway (see below).

Recently, genetic approaches have been used to elucidate the molecular basis for pexophagy. Titorenko *et al.* [11] used a screen based upon the retention of alcohol oxidase activity to isolate mutants in *H. polymorpha* that are peroxisome degradation deficient (*pdd*). The mutants can be placed into five complementation groups (JAKW Kiel, KB Rechinger, FA Salomons, VI Titorenko, M Veenhuis, personal communication). Morphological analyses of two of these mutants allow macropexophagy to be separated into two primary steps [11]. The *pdd1* mutant is defective in the sequestration of peroxisomes by the enwrapping membrane. In contrast, the *pdd2* mutant is able to sequester peroxisomes but is blocked in the subsequent fusion event. The *PDD1* gene has been found to encode a 116 kDa protein with a high similarity to the *Saccharomyces cerevisiae VPS34* gene product (JAKW Kiel, KB Rechinger, FA Salomons, VI Titorenko, M Veenhuis, personal communication). Vps34p is a PI3-kinase that is involved in subcellular targeting pathways [12]. Similarly, the *pdd1* mutant is defective in vacuolar protein sorting and endocytosis in *H. polymorpha* (JAKW Kiel, KB Rechinger, FA Salomons, VI Titorenko, M Veenhuis, personal communication). One prediction is that Pdd1p is involved in tagging peroxisomes for sequestration. Specificity may be achieved through interaction with another protein equivalent to the *S. cerevisiae* Vps15 protein [13]. Synthetic defects in growth on methanol were seen in a *pdd1 pdd2* double mutant, suggesting that the two gene products may physically interact [11].

A similar screen in *P. pastoris* resulted in the isolation of mutants defective in glucose-induced selective autophagy (*gsa*) [8]. These mutants display normal degradation of peroxisomes through macropexophagy during ethanol adaptation, providing further support for the presence of two independent pathways in this organism. The *gsa1* mutant is unable to form the vacuolar protrusions required for the engulfment of peroxisomes by micropexophagy. Cloning of the *GSA1* gene reveals that it is allelic to *PFK1*, the gene encoding the α subunit of phosphofructokinase [14••]. Mutation of the fructose 6-phosphate binding site of the *PFK1* gene, however, does not prevent complementation of the peroxisome degradation phenotype of the *gsa1* mutant [14••]. These results suggest that Pfk1p may have a function in regulating micropexophagy independent of its role in glycolysis.

Recently, Subramani and colleagues [9••] carried out another screen based on the retention of alcohol oxidase activity to isolate mutants blocked in peroxisome degradation via autophagy (*pag*). By double labeling cells with the vacuolar dye FM 4-64 and a green-fluorescent-protein–SKL construct targeted to the peroxisome by the Ser-Lys-Leu signal, the mutants were shown to be defective in several stages of the micropexophagic process. Interestingly, two of the *pag* mutants were shown to be defective for peroxisomal degradation during both glucose and ethanol adaptation, suggesting a partial overlap between these pathways.

Mitochondria may also be degraded by specific vacuolar uptake

Maintenance of the mitochondrial compartment is essential to cell viability. As with peroxisomes, mitochondrial levels fluctuate in response to growth conditions. Mitochondria are occasionally seen inside autophagic bodies, indicating that they can be degraded through macroautophagy [15].

Very little is known, however, about the mechanism used for the specific degradation of mitochondria in response to glucose adaptation. Thorsness and colleagues have studied the loss of mitochondrial DNA to the nucleus in *S. cerevisiae*. The *yme1* mutant displays elevated rates of mitochondrial DNA migration to the nucleus [16]. These rates are suppressed in strains having mutations in vacuolar hydrolases (P Thorsness, personal communication). Similarly, *yme1* yeast appear to display higher levels of mitochondrial turnover (P Thorsness, personal communication). The increased susceptibility to degradation may in part result from the mitochondrial fragmentation seen in the absence of Yme1p [17]. Along these lines, *yme1* mutant mitochondria are often found in close proximity to vacuoles (P Thorsness, personal communication). One interpretation of these results is that some aspect of mitochondrial degradation in the *yme1* strain allows the release of mitochondrial DNA, enhancing its escape to the nucleus.

Many questions concerning organellar degradation remain to be addressed. For example, it is not known how degradation is made specific for one type of organelle and how the extent of uptake of a particular organelle is regulated. It seems that there must be a mechanism for tagging, and in the case of peroxisomes this appears to involve the membrane [18]; however, the mechanism for recognition of specific cytosolic enzymes involved in methanol adaptation is completely unknown. Similarly, the signal transduction mechanism that triggers degradation, the origin of the sequestering membranes and their mode of enwrapping are not known.

Vacuolar import and degradation pathway

As discussed above, in some yeasts, particular environmental conditions lead to the degradation of cytosolic enzymes that are involved in methanol adaptation. This inactivation appears to be specific in that it is rapid and does not include bulk cytosol. Similarly, enzymes involved in other types of carbon metabolism are degraded when they are no longer needed. Fructose-1,6-bisphosphatase (FBPase) is a key regulatory enzyme in gluconeogenesis and is synthesized in response to glucose deprivation. Upon the readdition of glucose, this enzyme is selectively degraded, although there is some controversy regarding the mechanism [4]; it may be that FBPase is subject to degradation by both cytosolic and vacuolar enzymes.

In the case of vacuolar degradation, uptake may occur by two mechanisms. FBPase can be seen at the site of vacuolar membrane invagination, suggesting a microautophagic means of entry [3]. More recent studies provide convincing evidence that FBPase degradation also involves a vesicle-mediated pathway. Using a genetic screen based upon the detection of FBPase following transfer from acetate to glucose medium, vacuolar import and degradation (*vid*) mutants were isolated that are defective for FBPase degradation [19]. The *vid* mutants do not affect transit through the secretory pathway. In addition, analysis of the *vid* mutants by immunofluorescence reveals that they are blocked in at least two distinct stages of the uptake process. Most of the mutants accumulate FBPase in the cytosol. In the *vid14, 15* and *16* mutants, however, glucose readdition results in a punctate distribution of FBPase, suggesting that these mutants are blocked at a later stage in the pathway, subsequent to sequestration.

FBPase can be recovered from a vesicle fraction derived from wild-type cells [20••]. The association of FBPase with the vesicles kinetically precedes its delivery to the vacuole, indicating that the vesicles act as transport intermediates. The vesicles appear to be single membrane and are 30–40 nm in diameter. FBPase in the vesicle fraction is protected from exogenous protease indicating that it resides within the vesicle lumen. Recently, the *VID24* gene was cloned and was shown to encode a 41 kDa protein that is synthesized in response to glucose addition [21••]. Vid24p is a peripheral membrane protein that is primarily associated with the Vid vesicles that play a role in FBPase degradation. As with the *vid14, 15* and *16* mutants, *vid24* cells accumulate FBPase in intermediate vesicles, suggesting that Vid24p is required for targeting, docking and/or fusion with the vacuole. This function is in agreement with the location of Vid24p on the surface of FBPase-containing vesicles. The *vid24* mutant is not defective in autophagy or secretory pathway transit. This observation, coupled with the morphological differences between Vid vesicles and those used in other transport pathways, suggests that the uptake of FBPase represents a distinct cargo delivery mechanism.

The import of FBPase has been reconstituted *in vitro* [22••]. FBPase synthesized *in vitro* is taken up into the vacuole in semi-intact yeast cells. The efficiency of import parallels that seen *in vivo*; cells shifted to glucose for short periods of time prior to permeabilization showed faster rates and higher levels of FBPase uptake. This observation is consistent with the glucose-induced synthesis of proteins such as Vid24p. The *in vitro* uptake is dependent on ATP hydrolysis and stimulated by cytosol. *In vitro* reconstitution provides a means to biochemically identify and functionally assign additional components needed for FBPase import.

Among the most pressing questions regarding the Vid pathway is the process by which the cell is able to recognize specific cytosolic proteins destined for subsequent degradation. The identification of other potential cargo that are imported through the Vid pathway may provide insight into this topic.

Macroautophagy occurs in response to starvation

Macroautophagy enables the survival of starvation by non-selectively delivering cytosolic proteins and organelles to the lysosome/vacuole for degradation and recycling. This process has been examined in *S. cerevisiae* by electron microscopy [15,23,24]. In response to nutrient deprivation,

double-membrane autophagosomes surround bulk regions of cytosol encompassing mitochondria and cytosolic ribosomes as well as soluble enzymes [23]. These vesicles then fuse at the vacuole, delivering a single-membrane autophagic body into the vacuole lumen. Finally, the autophagic bodies are broken down and their contents digested [15]. The origin of the autophagosomal membrane is not completely clear. Marker analysis performed in mammalian cells indicates the presence of some rough endoplasmic reticulum (ER) markers in autophagosomes [25]. Because ribosomes are not present on autophagosomal membranes, ribosome-free regions of ER membrane have been proposed as the donor membrane source; however, the proportion of rough ER proteins in autophagosomes appears relatively low, leaving open the possibility that the membranes actually originate from a later secretory organelle such as a post-Golgi compartment, or alternatively that a specific donor membrane compartment called the phagophore serves as a membrane source [26]. Macroautophagy occurs in *S. cerevisiae* in response to starvation for nutrients such as nitrogen, carbon and sulfur [15], in cultured mammalian cells in response to serum withdrawal (reviewed in [27]), and in plants in response to sucrose starvation [28].

Molecular components of macroautophagy

Two groups of mutants defective in autophagy, called *apg* [29] and *aut* [30], have been obtained from studies in *S. cerevisiae*. Recently, the genes that complement several of these mutants have been cloned and sequenced. Apg5p [31], Apg13p [32] and Aut1p [33•] are predicted to be hydrophilic proteins of 33 kDa, 83 kDa, and 36 kDa, respectively. None of these polypeptides display significant homologies to other proteins in the database. Apg1p is predicted to be a novel serine/threonine kinase of 102 kDa [34•] and *APG1* overexpression suppresses *apg13*, suggesting that these two proteins interact [32]. The cloning of the remaining complementing genes together with localization studies and the characterization of how the gene products interact will continue to provide insights into the nature of the autophagic process.

To identify components involved in autophagy induction, a screen for mutants where autophagy is induced in rich media was performed [35••]. Two complementation groups of mutants called constitutive sequestration of cytosol by autophagy (*csc*) were identified. One mutant was cloned and found to be allelic to *end13/vps4/grd13*, a member of the AAA (ATPases associated with a variety of cellular activities) family of ATPases thought to be involved in endosome function. These data suggest a role for the endosome in macroautophagy.

In mammalian cells, insight into the molecular mechanism of autophagy induction has been obtained primarily from the use of chemical inhibitor studies (reviewed in [27,36,37]); for example, studies using GTPγS [37] and pertussis toxin [38] indicate the involvement of a heterotrimeric G_{i3} protein in autophagic sequestration. This finding was confirmed by molecular genetic analyses in transfected human cancer cells that resulted in either overexpression or depletion of the α_{i3} subunit [39]. Other studies indicate a correlation between high external concentrations of regulatory amino acids, phosphorylation of the 40S ribosomal protein S6 and inhibition of autophagic sequestration. This effect is blocked by rapamycin, an inhibitor of the phosphatidylinositol kinase that phosphorylates S6 [40]. Recent work in yeast supports these findings. Noda and Ohsumi found that rapamycin treatment caused autophagic induction even in rich media. In addition, the use of a mutant in the yeast phosphatidylinositol kinase homologues *tor1* and *tor2* allowed them to confirm the mechanism of action of rapamycin and suggest that induction of autophagy is regulated by the action of a phosphatidylinositol kinase [41•]. These studies provide additional evidence that autophagic mechanisms operate similarly in yeast and mammalian cells.

The vacuolar protease aminopeptidase I is targeted by a process that overlaps with macroautophagy

Initial investigation indicated that the vacuole-resident protease aminopeptidase I (API) is targeted from the cytosol to the vacuole, rather than via the secretory route utilized by most characterized vacuolar enzymes [42]. API is synthesized as a precursor containing an amino-terminal extension necessary for correct vacuolar sorting. This propeptide region is predicted to form two α helices [43]. The first helix is amphipathic in nature and is critical for the correct sorting of the enzyme [44]. Upon delivery of API to the vacuole, the proregion is cleaved resulting in the mature-sized enzyme.

The active form of API is a homododecamer of 600 kDa. A recent study indicates that the precursor form of the enzyme oligomerizes with a halftime of approximately 2 minutes. [45••]. This is significantly faster than vacuolar delivery, which has a halftime of 30 minutes. Kinetic analyses demonstrated that the oligomeric structure is, in fact, maintained throughout targeting [45••]. The large size of the API dodecamer precludes transport via a translocation channel, and suggests that API targeting occurs by a vesicle-mediated process.

Fifteen complementation groups of yeast mutants defective in API maturation were isolated [46,47]. Characterization of these mutants revealed that three are allelic to known vacuolar protein sorting (*vps*) mutants, and six are allelic to previously identified autophagy *apg* (mutants and *aut*) [46–48]. In addition, the majority of nonoverlapping autophagy mutants are also defective in API transport [47,48]. These data suggest that macroautophagy and API transport utilize many of the same molecular components. This is a surprising finding because API targeting is known to be a selective and constitutive event, whereas macroautophagy is clearly nonselective and is induced by starvation.

The biochemical examination of API precursor (prAPI) in mutant strains supports an autophagic mode of transport [49••]. First, the localization of an API propeptide mutant that is defective in vacuole delivery but not in the binding step of the targeting process was examined. The mutant prAPI was found to be in a pelletable form, but not associated with the surface of the vacuole, suggesting that the initial binding event is in a cytosolic location distinct from the vacuole surface. Second, the localization of prAPI was examined in a temperature-sensitive *vps18* mutant. A shift to the nonpermissive temperature causes an immediate block in API delivery. Fractionation studies localized the trapped pool of prAPI to cytosolic vesicles, again distinct from the vacuole itself. Finally, a mutant defective in the breakdown of vesicles, *cvt17*, was examined [49••]. In this strain, accumulated prAPI appeared in fractions containing purified vacuoles; however, these vacuoles could be lysed to release subvacuolar vesicles that still contained prAPI, but lacked other vacuolar marker proteins. The fact that prAPI can be recovered from cytosolic as well as subvacuolar vesicles supports an autophagic model for API transport and suggests that API is targeted by cytosolic double-membrane vesicles that fuse with the vacuole to release the inner membrane vesicle into the vacuolar lumen.

Autophagosomes formed during starvation and API-containing vesicles formed in rich media are morphologically similar

API import was examined by immunoelectron microscopy in both rich media and starvation conditions, to resolve the apparent discrepancy of how API could be transported both selectively and constitutively by macroautophagy, a mechanism thought to be nonselective and require starvation induction [50••]. In nutrient-rich conditions in wild-type cells, the vacuole signal of API was dispersed, as expected for a soluble enzyme; however, cytosolic pools of prAPI appeared clustered into specific regions of the cytosol called Cvt complexes. In some images, the clustered prAPI appeared enwrapped in double-membrane structures called Cvt vesicles that were similar in appearance to autophagosomes. These Cvt vesicles were also detected fusing at the vacuole membrane. In degradation-deficient strains such as *cvt17* or *pep4*, single-membrane subvacuolar vesicles called Cvt bodies were apparent. In starvation conditions, Cvt complexes still formed, but these were targeted to the vacuole by autophagosomes.

The Cvt structures observed in nutrient-rich conditions were morphologically similar to the corresponding vesicular structures formed in starvation conditions with a few exceptions [50••]. First, the contents of Cvt vesicles stained densely and appear to be devoid of ribosomes, whereas the contents of autophagosomes are indistinguishable from bulk cytosol [23,50••]. The diameter of the two types of vesicles also differed. Autophagosomes were 400–900 nm in diameter, whereas the Cvt vesicles measured just 150 nm. The cytosolic volume encompassed by autophagosomes is, therefore, between 19 and 152 times more than that of Cvt vesicles, making them a far more efficient mode for bulk delivery to the vacuole. These data suggest that during growing conditions, vesicles form that carry specific components such as API to the vacuole. On shifting to starvation, these vesicles are triggered to increase in size and gather in bulk cytosolic components in addition to their normal selective cargo. The fact that the Cvt complexes appear to be transported by autophagosomes in starvation conditions makes API an appropriate and useful marker for investigations of macroautophagy. Further, because the transport of API is selective even in starvation conditions, it is easier to follow than markers for bulk phase autophagy that are not enriched in autophagosomes.

Through the investigation of API transport, several additional proteins have been identified that are required for this process. One is the vacuolar t-SNARE Vam3p [51•]. This protein is required for the vacuolar delivery of vesicles originating from the endosome, as well as those delivered by the alkaline phosphatase pathway, and is likely to be a component of the general recognition/fusion machinery at the vacuole surface. Other general factors that have been identified include Sec19p, the yeast rab GDP dissociation inhibitor, and Ypt7p, a rab required for homotypic vacuole fusion (SV Scott, DJ Klionsky, unpublished data). In addition, several *vps* gene products are also involved in API delivery. *cvt4* and *cvt8*, alleles of *vam4/vps39* and *vam2/vps41* [46], respectively, have been found to be members of a protein complex on the vacuole membrane [52]. Finally, the vacuole protein Vps18p has been shown to be required for the delivery of both Cvt vesicles and autophagosomes to the vacuole [49••,53•].

Conclusions

The maintenance of organelles, each containing a defined set of resident proteins, represents a substantial energetic requirement. Accordingly, cells synthesize and degrade specific organelles in response to environmental conditions. In addition, organelles are designed to compartmentalize subcellular reactions, making them more efficient but also protecting the cell from harmful enzymes or byproducts. In particular, when organelles such as mitochondria and peroxisomes that are involved in oxidative reactions undergo damage, they must be degraded to prevent the release of reactive compounds into the cytosol. The mechanisms by which eukaryotic cells regulate the levels of organelles is not well understood.

Degradation by the lysosome/vacuole is a primary mechanism that enables the survival of starvation as well as providing for the timely turnover of metabolic enzymes and proliferated organelles under conditions where they are no longer required at high levels. Cellular pathways that deliver cytosolic substrates to the vacuole have been the focus of recent investigation. The available data suggest that there are overlaps among these pathways, in particular at the level of fusion with the vacuole. It is likely, however, that there are some specific differences reflecting the operation

of fundamentally different pathways; for example, the Vid pathway uses single-membrane vesicles of 30–40 nm diameter, whereas the Cvt and autophagic pathways use double-membrane vesicles that range from 150 up to 900 nm. Similarly, the conditions under which the various processes take place are varied and in some cases diametrically opposed; autophagy is induced by starvation while pexophagy is triggered by the readdition of glucose. The continued analysis of the molecular components of each pathway will ultimately reveal how distinct each mechanism is from one another. It is nonetheless clear that multiple means have developed to satisfy the degradative needs of the eukaryotic cell.

Acknowledgements

We would like to thank Hui-Ling Chiang, Suresh Subramani, Peter Thorsness and Marten Veenhuis for the communication of results prior to publication and/or helpful comments on the manuscript, and acknowledge the support of the National Institute of General Medical Sciences.

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