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Topical Review

Protein Transport from the Cytoplasm into the Vacuole

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Abstract. The fungal vacuole is integrally involved in various cellular processes that include protein and organellar degradation and recycling. The ability to sequester numerous hydrolases within the cell makes the hydrolytic capacity of the vacuole critical under certain environmental conditions. Accordingly, cellular constituents destined for degradation are delivered to the vacuole through the secretory pathway, by endocytosis and from the cytoplasm. Different mechanisms have evolved to accommodate these multiple transport pathways. Protein transport from the cytoplasm into the vacuole in particular relies on the dynamic nature of the vacuole membrane. This review describes recent research on this topic from yeast systems and points out the direction of future studies aimed at understanding this complex organelle.

Key words: Autophagy — Cytoplasm to vacuole targeting — Organelle degradation — Protein transport — Vacuole — Yeast

Introduction

The vacuole is the most undervalued organelle in the eukaryotic cell. Most people think of the vacuole as the cellular garbage disposal. Although this is a misleading viewpoint—the vacuole plays a role in numerous physiological processes (Klionsky, Herman & Emr, 1990)—even a cursory glance at the vacuole's resident proteins explains why this conception is so prevalent: aminopeptidase I, aminopeptidase Y; proteinase A, proteinase B; carboxypeptidase Y, carboxypeptidase S. The vacuole is an organelle that appears to be filled with degradative enzymes having redundant activities. An obvious conclusion from this observation is that the degradative capacity of the vacuole is critical to various cellular functions. In fact, the vacuole is integrally involved in many processes that are directly dependent on the organelle's ability to degrade and recycle macromolecular constituents. This turnover capacity is of major importance to yeast living in natural conditions; unlike most laboratory cultures growing logarithmically in rich medium, yeast in the wild routinely experience nitrogen limitation.

The need to degrade cytoplasmic contents, especially entire organelles, cannot be accommodated by the cytosolic degradation machinery, the most prominent being the proteasome (*see* Hilt & Wolf, 1996 for a review). Accordingly, eukaryotic cells have developed mechanisms that allow the direct targeting of proteins and membranes destined for degradation into the vacuole. In fact, a variety of mechanisms are used for degradative delivery: protein or organelle uptake may be specific or nonspecific, may occur at the vacuole surface or may rely on extravacuolar compartments, and can be triggered in response to nutrient limiting or nutrient rich conditions. In other words, several systems are used to ensure that appropriate types of degradation occur under a wide range of conditions (Table).

Delivery of constituents for degradation is useless without the presence of the necessary vacuolar hydrolases. Most vacuolar hydrolases transit through a portion of the secretory pathway. Recently, however, it has become apparent that alternate pathways are used in the localization of vacuolar enzymes; some resident vacuolar hydrolases are localized to the organelle by similar or overlapping mechanisms to those used for the import of

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Table. Pathways for the transport of proteins from the cytoplasm into the vacuole in yeast

Protein/organelle	Half-time	Condition	Mechanism	Mutants
Bulk cytoplasm/fatty acid synthase	12–24 hr	Starvation	Macroautophagy 400–900 nm vesic.	apg, aut
Alcohol oxidase/peroxisome	1–2 hr	Ethanol	Macropexophagy,	pdd
	1–2 hr	Glucose	Macropexophagy,	pdd
	2–3 hr	Glucose	Micropexophagy	gsa
Fructose-1,6-bisphosphatase	30 min	Glucose	Microautophagy, Vacuolar import and degradation 30–40 nm vesic.	vid
Aminopeptidase I	30 min	Constitutive	Cytoplasm to vacuole targeting 150–500 nm vesic.	cvt

degradative substrates. The need to ensure vacuolar hydrolytic capacity may have led to this redundancy in targeting mechanisms thus ensuring the delivery of at least a partial complement of vacuolar enzymes.

This review focuses on the various mechanisms of protein transport from the cytoplasm into the vacuole that have developed in eukaryotic cells. A substantial part of the review is concerned with autophagy, which is defined in a deceptively simple way as ''self-eating.'' Autophagy is a complex process that can be separated into two main types, nonspecific/bulk and specific. Even nonspecific autophagy fits in the category of targeting from the cytoplasm to the vacuole because the vesicles that deliver cytoplasmic contents to the vacuole must be correctly targeted to this organelle. With specific autophagy there are additional questions to be addressed including the mechanisms used for tagging and detecting correct substrates.

Much of the work on autophagy comes from mammalian studies which have focused on biochemical analyses and in particular, the action of various chemical inhibitors (*see* Seglen & Bohley, 1992; Dunn, 1994 for a review). Mammalian cells utilize both micro- and macroautophagic delivery mechanisms (Fig. 1). In addition, a pentapeptide-dependent (Chiang & Dice, 1988) direct translocation mechanism is used for the degradation of certain proteins upon serum deprivation (*see* Dice, 1990; Terlecky, 1994 for a review). Various approaches including the development of an in vitro system (Chiang et al., 1989; Terlecky & Dice, 1993) to analyze this type of lysosomal autophagic import have allowed the identification of some of the molecular components including a cytosolic and lysosomal chaperone, hsc73 (Chiang et al., 1989; Terlecky et al., 1992; Terlecky & Dice, 1993), and a receptor protein located on the lysosomal membrane (Cuervo & Dice, 1996). This review focuses on information from yeast systems where rapid advances are being made because of genetics and more recently due to the identification of useful marker proteins such as aminopeptidase I (API).

One final point concerns the current nomenclature.

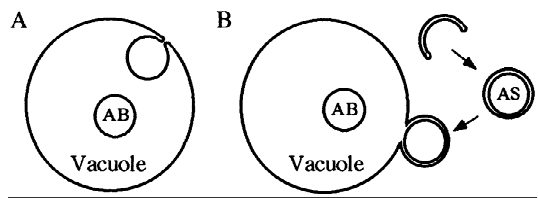

Fig. 1. Starvation-stimulated or nonspecific autophagy may occur by a (*A*) microautophagic or (*B*) macroautophagic process. Morphological data in yeast support macroautophagy where a double membrane autophagosome (AS) surrounds cytoplasmic contents and fuses with the vacuole to deliver the autophagic body (AB). The AB is broken down in a PrB-dependent manner.

Bulk or nonspecific autophagy involves the random sequestration of cytosolic proteins or cytoplasmic organelles. In some cases, autophagy is highly selective and probably occurs through substantially different mechanisms. These differences reflect the dramatically different signals that initiate the two processes: starvation versus transfer to an alternate and preferred carbon source. At this point we can see that the nomenclature in this field is limiting. The term ''autophagy'' is used to indiscriminately refer to any process whereby cytoplasmic constituents are degraded in a vacuole-dependent manner. For the purpose of this review, I propose the term ''starvation-stimulated autophagy'' to refer to the nonspecific/bulk process that is induced upon depletion of nitrogen or glucose. ''Nutrient-stimulated autophagy'' refers to those processes that are induced upon the transfer of cells to glucose or ethanol. Differences inherent in nutrient-stimulated autophagy of peroxisomes in the presence of glucose *vs.* ethanol suggest that more specific terms, glucose- or ethanol-stimulated autophagy or perhaps ''pexophagy,'' are also appropriate. The ''vacuolar import and degradation'' pathway refers specifically to the processes involved in vacuolar delivery of proteins such as fructose-1,6-bisphosphatase (FBPase). Last, I use the term ''cytoplasm to vacuole targeting'' to denote biosynthetic import of proteins such as API and α-mannosidase (AMS).

Starvation-stimulated Autophagy Employs the Formation of Cytosolic Double-membrane Structures

Morphological Studies

The levels of vacuolar hydrolases vary with nutritional conditions, and synthesis of many of the enzymes is induced by nutrient limitation. Several studies demonstrated the role of vacuolar proteases in protein turnover, in particular under starvation conditions (*see* Klionsky et al., 1990; Jones, 1991; Hilt & Wolf, 1992; Van Den Hazel, Kielland-Brandt & Winther, 1996 for review). To begin to understand the mechanism of this process, Ohsumi and colleagues carried out an extensive morphological examination of the yeast vacuole during starvation. Following a shift of yeast from nutrient-rich to a variety of nutrient-poor conditions, spherical membranous structures of 400 to 900 nm, termed ''autophagic bodies'' (ABs), are seen to accumulate in the vacuole of proteinase B (PrB)-deficient yeast (Simeon et al., 1992; Takeshige et al., 1992). The contents of these ABs are primarily indistinguishable from cytosol, suggesting they are involved in nonspecific sequestration of cytosolic proteins (Takeshige et al., 1992; Egner et al., 1993; Baba et al., 1994; Baba, Osumi & Ohsumi, 1995). These same types of structures are transiently seen in wild-type cells indicating that they result from the normal cellular response to nutrient deprivation but require PrB activity for breakdown within the vacuole. In addition to cytosolic proteins, the ABs contain mitochondria and endoplasmic reticulum, but these organelles appear to be sequestered in a nonspecific manner.

While nonspecific starvation-stimulated vacuolar degradation in yeast involves the formation of ABs within the vacuole (Takeshige et al., 1992), their origin could be due to either of two mechanisms. These structures could arise from a microautophagic process involving invagination of the vacuolar membrane, or a macroautophagic process involving fusion of a cytosolic double-membrane structure with the vacuole (Fig. 1). Morphological analyses provide support for the latter model (Baba et al., 1994; Baba et al., 1995). Following nutrient starvation, double-membrane structures termed ''autophagosomes'' are detected in the cytosol (Baba et al., 1994; Baba et al., 1995). These structures are of similar size to ABs and show a similar particle distribution, suggesting that the ABs are derived from the autophagosomes. The autophagosome and AB membranes are distinct from that of the vacuole; they contain fewer membrane proteins and glycoproteins (Baba et al., 1994; Baba et al., 1995). This morphological difference makes it unlikely that ABs derive from the vacuole membrane. Direct microscopic images demonstrating fusion of the autophagosome outer membrane with the vacuole further support the macroautophagic origin of the AB.

Formation of ABs is dependent on new protein synthesis and is also sensitive to NEM (Takeshige et al., 1992), suggesting a fusion-dependent event. AB formation is seen in both *sec17* and *sec18* mutants at the nonpermissive temperature, however, suggesting that the yeast α-SNAP and NSF homologues are not required in this process (Motizuki, Yokota & Tsurugi, 1995). Inhibition of the vacuolar ATPase with bafilomycin does not prevent AB formation (Takeshige et al., 1992) but does block maturation of API (*see below*) and Pho8Δ60p, a cytosolic construct that can be used to specifically measure bulk autophagy (Noda et al., 1995; Scott et al., 1996). Together, these results suggest that vacuolar acidification is required for the breakdown rather than the formation of ABs. In support of this, *vma* mutants (*see* Anraku, 1996 for a review) which are defective in the vacuolar ATPase accumulate ABs (Nakamura et al., 1997). To identify components involved in the breakdown of ABs, Ohsumi and colleagues screened for mutants that accumulate these structures within the vacuole. Most of these mutants defective in autophagic body disintegration (*abd*) are allelic to *vma* mutants, confirming the need for proper vacuolar pH in the breakdown of these subvacuolar compartments (Y. Ohsumi, *personal communication*).

One major unresolved question is the origin of the autophagosome membrane. In mammalian cells, autophagosomes are thought to be derived from the ER or Golgi. This is problematic, however, in that these membranes do not normally target to the vacuole and are unlikely to contain the appropriate targeting machinery such as vacuole-specific v-SNAREs (Rothman & Warren, 1994; Bennett, 1995). In yeast, autophagosomes do not appear to form from preexisting membrane because cycloheximide blocks AB formation (Takeshige et al., 1992). The autophagosomes may derive from restricted regions of the cytoplasm and preferentially form near the vacuole (Baba et al., 1994). The membrane protein profile as revealed by freeze-replica electron microscopy suggests that the autophagosome and AB membranes are distinct from the endoplasmic reticulum and Golgi. The outer membrane of the autophagosome is also different in appearance from the inner membrane. Unlike vesicles of the secretory pathway or endocytosis, both sides of the forming autophagosome face the cytoplasm; this makes it unclear how the proteins in both sides of this structure would be maintained as distinct populations.

Genetic Studies

Tsukada and Ohsumi (1993) undertook a genetic analysis to identify mutants defective in autophagy. In brief,

they screened mutagenized cells for loss of viability during nitrogen starvation, taking advantage of their observation that cells defective in autophagy lose viability more rapidly than wild type cells under these conditions. Potential mutants were subsequently screened by microscopy for the inability to accumulate ABs. A series of mutants defective in autophagy (*apg*) were identified. These mutants display decreased levels of total vacuolar-dependent protein degradation, are sensitive to nitrogen starvation and are sporulation-negative as diploids (Tsukada & Ohsumi, 1993). Transport to the vacuole through the secretory pathway and endocytosis are normal, indicating that the defects are specific to autophagic uptake. All of the *apg* mutants fail to accumulate ABs, suggesting that they are blocked in early (i.e., prevacuolar) steps of autophagy.

A different approach to identify autophagy defective mutants was utilized by Thumm et al. (1994). Screening for cells that lost the ability to degrade overproduced fatty acid synthase subunit β, and that did not accumulate ABs, they isolated a series of mutants named *aut* (Thumm et al., 1994). Similar to the *apg* mutants, the *aut* mutants fail to transport cytosolic proteins destined for degradation into the vacuole, are sporulation defective and in most cases show decreased survival upon starvation. The allelic overlap between the *aut* and *apg* mutants is not known, however, both sets of mutants show substantial overlap with the *cvt* mutants defective in API import (*see below*) (Harding et al., 1996; Scott et al., 1996). The differences in the screening procedures indicate that some *aut* mutants will be unique from the *apg* group; not all *aut* mutants are sensitive to starvation.

Several of the *APG* genes have been cloned, and the deduced amino acid sequences reveal essentially no homology to known proteins (Y. Ohsumi, *personal communication*). The *APG1* gene encodes a serine/threonine protein kinase; the kinase activity is needed for phosphorylation of Apg1p and for autophagy (Matsuura et al., 1997). Recently, the sequence of *APG5* has been reported (Kametaka et al., 1996). Although Apg5p function is critical under nutrient starvation conditions, there does not appear to be a change in expression level during growth in rich or nutrient–poor conditions. The sequence of the *AUT1* gene was also recently determined (Schlumpberger et al., 1997). Aut1p is a hydrophilic protein and, as with the *APG* gene products, has no homologies in the protein database.

Degradation of Peroxisomes Occurs through Micro- and Macropexophagic Processes and is Distinct from Starvation-stimulated Bulk Autophagy

The degradation of peroxisomes and peroxisomal enzymes when cells grown on methanol or oleic acid are shifted to glucose or ethanol, has been demonstrated in the methylotrophic yeasts *Hansenula polymorpha*, *Pichia pastoris* and *Candida boidinii* as well as in *Saccharomyces cerevisiae* (Bormann & Sahm, 1978; Veenhuis et al., 1983; Hill, Hann & Lloyd, 1985; Tuttle & Dunn, 1995; Chiang, Schekman & Hamamoto, 1996). Morphological analyses and more recent genetic studies have begun to elucidate the molecular basis of the degradation process. The current data support two different mechanisms for autophagic degradation. There are slight differences in the proposed mechanisms which may in part reflect differing interpretations of the electron microscopy data as well as actual differences among the various yeast studied. A simplified version that is based on a compilation of the available data is presented below.

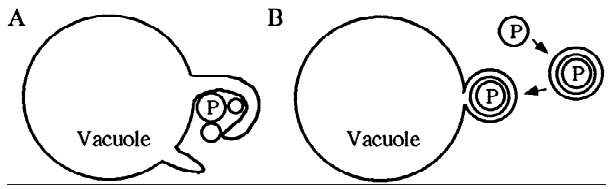

Fig. 2. Both (*A*) micro- and (*B*) macropexophagic processes are used for peroxisomal (P) degradation in some yeast strains. In micropexophagy, the vacuole engulfs clusters of peroxisomes. Individual peroxisomes are surrounded by a pexophagosome membrane during macropexophagy, followed by fusion with the vacuole.

Morphological Studies

When the methylotrophic yeasts *Hansenula polymorpha*, *Candida boidinii* or *Pichia pastoris* are shifted from methanol to glucose or ethanol medium, peroxisomes are rapidly degraded based on the loss of certain peroxisomal enzymes including alcohol oxidase (Bormann & Sahm, 1978; Veenhuis, Zwart & Harder, 1978; Veenhuis, Zwart & Harder, 1981; Veenhuis et al., 1983; Hill et al., 1985; Tuttle & Dunn, 1995). The specifics of the loss of enzyme activity as well as direct analyses by electron microscopy reveal that both micropexophagy and macropexophagy are involved (Fig. 2). Both of these processes are specific; the degradation is essentially limited to peroxisomes and does not include all of the peroxisomes within a particular cell. In macropexophagy, peroxisomes are surrounded by 2 or more membrane layers (Veenhuis et al., 1983; Tuttle & Dunn, 1995). In *Hansenula polymorpha* this pathway is induced by glucose or ethanol, while in *Pichia pastoris*, it is induced by ethanol but not glucose (Tuttle, Lewin & Dunn, 1993; Tuttle & Dunn, 1995). This macropexophagic process sequesters individual peroxisomes and excludes other organelles. In *Hansenula polymorpha*, two variations of the macropexophagic pathway have been proposed (Veenhuis et al., 1983). In either

case, a wrapping membrane forms around the peroxisome and subsequently engulfs or fuses with a vacuole to gain access to the vacuolar hydrolases.

Interestingly, in macropexophagy in *Hansenula polymorpha* it is proposed that the surrounding membrane is formed de novo at the site of the target as opposed to being the result of engulfment by nonspecific autophagosomes that form elsewhere within the cytoplasm (Veenhuis et al., 1983). Hence, the site of membrane formation could establish one difference between starvation-stimulated and nutrient-stimulated autophagy in this organism. In *Pichia pastoris,* however, ethanol-induced macropexophagy is not blocked by cycloheximide suggesting that preexisting membranes may be involved (Tuttle & Dunn, 1995).

A difference in sensitivity to cycloheximide suggests that the peroxisome-sequestering membranes are distinct from the autophagosomes that form during starvation-stimulated nonspecific autophagy. In addition, cytosolic formate dehydrogenase which is involved in methanol adaptation is not degraded during ethanol-induced macropexophagy in *Pichia pastoris* (Tuttle & Dunn, 1995); this contrasts with autophagosomal uptake which would presumably be unable to distinguish among cytoplasmic constituents. Whether the peroxisome-sequestering membranes form de novo around the organelle or arise from preexisting membranes, the means by which a specific peroxisome becomes tagged for destruction, the process of membrane recruitment, the origin of the membrane, and the mechanism by which it is targeted to the vacuole following completion are all unknown. There is some evidence that the signal for degradation involves peroxisomal membrane proteins (van der Klei, Harder & Veenhuis, 1991). Because the enwrapping membrane used in peroxisomal degradation is likely different from that used in starvation-stimulated autophagy, a name other than autophagosome, such as pexophagosome, may be more appropriate.

The second pathway for peroxisomal degradation is micropexophagy (Fig. 2). This pathway is seen in *Pichia pastoris* upon glucose adaptation and does not involve the formation of pexophagosomes (Tuttle & Dunn, 1995). Instead, extensions of the vacuole membrane surround clusters of peroxisomes rather than individual organelles. Other organelles are not sequestered into the vacuole along with the peroxisomes. This is equivalent to a microautophagic process but involves protrusion rather than invagination of the vacuolar membrane.

Enzyme Activity Studies

Examining the kinetics of vacuolar degradation of alcohol oxidase and formate dehydrogenase also reveals differences between the micro- and macropexophagy degradation pathways. In *Pichia pastoris,* ethanol adaptation results in the immediate loss of alcohol oxidase activity with a half-time of approximately 1 to 1.5 hr and this decrease in activity is insensitive to cycloheximide (Tuttle & Dunn, 1995). This is similar to the onset and rate of degradation seen for the glucose- and ethanol-induced pathways in *Hansenula polymorpha* (Veenhuis et al., 1981; Tuttle et al., 1993; Titorenko et al., 1995). In contrast, in the glucose-induced pathway in *Pichia pastoris,* there is a lag in the degradation of this protein, the kinetics of degradation are slower than for ethanol adaptation and the process is sensitive to cycloheximide. Although other organelles are apparently excluded, the cytosolic enzyme formate dehydrogenase is apparently included in the glucose-induced uptake process. It is not known if the process involved in the degradation of cytosolic proteins is similar to that used for the specific degradation of fructose-1,6-bisphosphatase (*see below*).

Genetic Studies

Using retention of alcohol oxidase activity following a shift of methanol-grown cells to glucose as a screen, mutants were identified that are defective in peroxisomal degradation. Analysis of peroxisome degradation-deficient (*pdd*) mutants (Titorenko et al., 1995) further separates macropexophagic peroxisomal degradation into discrete steps; the *pdd1* mutant is defective in the initial sequestration event while the *pdd2* mutant sequesters peroxisomes but is blocked in subsequent fusion with the vacuole (Titorenko et al., 1995). Both the ethanol- and glucose-induced pathways of degradation are blocked in the *pdd* mutants, suggesting that both nutrients may signal the same pathway in this organism.

In *Pichia pastoris,* the two pathways, macropexophagy induced by ethanol or micropexophagy induced by glucose, are more clearly delineated by the isolation of mutants which block only the glucose-induced selective autophagy (*gsa*) pathway (Tuttle & Dunn, 1995). Both the *gsa1* (W. Dunn, *personal communication*) and *gsa2* (Tuttle & Dunn, 1995) mutants are defective in vacuolar localization of peroxisomes upon glucose adaptation but are normal for the ethanol-induced pathway. Recent cloning of the gene complementing the *gsa1* mutation indicates that it encodes the regulatory subunit of phosphofructokinase (W. Dunn, *personal communication*). The observation that the *gsa1* mutant phenotype can be complemented by a catalytically inactive *PFK1* gene, however, suggests that phosphofructokinase activity per se is not involved in the micropexophagic pathway (W. Dunn, *personal communication*).

Fructose-1,6-Bisphosphatase is Specifically Targeted for Turnover by the Vacuolar Import and Degradation Pathway

Due to its role as a key regulatory enzyme in gluconeogenesis, FBPase has been the subject of much investigation. FBPase is subject to catabolite inactivation or nutrient regulation; it is degraded upon the readdition of glucose to glucose-depleted cells. There are conflicting interpretations regarding the site of degradation as being vacuolar (Funaguma, Toyoda & Sy, 1985) or nonvacuolar (Schäfer, Kalisz & Holzer, 1987). The varying conclusions from these studies may be due to different growth conditions and/or strain backgrounds (Schäfer et al., 1987; Teichert et al., 1989). In addition, FBPase may be degraded by more than one mechanism. This would be similar to the degradation of unassembled fatty acid synthase subunit β which is degraded by both the proteasome and vacuolar proteases depending on the growth conditions (Egner et al., 1993).

Cytological Studies

A detailed analysis of FBPase distribution by Chiang and Schekman (1991) provides further support for vacuolar degradation and demonstrates a vacuolar localization of the protein in a *pep4* (encoding vacuolar proteinase A) mutant by both subcellular fractionation and indirect immunofluorescence. Similarly, Egner et al. (1993) were able to identify FBPase in vacuoles from a protease-deficient strain although the experiment was carried out under starvation conditions. In this case, degradation of the protein may be due to nonspecific autophagy rather than the more rapid mechanism induced by glucose adaptation.

More recently, however, Wolf and colleagues (Schork et al., 1994) provide data that are in direct contrast to those of Chiang and Schekman. In their analysis, the *pra1* (*pep4*) mutant degrades FBPase upon glucose addition. In contrast, a *pre1 pre2* mutant defective in cytosolic proteasome activity shows a substantial increase in stability. Furthermore, they find no evidence for vacuolar localization based on immunofluorescence although these are negative results. An additional complication is that FBPase degradation occurs albeit more slowly in a *pep4* mutant (Chiang & Schekman, 1994), perhaps due to residual protease activity. The time course used by Schork et al. (1994) for immunofluorescence would not be optimal for detection of FBPase in the vacuole. More recently, it was demonstrated that degradation of FBPase is dependent on ubiquitination (Schork, Thumm & Wolf, 1995).

Some of the differences in the various sets of data, most notably the stability or lack thereof of FBPase in *pep4* mutants, are not possible to resolve at present. One explanation for some of the discrepancies is that the proteasome mutants have a pleiotropic effect and are not directly involved in FBPase degradation (Chiang & Schekman, 1994; Chiang et al., 1996). An answer to this question awaits an analysis with conditional mutants. FBPase is rapidly phosphorylated upon glucose addition and may also be ubiquitinated. One or both of these signals may allow the protein to be targeted for subsequent degradation; ubiquitinated proteins accumulate in the vacuole in proteinase-deficient yeast and may in part be associated with membranes (Simeon et al., 1992) of cytoplasmic origin. Analysis of FBPase uptake in vitro, however, indicates that the protein can be imported into the vacuole without ubiquitination (H.-L. Chiang, *personal communication*) again suggesting the possibility of more than one degradation mechanism.

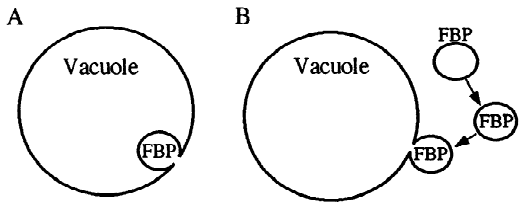

Fig. 3. Fructose-1,6-bisphosphatase (FBP) is degraded by multiple mechanisms. Both (*A*) nutrient-stimulated microautophagy and (*B*) the vacuolar import and degradation pathway may be utilized. In the latter process, FBPase may bind the surface of a cytosolic vesicle and subsequently translocate into the vesicle lumen prior to vacuolar fusion.

Substantial data indicate that at least some FBPase degradation occurs through a nutrient-stimulated autophagy mechanism (Fig. 3). Under derepressed conditions such as growth on acetate or ethanol, FBPase has a 90-hr half-life (Funayama, Gancedo & Gancedo, 1980). This decreases to 30 min upon addition of glucose. Immunofluorescent and immunoelectron microscopy indicate that FBPase enters the vacuole in a microautophagic process; the protein is seen at sites of vacuolar membrane invagination (Chiang et al., 1996). Uptake of FBPase is specific in that other cytosolic proteins are not taken into the vacuole under conditions that lead to FBPase degradation.

Genetic Studies

Analysis of mutants (vacuolar import and degradation, *vid*) that are defective in the degradation of FBPase upon readdition of glucose, indicates that import may also occur through a vesicle-mediated process. In some mutants, FBPase is detected in punctate structures in the cytosol that are pelletable at high speed (Hoffman & Chiang, 1996). Purification of the FBPase-associated compartments from wild-type cells demonstrates that they are 30–40 nm vesicles (Huang & Chiang, 1997). The FBPase associated with these vesicles is protected

from proteinase K suggesting that FBPase is contained within the vesicle lumen.

Because the FBPase-containing vesicles are substantially smaller than autophagosomes, Huang and Chiang (1997) speculate that FBPase translocates into the vesicles rather than being engulfed by them. If so, this may represent an interesting combination of the mammalian pentapeptide (KFERQ)-directed translocation and vesicular autophagic pathways. Some issues that await clarification are the following: Is FBPase degraded through both nutrient-stimulated microautophagy and the vesicle-mediated Vid pathway? How is FBPase selectively targeted for degradation? Is a cytosolic chaperone involved in FBPase degradation?

Two Resident Hydrolases are Localized to the Vacuole Independent of the Secretory Pathway through Cytoplasm to Vacuole Targeting

Most vacuolar enzymes are delivered to the organelle by transiting through a portion of the secretory pathway. These proteins enter the endoplasmic reticulum, transit to the Golgi complex and are then diverted to an endosomal compartment before being transported to the vacuole. The deduced amino acid sequences for the *AMS1* (encoding AMS; Kuranda & Robbins, 1987; Yoshihisa & Anraku, 1989) and *APE1* (encoding API; Chang & Smith, 1989; Cueva, Garcia-Alvarez & Suarez-Rendueles, 1989) genes reveal that both proteins lack either the cleavable N-terminal signal sequence or uncleaved internal hydrophobic domain that are typically used for translocation into the endoplasmic reticulum. Even though both proteins have potential sites for N-glycosylation, neither is glycosylated (Yoshihisa & Anraku, 1989; Klionsky, Cueva & Yaver, 1991). In addition, the vacuolar localization of both proteins is independent of several *SEC* gene products that are known to be required for initial steps of the secretory pathway (Yoshihisa & Anraku, 1990; Klionsky et al., 1992). Finally, overproduction of either protein does not lead to cell surface expression in contrast to vacuolar proteins that travel through the secretory pathway. These and other observations led to the proposal that AMS and API enter the vacuole by a secretory pathway-independent mechanism. Competition for import between API and AMS suggests that the two proteins may use the same machinery for vacuolar import (Klionsky et al., 1992).

Biosynthesis of α-Mannosidase

AMS is initially synthesized as a 107 kDa protein that is processed into 73 and 31 kDa species upon vacuolar delivery (Yoshihisa, Ohsumi & Anraku, 1988; Yoshihisa & Anraku, 1990). The processing is dependent on proteinase A and has a half-time of 2 to 10 hr. Fusion between AMS and the periplasmic protein invertase allow the mapping of a vacuolar targeting determinant to the C-terminal portion of the protein (Yoshihisa & Anraku, 1990). Overproduction of AMS results in substantial mislocalization suggesting a saturable component is involved in the targeting process. Vacuolar uptake of AMS is not substantially enhanced relative to other cytoplasmic proteins during starvation, suggesting that its localization within the organelle is not due primarily to a nonspecific starvation-stimulated autophagic pathway (Egner et al., 1993). The long half-time of processing and the low expression level of AMS make extensive analysis of this protein problematic.

Biosynthesis of Aminopeptidase I

API is synthesized as a 61 kDa precursor containing an N-terminal propeptide that is processed in a PrB-dependent reaction to generate the 50 kDa mature hydrolase (Klionsky et al., 1992). Secondary structure analysis predicts that the API propeptide forms two α-helices separated by a proline residue. The first potential helix is predicted to form an amphipathic structure (Chang & Smith, 1989). Mutational analysis of the API propeptide reveals that the first helix contains vacuolar targeting information (Oda et al., 1996). Mutations within this part of the protein prevent the precursor from binding the membrane and block subsequent vacuolar import (Segui-Real, Martinez & Sandoval, 1995; Oda et al., 1996). Import of API has been reconstituted in vitro and shown to require the hydrolysis of both ATP and GTP (Scott & Klionsky, 1995). Maturation is also dependent on correct vacuolar acidification, although this may reflect a block in AB breakdown rather than inhibition of import (*see above*). The temperature profile of the in vitro and in vivo (S.V. Scott and D.J. Klionsky, *unpublished data*) import reaction suggests that localization does not occur through a proteinaceous channel; vacuolar import of API is blocked at temperatures that do not interfere with translocation into the endoplasmic reticulum or mitochondria.

Genetic Studies

A genetic screen was used to identify components of the subcellular sorting machinery used in API targeting (Harding et al., 1995; Harding et al., 1996). An approach that relied on the accumulation of precursor API identified a series of mutants defective in cytoplasm to vacuole targeting (*cvt*). The unique *cvt* mutants are specific for this alternate targeting pathway in that they do not affect proteins such as proteinase A, PrB and carboxypeptidase Y that transit through the secretory pathway. Complementation studies suggest that the *cvt* mutants are allelic with many of the autophagy-defective *aut* (Thumm et al.,

1994) and *apg* (Tsukada & Ohsumi, 1993) mutants (Harding et al., 1996; Scott et al., 1996). In agreement with these analyses, it was shown that most of the autophagy mutants accumulate precursor API and most *cvt* mutants are defective in nonspecific autophagy (Harding et al., 1996; Scott et al., 1996), indicating at least a partial overlap between starvation-stimulated autophagy and the Cvt pathway.

The *cvt* mutants do not appear to be defective in degradation of FBPase and the currently characterized *vid* mutants are not blocked in API import (T. Harding, H.-L. Chiang and D.J. Klionsky, *unpublished results*), supporting a separation of the Cvt/starvation-stimulated autophagy and nutrient-stimulated autophagy/Vid pathways. Similarly, the size of the vesicles used for uptake of FBPase are substantially different from those used in API import (Huang & Chiang, 1997; Y. Ohsumi, M. Baba, *personal communication;* S.V. Scott and D.J. Klionsky, *unpublished results*). All but one of the unique *cvt* mutants accumulate protease-sensitive precursor API in the cytoplasm. It should be possible to isolate additional mutants that are defective in later stages of the delivery process including vesicle fusion and breakdown events.

Oligomerization of API

Recent studies examining the native state of API during import indicate that the precursor protein oligomerizes in the cytosol prior to binding a target membrane (Kim et al., 1997). Using an altered API protein bearing a temperature sensitive mutation in the targeting signal (Oda et al., 1996), it was possible to show that the membrane-bound precursor is subsequently imported in its oligomeric form (Kim et al., 1997). The API oligomer is a dodecamer of 732 kDa; the large size does not fit with translocation through a protein channel but would be accommodated by a vesicle-mediated mechanism. Direct isolation of precursor API from subvacuolar vesicles further indicates that API import involves a vesicular event (S.V. Scott and D.J. Klionsky, *unpublished results*).

Differences between the Cvt Pathway and Autophagy

It is surprising that the Cvt and starvation-stimulated autophagy pathways overlap because autophagy is degradative while API import is biosynthetic. There are differences, however, between these pathways. API import is rapid, occurring with a half-time of 30–45 minutes (Klionsky et al., 1992) while starvation-stimulated autophagy is slow, having a half-time of at least 12 hr (Scott et al., 1996). Starvation-stimulated autophagy occurs at a low basal level and is induced by starvation. API uptake is constitutive occurring under both starvation and nutrient-rich conditions but is upregulated by starvation (Scott et al., 1996). Finally, the Cvt pathway is specific and saturable while starvation-stimulated autophagy is nonspecific and not saturable. A direct comparison of vacuolar uptake of API and the nonspecific pathway substrate Pho8Δ60p indicates that vegetatively growing cells can differentiate between these two proteins (Scott et al., 1996).

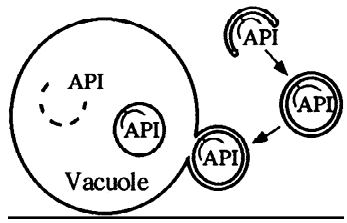

Fig. 4. Aminopeptidase I (API) is imported through the cytoplasm to vacuole targeting pathway. Precursor API oligomerizes in the cytosol and binds a membrane which may be similar to an autophagosome. The binding is made specific by a putative receptor protein. Following fusion, the subvacuolar vesicle is degraded in a PrB-dependent manner to allow release and processing of API in the lumen.

If API import utilizes components of the starvation-stimulated autophagic pathway, it presumably uses additional proteins that make the process rapid and specific (Fig. 4). While all of the characterized *apg* mutants accumulate precursor API (Scott et al., 1996), at least two *aut* mutants maintain normal levels of the mature hydrolase (Harding et al., 1996), indicating some separation between the two pathways. As with AMS (Yoshihisa & Anraku, 1990), overproduction of API results in precursor accumulation suggesting a saturable receptor (Klionsky et al., 1992). The identity of such a receptor and the nature of its interaction with API have not yet been determined. Finally, it is not known whether API is a passive cargo molecule or whether API membrane binding is detected and results in completion of an autophagosome or some other vesicular structure. In either case, the manner in which this transit compartment is targeted to and fuses with the vacuole remains to be examined. Because API is a resident vacuolar hydrolase that undergoes a convenient cleavage event upon vacuolar delivery, it will serve as a useful marker to study the cytoplasm to vacuole targeting and starvation-stimulated autophagic pathways.

Conclusions

As we learn more about eukaryotic cellular transport mechanisms, it becomes evident that numerous processes have evolved to ensure that proteins are targeted to the correct subcellular destination. Many of these processes are nonclassical, more recently discovered mechanisms that may be used by relatively fewer passenger proteins

or for which only a few of the specific cargo proteins have been identified (Scott & Klionsky, 1997). They are nonetheless important processes in overall cellular physiology. Of all the organelles, the vacuole appears to be the most adaptive with regard to delivery mechanisms; seven or more distinct processes (cytoplasm to vacuole targeting, vacuolar import and degradation, secretory pathway, endocytosis, starvation-stimulated autophagy and nutrient-stimulated macro- and micropexophagy) are used to localize cellular constituents to this organelle. This diversity in delivery mechanisms may reflect the overall importance of the vacuole in cellular physiology. In addition, the yeast vacuole has functions in common with multiple organelles of other eukaryotic cells including the mammalian lysosome, plant vacuole and possibly the contractile vacuole of organisms such as slime mold. The multiple targeting mechanisms that are used for vacuolar delivery in yeast may be represented by specific sorting pathways to these different organelles in other eukaryotes.

In recent years, great progress has been made in understanding the mechanisms by which proteins and organelles are delivered to the vacuole from the cytoplasm. Even a brief consideration of this problem, however, reveals many question that have yet to be answered: (i) What is the origin of the membrane that forms the various structures used to engulf organelles or cytosolic proteins? (ii) A related issue concerns the mechanism by which large amounts of phospholipids are mobilized for membrane formation. Autophagosomes are large structures and in the case of nutrient-stimulated autophagy, uptake is rapid. (iii) How are autophagosomes/pexophagosomes physically formed? How are double membrane structures generated and how are the two faces of these structures maintained separately during formation? In both micro- and macrophagic processes, is membrane deformation dependent on coat proteins or cytoskeletal elements? (iv) How are the signals for autophagy transduced? In nutrient-stimulated autophagy or pexophagy, how are substrates tagged as sites for membrane formation? How is membrane binding or engulfment of a substrate detected? (v) Following formation of an autophagosome, how does it target to and fuse with the vacuole? Are v- and t-SNAREs and rab proteins involved? (vi) Following fusion, does the autophagosome outer membrane become part of the vacuole membrane or is it removed in toto? If it is removed, how is mixing of the phospholipids prevented to maintain the correct composition of the vacuole membrane? How is the autophagosome-derived portion of the vacuole membrane protected from premature degradation which could cause the release of vacuolar hydrolases? (vii) In microautophagy or micropexophagy, it is also necessary to prevent premature breakdown of the forming autophagic/pexophagic body which could otherwise result in escape of vacuolar enzymes. How are these structures protected during formation, how is completion sensed, and how do they become sensitive to degradation? (viii) What enzymes are involved in breakdown of the AB and recycling of the membrane constituents?

The application of biochemical and genetic approaches to the topic of protein transport from the cytoplasm into the vacuole will begin to address these and other questions. The studies described in this review and those to come should make it obvious that the vacuole is one of the most dynamic and fascinating organelles in the eukaryotic cell.

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