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Vacuole Biogenesis in *Saccharomyces cerevisiae*: Protein Transport Pathways to the Yeast Vacuole

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INTRODUCTION

Subcellular compartmentalization is an important feature of eukaryotic cells, preventing inappropriate meetings of certain intracellular components as well as facilitating efficient ordered reactions. To maintain this compartmentalization, cells have evolved mechanisms to ensure that specific proteins are delivered to specific organelles.

The vacuole of *Saccharomyces cerevisiae* is central to much of the physiology of this organism. Among the many roles of this organelle are pH and osmoregulation, protein degradation, and storage of amino acids, small ions, and polyphosphates. To carry out all of its functions, it is essential that the vacuole contain its full complement of proteins. The functions of the vacuole in sporulation, protein turnover, osmoregulation, pH homeostasis, and ion transport have been well documented and reviewed recently (81). The focus of this review is the biogenesis of the vacuole in *S. cerevisiae* and the processes involved in the formation and transmission of this organelle. In particular, the transport pathways responsible for the delivery of proteins to the vacuole will be discussed.

Over the past two decades, genetic screens have identified many genes as being involved in vacuolar biogenesis in yeast. These have included screens to identify genes involved in the delivery of various marker proteins to the vacuole. In the yeast *S. cerevisiae* proteins get to the vacuole by a number of different pathways, including (i) sorting of vacuolar proteins away from those to be delivered to the cell surface following transit through the early stages of the secretory pathway; (ii) endocytosis of material from the plasma membrane; (iii) cytoplasm-to-vacuole targeting pathways, which do not pass through the early stages of the secretory pathway; and (iv) inheritance of vacuolar material by daughter cells during cell division.

These routes are obviously different from one another, but, interestingly, genetic screens designed to identify components involved in each of them have shown that although there are genes unique to each pathway, there is a significant overlap of the genetic requirements for the various processes. This demonstrates that although these processes each define indepen-

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dent pathways for protein delivery to the vacuole, they are all intimately related.

One of the features of vacuolar protein sorting in *S. cerevisiae* that makes it an attractive model system for studying protein trafficking is the array of convenient marker proteins whose delivery to the vacuole can be monitored easily. Vacuolar proteases tend to be synthesized as inactive zymogens whose activation requires proteolytic cleavage to remove a propeptide (84). Processing occurs in the vacuole and requires an intact copy of the structural gene for proteinase A (PrA), *PEP4*, to initiate a vacuolar protease activation cascade (103, 108). The *PEP4*-dependent cleavage of many vacuolar proteins results in the formation of a lower-molecular-weight form of the protein, which can be distinguished from its precursor form through an increased mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Other proteins, such as endocytosed proteins, are completely degraded upon delivery to the vacuole. Such detectable proteolytic events provide convenient means to monitor the kinetics of vacuolar delivery. This, combined with the more classical tools of cell biology such as immunofluorescence microscopy and the ease with which *S. cerevisiae* can be manipulated genetically, makes the study of protein trafficking to the yeast vacuole a very attractive system.

SORTING OF PROTEINS FROM THE LATE GOLGI TO THE VACUOLE

Many vacuolar proteins such as the soluble vacuolar hydrolases carboxypeptidase Y (CPY), PrA, and proteinase B (PrB), as well as membrane proteins such as the 100-kDa subunit of the vacuolar ATPase (Vph1p), alkaline phosphatase (ALP or Pho8p), and dipeptidyl aminopeptidase B (Dap2p), travel through the early stages of the secretory pathway (66, 84, 123). Once these proteins reach the last compartment of the Golgi apparatus, they are sorted away from proteins destined for delivery to the cell surface (54). Soluble hydrolases require a sorting signal to mediate their vacuolar delivery from the Golgi apparatus (see below), and in the absence of such information, these proteins are delivered to the cell surface. In contrast to this, integral membrane proteins lacking information to localize them to a specific intracellular organelle are not delivered to the cell surface. Instead, these proteins are transported to the vacuole (130, 187).

Receptor-Mediated Sorting of Soluble Vacuolar Hydrolases

The posttranslational modifications received by CPY as it transits from the endoplasmic reticulum (ER) to the vacuole have been well characterized and have allowed its use as a marker protein to monitor protein trafficking to the vacuole (78, 159). CPY is synthesized as an inactive precursor, prepro-CPY, which is translocated into the lumen of the ER, where its signal sequence is removed by signal peptidase and it undergoes N-linked glycosylation to become the 67-kDa, ER (or p1) form of the protein. By means of vesicular trafficking, in a *SEC*-dependent manner, CPY is transported to and subsequently through the Golgi apparatus, where it receives further oligosaccharide modifications to produce the 69-kDa Golgi-modified (or p2) form of the zymogen. The last definable subcompartment of the Golgi apparatus (54) is that which contains the three processing proteinases involved in the maturation of the mating pheromone α-factor (Kex2p, Kex1p, and Ste13p) (15) before it is secreted from the cell. It is in this compartment that p2 CPY is diverted away from the secretory pathway through a receptor-mediated process that leads to its delivery to the vacuole, where it is cleaved by vacuolar proteases into its active, mature, 61-kDa form (mCPY).

The sequence QRPL (residues 24 to 27) in the propeptide region of pro-CPY forms the core of the targeting signal required to divert p2 CPY away from the secretory pathway, into the vacuolar biogenesis pathway. Alteration of this signal results in the entry of the protein into Golgi-derived secretory vesicles and its subsequent secretion from the cell (78, 169, 170). Overproduction of CPY also results in its secretion from the cell, an observation that first suggested the involvement of a saturable receptor in CPY sorting (160). The receptor has been identified as being encoded by the *VPS10* gene (98). Cells lacking Vps10p missort more than 90% of their CPY, and Vps10p can be cross-linked to pro-CPY but not to a sorting-defective form of pro-CPY. The current model of the pathway taken by CPY from the late Golgi to the vacuole, depicted in Fig. 1, is based largely on the delivery of proteins to the lysosome of mammalian cells by the mannose-6-phosphate receptor (90) and is as follows (19, 29): (i) p2 CPY binds to Vps10p in the late Golgi; (ii) the receptor-ligand complex travels to an endosomal or prevacuolar compartment (PVC) in Golgi-derived transport vesicles; (iii) and CPY dissociates from its receptor in the PVC and is transported to the vacuole, while Vps10p is recycled back to the Golgi to bind more CPY. This model accounts for the observation that Vps10p is synthesized at a 20-fold-lower rate than is CPY yet it binds its ligand with a 1:1 stoichiometry in vitro (29). In addition, Vps10p carries a tyrosine-based localization signal that is essential both for its steady-state localization to the *trans*-Golgi and its function as the CPY receptor (19, 29). Aromatic-residue-containing motifs are essential for the Golgi localization of membrane proteins, which continually cycle between the Golgi and the PVC (reviewed in reference 113), and it is evident that Vps10p also follows this itinerary (see below). As well as secreting CPY, *vps10*Δ cells secrete 50% of their newly synthesized PrA (29), suggesting that despite the lack of similarity between CPY and PrA, Vps10p can recognize both proteins as being destined for vacuolar delivery (29, 185). In addition, Vps10p seems to be responsible for targeting misfolded proteins that exit the ER to the vacuole (70). Therefore, while it is clear that the QRPL sequence is responsible for mediating interactions between pro-CPY and Vps10p, the receptor does seem to recognize a broad range of signals, whose exact nature remains unclear. While it has been found that the 54-residue propeptide of PrA can direct the normally secreted protein invertase to the vacuole in a *VPS10*-dependent manner, it has also been found that the propeptide is not essential for vacuolar targeting of PrA, suggesting that the protease can also reach the vacuole through a Vps10p-independent route (185). In addition, homologs of Vps10p have been identified within the *S. cerevisiae* genome, and there is evidence to suggest that at least one of these (*VTH2*) can act as a functional receptor for both CPY and PrA. These data suggest that *S. cerevisiae* contains a family of receptors that participate to various degrees in the sorting of soluble hydrolases to the vacuole (29, 185).

VPS10 is identical to *PEP1*, and *PEP1* is one of the group of genes identified in several genetic screens that were performed to identify functions required for vacuolar biogenesis. The *pep* mutants were isolated as being defective in CPY enzymatic activity (80). The *vps* mutants were isolated as a collection of mutants that fail to properly sort CPY or a CPY-invertase fusion protein to the vacuole and secrete the soluble hydrolase as a result of some block in the vacuolar biogenesis pathway (5, 137). Complementation analysis revealed extensive overlap between *pep* mutants and the *vps* mutants (136). Whereas the vast majority of *PEP* genes are required for the transport of pro-

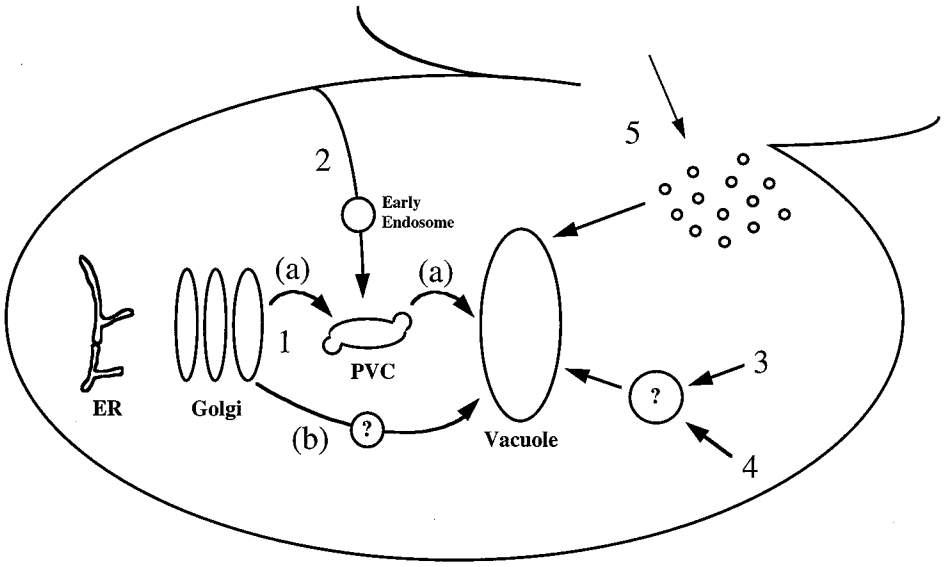

FIG. 1. Protein transport pathways to the yeast vacuole. Depicted are six pathways used by proteins to reach the vacuole in *S. cerevisiae*: 1, sorting of vacuolar proteins in the late Golgi (a) through a PVC and (b) via an alternative route; 2, endocytosis of proteins from the cell surface; 3, biosynthetic cytoplasm to vacuolar targeting; 4, autophagy (degradative cytoplasm to vacuolar targeting); 5, vacuolar inheritance from mother to daughter cells, using homotypic fusion to fuse vacuolar vesicles.

teins to the vacuole (reflected by the observation that most *pep* mutants secrete CPY), the *PEP4* gene encodes PrA, a protease required for the activation of numerous vacuolar hydrolases (1, 188).

Trafficking Steps between the *trans*-Golgi Network and the Vacuole

The model outlined in Fig. 1 can be broken down into a number of different steps, and a variety of assays have been used to gain insight into the steps at which the various *VPS*/*PEP* genes function. This endeavor has been greatly enhanced by the cloning and molecular characterization of many *VPS*/*PEP* genes. This approach has revealed that many of the gene products involved in vacuolar protein sorting are homologous to those involved in protein trafficking in many other systems and has also allowed the isolation of conditional alleles. The use of conditional alleles that produce rapidly inactivatable proteins has proved particularly useful in determining the primary function(s) of these gene products.

An important stage in the transport of proteins to the vacuole is their segregation from secreted proteins and packaging into vesicles distinct from those bound for the cell surface. It is clear that this occurs at the level of the late Golgi, the yeast equivalent of the mammalian *trans*-Golgi network (TGN) (54), and that vacuolar proteins are not first delivered to the cell surface and then transported to the vacuole by endocytosis. Evidence that vacuolar delivery of CPY and the vacuolar membrane protein ALP occurs through an intracellular route comes from studies with mutants known to block the fusion of Golgi-derived secretory vesicles with the plasma membrane and mutants defective in early stages of endocytosis (112, 159). Both CPY and ALP reach the vacuole in these late-acting *sec* and *end* mutants, indicating that they do not require transit through the plasma membrane to reach their final destination. Recent studies have revealed that proteins can be sorted into one of at least five routes from the TGN: two to the cell surface (62), one that goes back to earlier Golgi subcompartments (61), and two that carry proteins to the vacuole (32, 117). This complexity demands a high level of regulation at the molecular level, and the factors involved in the formation of vacuolar-bound vesicles from the late Golgi are beginning to be uncovered.

Vesicle Formation at the *trans*-Golgi Network

Three *VPS* genes have been implicated in the budding of vesicles carrying vacuolar cargo from the Golgi apparatus. The observation that these genes are required for the formation of Golgi-derived vesicles that contain vacuolar cargo but not for the formation of those destined for the cell surface implies that they might somehow be involved in creating the specificity of these vesicles. *VPS1* encodes a homolog of the mammalian protein dynamin (reviewed in reference 26), which is required for endocytosis and appears to be involved in the pinching off of vesicles from the plasma membrane (reviewed in reference 177). Yeast cells lacking Vps1p are not defective in endocytosis but instead secrete the Golgi-modified form of CPY and transport vacuolar membrane proteins to the plasma membrane (112). Such observations and the homology of Vps1p to dynamin suggest a role for it in the formation of vesicles that divert vacuolar proteins away from the secretory pathway in wild-type cells. Clathrin has also been implicated in the formation of these vesicles, and, indeed, a sudden loss of clathrin function also results in the mislocalization of vacuolar proteins to the cell surface (146). This shared phenotype is consistent with models in which Vps1p and clathrin act together to form vesicles carrying vacuolar cargo, and an absence of their function results in this cargo spilling over into secretory vesicles that transport them to the cell surface (26).

It has been proposed that Vps15p and Vps34p act together as part of a regulatory complex that functions at the late Golgi to drive vesicle formation (reviewed in reference 158). Vps15p exhibits homology to the Ser/Thr family of protein kinases and has been demonstrated to have kinase activity both in vitro and in vivo. The protein is myristylated at its N terminus and is thought to reside on the cytosolic face of Golgi membranes. Under restrictive conditions, *vps15*-ts mutants accumulate p2

CPY in membrane fractions enriched for Golgi markers. Vps15p is necessary for the membrane association of Vps34p. Vps34p is homologous to the 110-kDa catalytic subunit of mammalian phosphoinositide 3-kinase (PI3-kinase) (157). In mammalian cells, this subunit acts as part of a complex to phosphorylate phosphatidylinositol and other phosphorylated phosphoinositides in response to activated tyrosine kinases. Vps34p has also been demonstrated to have kinase activity, and, interestingly, *vps15* mutant strains are defective in Vps34p PI3-kinase activity. These data have led to the model that Vps15p is responsible for recruiting Vps34p onto the membrane of the TGN and activating its lipid kinase activity, which, in turn, leads to the formation of vesicles carrying vacuolar cargo. This model predicts that *vps15* and *vps34* mutants will divert vacuolar traffic (such as Vps10p) to the cell surface as is the case in *vps1* mutants, a prediction that remains to be tested. Vps15p and Vps34p are found in a hetero-oligomeric complex along with a number of as yet unidentified proteins. Identification of these proteins should aid our understanding of the link between kinase activity and membrane-trafficking events.

Vacuolar Delivery via a Prevacuolar/Endosomal Compartment

Subcellular fractionation studies have revealed that CPY transits another membrane-bound compartment on its journey from the late Golgi to the vacuole (172). This helped to form the model that Golgi-derived vesicles carrying pro-CPY fuse with a PVC in a manner analogous to the sorting of lysosomal proteins in mammalian cells (reviewed in references 72 and 158).

A large collection of *vps* mutants were divided into six classes (A to F) on the basis of a number of criteria including vacuolar morphology (6, 123). *vps* mutants exhibit one of a number of vacuolar morphologies, ranging from cells with near-wild-type vacuoles to those with highly fragmented vacuolar membranes. In many cases, it appears that mutants that fall into the same class are blocked in the same stage of vacuolar protein sorting (81, 158). For example, mutants from any of the 13 class E *vps* groups accumulate an exaggerated form of the PVC through which CPY transits en route to the vacuole. In addition to CPY, this class E compartment accumulates endocytosed proteins, such as the mating pheromone receptor Ste3p, as well as vacuolar membrane proteins, such as Vph1p, indicating that it represents a point of overlap between the endocytic pathway and the vacuolar biogenesis pathway (118) (see Fig. 3).

Studies with *vps27*, *vps28*, and *vps4* mutants indicate that these class E *vps* mutants are unaffected in anterograde traffic between the Golgi and the PVC and are competent for endocytosis as far as the PVC (4, 118, 127). An exaggerated form of this PVC can be seen as a multilamellar structure by electron microscopy (4, 127). The accumulation of this compartment seems to result from a block in membrane traffic out of the PVC both on to the vacuole and back to the Golgi apparatus. Such a block causes the entrapment of vacuolar proteins and recycling late-Golgi membrane proteins, as well as endocytosed proteins whose normal cellular itinerary includes transit through the PVC (16, 118).

Studies with the class E *vps* gene *VPS27* provided evidence for the receptor-recycling model of CPY sorting outlined above (29, 118). As described above, *VPS10* encodes a receptor that carries CPY from the late Golgi to the PVC (Fig. 1). By using a reversible, temperature-sensitive allele of *VPS27*, it has been shown that Vps10p, which accumulates in the class E compartment upon loss of *VPS27* function, returns to the Golgi apparatus with a concomitant restoration of ability to properly sort CPY, upon restoration of *VPS27* function, while Vph1p travels on to the vacuole.

Whereas class E Vps proteins appear to control traffic out of the PVC, class D Vps proteins act before vacuolar proteins gain entry into the PVC. *VPS15* and *VPS34* (described above) are examples of class D *VPS* genes that appear to act at the level of vesicle formation from the late Golgi, and thus a loss of function of these genes blocks traffic between the late Golgi and the PVC. Other class D mutations also block traffic between the Golgi and the PVC, but whereas *vps15* and *vps34* mutations appear to prevent the formation of Golgi-derived transport vesicles, these other class D mutations prevent the fusion of such vesicles with their acceptor membrane, the PVC.

SNARE Components of the *VPS*-Dependent Carboxypeptidase Y Pathway

Many lines of investigation suggest that the molecular mechanisms underlying vesicular docking and fusion are conserved across both species and organelles. For example, members of the highly conserved syntaxin-, Rab-, and Sec1p-like protein families are required for most, if not all, vesicle-mediated transport steps identified to date. Second, a single protein, NSF (NEM sensitive fusion protein) (Sec18p in yeast), seems to be required for the docking and/or fusion of transport vesicles derived from different organelles with their appropriate target membranes in many different species. The SNARE (soluble NSF receptors) hypothesis was proposed as a model to explain the specificity of vesicle targeting (44, 135, 156). It states that vesicles are identified by the possession of unique members of the v-SNARE family, which interact specifically with particular t-SNAREs on the appropriate target membrane. The complex of proteins formed when a vesicle docks generates a binding site for soluble NSF attachment proteins, which then recruit NSF to facilitate membrane fusion through an ATP hydrolysis-requiring step. The specificity of vesicular targeting is proposed to be inherent in the complementary molecular interactions between unique SNARE complex components, including Rab- and Sec1p-like proteins, which then recruit general membrane fusion factors that function at multiple points throughout the cell. Some aspects of the SNARE hypothesis, such as the role of SNARE proteins in determining the specificity of targeting, have recently been called into question (see below for examples of this); however, they are an integral part of the docking/fusion machinery.

The molecular mechanisms mediating transport between the *trans*-Golgi and the PVC are predicted to be similar to those involved in other vesicle-mediated transport steps. Among the proteins encoded by the class D genes are a syntaxin homolog (Pep12p/Vps6p), a Rab GTPase (Vps21p), and a Sec1p family member (Vps45p). Consistent with its homology to Sec1p, which is required for the fusion of Golgi-derived secretory vesicles with the plasma membrane, loss of Vps45p function results in the rapid accumulation of transport vesicles (~60 nm in diameter) within the cytoplasm (30, 119). Immunofluorescence studies indicate that vacuolar proteins, such as Vph1p, do not reach the vacuole in *vps45* mutants because they are trapped within these vesicles (117). The model that Vps45p is required for the fusion of Golgi-derived transport vesicles with the PVC is also supported by data from pulse-chase analyses of both soluble and integral membrane vacuolar proteins. These accumulate in their Golgi-modified, precursor forms in *vps45* mutants, indicating that they do not reach a proteolytically active compartment (30, 117, 119). Other class D mutants also accumulate transport vesicles and Golgi-modified pro-CPY,

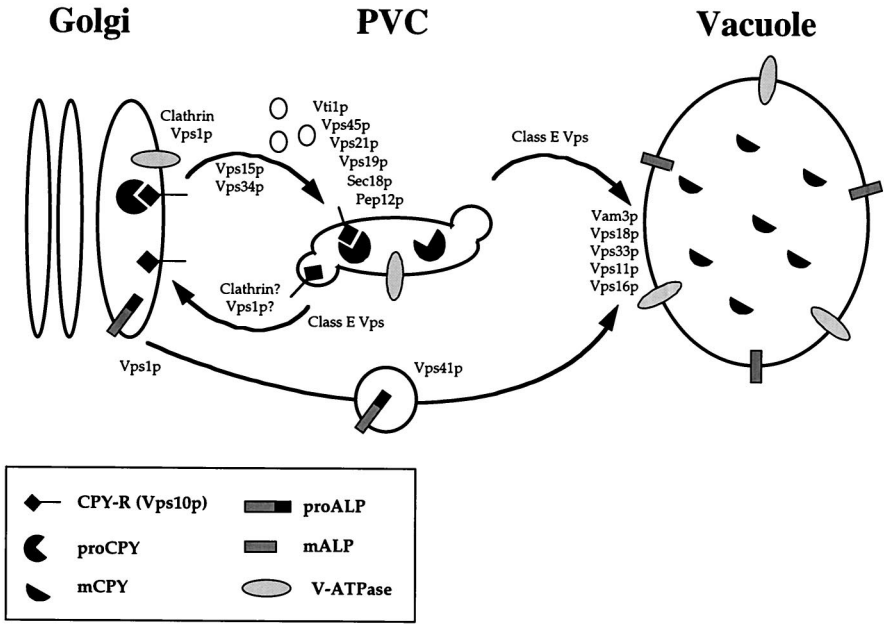

FIG. 2. Sorting of proteins from the late Golgi to the vacuole. Vacuolar proteins are sorted in vesicles bound for the vacuole at the level of the TGN. The soluble hydrolase, CPY, binds to its membrane receptor (Vps10p) in this compartment, and the receptor-ligand complex is delivered to the PVC. In the PVC, CPY dissociates from its receptor and travels on to the vacuole while Vps10p returns to the Golgi to bind more ligand. A second pathway from the TGN to the vacuole does not pass through this compartment and is taken by the vacuolar membrane proteins ALP and Vam3p. Interestingly, the two pathways converge at, or before, the vacuolar membrane.

and genetic and biochemical data suggest the existence of a complex containing the SNARE complex components Vps45p, Pep12p, Vps21p, and Sec18p, as well as Vac1p/Vps19p, which is believed to function in Golgi to PVC transport (18).

Pep12p is the presumed t-SNARE of the PVC and is required for the sorting of CPY. Although it has been shown that this syntaxin homolog fractionates away from Golgi and vacuolar markers (7), its positive assignment to the PVC remains to be assessed. Such studies are hindered by the lack of marker proteins for this compartment. Since the PVC is proposed to be the point at which the endocytic and Vps pathways intersect (118), experiments aimed at colocalizing Pep12p and other proposed markers of endosomes (such as Ypt51p [154; see below]) with endocytosed proteins in transit to the vacuole might resolve this issue.

The member of the v-SNARE family thought to direct Golgi-derived transport vesicles to the PVC is encoded by *VTI1* (45). While many *vti1* mutants secrete CPY, *VTI1* is different from the other *VPS* genes in that it is essential for yeast cell viability. By isolating alleles of *VTI1* that are temperature sensitive for either CPY secretion or viability, the essential function of the gene has been separated from its role in the Vps pathway. In addition to its interaction with Pep12p, Vti1p binds the t-SNARE Sed5p, which functions in traffic between the ER and the *cis*-Golgi. *SED5*, first isolated as a suppressor of the *erd2* defect (60), was found to be a multicopy suppressor of a conditionally lethal *vti1* mutation, an allele that blocks ER-to-Golgi traffic. This involvement of a single v-SNARE in distinct membrane-trafficking steps through interaction with two different t-SNAREs demands a modification of the original SNARE hypothesis. Besides interacting with Vti1p, Sed5p also recognizes the v-SNAREs Sec22p/Sly2p, Bet1p/Sly12p, and Bos1p, which are required for ER-to-*cis*-Golgi traffic, and the v-SNARE Sft1p, which is involved in retrograde traffic from later Golgi compartments back to the *cis*-Golgi. These data suggest that a t-SNARE defines a target organelle and can interact with v-SNAREs of all incoming vesicles rather than being restricted to interaction with one specific v-SNARE.

Transport from the Prevacuolar Compartment to the Vacuole

The mechanism of protein transport from the PVC on to the vacuole has remained something of an enigma. The fragmentation of vacuoles in class B and C *vps* mutants (6, 123) makes it tempting to speculate that the genes represented by these classes are required for the final trafficking step into the vacuole. Indeed, cells lacking the vacuolar t-SNARE Vam3p display a class B *vps* phenotype (34, 174) and are defective for traffic into the vacuole not only from the CPY pathway but also from the alternative pathway taken by ALP and Vam3p itself (see below). In addition, Vam3p is required for homotypic vacuole fusion (see below), as well as the Cvt pathway taken by aminopeptidase I (API) (see below). The four known class C *VPS* gene products (Vps11p, Vps16p, Vps18p, and Vps33p) have recently been localized to the vacuolar membrane and have been shown to interact with each other both physically and genetically (126). In this same study, a conditional allele of *VPS18* was used to demonstrate an interaction between the class C *VPS* genes and *VAM3* (126). Characterization of a *vps18*-ts mutant revealed that, like Vam3p, Vps18p functions in multiple protein transport pathways to the vacuole. Interestingly, rapid loss of Vps18p function leads to the accumulation of small vesicles, autophagosomes, and other membrane compartments. Taken together, these data suggest that the class C Vps proteins act as part of a complex to facilitate the delivery of multiple transport intermediates to the vacuole (Fig. 2).

The presence of SNARE complex homologs encoded by the

class B and C *VPS* genes (e.g., Vps33p, Ypt7p, and Vam3p) makes it tempting to suggest that protein traffic from the PVC to the vacuole is vesicle mediated, and, as mentioned above, *vps18*-ts mutants do accumulate vesicles at the restrictive temperature (126). It has been proposed that in mammalian cells, endosomes mature with time and then fuse directly with lysosomes to deliver their contents there rather than utilizing a vesicle-mediated step (46). The involvement of SNARE complex proteins in PVC-to-vacuole trafficking does not argue against a similar maturation model since these proteins, as well as Sec18p (yeast NSF), have also been implicated in the homotypic fusion of vacuolar membranes (see below). Due to the apparent role of class C *VPS* genes in multiple protein transport pathways to the vacuole, *vps18*-ts mutants accumulate an array of intermediates upon exposure to restrictive conditions, and it therefore remains unclear whether the PVC-to-vacuole step is vesicle mediated. Among the intermediates that accumulate are 40- to 50-nm vesicles, but the identity of the cargo they carry awaits further analysis, perhaps by immunoelectron microscopy.

Trafficking of Alkaline Phosphatase to the Vacuole

Unlike most other vacuolar proteins that travel from the TGN to the vacuole that have been studied to date, the integral membrane protein ALP (encoded by *PHO8*) does not travel through the PVC defined by mutations in the class E genes *VPS27* and *VPS4* or in the Golgi-derived transport vesicles defined by *vps45* mutations that require Pep12p for their targeting (32, 117). ALP is consistently delivered to the vacuole in cells that are blocked in the Vps pathway at these stages through a route that does not involve its transport to the cell surface and subsequent endocytosis (32, 117). The observation that ALP is not carried from the *trans*-Golgi by vesicles defined by *vps45* mutations implies that there is a second class of vesicles that carry vacuolar cargo out of this organelle. The diversion of ALP, as well as proteins that utilize the Vps pathway, to the plasma membrane in *vps1* mutant cells (112) provides evidence that Vps1p is involved in the formation of both classes of vesicles from the *trans*-Golgi, but it seems likely that clathrin is involved in the formation only of those bound for the PVC, since ALP is not significantly mislocalized to the cell surface of *chc1*-ts cells under restrictive conditions (147). Such a dual role for Vps1p is not surprising, since it is clear that its mammalian homolog, dynamin, participates both in rapid endocytosis from the plasma membrane and in the formation of caveolae (2).

The 33-residue N-terminal cytosolic tail of ALP is both necessary and sufficient for the diversion of membrane proteins from the Vps-dependent CPY pathway into the alternative route taken by ALP to the vacuole (32, 117). Although the precise signal required to mediate entry into this pathway remains to be defined, it is clear that ablation of this information from ALP results in trafficking of the protein to the PVC (and subsequently to the vacuole) in *VPS45*-controlled vesicles. Consistent with the identification of an ALP pathway targeting signal is the observation that this pathway is saturable. Overproduction of ALP leads to a portion that is now transported to the vacuole in a Pep12p-dependent manner (32).

Mutations in a number of class B genes, such as *VAM3*, which encodes a vacuole-localized syntaxin homolog (174) and also travels to the vacuole independently of the PVC (117), not only block the trafficking of ALP to the vacuole but also prevent transport of proteins by the Vps-dependent pathway. As well as being defective in both transport pathways from the TGN to the vacuole, *vam3*-ts cells are blocked in the cytoplasm-to-vacuole targeting pathway taken by API (see below) at the restrictive temperature, demonstrating that Vam3p acts at a convergence point for these three pathways (34). Similarly, *YPT7* is required for the delivery of ALP to the vacuole and also for traffic from the late endosome/PVC to the vacuole (139, 186), in addition to its role in homotypic vacuolar fusion (see below).

Another class B *VPS* gene, *VPS41*, has been identified as being required for the vacuolar delivery of ALP but not carboxypeptidase S (CPS), which travels through the PVC via *VPS45* vesicles (32). *vps41* mutants accumulate structures that resemble mammalian multivesicular bodies (71), and these cells exhibit a block in the processing of ALP but process CPS and CPY with near-wild-type kinetics. In wild-type cells, newly synthesized ALP is found in a low-speed pellet fraction (containing vacuolar membranes) following a 30-min chase period and subcellular fractionation. In contrast, following exposure of *vps41*-ts mutant cells to restrictive conditions, ALP fractionates in the high-speed pellet, probably reflecting its entrapment in some transport intermediate defined by this mutation (32). The interpretation that the *vps41*-ts mutation selectively blocks the ALP pathway without affecting the CPY pathway is based on the assumption that the observed maturation of CPY and CPS reflects vacuolar delivery of these proteins. Unless confirmed by immunolocalization data, this assumption may be misleading, since it is clear that processing by vacuolar proteases does not always occur in the vacuole; for example, *vps27* mutants (both null and temperature sensitive) process proteins in a *PEP4*-dependent manner in the PVC (118).

A genetic screen for factors specifically involved in transport of ALP to the vacuole was performed with an ALP-Ste13p fusion protein and identified Ap16p and Ap15p of the AP-3 adaptor protein complex (31). Adaptor proteins are believed to provide membrane binding sites for vesicle coat components such as clathrin and also interact with trafficking-membrane proteins to recruit specific cargo into transport vesicles (132). Recently, a new adaptor complex associated with the TGN and endosomes in mammalian cells has been described (36, 37, 150, 151), and a yeast equivalent of this complex has been identified by genetic screens aimed at suppressing the growth defects associated with endocytosis-deficient casein kinase I mutants (115). AP-3 is not found in association with clathrin, nor does it show genetic interaction with clathrin, and thus AP-3 most probably associates with other as yet unidentified coat proteins (115, 150). Interestingly, AP-3 has been shown to be involved in the trafficking of ALP, since disruption of genes encoding subunits of AP-3 result in the accumulation of pro-ALP (31). Although it is clear that the AP-3 complex functions along the alternative pathway, its precise site of action is unclear. While it is possible that AP-3 proteins function at the level of the TGN to sort proteins such as ALP into the alternative pathway, it is equally likely that the AP-3 complex functions at some intermediate compartment along the pathway. Nevertheless, these observations are provocative, since they imply that the alternative pathway followed by ALP defines a novel sorting pathway from the TGN that is present in all eukaryotes.

ENDOCYTOSIS OF PROTEINS FROM THE PLASMA MEMBRANE TO THE VACUOLE

Endocytosis is the uptake of extracellular and plasma membrane material from the cell surface into the cell. Endocytosis has been well characterized at the morphological level in mammalian cells, where both fluid-phase endocytic markers, such as horseradish peroxidase, and membrane-bound endocytic markers, such as colloidal gold-labeled transferrin receptors,

can be internalized and visualized by electron microscopy. The best-characterized endocytic mechanism to date is clathrin-dependent endocytosis (141), in which markers are initially seen in invaginations of the plasma membrane, known as coated pits, coated on the cytoplasmic side of the plasma membrane with polymerized clathrin. In many cases, it has been shown that receptors are concentrated into these pits via an interaction between their cytosolic tails and clathrin adaptor proteins, which in turn associate with clathrin triskelions (116). After longer incubation times, endocytic markers label early endosomes and subsequently multivesicular late endosomes. It is through late endosomes that the mannose-6-phosphate receptor cycles as it targets proteins from the TGN to the lysosome, and these organelles seem to be the point at which the endocytic and lysosomal biogenesis pathways intersect (90, 101). After prolonged internalization, the marker proteins are delivered to lysosomes, the mammalian equivalent of the yeast vacuole. Endocytosis of extracellular fluid by *S. cerevisiae* has been demonstrated by using lucifer yellow (128) and the lipophilic dye FM4-64 (171), and receptor-mediated endocytosis can be monitored by using the mating pheromone receptors Ste2p (24, 77) and Ste3p (35).

Endocytosis of Mating-Pheromone Receptors

Yeast pheromone receptors function in cell-to-cell communication prior to mating. Each of the two haploid yeast cell types, **a** and α, secretes unique peptide pheromones and expresses a cell surface receptor for the pheromone secreted by the other cell type (64). Binding of pheromone to its receptor activates an intracellular signal transduction pathway that leads to the physiological alterations that facilitate mating. Following binding, the pheromone-receptor complex is internalized by endocytosis and degraded in the vacuole in a *PEP4*-dependent manner (35, 138). Riezman and colleagues used radiolabeled α-factor to monitor the uptake of the receptor-ligand complex from the cell surface to the vacuole and showed that the pheromone passes through at least two internal membrane-bound compartments with distinct densities, termed early and late endosomes, before being delivered to the vacuole and degraded by resident proteases (152, 153).

Ligand-Dependent versus Constitutive Endocytosis

Two distinct modes of endocytosis operate on both Ste3p and Ste2p (35): a constitutive (ligand-independent) mechanism and a ligand-dependent mechanism. In both cases, receptors follow the same intracellular itinerary from the cell surface to the vacuole, where they are degraded. The two modes are likely to be distinct mechanistically, since they can be dissected genetically: for example, a version of Ste3p lacking 105 of the 185 residues that make up the C-terminal cytosolic tail does not undergo constitutive endocytosis but is still susceptible to the ligand-induced mechanism (35). The failure of the truncated receptors to undergo constitutive endocytosis suggests that the constitutive pathway is not due to bulk endocytosis of the plasma membrane but, instead, requires some signal lacking in these truncated mutant receptors. Furthermore, not all plasma membrane proteins in yeast turn over as rapidly as the pheromone receptors do (e.g., the plasma membrane ATPase Pma1p has a half-life of >10 h [11], compared with 20 min and ~2 h for Ste3p and Ste2p, respectively). Constitutive endocytosis of pheromone receptors may be necessary to remove receptor from the cell surface, since cells switch mating type in the wild as often as every cell division under some conditions (9). Along the same lines, endocytosis may aid in the process of cell orientation in response to a gradient of mating pheromone by clearing the receptor from regions of the cell not oriented toward the partner (74).

Ubiquitination as a Signal for Endocytosis

Both Ste2p and Ste3p become phosphorylated and ubiquitinated in response to ligand binding, prior to internalization (65, 134). The signal SINNDAKSS within the cytosolic tail of Ste2p has been identified as being sufficient for ligand-dependent receptor internalization (133). Although this signal is sufficient for the internalization of a truncated version of the receptor, it appears that the cytosolic tail of Ste2p contains other, perhaps redundant, internalization information, since this signal is not essential for internalization of the full-length receptor. The lysine residue contained within this signal is one of the eight lysines in the cytosolic tail that is modified by ubiquitin-conjugating enzymes in response to ligand binding (65). Abolition of this ubiquitination, either through mutation of the lysine to an arginine residue or by using *ubc* mutants, prevents internalization of Ste2p (reviewed in reference 129). In mammalian cells, surface receptors, including the epidermal growth factor receptor and the platelet-derived growth factor receptor, become ubiquitinated in response to ligand binding. These receptors are also internalized and are subsequently degraded in the lysosome in response to ligand binding, suggesting a role for ubiquitination in endocytosis in animal cells (49, 104). More direct evidence for this comes from studies with CHO cells carrying a temperature-sensitive form of the ubiquitin-activating enzyme. These cells are unable to internalize growth hormone receptor under restrictive conditions (161).

The role of phosphorylation in the internalization of the mating-pheromone receptors is poorly understood. Mutational analysis had demonstrated that phosphorylation of one of the three serine residues within the SINNDAKSS sequence is necessary for the internalization function of this sequence (65). It has been proposed that phosphorylation acts as a signal to mediate ubiquitination, as observed for certain cytosolic proteins (129).

The **a**-factor transporter, Ste6p, is constitutively endocytosed from the cell surface to the vacuole. Ste6p does not follow this itinerary in mutants lacking ubiquitin-conjugating enzymes (*ubc* mutants) but instead accumulates at the cell surface. Similarly, ubiquitinated forms of the protein accumulate at the cell surface in mutants blocked in early stages of endocytosis (see below) (14, 41, 88, 89). Such a role for ubiquitination in the endocytosis of Ste6p, as well as in the internalization of various permeases (47, 48, 94), implies that ubiquitination signals mediate constitutive as well as ligand-induced endocytosis in yeast cells. Consistent with this is the finding that Ste3p requires ubiquitination of the receptor for constitutive uptake as well as ligand-induced endocytosis (134).

Although ubiquitination is a well-characterized signal for the degradation of cytosolic protein by the proteasome (25), turnover of ubiquitinated pheromone receptors is not mediated by the proteasome but, rather, requires active vacuolar hydrolases (65, 129). It may be that the type of ubiquitination that proteins receive determines whether they are targeted for degradation by the proteasome or the vacuole. Multiubiquitination of substrates is generally required for recognition by the proteasome, but it seems that a monoubiquitinated species is the predominant form of modified Ste2p induced by ligand binding. These data suggest that a single ubiquitin moiety attached to a single lysine residue may lead to preferential recognition by the endocytic machinery instead of the proteasome (65). While the mechanism by which ubiquitin induces receptor internalization

is unknown, it is clear that ubiquitination does not promote internalization by modulating the pheromone-stimulated signal transduction pathway (65). Models in which ubiquitin is recognized directly by the endocytic machinery or promotes the migration of receptors into domains of the plasma membrane that actively endocytose have been proposed. Another possibility is that ubiquitination distinguishes proteins to be degraded after endocytosis from those to be recycled to the cell surface after internalization (173).

Identification of Endocytic Machinery

The first genes identified as being required for the internalization of α-factor were *END3* and *END4*. *end3* and *end4* mutants were identified as being deficient in pheromone uptake as well as in fluid-phase endocytosis (122). *END4* is allelic to *SLA2* (69), which was isolated as being synthetically lethal with the actin binding protein, Abp1p, and encodes a protein that localizes to cortical actin patches and exhibits homology to the mammalian focal adhesion protein talin (69). *END3* is also required for the proper functioning of the actin cytoskeleton (10) and encodes a protein displaying similarity to domain I of eps15, a protein believed to facilitate interactions between clathrin and the epidermal growth factor receptor through the adaptor protein AP2 in mammalian cells (12, 13). Within this region (the EH domain, for eps15 homology), End3p contains a putative Ca^{2+} binding site, but no requirement for calcium in endocytosis has been shown. Interestingly, calmodulin is required for the internalization step of endocytosis (92), but the calcium binding function of the protein does not seem to be involved since calmodulin mutants that have lost their high-affinity calcium binding sites are not defective in endocytosis.

The endocytic tracer FM4-64 travels from the plasma membrane to the vacuole via punctate endocytic intermediates in a time-, temperature-, and energy-dependent manner (171). This molecule has been used in a fluorescence-activated cell sorter-based screen to isolate mutants defective in its uptake. The idea behind this screen was to isolate mutants defective in both clathrin-independent and clathrin-dependent endocytosis (see below), and although it is still possible that such mutants can be isolated by this approach, the two mutants reported to date, *dim1* and *dim2*, also affect α-factor internalization (184), which occurs through the clathrin-dependent mechanism.

Like End3p, Dim2p/Pan1p contains an EH domain and localizes to cortical actin patches (10, 165). End3p and Pan1p interact genetically and physically both in vitro and in vivo (164). Since the localization of Pan1p is affected by *end3* mutations, it has been proposed that End3p is important for the localization of Pan1p to the cortical actin skeleton (see below) (164), and perhaps the End3p/Pan1p-containing complex is involved in the formation of clathrin-coated pits at the cell surface through interactions with clathrin adaptor proteins (155) via EH domains. *DIM1* is allelic to *SHE4* and *MYO4*; mutants with mutations in *MYO4* were isolated as having cytoskeletal defects (55), and those with mutations in *SHE4* were isolated as having defects in mating-type switching (75). Both *dim1* and *dim2* mutants were shown to accumulate aberrant membranous structures that can be labeled with cationized ferritin as a marker of the endocytic pathway, suggesting that they represent intermediates in the internalization of plasma membrane material (184).

Other studies also suggest that the actin cytoskeleton plays a role in the internalization step of endocytosis in yeast (91). The *act1-1* mutation leads to the production of an altered form of actin, and cells harboring this temperature-sensitive mutation are subjected to a rapid block in the internalization of α-factor by receptor-mediated endocytosis upon exposure to restrictive conditions. Also, an extension of the *end* mutant screen identified three genes that affect actin organization and are required for the internalization step in the endocytosis of Ste2p (107), and the yeast homolog of the actin-bundling protein fimbrin (Sac6p) is also required for the same stage in endocytosis (91).

Involvement of the Cytoskeleton in Endocytosis

Actin filaments assemble into two different structures in *S. cerevisiae*: actin cables, which extend through the cytoplasm, and cortical patches, which are found at the cell surface. While it has been proposed that cortical patches provide osmotic support during the insertion of new cell wall material, the physiological roles of both actin cables and the patches remain unclear. The small actin binding protein cofilin stimulates actin filament disassembly and thus stimulates a cycle of filament assembly and disassembly (96). Cofilin mutants are defective in the uptake of lucifer yellow suggesting that endocytosis requires the rapid turnover of actin cables induced by cofilin (96). Morphological evidence for the involvement of the actin cytoskeleton in endocytosis comes from immunoelectron microscopy experiments that demonstrate that actin in the cortical patches is associated with fingerlike invaginations of the plasma membrane (105). It has been suggested that organized bundles of actin filaments may be required to drive the scission of vesicles from invaginations of the plasma membrane (91) and that actin cables may be involved in producing the plasma membrane invaginations from which endocytic vesicles form (105). A role for actin in the scission of vesicles from the plasma membrane is consistent with observations following the treatment of mammalian cells with the drug cytochalasin D (52), when cells accumulate elongated clathrin-coated pits.

While not all mutations that affect cortical actin patches abolish endocytosis (e.g., the *myo2-66* mutants endocytose α-factor with the same kinetics as do wild-type cells [97] despite having extensively delocalized cortical actin [79]), it remains possible that these structures play a role in the uptake of molecules from the cell surface. It has been suggested that mutations that affect the structure of cortical actin patches abolish endocytosis whereas those that affect the localization of these structures during the cell cycle do not (107). Unlike Myo2p, the type I yeast myosins Myo3p and Myo5p are involved in endocytosis. These molecules possess an N-terminal actin/ATP-dependent motor domain and a phospholipid binding, acidic C-terminal tail and have been postulated to be involved in vesicle movement (reviewed in reference 129).

Clathrin-Dependent and Clathrin-Independent Endocytosis

A role for clathrin in endocytosis in yeast has been identified in both constitutive and ligand-induced endocytosis by using strains expressing a temperature-sensitive clathrin heavy chain (163). In mammalian cells, endocytosis appears to operate through both a clathrin-dependent mechanism and a clathrin-independent mechanism (95); interestingly, yeast cells lacking clathrin heavy chain are capable of endocytosis at 30 to 50% of wild-type levels. Whether this residual uptake occurs through a second clathrin-independent pathway or because other elements of clathrin coats are still capable of limited vesiculation in the absence of clathrin heavy chain remains unclear.

Intersection of the Endocytic and *VPS* Pathways

Endocytosis mutants that disrupt actin organization are not required for the later stages of endocytosis, that is, the trans-

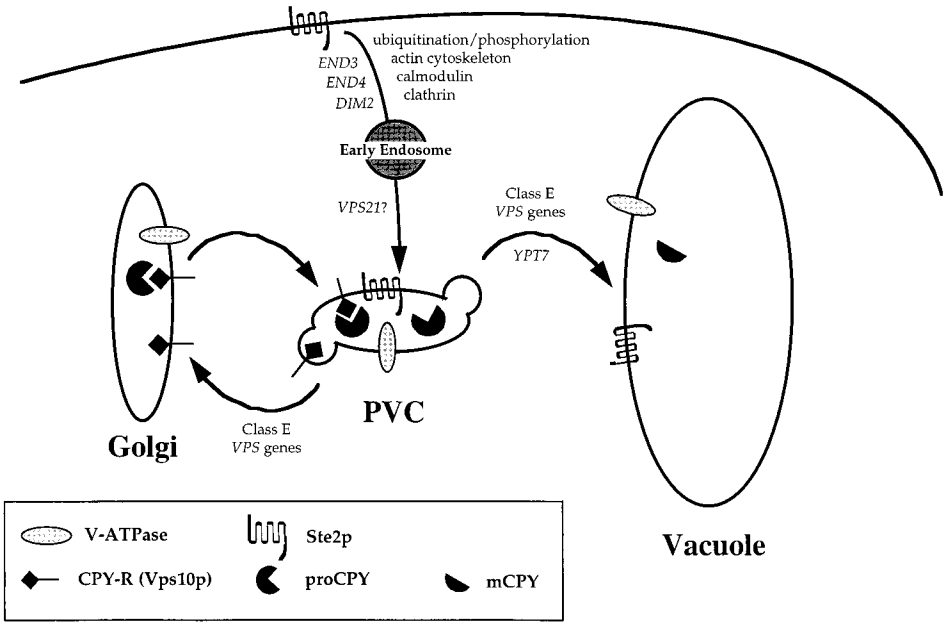

FIG. 3. Endocytosis of proteins from the plasma membrane to the vacuole. Proteins such as the mating pheromone receptor Ste2p are endocytosed to the vacuole from the plasma membrane, passing through at least two membrane-bound compartments en route. The requirements for this process are indicated here. The requirement of class E *VPS* genes for endocytosis pinpoints the PVC as a convergence point for the biosynthetic and endocytic pathways.

port of endocytosed proteins from endosomal compartments to the vacuole (91). In a manner similar to the endocytic and lysosomal pathways in mammalian cells, the endocytic and vacuolar biogenesis pathways intersect at a post-Golgi PVC (118). A number of genes have been implicated as being required for trafficking of proteins out of this compartment and on to the vacuole. Class E *vps* mutants (118, 123) accumulate an exaggerated form of the PVC that contains endocytosed proteins as well as proteins en route to the vacuole via the biosynthetic pathway (118). Indeed, it is common for class E *VPS* genes to be identified in screens for endocytosis mutants: *ren1* is allelic to *vps2* and was isolated as being required for the endocytosis of Ste3p (35), and *end13* is allelic to *vps4* (106), confirming that transport from the PVC to the vacuole is part of the endocytic pathway. It is through the same compartment that Vps10p cycles in its role as the CPY receptor (29), and it is from here that late-Golgi membrane proteins are retrieved to achieve steady-state localization in the TGN (16) (Fig. 3).

Involvement of Rab Proteins in Endocytosis

Members of the small GTPase or Rab family of proteins have been implicated as being key regulators of endocytosis in mammalian cells at postinternalization stages (193). Rab7 is associated with late endosomes, and Rab5 is associated with early endosomes (21). The yeast homolog of Rab7, Ypt7p, is believed to control traffic between the PVC and the vacuole (139, 186), whereas homologs of Rab5, i.e., Ypt51p, Ypt52p, and Ypt53p, are proposed to be involved in an earlier transport step, such as between early endosomes and the PVC (154). Consistent with this model is the observation that the degradation of α-factor is more severely inhibited in *ypt51* mutants than in *ypt7* mutants (154), suggesting that in these cells the pheromone is trapped inside a proteolytically inactive compartment. In addition, the pheromone that accumulates in *ypt7* cells fractionates to the same position in a density gradient as the kinetic intermediate defined by Singer et al. (152) as late endosomes, while the pheromone that accumulates in *ypt51* mutants is found in fractions enriched for early endosomes (139). *YPT51* is allelic to *VPS21*, a class D *VPS* gene, believed to function in the fusion of Golgi-derived transport vesicles with the PVC (18). The involvement of a member of the Rab family of GTPases in more than one transport step has two well studied precedents: Rab1 facilitates transport between the ER and the Golgi apparatus and also seems to be required for transport between Golgi stacks (120), and Rab5 is involved in endosome-to-endosome fusion as well as in transport from the plasma membrane to early endosomes (17, 51). Similarly, in yeast, the small GTPase Ypt1p is required for ER–to–*cis*-Golgi transport as well as for *cis*- to *medial*-Golgi transport (76). However, conditional alleles of both *VPS21/YPT51* and *YPT7* are needed before we can convincingly assess the primary function of these Rab proteins in endocytic and biosynthetic vacuolar membrane traffic.

Most mutants defective in endocytosis isolated to date fall into two categories: those that block internalization of material from the plasma membrane and those that inhibit transport from the PVC to the vacuole. The latter category also shows defects in vacuolar protein sorting, pointing to an overlap between the endocytic and biosynthetic pathways. This model (Fig. 3) reconciles data from studies with class E *vps* mutants (discussed above), but the isolation of mutants such as *vps34* as being defective in endocytosis (106) is perhaps harder to reconcile, since *VPS34* has been implicated in the generation of Golgi-derived transport vesicles (see above).

CYTOPLASM-TO-VACUOLE TARGETING

Biosynthetic Pathway

Like many vacuolar proteins, the soluble vacuolar hydrolase API is synthesized as an inactive precursor (61 kDa) and converted to its active form (50 kDa) following a *PEP4*-dependent cleavage event (87). However, unlike most vacuolar proteins that have been characterized to date, API does not travel along

the early stages of the secretory pathway to reach the vacuole. The delivery of both CPY and ALP to the vacuole is blocked in cells bearing mutations that block early stages in the secretory pathway (159), either by preventing traffic out of the ER or by blocking traffic through the Golgi apparatus (although not in *sec* mutants, which are specifically blocked in traffic from the Golgi apparatus to the cell surface). In contrast, the processing of API shows essentially the same kinetics in cells bearing temperature-sensitive alleles of *sec23*, *sec12*, *sec18*, or *sec7* at the restrictive temperature as it does in wild-type cells, indicating that the delivery of API to the vacuole does not require trafficking out of the ER or through the Golgi apparatus (87). Further evidence in support of a model in which API reaches the vacuole through a route distinct from that taken by CPY and ALP comes from the time taken by these proteins to reach their final destination following synthesis. Both ALP and CPY are processed in a *PEP4*-dependent manner with half-lives of less than 10 min, whereas API is similarly processed with a much longer half-life of 45 min (87).

CPY is delivered to the vacuole by a receptor-mediated process (see above), and overproduction of CPY causes saturation of the receptor, resulting in a spillover of CPY into secretory vesicles in the late Golgi (160). Consequently, overproduction results in secretion of CPY from the cell. In contrast, overproduction of API does not cause secretion of the protein, but the unprocessed form instead accumulates in the cytosol; indeed, even without overproduction, it is evident that the precursor form of API does not reside within a membrane-bound compartment, since it is sensitive to proteases under conditions where mature API and all forms of CPY are not (87).

The above observations led to the model that API is synthesized in the cytosol and then transported into some "post-Golgi" compartment, originally postulated to be the vacuole itself, directly from the cytosol through the Cvt (cytoplasm-to-vacuole targeting) pathway. Such a route also appears to be taken by the product of the *AMS1* gene, α-mannosidase I (Ams1p) (93, 191). Ams1p is initially synthesized as a 107-kDa precursor and is processed in a *PEP4*-dependent manner into 73- and 31-kDa forms upon delivery to the vacuole (192). Although the *AMS1* gene product has seven consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr) the protein is not glycosylated (192). By constructing a prepro-CPY–Ams1p fusion protein, which is delivered to the vacuole by the secretory pathway, it has been shown that the glycosylation sites within Ams1p are competent substrates for N-linked glycosylation and that the lack of such carbohydrate modification on Ams1p is probably because the protein is not exposed to the ER lumen. Similarly, API has four consensus N-linked glycosylation sites, and although initial characterization of the protein indicated that it was glycosylated (102), more recent studies involving lectin binding and tunicamycin treatment of cells demonstrate that the protein does not carry carbohydrate modifications (20, 87).

Ams1p and API have other features in common that set them apart from other vacuolar hydrolases and lend credence to the Cvt model. While having no effect on the sorting of CPY, overproduction of Ams1p increases the half-life of processing of API (as does overproduction of API itself), suggesting that the two have a common import mechanism and perhaps use the same saturable component to facilitate their entry into the vacuole (87). The converse experiment is hard to perform due to the long half-life of processing of Ams1p (10 h in wild-type cells at 30°C [192]).

Like API, the delivery of newly synthesized Ams1p to the vacuole is not dependent on the function of *SEC12* or *SEC23*, as demonstrated by the observation that processing occurs in cells bearing temperature-sensitive mutations in these genes at the restrictive temperature (192). The other *sec* mutants analyzed in this study (*sec7* and *sec18*) did not process Ams1p, but this is probably due to death of these mutants during the long incubation times at the restrictive temperature required in these studies (again due to the long half-life of processing of the protein).

Consistent with the data that neither API nor Ams1p is glycosylated, neither possesses a standard signal sequence as found in CPY, PrA, and PrB, specifying translocation into the ER lumen. They do, however, contain information targeting them to the vacuole (87, 192). For Ams1p, it has been shown that the extreme C terminus of the protein is capable of diverting the normally secreted protein invertase to the vacuole. The propeptide of API, which is removed upon delivery of the protein to the vacuole, is required for the targeting of the protein to the vacuole, and a mutant form of the protein lacking this pro region accumulates in the cytosol (114). This propeptide lacks any similarity to a standard signal sequence, because it is not hydrophobic in nature, and secondary-structure analysis predicts that it is composed of two α-helices separated by a β-turn, with the first of the two helices being amphipathic (20). Mutations within this amphipathic helix that substitute a charged amino acid for a nonpolar residue prevent membrane association of pro-API, suggesting that the amphipathic nature of this helix is vital for vacuolar targeting. In contrast, the periodicity of the second helix does not seem to be as important, and it has been suggested that its role might be to present the first helix in the correct context to allow it to facilitate Cvt transport (114).

Recent studies indicate that the precursor API oligomerizes in the cytosol prior to membrane binding (83), and a form of the protein carrying a temperature-sensitive mutation in the targeting signal has been used to show that the membrane-bound precursor is imported in its oligomeric form (83). The API oligomer is a dodecamer of 732 kDa, and this large size does not fit with translocation through a protein channel but would be accommodated by a vesicle-mediated mechanism such as macroautophagy (see below). Similarly, the temperature profiles of the import reaction both in vivo and in an in vitro reconstitution suggest that import does not occur through a proteinaceous channel, since the Cvt pathway is not operational at temperatures at which translocation into the ER and mitochondria occurs normally (144).

To identify components of the machinery involved in the Cvt pathway, Harding et al. used a genetic screen relying on the accumulation of precursor API to identify mutants defective in the cytoplasm to vacuole targeting of API (59). The original *cvt* mutants fall into eight complementation groups, five of which do not have a significant effect on vacuolar protein sorting through the secretory pathway and, by this criterion, appear to be specific for the Cvt targeting pathway. The *cvt* screen also identified *VPS39* and *VPS41* as being involved in the targeting of API, and this observation prompted a systematic screening of *vps* mutants. The discovery that *vps1*, *vps8*, *vps15*, *vps16*, *vps17*, *vps18*, and *vps26* mutants all show defects in API processing as well as in α-mannosidase targeting suggests that there is overlap between the machinery used by the *vps* pathway taken by CPY and the Cvt pathway.

Degradative Pathway: Starvation-Induced Autophagy

Autophagy was first identified as the transport of cytoplasmic proteins to the vacuole as part of a response to nutrient

deprivation in which cells degrade large amounts of intracellular proteins (reviewed in reference 40). Morphological studies have demonstrated that autophagocytosis in mammalian cells is used for bulk, nonselective transport of cytosol and organelle fragments to the lysosome, and it appears that there is also a more selective form of autophagocytic uptake that depends on the presence of a peptide signal and a membrane receptor (33, 38). Microautophagy is the sequestration of small portions of cytosol (including large molecules such as glycogen and also ribosomes) by an invagination of the lysosome membrane, resulting in the formation of membrane-bound intralysosomal vesicles, whose contents are subsequently degraded by lysosomal hydrolases. Macroautophagy is defined as the sequestration of organelles for degradation by the lysosome. Electron microscopy suggests that early autophagosomes are formed from the ER and that these then mature into late autophagosomes and subsequently into autolysosomes.

Under conditions of nitrogen starvation, *S. cerevisiae* cells degrade nearly half of their total cellular protein content within 24 h, with approximately 80% of this degradation taking place in the vacuole (166). As in mammalian cells, the nonselective component of this process has been demonstrated by monitoring the vacuolar uptake of several cytosolic enzymes under these conditions (42). Autophagy can be monitored morphologically in yeast and is characterized by the appearance of autophagic vesicles (whose contents are indistinguishable from cytosol, again demonstrating the nonselective nature of this uptake) inside vacuoles of protease-deficient strains under starvation conditions (149, 162). Two genetic screens have isolated mutants that fail to accumulate autophagic vesicles during starvation. The *apg* mutants were isolated through their reduced ability to survive during starvation (168), and the *aut* mutants were isolated as being defective in the breakdown of the cytosolic enzyme fatty acid synthase, whose degradation during starvation is dependent on vacuolar proteases (167).

Overlap between the Biosynthetic and Degradative Pathways

Autophagy appears to be a slow, nonselective, unsaturable pathway taken by proteins destined for degradation, whereas the Cvt pathway defined by API targeting delivers specific resident proteins to the vacuole in a saturable manner with relatively rapid kinetics. Despite the obvious differences between the two processes, both pathways transport fully synthesized proteins from the cytoplasm into the vacuole, and complementation analysis between the autophagy mutants and the *cvt* mutants was performed to identify overlap between API targeting and autophagy (58, 145). There is a significant amount of phenotypic and genetic (by using precursor API accumulation in diploids as an indication of noncomplementation) overlap between the two sets of mutants. For example, most of the autophagy mutants are blocked in the maturation of API without affecting the targeting of PrA or CPY. Despite this extensive overlap, a few mutants in each group showed little or no phenotypic overlap, implying that the pathway is only partially shared. These data are consistent with the model that API uptake from the cytoplasm to the vacuole is a selective process (see below), as seen for RNase A in mammalian cells, which requires the presence of a consensus sequence motif, KFERQ, in the protein (39).

Nutrient-Induced Autophagy

Peroxisomes are important for the oxidation of fatty acids and are induced when yeast cells are grown on oleic acid (43). Immunoelectron microscopy studies demonstrate that peroxisomes are transported to the vacuole when cells are shifted to glucose, resulting in destruction of the whole organelle in the vacuole (22). Similarly, other proteins are delivered to the vacuole in response to glucose exposure. For example, plasma membrane proteins such as the galactose and maltose transporters are endocytosed to the vacuole (22, 125). The key regulatory enzyme of the gluconeogenic pathway, fructose-1,6-bisphosphatase (FBPase), is also rapidly degraded when cells grown on poor carbon sources are provided with glucose to prevent a futile cycle of glucose anabolism and catabolism. FBPase has been localized to vacuoles both by immunofluorescence and by subcellular fractionation experiments by Chiang and Schekman under conditions that induce its degradation (23). The same group also found that this degradation requires *PEP4* but not subunits of the proteasome, another major degradative site of the cell. In direct contrast to this, Schork et al. found that FBPase degradation does not require *PEP4*, but instead requires components of the proteasome (143). However, the results of Schork et al. are difficult to reconcile with immunolocalization of FBPase to the vacuolar lumen of glucose-treated *pep4*Δ yeast cells. Although this controversy awaits resolution (142), it does appear that autophagy can account for at least part of the FBPase degradation observed upon the addition of glucose.

Following glucose replenishment of protease-deficient cells, FBPase displays a punctate staining of the vacuole by indirect immunofluorescence (22). Electron microscopy studies showed that FBPase can be found at sites on the vacuolar membrane where invaginations occur, as well as being associated with vesicles inside the vacuole of protease-deficient strains. These results indicate that the enzyme is taken up into the vacuole by some sort of autophagic mechanism. Mutants that fail to degrade FBPase in response to glucose replenishment were isolated (68). Under such conditions, FBPase is found in the cytosol of most of these *vid* mutants, but in some *vid* mutants, it is found in punctate structures within the cytosol, consistent with its sequestration in membrane-bound vesicles (68). Glucose-induced FBPase degradation occurs more slowly at 22°C, and under such conditions it is possible to purify the vesicles that accumulate in the *vid* mutants from wild-type cells (73). FBPase associates transiently with these 30- to 40-nm vesicles upon glucose addition, suggesting that they represent an intermediate in the FBPase degradation pathway. The FBPase associated with these cytoplasmic vesicles is in a form protected from proteases, consistent with the model that the protein enters these membrane-enclosed structures before being taken up by the vacuole. Fractionation experiments show that these vesicles are distinct from the vacuole, peroxisomes, mitochondria, ER, Golgi, endosomes, and COPI or COPII vesicles. In addition, morphological studies suggest that they are distinct from early or late endosomes, which are 100 to 400 nm in diameter (153), the COPI and COPII vesicles, which are 50 nm in diameter (8, 82), and the post-Golgi vesicles, which are approximately 100 nm (175). These *vid* vesicles are also a substantially different size from the vesicles associated with API import (86), an observation in agreement with the fact that despite the substantial overlap between the *cvt* mutants and the *apg*/*aut* mutants, there is no overlap between the *cvt* mutants and the *vid* mutants. *cvt* mutants are not blocked in glucose-stimulated FBPase degradation, and *vid* mutants do not accumulate pro-API, indicating a separation of the Cvt/starvation-induced autophagy and nutrient-stimulated autophagy pathways (reviewed in reference 85).

The identification of many *VPS* genes as being required for the Cvt pathway as well as for the convergence of the CPY

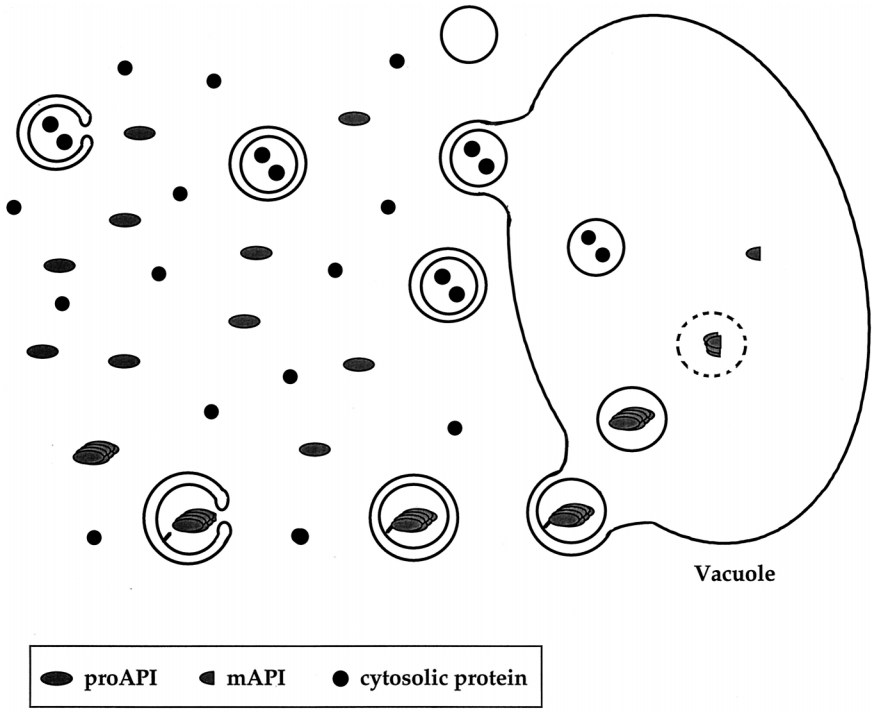

FIG. 4. Cytoplasm-to-vacuole targeting. API reaches the vacuole through the Cvt pathway. There is extensive overlap between the Cvt and autophagic pathways to the vacuole (see the text for details). Oligomerized pro-API is sequestered into cytoplasmic vesicles through a saturable, perhaps receptor-mediated process. These vesicles are then taken up by the vacuole via an autophagic mechanism. Breakdown of these vesicles requires PrB, resulting in the release of API into the vacuole lumen and its subsequent maturation.

pathway, the ALP pathway, and the Cvt pathway on Vam3p (34) indicates an overlap between these protein-trafficking pathways. Similarly, an overlap between endocytosis and autophagocytosis can be demonstrated in mammalian cells by the appearance of endocytosed material in autophagic vesicles (50). The exact convergence point(s) between these pathways remains unclear. Ultrastructural analysis of yeast cells reveals double-membrane-layered autophagosome-like structures that can be found with their outer membranes in continuity with the vacuolar membrane (3) (Fig. 4). Membrane fusion following uptake of autophagic bodies by the lysosome through macroautophagy could also explain the appearance of single-membrane-surrounded vesicles in the vacuolar lumen (149, 162); therefore, the mechanism by which these membrane enclosed bodies enter the vacuole awaits elucidation.

Models of Aminopeptidase I Import

Direct isolation of precursor API from subvacuolar vesicles indicates that API import involves a vesicular event (85, 144). The extensive overlap between the autophagic and Cvt pathways supports a model whereby API is taken up into a population of vesicles by macroautophagy and these vesicles subsequently fuse with the vacuole (Fig. 4). This model is supported by detection of double-membrane vesicles in the vacuole (3) and also by the observation that *cvt1* and *cvt17* mutants accumulate pro-API inside vesicles within the vacuole (58, 144). In addition, *vps18*-ts cells accumulate pro-API in a population of vesicles proposed to be an intermediate in the Cvt pathway (144). Vps18p/Pep3p is a class C Vps protein, localized to the vacuolar membrane and proposed to function in PVC-to-vacuole traffic (121, 131), and these data demonstrate another example of overlap between the Cvt and Vps pathways. The model outlined in Fig. 4 accommodates the large size of the oligomer imported into a membrane-bound compartment and explains the overlap between the two pathways. Since autophagy has been characterized as a slow process, targeting of API via this pathway might somehow concentrate the protein prior to targeting. It is possible that some sort of receptor-mediated macroautophagy increases the efficiency of the process, which would explain why only a small number of *cvt* mutants is not also defective in autophagy.

Molecular characterization of genes involved in cytoplasm-to-vacuole targeting is in its early stages (99, 140). The power of yeast genetics combined with the classical techniques of cell biology afforded through electron microscopy and biochemical studies should eventually heighten our understanding of the pathways described above.

VACUOLAR INHERITANCE

Cells do not synthesize organelles de novo but, instead, build upon material inherited from their mother cell. Cytological studies of mammalian cells have demonstrated vesiculation of nuclear, ER, and Golgi membranes at the beginning of mitosis and subsequent fusion of these vesicles to reconstitute these organelles after cytokinesis (reviewed in references 178 and 179). While not all organelles in *S. cerevisiae* follow this pattern of inheritance, yeast cells do inherit vacuoles from their mothers through a mechanism that involves the projection of vesicles and tubules, known as segregation structures, from the mother-cell vacuole into the growing daughter cell (124, 182) (Fig. 5). As with the inheritance of other organelles (reviewed in reference 179), vacuolar inheritance is coordinated by the cell cycle. Segregation structures are formed early in the cell cycle (67, 180), and then, sometime after cytokinesis, the ves-

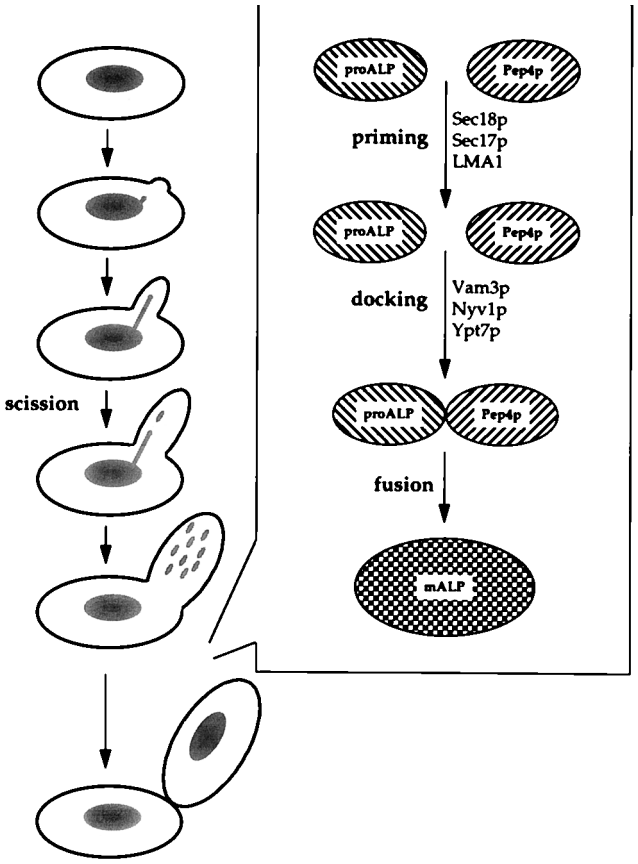

FIG. 5. Vacuolar inheritance. Mother cells donate approximately 50% of their vacuolar material to growing buds. Protrusions from the vacuole grow toward the emerging bud, and a scission event leads to the formation of vesicles, which travel into the bud. A homotypic fusion event between these vesicles (consisting of a priming event, docking, and, finally, fusion) forms the vacuole of the daughter cell.

icles deposited in the growing bud by these segregation structures use a homotypic fusion process to form one or a few larger vacuoles within the daughter cell (28) (Fig. 5).

Mutants defective in vacuolar inheritance were isolated initially by microscopic examination of cells that secrete CPY to identify those that lack segregation structures (180, 181). More recently, a fluorescence-activated cell sorter-based screen has been used to isolate cells that are defective in vacuolar inheritance (176). Most of these *vac* mutants secrete CPY, and many of the class C and D *vps* mutants lack segregation structures (6, 123), demonstrating that the sorting of proteins from late Golgi to vacuole and the process of vacuolar inheritance are intimately tied. It is apparent that the lack of vacuolar inheritance seen in *vac* mutants is not due to some gross perturbation of cell division, since many of the *vac* mutants are normal for the inheritance of other organelles such as the nucleus and mitochondria, an interpretation supported by the observation that the buds of many *vac* mutants grow to a normal size and have a normal complement of mitochondria and nuclear DNA but little or no vacuolar material (181). While it is clear that the inheritance of different organelles is individually controlled, it is likely that these processes are intimately related and indeed have common mechanisms; for example, the inheritance of many organelles requires actin (reviewed in references 63, 178, and 179).

Cell-Free Assays

A second approach to enhance the understanding of vacuolar inheritance at the molecular level has been undertaken by Wickner and coworkers with the development of cell-free assays for vacuolar inheritance (27, 28, 56). The formation of vacuole-derived tubules and vesicles can be observed in semi-intact cells by using the fluorescein derivative CDCFDA (28). In addition, a biochemical assay has been used to quantify the homotypic fusion of vacuoles isolated from strains carrying a gene disruption in either *PEP4* or *PHO8*, by measuring ALP activity in vitro based on the maturation and activation of the unprocessed form of the vacuolar membrane protein ALP (encoded by *PHO8*) by the vacuolar protease PrA (Pep4p).

These assays have been used to demonstrate requirements for vacuolar inheritance, and it has been shown that the formation of segregation structures requires energy in the form of ATP and also the proteins encoded by *VAC1* and *VAC2* (28, 148). Such a link between a vacuolar inheritance phenotype in vivo and the requirement of the morphological cell-free assay for the proteins represented by these mutants is also seen in the biochemical assay. For example, cytosol from a *vac5-1* mutant does not support the reaction with vacuoles isolated from wild-type cells, and vacuoles isolated from a *vac1-1* mutant will not fuse in the reaction even in the presence of cytosol isolated from wild-type cells (110, 111).

Actin Cytoskeleton

vac15 mutations are allelic to mutations in *MYO2* (67). Myo2p is an unconventional myosin (type V), which is required for polarized growth in *S. cerevisiae*. It has been proposed that Myo2p acts in the transport of small vesicles from the mother cell into a growing bud (53, 79). A mutation in *MYO2* that is known to disrupt interactions between Myo2p and actin causes defects in vacuolar inheritance, and mutations in *ACT1* that prevent the same interaction cause similar defects (67). These observations have been instrumental in the proposal of a model in which Myo2p serves as a motor for vacuole transport along actin filaments from the mother cell into the bud in a similar fashion to its proposed role in the movement of Golgi-derived secretory vesicles to the cell surface (53). Hill et al. undertook a systematic screening of various actin mutants to investigate the role of the actin cytoskeleton in vacuolar inheritance (67). As well as identifying the requirement of the myosin-actin interaction, they observed that filamentous actin is found in association with vacuolar membranes within the mother cell. Cortical actin is found at the site of bud emergence in unbudded cells, and a portion of the mother vacuole is also found there; it seems that this region of the vacuole travels into the bud as it emerges.

Machinery Involved

As mentioned above, there is significant overlap between the *vac* mutants and the *vps* mutants. Interestingly, *vac2-1* mutants do not secrete soluble vacuolar hydrolases but are clearly unable to partition maternal vacuolar material into the emerging bud. Fluorescence microscopy studies indicate that *vac2-1* cells are unable to initiate early steps in segregation structure formation, and it has been postulated that the mutation interferes with a cytoskeletally based transport system (148). However, it seems likely that the role of *VAC2* in inheritance is also required at later stages, since vacuoles prepared from *vac2-1* mutants are defective in the homotypic fusion assay (56).

Another *VAC* gene proposed to function early in the inheritance pathway is *VAC8*. *VAC8* encodes a protein with homol-

ogy to armadillo in *Drosophila* (and β-catenin in mammalian cells), which is found in cell adherence junctions. *vac8* mutants show vacuolar inheritance defects similar to those of *myo2* mutants. The buds receive very little vacuolar material from the mother, and vacuoles fail to migrate to the bud (14a). The removal of palmitoylation sites from the protein abolishes its function in vacuolar inheritance, and it has been proposed that the protein, which has been immunolocalized to the vacuolar membrane, may attach the vacuole to the bud site during cell division. Consistent with a role in the early stages of inheritance, anti-Vac8p antibodies do not inhibit the vacuolar fusion assay.

Mutations in *VAC1* are allelic to *vps19* mutations (183). *VPS19* is a class D *vps* gene, which encodes a ring-finger-motif-containing protein that has been proposed to act as part of a Sec18p-dependent complex in Golgi-to-PVC traffic (18). Like other *vac* mutants that missort CPY, *vac1* mutant cells show only a partial defect in vacuolar inheritance, in that they have a normal-size vacuole before they bud even though they inherit little vacuolar material from their mother (183). Whether the role of *VAC1* in inheritance is related to its role in Golgi-to-vacuole transport or whether Vac1p is independently involved in protein sorting and vacuolar inheritance remains to be elucidated.

One subset of *vac* mutants seems to be defective in vacuole membrane scission (fusion of the internal faces of the vacuole membrane) (14a). Instead of a multilobed vacuole, these mutants contain a single, extremely large vacuole. Populations of these mutants contain cells in which the vacuole does extend into the bud, but even in the narrow confines of the bud neck there is a clear gap between opposing vacuolar membranes due to a lack of scission (it is worth noting that other mutants that have large vacuoles, such as class D *vps* mutants, do not form these "open figure eights"). Three mutants of this type have been identified to date: mutants with mutations in *vac7-1*, *vac14-1*, and *fab1-2*. Fab1p has significant homology to mammalian PI4 kinase, and since PI kinases have been implicated in other membrane fusion events (see above), it is tempting to draw analogies between vacuolar scission and bud formation. Indeed, since the scission event involved in the formation of clathrin-coated vesicles in mammalian cells seems to be mediated by dynamin (homologous to Vps1p), one might speculate that a dynamin homolog could be involved in vacuolar scission.

Dissection of Homotypic Vacuolar Fusion

By carefully studying the requirements of the biochemical assay for vacuolar fusion, the reaction was divided into four distinct stages (27). Stage I requires exposure of vacuoles to solutions of moderate ionic strength. It has been proposed that this somehow prepares the vacuoles for stage II, perhaps by removing some inhibitory molecule from the membrane. Stage II requires stage I vacuoles and cytosol. It seems likely that cytosolic factors bind to the vacuolar membrane during this stage, rendering them competent for fusion. Fusion is initiated in stage III and requires ATP, but it does not proceed until stage IV, when vacuoles must be present at or above a minimal concentration for the reaction to proceed. Stage III of the reaction is sensitive to inhibitors of the vacuolar ATPase as well as to proton ionophores, suggesting a requirement for an electrochemical gradient across the vacuolar membrane at this stage. Similarly, GTP-hydrolyzing proteins have been implicated at this stage through the inhibitory effect of GTPγS.

By using an in vitro assay for early endocytic events, it was demonstrated that rab5 is required for homotypic endosome fusion in mammalian cells (51); similarly, it has been demonstrated that Ypt7p functions in homotypic vacuolar fusion in *S. cerevisiae*. *YPT7* was seen as being a prime candidate for the GTPase regulator of vacuolar fusion (whose existence was uncovered by the in vitro assay), since *ypt7* cells have a class B vacuolar morphology phenotype in that they possess numerous small vacuoles (186), which may result from a failure of homotypic fusion. With antibodies to Ypt7p, a direct involvement for the small GTPase in the vacuole fusion reaction was demonstrated (57). In addition, purified Sec19p, the yeast homolog of the GDPase inhibitor protein Gdi1p, blocks homotypic fusion but not segregation structure formation, suggesting a role for Ypt7p in vacuolar inheritance (186).

Kinetic studies of the inhibitory affects of anti-Ypt7p antibodies on homotypic fusion suggest that the GTPase acts at the docking stage of the reaction (100), a stage that also requires the vacuolar SNARE proteins Vam3p and Nyv1p (109). The reaction has also been shown to require Sec18p and Sec17p (yeast homologs of the mammalian proteins NSF and α-SNAP [soluble NSF attachment protein]) and a low-molecular-weight heterodimer consisting of a thioredoxin subunit and an inhibitor of proteinase B (189) termed LMA1 (190) prior to the action of Ypt7p (99). According to the SNARE hypothesis, Sec18p is required after vesicles have docked with their acceptor membrane, directed by the binding of a v-SNARE with its cognate t-SNARE. In contrast to this, Sec18p and Sec17p are required in vacuolar homotypic fusion at a predocking stage (100), as determined both biochemically and microscopically. Therefore, it has been proposed that they are required to transiently modify the structure of the SNARE proteins involved in the fusion into a binding-competent state, which is stabilized by LMA1. Consistent with this theory is the finding that both partners must be exposed to Sec17p/Sec18p to render them competent for fusion.

OVERVIEW

Although we are beginning to gain some insight into the molecular mechanisms that underlie the biogenesis of the vacuole in *S. cerevisiae* by studying the delivery of its proteins through their various pathways, much obviously remains to be learned. Genetic screens that led to the isolation of mutants defective in these various pathways have greatly enhanced our knowledge, and the overlap of mutants identified by these screens has also uncovered much information. For example, the isolation of many class E *vps* mutants as being defective in endocytosis helped designate the PVC as the point of intersection between the endocytic and Vps pathways. The identification of the vacuolar syntaxin, Vam3p, as being necessary for the delivery of CPS, ALP, and API, which reach the vacuole through different routes, tells us that these pathways converge at or before the vacuole. Interestingly, *VPS41* seems to be required for correct targeting of ALP and API but not CPS, perhaps indicating that the routes taken by the first two proteins converge before meeting up with that taken by CPS. However, such overlap is not always as immediately informative; for example, *VPS34* was identified as being required for endocytosis, and it is not easy to see how this fits in with its proposed role in vesicle formation at the *trans*-Golgi. Such a block could arise from the lack of delivery of some component essential for endocytosis to the PVC or the vacuole, or *VPS34* could function at more than one trafficking step. It should be possible to distinguish between such models through the use of conditional alleles, which allow the determination of which phenotypes arise as a direct effect of the loss of gene function.

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