GENETIC, MOLECULAR AND BIOCHEMICAL STUDIES OF VACUOLE BIOGENESIS AND MAINTENANCE IN THE YEAST *Saccharomyces cerevisiae*

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Dedicated to Patches

ABSTRACT

Using a selection for spontaneous mutants that mislocalize a vacuolar CPY-Inv fusion protein to the cell surface, 505 *Saccharomyces cerevisiae* mutants with defects in protein sorting were identified. Seventeen of these mutants were dominant; the others defined 25 new *vps* (for *v*acuolar *p*rotein *s*orting) complementation groups. Alleles of each *vps* complementation group exhibit defects in the targeting and final processing of soluble vacuolar enzymes (CPY, PrA and PrB). Two of the genes, *VPS17* and *VPS15*, map to ChXV and ChII, respectively. The *vps11*, *vps16*, *vps18* and *vps33* mutants exhibit morphological defects, their cells containing debris of a membranous nature and highly abnormal vacuole remnants. Intercrosses with other vacuole function-defective mutants (*vpl*, *pep*, *slp* and *end*), revealed genetic overlaps. It is evident that more than 50 gene products are involved in biogenesis and maintenance of the yeast vacuole. Alleles of 7 of the *vps* complementation groups are temperature-sensitive for vegetative cell growth at 37°C, and this recessive, Ts phenotype cosegregated with the *vps* defect in each case. This easily complemented phenotype has facilitated cloning of six of the genes.

The *VPS18* gene was chosen for further studies. A plasmid complementing the Ts growth defect of *vps18-1* was isolated and shown by integrative mapping to carry DNA from the *VPS18* locus. Yeast strains with a deletion of the entire *VPS18* coding region (*Δvps18*), are viable and exhibit the same phenotypes as *vps18-1*. *Δvps18*, α strains have smaller α-factor halos on *sst2,a* lawns than do *VPS18*, α strains. Immunoprecipitation of α-factor indicated that it is secreted from the *Δvps18* mutant in precursor form. Several of the other severely defective *vps* mutants also show this α-factor processing defect. DNA sequencing of *VPs18* showed an open reading frame encoding a 918aa protein, hydrophilic in nature. The protein sequence revealed a zinc

finger like, cysteine-rich motif in its C-terminal region. A synthetic mutant with a cysteine to serine alteration has a temperature-conditional CPY sorting defect with very rapid onset. Therefore, Vps18p may be a zinc-binding protein, directly necessary for correct vacuolar protein sorting and proper functioning of the Golgi compartment that contains Kex2p.

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SUMMARY

Yeast cells, like those of other eukaryotes, possess a series of membrane-enclosed organelles through which secreted proteins pass on their way to the cell surface. These secretory proteins receive precise modifications characteristically accomplished in certain of the organelles through which they pass. For example, signal sequence cleavage and core glycosylation take place in the endoplasmic reticulum (ER), while oligosaccharide side chain elongations take place in the cisternae of the Golgi apparatus. These specific modifications therefore provide diagnostic markers for whether the secretory protein examined has reached and been modified in a particular organelle of the pathway. Many of the proteins secreted by yeast cells have been characterized in some detail and are useful for studying this transport pathway. A large number of mutants with defects in the secretion of such proteins has been identified. These *sec* mutants block transport at various stages along the secretion pathway, allowing the accumulation of specific organelles and blocking the procession of secretory enzymes at certain defined stages.

Several proteins found in the yeast vacuole have also been thoroughly characterized. The transport of these vacuolar hydrolases is defective in the *sec* mutants blocked at early (ER and Golgi) stages of the secretion pathway, but occurs normally in *sec* mutants blocked at later, plasma membrane delivery stages of the pathway. This indicates that delivery of soluble hydrolases to the vacuole occurs via the first half of the secretion pathway. Many mutants in which correct vacuolar localization fails to occur have been isolated. These do not genetically overlap with the *sec* mutants. Studies of these *vps* (*v*acuolar *p*rotein *s*orting) mutants indicated that vacuolar delivery is a complex process requiring more than 50 gene products.

At each organelle along the secretion pathway, resident proteins remain to carry out their functions in that organelle, whereas secretory proteins must efficiently pass through that compartment on their journey to the plasma membrane or to other

destinations. The favored model is that secreted proteins move forward passively by bulk flow, whereas organelle-specific proteins possess retention signals of some sort. In addition to the retention versus flowthrough decision taken at every point along the secretion pathway, there is a branch point in the pathway where proteins destined for the vacuole are sorted away from the secretory traffic bound for the plasma membrane. This sorting event is thought to take place at a late, or trans, compartment of the Golgi apparatus. The plasma membrane-directed branch appears to be the default pathway, so it is most probable that the sorting of vacuolar proteins is an active event. Some of the *VPS* gene products may be directly involved in carrying out this sorting process.

I: Genetics of protein localization via the secretion pathway

i. Outline

Genetic studies of the secretion pathway in the yeast *Saccharomyces cerevisiae* have permitted this pathway to be subdivided into sections for the purposes of study. Each section is demarcated by one or more mutations that cause the arrest of transport through the pathway at this point. For example, *sec12* blocks the exit of proteins from the endoplasmic reticulum (ER) and causes ER-like structures to accumulate within the cells. Secretory proteins, many of which undergo sequential modifications as they pass through the pathway, are frozen in a characteristic ER-modified form in the *sec12* mutant. Likewise, *sec7* blocks the transport pathway at a later stage, causing the accumulation of Golgi-like organelles and Golgi-modified proteins, and so on.

It is clear that in order to study secretory transport, proteins that are biosynthesized by this pathway must be familiar to the investigator. Our knowledge about secreted proteins has come from both biochemical and genetic studies in yeast. Information about the modifying enzymes and proteins resident along the route that

secreted proteins travel has also played a major role in defining this pathway. Mutants with defects in asparagine-linked glycosylation and cell wall mannoprotein synthesis have been important in defining the glycosylation events that take place in the ER and Golgi, respectively. Likewise, mutants defective in the proteolytic maturation of the soluble, secreted proteins α-factor and killer toxin have defined the proteases present at late stages of the Golgi apparatus.

ii. Secreted proteins

The yeast cell localizes many proteins to the plasma membrane and beyond: to the periplasmic space, the cell wall and the extracellular growth medium. These proteins have diverse functions and include structural components of the cell wall, mating pheremones, mating pheremone receptors and enzymes for the extracellular breakdown of unassimilable nutrient sources. The most useful markers for studies of the secretion pathway in yeast have been invertase and α-factor. Other proteins secreted through the pathway are briefly described; as more becomes known about them, they should also add to our understanding of this biosynthetic pathway.

A) Plasma membrane proteins: Proteins found attached to the yeast plasma membrane include Fus1, plasma membrane ATPase, uracil permease, chitin synthetase and mating pheremone receptors. Such cell surface proteins are synthesized in the rough ER, transit the Golgi apparatus and enter secretory vesicles that fuse with the plasma membrane, externalizing their contents and interior membrane faces.

A pheremone-induced surface protein, Fus1,the product of the *FUS1* gene, is required for efficient cell fusion during conjugation. This 80 kDa protein spans the plasma membrane and is modified with O-linked mannose oligosaccharides in the secretion pathway. The short periplasmic amino terminus is the only region of the protein that is glycosylated. The addition of mannose is completely abolished in the early secretion defective mutant *sec53*, attenuated in the late ER-blocked *sec18*, and

unaffected in *sec7*, which is blocked late in the Golgi phase of secretion. These data indicate that Fus1 progresses through the secretion pathway (Trueheart and Fink 1989).

Two plasma membrane proteins are known that apparently do not undergo glycosylation or proteolytic processing, yet appear to transit through the secretory pathway. Plasma membrane ATPase (PM ATPase) functions in the generation of an electrochemical proton gradient across the membrane, and probably in the control of intracellular pH (Bowman and Bowman 1986). Yeast PM ATPase is synthesised as a 106 kDa polypeptide that is not noticeably modified by glycosylation or proteolysis during transit to the plasma membrane. However, export of this protein requires the secretory pathway, and it has been demonstrated that it is transported in the same secretory vesicles as the soluble secreted enzyme, acid phosphatase (Brada and Schekman 1988; Holcomb et al. 1988). Uracil permease is a 633aa protein containing 12 potential membrane-spanning segments, which functions in the uptake of uracil by the yeast cell. It undergoes neither N-linked glycosylation nor proteolytic processing, but experiments with gene fusions suggest that the protein enters and transits the secretion pathway (Silve et al. 1991).

Some additional plasma membrane proteins that probably also transit the secretory pathway are as follows. The purine-cytosine transport protein of *S. cerevisiae*, which functions in the uptake of purine and pyrimidine bases into the cell, has been identified and shown to be a glycoprotein (Schmidt et al. 1984). As yeast glycoproteins are biosynthesised in the secretion pathway (Kukuruzinska et al. 1987), this is the most probable route for membrane delivery of the purine-cytosine transporter. Chitin synthase is present on the cell surface at sites of chitin deposition. It is also found within the cell in the same fraction (probably secretory vesicles) as the soluble, secreted protein, acid phosphatase; thus it probably transits through the secretory pathway (Schekman and Brawley 1979). The mating pheremone receptor, Ste2, is a 49 kDa protein containing seven hydrophobic segments, which resides in the

plasma membrane. It is responsible for the ligand recognition function of the yeast a-factor receptor (Blumer et al. 1988). It remains to be determined whether this protein is delivered to the plasma membrane by the secretion pathway.

Several otherwise uncharacterized plasma membrane proteins have been shown to be exported by the secretory pathway; this was done by specifically tagging cell surface proteins (Novick and Schekman 1983).

B) Cell wall components: The main structural components of the yeast cell wall are glucan, mannoprotein and chitin. The glucan has not been well characterized, but a number of mutants defective in mannoprotein biosynthesis (*mnn*) have been isolated and studied (Ballou 1982).

The most thououghly investigated cell wall mannoprotein with respect to its transport through the secretory pathway is the 33 kDa cell wall protein (Sanz et al. 1987). This abundant 33 kDa protein consists of a single oligomannose chain N-glycosidically linked to a 30.5 kDa peptide moiety, and has been purified from the cell walls of *S. cerevisiae*. Antisera raised against the purified protein were used to study the passage of this protein through various secretion blocked mutants. These studies indicated that the 33 kDa protein travels through the secretion pathway and receives its only sugar modification in the ER. Before being incorporated into the cell wall, this protein spends a short time in soluble form in the periplasmic space. The secretion of an unrelated, and otherwise uncharacterized, 120 kDa mannoprotein was also blocked in the *sec* mutants (Sanz et al. 1987).

C) Soluble proteins of the periplasmic space and growth medium: The two most characterised of the external, soluble proteins of the yeast cell are invertase and acid phosphatase; these proteins are found largely in the periplasmic space. In addition, *MATα* cells secrete α-factor, *MATa* cells secrete a-factor and barrier protein (Bar1), and killer virus infected cells secrete killer toxin. These four proteins are

secreted into the growth medium beyond the cell wall; the first three are small peptides, but barrier protein is rather large.

Invertase (B-D-fructofuranoside fructohydrolase) functions to cleave extracellular sucrose to fructose and glucose; unlike sucrose, these are sugars that yeast can import. Secreted invertase consists of a homo-octomer of 60 kDa polypeptide chains, which are extensively glycosylated during their transit through the secretory pathway. Glycosylation and octamer formation are thought to function in the retention of invertase in the periplasmic space behind the cell wall (Esmon et al. 1987). It is interesting that oligomerization of invertase takes place in the ER as this is also the case for most oligomeric secretory proteins of mammalian cells (Rose and Doms 1988). The same *SUC2* gene encodes two forms of invertase: the secreted, glycosylated form described above, the synthesis of which is regulated by glucose repression, and an intracellular, nonglycosylated enzyme that is produced constitutively (Carlson and Botstein 1982).

Acid phosphatase (orthophosphoric monoester phosphohydrolase) is secreted by the yeast cell in response to conditions of low phosphate and is found mainly in the periplasmic space (Van-Rijn et al. 1972). The *PHO5* gene has been shown to encode this repressible acid phosphatase, and antisera have been raised against the purified protein. The protein undergoes proteolytic cleavage of a 17 amino acid signal peptide, core glycosylation and outer chain glycosylation during its transit through the secretory pathway (Haguenauer-Tsapis and Hinnen 1984). By staining for the enzyme activity in growing cells that have been recently repressed or derepressed, it has been found that secretion of acid phosphatase takes place in the newly growing portion of the bud (Field and Schekman 1980).

The mating pheremone, α-factor, is released form *MATα* cells and interacts in a receptor mediated process with cells of the opposite mating type to arrest cells in late G1 and initiate conjugation with the eventual formation of diploid zygotes (Bussey

1988). The initial α-factor precursor contains 165aa residues, of which an amino terminal signal is cleaved off in the ER. A 60aa pro region is glycosylated in the ER and cleaved off after passage through the Golgi, leaving a COOH-terminal section containing four repeats of the mature α-factor. These are released by further proteolytic cleavage before reaching the cell surface (Julius et al. 1984b).

Barrier protein, Bar1, is produced and released into the growth medium by *MATa* cells and probably functions as a pepsinlike protease to cleave α-factor. The primary translation product deduced from the gene sequence has a putative signal peptide of 24aa and nine potential asparagine-linked glycosylation sites, features that are consistent with the export of the polypeptide through the secretory pathway (MacKay et al. 1988). *MATa* cells also produce a mating pheremone, a-factor, but this protein is unusual in that it reaches the cell surface by a route alternative to the secretory pathway (Kuchler et al. 1989; Mcgrath and Varshavsky 1989; Powers et al. 1986).

Killer toxin is secreted by yeast strains harboring killer virus. These strains kill sensitive yeast strains by secreting a protein toxin, and they express a killer immunity component on their own plasma membrane that is derived from the same peptide precursor as the toxin. The 316aa precursor has a 26 residue signal sequence that is cleaved by signal peptidase in the ER. Glycosylation is completed by exit from the ER (i.e., before *sec18* block); further proteolytic processing does not occur until late or post Golgi organelles have been reached (Bussey 1988; Lolle and Bussey 1986). The immunity is thought to be conferred by killer toxin precursor that escapes processing and ends up attached to the plasma membrane at the cell surface (Bussey 1988).

iii. Modifying enzymes resident in the secretion pathway

A) Enzymes responsible for proteolytic cleavage: Soon after soluble secretory proteins are translocated into the endoplasmic reticulum, their signal sequences are removed. A signal peptidase has been purified from dog pancreas

microsomes; it is part of a complex of six small subunits, two of which are glycoproteins (Evans et al. 1986).

Since amino acid substitutions at the signal peptide cleavage site of yeast invertase result in accumulation of core glycosylated precursor forms of the enzyme within the lumen of the ER (Schauer et al. 1985), one might expect to find a signal peptidase mutant among the collection of early *sec* mutants. The *SEC11* gene is the best candidate for the signal peptide cleavage function of the ER as *sec11* is unique among these mutants in that signal peptide cleavage (from both invertase and acid phosphatase) is not carried out. In addition, it it the only one of the original *sec* mutants that does not accumulate amounts of secretory organelles of some sort upon incubation at restrictive temperature (Novick et al. 1980). The gene has been sequenced and the predicted Sec11 protein resembles canine and hen signal peptidases in several respects (Bohni et al. 1988).

The enzymes responsible for the final maturation of α-factor and killer toxin that are located in the trans Golgi or a compartment immediately distal to this, have been better characterized than the signal peptidases. This is mainly because mutants with defects in such functions (*kex1*, *kex2*, *ste13*) were isolated a long time ago. The *kex* (for ***k***iller ***ex***pression) mutants are defective in the production of active killer toxin and *ste13* is a *MATα*-specific *ste*rile mutant (Wickner 1974); Rine, cited in (Herskowitz and Oshima 1981). These genes were subsequently shown to encode enzymes that function in α-factor and killer toxin processing (Julius et al. 1984a); reviewed in (Bussey 1988). In both α-factor and killer toxin, the mature enzyme repeats are flanked by basic residues; two cleavages are necessary to remove these; a dibasic endoprotease activity, Kex2, and a carboyxpeptidase B like activity, Kex1. The α-factor additionally needs to be matured at the N-terminus; this is done by the dipeptidyl amino peptidase (DPAP A or Ste13p) encoded by the *STE13* gene. The well characterized periplasmic and cell wall proteins secreted from yeast do not appear to undergo any of the late Golgi

proteolytic modifications described above, but certain otherwise uncharacterized secreted glycoproteins have altered sizes in the *kex2* mutant as opposed to wild-type cells (Rogers et al. 1979). An additional phenotype of *kex2* mutants is that homozygous *kex2* diploids fail to sporulate (Leibowitz and Wickner 1976). As it has been found that sporulating yeast cells release proteolytic activities into the growth medium (Chen and Miller 1968), it is possible that Kex2 activity is also required for the final processing of such secreted proteases.

B) Glycosylation and deglycosylation activities in the secretion pathway: Most secreted proteins receive carbohydrate modifications as they transit through the pathway. These carbohydrate chains are linked to proteins at specific amino acid residues; asparagine in the case of N-linked oligosaccharides, and serine or threonine for O-linked species. In general, the cell wall mannoproteins receive long, N-linked carbohydrate chains (100-200 mannose), although for the 33 kDa CWP described above, this is not the case; some of them also get short, O-linked chains. The periplasmic space proteins such as invertase receive intermediate-sized N-linked chains (50 mannose), and vacuolar glycoproteins (see below) get short, mannose chains (Kukuruzinska et al. 1987). Some plasma membrane proteins such as Fus1 appear to be only O-glycosylated (Trueheart and Fink 1989). The functional significance of the various carbohydrate attachments received by different proteins biosynthesised in the secretory pathway is unclear at present. Secretory and vacuolar proteins appear to be correctly localized even when they have been incorrectly glycosylated. The mannose found on many cell wall proteins may have a structural role, and the hyperglycosylation of periplasmic proteins like invertase may serve to keep them from diffusing quickly out of the cytoplasm and being lost to the cell in the growth substrate.

In the ER, oligosaccharides consisting of 14 monosaccharides ($GlcNAc_2Man_9Glc_3$) are assembled on a carrier that is embedded in the membrane of the ER. The complex pathway of lipid-linked assembly is defined by the *alg*

(asparagine-linked, glycosylation defective) mutants that were isolated in an elegant mannose suicide selection scheme (Huffaker and Robbins 1982) and was reviewed recently (Kukuruzinska et al. 1987). Transfer of the lipid-linked oligosaccharide to the appropriate asparagine on the polypeptide is catalyzed by oligosaccharyltransferase. Such an activity has been identified and partially characterized in yeast (Sharma et al. 1981). From studies with the *sec* mutants it is known that this transfer is accomplished in the ER; in mammalian systems it even occurs as the nascent polypeptide is entering the ER lumen.

Mutations in the *SEC53* gene interfere with secretory protein folding and glycosylation in the ER lumen but do not affect protein translocation or signal peptide processing (Feldman et al. 1987). This gene encodes a protein that is probably the phosphomannomutase of yeast (Kepes and Schekman 1988). The asparagine-linked, glycosylation defective mutant, *alg4*, is allelic to *sec53* and shows a general defect in the assembly of oligosaccharides on the dolichol carrier (Huffaker and Robbins 1983). *SEC59* is another gene encoding a product required for core glycosylation in the ER. It is a membrane protein apparently involved in the transfer of mannose to dolichol-linked oligosaccharide (Bernstein et al. 1989). Many of the *ALG* genes are also implicated in this ER oligosaccharide assembly process (Kukuruzinska et al. 1987).

After attachment to the protein, the oligosaccharide is trimmed, one mannose and three glucose residues being removed. Two glucosidases and one mannosidase are involved. There is a mutant defective in α1,2 glucosidase activity, *gls1*, that fails to remove the three glucose residues. Even in the absence of this step the mannose can still be removed and outer chain elongation can take place. There is no mannosidase mutant available yet, and there is some disagreement about whether the removal of the terminal mannose is essential for initiation of outer-chain synthesis (Kukuruzinska et al. 1987). The function of these trimming events is unclear.

Outer-chain formation takes place in the Golgi compartments in a stepwise manner. Several mutants (*mnn*) blocked at various stages of outer-chain elongation were initially isolated because of their defects in cell wall carbohydrate synthesis. This was possible because mannose is the major cell wall antigen of yeast; antibodies against such antigens agglutinate wild-type yeast but not mutant cells in which specific determinants are missing. In this manner a mutagenized yeast culture could be enriched for mutants with antigenically altered cell walls. Nine genes were identified and have been reviewed in Ballou (1982). Briefly, the *mnn9* mutant is defective in the formation of α1-6 linkages; the *mnn2* and *mnn5* mutants are defective in the formation of α1-2 linkages; the *mnn4* and *mnn6* mutants fail to transfer mannosylphosphate to mannoses of the outer chain; and *mnn1* mutants are defective in the formation of the final α1-3 linked mannose.

Several of these genes encode mannosyltransferases specific to distinct Golgi compartments. Biochemical analysis has revealed that the product of the *MNN1* gene, mannosyltransferase I, responsible for the formation of a1-3 linkages, is localized in the Golgi in a compartment distinct from that in which the Kex2 protease resides (Cunningham and Wickner 1989). Recent studies of secretion defective mutants, that made use of antisera against the α1-6 and α1-3 mannose linkages, have indicated that from *cis* to *trans* the Golgi compartments sequentially contain α1-6 mannosyltransferase activity, α1-2 mannosyltransferase activity and finally, α1-3 mannosyltransferase activity (Franzusoff and Schekman 1989; Graham and Emr 1991). Studies of HDEL-tagged α-factor (see below), confirm that the α1-6 mannosyl-transferase is the first such activity encountered in the Golgi (Dean and Pelham 1990). As the α1-2 linkages have not been directly examined in these studies, the assignment of this transferase to a distinct *medial* Golgi compartment may be premature.

O-linked glycosylation has been less well characterized but follows the same general progression as described above. The first mannose is transferred to serine or

threonine residues of the protein substrate in the ER. Subsequently, up to four additional O-linked mannose residues are added in the Golgi apparatus (Kukuruzinska et al. 1987). Fatty acylation of certain proteins also takes place in the secretory pathway; this activity has been localized to the ER by examining fatty acylation in *sec* mutants blocked at different stages (Wen and Schlesinger 1984).

iv. Secretion defective mutants

A) Isolation and initial characterization: The first of the *sec*retion defective mutants of yeast (*sec1* and *sec2*) were isolated in a screen for temperature-sensitive (Ts) mutants blocked in the secretion of repressible acid phosphatase (Novick and Schekman 1979). Ts mutants were screened as it was reasoned that the cessation of secretion and presumably plasma membrane expansion would be lethal to the cell. By electron microscopic examination, the two mutants were found to accumulate large numbers of membrane-bound vesicles at the restrictive temperature. The observation that the cells become dense after incubation at the nonpermissive temperature allowed the selection of additional secretory mutants. Dense cells, separated from normal cells on a density gradient, were screened for conditional growth and secretion phenotypes (Novick et al. 1980). This approach permitted the identification of an additional twenty-one complementation groups, all of which accumulate secretory proteins inside the cell at the restrictive growth temperature. All except one of the complementation groups also accumulated excess amounts of secretory organelles at 37°, as determined by electron microscopy. The exception was *sec11*; the *SEC11* gene was subsequently found to encode a protein that is probably a signal peptidase (Bohni et al. 1988), (see below). The other *sec* mutants accumulated either ER, ER and small vesicles, Berkeley bodies (Golgi), vesicles, or some combination of the above (Novick et al. 1980). The distribution of the alleles identified in this screen suggested that complementation groups existed for which no mutant had been found. More recently, the [^{3}H]-mannose

suicide selection, previously used for isolating the *alg* mutants (above), has been used to isolate a further two complemention groups, *bet1* and *bet2*, blocked at the ER stage of the secretion pathway (Newman and Ferro-Novick 1987).

A requirement of the initial screen for *sec* mutants was that secretory proteins such as invertase be accumulated inside the cell in enzymatically active form; this was to eliminate mutants defective in the expression of, for example, the *SUC2* gene for invertase. Among the collection of mutants that failed to secrete invertase and acid phosphatase, there were several that had wild-type protein synthesis levels but accumulated inactive, incompletely glycosylated invertase within the cell (Ferro-Novick et al. 1984b). These genes, *SEC53* and *SEC59*, are required at early stages of secretory protein biosynthesis in the ER (Ferro-Novick et al. 1984a).

An elegant approach was used in two different laboratories to obtain Ts mutants defective in protein translocation into the ER. Such mutants were selected in *his4* or *trp1* yeast on the basis of their ability to retain a fusion protein in the cytoplasm. The fusion protein comprised the prepro region of α-factor, fused N-terminal to His4 or Trp1, respectively. In wild-type cells the fusions, and thus the His or Trp activities, are sequestered in the secretory pathway, and it requires translocation incompetent mutants or signal sequence alterations to retain the marker activities in the cytoplasm where they are of use to the cell (Deshaies and Schekman 1987; Rothblatt et al. 1989; Toyn et al. 1988).

By examining haploid double mutants with different phenotypes in which each member of the pair represents a distinct morphological block, the order of organelles transited in the yeast secretion pathway was determined to be ER→small vesicles→Golgi→vesicles→Cell surface. Secretory proteins enter the ER, where initial glycosylation steps occur. Nine or more *SEC* gene products are required to transfer material from the ER to the Golgi apparatus where further glycosylation events take place. Two or more gene products are necessary for exit from this Golgi organelle into

secretory vesicles. The secretory vesicles require ten more gene products to reach and fuse with the plasma membrane (Novick et al. 1981). A more recent study of *sec* mutants that are blocked at the ER to Golgi stage has made use of the observation that some of these mutants accumulate small vesicles as well as ER at the restrictive temperature. Vesicle accumulation in these mutants is blocked by other ER-accumulating mutations as shown by analysis of double mutants (Kaiser and Schekman 1990). Thus, the gene products that act in transport between the ER and Golgi can be divided into the three responsible for vesicle formation and four others that function in vesicle consumption.

B) Endoplasmic reticulum: Mechanisms must exist in the cell to ensure that proteins are targeted to and subsequently translocated across the ER membrane and not some other membrane accessible to the cell cytoplasm such as the plasma membrane or vacuolar membrane. It is possible that some or all of the translocation Sec proteins (Sec61, Sec62, Sec63) function in the recruitment or adherence of secretory precursors to the ER membrane (Rothblatt et al. 1989). The SRP (signal recognition particle) is a soluble factor required in mammalian cells for targeting nascent secretory proteins to the ER. A yeast homologue 47% identical to one of the mammalian SRP subunits (SRP54) has been isolated, and gene disruption shows that it is essential for growth (Hann et al. 1989). The function of this SRP-related protein in yeast is not known at present. Studies with hybrid proteins have indicated that a signal peptide will suffice to direct some cytoplasmic proteins into the ER (Emr et al. 1984).

Most secreted and vacuolar proteins, as well as the proteins destined to reside at various organelles along the secretory pathway, are translocated into the ER while they are being translated from the messenger RNA, or immediately after they have been translated. The *sec61*, *sec62* and *sec63* mutants all accumulate unglycosylated precursor forms of several different secretory and vacuolar proteins; in the case of invertase and a-factor, translocation is blocked before the signal peptide cleavage stage.

The secretory proteins were found to be membrane-bound but accessible to proteases, suggesting that they had not yet been fully inserted into the lumen of the ER. Two additional alleles *ptl1* and *nlp1* of *sec63* were also isolated. Surprisingly, the *nlp1* mutant was obtained from a selection for mutants with defects in import into the nucleus (Sadler et al. 1989). *NLP1* (*SEC63*) encodes a protein with homology to DnaJ, an *E. coli* heat shock protein. Other heat shock proteins have also been implicated in the translocation of precursor peptides into the ER (Chirico et al. 1988; Deshaies et al. 1988). *SEC62* encodes a 32 kDa protein with potential membrane-spanning regions (Deshaies and Schekman 1989). The *SEC62* gene sequence has not yet been determined. These three mutants show some synthetic growth defects in double mutant combinations, possibly suggesting interactions between them.

Three other *SEC* genes, defined as ER-specific in early studies, have been found to encode proteins that are probably modifying enzymes of the ER. These are *SEC11*, *SEC53* and *SEC59* and were described in Section I iii of this review.

C) Vesicle production and consumption: Exit from the endoplasmic reticulum require a large number of gene products. It was found that the exit from the ER and transit to the Golgi body requires at least two steps: the formation of small transport vesicles (blocked by the *sec12*, *sec13*, *sec16*, and *sec23* mutants at restrictive temperature) and the consumption of these vesicles, which is blocked in *sec17*, *sec18* and *sec22* at restrictive temperature (Kaiser and Schekman 1990).

The *SEC12* gene, defined above as one of those necessary for vesicle production, has been sequenced. It encodes an integral membrane glycoprotein that faces the cytoplasm where it functions to promote protein transport to the Golgi apparatus and perhaps beyond (Nakano et al. 1988). A GTP-binding protein, Sar1p, was isolated as a multicopy suppressor of the *sec12* mutant; gene disruption showed that it is essential for cell growth. With the *SAR1* gene under *GAL1* promoter control, ER precursor forms of secretory proteins are seen to accumulate upon shutoff of

expression in glucose medium (Nakano and Muramatsu 1989). Thus, it is thought that Sar1p and Sec12p cooperate in directing ER to Golgi protein transport, probably at the point of transport vesicle production.

The *SEC23* gene encodes an 84 kDa unglycosylated protein that resides in the cytoplasm as part of a large membranous or cytoskeletal structure (Hicke and Schekman 1989). One *sec23* allele has a rapid onset of the Ts defect, allowing the observation that Sec23p acts at an ER proximal compartment in which α1-6 linked mannose is added to secretory proteins such as α-factor. Using the *sec23* mutant, it has been observed that intercompartmental protein transport events within the Golgi complex and beyond can occur efficiently in the absence of ER to Golgi transport (Graham and Emr 1991).

As mentioned above, *sec18* is thought to act at the vesicle-consumption stage of ER to Golgi transport. Recent studies have implicated the Sec18 protein in transport events through the Golgi apparatus from one stack to the next and in plasma membrane delivery, as well as in the transit from ER to Golgi (Graham and Emr 1991). These observations were possible because the Ts *vps18-1* allele shows remarkably rapid onset of the sorting defect upon shift to 37°C. Thus, radioactively labeled protein could be dispersed throughout the secretory pathway and (upon temperature shift) frozen in place wherever the *VPS18* gene product was necessary for further transport. The *SEC18* gene encodes a protein (Eakle et al. 1988), very similar in sequence to the mammalian NSF (N-ethylmaleimide-sensitive factor) required for vesicle fusion during intercisternal Golgi transport in *in vitro* systems (Wilson et al. 1989).

It is probable that several of the other *sec* mutants originally defined as being necessary for transport from the ER to the Golgi function in the formation and consumption of vesicles for later transport steps as well. In particular, the SNAP components purified from bovine brain cytosol appear to interact with NSF in mammalian *in vitro* systems; the yeast *sec17* mutant is deficient in an activity that can be

replaced with mammalian α-SNAP (Clary et al. 1990). Thus, it is likely that the *SEC18* and *SEC17* gene products will interact in yeast, perhaps to promote transport steps throughout the pathway.

D) Golgi apparatus, transit and exit: As discussed above, several *SEC* gene products appear to function at multiple transport steps along the pathway. This may account for the dearth of *sec* mutants specifically affecting the Golgi apparatus in the original screen (Novick et al. 1980). The only two mutants specifically affecting the Golgi were *sec7* and *sec14*. These mutants accumulate Golgi-like structures and Golgi-modified secretory proteins upon incubation at the restrictive temperature.

The *SEC7* gene encodes a 230 kDa hydrophillic protein that may be peripherally assosiated with the cytoplasmic surface of Golgi membranes (Achstetter et al. 1988; Franzusoff and Schekman 1989). The protein contains an unusual, highly charged acidic domain of 125 amino acids. It is thought that the Sec7p facilitates transport between distinct cisternae of the Golgi apparatus, as at least two stages in Golgi-specific, N-linked glycoprotein maturation depend on *SEC7* (Franzusoff and Schekman 1989).

The *SEC14* gene has been sequenced and found to encode a 37 kDa cytosolic factor (Bankaitis et al. 1989). This has been recently shown to be a phosphatidylinositol/phosphatidylcholine transfer protein in yeast (Bankaitis et al. 1990). A genetic screen for suppressors of the *sec14-1*^{ts} allele yielded mutations in the CDP-choline pathway for phospholipid biosynthesis. One of these suppressor genes was the structural gene for the choline kinase of yeast, disruption of which suppressed the *sec14-1* Ts growth defect (Cleves et al. 1991). These findings are consistent with a phospholipid equilibration function for Sec14p.

The screen for extragenic suppressors of *sec14-1*^{ts} also identified alleles of the *SAC1* gene capable of suppressing the *sec14* growth defect (Cleves et al. 1989). *sac1* mutants were originally identified as suppressors of the yeast *act1-1*^{ts} mutant.

Curiously, the *sac1* suppressor properties also extend to *sec6* and *sec9* defects, although this suppression is not as efficient, being effective only at temperatures lower than 37°. The *sec6* and *sec9* mutants act late in the pathway, accumulating secretory vesicles at restrictive temperature (Novick et al. 1980). These data imply a relationship between Sec14p, Sec6p, Sec9p and actin function in the yeast cell. *sac1* mutants appear to exhibit negative genetic interactions with some other yeast secretory defects that act at the vesicle production/consumption stage described above, the most severe effect being on *sec13-1* (Cleves et al. 1989). The *SAC1* gene was sequenced but no known functional domains were identified; it would be very interesting to know more details of Sac1p action and function.

The *YPT1* gene of *S. cerevisiae* encodes a GTP-binding protein that appears to be located at the Golgi apparatus (Segev et al. 1988). From studies of the accumulated secretory proteins in two different alleles of *ypt1*, it has been suggested that Ypt1p is required for transport from an early stage to a later stage of the Golgi apparatus (Bacon et al. 1989).

Some of the *VPS* (*v*acuolar *p*rotein *s*orting) gene products (see below) may also act in the Golgi apparatus, thought to be the site of vacuolar or lysosomal protein sorting in the secretion pathway (Graham and Emr 1991; Griffiths and Simons 1986).

E) Plasma membrane delivery: Ten *SEC* gene products have been implicated in the final stages of the yeast secretory pathway, blocking transport at the post Golgi stage (Novick et al. 1980). Since all vesicular transport events seem to share the same basic mechanism, some secretory components may be required for all these vesicle transport events; the *SEC18* gene product appears to be such a component (Graham and Emr 1991; Wilson et al. 1989). The nature of the phenotype of the late *sec* mutants precludes them from functioning at earlier stages of the transport pathway, so they are candidates for specific events such as vesicle target recognition.

One of the *SEC* gene products specific for the post-Golgi vesicle transport stage, is the GTP-binding protein, Sec4 (Salminen and Novick 1987). This protein has a cycle of attachment to, and detachment from, the cytoplasmic surface of secretory vesicles that may be coupled to the hydrolysis of GTP (Bourne 1988; Goud et al. 1988). Sec4 shows homology to Ypt1, which is thought to act in the Golgi (see above). Duplication of the *SEC4* gene suppresses post-Golgi blocked mutations in three other *SEC* genes; these are *sec15*, and more weakly, *sec2* and *sec8*. In addition, double mutant combinations of *sec4* with seven other late acting *sec* mutants, including *sec15*, *sec2* and *sec8*, resulted in growth defects or synthetic lethality (Salminen and Novick 1987).

GTP-binding proteins often carry out their function by regulating an effector protein. In view of the genetic studies discussed above, Sec15 was a possible candidate for this effector. The *SEC15* gene was sequenced and found to encode a protein of 116 kDa that has pH-dependent association with yeast microsomal fractions (Salminen and Novick 1989). Interestingly, overproduction of Sec15p interferes with the secretion pathway, resulting in the formation of a cluster of secretory vesicles and a patch of Sec15p as seen by immunofluorescence. The *sec2* and *sec4* mutations prevent formation of this protein patch.

The *SEC2* gene has also been sequenced and found to encode a 759aa protein with an α-helical amino terminal region. This protein is present in the cell in a soluble, high molecular mass complex (Nair et al. 1990). The components of this large complex are not yet known.

II. Biogenesis and maintenance of *Saccharomyces cerevisiae* vacuoles; delivery of components via the secretion pathway.

i. Outline

The process of vacuolar protein delivery is allied to that of secretion in that most of the known vacuolar proteins are biosynthesised in the secretion pathway and transit together with the secreted proteins through the first half of the pathway. At some point, probably in or just after the trans-Golgi compartment, the pathways diverge into two, one for vacuole delivery and one for plasma membrane delivery.

The vacuole of *Saccharomyces cerevisiae* is a membrane-enclosed organelle that contains a variety of hydrolases and other proteins with diverse functions. In addition, the yeast vacuole is the compartment in which metabolites are stored. It also appears to sequester toxic compounds, such as heavy metals and drugs, that have been accumulated by the cell. The yeast vacuole is analogous, but not identical, to the mammalian lysosome. The reader is referred to (Klionsky et al. 1990) for a detailed review of the composition and functions of fungal vacuoles.

Vacuolar constituents can be delivered to this organelle via at least two separate routes. The major and most studied route is the secretory pathway and a branch of this, sometimes referred to as the vacuolar protein targeting or localization pathway. Several soluble vacuolar hydrolases such as CPY and PrA are known to travel via this pathway. Many mutants affecting this branch pathway have been identified. These mutants are collectively known as the *vps*, or *v*acuolar *p*rotein *s*orting mutants. Some other vacuolar components enter the compartment directly from the cytoplasm. Vacuolar proteins such as α-mannosidase and ATPase in addition to toxic compounds and amino acids should probably be included in this group. It is thought that there is a third route of entry to the vacuole from the cell surface via endocytic vesicles.

ii Vacuolar hydrolases

A) Soluble: Many soluble vacuolar hydrolases have been identified. Surprisingly, there appears to be no major shared characteristic that would tell how they

are transported, recognized and sorted from secretory proteins. The more extensively studied of these proteins include Carboxypeptidase Y (CPY), Proteinase A (PRA) and Proteinase B (PrB), reviewed in (Klionsky and Emr 1990). These are all hydrolytic enzymes and presumably aid the vacuole in carrying out its general degredative functions in the cell. Proteinase A is responsible for the activation of a number of vacuolar hydrolases by proteolytically removing the prosegment of their precursors (Zubenko et al. 1983). Some of these vacuolar proteases also function in the process of sporulation (Zubenko and Jones 1981).

These luminal vacuolar proteins pass through the secretory pathway, receiving a series of modifications along the way. Like the secretory proteins, they are translocated into the ER while they are being synthesised from the mRNA, or immediately upon completion of this process. Their NH_2-terminal hydrophobic signal sequence of 18-30 amino acids is similar to that of secreted proteins and functions as a sorting signal for ER targeting and translocation; it is cleaved off within the ER (Blachly-Dyson and Stevens 1987; Johnson et al. 1987; von Heijne 1986). The proteins receive core oligosaccharides in the ER and then pass on through the compartments of the Golgi apparatus where they recieve a series of outer-chain oligosaccharide additions. Upon arrival at the vacuole, the pro segment is cleaved off, releasing the active enzyme. The characteristic precursor forms of the proteins have been useful as markers for secretory pathway and clathrin function, for example (Payne et al. 1988; Stevens et al. 1982). The details of the biosynthesis of these proteins as they pass through the secretory pathway are given in Figure I.

B) Membrane associated: The membrane-associated vacuolar proteins have been less well studied but include Dipeptidyl aminopeptidase B (DPAP B) and Alkaline phosphatase (ALP).

DBAP B is a dipeptidyl aminopeptidase associated with the vacuolar membrane and encoded by the *DAP2* gene (Roberts et al. 1989); it is distinct from the Golgi

localized DPAP A that is encoded by the *STE13* gene. DPAP B is prevented from entering the vacuole in *sec18* and *sec7* cells but not in *sec1* cells, indicating that it is transported to the vacuole via the same portions of the secretion pathway followed by soluble vacuolar hydrolases. DPAP B is 120 kDa glycoprotein that does not appear to undergo proteolytic cleavage upon delivery to the vacuole. The short, NH_2-terminal cytoplasmic tail and transmembrane region of DPAP B are sufficient to target invertase reporter activity to the vacuole (Roberts and Stevens 1990). The *vps* mutants apear to have little effect on the vacuolar delivery of DPAP B (Roberts et al. 1989).

The repressible alkaline phosphatase (ALP) of yeast is the product of the *PHO8* gene (Kaneko et al. 1987). It appears to be an integral membrane protein that is anchored by a hydrophobic domain near its NH_2-terminus. ALP transits through the early stages of the secretory pathway and is activated upon delivery to the vacuole in a *PEP4*-dependent manner. This is achieved by cleavage of the COOH-terminal propeptide (Klionsky and Emr 1989). Gene fusion studies with invertase have indicated that a 52 amino acid NH2-terminal portion of the ALP protein, that includes the transmembrane domain, is necessary and sufficient for vacuolar localization (Klionsky and Emr 1990). The vacuolar localization of ALP appears to be defective in some of the *vps* mutants, though not to the same extent as the soluble enzymes whose vacuolar delivery was measured in the same alleles.

iii. Genetic screens and selections that have yielded vacuole defective mutants

A large number of different mutant selection approaches has yielded a genetically overlapping set of yeast mutants that affect vacuole biogenesis or vacuolar enzyme biosynthesis and function. Many of the selection schemes have made use of our knowledge about the soluble hydrolytic enzymes discussed above, and their activities.

An early screen at a time when not much was known about the secretion pathway for vacuole biogenesis in yeast, made use of the Carboxypeptidase Y activity. Mutants with decreased cellular peptidase (CPY) activity were identified. The screen was for strains with reduced ability to cleave the chymotrypsin substrate N-acetyl-DL-phenylalanine B-naphthyl ester (APE), and was thus a screen for decreased peptidase activity. EMS mutagenesis was carried out. This screen yielded the structural genes for two vacuolar hydrolases; the *PRC1* gene encodes CPY and *PEP4* encodes PrA. Fourteen additional mutants with reduced levels of vacuolar hydrolases and some other vacuole defects (*pep1* to *pep15*) were isolated (Jones 1977).

More recent selection schemes demanded the secretion of vacuolar proteases from the cell. The reasoning was that if passive bulk flow was to the cell surface and vacuolar targeting was by an active process, perturbations in this active sorting process should result in the passive secretion of vacuolar proteins. The first involved a CPY-Inv fusion protein. The selection was for invertase activity of the fusion protein at the cell surface, on the basis of the inaccesibility of uncleaved sucrose to the cell. All the mutants were spontaneous isolates. The original gene designations were *vpt1* to *vpt33*, later altered to *vps1* to *vps35* to accommodate the overlaps with the *vpl* mutants (Table 1; Bankaitis et al. 1986; Robinson et al. 1988; Chapter Two).

The other approach involved the selection for CPY activity at the cell surface of *pep4* cells on the basis of the ability of external CPY to cleave the N-blocked dipeptide

CBZ-pheleu and to release leucine that can be taken up by the cell, allowing *leu* minus auxotrophs to grow. The cells were subjected to EMS mutagenesis to 50% killing for the isolation of *vpl1* to *vpl8*, whereas *vpl9* to *vpl19* were spontaneous isolates (Rothman and Stevens 1986; Rothman et al. 1989).

Several other screens for vacuole defective mutants concentrated on the storage or sequestering activities of the vacuole. Mutants with small amino acid pools were selected on the basis of sensitivity to high concentrations of lysine and screened for small lysine pools by amino acid analysis. The *slp1* mutant was identified (Kitamoto et al. 1988). Calcium sensitive mutants were also isolated and found to have vacuole associated defects.

In an attempt to study endocytosis in yeast, a collection of Ts mutants isolated after EMS mutagenesis to 50% survival was screened for defects in the accumulation of lucifer yellow in the vacuole). The *end1* and *end2* mutants were identified (Chvatchko et al. 1986), but these were subsequently found to have their primary defect in vacuole biogenesis, rather than in endocytosis (Dulic and Riezman 1989).

Screens for mutants with altered vacuolar pH yielded a set of mutants that overlapped with the *pep* and *vps* mutants. These were termed the *vph* mutants (Preston et al. 1989). Genes encoding subunits of the vacuolar ATPase have also been identified, mutations in which lead to a soluble vacuolar protein sorting defect. The subject of vacuole acidification and its relation to sorting has been recently reviewed in (Klionsky et al. 1990).

It has transpired that two mutants originally identified as being sporulation defective, also have a *vps* defect. This is not surprising, given the inability of *pep4* mutants that lack PrA to sporulate. These sporulation defective mutants were *spoT7* and *spo15* (Woolford et al. 1990; Yeh et al. 1991), and references therein.

The genetic overlaps between these different sets of mutants are given in Table 1. The *vpt* and *vpl* sets of mutants were combined and renamed as the *vps* mutants;

they were assigned new numbers where necessary to avoid overlaps. The new *vps* gene designation is also shown in Table 1.

iv. Phenotypic studies of the *vps* and related mutants

Initial phenotypic analysis of the *vps* mutants from this lab indicated that the mutants could be divided into several categories, given their morphological appearance. The most severely defective of the mutants (*vps11*, *vps16*, *vps18* and *vps33*) were termed class C and lacked the large, central vacuole that in wild-type cells can be stained with diagnostic, fluorescent markers. Upon examination by electron microscopy, these mutant cells were seen to accumulate various forms of membranous debris, including probable lipid droplets.

Three other complementation groups (*vps1*, *vps5* and *vps17*) showed fragmented vacuoles; this phenotype was termed Class B morphology. The remaining complementation groups appeared to contain normal, wild-type vacuoles (Class A) in spite of the fact that some of the more defective mutants secrete up to 95% of the soluble vacuolar hydrolase, CPY.

The mutants could be further subdivided on the basis of growth characteristics and on the severity of their vacuolar protein sorting defect. These studies showed that all the Class C mutants were Ts for growth, even though they were already severely defective in vacuolar protein sorting when grown at low temperatures. Two of the complementation groups that had wild-type vacuolar appearance were also Ts for growth (*vps15* and *vps34*). These mutants showed multiple vacuole-associated defects and were >95% defective in the vacuolar targeting and processing of soluble hydrolases.

The remaining non Ts mutants of wild-type vacuolar appearance could be divided into profoundly defective (e.g., *vps35*) and weakly defective (e.g., *vps28*)

categories, given the extent of their sorting defect for the soluble vacuolar protein, CPY.

All the above-mentioned properties of the *vps* mutants are described and documented in greater detail in Chapters Two and Three of this thesis. Recently, many of the *vps* mutants have been sequenced and studied in some detail. One such analysis of a Class C complementation group, *vps18*, is the subject of Chapter Four. A brief discription of recent investigations on *vps* genes and their products, and a discussion of the possible modes of action for some of these gene products is presented in Section III iii B of this Chapter.

III: Protein sorting in the secretion pathway

i. Outline

At every organelle along the secretion pathway, certain proteins must be retained to carry out their functions in that organelle. Other proteins destined for retention in later stages of the pathway or for eventual secretion must pass through the organelle efficiently, lingering only long enough to receive the appropriate modifications. When one considers the complexity of the pathway and the variety of its traffic, described in the preceeding sections of this review, it becomes apparent that this is a complicated problem. In addition to this retention versus flow-through decision taken at every point along the secretion pathway, there is a branch point in the pathway. This is the place at which the secretion pathway diverges into two pathways, one for vacuolar protein delivery to the vacuole and the other for secretory protein delivery to the plasma membrane and beyond. The sorting of proteins into these alternate pathways probably takes place in the last or "*trans*" compartment of the Golgi apparatus or immediately after exit from this compartment. As several pieces of evidence suggest that the plasma membrane-directed branch is the default pathway, it is probable that the sorting of

vacuolar proteins is an active event. The research described in this thesis has been directed toward furthering our knowledge of the vacuole delivery section of the secretion pathway, with the eventual aim of discovering how proteins are sorted into this pathway away from the plasma membrane-delivery pathway.

ii. Retention versus flowthrough

A) Endoplasmic reticulum. Numbers of secretory proteins pass rapidly through the ER on their way to destinations such as the cell surface. However, another set of proteins, translocated into the ER in the same manner, does not continue through the later stages of the secretory pathway. Several observations on yeast and mammalian systems have lent support to the hypothesis that the passage of soluble proteins through the secretory pathway is passive and is probably achieved by bulk flow (see Rothman 1987 for a brief review). If this is indeed the case, there must be some active method by which the cell retains specific proteins in the lumen of the ER. Work with mammalian cells has revealed that certain abundant ER localized proteins such as GRP78 (which is a member of the HSP70 heat shock protein family and is also known as BiP) have a short, conserved sequence at their C-terminus (Munro and Pelham 1987). It has been postulated that this C-terminal KDEL sequence is the retention signal and that proteins with this signal are recycled into the ER from a later compartment such as the cis-Golgi rather than held in place in the ER (Pelham 1988).

To determine whether this was also the case in yeast, and to establish a genetic system for the study of ER retention, the C-terminal sequence of the yeast homologue of GRP78 was studied. This protein has the C-terminal sequence HDEL instead of KDEL (Rose et al. 1989). Experiments with complicated gene fusions from heterologous systems, with yeast invertase as the reporter activity, suggested that the HDEL (rather than KDEL) sequence plus some more amino acids (from mammalian GRP78) functioned in the ER retention of this manmade protein in yeast (Pelham et al.

1988). The fusion protein was used to select for mutants that released invertase activity into the growth medium, instead of retaining it in the ER, allowing the cells to grow on sucrose as their sole carbon source (Pelham et al. 1988). Two complementation groups, *erd1* and *erd2* (for *E*R *r*etention *d*efective) were identified.

The *ERD1* gene encodes a nonessential integral membrane protein of 362 amino acids that appears to enter the secretory pathway (Hardwick et al. 1990). The *erd1* mutant cells show a serious disruption of the ER retention system. In addition, they show defects in the Golgi-dependent modification of glycoproteins. The precise function of Erd1 is unknown, but it has been suggested to facilitate the interaction of the HDEL sequence and its receptor in the cis-Golgi.

The *ERD2* gene is also required for ER retention of HDEL-containing proteins. It encodes a 26 kDa integral membrane protein. Deletion of the *ERD2* gene results in lethality, and ER-like membranes accumulate in *ERD2*-depleted cells. The vacuolar hydrolase CPY is accumulated in the p2 (Golgi-associated) form in the mutant cells, suggesting an additional function in Golgi transport for the Erd2 protein (Semenza et al. 1990). The expression level of *ERD2* in the cell determines the capacity of the HDEL retention system, and therefore Erd2 has been proposed to be the receptor for HDEL. Experiments with the homologue of *ERD2* from *Kluyveromyces lactis* support this hypothesis (Lewis et al. 1990).

The glycosylation patterns of two different secretory proteins of yeast tagged with HDEL were examined. These were the tagged invertase described above and a construct that encodes prepro α-factor attached to a C-terminal HDEL sequence; neither is secreted from the cell. Both the proteins are partially modified by Golgi-specific mannosyltransferases. The α-factor construct receives α1-6 mannose linkages in a *SEC18*-dependent manner and receives no detectable α1-3 additions, suggesting that it penetrates no further than an early compartment of the Golgi. Cell fractionation indicated that the Golgi-modified form of α-factor was in the ER fraction, suggesting

that it had been recycled to the ER from the compartment in which it received the α1-6 modification (Dean and Pelham 1990). A flaw of this experiment is that both the proteins tagged with HDEL were originally secreted proteins, and might have had some characteristic encouraging their forward movement through the secretory pathway.

Antisera raised against a short peptide containing the HDEL sequence, recognise only a few protein species in yeast (Hardwick et al. 1990). This indicates that additional ER proteins probably have other ways of staying in the ER. For example, it has been suggested that many ER membrane proteins remain in the ER by aggregating to form large complexes that cannot exit from this organelle (Pelham 1989).

B) Golgi: The problem of retaining proteins in the Golgi is conceptually the same as that for ER resident proteins. Aspects of the problem have been discussed in (Pfeffer and Rothman 1987); the authors favor the model in which Golgi-retained proteins are aggregated into moderate-sized patches in the membrane bilayer. These aggregates or immobile phases would be too large to become incorporated into exiting secretory vesicles. As there are several Golgi compartments, each compartment would need its own immobile phase specifically able to accept resident proteins of that particular compartment. Clearly, this is a problem of great complexity, and our knowledge on this topic is not very advanced at present.

In mammalian cells, clathrin has been implicated in the concentration and sorting of various receptors in Golgi and post-Golgi membranes, including the plasma membrane. It appears to be involved in the processes of mannose-6-phosphate receptor-mediated transport of lysosomal enzymes and in the formation of hormone-containing secretory granules. In addition, clathrin appears to increase the efficiency of receptor-mediated endocytosis. It is probably not involved in the export of membrane and constitutively secreted proteins (reviewed in Brodsky 1988).

In contrast, the role of clathrin in yeast, as determined by the examination of strains in which the clathrin heavy chain homologue had been deleted (Payne et al.

1987), appears to be somewhat different. The main function attributed to clathrin in yeast has been that of keeping the late Golgi protein Kex2 resident at its correct location. In *chc1* (*c*lathrin *h*eavy *c*hain-deleted) cells, the Kex2 protein is inefficient at performing its normal function of α-factor processing. This is not surprising, as Kex2p is found at the plasma membrane in *chc1* mutant cells, whereas it remains intracellular in wild-type cells (Payne and Schekman 1989). In *chc1* cells, secretion of Invertase occurs normally, indicating that the constitutive secretion pathway of yeast does not require clathrin (Payne and Schekman 1985). Protein transport to the vacuole in clathrin heavy chain deficient yeast also appears normal, as assessed by pulse-chase examination of the soluble vacuolar hydrolases CPY and PrA (Payne et al. 1988).

On the basis of this information, several models have been proposed for the action of clathrin in yeast cells (Payne and Schekman 1989). The feature common to all of these models is that clathrin functions in some manner to retain the membrane-associated Kex2 endoprotease in the organelle where it is active, probably the last, or trans, cisterna of the Golgi apparatus (Cunningham and Wickner 1989; Graham and Emr 1991).

The retention of other resident Golgi proteins of yeast has not yet been investigated in any depth. At present the gene fusion approach is being applied to study the retention of the *MNN1* gene product, α1-3 mannosyltransferase, in the Golgi apparatus and to select mutants defective in the Golgi retention of this protein (T. Graham, personal communication). As discussed above, this protein appears to reside in a Golgi compartment distinct from that occupied by the Kex2 protein (Cunningham and Wickner 1989; Graham and Emr 1991).

iii. Vacuolar protein sorting

A) Sorting signals of vacuolar proteins. In mammalian cells the process of protein sorting to the lysosome seems relatively orderly and logical. The main step of

the sorting process is the generation of phosphomannosyl residues on soluble proteins destined for the lysosome. This is achieved by a phosphorylating enzyme that must recognise a protein determinant shared by lysosomal enzymes. Thus far, the exact nature of this protein domain is not known (Lang et al. 1984; Valls et al. 1990). A further modification provides the proteins destined for the lysosome with the characteristic mannose-6-phosphate marker (Pfeffer and Rothman 1987). The marked proteins are efficiently separated from secreted proteins by high-affinity binding to the mannose-6-phosphate receptors and exit the Golgi via clathrin coated vesicles from which they are delivered to the lysosome (Dahms et al. 1989). Membrane-bound lysosomal proteins appear to be sorted by a different mechanism, as none of those examined possess mannose-6-phosphate additions (von Figura and Hasilik 1986).

In contrast, the sorting of proteins to the vacuole of yeast does not rely on tagging with mannose-6-phosphate. CPY that has been deprived of its carbohydrate still arrives in the vacuole, suggesting that no carbohydrate modifications of any kind play a part in yeast vacuolar protein sorting (Schwaiger et al. 1982).

It has not yet been possible to identify any distinctive protein sequence or structure, common to the known vacuolar proteins, that might act as a sorting and targeting signal specific for the vacuole. This is in spite of the fact that this problem has been intensively studied, reviewed in (Klionsky et al. 1990). For example, recent mutational studies of the *PRC1* gene that encodes CPY have demonstrated that four contiguous amino acids of the propeptide define the vacuolar targeting signal for this particular protein (Valls et al. 1990). From the lack of an identifiably conserved sorting signal, it is tempting to imagine that each vacuolar protein may have its own sorting receptor. If it were the case, one might have expected at least one of the *vps* mutants to be defective in the sorting of CPY, but not of other vacuolar hydrolases. However, there is no clear-cut example of this phenotype; instead, many of the *vps* mutants have their most severe defect in the localization of CPY and are slightly less defective, but

not wild-type, in their PrA localization phenotype. This difference appears most marked in the *vps35* mutant (Paravicini, submitted).

The idea that vacuolar membrane proteins are sorted in a manner that is different from the sorting of soluble vacuolar components, or that they transit to the vacuole in separate transport vesicles or structures is regarded as a possibility. This is based on the following observations. 1) Most *vps* mutants possess a vacuolelike compartment enclosed by a membrane (Banta et al. 1988), in which DPAP B can be seen by immunofluorescence (C. Roberts, personal communication), while they mislocalize soluble vacuolar proteins such as CPY to the cell surface. 2) ALP is apparently not mislocalized to as great an extent as CPY in most of the *vps* mutants. 3) Modifications in the acidification state of vacuolar and possibly prevacuolar compartments, either by mutation of particular genes or by incubation with certain chemicals, result in the missorting of soluble vacuolar hydrolases but not of the membrane-bound ALP and DPAP B, reviewed in (Klionsky et al. 1990). 4) Overproduction of the soluble vacuolar protein CPY results in its secretion, possibly because of the saturation of some component of the sorting apparatus (Stevens et al. 1986). In contrast, 20 fold overproduction of the membrane protein, DPAP B, does not result in surface activity of this enzyme (Roberts et al. 1989). At this level of expression, however, DPAP B does process α-factor in a *ste13* mutant, suggesting that the DPAP B is mislocalized into the *trans* Golgi or a later compartment of the secretory pathway and substitutes there for the DPAP A activity that is absent in the *ste13* mutant.

This last point may indicate a difference in the default pathways for soluble versus membrane protein transport (Roberts and Stevens 1990), rather than a difference in the vacuolar protein-targeting pathways. But as little is known about the recycling of membrane and membrane proteins from the plasma membrane of yeast into the interior of the cell, it seems premature to speculate on this point. For example, by analogy to the apparent recycling of HDEL signal-containing ER proteins from the Golgi, Golgi

resident proteins could well have a signal that allows them to be salvaged from the plasma membrane. As DPAP B is closely related to the DPAP A protein (DPAP A is probably a resident of the *trans* Golgi (Bussey 1988)), DPAP B may be recognized by this Golgi protein-specific salvage mechanism if the mislocalized vacuolar membrane protein were to reach the cell surface (in a *vps* mutant or after being overproduced). This may account for the different behavior of the two vacuolar membrane proteins ALP and DPAP B in the *vps* mutants (Klionsky and Emr 1989; Roberts et al. 1989), in that no known relative of the ALP protein normally resides in the Golgi apparatus.

B) Components (Vps proteins) necessary for transport and sorting of vacuolar proteins. In an effort to begin to understand the mechanism of vacuolar protein sorting, most of the more phenotypically interesting *vps* mutants that were isolated in our screen (Chapter Two) have been cloned and sequenced. These are as follows. Class C, *VPS11* (=*PEP5*=*END1*) (Dulic and Riezman 1989; Woolford et al. 1990); *VPS16* (Horazdovsky and Emr, unpublished); *VPS18* (=*PEP3*)(Robinson, Chapter Four; Preston and Jones, unpublished); *VPS33* (=*SLP1*) (Banta et al. 1990; Wada et al. 1990). Class B, *VPS1* (=*SPO15*) (Rothman et al. 1990; Yeh et al. 1991); *VPS5* (Horazdovsky and Emr, unpublished); *VPS17* (Koorer and Emr, unpublished). Class A and Ts, *VPS15* (Herman et al. 1991); *VPS34* (Herman and Emr 1990). Class A, *VPS3* (Raymond et al. 1990); *VPS35* (Paravicini and Emr, submitted for publication). It is interesting to note that in no case does disruption of these genes lead to cell inviability under optimal growth conditions. However, in several cases a Ts growth defect has been observed, this may indicate that the vacuole is essential to the cell only under suboptimal growth conditions. This subject is discussed in Chapter Four.

On the basis of the phenotypes observed for the *vps* mutants, the various *VPS* gene products are expected to act in the secretion pathway, the vacuolar targeting pathway and the branch point of these routes..A brief description of the known

properties of some of the *VPS* genes and gene products is given below. On the basis of these data, a speculative model for the mode and place of action of each of these gene products is given in Figure 2. This model should be regarded as a provocation for discussion and future experiments, rather than as a cautious and sober interpretation of the data available at present.

The *vps8* and *vps9* mutants were seen to accumulate and secrete some p1 CPY (Chapter Two; Robinson, unpublished observations). p1 CPY is normally converted to p2 CPY in a *cis* or *medial* Golgi compartment (see Figure 1), so this mutant phenotype suggests an early site of action for the *VPS8* and *VPS9* gene products. On the basis of this information, Vps8 and Vps9 are proposed to have a general sorting and retention function as indicated in the model shown in Figure 2. They could function in retention/sorting at all compartments of the Golgi, either directly or indirectly, maybe by altering the local environment in the Golgi; alternatively, they may promote secretion to the cell surface from an earlier compartment of the Golgi than is normal. These potentially interesting genes have not been studied further.

The *vps16* mutants display pleiotropic phenotypes that include greatly reduced or absent vacuoles (Banta et al. 1988) and a defect in the processing of α-factor (Robinson and Graham, unpublished observations). The *VPS16* gene encodes a hydrophillic, nonglycosylated protein of 92 kDa. Fractionation studies indicate that the *VPS16* protein is associated with a non-membranous proteinaceous or cytoskeletal complex in the cell cytoplasm (Horazdovsky, personal communication). Thus, it appears that Vps16 may function at the *trans* Golgi compartment where vacuolar protein sorting takes place. Because of its association with a large nonmembranous component of the cell, the Vps16 protein has been given the role of an anchor or address for the *trans* Golgi compartment in the model presented in Figure 2. Without Vps16 one might speculate that the *trans* Golgi compartment would not form correctly, and protein sorting that was supposed to take place in this compartment would not happen.

The *vps18* and *vps33* mutants display pleiotropic phenotypes identical to those of *vps16*, described above. The predicted protein encoded by the *VPS18/PEP3* gene is 918 amino acids in size and hydrophillic in nature. It has a COOH-terminal domain with a cysteine-rich repeat that has been shown by site-directed mutagenesis to be functional in vacuolar protein sorting. The loss of the Vps18 function not only affects vacuolar protein targeting and biogenesis but also leads to a partial defect in α-factor processing by Kex2. This suggests that Vps18 is required to maintain the functional organization or integrity of a *trans* Golgi compartment containing the Kex2 protease (Chapter Four). The *VPS33/SLP1* gene encodes a protein of 75 kDa that behaves as a cytosolic protein in cell fractionation studies (Banta et al. 1990; Wada et al. 1990). The sequence of Vps33 shows a region of similarity with ATP-binding proteins (Banta et al. 1990). However, site-directed mutagenesis of an amino acid in this motif does not lead to a *vps* defect (Banta, personal communication). Based on these observations, the Vps18 and Vps33 proteins are shown next to the *trans* Golgi in the model in Figure 2 to indicate that they may function in maintaining the integrity or the organization of this organelle. Maybe in the absence of these gene products, the *trans* Golgi becomes disorganised such that transport vesicles bud from the wrong places and at the wrong times leading to all the pleiotripic phenotypes displayed by these mutants.

vps35 mutants have a vacuole of wild-type appearance. They secrete all of their CPY, but only 50% of another soluble vacuolar hydrolase, PrA. The*VPS35* gene codes for a protein 110 kDa in size, most of which associates with a particulate cell fraction that, similar to the complex that Vps16 associates with, is not composed of membranes (Paravicini et al., submitted for publication). Thus it is possible that the Vps35 protein is a signal or receptor for efficient sorting of particular soluble hydrolases away from the cell surface and towards the vacuolar protein packaging apparatus (Figure 2). In the absence of *VPS35* function the filling and transport of vacuole directed vesicles would occur normally, but these vesicles would lack certain

soluble vacuolar proteins that were not removed from the bulk flow pathway, and were thus transported efficiently to the cell surface.

The *vps15* and *vps34* mutants display interesting common phenotypes unique to these two complementation groups. They are severely defective in the localization of many soluble vacuolar hydrolases to the vacuole and display genetically linked Ts and Os growth defects (Robinson et al. 1988). In spite of these severe defects, the vacuole of these mutants has a normal, wild-type appearance (Banta et al. 1988). *VPS15* encodes a nonglycosylated 170 kDa protein with homology to protein kinases; mutations within the Vps15 kinase domain result in the secretion of vacuolar hydrolases. Fractionation studies indicate that the Vps15 protein is associated with the cytoplasmic face of a membranous compartment, either Golgi or vesicles, possibly by NH_2-terminal myristate. It has been proposed that protein phosphorylation involving Vps15p may play a role in vacuolar protein sorting (Herman et al. 1991). A protein of 95 kDa is encoded by the *VPS34* gene, and like vps16, it is associated with a particulate fraction of yeast cells (Herman and Emr 1990). The Vps34p may interact with Vps15p as determined by both genetic and biochemical studies (Stack, personal communication). It is possible that this Vps15/Vps34 protein complex is a central component that carries out the function of packaging or filling vacuolar transport vesicles with soluble proteins (Figure 2).

Several of the *vps1* alleles have a very interesting morphological phenotype in that they contain many small fragmented vacuole like structures and do not display a large central vacuole upon entry into stationary phase, in contrast to wild-type cells (Banta et al. 1988). The *VPS1/SPO15* gene encodes a GTP-binding protein of 85 kDa with sequence similarity to Mx proteins of mammals (Rothman et al. 1990) and to dynamin (Yeh et al. 1991). Antisera against Vps1p label punctate cytoplasmic structures that condense to larger structures in the Golgi-accumulating *sec7* mutant (Rothman et al. 1990). It is possible that the cytoplasmic structures with which Vps1 is

associated are cytoskeletal in nature (Yeh et al. 1991). On the basis of these observations, it is proposed that Vps1 is responsible for the correct movement of vacuolar transport vesicles along a cytoskeletal "track" to the vacuole (Figure 2). Because Vps1 is an abundant protein compared to most of the other Vps proteins described here, a structural function for Vps1 in anchoring transport vesicles to a cytoskeletal track seems to be an attractive hypothesis.

Two other complementation groups had alleles with vacuolar morphologies the same as *vps1* mutants. These were *vps5* and *vps17*. On the basis of this vacuole morphology, the model presented in Figure 2 suggests that these two proteins have a role in the coalesence of the vacuole; i.e., not only are they necessary for the coalesence of the small vacuole fragments to form a large central vacuole, but also for the fusion of transport vesicles with the vacuolar compartment(s). Unpublished observations on the Vps5 and Vps17 proteins indicate that they are both of relatively high abundance in the cell (Horazdovsky and Kohrer, personal communication). Again, they could have some structural role, perhaps in seeding of anchoring the nascent vacuole and thus inducing the formation of a single central vacuole.

vps3 mutants appear to have a defect in vacuole acidification and mislocalize >90% of their newly synthesised CPY. They may also have a defect in the partioning of vacuoles into the newly growing bud. The Vps3p is a cytoplasmic protein of 110 kDa (Raymond et al. 1990). In addition, perturbations of the function of vacuolar ATPase and proteolipid appear also to cause vacuolar protein sorting defects. These proteins are probably localized at the vacuole surface. It is unclear why the acidification state of the vacuole would affect the sorting step that takes place at an earlier stage in the pathway. It is possible that vacuole directed vesicles cannot fuse efficiently with an incorrectly acidified vacuolar compartment and are thus shunted to the cell surface. Alternatively, the proteins responsible for the correct acidification of the vacuole may also involved in the maintenance of correct pH throughout the secretory pathway or, at

least, in the *trans* Golgi compartment. If the *trans* Golgi pH were perturbed, it is possible that sufficient disorganization would result to account for the incorrect sorting of some vacuolar proteins and their delivery to the cell surface.

The *vps11* mutants showed the same vacuole negative phenotype as the *vps16*, *vps18* and *vps33* mutants discussed above. The *VPS11/PEP5/END1* gene encodes a hydrophillic protein of 107-118 kDa. The predicted protein sequence shows a short region of similarity to the ATP-binding sites shared by ATPases (Dulic and Riezman 1989). The COOH-terminal region of the protein sequence shows a cysteine-rich repeat (Chapter Four). This protein was detected in extracts from logarithmically growing cells, but not from stationary phase cells. Cell fractionation studies indicate that it may be peripherally attached to the vacuolar membrane (Woolford et al. 1990). Therefore, the Vps11 protein is proposed to function at the vacuole to promote recycling of important sorting components to the *trans* Golgi compartment (Figure 2). With the depletion of these components a serious disruption in the organization and sorting capabilities of the sorting compartment may occur. As it is not yet known if recycling of proteins from the vacuole to the Golgi apparatus takes place in yeast this model is speculative, but it is the most straightforward way of reconciling the vacuole localization of the Vps11p with the Golgi defective phenotype observed in the *vps11* mutants.

REFERENCES

Achstetter, T., A. Franzusoff, C. Field and R. Schekman. 1988. *SEC7* encodes an unusual, high molecular-weight protein required for membrane traffic from the yeast Golgi apparatus. *J. Biol. Chem.* **263**: 11711-11717.

Bacon, R. A., A. Salminen, H. Ruohola, P. Novick and S. Ferro-Novick. 1989. The GTP-binding protein Ypt1 is required for transport *in vitro*: The Golgi apparatus is defective in *ypt1* mutants. *J. Cell Biol.* **109**: 1015-1022.

Ballou, C. (1982). Yeast cell wall and cell surface. *in* The Molecular Biology of the Yeast Saccharomyces cerevisiae: Metabolism and Gene Expression. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory.

Bankaitis, V., J. Aitken, A. Cleves and W. Dowhan. 1990. An essential role for a phospholipid transfer protein in yeast Golgi function. *Nature*. **347**: 561-562.

Bankaitis, V., D. Malehorn, S. Emr and R. Green. 1989. The *Saccharomyces cerevisiae SEC14* gene encodes a cytosolic factor that is required for transport of secretory proteins from the yeast Golgi complex. *J. Cell Biol.* **108**: 1271-1281.

Bankaitis, V. A., L. M. Johnson and S. D. Emr. 1986. Isolation of yeast mutants defective in protein targeting to the vacuole. *Proc. Natl. Acad. Sci. USA*. **83**: 9075-9079.

Banta, L. M., J. S. Robinson, D. J. Klionsky and S. D. Emr. 1988. Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting. *J. Cell Biol.* **107**: 1369-1383.

Banta, L. M., T. A. Vida, P. K. Herman and S. D. Emr. 1990. Characterization of Yeast Vps33p, a Protein Required for Vacuolar Protein Sorting and Vacuole Biogenesis. *Mol. Cell. Biol.* **1990**: 4638-4649.

Bernstein, M., F. Kepes and R. Schekman. 1989. *SEC59* encodes a membrane protein required for core glycosylation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **9**(3): 1191-1199.

Blachly-Dyson, E. and T. H. Stevens. 1987. Yeast carboxypeptidase Y can be translocated and glycosylated without its amino-terminal signal sequence. *J. Cell Biol.* **104**: 1183-1191.

Blumer, K., J. Reneke and J. THorner. 1988. The *STE2* gene product is the ligand-binding component of the α-factor receptor of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **263**: 10836-10842.

Bohni, P., R. Deshaies and R. Schekman. 1988. *SEC11* is required for signal peptide processing and yeast cell growth. *J. Cell Biol.* **106**: 1035-1042.

Bourne, H. 1988. Do GTPases direct membrane traffic in secretion? *Cell.* **53**: 669-671.

Bowman, B. J. and E. J. Bowman. 1986. H+-ATPases from mitochondria, plasma membrane, and vacuoles of fungal cells. *J. Membrane Biol.* **94**: 83-97.

Brada, D. and R. Schekman. 1988. Coincident localization of secretory and plasma membrane proteins in organelles of the yeast secretory pathway. *J. Bact.* **170**: 2775-2783.

Brodsky, F. 1988. Living with clathrin: its role in intracellular membrane traffic. *Science.* **242**: 1396-1402.

Bussey, H. 1988. Proteases and the processing of precursors to secreted proteins in yeast. *Yeast.* **4**: 17-26.

Carlson, M. and D. Botstein. 1982. Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. *Cell.* **28**: 145-154.

Chen, A. and J. Miller. 1968. Proteolytic activity of intact yeast cells during sporulation. *Can. J. microbiol.* **14**: 957-963.

Chirico, W. J., M. G. Waters and G. Blobel. 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature.* **332**: 805.

Chvatchko, Y., I. Howald and H. Riezman. 1986. Two yeast mutants defective in endocytosis are defective in pheromone response. *Cell.* **46**: 355-364.

Clary, D., I. Griff and J. Rothman. 1990. SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell.* **61**: 709-721.

Cleves, A., T. McGee, E. Whitters, K. Champion, J. Aitken, W. Dowhan, M. Goebel and V. Bankaitis. 1991. Mutations in the CDP-choline pathway for phospholipid biosynthesis bypass the requirement for an essential phospholipid transfer protein. *Cell.* **64**: 789-800.

Cleves, A., P. Novick and V. Bankaitis. 1989. Mutations in the *SAC1* gene suppress defects in yeast Golgi and yeast actin function. *J. Cell Biol.* **109**: 2939-2950.

Cunningham, K. W. and W. T. Wickner. 1989. Yeast Kex2 protease and mannosyltransferase I are localized to distinct compartments of the secretory pathway. *Yeast.* **5**: 25-33.

Dahms, N. M., P. Lobel and S. Kornfeld. 1989. Mannose 6-phosphate receptors and lysosomal enzyme targeting. *J. Biol. Chem.* **264**: 12115-12118.

Dean, N. and H. R. B. Pelham. 1990. Recycling of proteins from the Golgi compartment to the ER in yeast. *J. Cell Biol.* **111**: 369-377.

Deshaies, R. and R. Schekman. 1989. *SEC62* encodes a putative membrane protein required for protein translocation into the yeast endoplasmic reticulum. *J. Cell Biol.* **109**: 2653-2664.

Deshaies, R. J., F. Kepes and P. C. Bohni. 1989. Genetic dissection of the early stages of protein secretion in yeast. *Trends in Genetics.* **5**(3): 87-93.

Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig and R. Schekman. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature*. **332**: 800.

Deshaies, R. J. and R. Schekman. 1987. A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J. Cell Biol.* **105**: 633-645.

Dulic, V. and H. Riezman. 1989. Characterization of the *END1* gene required for vacuole biogenesis and gluconeogenic growth of budding yeast. *EMBO J.* **8**(5): 1349-1359.

Eakle, K. A., M. Bernstein and S. D. Emr. 1988. Characterization of a component of the yeast secretion machinery: Identification of the *SEC18* gene product. *Mol. Cell. Biol.* **8**: 4098-4109.

Emr, S. D., R. Schekman, M. C. Flessel and J. Thorner. 1984. Invertase beta-galactosidase hybrid proteins fail to be transported from the endoplasmic reticulum in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **4**: 2347-2355.

Esmon, P. C., B. E. Esmon, T. E. Schauer, A. Taylor and R. Schekman. 1987. Structure, assembly, and secretion of octameric invertase. *J. Biol. Chem.* **262**: 4387-4394.

Evans, E., R. Gilmore and G. Blobel. 1986. Purification of microsomal signal peptidase as a complex. *Proc. Natl. Acad. Sci., USA*. **83**: 581-585.

Feldman, R. I., M. Bernstein and R. Schekman. 1987. Product of *SEC53* is required for folding and glycosylation of secretory proteins in the lumen of the yeast endoplasmic reticulum. *J. Biol. Chem.* **262**(19): 9332-9339.

Ferro-Novick, S., W. Hansen, I. Schauer and R. Schekman. 1984a. Genes required for completion of import of proteins into the endoplasmic reticulum in yeast. *J. Cell Biol.* **98**: 44-53.

Ferro-Novick, S., P. Novick, C. Field and R. Schekman. 1984b. Yeast secretory mutants that block the formation of active cell surface enzymes. *J. Cell Biol.* **98**: 35-43.

Field, C. and R. Schekman. 1980. Localized secretion of acid phosphatase reflects the pattern of cell surface growth in *Saccharomyces cerevisiae*. *J. Cell Biol.* **86**: 123-128.

Franzusoff, A. and R. Schekman. 1989. Functional compartments of the yeast Golgi apparatus are defined by the *sec7* mutation. *EMBO J.* **8**: 2695-2702.

Goud, B., A. Salminen, N. Walworth and P. Novick. 1988. A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. *Cell.* **53**: 753-768.

Graham, T. and S. Emr. 1991. Sec18/NSF protein is required for multiple steps in the secretory pathway: evidence for the compartmental organization. *J. Cell Biol.* (in press).

Griffiths, F. and K. Simons. 1986. The trans Golgi network: sorting at the exit site of the Golgi complex. *Science.* **234**: 438-443.

Haguenauer-Tsapis, R. and A. Hinnen. 1984. A deletion that includes the signal peptidase cleavage site impairs processing, glycosylation, and secretion of cell surface yeast acid phosphatase. *Mol. Cell. Biol.* **4**: 2668-2675.

Hann, B., M. Poritz and P. Walter. 1989. *Saccharomyces cerevisiae* and *Scizosaccharomyces pombe* contain a homologue to the 54-kD subunit of the signal recognition particle that in *S. cerevisiae* is essential for growth. *J. Cell Biol.* **109**: 3223-3230.

Hardwick, K., M. Lewis, J. Semenza, N. Dean and H. Pelham. 1990. *ERD1*, a yeast gene required for the retention of luminal endoplasmic reticulum proteins, affects glycoprotein processing in the Golgi apparatus. *EMBO J.* **9**: 623-630.

Hasilik, A. and W. Tanner. 1978. Biosynthesis of the vacuolar yeast glycoprotein carboxypeptidase Y. Conversion of precursor into the enzyme. *Eur. J. Biochem.* **85**: 599-608.

Hemmings, B. A., G. S. Zubenko, A. Hasilik and E. W. Jones. 1981. Mutant defective in processing of an enzyme located in the lysosomelike vacuole of *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA.* **78**: 435-439.

Herman, P. K. and S. D. Emr. 1990. Characterization of *VPS34*, a gene required for vacuolar protein sorting and vacuole segregation in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **10**: 6742-6754.

Herman, P. K., J. H. Stack, J. A. DeModena and S. D. Emr. 1991. A novel protein kinase homolog essential for protein sorting to the yeast lysosomelike vacoule. *Cell.* **64**: 425-437.

Herskowitz, I. and Y. Oshima. (1981). Control of cell type in *Saccharomyces cerevisiae*: mating type and mating type interconversion. *in* The Molecular Biology of the Yeast *Saccharomyces*: Life Cycle and Inheritance. CSH, NY, Cold Spring Harbor Laboratory.

Hicke, L. and R. Schekman. 1989. Yeast Sec23p acts in the cytoplasm to promote protein transport from the endoplasmic regiculum to the Golgi complex *in vivo* and *in vitro*. *EMBO J.* **8**: 1677-1684.

Holcomb, C., W. Hansen, T. Etcheverry and R. Schekman. 1988. Secretory vesicles externalize the major plasma membrane ATPase in yeast. *J. Cell Biol.* **106**: 641-648.

Huffaker, T. and P. Robbins. 1982. Temperature-sensitive yeast mutants deficient in asparagine-linked glycosylation. *J. Biol. Chem.* **257**: 3203-3210.

Huffaker, T. and P. Robbins. 1983. Yeast mutants deficient in protein glycosylation. *Proc. Natl. Acad. Sci., USA.* **80**: 7466-7470.

Johnson, L. M., V. A. Bankaitis and S. D. Emr. 1987. Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protein. *Cell.* **48**: 875-885.

Jones, E. W. 1977. Proteinase Mutants of *Saccharomyces cerevisiae*. *Genetics*. **85**: 23-33.

Julius, D., A. Brake, L. Blair, R. Kunisawa and J. Thorner. 1984a. Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-α-factor. *Cell*. **37**: 1075-1089.

Julius, D., R. Schekman and J. Thorner. 1984b. Glycosylation and processing of prepro-α-factor through the yeast secretory pathway. *Cell*. **36**: 309-318.

Kaiser, C. A. and R. Schekman. 1990. Distinct sets of *SEC* genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell*. **61**: 723-733.

Kaneko, Y., N. Hayashi, A. Toh-e, I. Banno and Y. Oshima. 1987. Structural characteristics of the *PHO8* gene encoding repressible alkaline phosphatase in *Saccharomyces cerevisiae*. *Gene*. **58**: 137-148.

Kepes, F. and R. Schekman. 1988. The yeast *SEC53* gene encodes phosphomannomutase. *J. Biol Chem*. **263**: 9155-9161.

Kitamoto, K., K. Yoshizawa, Y. Ohsumi and Y. Anraku. 1988. Mutants of *Saccharomyces cerevisiae* with defective vacuolar function. *J. Bact*. **170**: 2687-2691.

Klionsky, D. J. and S. D. Emr. 1989. Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. *EMBO J*. **8**: 2241-2250.

Klionsky, D. J. and S. D. Emr. 1990. A new class of lysosomal/vacuolar protein sorting signals. *J. Biol. Chem.* **265**: 5349-5352.

Klionsky, D. J., P. K. Herman and S. D. Emr. 1990. The fungal vacuole: Composition, function, and biogenesis. *Microbiol. Rev.* **54**: 266-292.

Kominami, E., H. Hoffschulte and H. Holzer. 1981. Purification and properties of proteinase B from yeast. *Biochim. Biophys. Acta.* **661**: 124-135.

Kuchler, K., R. Sterne and J. Thorner. 1989. *Saccharomyces cerevisiae STE6* gene product: a novel pathway for protein export in eukaryotic cells. *EMBO J.* **8**: 3973-3984.

Kukuruzinska, M., M. Bergh and B. Jackson. 1987. Protein glycosylation in yeast. *Ann Rev Biochem.* **56**: 915-944.

Lang, L., M. Reitman, J. Tang, R. M. Roberts and S. Kornfeld. 1984. Lysosomal enzyme phosphorylation. *J. Biol. Chem.* **259**: 14663-14671.

Leibowitz, M. and R. Wickner. 1976. A chromosomal gene required for killer plasmid expression, mating, and spore maturation in *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci., USA.* **73**: 2061-2065.

Lewis, M., D. Sweet and H. Pelham. 1990. The *ERD2* gene determines the specificity of the lumenal ER protein retention system. *Cell.* **61**: 1359-1363.

Lolle, S. and H. Bussey. 1986. *In vivo* evidence for posttranslational translocation and signal cleavage of the killer preprotoxin of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **6**: 4274-4280.

MacKay, V., S. Welch, M. Insley, T. Manney, J. Holly, G. Saari and M. Parker. 1988. The *Saccharomyces cerevisiae BAR1* gene encodes an exported protein with homology to pepsin. *Proc. Natl. Acad. Sci., USA*. **85**: 55-59.

Mcgrath, J. and A. Varshavsky. 1989. The yeast *STE6* gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. *Nature*. **340**: 400-404.

Mechler, B., H. Hirsch, H. Muller and D. H. Wolf. 1988. Biogenesis of the yeast lysosome (vacuole): biosynthesis and maturation of proteinase yscB. *EMBO J.* **7**: 1705-1710.

Mechler, B., M. Muller, H. Muller, F. Meussdoerffer and D. F. Wolf. 1982. *In vivo* biosynthesis of the vacuolar proteinases A and B in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **257**: 11203-11206.

Moehle, C. M., M. W. Aynardi, M. R. Kolodny, F. J. Park and E. W. Jones. 1987a. Protease B of *Saccharomyces cerevisiae*: isolation and regulation of the *PRB1* structural gene. *Genetics*. **115**: 255-263.

Moehle, C. M., C. K. Dixon and E. W. Jones. 1989. Processing pathway for protease B of *Saccharomyces cerevisiae*. *J. Cell Biol.* **108**: 309-324.

Moehle, C. M., R. Tizard, S. K. Lemmon, J. Smart and E. W. Jones. 1987b. Protease B of the lysosomelike vacuole of the yeast Saccharomyces cerevisiae is homologous to the subtilisin family of serine proteases. *Mol. Cell. Biol.* **7**: 4390-4399.

Munro, S. and H. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell.* **48**: 899-907.

Nair, J., H. Muller, M. Peterson and P. Novick. 1990. Sec2 protein contains a coiled-coil domain essential for vesicular transport and a dispensable carboxy terminal domain. *J. Cell Biol.* **110**: 1897-1909.

Nakano, A., D. Brada and R. Schekman. 1988. A membrane glycoprotein, Sec12p, required for protein transport from the endoplasmic reticulum to the Golgi apparatus in yeast. *J. Cell Biol.* **107**: 851-863.

Nakano, A. and M. Muramatsu. 1989. A novel GTP-binding protein, Sar1p, is involved in transport from the endoplasmic reticulum to the Golgi apparatus. *J. Cell Biol.* **109**: 2677-2691.

Newman, A. and S. Ferro-Novick. 1987. Characterization of new mutants in the early part of the yeast secretion pathway isolated by a [3H]-mannose suicide selection. *J. Cell Biol.* **105**: 1587-1594.

Novick, P., S. Ferro and R. Schekman. 1981. Order of events in the yeast secretory pathway. *Cell.* **25**: 461-469.

Novick, P., C. Field and R. Schekman. 1980. Identification of 23 complementation groups required for posttranslational events in the yeast secretory pathway. *Cell.* **21**: 205-215.

Novick, P. and R. Schekman. 1979. Secretion and cell surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci., USA.* **76**: 1858-1862.

Novick, P. and R. Schekman. 1983. Export of major cell surface proteins is blocked in yeast secretory mutants. *J. Cell Biol.* **96**: 541-547.

Payne, G. and R. Schekman. 1985. A test of clathrin function in protein secretion and cell growth. *Science.* **230**: 1009-1014.

Payne, G. and R. Schekman. 1989. Clathrin: a role in the intracellular retention of a Golgi membrane protein. *Science.* **245**: 1358-1365.

Payne, G. S., D. Baker, E. van Tuinen and R. Schekman. 1988. Protein transport to the vacuole and receptor-mediated endocytosis by clathrin heavy chain-deficient yeast. *J. Cell Biol.* **106**: 1453-1461.

Payne, G. S., T. B. Hasson, M. S. Hasson and R. Schekman. 1987. Genetic and biochemical characterization of clathrin-deficient Saccharomyces cerevisiae. *Mol. Cell. Biol.* **7**: 3888-3898.

Pelham, H. 1988. Evidence that lumenal ER protteins are sorted from secreted proteins in a post-ER compartment. *EMBO J.* **7**: 913-918.

Pelham, H. 1989. Control of protein exit from the endoplasmic reticulum. *Ann. Rev. Cell Biol.* **5**: 1-23.

Pelham, H. R. B., K. G. Hardwick and M. J. Lewis. 1988. Sorting of soluble ER proteins in yeast. *EMBO J.* **7**: 1757-1762.

Pfeffer, S. R. and J. E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Ann. Rev. Biochem.* **56**: 829-852.

Powers, S., S. Michaelis, D. Broek, S. SantaAnna, J. Field, I. Herskowitz and M. Wigler. 1986. *RAM*, a gene of yeast required for a functional modification of *RAS* proteins and for production of mating pheremone a-factor. *Cell.* **47**: 413-422.

Preston, R. A., R. F. Murphy and E. W. Jones. 1989. Assay of vacuolar pH in yeast and identification of acidification-defective mutants. *Proc. Natl. Acad. Sci. U.S.A.* **86**: 7027-7031.

Raymond, C. K., P. J. O'Hara, G. Eichinger, J. H. Rothman and T. H. Stevens. 1990. Molecular analysis of the yeast *VPS3* gene and the role of its product in vacuolar protein sorting and vacuolar segregation during the cell cycle. *J. Cell Biol.* **111**: 877-892.

Roberts, C. and T. Stevens. 1990. The sorting of membrane proteins in *Saccharomyces cerevisiae*. *J. Cell Biol.* **111**: 202a.

Roberts, C. J., G. Pohlig, J. H. Rothman and T. H. Stevens. 1989. Structure, biosynthesis, and localization of dipeptidyl aminopeptidase B, an integral membrane glycoprotein of the yeast vacuole. *J. Cell Biol.* **108**: 1363-1373.

Robinson, J. S., D. J. Klionsky, L. M. Banta and S. D. Emr. 1988. Protein sorting in *Saccharomyces cerevisiae*: Isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol. Cell. Biol.* **8**: 4936-4948.

Rogers, D., D. Saville and H. Bussey. 1979. *Saccharomyces cerevisiae* killer expression mutant *kex2* has altered secretory proteins and glycoproteins. *Biochem. Biophys. Res. Com.* **90**: 187-193.

Rose, J. and R. Doms. 1988. Regulation of protein export from the endoplasmic reticulum. *Ann. Rev. Cell Biol.* **4**: 257-288.

Rose, M., L. Misra and J. Vogel. 1989. *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. *Cell.* **57**: 1211-1221.

Rothblatt, J., R. Deshaies, S. Sanders, G. Daum and R. Schekman. 1989. Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum in yeast. *J. Cell Biol.* **109**: 2641-2652.

Rothman, J. 1987. Protein sorting by selective retention in the endoplasmic reticulum and Golgi stack. *Cell.* **50**: 521-522.

Rothman, J. H., I. Howald and T. H. Stevens. 1989. Characterization of genes required for protein sorting and vacuolar function in the yeast *Saccharomyces cerevisiae*. *EMBO J.* **8**: 2057-2065.

Rothman, J. H., C. K. Raymond, T. Gilbert, P. J. O'Hara and T. H. Stevens. 1990. A putative GTP-binding protein homologous to interferon-inducible Mx proteins performs an essential function in yeast protein sorting. *Cell.* **61**: 1063-1074.

Rothman, J. H. and T. H. Stevens. 1986. Protein sorting in yeast: mutants defective in vacuole biogenesis mislocalization vacuolar proteins into the late secretory pathway. *Cell.* **47**: 1041-1051.

Sadler, I., A. Chiang, T. Kurihara, J. Rothblatt, J. Way and P. Silver. 1989. A yeast gene important for protein assembly into the endoplasmic reticulum and the nucleus has homology to DnaJ, an *Eschericia coli* heat shock protein. *J. Cell Biol.* **109**: 2665-2675.

Salminen, A. and P. Novick. 1987. A ras-like protein is required for a post-Golgi event in yeast secretion. *Cell.* **49**: 527-538.

Salminen, A. and P. Novick. 1989. The Sec15 protein responds to the function of the GTP-binding protein, Sec4, to control vesicular traffic in yeast. *J. Cell Biol.* **109**: 1023-1036.

Sanz, P., E. Herrero and R. Sentandreu. 1987. Secretory pattern of a major integral mannoprotein of the yeast cell wall. *Biochem. Biophys. Acta.* **924**: 193-203.

Schauer, I., S. Emr, C. Gross and R. Schekman. 1985. Invertase signal and mature sequence substitutions that delay intercompartmental transport of active enzymes. *J. Cell Biol.* **100**: 1664-1675.

Schekman, R. and V. Brawley. 1979. Localized deposition of chitin on the yeast cell surface in response to mating pheremone. *Proc. Natl. Acad. Sci., USA.* **76**: 645-649.

Schmidt, R., M. Manolson and M.-R. Chevallier. 1984. Photoaffinity labeling and characterization of the cloned purine-cytosine transport system in *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci., USA.* **81**: 6276-6280.

Schwaiger, A. Hasilik, K. von Figura, A. Wiemken and W. Tanner. 1982. Carbohydrate-free carboxypeptidase Y is transferred into the lysosomelike yeast vacuole. *Biochem. Biophys. Res. Com.* **104**: 950-956.

Segev, N., J. Mulholland and D. Botstein. 1988. The yeast GTP-binding Ypt1 protein and a mammalian counterpart are associated with the secretion machinery. *Cell.* **52**: 915-924.

Semenza, J., K. Hardwick, N. Dean and H. Pelham. 1990. *ERD2*, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell.* **61**: 1349-1357.

Sharma, C., L. Lehle and W. Tanner. 1981. N-glycosylation of yeast proteins. *Eur. J. Biochem.* **116**: 101-108.

Silve, S., C. Volland, C. Garnier, R. Jund, M. Chevallier and R. Haguenauer-Tsapis. 1991. Membrane insertion of uracil permease, a polytopic yeast plasma membrane protein. *Mol. Cell. Biol.* **11**: 1114-1124.

Stevens, T., B. Esmon and R. Schekman. 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell.* **30**: 439-448.

Stevens, T. H., J. H. Rothman, G. S. Payne and R. Schekman. 1986. Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y. *J. Cell Biol.* **102**: 1551-1557.

Toyn, J., A. R. Hibbs, P. Sanz, J. Crowe and D. I. Meyer. 1988. *In vivo* and *in vitro* analysis of *ptl1*, a yeast Ts mutant with a membrane-associated defect in protein translocation. *EMBO J.* **7**(13): 4347-4353.

Trueheart, J. and G. Fink. 1989. The yeast cell fusion protein Fus1 is O-glycosylated and spans the plasma membrane. *Proc. Natl. Acad. Sci., USA.* **86**: 9916-9920.

Valls, L., J. Winther and T. Stevens. 1990. Yeast carboxypeptidase Y vacuolar targeting signal is defined by four propeptide amino acids. *J. Cell Biol.* **111**: 361-368.

Van-Rijn, H., P. Boer and E. Steyn-parve. 1972. Biosynthesis of acid phosphatase of baker's yeast. Factors influencing its production by protoplasts and characterization of the secreted enzyme. *Biochem. Biophys. Acta.* **268**: 431-441.

von Figura, K. and A. Hasilik. 1986. Lysosomal enzymes and their receptors. *Ann. Rev. Biochem.* **55**: 167-193.

von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucl. Acids Res.* **14**: 4683-4690.

Wada, Y., K. Kitamoto, T. Kanbe, K. Tanaka and Y. Anraku. 1990. *SLP1* gene of *Saccharomyces cerevisiae* is essential for morphogenesis and function of the vacuole. *Mol. Cell. Biol.* : **10**: 2214-2223.

Wen, D. and M. Schlesinger. 1984. Fatty acid-acylated proteins in secretory mutants of *Saccharomyces cerevisiae. Mol. Cell. Biol.* **4**: 688-694.

Wickner, R. 1974. Chromosomal and nonchromosomal mutations affecting the "killer character" of *Saccharomyces cerevisiae. Genetics.* **76**: 423-432.

Wilson, D. W., C. A. Wilcox, G. C. Flynn, E. Chen, W.-J. Kuang, W. J. Henzel, M. R. Block, A. Ulrich and J. E. Rothman. 1989. A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature.* **339**: 355-359.

Woolford, C. A., C. K. Dixon, M. F. Manolson, R. Wright and E. W. Jones. 1990. Isolation and characterization of *PEP5*, a gene essential for vacuolar biogenesis in *Saccharomyces cerevisiae. Genetics.* **125**: 739-752.

Yeh, E., R. Driscoll, M. Coltrera, A. Olins and K. Bloom. 1991. A dynaminlike protein encoded by the yeast sporulation gene *SPO15. Nature.* **349**: 713-715.

Zubenko, G. and E. Jones. 1981. Protein degradation, meiosis and sporulation in proteinase-deficient mutants of *Saccharomyces cerevisiae*. *Genetics*. **97**: 45-64.

Zubenko, G. S., F. J. Park and E. W. Jones. 1983. Mutations in *PEP4* locus of *Saccharomyces cerevisiae* block final step in maturation of two vacuolar hydrolases. *Proc. Natl. Acad. Sci., USA*. **80**: 510-514.

FIGURE 1 Biosynthesis of soluble vacuolar hydrolases

Biosynthesis of the soluble vacuolar hydrolases CPY, PrA and PrB during transit through the secretory pathway and delivery to the vacuole. The proteins are represented by arrows, the parts remaining after the final processing step are black, the signal sequences are white and the other portions removed by proteolytic cleavage are shown in grey. N-linked oligosaccharide additions are represented by: core = black dot on stick, elongated chain = hatched line attached to this. O-linked oligosaccharides are represented by: core = open circle, outer chain = slanted line attached to this. The progressive proteolytic cleavages are indicated by trimming in the appropriate places. The data were taken from the following sources, CPY (Deshaies et al. 1989; Hasilik and Tanner 1978; Hemmings et al. 1981; Johnson et al. 1987; Stevens et al. 1982); PrA (Deshaies et al. 1989; Hasilik and Tanner 1978; Hemmings et al. 1981; Johnson et al. 1987; Stevens et al. 1982); and Prb (Klionsky et al. 1990; Kominami et al. 1981; Mechler et al. 1988; Mechler et al. 1982; Moehle et al. 1987a; Moehle et al. 1989; Moehle et al. 1987b).

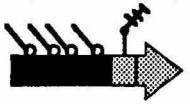
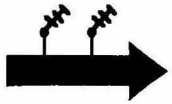
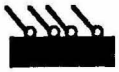
COMPARTMENT	MODIFICATIONS	*PRC1* 1596bp **Carboxypeptidase Y**	*PEP4* 1215bp **Proteinase A**	*PRB1* 1905bp **Proteinase B**
pretranslocation (Cytoplasm)	protein synthesis translocation	ppCPY (20 aa signal sequence) 	ppPrA (22 aa signal sequence) 	ppPrB (19aa signal sequence + 261aa) 
Endoplasmic Reticulum	signal sequence cleavage core glycosylation	p1CPY (67kDa) 	p1PrA (48kDa) 	p1PrB (39-41kDa) 
Golgi (cis) Golgi (medial) Golgi (trans)	elongation of core oligosaccharides	p2CPY (69kDa) 	p2PrA (52kDa) 	p2PrB (40-42kDa)  p3PrB (37kDa) 
Vacuole	proteolytic removal of propeptide segment	mCPY (61kDa) 	mPrA (42kDa) 	mPrB (31-33kDa) 

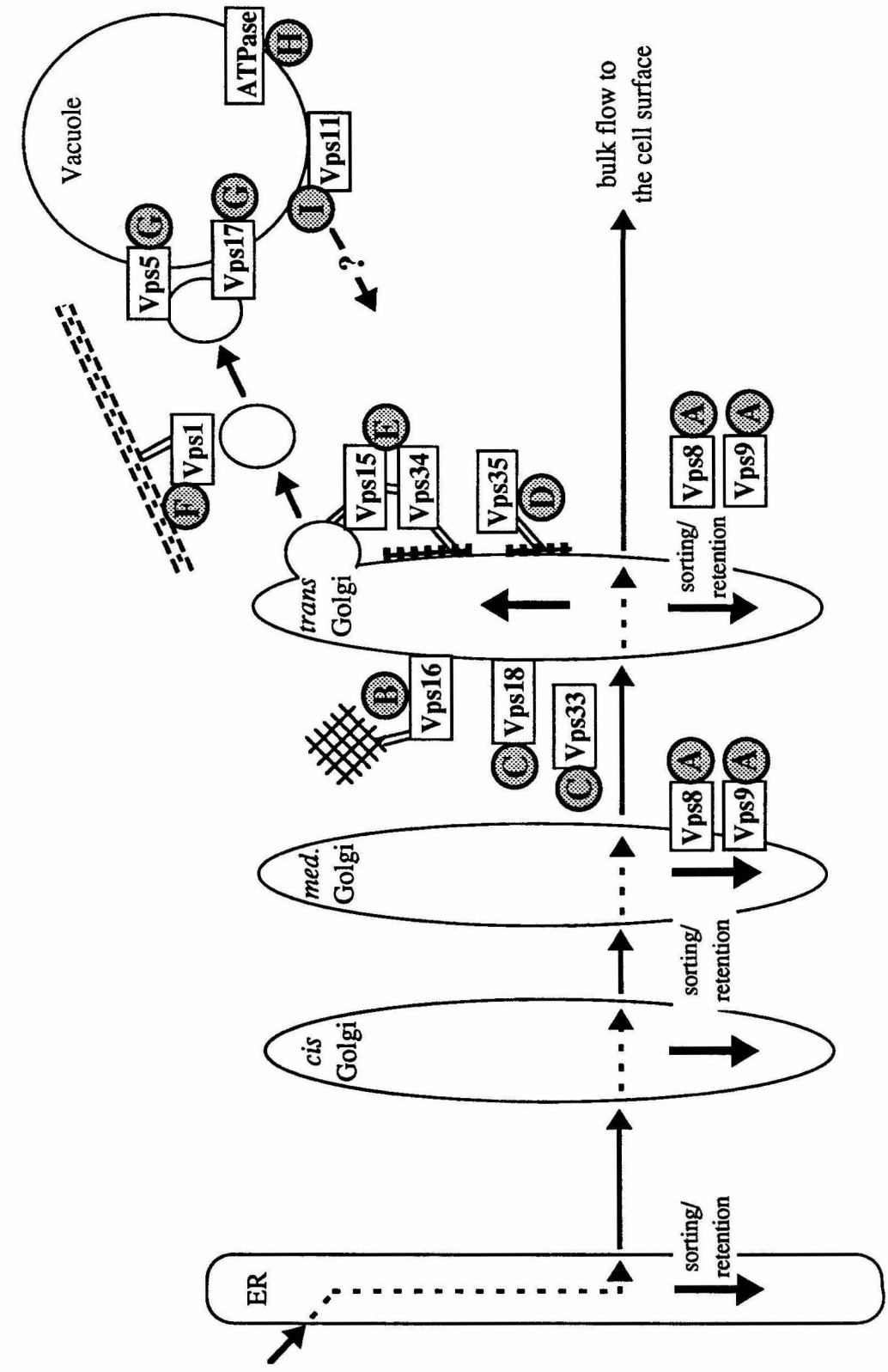
TABLE 1 Genetic overlaps among the vacuole defective mutants

Genetic overlaps among the vacuole defective mutants of yeast (Klionsky et al. 1990; Robinson et al. 1988; Rothman et al. 1989).

vps	*vpt*	*vpl*	*pep*	other
vps1	*vpt26*	*vpl1*	-	*spo15*
vps2	-	*vpl2*	-	-
vps3	*vpt17*	*vpl3*	*pep6*	-
vps4	*vpt10*	*vpl4*	-	-
vps5	*vpt5*	*vpl5*	*pep10*	-
vps6	*vpt13*	*vpl6*	*pep12*	-
vps7	-	*vpl7*	*pep15*	-
vps8	*vpt8*	*vpl8*	-	-
vps9	*vpt9*	-	-	-
vps10	*vpt1*	-	-	-
vps11	*vpt11*	*vpl9*	*pep5*	*end1,vam1,cls13*
vps12	*vpt12*	-	-	-
vps13	*vpt2*	-	-	-
vps14	*vpt14*	-	-	-
vps15	*vpt15*	-	-	-
vps16	*vpt16*	-	-	*vam9*
vps17	*vpt3*	-	*pep21*	-
vps18	*vpt18*	-	*pep3*	*vam8*
vps19	*vpt19*	-	*pep7*	-
vps20	*vpt20*	*vpl10*	-	-
vps21	*vpt21*	-	-	-
vps22	*vpt22*	*vpl14*	-	-
vps23	*vpt23*	*vpl15*	-	-
vps24	*vpt24*	-	-	-
vps25	*vpt25*	*vpl12*	-	-
vps26	*vpt4*	-	*pep8*	-
vps27	*vpt27*	-	-	-
vps28	*vpt28*	*vpl13*	-	-
vps29	*vpt6*	-	-	-
vps30	*vpt30*	-	-	-
vps31	*vpt31*	-	-	-
vps32	*vpt32*	-	-	-
vps33	*vpt33*	-	*pep14*	*slp1,vam5,cls14*
vps34	*vpt29*	-	-	-
vps35	*vpt7*	-	-	-
vps36	-	*vpl11*	-	-
vps37	-	*vpl16*	-	-
vps38	-	*vpl17*	-	-
vps39	-	*vpl18*	-	-
vps40	-	*vpl19*	-	-
-	-	-	*pep1*	-
-	-	-	*pep2*	-
-	-	-	*pep9*	-
-	-	-	*pep11*	-
-	-	-	*pep13*	-
-	-	-	*pep16*	-
-	-	-	-	*tfp1,vma1*
-	-	-	-	*vat2,vma1*
-	-	-	-	*proteolipid*

FIGURE 2 Speculative model for vacuolar protein sorting

A very speculative model for vacuolar protein sorting in the secretion pathway of yeast based on the currently available data. The figure is labeled with letters within grey circles to represent the following proposed fuctions that might be carried out by the Vps proteins as indicated on the figure. **(A)** General retention and sorting function in the Golgi apparatus, not necessary for the integrity of the organelle, just for the sorting/retention step. **(B)** A specific label on the structural element that anchors the *trans* Golgi compartment, without it the transport vesicles arriving from the *medial* Golgi do not have an address to accumulate in, leading to a profound disruption in *trans* Golgi function and default secretion of many proteins. **(C)** Additional elements that direct the functional integrity of the *trans* Golgi compartment by sorting, retaining and organizing membrane associated and soluble proteins at this organelle. **(D)** Signal or receptor for efficient sorting of particular soluble vacuolar hydrolases, especially CPY, away from the cell surface directed bulk flow and towards the packaging apparatus. **(E)** Protein complex involved in packaging soluble vacuolar hydrolases that have already been "labeled" and sorted from the secretory bulk flow. May function by phosphorylating the "label" or some element of the transport vesicle, to promote packaging or departure of this vesicle to its vacuolar destination. With this function impaired, empty vesicles go to the vacuole. **(F)** Correct tracking of vacuolar transport vesicles along a cytoskeletal element towards the vacuole. **(G)** Coalescence of vesicles to form vacuole. **(H)** Maintanance of correct vacuolar environment. **(I)** Maintains integrity of vacuole and pre-vacuolar compartments; maybe by promoting the recycling of sorting components to the Golgi; without this function a profound breakdown in Golgi organization/sorting may occur as a secondary result of a defect in sorting back from the vacuole.


ER
sorting/
retention
cis
Golgi
sorting/
retention
med.
Golgi
sorting/
retention
trans
Golgi
bulk flow to
the cell surface
Vacuole
ATPase
H
Vps11
I
?
Vps5
G
Vps17
G
F
Vps1
Vps15
E
Vps34
Vps35
D
B
Vps16
C
Vps18
C
Vps33
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Vps9
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Vps9
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Chapter 2

Protein sorting in *Saccharomyces cerevisiae*: Isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases.

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Protein Sorting in *Saccharomyces cerevisiae*: Isolation of Mutants Defective in the Delivery and Processing of Multiple Vacuolar Hydrolases

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Using a selection for spontaneous mutants that mislocalize a vacuolar carboxypeptidase Y (CPY)-invertase fusion protein to the cell surface, we identified vacuolar protein targeting (*vpt*) mutants in 25 new *vpt* complementation groups. Additional alleles in each of the eight previously identified *vpt* complementation groups (*vpt1* through *vpt8*) were also obtained. Representative alleles from each of the 33 *vpt* complementation groups (*vpt1* through *vpt33*) were shown to exhibit defects in the sorting and processing of several native vacuolar proteins, including the soluble hydrolases CPY, proteinase A, and proteinase B. Of the 33 complementation groups, 19 were found to contain mutant alleles that led to extreme defects. In these mutants, CPY accumulated in its Golgi complex-modified precursor form which was secreted by the mutant cells. Normal protein secretion appeared to be unaffected in the *vpt* mutants. The lack of significant leakage of cytosolic markers from the *vpt* mutant cells indicated that the vacuolar protein-sorting defects associated with these mutants do not result from cell lysis. In addition, the observation that the precursor rather than the mature forms of CPY, proteinase A, proteinase B were secreted from the *vpt* mutants was consistent with the fact that mislocalization occurred at a stage after Golgi complex-specific modification, but before final vacuolar sorting of these enzymes. Vacuolar membrane protein sorting appeared to be unaffected in the majority of the *vpt* mutants. However, a subset of the *vpt* mutants (*vpt11*, *vpt16*, *vpt18*, and *vpt33*) was found to exhibit defects in the sorting of a vacuolar membrane marker enzyme, α-mannosidase. Up to 50% of the α-mannosidase enzyme activity was found to be mislocalized to the cell surface in these *vpt* mutants. Seven of the *vpt* complementation groups (*vpt3*, *vpt11*, *vpt15*, *vpt16*, *vpt18*, *vpt29*, and *vpt33*) contained alleles that led to a conditional lethal phenotype; the mutants were temperature sensitive for vegetative cell growth. This temperature-sensitive phenotype has been shown to be recessive and to cosegregate with the vacuolar protein-sorting defect in each case. Tetrad analysis showed that *vpt3* mapped to the right arm of chromosome XV and that *vpt15* mapped to the right arm of chromosome II. Intercrosses with other mutants that exhibited defects in vacuolar protein sorting or function (*vpl*, *sec*, *pep*, and *end* mutants) revealed several overlaps among these different sets of genes. Together, these data indicate that more than 50 gene products are involved, directly or indirectly, in the process of vacuolar protein sorting.

A basic and fundamental question in biology is that of how a cell is constructed spatially from the information encoded by its genes and from maternally inherited cues and structures. This spatially segregated cellular organization is critical for normal cell functioning. To understand the process, it is important to know not only how the cytoskeletal elements fit together and define the overall shape and structure of the cell but also how proteins, structural and otherwise, find their way to appropriate locations within the cell. An analysis of the assembly and maintenance of each organelle individually could provide a view of the mechanism underlying the cellular construction process.

Protein sorting to mammalian lysosomes represents one of the best-characterized protein delivery pathways (19, 37). Much is known about the route that is followed by lysosomal enzymes, and a few components of the sorting machinery have been characterized at the biochemical and molecular levels. However, this is a complex process and we still lack a detailed understanding of the molecular mechanisms underlying this sorting pathway. We chose to work on protein sorting to the yeast equivalent of the mammalian lysosome, the vacuole. The yeast *Saccharomyces cerevisiae* is the organism of choice because we wished to take a genetic approach to solving the vacuolar protein targeting problem. The vacuole is an acidic compartment that is involved in amino acid and inorganic ion storage, as well as various degradative and nutrient recycling functions, especially under starvation conditions (20, 43). It contains a variety of hydrolytic enzymes, including the soluble glycoproteins carboxypeptidase Y (CPY), proteinase A (PrA), and proteinase B (PrB), and several membrane-bound enzymes, including an α-mannosidase, a proton-translocating ATPase, dipeptidylaminopeptidase B, and several permeases (43, 44).

Vacuolar proteins transit along with proteins destined for secretion through the early stages of the secretory pathway (38). The transport of the luminal enzymes CPY and PrA has been studied in the greatest detail (18, 18a, 35a). Each of these enzymes is synthesized initially as an inactive preproenzyme that is translocated into the lumen of the endoplasmic reticulum, where the transient N-terminal signal peptide is removed and the proteins are modified with an N-linked core oligosaccharide (12). These intermediate precursor forms are referred to as p1CPY and p1PrA. The p1 forms are then delivered to the Golgi complex, where further glycosyl modifications convert them to larger p2 forms. At this stage, it is presumed that proteins on their way to the vacuole are sorted from other secretory proteins destined for

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secretion or assembly into the plasma membrane (9, 11, 38). Vesicular carriers are presumed to mediate Golgi complex-vacuole transfer of these enzymes. At present there is no evidence concerning the existence of an intermediate compartment between the Golgi complex and the vacuole in *S. cerevisiae* analogous to the endosomal compartment detected in mammalian cells. Just prior to, or more likely upon, arrival in the vacuole, the p2 forms of CPY and PrA are proteolytically cleaved to their mature forms (22, 23). This proteolytic maturation is dependent on the presence of active vacuolar PrA, and involves the removal of N-terminal prosegments from each protein (13). Similarly, other vacuolar enzymes such as PrB also appear to be made and transported in their precursor forms and to undergo PrA-dependent proteolytic maturation in the vacuole (21, 22; C. Moehle, C. K. Dickxon, and E. Jones, J. Cell Biol., in press).

The observations that mutations in the CPY vacuole sorting signal or high gene dosage-induced overproduction of CPY and PrA lead to missorting and secretion of these proteins indicate that a failure to sort vacuolar proteins results in their delivery to the cell surface (15, 39, 41). This rationale was used by Rothman and Stevens (35) to identify mutants that were defective in vacuolar protein sorting by isolating yeast strains that secreted CPY. Through this approach, they identified mutants that were assigned to eight complementation groups (referred to as *vpl*, for vacuolar protein localization-defective mutants). Independently, we used a gene fusion-based selection scheme to isolate mutants defective in vacuolar protein targeting (*vpt*) (2). By this approach, mutants defective in the vacuolar sorting of a CPY-invertase (Inv) hybrid protein (CPY-Inv433) (15) were selected. Eight *vpt* complementation groups were identified and shown to missort and secrete the CPY-Inv hybrid protein as well as the wild-type CPY protein (2). The advantages of the Inv fusion approach include the following. (i) The identification of mutants is not dependent on their secretion of active CPY; (ii) the gene fusion approach, in theory, could be applied to any vacuolar protein whether its biochemical activity was known or not; and (iii) the absence or presence of external Inv activity can be easily assayed, and powerful genetic selection procedures have been developed that demand the presence of external Inv activity.

In this study we greatly extended the gene fusion approach to isolate and biochemically characterize some 500 new *vpt* mutants. Here we describe the genetic and biochemical analysis of these vacuolar protein-targeting mutants. Our results indicate that many different gene functions participate in the events associated with normal vacuolar protein sorting. This process is likely to involve several distinct steps, including the selective recognition of vacuolar proteins, their packaging into transport vesicles, delivery of these vesicles to the correct target organelle, recognition and fusion with the target, release of the vacuolar proteins, and recycling of transport components for additional rounds of protein sorting.

MATERIALS AND METHODS

Yeast strains. *S. cerevisiae* SEY6210 (*MATα leu2-3,112 ura3-52 his3-Δ200 trpl-Δ901 lys2-801 suc2-Δ9 GAL*) and SEY6211 (*MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 GAL*) were constructed by standard genetic methods (36). Both strains contained a complete deletion (*suc2-Δ9*) of the chromosomal copy of *SUC2* and contained no other unlinked Inv structural genes (7). SEY6210 and SEY6211 were transformed to uracil independence with either of two CPY-Inv fusion-encoding plasmids, pCYI-50 and pCYI-433 (15). *vpt* mutant alleles *vpt1-1* through *vpt8-1* (2) in the SEY2101 (*MATa ura3-52 leu2-3,112 suc2-Δ9 ade2-101*) (7) strain background were used for complementation tests with new *vpt* mutants isolated in strain SEY6210 (this study). They were also crossed with strain SEY6210 to give *MATα lys2-801 vpt1-1* through *vpt8-1* strains for crossing with new *vpt* mutants isolated from SEY6211.

Media and genetic methods. YPD medium, minimal yeast medium, sporulation medium, and bromocresol purple indicator plates were prepared as described previously (36). The bromocresol purple plates contained 2% sucrose as the sole carbon source and were spread with 0.2 mg of antimycin A in 95% ethanol just before use (2). The minimal medium of Wickerham (42), which was modified as described previously (15), was used for cell growth before and during labeling with $Na^{35}SO_4$ (ICN Radiochemicals). Standard genetic methods (36) were used throughout this study. Replica plating to test phenotypes of segregants was done with a 48-pronged replicator by using dilution in sterile 96-well microtiter dishes (24). Rapid complementation analysis was carried out by using the pronged replicator to transfer arrays of 48 new *vpt* mutants to microtiter dishes containing *vpt* tester strains of the opposite mating type suspended in liquid YPD medium. Mating and diploid growth were allowed to occur in the wells for 2 days at 26°C. Replicas were then made onto minimal medium, which was supplemented to select for diploid strains. The resulting patches of diploid strains were transferred without dilution to YP fructose and assayed for external Inv activity by using a rapid filter replica assay method (15, 18) to score complementation.

Enzyme assays. The procedures and unit definitions used for assays of invertase and α-glucosidase (15), CPY (39), PrA (1), α-mannosidase (28), and glyceraldehyde-3-phosphate dehydrogenase (Worthington Enzyme Manual; Worthington Diagnostic, Freehold, N.J.) (3) have been described previously.

Labeling, fractionation, and immunoprecipitation. Spheroplasts were prepared and labeled by a modification of a previously described method (32). Cells were grown to the mid-log phase in the minimal medium of Wickerham (42) that was supplemented with 0.2% yeast extract. Four units of cells at an optical density at 600 nm were then centrifuged and suspended in 0.1 M Tris sulfate (pH 9.4)–10 mM dithiothreitol and incubated at 30°C for 5 min, centrifuged again, and suspended in the minimal medium of Wickerham (42) that was adjusted to pH 7.4 and that contained 1.3 M sorbitol. Lyticase was added to a final activity of 30 U per optical density unit at 600 nm, and the cultures were incubated at 30°C for 20 min to remove the cell wall. Spheroplasts were pelleted, suspended in the minimal medium of Wickerham (42) containing 1.3 M sorbitol, and labeled with Tran^{35}S label (0.2 mCi/ml) for 20 min at 30°C. Chase was initiated by the addition of methionine (final concentration, 50 μg/ml). The labeled culture was separated into spheroplast (intracellular) and medium (extracellular) fractions. To examine proteins in the medium fraction, cells were grown and labeled as described previously (15). The whole cells were then separated from the medium fraction by centrifugation, precipitated with 5% trichloroacetic acid, suspended in loading buffer, and run on sodium dodecylsulfate–10% polyacrylamide gels. Antisera to PrA, CPY, and Inv were prepared as described previously (18a).

Materials. Lyticase was obtained from Enzogenetics,

Tran^{35}S label was from ICN Radiochemicals and all other chemicals were from Sigma Chemical Co. (St. Louis, Mo.). Antiserum to PrB was a gift from C. Moehle and E. Jones.

RESULTS

Isolation of 25 new *vpt* complementation groups. Previously, we observed that short amino-terminal domains of the CPY sequence, when fused to mature enzymatically active Inv, can quantitatively divert delivery of this normally secreted enzyme to the yeast vacuole (15). Yeast cells require external Inv activity to grow on sucrose as a sole fermentable carbon source, so we could exploit the sorting behavior of the CPY-Inv hybrid protein to select mutants that missort and secrete the fusion protein. Yeast strains deleted for all endogenous genes encoding invertase (Δ*suc*) but containing a low-copy-number (*CENIV*, *ARSI*) plasmid encoding an appropriate CPY-Inv hybrid protein as their only source of Inv activity could not grow on sucrose, because all the Inv activity was sequestered in the vacuole (Fig. 1). Suc$^+$ derivatives of such cells can be isolated simply by selecting for growth on sucrose-containing medium. Based on earlier results, many of these mutants were expected to mislocalize the native vacuolar enzyme CPY as well, because targeting of the fusion proteins to the vacuole is dependent on the presence of the CPY vacuolar sorting signal at the amino-terminal end of the CPY-Inv hybrid proteins (15). In the present study, we decided to select such mutants using two different CPY-Inv hybrid proteins: CPY-Inv50 (encoded by plasmid pCYI-50) and CPY-Inv433 (encoded by plasmid pCYI-433) (Fig. 1). These gene fusions were composed of the coding sequence for the N-terminal 50 and 433 amino acids of proCPY, respectively, which were fused in-frame to a truncated form of the *SUC2* gene carried on the *Escherichia coli-S. cerevisiae* shuttle vector pSEYC306 (15). This modified *SUC2* gene lacked its 5′-regulatory sequences and the coding sequence for its amino-terminal signal peptide (15) (Fig. 1).

Suc$^+$ mutant strains were isolated from the Δ*suc2* strains SEY6210 and SEY6211 harboring either pCYI-50 or pCYI-433. To select Suc$^+$ mutants, single colonies of the four parental strains were plated onto YP medium with sucrose as the sole carbon source, antimycin A as an inhibitor of respiration, and bromocresol purple as a pH indicator; bromocresol purple turns yellow when sucrose is being fermented. Only one spontaneous mutant derived from each parental colony was picked, to ensure that each mutation event was independent. A total of 505 Suc$^+$ mutants were obtained and characterized: 241 *MAT***a** Suc$^+$ *vpt* mutants (isolated from SEY6211) and 264 *MAT*α Suc$^+$ *vpt* mutants (isolated from SEY6210). We confirmed that these *vpt* mutants secreted the CPY-Inv hybrid protein by carrying out a rapid filter assay for external Inv activity. This assay involved patching the mutants onto YP plates containing fructose, incubating the plates at 26°C for 24 to 36 h, and then making replicas onto Whatman no. 1 filter papers (Whatman, Inc., Clifton; N.J.) that were presoaked in an Inv assay solution (15, 18). External Inv reacted with the assay mixture to produce a red spot at the position of the appropriate cell patch. The presence of internal Inv activity was confirmed by exposing the cell patches to chloroform vapor, which lyses the yeast cells, prior to transfer onto the assay filter. This simple method permitted us to get an initial indication of the extent of the mislocalization defect for each new *vpt* mutant. All of the *vpt* mutants were found to express external Inv activity by this assay. In the parental control strains, all of the Inv activity was internal, as determined by the assay.

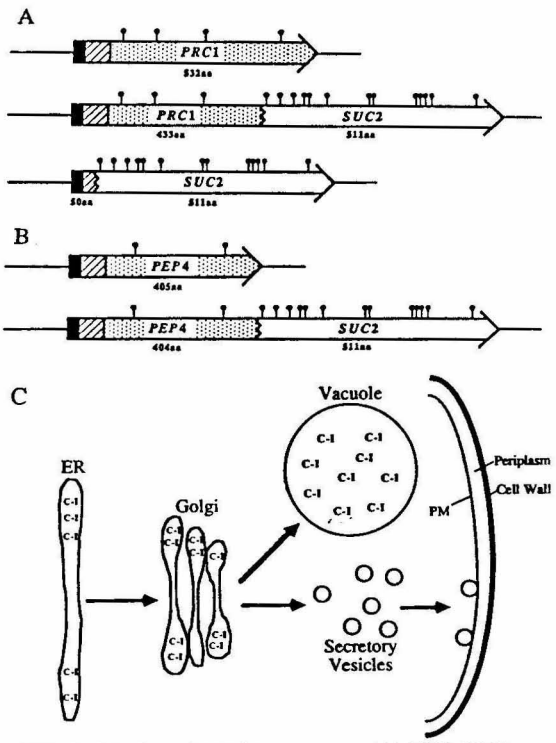

FIG. 1. Selection scheme for *vpt* mutants. (A) *PRC1-SUC2* gene fusions encoding CPY-Inv hybrid proteins. At the top is a schematic representation of the gene encoding CPY (*PRC1*). The signal sequence is indicated by a solid black box, the prosegment is indicated by hatched lines, and the mature sequences are indicated by dots (15). The positions of the oligosaccharide addition sites are indicated by tadpole-shaped forms. The two fusions of *PRC1* sequences to *SUC2* used to select *vpt* mutants are shown below. The 50-amino-acid fusion received extensive glycosyl modification, whereas the 433-amino-acid fusion did not become hyperglycosylated. (B) The gene encoding PrA (*PEP4*) and a representation of the *PEP4-SUC2* gene fusion tested for its localization in the *vpt* mutants (Table 1). (C) A simple diagram showing the vacuolar protein delivery route followed by the CPY-Inv hybrid protein (C-I). The *vpt* mutants were selected for their mislocalization of some or all of this CPY-Inv hybrid protein to the cell surface. For some of the *vpt* mutants, secretion of the mislocalized CPY-Inv was shown to be dependent on *SEC1* gene function, indicating that delivery to the surface is mediated by secretory vesicles (2). ER, Endoplasmic reticulum; aa, amino acid; PM, plasma membrane.

The Inv filter assay was also used to determine whether the *vpt* mutations were dominant or recessive in diploid strains derived by backcrossing each of the *vpt* mutants to the appropriate parental strain (SEY6210 or SEY6211). Recessive *vpt* mutants were then intercrossed, diploid strains were isolated, and the filter assay was used to assign the *MAT***a** *vpt* × *MAT*α *vpt* strains to complementation groups (Fig. 2). Complementing pairs of recessive *vpt* mutants exhibited no external Inv activity (white patch on Inv filter assays), whereas noncomplementing pairs of *vpt* mutants gave rise to red patches on the filters corresponding to

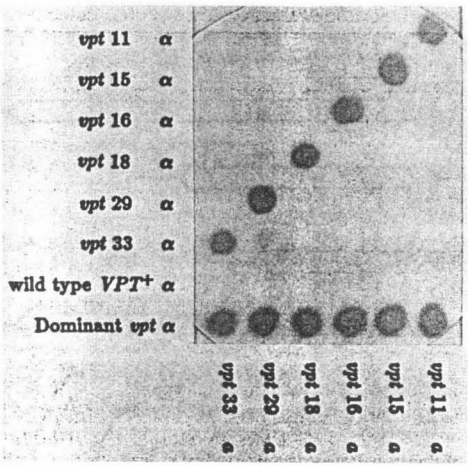

FIG. 2. Complementation analysis of *vpt* mutants. Secreted Inv activity of intact cells. Diploids were constructed by crossing *vpt MATα lys2* with *vpt MAT***a** *ade2* mutants and selecting Ade^+ Lys^+ diploid strains on minimal medium. These diploid strains were patched in an array onto YP medium containing fructose and screened by the Inv filter assay. Noncomplementation was seen as a red spot, indicating secretion of the CPY-Inv hybrid protein, while complementation was observed as no reaction (white).

the secretion of Inv activity. In general, the assay gave unambiguous results, as shown in Fig. 2. Of the 505 new *vpt* mutants, 17 were found to contain mutations that led to a dominant Suc^+ phenotype. Of six of these dominant mutants tested, only one was found to harbor a mutation linked to the fusion plasmid. The other mutants apparently contained dominant chromosomal mutations. These have not yet been characterized further. The remaining 488 mutants contained recessive mutations. Of these, 184 (84 *MAT***a** and 100 *MATα*) could be assigned to the previously identified *vpt* complementation groups (*vpt1* through *vpt8*) (2). The remaining *MAT***a** *vpt* mutants were systematically crossed with new *MATα vpt* mutants until they could be assigned to new complementation groups (arbitrarily defined as containing one *MAT***a** and one *MATα* allele). Twenty-five new *vpt* complementation groups were identified (*vpt9* through *vpt33*) (Table 1). Mutant alleles in each of the 33 complementation groups identified were obtained with both the pCYI-50 and the pCYI-433 fusion plasmids. A total of 21 recessive *MAT***a** *vpt* mutants and 36 recessive *MATα vpt* mutants were crossed with at least one allele from each of the 33 *vpt* complementation groups and, in addition, were crossed with all the remaining unassigned *vpt* mutants of the opposite mating type. All 21 unassigned *MAT***a** mutants complemented each of the remaining 36 unassigned *MATα vpt* mutants. We did not construct *MAT***a** and *MATα* alleles of each of these 57 unassigned *vpt* mutants and continue the complementation analysis further.

Because the *vpt* mutants were selected as spontaneous events, we expected that they would correspond to single-site mutations. To obtain further support of this, many of the *vpt* mutants (*vpt3*, *vpt4*, *vpt7*, *vpt9*, *vpt11*, *vpt14*, *vpt15*, *vpt16*, *vpt18*, *vpt29*, and *vpt33*) were crossed with parental *VPT* strains and subjected to tetrad analysis. For each mutant tested, the *vpt* defect and all the other markers scored in the cross segregated with the expected 2:2 Mendelian inheritance pattern of simple single-locus mutations. This confirmed that these *vpt* mutants represent single-site changes and further indicated that the genetic defects associated with these mutants do not result in any gross mating or chromosomal abnormalities. In the course of these analyses, we noticed that two of the *vpt* mutants appeared to be linked to auxotrophic markers that were heterozygous in the crosses. The segregation data for *vpt3* and *ade2* (17 parental ditype:1 tetratype:0 nonparental ditype) indicated that *vpt3* was linked to *ade2*. The approximate map distance (calculated as described previously [36]) between *ade2* and *vpt3* was ~2.8 centimorgans. This locates the *VPT3* locus on the right arm of chromosome XV, where *ADE2* has been mapped (25). Another of the *vpt* mutants, *vpt15*, appeared to be linked to *lys2* (17 parental ditype:5 tetratype:0 nonparental ditype), in this case less tightly (~11.4 centimorgans). The *LYS2* gene has been mapped to the right arm of chromosome II (25).

Mislocalization of vacuolar enzymes in the *vpt* mutants. At least one mutant from each of the 33 *vpt* complementation groups was subjected to a series of enzyme assays to quantitate the extent of the mislocalization defect and to test whether mislocalization of the CPY-Inv fusion protein was due to cell lysis.

The extent of mislocalization of the CPY-Inv hybrid protein was determined for several alleles of each complementation group by assaying external Inv (in whole cells) as well as total Inv (in lysed cells) and then calculating the percentage of Inv activity that was secreted (15). The results obtained for a representative *vpt* mutant allele from each complementation group are presented in Table 1. In most cases other alleles from the same complementation group secreted the CPY-Inv hybrid protein to a similar extent. Two to five alleles from each *vpt* complementation group were assayed. The alleles for which assay data are shown in Table 1 were used in all subsequent analyses.

The most obvious unrelated defect that might lead to the apparent secretion of vacuolar proteins is cell lysis. For this reason, assays of a cytoplasmic marker enzyme, glyceraldehyde-3-phosphate dehydrogenase, were carried out to determine whether any of the *vpt* mutants exhibited significant levels of external glyceraldehyde-3-phosphate dehydrogenase activity. In no case was there evidence of significant leakage of this cytoplasmic marker enzyme into an extracellular fraction. Furthermore, the level of cell-associated glyceraldehyde-3-phosphate dehydrogenase activity detected after detergent lysis of *vpt* mutant cells was similar to that detected in the parental strains (SEY6210 and SEY6211) (Table 1). In addition, the mutants were tested for the presence of external α-glucosidase, another cytoplasmic enzyme. Again, no evidence for lysis was found (data not shown). As discussed below, the *vpt* mutants secreted precursor forms of the vacuolar proteins rather than the mature forms, as would have been expected if cell lysis were responsible for the observed secretion.

As an initial crude test to determine whether the new *vpt* mutants also secrete CPY, we assayed CPY enzyme activity in each mutant. Since CPY is normally processed from the inactive p2 zymogen to the mature, active form inside the vacuole, it is expected that if CPY is missorted into the media, it will not become activated or will be activated poorly (38, 39). Thus, the more extreme the *vpt* mislocalization defect, the lower the cell-associated CPY activity is expected to be relative to the activities of the parental strains SEY6210 and SEY6211. CPY activity levels ranged from 2% of parental activity in *vpt5* up to 82% in *vpt23* (Table 1). CPY

TABLE 1. *vpt* complementation groups and enzyme assay data[a]

Complementation groups	Total no. of alleles	No. of *ts* alleles	% CPY-Inv secreted	% of parent CPY activity	% PrA-Inv secreted	% External G3PDH	% External α-mannosidase
vpt1	46		85	10	45	5	<5
vpt2	29		25	65	ND	ND	ND
vpt3	11	1	80	10	75	<5	<5
vpt4	15		75	5	65	<5	<5
vpt5	9		100	<5	65	<5	5
vpt6	6		70	5	45	<5	<5
vpt7	32		90	30	55	<5	10
vpt8	33		65	25	65	<5	<5
vpt9	20		65	35	55	<5	<5
vpt10	29		50	30	35	<5	20
vpt11	8	2	90	10	60	<5	25
vpt12	3		65	70	55	<5	<5
vpt13	26		100	25	110	<5	10
vpt14	8		50	45	35	<5	15
vpt15	14	8	80	25	65	5	20
vpt16	5	2	75	10	60	<5	30
vpt17	40		80	20	65	<5	<5
vpt18	8	3	100	30	90	<5	30
vpt19	16		80	20	100	<5	<5
vpt20	5		35	75	30	5	5
vpt21	2		85	45	105	<5	5
vpt22	4		35	50	25	<5	<5
vpt23	4		25	80	30	<5	<5
vpt24	8		40	50	50	<5	10
vpt25	4		35	50	30	5	<5
vpt26	4		95	15	70	<5	15
vpt27	9		40	40	40	<5	20
vpt28	7		35	65	40	<5	5
vpt29	8	2	70	15	90	<5	10
vpt30	4		60	40	70	<5	5
vpt31	2		15	65	30	<5	5
vpt32	5		35	55	35	<5	5
vpt33	7	5	90	10	85	<5	20
VPT$^+$			5	100	5	<5	<5

[a] The *vpt* mutants were assigned to 33 complementation groups on the basis of standard complementation analysis. Also shown is the number of temperature-sensitive *vpt* mutants in each of the seven complementation groups that had Ts alleles. Inv assays were performed on several alleles of each complementation group. Whole cells were assayed for external Inv activity, and lysed cells were assayed for total Inv activity. The percentage of external Inv activity was calculated for each mutant allele. A strongly defective allele (high extracellular percentage of Inv) was chosen for further analysis. Levels of other enzyme activities secreted by, or associated with, these representative alleles are presented. Abbreviations: G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ND, not determined.

activities were inversely proportional to the level of the CPY-Inv hybrid protein secreted from most of the *vpt* mutants (Table 2). For example, some mutants such as *vpt5* and *vpt33* were very defective by both criteria, whereas others, including *vpt2* and *vpt20*, did not show major defects by either assay. This result indicates that the behavior of the CPY-Inv hybrid protein in the *vpt* mutants accurately reflects the effect that these mutations have on CPY activity and, presumably, on its localization.

The availability of PrA-Inv hybrid proteins that were efficiently targeted to the vacuole (Fig. 1) (18, 18a) offered a simple way for us to test the effect of *vpt* mutations on the localization of another protein that is found in the vacuole in wild-type yeast. To this end, the representative *vpt* alleles were cured of the plasmids encoding CPY-Inv fusions and retransformed with a vector (pP4I-404) encoding a fusion protein, PrA-Inv404, consisting of the N-terminal 404 amino acids of PrA fused to mature active Inv. The hybrid protein expressed by pP4I-404 is delivered efficiently to the vacuole in *S. cerevisiae VPT* strains (18a). Inv assays showed that in most of the *vpt* mutants significant amounts of PrA-Inv hybrid protein were secreted instead of being targeted to the

TABLE 2. Grouping of *vpt* mutants based on CPY and CPY-Inv localization[a]

% of CPY-Inv activity secreted	*vpt* complementation group with the following CPY activities (% of parent):			
	<15%	15–30%	30–50%	>50%
>90%	**5, 26, 33**	**7, 13, 18**		
65–90%	**1, 3, 4, 6, 11, 16**	**15, 17, 19, 29**	21	
40–65%		**8**, 10	**9, 30**, 14, 24	12
<40%			27	2, 20, 22, 23, 25, 28, 31, 32

[a] Grouping of *vpt* mutants based on severity of CPY and CPY-Inv fusion protein localization defects. The percentage of CPY enzyme activity was broken into four levels, as was the percentage of CPY-Inv hybrid protein secreted. The 33 *vpt* complementation group numbers are shown in the appropriate spaces. *vpt* mutants that showed severe mislocalization defects, processing defects, or both types of defect for both CPY and PrA, as shown by cell fractionation and immunoprecipitation of these proteins (Fig. 3), are given in boldface.

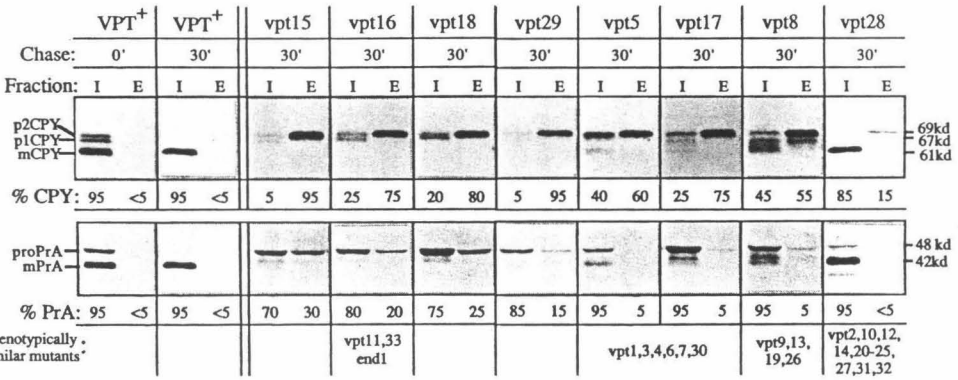

FIG. 3. Immunoprecipitation of CPY and PrA in wild-type (SEY6210 and SEY6211) and *vpt* mutant yeast strains. Spheroplasts were labeled (as described in the text) and chased for the indicated times (in minutes). The spheroplast pellet made up the internal (I) fraction and the supernatant constituted the external (E) fraction in each case. Immunoprecipitations were carried out with CPY or PrA antisera. Densitometer tracings were used to calculate the approximate percentages of CPY and PrA in the internal and external fractions for each mutant shown. The *vpt* mutants with similar phenotypes to those shown are listed. Distribution patterns of CPY and PrA for *vpt1*, *vpt3*, *vpt4*, *vpt6*, *vpt7*, and *vpt30* ranged between those shown for *vpt5* and *vpt17*. The migration positions of precursor and mature forms of CPY (mCPY) and PrA (mPrA) and their approximate molecular sizes (in kilodaltons [kd]) are indicated. For many of the mutants a protein form intermediate in size between p2CPY and mature CPY was immunoprecipitated by anti-CPY antisera. This band migrated at approximately the same position as p1CPY on our gels. The p1 and p2 forms of PrA could not be resolved under the gel conditions used.

vacuole (Table 1). The most defective *vpt* mutants, as estimated by the reduction in CPY activity levels and secretion of the CPY-Inv hybrid proteins, were also the mutants that exhibited the largest defects in PrA-Inv sorting (Table 1). It is possible that both PrA and CPY use a common delivery pathway.

We were also interested in determining whether, in addition to the sorting of soluble vacuolar hydrolases, sorting of vacuolar membrane proteins was affected in any of the *vpt* mutants. One vacuolar membrane enzyme that has been well characterized is α-mannosidase. This enzyme is not dependent on PrA processing for its activation (17), and thus would be expected to remain active even when it was mislocalized (to the cell surface, for example). The *vpt* mutants were tested for the presence of cell surface α-mannosidase activity by assaying whole, as well as detergent-lysed, cells. In all of the *vpt* mutants, total α-mannosidase activity levels were comparable to that of the wild type (*VPT*). The only *vpt* mutants that showed significant levels of external cell-associated α-mannosidase activity were *vpt10*, *vpt11*, *vpt15*, *vpt16*, *vpt18*, *vpt27*, and *vpt33* (Table 1). It is interesting that these mutants, with the exception of *vpt10* and *vpt27*, were severely defective by several other criteria, including CPY and PrA mislocalization (Fig. 3), and the possession of additional phenotypes such as temperature sensitivity (Table 1) and abnormal vacuolar morphology (L. M. Banta, J. S. Robinson, D. J. Klionsky, and S. D. Emr, J. Cell. Biol., in press). All of the representative *vpt* alleles shown in Table 1 were non-temperature-sensitive alleles. To further address the α-mannosidase localization defect, we also assayed the potentially more severe temperature-sensitive (Ts) alleles of each complementation group for which they were available (*vpt11*, *vpt15*, *vpt16*, *vpt18*, and *vpt33*). Interestingly, we observed as much as 40 to 50% of the α-mannosidase activity at the cell surface in some Ts alleles of *vpt11*, *vpt16*, *vpt18*, and *vpt33*, even when the cells were grown at the permissive growth temperature (25°C). No evidence for cell lysis of the Ts alleles was obtained.

We suspected that some of the *vpt* mutants might secrete an altered spectrum of proteins compared with that of wild-type *S. cerevisiae* for two reasons. First, since secretory and vacuolar proteins transit together through portions of the secretory pathway (38), some gene products affecting the delivery of molecules to the vacuole might also affect the transport of other molecules destined for secretion. Thus, if the *vpt* mutations affected the secretion pathway, some extracellular proteins might be missing or reduced in the medium isolated from certain of the *vpt* mutant strains. Second, and conversely, additional protein species may accumulate in the growth medium, since the *vpt* mutants were selected for their ability to secrete vacuolar contents. To address these questions, we examined the secretion of radioactively labeled proteins by some *vpt* mutants into the growth medium. Cells were labeled with $^{35}SO_4$ for 20 min and then chased for 20 min in the presence of excess unlabeled SO_4. Media proteins were precipitated in trichloroacetic acid, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and examined by autoradiography (Fig. 4). No striking differences between the media fractions isolated from wild-type or *vpt* mutant cells were observed. Comparable amounts of a similar set of proteins were observed in extracts from both wild-type and *vpt* mutant cells. Two of the mutants (*vpt7* and *vpt11*) exhibited an extra protein band, in addition to the set observed in *VPT S. cerevisiae* (Fig. 4). We did not determine whether either of these new bands corresponded to a known vacuolar protein. Based on their apparent molecular weights, these proteins were not CPY or PrA. Because we analyzed the medium from pulse-labeled cells, we also inferred that the rate of normal protein secretion was not grossly altered in the *vpt* mutants. The lack of new proteins corresponding to CPY or PrA in the medium was probably due to the instability of these mislocalized proteins (2, 38), as well as to their relatively low abundance compared with that of the proteins in the medium observed under these experimental conditions.

In addition, many of the mutants were transformed with

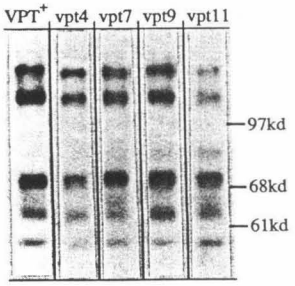

FIG. 4. Proteins secreted from wild-type and *vpt* mutant strains. Strains were labeled with $^{35}SO_4$ for 20 min and chased for 20 min with $(NH_4)_2SO_4$ (1 mM). Cells were removed by centrifugation. The supernatant (medium) proteins were precipitated with trichloroacetic acid and run on a 10% polyacrylamide gel. Size standards (in kilodaltons [kd]) are as indicated.

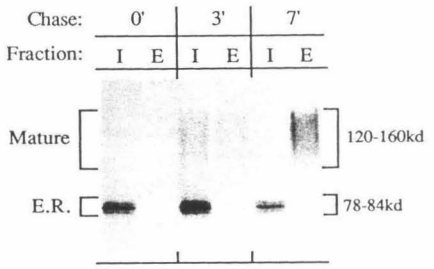

FIG. 5. Immunoprecipitation of Inv from labeled spheroplasts. *VPT S. cerevisiae* (SEY6211) containing the Inv-encoding plasmid pRB58 was derepressed by growth in 0.1% glucose for 30 min, converted to spheroplasts, and labeled for 5 min with [^{35}S]methionine. Chase with cold methionine was for 0, 3, or 7 min, as indicated. Spheroplasts were separated into pellet (I) and supernatant (E) fractions by centrifugation and immunoprecipitated with Inv antisera. The core glycosylated Inv found in the endoplasmic reticulum (E.R.) migrated as a set of bands that were converted to a heterogeneous mixture of highly glycosylated molecules in the Golgi complex prior to secretion from the cell. kd, Kilodaltons.

an Inv-encoding plasmid (pRB58) and tested for normal Inv secretion (assay and calculations were as described previously [27]). The *vpt* mutants were found to secrete Inv at the same levels as wide-type *S. cerevisiae*. This provides further evidence that the *vpt* mutants carry out secretion normally.

***vpt* mutants exhibit defects in the sorting and processing of several vacuolar proteins.** To assess directly the extent of the sorting defect in the various *vpt* mutants, we labeled and fractionated *vpt* mutant cells. Antisera directed against CPY, PrA, and PrB were used to detect the amount and form of these different vacuolar proteins in each cell fraction. Initially, cells were labeled and fractionated into medium, periplasm, and spheroplast fractions, with subsequent immunoprecipitation by anti-CPY and anti-PrA antisera. These data (not shown) allowed us to categorize the *vpt* mutants based on the severity of their CPY and PrA localization and processing defects. However, we noticed that during the fractionation, presumably because of the incubation time required to remove the cell wall enzymatically, some fraction of these proteins was being degraded or modified. To avoid this, we converted the mutant cells to spheroplasts before they were labeled and fractionated. In this way we were able to look at mislocalization by isolating spheroplast (pellet) and medium fractions by centrifugation. The medium fraction corresponded to all material secreted from the cells, including those proteins that are normally retained in the periplasm. A further advantage of labeling spheroplasts is that because lysed spheroplasts do not incorporate the label, it reduces the background of labeled vacuolar proteins in the medium resulting from cell lysis during spheroplast preparation. To confirm that the spheroplast-labeling technique did not alter normal protein secretion or protein sorting to the vacuole, we initially analyzed the modification and processing of the secreted enzyme (Inv) and the vacuolar proteins (CPY and PrA) in spheroplasts prepared from a wild-type strain. Both protein secretion and vacuolar protein sorting and processing appeared to be normal (Fig. 3 and 5). Indeed, we observed that the level of protein expression and the kinetics of protein modification and delivery appeared to be normal by the spheroplast-labeling technique (8, 15, 18, 35b). This argues that secretory protein traffic occurs normally in yeast spheroplasts.

Wild-type and *vpt* mutant spheroplasts were labeled with [^{35}S]methionine for 20 min and chased with cold methionine for 30 min. Supernatant (external) and pellet (internal) fractions were immunoprecipitated with anti-CPY antisera. We observed two significant effects of the *vpt* mutants on the biogenesis of the vacuolar protein CPY (Fig. 3). First, processing was altered; 14 of the mutants exhibited no mature CPY at all (*vpt1*, *vpt3*, *vpt4*, *vpt5*, *vpt6*, *vpt7*, *vpt11*, *vpt15*, *vpt16*, *vpt17*, *vpt18*, *vpt29*, *vpt30*, *vpt33*), and an additional 5 *vpt* mutants processed less than 30% of CPY to the mature forms (*vpt8*, *vpt9*, *vpt13*, *vpt19*, *vpt26*). Second, missorting and secretion of CPY were observed. Two of the *vpt* mutants (*vpt15* and *vpt29*) secreted more than 95% of CPY into the periplasmic and medium fraction. An additional 12 *vpt* mutants (*vpt1*, *vpt3*, *vpt4*, *vpt5*, *vpt6*, *vpt7*, *vpt11*, *vpt16*, *vpt17*, *vpt18*, *vpt30*, *vpt33*) were almost as defective; they secreted between 60 and 80% of CPY (Fig. 3). These 14 most defective mutants exhibited a complete defect in maturation of the small amount of CPY that remained associated with the spheroplast fraction, as discussed above, indicating that the accumulated proCPY was not sorted to the vacuole or, possibly, that the vacuole of these mutants was so defective that it was not competent for the processing of proCPY. Many of the remaining *vpt* mutants exhibited less severe defects in the sorting of the CPY protein. In these mutants, a substantial portion of CPY remained associated with the spheroplast and was processed to the mature active enzyme, indicating that some of the proCPY was sorted correctly to the vacuole (Fig. 3).

To determine whether this protein-sorting defect is specific for CPY or extends to other vacuolar proteins, we examined sorting and processing of two additional vacuolar proteins, PrA and PrB, in the *vpt* mutants. Relative to CPY, lower levels of both PrA and PrB were secreted by the *vpt* mutants. However, we noted that the extent of the defect in maturation of these proteins paralleled that observed for CPY in each of the *vpt* mutants (Fig. 3 and 6). The absence of CPY in the vacuole cannot explain the accumulation of precursor forms of PrA and PrB, as maturation of these proteases is not dependent on CPY function. This suggests that the defect in vacuolar sorting of PrA and PrB may be similar to that observed for CPY, but that secretion of the missorted PrA and PrB is blocked. We reasoned that the reduced level of proPrA and proPrB in the media fraction

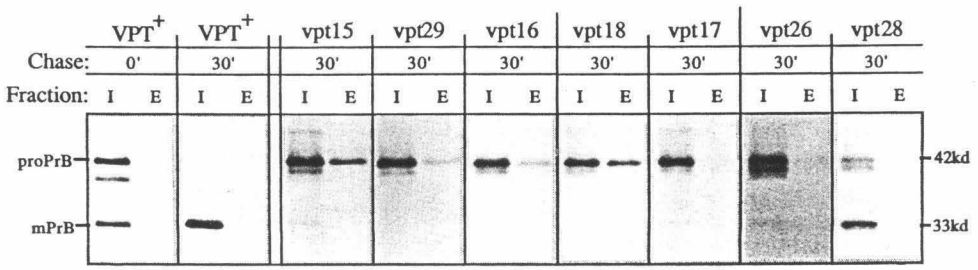

FIG. 6. Immunoprecipitation of PrB in wild-type (SEY6210 and SEY6211) and *vpt* mutant yeast strains. Spheroplasts were labeled as described in the text and chased for the indicated times (in minutes). I, Internal (spheroplast pellet) fraction; E, external (supernatant) fraction. PrB antiserum was used for the immunoprecipitations. The migration positions of precursor (proPrB) and mature (mPrB) forms of PrB and their approximate molecular sizes (in kilodaltons [kd]) are indicated. Note the extra band at 0 min; presumably, it was an intermediate in PrB maturation.

might be due to a reduced rate in secretion of these missorted proteins. To address this possibility, we extended the length of the chase period from 30 to 90 min and otherwise performed the fractionation and immunoprecipitation procedures as described above. When this experiment was carried out on *vpt5*, *vpt11*, and *vpt15*, no increases in the levels of proPrA or proPrB were observed in the medium after this extended chase period (data not shown). In this experiment we also observed that no additional processing of the proPrA and proPrB occurred during the chase.

An alternative explanation is that the lack of these proteins in the medium fraction might not be due to a block in secretion of the mislocalized zymogens, but rather to the specific or nonspecific association of these proteins with the spheroplast membrane surface. To test this possibility, we treated the labeled spheroplasts with 500 μg of proteinase K per ml for 30 min at 0°C to determine whether any of the cell-associated proPrA and proPrB was accessible to the exogenously added protease. Several *vpt* mutants (*vpt5*, *vpt17*, and *vpt26*) were analyzed in this way, and in each case, PrA and PrB were unaffected by the addition of proteinase K. As expected, secreted CPY was degraded under these conditions. Both PrA and PrB were, however, subject to degradation if the detergent Triton X-100 (1%) was added prior to protease addition. Taken together, these experiments indicate that proPrA and proPrB accumulate in some intracellular compartment. The present data do not rule out the vacuole as the site in which these proteins reside; however, the lack of processing of the accumulated proteins suggests that this may not be case. This lack of processing does not eliminate the vacuole as the site of accumulation of PrA and PrB, because of the observation that in missense PrA mutants, CPY activity is normal but PrA activity is decreased (23).

Several *vpt* mutants are temperature sensitive for growth. It is not clear whether vacuolar protein sorting is essential for yeast cell growth. The *pep4* mutants (45) are defective in enzyme activities of many soluble vacuolar hydrolases, yet they grow normally at all temperatures. We selected our mutants at 26°C to allow us to score for potential temperature-conditional phenotypes. Thus, after the mutants were selected for their ability to grow on sucrose-containing medium at 26°C, they were tested for their ability to grow at 37°C on YPD medium. Of the 505 mutants isolated, 23 (approximately 5%) were Ts for growth at 37°C (i.e., they did not form colonies at 37°C). These Ts mutants belonged to six *vpt* complementation groups (*vpt11*, *vpt15*, *vpt16*, *vpt18*, *vpt29*, and *vpt33*). In addition, one allele of *vpt3* had a partial Ts phenotype (very small colonies at 37°C). Genetic crosses and tetrad analysis were used to confirm that in each case the Ts phenotype cosegregated with the *vpt* protein sorting defect. One Ts allele from each complementation group was crossed to the parent strain (SEY6210 or SEY6211), sporulated, and subjected to tetrad dissection. Ten or more tetrads were examined for each cross (an example of the cosegregating phenotypes is shown in Fig. 7). The remaining Ts alleles in a given complementation group were inferred to be linked to the appropriate *vpt* locus by complementation testing at 37°C. It is interesting that of the *vpt* complementation groups that contained Ts alleles (not including *vpt3*), 22 of 50 total alleles (i.e., 44%) in these complementation groups were Ts. Thus, the Ts alleles are not scattered at

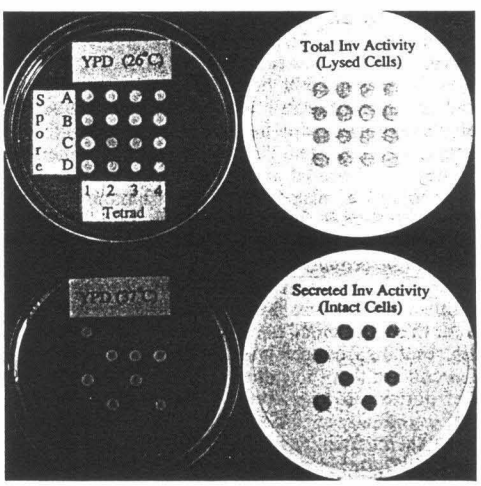

FIG. 7. Demonstration that the Ts growth phenotype and the *vpt* mislocalization phenotype cosegregate in four representative tetrads from a cross of a Ts allele of *vpt16* with strain SEY6210. The filter assay of the lysed segregant patches showed that each spore had Inv activity. Below, the Inv filter assay of intact segregant patches showed that the ability to secrete Inv activity (i.e., the CPY-Inv hybrid protein) segregated 2:2. Similar cosegregation of the Ts and *vpt* defects was seen for *vpt3*, *vpt11*, *vpt15*, *vpt18*, *vpt29*, and *vpt33*. At least 10 tetrads were analyzed in each case.

random throughout the total set of *vpt* complementation groups. In addition, the complementation groups which possess Ts alleles all tend to exhibit more severe defects by several criteria. These defects include missorting of vacuolar proteins, sensitivity to high osmotic pressure, and inability to assemble morphologically normal vacuoles (Banta et al., in press).

As described above, the Ts *vpt* mutants exhibited a vacuolar protein-sorting defect at the permissive growth temperature. We examined the Ts alleles at the restrictive temperature to see whether the transport defect was exaggerated further and thereby might explain their inviability at 37°C. The mutants were preincubated for 60 min at 37°C and then labeled for 20 min with $^{35}SO_4$ and chased for 20 min, both at 37°C. Immunoprecipitation with CPY antibody showed that there was no apparent difference in CPY processing or secretion at the restrictive temperature (data not shown), but it should be noted that all of these Ts *vpt* mutants exhibited extreme defects in vacuolar protein sorting, even at the permissive temperature. Since the Ts *vpt* mutants secreted Inv at 37°C, secretion apparently is not blocked at this temperature (see below). Because the defect in CPY sorting and processing was nearly complete even at the permissive growth temperature, the absence of growth at the nonpermissive temperature may be explained by the appearance of a more complete sorting defect at the high temperature, which alters the sorting of additional vacuolar proteins and possibly also affects secreted proteins that are essential for growth. Alternately, it may be that the combined stress of growth at a high temperature together with a defect in vacuolar protein sorting may, in an additive fashion, prevent cell growth at 37°C. For example, it is possible that a loss of vacuole protein localization at a high temperature may result in a block in secretion or endocytosis, or both, since these defects have been shown to be lethal (27, 34). To address these questions, the Ts *vpt* mutants were tested in several ways for possible secretory or endocytic defects. First, the Ts *vpt* mutants were transformed with a plasmid (pRB58) carrying the entire wild-type structural gene and regulatory sequences of *SUC2* and tested for Inv secretion at restrictive and permissive temperatures. For the experiment at the restrictive temperature, cells were shifted to 37°C for 60 or 120 min prior to the assay. Activities in whole and lysed cells were measured and compared to determine the percentage of Inv secreted (27). The mutants that were tested were not found to be significantly different from the parental strain SEY6210 harboring the same *SUC2* plasmid (data not shown). This implies that the Ts *vpt* mutants do not exhibit an Inv secretion defect at 37°C. Second, the Ts *vpt* mutants were tested for genetic overlap with the Ts secretion-defective mutants *sec1-sec23* (27) and *sec53* (10). The *sec* mutants are defective for the secretion of Inv at 37°C and are blocked for transport at various stages of the secretory pathway in *S. cerevisiae* (26). *sec* × *vpt* diploid strains were selected and tested for complementation of the temperature sensitivity at 37°C. In each case complementation was observed, implying that there is no genetic overlap between these two sets of mutants. The Ts *vpt* mutants were also crossed with the Ts endocytosis-defective mutant *end1* (5). Diploid strains were again tested for Ts growth defects. It was found that *vpt11* did not complement *end1*, implying that these two mutants are allelic to one another. In addition, the *END1* gene (a gift from H. Riezman) was found to rescue the Ts defect of the *vpt11* allele tested (P. Herman, unpublished data). This genetic overlap raises the possibility that some of the *vpt* mutants may have a defect in endocytosis, possibly at the restrictive temperature, as discussed above.

Overlap between the *vpt* and *vpl* mutants. Independent of our *vpt* mutant screen, Rothman and Stevens (35) have also isolated mutants that are defective in vacuolar protein localization, *vpl1* through *vpl8*. Recently, they extended this work and selected new *vpl* mutants and assigned them to 11 new *vpl* complementation groups (T. Stevens, personal communication). Intercrosses of the *vpt* mutants with all the *vpl* mutants allowed us to determine the overlap between these two sets of presumably related mutants. Each of the *vpl* mutants (*vpl1* through *vpl19*) was crossed with our Suc$^-$ parental strain, diploid strains were selected and sporulated, and Suc$^-$ *vpl* segregants were identified. These Δ*suc vpl* mutants were then crossed to representative *vpt* mutant alleles from each complementation group, which harbored the CPY-Inv433 hybrid protein-encoding plasmid. Mislocalization of the hybrid protein from the diploid strains was scored by Inv filter assays. The results of these complementation studies are shown in Table 3. We observed that 12 of the 33 *vpt* mutants overlapped with *vpl* complementation groups. This overlap was confined in most cases to *vpt* complementation groups with the less severe vacuolar protein localization defects (Table 1 and 3). At present, we do not have an explanation for this observation. Differences in the selection schemes used or in the starting strains may have affected in part the final set of mutants obtained. J. H. Rothman and T. H. Stevens (unpublished data) have used another complementation assay technique and have independently confirmed each of these results. To avoid future confusion regarding these two classes of mutants, we have decided to use the general term *vps* for vacuolar protein sorting to describe both classes of mutants (Table 3).

Some differences were seen between *vpl* and *vpt* mutants in terms of the levels of missorting of vacuolar proteins. For example, *vpt5* and *vpl5* were in the same complementation group (*vps5*). The *vpl5* allele analyzed by Rothman and Stevens (35) showed a weaker protein sorting defect for CPY and PrA than did *vpt5* (2) (Fig. 3). This result may indicate allele differences. However, since the two mutations were in unrelated genetic backgrounds, the discrepancy also could be due to strain background differences.

Another class of pleiotropic mutants, the *pep* mutants, also has been shown to affect vacuolar protein processing (16, 17). Complementation analysis has shown that there is genetic overlap between subsets of the *pep*, *vpt*, and *vpl* mutants (T. Stevens and E. Jones, personal communication). The overlap between the *vpt* and *pep* mutants is as follows: *vpt3* = *pep21*, *vpt4* = *pep8*, *vpt5* = *pep10*, *vpt11* = *pep5*, *vpt13* = *pep12*, and *vpt17* = *pep6*.

DISCUSSION

In an effort to identify additional mutants that exhibit novel defects in vacuolar protein sorting and to develop an appreciation for the number of gene functions that may contribute to this process, we exploited a CPY-Inv fusion-based selection scheme (2) to isolate more than 500 new *vpt* mutants. The mutants were assigned to 33 different complementation groups, 7 of which contained Ts alleles. Even though this was an unexpectedly large number of complementation groups, our data suggest that many additional gene functions are likely to influence the vacuolar protein delivery pathway. Several of the newly isolated *vpt* mutants could not be assigned to any of these 33 complementation groups. In addition, genetic comparison of the *vpt* mutants

TABLE 3. Complementation analysis of *vpt* and *vpl* mutants

vpt mutant	*vpl* mutant	Common complementation group name
vpt26	*vpl1*	*vps1*
	vpl2	*vps2*
vpt17	*vpl3*	*vps3*
vpt10	*vpl4*	*vps4*
vpt5	*vpl5*	*vps5*
vpt13	*vpl6*	*vps6*
	vpl7	*vps7*
vpt8	*vpl8*	*vps8*
vpt9		*vps9*
vpt1		*vps10*
vpt11	*vpl9*	*vps11*
vpt12		*vps12*
vpt2		*vps13*
vpt14		*vps14*
vpt15		*vps15*
vpt16		*vps16*
vpt3		*vps17*
vpt18		*vps18*
vpt19		*vps19*
vpt20	*vpl10*	*vps20*
vpt21		*vps21*
vpt22	*vpl14*	*vps22*
vpt23	*vpl15*	*vps23*
vpt24		*vps24*
vpt25	*vpl12*	*vps25*
vpt4		*vps26*
vpt27		*vps27*
vpt28	*vpl13*	*vps28*
vpt6		*vps29*
vpt30		*vps30*
vpt31		*vps31*
vpt32		*vps32*
vpt33		*vps33*
vpt29		*vps34*
vpt7		*vps35*
	vpl11	*vps36*
	vpl16	*vps37*
	vpl17	*vps38*
	vpl18	*vps39*
	vpl19	*vps40*

[a] The *vpt* mutants were crossed with the *vpl* mutants (that had been made Δ*suc2* by crossing them with strain SEY6210) and tested for their ability to complement the CPY-Inv433 sorting defect. Extensive overlapping was seen, and it is proposed that to avoid confusion in the future, these strains should be referred to as *vps* mutants (for vacuolar protein sorting) and the numbers should be as listed here (see also J. H. Rothman and T. H. Stevens, manuscript in preparation).

with similar mutants from other laboratories (*sec*, *pep*, *vpl*, *end* [5, 10, 16, 27, 35]), affecting secretory protein delivery or processing, indicated that although there was overlap among some of these different sets of mutants, more than 50 gene functions are implicated in the vacuolar protein sorting process.

This large number of genes can be interpreted to indicate that vacuolar protein sorting is a very complex process involving a series of distinct and precisely controlled sorting events. These include translocation into the endoplasmic reticulum, sorting to and through the Golgi complex, separation from proteins destined for secretion or Golgi complex retention, and selective delivery to the vacuole, possibly via an intermediate endosomelike compartment. Because each of these steps is likely to be catalyzed by proteins that are reused through several rounds of protein transfer, any mutations that affect their level of expression, stability, activity, or ability to recycle back to the appropriate compartment of action may lead to sorting defects. Alternatively, the large number of mutants identified may suggest that many unrelated physiological and cellular defects can interfere with vacuolar protein sorting by indirectly influencing the process. That the former argument (that the *vpt* mutations cause specific changes in the targeting machinery) may indeed be the case for the more defective *vpt* mutants is supported by the following observations. (i) All of the late-acting *sec* mutants, which exhibited major defects in protein secretion, did not alter the delivery or maturation of CPY (38). (ii) Yeast strains deleted for the gene encoding the clathrin heavy chain did not exhibit defects in vacuolar protein biogenesis, even though the growth rate and intracellular morphology of these mutants were dramatically different from those of wild-type cells (29, 30). (iii) Cell fractionation and enzyme assay data ruled out the release of vacuolar proteins as a consequence of yeast cell lysis. (iv) Though many of the *vpt* mutants exhibited major defects in the targeting and maturation of vacuolar proteins, protein secretion as well as many other cell functions, including cell division, mating, and respiratory growth, remained largely unaffected. Direct evidence concerning the specificity of the events affected by the *vpt* mutants depends on the identification of the various *VPT* gene products, their site of action in the cell, and their individual biochemical activities.

Several observations led us to suspect that if vacuolar enzymes were not efficiently targeted to the vacuole, they would appear in the medium. Both CPY and PrA travel through the initial part of the secretory pathway (38, Klionsky et al., in press), so it can be postulated that if CPY or PrA fails to be diverted to the vacuole at the appropriate step, they might continue to be carried through the secretory pathway and end up at the cell surface. Indeed, much evidence has accumulated suggesting that many proteins are secreted by a default mechanism, while vacuolar and lysosomal enzymes must be actively sorted away from these proteins (4, 31). In addition, gene dosage-induced overproduction of CPY or PrA or mutational changes in the vacuolar sorting signals of these proteins lead to their missorting and secretion (15, 18a, 39, 41), suggesting that if the vacuolar targeting apparatus is overloaded or unable to recognize vacuolar proteins, the default reaction for these proteins is secretion. A similar overproduction-induced mislocalization phenotype has been observed for the vacuolar CPY-Inv and PrA-Inv hybrid proteins (2, 18a). Finally, an initial set of vacuolar protein mislocalization mutants which missort and secrete vacuolar enzymes has already been obtained by this scheme (2, 35).

The *vpt* mutants were selected on the basis of their ability to missort and secrete a hybrid vacuolar protein, CPY-Inv. The sorting of the artificial hybrid protein CPY-Inv fairly accurately reflects the sorting of the native CPY protein. Several lines of evidence support this conclusion. CPY-Inv hybrid proteins, carrying the CPY sorting signal, are very efficiently localized to the vacuole, as is true for native CPY (15, 18a). Mutants selected as defective in the vacuolar sorting of CPY-Inv hybrid proteins (*vpt*) also exhibit similar defects in the sorting of wild-type CPY (2). Enzyme assay data from this and previous studies (2) demonstrate that the extent of mislocalization of both the CPY-Inv hybrid proteins and native CPY protein correlate closely in each of the *vpt* mutants (Table 2). The *vpt* mutants genetically overlapped with the *vpl* mutants, which were selected by an independent scheme for defects in CPY sorting (35) (Table 3). The gene fusion approach also offers the advantage that it can be generally applied to the study of other gene products

independent of any knowledge of their biochemical activity (6, 7, 14).

It is of interest that although the extents of the sorting defects seen in different *vpt* mutants may vary, each mutant individually appears to exhibit qualitatively similar defects in the sorting and processing of the three proteins that were analyzed: CPY, PrA, and PrB. Although the *vpt* mutants did not secrete the same level of proCPY, proPrA, or proPrB, they all appeared to exhibit similar proteolytic processing defects for each of these proteins (Fig. 3 and 6). The *vpt* mutants caused significantly higher levels of proCPY to be mislocalized to the medium than the levels observed for either proPrA or proPrB. Most of the *vpt* mutants secreted p2CPY forms, indicating that this protein passes correctly through the Golgi complex. Although some level of secretion of proPrA and proPrB was observed in many of the *vpt* mutants, the bulk of each of these precursor proteins appeared to accumulate in an intracellular compartment distinct from the vacuole. They remained associated with the spheroplast cell fraction and were not degraded when extracellular proteases were added to the labeled spheroplasts. The proteins accumulated in their precursor forms, even though in many *vpt* mutants mature forms were also present, indicating that the vacuoles of these mutants were still competent for precursor maturation (Fig. 3 and 6). We do not know where in the cells the precursors accumulated or why they were not secreted like proCPY. The PrA-Inv hybrid protein was efficiently secreted by many of the *vpt* mutants (Table 1), suggesting that PrA itself is missorted in the mutants but cannot transit through later stages of the secretory pathway as efficiently as the PrA-Inv hybrid protein can. (Inv is normally secreted by *S. cerevisiae* and may facilitate transit of the PrA-Inv hybrid.) proPrA and proPrB may associate specifically or nonspecifically with some component of an intracellular membrane, or they may be diverted into a compartment that does not communicate with the cell surface. The vacuolar protein-sorting signals in both proCPY and proPrA have been identified, but they appear to share no significant primary sequence similarities (15, 18, 41) and therefore may be recognized by different carrier systems.

An important unresolved question regarding protein sorting to mammalian lysosomes or to the yeast vacuole is whether luminal proteins transit together with integral membrane proteins, and if so, does a common sorting apparatus participate in directing the delivery of these two distinct sets of proteins? In the case of I-cell disease, many lysosomal luminal enzymes are missorted; however, certain membrane proteins continue to be faithfully sorted to the vacuole (33, 37, 40). When we assayed for the location of the vacuolar membrane marker enzyme α-mannosidase, we found that it was not mislocalized to the cell surface in most of the *vpt* mutants. Vacuoles isolated from two of these mutants (*vpt3* and *vpt4*) retained normal levels of this enzyme activity (2). However, a small subset of the *vpt* mutants did exhibit significant defects in the sorting of α-mannosidase activity. Alleles of *vpt11*, *vpt16*, *vpt18*, and *vpt33* secreted up to 50% of their α-mannosidase activity to the cell surface. Interestingly, this same set of *vpt* mutants also exhibited other phenotypes that were consistent with the fact that these mutants have defects in both luminal and membrane vacuolar protein sorting. These phenotypes include the absence of a detectable vacuole in the mutant cells, as determined by light or electron microscopy, and accumulation of novel membrane-enclosed structures that may correspond to intermediates in vacuole membrane biogenesis (Banta et al., in press). These results indicate that at least some vacuolar membrane proteins may share a common sorting step or compartment with soluble vacuolar proteins en route to the vacuole.

A useful observation is that seven of the *vpt* complementation groups had alleles exhibiting a Ts growth defect (on YPD medium at 37°C). In each case, this Ts growth defect was shown to be genetically linked to the *vpt* phenotype. It is probably not coincidental that these mutants all exhibited interesting additional vacuole-related defects. These Ts *vpt* mutants could be divided into three groups on the basis of their additional phenotypes and characteristics. (i) *vpt15* and *vpt29* were the two complementation groups with the most severe localization defect for CPY and PrA (Fig. 3). In addition, they both exhibited abnormal morphologies, including the accumulation of vesicular and Golgi complex-related structures, and apparently enlarged vacuoles relative to wild-type *S. cerevisiae* (Banta et al., in press). (ii) *vpt11*, *vpt16*, *vpt18*, and *vpt33* also exhibited severe defects in the sorting of CPY and showed the accumulation of most of their PrA in the precursor form, indicating that it probably also did not reach the vacuole. These mutants exhibited the most striking structural defects of any of the *vpt* mutants, since they did not contain a morphologically identifiable vacuole. (iii) *vpt3* had one Ts allele and was moderately defective in CPY and PrA localization, as assessed by immunoprecipitation (Fig. 3). The *vpt3* mutant is interesting morphologically because it appears to contain highly fragmented vacuoles (Banta et al., in press).

One of the *vpt* mutants (*vpt11*) was found to be allelic to the Ts endocytosis-defective mutant *end1* (5). This observation is interesting because it provides the first genetic evidence for a link between the endocytotic and biosynthetic delivery routes to the yeast vacuole. This result was not unexpected, because some mutants defective in the secretory pathway (*sec*) are also defective in endocytosis (34), and as discussed above, early stages of vacuolar protein targeting are *sec* dependent (38). It will be of interest to address the endocytic competence of the *vpt* mutants.

We identified many new genes whose products are involved in the process of vacuolar protein targeting. We expect that many of the gene products act directly in the targeting and transport of molecules to the yeast vacuole. More detailed information about how the vacuolar targeting pathway operates is anticipated on cloning of the *VPT* genes and detailed biochemical characterization of the gene products.

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LITERATURE CITED

1. **Ammerer, G., C. P. Hunter, J. H. Rothman, G. C. Saari, L. A. Valls, and T. H. Stevens.** 1986. *PEP4* gene of *Saccharomyces cerevisiae* encodes proteinase A, a vacuolar enzyme required for processing of vacuolar precursors. Mol. Cell. Biol. **6**:2490–2499.
2. **Bankaitis, V. A., L. M. Johnson, and S. D. Emr.** 1986. Isolation of yeast mutants defective in protein targeting to the vacuole. Proc. Natl. Acad. Sci. USA **83**:9075–9079.
3. **Bernstein, M., W. Hoffman, G., Ammerer, and R. Schekman.** 1985. Characterization of a gene product (Sec53p) required for protein assembly in the yeast endoplasmic reticulum. J. Cell Biol. **101**:2374–2382.
4. **Burgess, T. L., and R. B. Kelly.** 1987. Constitutive and regulated secretion of proteins. Annu. Rev. Cell Biol. **3**:243–293.
5. **Chvatcho, Y., I. Howald, and H. Riezman.** 1986. Two yeast mutants defective in endocytosis are defective in pheromone response. Cell **46**:355–364.
6. **Douglas, M. G., B. L. Geller, and S. D. Emr.** 1984. Intracellular targeting and import of an F_1-ATPase β-subunit-β-galactosidase hybrid protein into yeast mitochondria. Proc. Natl. Acad. Sci. USA **81**:3983–3987.
7. **Emr, S. D., R. Schekman, M. C. Flessel, and J. Thorner.** 1983. An MFα1-SUC2(α-factor invertase) gene fusion for study of protein localization and gene expression in yeast. Proc. Natl. Acad. Sci. USA **80**:7080–7084.
8. **Esmon, P. C., B. E. Esmon, T. E. Schauer, A. Taylor, and R. Schekman.** 1987. Structure, assembly, and secretion of octameric invertase. J. Biol. Chem. **262**:4387–4394.
9. **Farquhar, M. G., and G. E. Palade.** 1981. The Golgi apparatus (complex)-(1954–1981)—from artifact to center stage. J. Cell Biol. **81**:77s–103s.
10. **Ferro-Novick, S., P. Novick, C. Field, and R. Schekman.** 1984. Yeast secretory mutants that block the formation of active cell surface enzymes. J. Cell Biol. **98**:35–43.
11. **Griffiths, F., and K. Simons.** 1986. The *trans* Golgi network: sorting at the exit site of the Golgi complex. Science **234**:438–443.
12. **Hasilik, A., and W. Tanner.** 1978. Biosynthesis of the vacuolar yeast glycoprotein carboxypeptidase Y. Eur. J. Biochem. **85**: 599–608.
13. **Hemmings, B. A., G. S. Zubenko, A. Hasilik, and E. W. Jones.** 1981. Mutant defective in processing of an enzyme located in the lysosome-like vacuole of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **78**:435–439.
14. **Hoffmann, W.** 1987. *CAN1-SUC2* gene fusion studies in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **210**:277–281.
15. **Johnson, L. M., V. A. Bankaitis, and S. D. Emr.** 1987. Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. Cell **48**:875–885.
16. **Jones, E. W.** 1977. Proteinase mutants of *Saccharomyces cerevisiae*. Genetics **85**:23–33.
17. **Jones, E. W., G. S. Zubenko, and R. R. Parker.** 1982. *PEP4* gene function is required for expression of several vacuolar hydrolases in *Saccharomyces cerevisiae*. Genetics **102**:665–677.
18. **Klionsky, D. J., L. M. Banta, and S. D. Emr.** 1988. Intracellular sorting and processing of a yeast vacuolar hydrolase: proteinase A propeptide contains vacuolar targeting information. Mol. Cell. Biol. **8**:2105–2116.

18a. **Klionsky, D. J., L. M. Banta, J. S. Robinson, and S. D. Emr.** 1988. Vacuolar protein targeting and organelle biogenesis in yeast, p. 173–186. *In* R. Bradshaw, L. McAlister-Henn, and M. Douglas (ed.), Molecular biology of intracellular protein sorting and organelle assembly. Alan R. Liss, Inc., New York.

19. **Kornfeld, S.** 1986. Trafficking of lysosomal enzymes in normal and diseased states. J. Clin. Invest. **77**:1–6.
20. **Matile, P., H. Moor, and C. F. Robinow.** 1969. Yeast cytology, p. 219–302. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts. Academic Press, Inc., London.
21. **Mechler, B., H. H. Hirsch, H. Müller, and D. H. Wolf.** 1988. Biogenesis of the yeast lysosome (vacuole): biosynthesis and maturation of proteinase yscB. EMBO J. **7**:1705–1710.
22. **Mechler, B., M. Müller, H. Müller, F. Meussdoerffer, and D. H. Wolf.** 1982. *In vivo* biosynthesis of the vacuolar proteinases A and B in the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **257**:11203–11206.
23. **Mechler, B., H. Müller, and D. H. Wolf.** 1987. Maturation of vacuolar (lysosomal) enzymes in yeast: proteinase yscA and proteinase yscB are catalysts of the processing and activation event of carboxypeptidase yscY. EMBO J. **6**:2157–2163.
24. **Moir, D., S. Stewart, B. Osmond, and D. Botstein.** 1982. Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties and pseudoreversion studies. Genetics **100**:547–563.
25. **Mortimer, R. K., and D. C. Hawthorne.** 1986. Genetic mapping in *Saccharomyces*. Genetics **53**:165–173.
26. **Novick, P., S. Ferro, and R. Schekman.** 1981. Order of events in the yeast secretory pathway. Cell **25**:461–469.
27. **Novick, P., C. Field, and R. Schekman.** 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell **21**:205–215.
28. **Opheim, D. J.** 1978. α-D-Mannosidase of *Saccharomyces cerevisiae*, characterization and modulation of activity. Biochim. Biophys. Acta **524**:121–130.
29. **Payne, G. S., D. Baker, E. van Tuinen, and R. Schekman.** 1988. Protein transport to the vacuole and receptor-mediated endocytosis by clathrin heavy chain-deficient yeast. J. Cell. Biol. **106**: 1453–1461.
30. **Payne, G. S., T. B. Hasson, M. S. Hasson, and R. Schekman.** 1987. Genetic and biochemical characterization of clathrin-deficent *Saccharomyces cereivisiae*. Mol. Cell. Biol. **7**:3888–3898.
31. **Pfeffer, S. R., and J. E. Rothman.** 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. Annu. Rev. Biochem. **56**:829–852.
32. **Reid, G. A.** 1983. Pulse labeling of yeast cells and spheroplasts. Methods Enzymol. **97**:324–329.
33. **Reitman, M. L., A. Varki, and S. Kornfeld.** 1981. Fibroblasts from patients with I-cell disease and pseudo-Hurler polydystrophy are deficient in uridine 5'-diphosphate-*N*-acetylglucosamine:glycoprotein *N*-acetylglucosaminylphosphotransferase activity. J. Clin. Invest. **67**:1574–1579.
34. **Riezman, H.** 1985. Endocytosis in yeast: several of the yeast secretory mutants are defective in endocytosis. Cell **40**:1001–1009.
35. **Rothman, J. H., and T. H. Stevens.** 1986. Protein sorting in yeast: mutants defective in vacuole biogenesis mislocalize vacuolar proteins into the late secretory pathway. Cell **47**:1041–1051.

35a. **Rothman, J. H., and T. H. Stevens.** 1988. Protein sorting and biogenesis of the lysosome-like vacuole in yeast. p. 317–362. *In* R. C. Das and P. W. Robins (ed.), Protein transfer and organelle biogenesis. Academic Press, San Diego, Calif.

35b. **Schauer, I., S. Emr, C. Gross, and R. Schekman.** 1985. Invertase signal and mature sequence substitutions that delay intercompartmental transport of active enzymes. J. Cell Biol. **100**:1664–1675.

36. **Sherman, F., G. R. Fink, and C. W. Lawrence.** 1979. Methods in yeast genetics: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Sly, W. S., and H. D. Fischer.** 1982. The phosphomannosyl recognition system for intracellular and intercellular transport of lysosomal enzymes. J. Cell. Biochem. **18**:67–85.
38. **Stevens, T., B. Esmon, and R. Schekman.** 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. Cell **30**:439–448.
39. **Stevens, T. H., J. H. Rothman, G. S. Payne, and R. Schekman.** 1986. Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y. J. Cell. Biol. **102**:1551–1557.
40. **Tager, J. M.** 1985. Biosynthesis and deficiency of lysosomal enzymes. Trends Biochem. Sci. **10**:324–326.
41. **Valls, L. A., C. P. Hunter, J. H. Rothman, and T. H. Stevens.** 1987. Protein sorting in yeast: the localization determinant of yeast vacuolar carboxypeptidase Y resides in the propeptide. Cell **48**:887–897.
42. **Wickerham, L. J.** 1946. A critical evaluation of the nitrogen

assimilation tests commonly used in the classification of yeasts. J. Bacteriol. **52**:293–301.

43. **Wiemken, A., M. Schellenberg, and K. Urech.** 1979. Vacuoles: the sole compartments of digestive enzymes in yeast (*Saccharomyces cerevisiae*)? Arch. Microbiol. **123**:23–35.
44. **Yoshihisa, T., Y. Otsumi, and Y. Anraku.** 1988. Solubilization and purification of vacuolar membranes in *Saccharomyces cerevisiae*. J. Biol. Chem. **263**:5158–5163.
45. **Zubenko, G. S., F. J. Park, and E. W. Jones.** 1983. Mutations in *PEP4* locus of *Saccharomyces cerevisiae* block final step in maturation of two vacuolar hydrolases. Proc. Natl. Acad. Sci. USA **80**:510–514.

Chapter 3

Organelle assembly in yeast: Characterization of yeast mutants defective in vacuolar biogenesis and protein sorting.

Contributions of the authors of this chapter.

L. Banta did all the microscopy, and observed the Os and pH-sensitive phenotypes. J. Robinson isolated all the mutants (this is the first report of many of the phenotypes associated with these mutants). She also observed the *ade2*-white phenotype and growth defects of Class C mutants, made Figure 10, and carried out all the genetic analyses reported in the paper. D. Klionsky did the immunoprecipitations shown in Figure 4. S. Emr supervised this work in his laboratory and was responsible for the writing of the paper.

Organelle Assembly in Yeast: Characterization of Yeast Mutants Defective in Vacuolar Biogenesis and Protein Sorting

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Abstract. Yeast vacuole protein targeting (*vpt*) mutants exhibit defects in the sorting and processing of multiple vacuolar hydrolases. To evaluate the impact these *vpt* mutations have on the biogenesis and functioning of the lysosome-like vacuole, we have used light and electron microscopic techniques to analyze the vacuolar morphology in the mutants. These observations have permitted us to assign the *vpt* mutants to three distinct classes. The class A *vpt* mutants (26 complementation groups) contain 1–3 large vacuoles that are morphologically indistinguishable from those in the parental strain, suggesting that only a subset of the proteins destined for delivery to this compartment is mislocalized. One class A mutant (*vpt*13) is very sensitive to low pH and exhibits a defect in vacuole acidification. Consistent with a potential role for vacuolar pH in protein sorting, we found that bafilomycin A_1, a specific inhibitor of the vacuolar ATPase, as well as the weak base ammonium acetate and the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone, collapse the pH gradient across the vacuolar membrane and cause the missorting and secretion of two vacuolar hydrolases in wild-type cells. Mutants in the three class B *vpt* complementation groups exhibit a fragmented vacuole morphology. In these mutants, no large normal vacuoles are observed. Instead, many (20–40) smaller vacuole-like organelles accumulate. The class C *vpt* mutants, which constitute four complementation groups, exhibit extreme defects in vacuole biogenesis. The mutants lack any organelle resembling a normal vacuole but accumulate other organelles including vesicles, multilamellar membrane structures, and Golgi-related structures. Heterozygous class C zygotes reassemble normal vacuoles rapidly, indicating that some of the accumulated aberrant structures may be intermediates in vacuole formation. These class C mutants also exhibit sensitivity to osmotic stress, suggesting an osmoregulatory role for the vacuole. The *vpt* mutants should provide insights into the normal physiological role of the vacuole, as well as allowing identification of components required for vacuole protein sorting and/or vacuole assembly.

EUKARYOTIC cells are distinguished by their several discrete membrane-enclosed organelles. Each of these subcellular compartments has unique structural and functional characteristics which are conferred in large part by the distinct set of proteins that constitute that organelle. Thus, accurate sorting and trafficking of proteins from their site of synthesis in the cytoplasm to their correct noncytoplasmic destinations are essential for maintaining the functional and structural identity of each organelle.

In mammalian cells, the secretory pathway has been shown to mediate the modification, processing, and delivery of proteins destined for a variety of intracellular and extracellular compartments. Proteins destined for secretion, assembly into the plasma membrane, delivery to lysosomes, or retention within endoplasmic reticulum (ER)[1] and Golgi compartments transit through all or a portion of the secretory pathway (11). Presently, available data are consistent with a model in which much of protein secretion occurs via a default mechanism (5). Proteins competent for entry into the ER but lacking any additional sorting information passively transit from the ER to the Golgi complex and then are secreted via a nonspecific bulk flow mechanism (58). However, proteins that depart from this pathway, such as lysosomal enzymes, contain additional sorting signals that permit specific recognition, modification, and subsequent delivery of these proteins from late Golgi compartments to the lysosome (53).

In the yeast *Saccharomyces cerevisiae* most protein secretion also appears to occur via a constitutive default pathway (55). However, like mammalian lysosomal enzymes, proteins destined for the yeast vacuole depend on the presence of additional protein sorting information (21, 25, 56). The yeast vacuole is a prominent intracellular organelle which shares functional characteristics with both mammalian lysosomes and plant central vacuoles. This organelle is believed

1. *Abbreviations used in this paper*: Bb, Berkeley body; CPY, carboxypeptidase Y; ER, endoplasmic reticulum; FD, FITC-conjugated dextran; Inv, invertase; PrA, proteinase A; PrB, proteinase B.

to play an important role in the storage of amino acids and other small molecules (30). Like mammalian lysosomes, the yeast vacuole is an acidic compartment and contains a number of hydrolytic enzymes (33, 59). Certain of these hydrolases, including the glycoproteins proteinase A (PrA), proteinase B (PrB), and carboxypeptidase Y (CPY) have been shown to be synthesized at the level of the ER as inactive precursors. These proenzymes transit through the Golgi complex and are sorted to the vacuole, where they are processed to the mature active enzymes (16, 19, 32, 54). Sequence determinants have been defined within proCPY and proPrA that are necessary and sufficient to target these proteins to the vacuole (21, 25, 56). Mutational alterations in the proCPY sorting signal lead to missorting and secretion of the precursor form of this enzyme. Although the sorting signals in proCPY and proPrA lack any obvious primary sequence similarity, it is presumed that a common cellular protein-sorting apparatus mediates specific recognition and subsequent vacuolar localization of these enzymes. Each undergoes a similar set of compartment-specific modifications, and the kinetics for vacuolar delivery of both proteins are essentially the same (25). It seems likely that many soluble vacuolar proteins are segregated to this compartment via the same targeting mechanism.

In an effort to identify components of the vacuolar protein sorting apparatus, we recently isolated a number of mutants that exhibit defects in the proper localization and processing of several vacuolar proteins. These vacuolar protein targeting (*vpt*) mutants were identified using a gene fusion-based selection scheme. In wild-type cells, proCPY sequences fused to the gene for the normally secreted enzyme invertase (Inv) contain sufficient sorting information to divert delivery of enzymatically active Inv to the yeast vacuole (2, 21). Mutants have been selected that missort and secrete such CPY-Inv hybrid proteins. The ~600 mutants isolated thus far have been assigned to more than 33 complementation groups. The mutants exhibit hybrid protein-independent defects in the sorting of normal vacuolar enzymes including CPY, PrA, and PrB (46). Upon missorting, the precursor forms of these proteins are secreted, presumably because the selective vacuole protein delivery pathway is defective and the proteins then follow the default secretion pathway (2).

Given this large number of potential gene products that can influence the vacuolar protein-sorting process, it seemed likely that mutations in at least some of these genes might also affect biogenesis of a normal vacuole. Specifically, some of the proteins that are mislocalized in the *vpt* mutants may be essential in defining important structural and functional characteristics of this organelle. In addition, one might expect that lipid and protein constituents of the vacuole membrane would transit together with soluble vacuolar enzymes via a common vesicle carrier. Defects in vacuole membrane assembly therefore might also be expected in certain of the *vpt* mutants. To address these questions, we have assessed the vacuolar as well as other organellar morphologies in each of the *vpt* mutants. Using both light and electron microscopic techniques, we found that mutants in most of the *vpt* complementation groups still assembled morphologically normal vacuoles. However, mutants in three complementation groups accumulated what appeared to be multiple small vacuoles. Cells in four other *vpt* complementation groups exhibited extreme defects in vacuole biogenesis; these mutants accumulated vesicles and membrane-enclosed compartments that bore no resemblance to a normal vacuole. In addition, certain of the *vpt* mutants exhibited other phenotypes such as sensitivity to low pH or to osmotic stress. These observations provide insights into the mechanism(s) of vacuole biogenesis as well as the normal physiological role of this organelle.

Materials and Methods

Strains and Media

Mutants were isolated as described (46) from parental strains SEY6210 *MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9* and SEY6211 *MATa ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9*. Other strains used were HMSF1 *MATα sec1-1* (37), SEY5078 *MATα sec7-1 suc2-Δ9 leu2-3,112 ura3-52* (this study), and SEY5186 *MATα sec18-1 leu2-3,112 ura3-52* (this study). Cells were grown on standard yeast extract peptone dextrose (YPD) or synthetic dextrose (SD) (synthetic minimal, supplemented as necessary) media (49). *ade2* strains were scored for the presence of red pigment after growth for 3–5 d on both standard YPD (containing 2% glucose) and YPD containing 8% glucose. Sensitivity to low pH was assessed on YPD adjusted to pH 3.5, 3.0, or 2.5 with 6 N HCl. Sensitivity to high osmotic pressure was determined on YPD containing 1.0 or 1.5 M NaCl, 1 M KCl, or 2.5 M glycerol (osmosity ~1.7s). As noted previously by Singh (51), certain batches of hypertonic media stored more than a few days inhibited growth of the wild-type strains; therefore it was important to test the media with a strain known to be resistant to the osmotic stress conditions present.

Genetic crosses, sporulation of diploids, and dissection of tetrads were performed as described by Sherman et al. (49).

Labeling of Cells with Fluorescent Dyes

All manipulations were performed at room temperature unless otherwise noted. Cells were labeled in the presence of FITC–dextran (FD) as described by Makarow (27), with the following modifications. Cells (5 ml) were grown in YPD to early log phase (1–2 × 10^7 cells/ml), centrifuged for 5 min in a clinical centrifuge (International Equipment Co., Needham Heights, MA), and washed once in YPD, pH 4.5. The cell pellet was resuspended to a concentration of 1.5 × 10^8 cells/ml in YPD, pH 4.5, containing 100 mg/ml 70 S FD, and incubated for 90 min at 37°C (or 25°C for temperature-sensitive (*ts*) strains) on a rotary shaker. The cells were centrifuged for 2 min at 6,000 rpm in a microcentrifuge (Savant Instruments Inc., Hicksville, NY), washed twice in PBS (10 mM Na-phosphate, pH 7.4, 140 mM NaCl), and resuspended in 0.4 ml PBS. The resuspended cells were mixed with low melting point agarose (0.5% final concentration) at 37°C, mounted on glass slides, covered with a coverslip which was sealed with nail polish, and observed immediately. Alternatively, cells were labeled with FITC as described previously (41). Cells (2 × 10^7) were resuspended in 1 ml YPD containing 50 mM Na-citrate, pH 5.5, and 10 μg/ml FITC in DMSO. After a 10-min incubation at 25°C with shaking, cells were centrifuged, washed once, and resuspended in 0.1 ml 100 mM K-phosphate, pH 7.5, containing 2% glucose. Cells were mounted as above.

For quinacrine labeling, cells were grown as described above and quinacrine was added to a final concentration of 175 μM in YPD, pH 7.6. After a 5-min incubation at 30°C, cells were centrifuged and mounted as above without washing. Ammonium acetate (200 μM final concentration) was added to the incubation mix where indicated (57).

For observation of the *ade2* endogenous fluorophore, cells were grown as described by Weisman et al. (57) and mounted as for FD.

Microscopy

Cells were observed using a Carl Zeiss Inc. microscope (Thornwood, NY), with a 100× oil-immersion objective, equipped for Nomarski optics and epifluorescence. Fluorescence filters used were Carl Zeiss Inc. BP450-490 (excitation), FT510 (beam splitter), and BP520-560 (emission barrier). All fluorescent images were photographed for 40–60 s using Eastman Kodak Co. (Rochester, NY) Tri-X Pan ASA400 film, increased to ASA1600 by using Diafine developer.

Electron Microscopy

Cells were prepared using a modification of the procedure of Byers and Goetsch (6). Cells (100 ml) were grown in synthetic minimal medium to an OD_{600} of ~0.3, shifted to YPD medium, and allowed to grow for one generation. The cells were harvested by centrifugation (5 min in an International Equipment Co. clinical centrifuge), washed in dH_2O, and fixed for 2 h at room temperature in 2 ml of 0.1 M Na-cacodylate, pH 6.8, 5 mM $CaCl_2$ (Buffer A), containing 3% gluteraldehyde. The fixed cells were washed once in 100 mM Tris-HCl, pH 8.0, 25 mM dithiothreitol, 5 mM Na_2-EDTA, 1.2 M sorbitol, and then incubated in the same buffer for 10 min at 30°C. To remove cell walls, the cells were centrifuged, washed once with 1 ml 0.1 M K-phosphate (adjusted to pH 5.8 with citric acid) containing 1.2 M sorbitol, and resuspended in 0.5 ml of the same buffer containing 0.05 ml β-glucuronidase type H-2 and 2.5 mg zymolyase. This cell suspension was incubated for 2 h at 30°C. The spheroplasts were centrifuged and washed in 1 ml buffer A. *ts* strains were shifted to 37°C for 3 h before harvesting and were fixed at 37°C for analysis at the nonpermissive temperature; the rest of the procedure was identical to that for non-*ts* strains. The reduced osmium–thiocarbohydrazide-reduced osmium membrane–enhancement staining technique was adapted from Willingham and Rutherford (60). Spheroplasts prepared as described above were resuspended in 0.5 ml 1% OsO_4, 1% K-ferrocyanide in buffer A, and incubated for 30 min at room temperature. After four washes in dH_2O (1 ml each), the cells were resuspended in 1% thiocarbohydrazide, incubated for 5 min at room temperature, and washed again four times in dH_2O. The cells were stained with 1% OsO_4, 1% K-ferrocyanide in buffer A for 3 min at room temperature, and washed in dH_2O as before. The samples were dehydrated through an ethanol series and embedded in London Ross White, which was allowed to polymerize for 3 d at 4°C with exposure to UV light. Thin sections were collected on 200-mesh copper grids, stained for 30–45 s with lead citrate (42), and observed on a transmission electron microscope (model 420; Philips Electronic Instruments, Inc., Mahwah, NJ).

Immunoprecipitations

Immunoprecipitations on labeled spheroplasts (46) were performed as described previously (25).

Materials

Bafilomycin A_1 was the generous gift of K. Altendorf, Universität Osnabrück, Osnabrück, FRG. Tran ^{35}S-label was purchased from ICN Radiochemicals, Irvine, CA. Gluteraldehyde, OsO_4, thiocarbohydrazide, and K-ferrocyanide were purchased from Polysciences, Inc., Warrington, PA. Zymolyase was purchased from Seikagaku Kogyo Co., Ltd., Japan, and low melting point agarose was purchased from Bethesda Research Laboratories, Gaithersburg, MD. Diafine developer was the product of Acufine, Chicago, IL. London Ross White embedding resin was purchased from Ted Pella Inc., Irvine, CA. FD (70 S), FITC, quinacrine, β-glucuronidase (type H-2), and all other chemicals not listed above were purchased from Sigma Chemical Co., St. Louis, MO.

Results

Vacuole Morphology in vpt Mutants

We have used light and fluorescence microscopy to determine the state of the vacuole in multiple *vpt* alleles from each of 33 complementation groups. In a wild-type strain grown in rich medium, yeast vacuoles occupy ~20% of the yeast cell volume and can often be visualized in the light microscope using Nomarski optics. However, small vacuoles can not be visualized by this technique and some cells do not appear to have any vacuole when observed by Nomarski optics (57). Therefore, in order to visualize even small vacuoles, we have taken advantage of a number of fluorescent dyes that specifically accumulate in this organelle.

FD as well as FITC[2] by itself have been used to label yeast vacuoles (27, 41). In wild-type cells, the vacuole appeared as a single large fluorescent spot or 2–3 spots of approximately equal size under the labeling conditions used (Fig. 1 *A*). A large vacuole was also visible in these cells using Nomarski optics. Unexpectedly, when the *vpt* mutants were examined using this dye, most of the mutants (26 complementation groups, see Table I) exhibited a vacuolar morphology indistinguishable from that of the parental strains (Fig. 1 *B*). We have grouped these mutants together and refer to them as class A *vpt* mutants. The remaining seven complementation groups exhibited an altered vacuolar morphology. In three of the groups (*vpt*3, 5, and 26), cells had multiple smaller organelles which were visible using Nomarksi optics and which stained with FITC (Fig. 1 *C*). This group of mutants has been designated class B. The remaining four *vpt* complementation groups (*vpt*11, 16, 18, and 33) had no intracellular structures which stained in the presence of FD. When observed by Nomarski optics, cells in these complementation groups appeared to have rough surfaces, and no vacuoles were visible (Fig. 1 *D*). We have defined this group of mutants as class C *vpt* mutants (Table I). These observations have been confirmed using two other fluorescent dyes that also accumulate in yeast vacuoles (see below).

Class A vpt Mutants Exhibit Wild-type Vacuole Morphology

We have studied the *vpt* mutants exhibiting each of the three vacuolar morphologies in more detail. In *ade*2 strains of *S. cerevisiae*, an endogenous fluorophore accumulates in the vacuole and can be visualized using fluorescence microscopy (57). By this method, the parental strains and the class A *vpt* mutants had vacuolar morphologies identical to those observed using FD (Fig. 1, *E* and *F*).

Like mammalian lysosomes (38), yeast vacuoles have an acidic pH (33) and can be labeled by dyes, such as quinacrine (1, 57), chloroquine (26), and neutral red (20, 34), which accumulate in a pH-dependent manner. These weak bases are presumed to diffuse through membranes and accumulate in acidic compartments (9). The vacuole morphology of wild-type cells as observed using quinacrine was identical to that seen with FD or the *ade*2 endogenous dye (Figs. 2 *A* and 4 *A*). Most of the class A *vpt* mutants also contained 1–3 large vacuoles which accumulated quinacrine, although in many cases the fluorescence was less intense than that in the parental strain (not shown). Multiple alleles of three complementation groups (*vpt*10, 13, and 24), exhibited very little or no vacuolar staining with quinacrine, although vacuoles were clearly visible by Nomarski optics in these cells (Fig. 2 *E*). These *vpt* mutants, which had morphologically normal vacuoles but exhibited no pH-dependent accumulation of dye, might carry mutations which affect vacuole acidification. In plant cells, the vacuole plays an important role in pH homeostasis; a decrease in external pH results in a lowered vacuolar pH, while the cytoplasmic pH remains constant (3). We reasoned that mutants defective in vacuole acidification might also exhibit defects in the regulation of intracellular pH. To address this issue, we tested whether any of the *vpt* mutants were sensitive to low pH. Growth was assayed on

2. While these studies were in progress, Preston et al. (41) reported that the vacuolar staining associated with FD was in fact due to nonendocytic uptake of FITC and other contaminating impurities in the FD. We have repeated the vacuole labeling experiments in a few of the *vpt* mutants using FITC and have observed vacuolar morphologies similar to those reported here for FD.

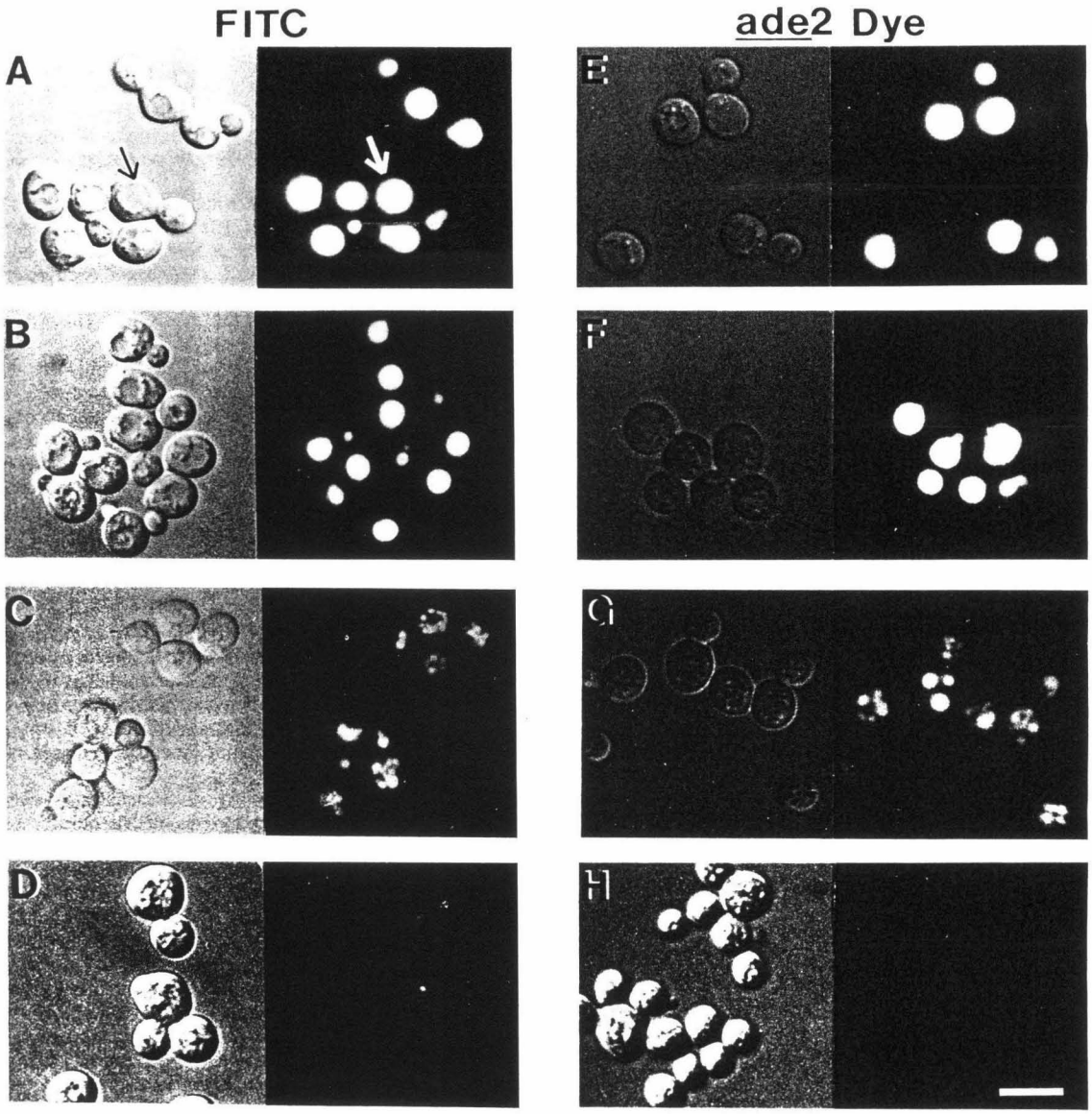

Figure 1. Vacuole morphology in *vpt* mutants labeled with FITC or the *ade*2 endogenous fluorophore. (*A*, *B*, *C*, and *D*) Nomarski (left) and fluorescence (right) photomicrographs of cells labeled in the presence of FD (see Materials and Methods). (*E*, *F*, *G*, and *H*) Nomarski (left) and fluorescence (right) photomicrographs of cells grown in SD containing limiting adenine (12 μg/ml) to allow production of the *ade*2 fluorophore. In each cell, the fluorescent spot corresponds to the vacuole, which appears as a large circular indentation using Nomarski optics (*arrows*). (*A* and *E*) Wild-type vacuole morphology as seen in the parental strains SEY6210 (*A*) or SEY6211 (*E*). (*B* and *F*) Representative class A *vpt* mutants, *vpt*10 (*B*) and *vpt*29 (*F*), in which the vacuole morphology is indistinguishable from that of the parent. (*C* and *G*) The class B *vpt* mutants, *vpt*5 (*C*) and *vpt*3 (*G*), contain multiple small vacuoles. (*D* and *H*) Representative class C *vpt* mutants, *vpt*18 (*D*) and *vpt*11 (*H*), which contain no structures that stain like vacuoles. Bar, 10 μm.

YPD media adjusted to pH 3.5, 3.0, or 2.5. The parental strains grew, although more slowly than on standard YPD, under these conditions. Strains in one complementation group, *vpt*13, were found to be extremely sensitive to low pH. Fourteen of 21 *vpt*13 alleles were unable to grow at pH 3.5, and 19 alleles of *vpt*13 were inhibited for growth at pH 3.0 (Fig. 3 *A*). Some alleles of other *vpt* mutants were also weakly sensitive to low pH (data not shown).

The existence of *vpt* mutants that exhibited possible defects in vacuole acidification led us to investigate the role of vacuo-

Table I. Summary of vpt Mutant Phenotypes

	Vacuole morphology	Abnormal growth phenotypes		Aberrant organelles accumulated
		Osmotic sensitive	Low pH sensitive	
Class A (*vpt*1, 2, 4, 6, 7, 8, 9, 10, 12, 13, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 27, 28, 29, 30, 31, 32)	Wild-type vacuoles	*vpt*15, 29	*vpt*13	*vpt*15, 29-Bbs, vesicles *vpt*7, 28-"Golgi" *vpt*12-vesicles
Class B (*vpt*3, 5, 26)	Fragmented vacuoles	*vpt*26		
Class C (*vpt*11, 16, 18, 33)	No vacuoles	*vpt*11, 16, 18, 33		Multilamellar membrane structures, vesicles, Bbs

lar pH in the localization of proteins to the vacuole in wild-type cells. The vacuolar membrane contains a proton-translocating Mg^{++}-dependent ATPase which produces a proton gradient across the vacuolar membrane and acidifies the interior of the vacuole (24). The drug bafilomycin A_1 has been shown to be a specific inhibitor of the vacuolar membrane proton-translocating ATPase of *Neurospora crassa* (4). When wild-type yeast cells were treated with 10 μM bafilomycin A_1 for 10 min before staining with quinacrine, no vacuolar fluorescence was observed (Fig. 4 *B*). The inhibition of the pH-dependent quinacrine staining in these cells indicates that bafilomycin eliminates the pH gradient across the yeast vacuolar membrane, presumably by inhibiting the vacuolar membrane ATPase. To assess the role of the pH of the yeast vacuole in vacuolar protein localization, we next examined the effect of bafilomycin on the sorting and processing of vacuolar hydrolases. Spheroplasts were pretreated with bafilomycin (10 μM final concentration) for 10 min, radioactively labeled, and separated into intracellular and extracellular fractions before immunoprecipitation with CPY- and PrA-specific antisera. As shown in Fig. 4 *C*, in the absence of bafilomycin all of the CPY and PrA was processed to the mature enzyme form and remained associated with the yeast spheroplast fraction (0 μM, lanes *I* and *E*), indicating that these enzymes had been delivered to the vacuole (46, 59). In contrast, in the presence of bafilomycin, ~50% of the CPY was present in the proenzyme form, and most of this proCPY was secreted into the extracellular fraction (Fig. 4 *C*, 10 μM, lanes *I* and *E*). Bafilomycin caused a similar defect in the processing and targeting of PrA. Other agents known to raise vacuolar pH, including the weak base ammonium acetate (200–400 mM; reference 57) and the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 40 μM) also caused the mislocalization of CPY and PrA (data not shown). Significantly, protein traffic to the cell surface was not disrupted under these conditions, and the concentration of bafilomycin used in these experiments did not inhibit yeast cell growth. Together these data indicate that vacuole pH plays a role in the efficient delivery and maturation of at least some vacuolar hydrolases.

We next examined the class A *vpt* mutants by transmission EM, using a technique that results in enhanced staining of biomembranes and structures containing glycomolecules (see Materials and Methods). As seen in Fig. 5 *A*, the vacuole stained as a large electron-dense compartment using this procedure. Thin sections of wild-type cells typically contained one large or two to three smaller vacuoles per cell. Other intracellular structures such as the nucleus, mitochondria, and ER were also readily visible. The majority of the class A *vpt* strains, such as the ones shown in Fig. 5, *B* and *C*, exhibited typical wild-type morphology. In thin sections of both wild-type and class A strains, we often observed an apparent substructure within the vacuole that did not stain like the rest of the vacuole and remained electron transparent (e.g., see Fig. 5 *B*). The significance of this structure is unclear, although it may represent the polyphosphate precipitate often observed in freeze-etched yeast cells (30).

Electron microscopic analysis also revealed that certain class A *vpt* mutants accumulated aberrant structures in addition to the normal vacuole. Mutants in two complementation groups, *vpt*15 and *vpt*29, contained organelles similar to those seen in yeast protein secretion (*sec*) mutants (37), including vesicles and Berkeley bodies (Bbs, structures presumably related to the Golgi complex; see reference 36). The electron micrographs in Fig. 6 show typical *vpt*15 and *vpt*29 cells and, for comparison, *sec*1 (accumulates vesicles), *sec*18 (accumulates ER), and *sec*7 (accumulates Bbs), prepared using the membrane-enhancement technique. The vacuoles in these *vpt*15 and *vpt*29 cells were abnormally large and occasionally contained inclusions (Fig. 6 *C*). This aberrant morphology was seen in every *vpt*15 and *vpt*29 allele examined. *vpt*13 and *vpt*26 cells also occasionally contained vesicles and Bbs (not shown).

In two complementation groups, *vpt*7 and *vpt*28, stacks of lamellae and reticular membrane arrays were observed at a high frequency (Fig. 7). These organelles were more prevalent in *vpt*7 and *vpt*28 than in the parental strain (i.e., 0.8–1.0 structure per cell section vs. 0.4 for the parent in 30–50 sections examined for each strain). These structures, which were usually not associated with any other organelle, are likely to correspond to exaggerated Golgi complexes. Finally cells in one class A mutant, *vpt*12, accumulated vesicles similar to those seen in the class C mutants (see below).

Class B vpt Mutants Exhibit an Altered Vacuole Morphology

Unlike the class A *vpt* mutants, cells in the three class B complementation groups (*vpt*3, 5, and 26) contained multiple compartments that stained in the presence of FD and were visible using Nomarski optics (Fig. 1 *C*). These small "vacuoles" also accumulated the *ade*2 endogenous fluorophore (Fig. 1 *G*). To determine whether these organelles had the lowered pH characteristic of wild-type vacuoles, we tested their ability to accumulate quinacrine. As shown in Fig. 2 *C*, the structures in the class B *vpt* mutants stained with quina-

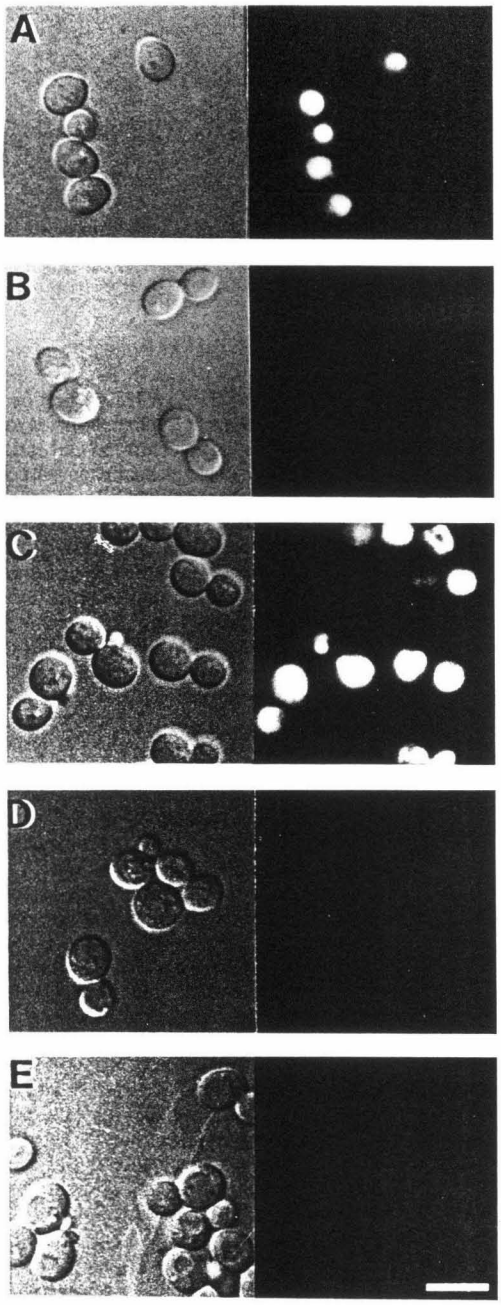

Figure 2. Quinacrine accumulation in the vacuoles of wild-type cells and *vpt* mutants. Cells were incubated in YPD, pH 7.6, containing 175 μM quinacrine for 5 min in the absence (*A*, *C*, and *E*) or presence (*B* and *D*) of 200 mM ammonium acetate. (*A* and *B*) Nomarski (left) and fluorescence (right) images of the parental strain SEY6210. (*C* and *D*) Nomarski (left) and fluorescence (right) images of *vpt5*, which exhibits a typical class B vacuole morphology. (*E*) *vpt13* exhibits no quinacrine staining (right), although vacuoles are clearly visible using Nomarski optics (left). Bar, 10 μm.

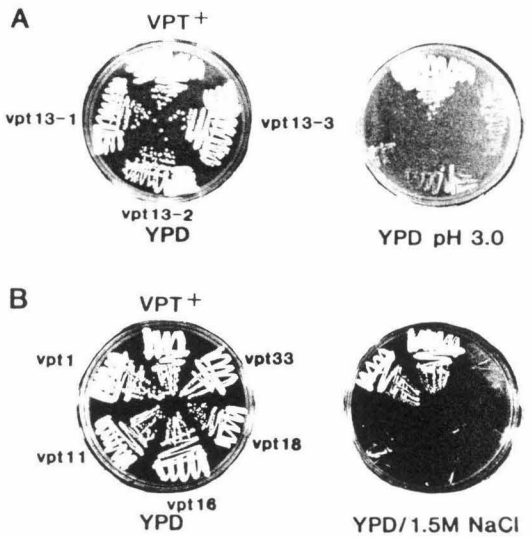

Figure 3. Growth defects associated with *vpt13* and the class C *vpt* mutants. (*A*) Three alleles of *vpt13* and the parental strain SEY6210 were streaked on YPD medium (left) or YPD medium adjusted to pH 3.0 with 6 N HCl (right). The plates were incubated at 30°C for 2 and 4 d, respectively. (*B*) A representative allele from each of the four class C *vpt* complementation groups was streaked on YPD medium (left) or YPD medium containing 1.5 M NaCl (right). The plates were incubated at 30°C for 2 and 5 d, respectively. The parental strain SEY6211 and a class A *vpt* mutant, *vpt1*, are shown for comparison.

crine, suggesting that these compartments had a pH similar to that of the vacuole in the parental strain. This hypothesis was further tested by labeling with quinacrine in the presence of ammonium acetate (57). Under these conditions, no fluorescent staining was observed in the parental strain or the class B *vpt* mutant (Fig. 2, *B* and *D*). This supports the hypothesis that the fluorescent staining observed in class B cells is due to the acidic pH of the compartments stained rather than to some nonspecific accumulation of dye.

The class B *vpt* mutants were also examined at the ultrastructural level to confirm the multiple-vacuole morphology observed by light and fluorescence microscopy. As seen in Fig. 5 *D*, a representative class B *vpt5* mutant allele contained multiple small organelles that stained like wild-type vacuoles. The number of vacuoles per cell section was quantitated for *vpt5* and for the parental strain (using 30–40 cell sections per strain). The average number of vacuole-like structures per cell section for the class B *vpt* was 5.7, while the number for the wild-type strain was 1.8. 71% of the class B cells, as compared to 16% of the parental cells, had 3 or more vacuoles. On the basis of the size of the vacuoles and the thickness of the sections, we have calculated that an average class B cell contains ~35 of these small vacuoles, while wild-type cells contain one to four vacuoles per cell. Representatives of the other two class B complementation groups (*vpt3* and *vpt26*) exhibited similar vacuolar morphologies when observed by EM.

Yeast which carry a mutation in the β-tubulin gene (*tub2*)

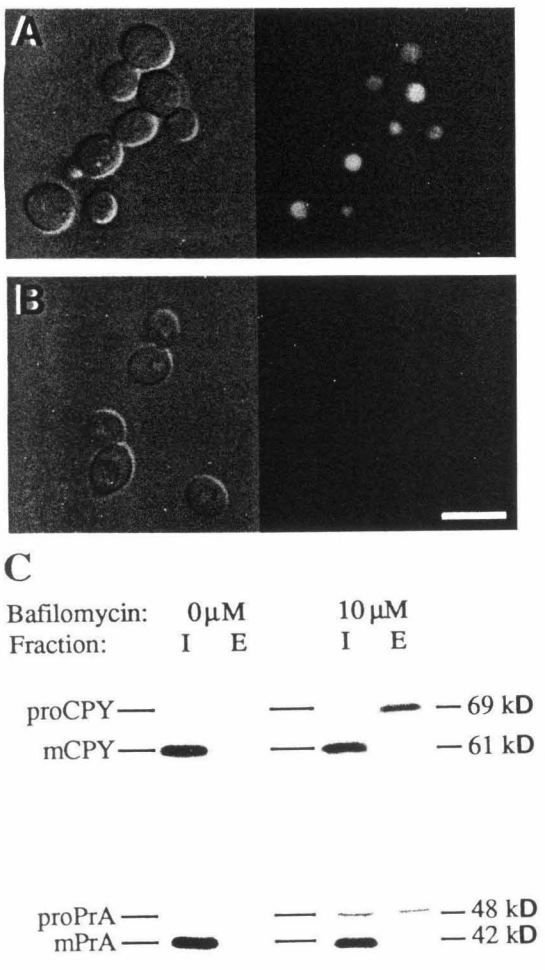

Figure 4. Effect of bafilomycin on vacuole staining with quinacrine and on vacuolar protein sorting. (*A* and *B*) Nomarski (left) and fluorescence (right) images of parental strain SEY6210 stained with quinacrine. Cells (2.5×10^7 in 0.25 ml) were preincubated for 10 min at 25°C in YPD, pH 7.6, in the absence (*A*) or presence (*B*) of 10 μM bafilomycin (in DMSO). An aliquot of cells (50 μl = 5×10^6 cells) from each sample was added to 400 μl of YPD, pH 7.6, containing 200 μM quinacrine without (*A*) or with (*B*) 10 μM bafilomycin. Cells were incubated for 5 min at 30°C, resuspended, and mounted as described in Materials and Methods. Bar, 10 μm. (*C*) Strain SEY6210 was enzymatically converted to spheroplasts as described (46). Bafilomycin was added to 10 μM final concentration as indicated 10 min before the addition of radioactive label. Cells were labeled with Tran ^{35}S-label for 20 min at 30°C and chased for 30 min by the addition of 5 mM methionine. Cultures were then separated into (*I*) intracellular (spheroplast) and (*E*) extracellular (periplasm and media) fractions (46). Immunoprecipitations with antisera to CPY and PrA were performed as described (25). The predicted locations and approximate molecular sizes of the different forms of CPY and PrA are indicated. Increased concentrations (100 μM) of bafilomycin did not increase the amount of missorted precursor CPY or PrA. Importantly, bafilmyocin had no effect on mitochondrial protein import (data not shown), which requires an electrochemical potential across the mitochondrial inner membrane (17).

or which have been treated with microtubule-disrupting drugs have a fragmented vacuole phenotype similar to that of the class B *vpt* mutants (15). We examined representative alleles of each complementation group by immunofluorescence, using anti-tubulin antibodies. No evidence of abnormal microtubule structures in any of the *vpt* mutants was observed (data not shown).

Class C vpt Mutants are Defective in Vacuole Assembly

Cells in four *vpt* complementation groups (*vpt*11, 16, 18, and 33) lacked any intracellular structures which stained in the presence of FD (Fig. 1 *D*). This phenotype was not simply due to an inability to sequester FITC in the vacuole; these mutants also failed to accumulate quinacrine or the *ade*2 endogenous fluorophore (Fig. 1 *H*). The *ade*2 dye is produced when a purine biosynthetic intermediate concentrates in the vacuole and undergoes oxidation and polymerization to produce a naturally fluorescent red pigment (23, 50). As a result, *ade*2 mutant yeast exhibit a red colony color when supplied with limiting amounts of adenine. All class A and B *ade*2 *vpt* mutants grown under conditions of limiting adenine were red. However, several *ade*2 alleles in each of the class C complementation groups were white. This phenotype was shown to be genetically linked to the *vpt* defect (see below). These observations suggest that in the absence of a functional vacuole, the purine biosynthetic intermediate is unable to undergo the reactions necessary to form the red color. We do not know whether the precursor accumulates in the cytoplasm or in other intracellular compartments, or whether it is secreted from the cell.

By EM, all class C mutants examined (at least two alleles of each of the four complementation groups) exhibited the same morphology (Fig. 8, *A–C*). Even at high magnification, these mutants appeared to lack any structure exhibiting the characteristic staining properties of a wild-type vacuole. Ultrastructural analysis also revealed that the class C *vpt* mutants accumulated a variety of novel membrane-enclosed organelles, including vesicles and Bbs. Fig. 8, *D* and *E* show higher magnification views of some of the structures that were exaggerated in these cells. The vesicles that accumulated in these mutants (Fig. 8 *D*) were enclosed by a membrane bilayer and were ~80 nm in diameter. In comparison, the vesicles that accumulate in the secretory mutant *sec*1 at nonpermissive temperature are ~100 nm in diameter and have a very different appearance from the vesicles seen in class C *vpt* mutants (37; see Fig. 6 *F*). As shown in Fig. 8 *E*, class C mutants also accumulated large, multilamellar, membrane-enclosed structures. Somewhat surprisingly, these structures were electron transparent, suggesting that they do not contain significant amounts of glycoproteins or sugars.

Several of the *vpt* complementation groups contain *ts* alleles (46). Four of these groups (*vpt*11, 16, 18, and 33) are class C, one (*vpt*3) is class B, and two (*vpt*15 and *vpt*29) are class A. These two class A mutants contained vacuoles but also accumulated organelles similar to those seen in the class C mutants (see above). We have examined the morphology

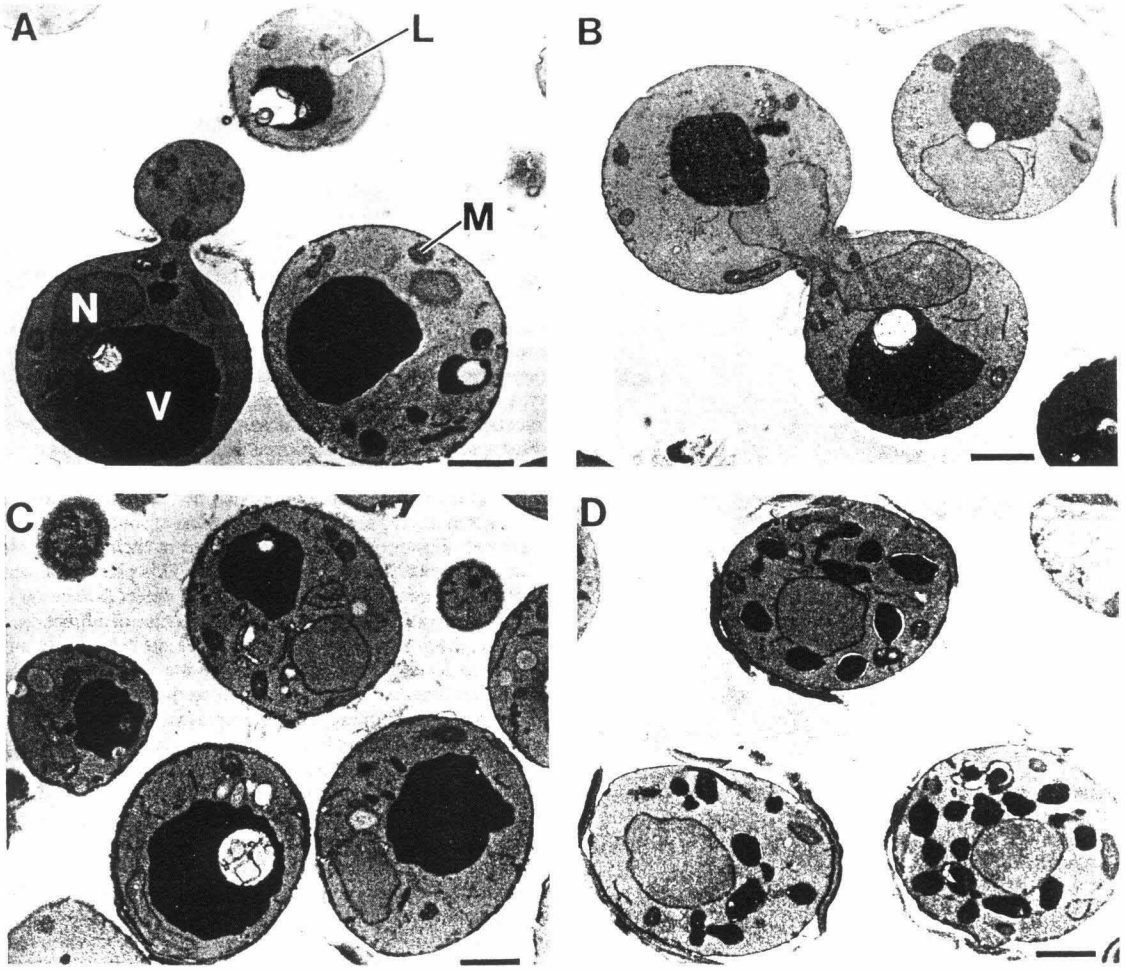

Figure 5. Electron micrographs of cells exhibiting wild-type, class A, and class B vacuole morphologies. Cells were prepared using the reduced osmium–thiocarbohydrazide-reduced osmium membrane-enhancement technique as described in Materials and Methods. (*A*) Parental strain SEY6210. Representative class A *vpt* mutants *vpt*17 (*B*) and *vpt*9 (*C*) also exhibit wild-type vacuolar and overall cellular morphology. *vpt*5 (*D*), a representative class B mutant, contains many small membrane-enclosed compartments which stain like wild-type vacuoles. *V*, vacuole; *N*, nucleus; *M*, mitochondrion; *L*, lipid droplet. Bars, 1 μm.

of these conditional lethal strains at both permissive (25°C) and nonpermissive (37°C) temperatures. Surprisingly, the vacuolar morphology of each of the *ts* strains examined was identical at 25°C and 37°C; aberrant organelles accumulated to the same extent at either temperature.

The existence of mutants in several complementation groups that lacked any apparent vacuole allowed us to begin to analyze the requirements for vacuole biogenesis in vivo. If a vacuole can be formed via a de novo synthetic pathway, diploids made by mating two class C mutants from different complementation groups should contain a normal vacuole. If, however, a preexisting normal template vacuole is necessary to direct the synthesis of the new organelle, diploids heterozygous for two class C mutations may be incapable of generating a vacuole despite the presence of both wild-type gene products. When we examined diploids made by crossing a *ts vpt*11 allele with a *ts vpt*18 allele, we observed that the diploid was temperature resistant, competent to sort vacuolar proteins, and contained a normal-appearing vacuole which stained with FITC. Homozygous diploids made by crossing two *ts* alleles of the same class C complementation group exhibited typical class C morphology and were *ts*, as expected. Furthermore, when the mating pairs were examined within 4–6 h of mixing, heterozygous class C zygotes were observed which clearly contained a vacuole in each of the conjugating cells, as well as in the diploid bud emerging from the zygote (Fig. 9). Ultrastructural analysis confirmed the presence of vacuoles in these heterozygous mating pairs

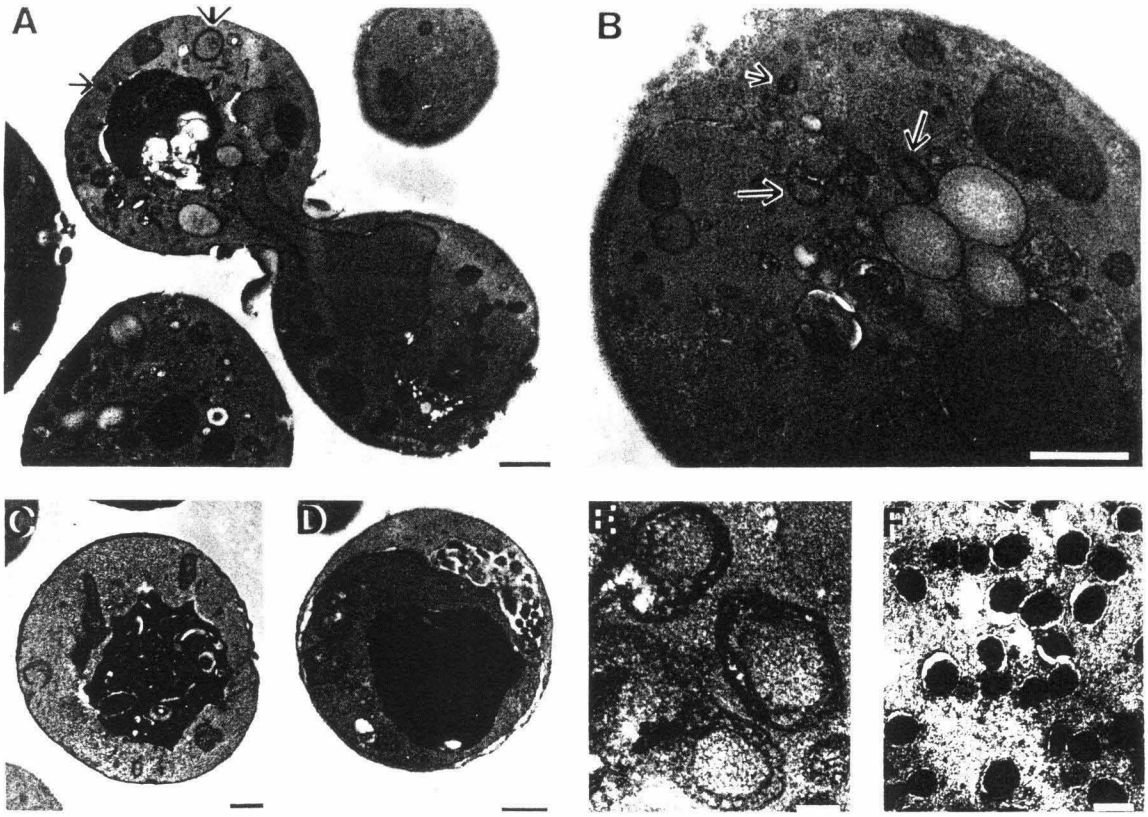

Figure 6. Electron micrographs of *vpt*15 and *vpt*29, class A mutants which exhibit aberrant organelles similar to those seen in certain of the *sec* mutants. Cells were prepared as described in the legend to Fig. 5. *vpt*15 (*A*) and *vpt*29 (*B*) cells contain Bbs (*large arrows*) and vesicles (*small arrows*) throughout the cytoplasm. (*C*) A typical *vpt*15 cell in which inclusions are seen in the vacuole. (*D*, *E*, and *F*) *sec* mutants (37) prepared using the membrane-enhancement technique, are shown for comparison. Each of the mutant strains was incubated at 37°C for 3 h before fixation. (*D*) Exaggerated tubular networks of membranes, presumably corresponding to ER, are clearly visible in this typical *sec*18 cell. (*E*) Accumulated Bbs are seen in this high magnification view of a portion of a representative *sec*7 cell. (*F*) A high magnification view of part of a *sec*1 cell shows an accumulation of secretory vesicles. These vesicles have a different appearance than those seen in *vpt*15 and *vpt*29 (compare with *A* and *B*). Bars: (*A*, *B*, *C*, and *D*) 0.5 μm; (*E* and *F*) 0.1 μm.

(not shown). These observations suggest that vacuole biogenesis can occur de novo in the absence of a normal template organelle. This assembly is rapid, occurring within one generation after conjugation. We cannot at present, however, exclude the possibility that the class C mutants contain tiny degenerate vacuole forms that are capable of functioning as templates or targets for new vacuolar protein and membrane delivery.

Class C Mutants are Defective in Several Vacuolar Functions

The vacuole has been implicated in a number of diverse cellular functions in wild-type cells, including osmoregulation (29), storage of amino acid reserves (30), endocytosis (43), and adaptation to adverse growth conditions (48). Like mammalian lysosomes and plant vacuoles (10, 28), the yeast vacuole may also mediate the normal intracellular turnover of macromolecules. Protein degradation increases dramatically during sporulation, and mutants lacking PrA or PrB activity are partially or completely defective in sporulation (22). We postulated that the class C *vpt* mutants, which exhibited extreme aberrations in vacuole assembly and morphology, might be defective in other cellular functions that may be vacuole related.

If the vacuole is required for adaptation to a change in external osmosity, one might expect that cells that lack a vacuole would be unable to survive in the presence of even a small increase in ionic or osmotic pressure. We assessed the growth of representative *vpt* alleles on YPD medium supplemented with 1.0 or 1.5 M NaCl, 1.0 M KCl, or 2.5 M glycerol. None of the class C mutants were able to survive, although the parental strains grew, under these conditions (Fig. 3 *B*). In addition, *vpt* 15, 26, and 29 were somewhat sensitive to the presence of 1.0 M NaCl or 2.5 M glycerol and were completely unable to grow on YPD medium containing 1.5 M NaCl. None of the other *vpt* mutants were sensitive to any of the osmotic stress conditions tested.

The class C mutants also exhibited other physiological

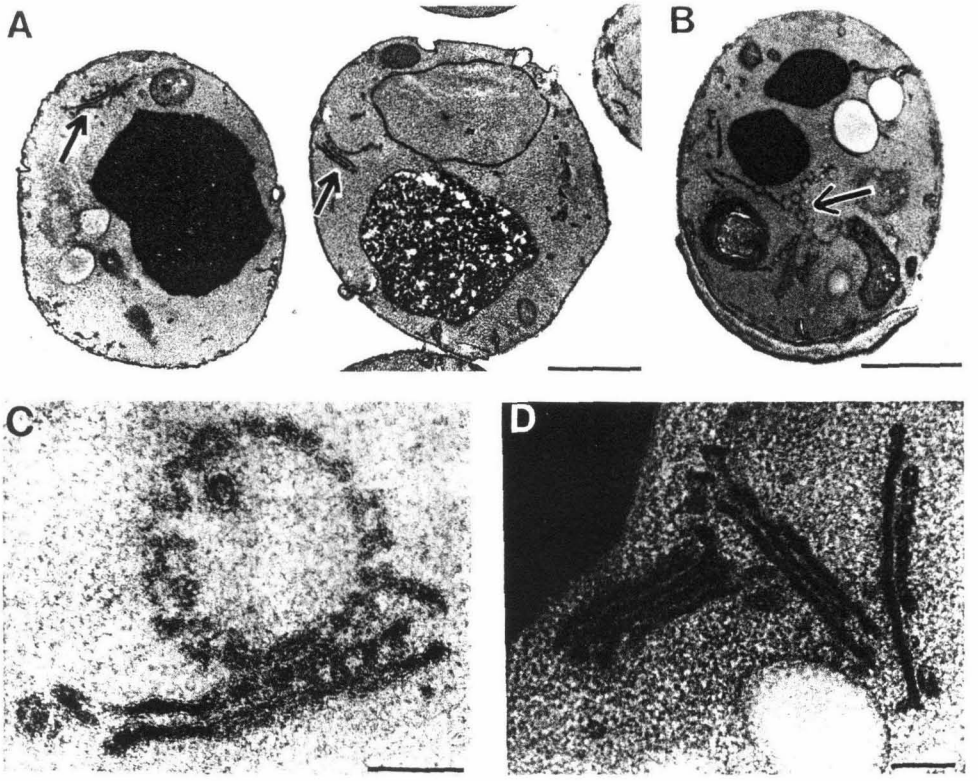

Figure 7. A gallery of electron micrographs of *vpt28* and *vpt7*, which accumulate Golgi-like structures (*arrows*). Cells were prepared as described in the legend to Fig. 5. *A* shows *vpt7* cells; *B* shows a *vpt28* cell. *C* and *D* are high magnification views of two Golgi-like structures representative of those seen in these mutants. Bars: (*A* and *B*) 1 μm; (*C* and *D*) 0.2 μm.

defects, including poor growth on nonfermentable carbon sources such as glycerol or lactate, poor growth in minimal media containing proline as the sole nitrogen source, poor sporulation of homozygous diploids, and low frequencies of DNA transformation. Furthermore, the class C *vpt* mutants contained smaller pools of basic amino acids than the class A or B *vpt* mutants, as judged by a filter assay for basic amino acids (reference 8; data not shown). Other cellular functions in the class C mutants, however, appeared to be normal. Based on the ultrastructural analysis, these mutants exhibited wild-type morphology of organelles such as the nuclei and mitochondria (see Fig. 8 *B*). The microtubules in these mutants appeared normal, as judged by immunofluorescence. Protein secretion also appeared to be unaffected in the class C as well as the other *vpt* mutants (46).

All vpt Phenotypes Cosegregate in Genetic Crosses

If the pleiotropic phenotypes discussed above all result from the *vpt* mutation in question, each of the phenotypes should cosegregate in genetic crosses of the mutants with wild-type cells. To confirm this, representative *vpt* mutants were backcrossed to the parental strain of the opposite mating type, the diploids were sporulated and tetrads were dissected. In this way, the vacuole morphology associated with each of the class B and class C *vpt* mutants was shown to cosegregate with the *vpt* defect (data not shown). The segregation of each of the other phenotypes associated with the class C mutants was also examined. In crosses between *ts* class C mutants and parental strains, all phenotypes showed the expected 2:2 segregation pattern. In each cross, the temperature sensitivity, osmotic sensitivity, and block in *ade2* red pigment formation all cosegregated with the vacuole protein sorting defect. The results of a typical tetrad analysis for a *ts* class C mutant, *vpt16*, are shown in Fig. 10. Similar tetrad analyses also demonstrated that the low pH sensitivity exhibited by *vpt13* cosegregated with the *vpt* sorting defect (data not shown).

Discussion

We have analyzed in detail the morphology and growth properties of a number of mutants defective in vacuole protein targeting. Three distinct vacuolar morphologies associated with the *vpt* mutants have been observed. The class A mutants, constituting 26 complementation groups, resembled the wild-type parent strains in that they had one or a few large vacuoles which were easily observed using light and fluorescence microscopy (Fig. 1, *A* and *B*). A second class of mutants,

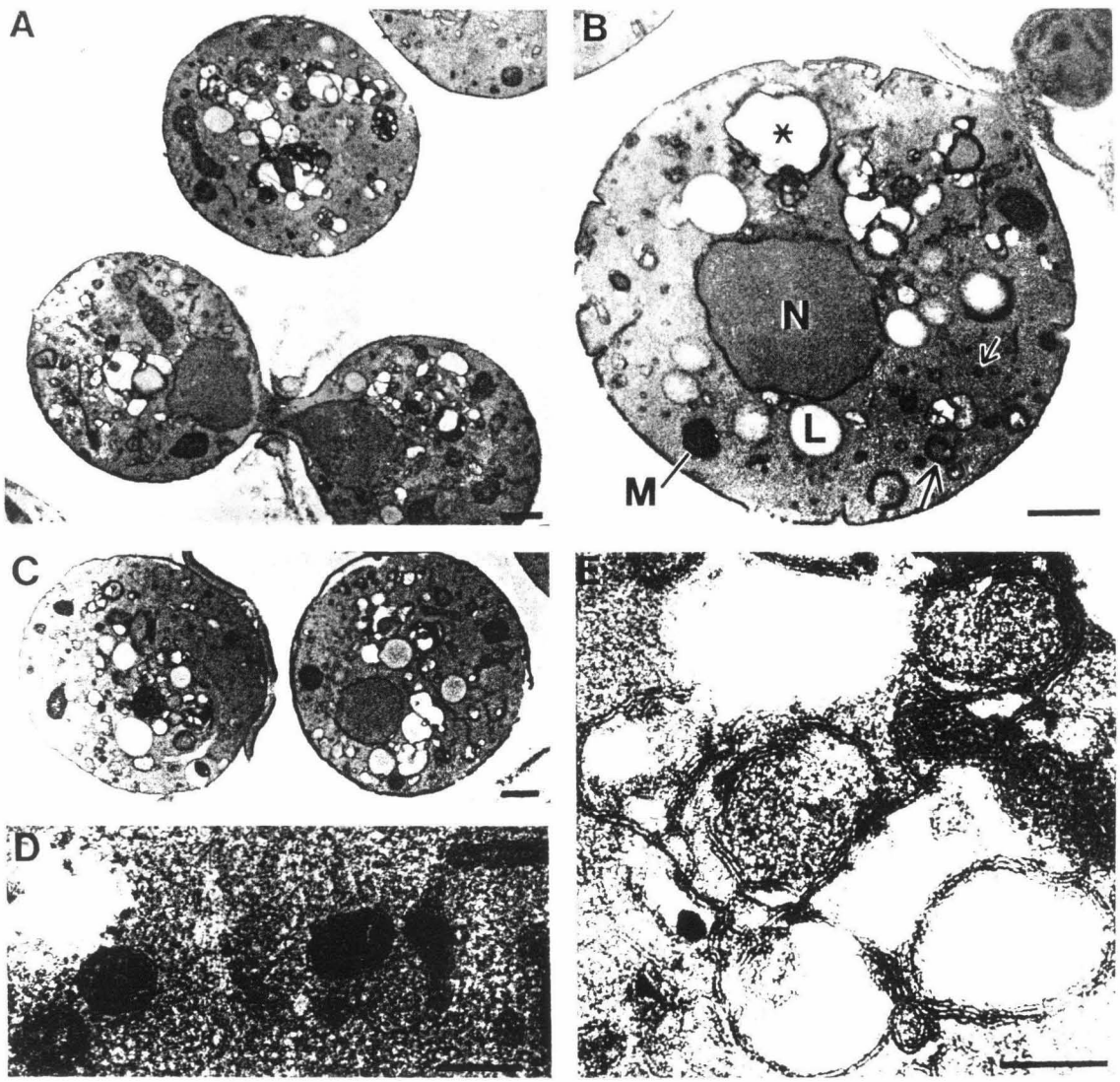

Figure 8. Electron micrographs of class C *vpt* mutants, which exhibit extreme defects in vacuole biogenesis. Cells were prepared as described in the legend to Fig. 5. (*A*, *B*, and *C*) Typical class C cells lack a discernible vacuole but accumulate aberrant organelles, including vesicles (*small arrow*), Bbs (*large arrow*), and large membranous structures (*asterisk*). *A* and *B* show *vpt*16 cells; *C* shows *vpt*11 cells. *D* shows a high magnification view of the vesicles that accumulate in class C cells (compare with vesicles in *sec*1 mutant, Fig. 6 *F*). *E* shows a high magnification view of the complex multilamellar arrays that accumulate in the cytoplasm in the class C *vpt* mutants. *N*, nucleus; *L*, lipid droplet; *M*, mitochondrion. Bars: (*A*, *B*, and *C*) 0.5 μm; (*D* and *E*) 0.1 μm.

class B, consisted of three complementation groups and was characterized by an altered morphology in which the vacuole was highly fragmented (Fig. 1 *C*). Mutants in the four class C complementation groups had no discernible vacuoles (Fig. 1 *D*), but accumulated small vesicles and other novel membrane-enclosed structures throughout the cytoplasm (Fig. 8).

The majority of the class A *vpt* mutants showed no apparent abnormalities in the vacuole itself or in other cellular features, as determined by electron microscopic analysis. In these mutants, at least some proteins presumably must continue to be properly targeted to the vacuole. Consistent with this idea, many of the class A *vpt* mutants mislocalize only a small fraction of CPY, PrA, PrB, or a CPY-Inv hybrid protein that contains vacuolar sorting information (46). It is possible that these mutants define functions which are only peripherally involved in vacuole protein targeting. However, other class A *vpt* mutants (*vpt* 1, 4, 6, 7, 15, 17, 29, and 30) exhibit gross defects in the localization and processing of

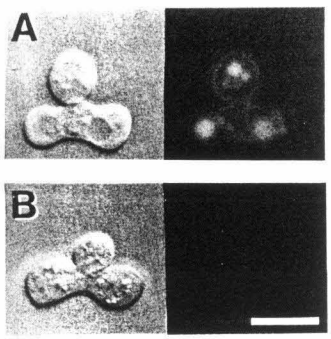

Figure 9. Heterozygous class C *vpt* zygotes form vacuoles within one generation after mating. (*A*) *vpt*18 (*MAT*α) and *vpt*33 (*MAT*à) cells were patched together on YPD medium and allowed to mate for 7 h at 25°C. Cells were scraped off the plate, resuspended in 1 ml YPD containing 50 μM Na-citrate, pH 5.5, and 1 μl of 10 mg/ml FITC was added. Cells were incubated for 10 min at 25°C, washed, and mounted as described in Materials and Methods. (*B*) Two *vpt*33 alleles were mated and stained as described in *A*. Bar, 10 μm.

vacuolar proteins, secreting as much as 70–100% of the CPY (46). These class A mutants still contain intact vacuoles and secrete <5% of the activity of a vacuolar membrane marker enzyme, α-mannosidase, suggesting that different pathways may exist for the sorting of soluble and membrane vacuolar proteins (see below).

Mutants in one of the class A complementation groups, *vpt* 13, exhibited extreme sensitivity to low pH (Fig. 3 *A*), suggesting that these cells may be unable to regulate their intracellular pH. The *vpt* 13 mutants were also defective in the pH-dependent accumulation of quinacrine in the vacuole (Fig. 2 *E*). In mammalian cells, endosomal acidity has been implicated in the proper localization of proteins to this organelle. Compounds such as amines that raise intralysosomal and endosomal pH (40) cause lysosomal enzymes to be secreted. The increase in pH appears to inhibit the uncoupling of lysosomal enzymes from their receptor carrier(s), resulting in a saturation of the available receptor sites (13). Furthermore, mammalian cell mutants have been described which appear to be defective in acidification of the endosome; these mutants secrete increased amounts of lysosomal hydrolases (45). In yeast, vacuolar pH may also play an important role in vacuole protein targeting. Treatment of wild-type yeast cells with ammonium acetate, the proton ionophore CCCP, or bafilomycin A_1, a drug which specifically inhibits the vacuolar ATPase, resulted in a block in the pH-dependent accumulation of quinacrine in the vacuole as well as the missorting of CPY and PrA (Fig. 4). Therefore, it seems likely that one or more of the *VPT* genes (e.g., *VPT*13) encode subunits of the vacuolar Mg^{++}-ATPase or other proteins involved in maintaining the vacuolar (or prevacuolar/endosomal) pH. Indeed, Stevens and colleagues (personal communication) have recently obtained data indicating that mutants in the *vpt* 13 complementation group exhibit levels of vacuolar ATPase activity that are tenfold lower than those in the wild-type strain.

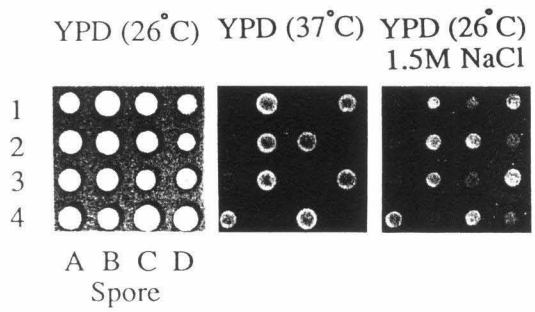

Figure 10. Cosegregation of the *ts* and osmotic-sensitive phenotypes in tetrads resulting from the sporulation of a *MAT*α *vpt*16/*MAT*à VPT⁻ diploid. *vpt*16 was crossed with parental strain SEY6211 and diploids were selected. Diploids were sporulated and tetrads dissected as described in reference 49. Segregants were patched onto YPD medium and replica-plated onto YPD medium which was incubated at 25°C or 37°C, or onto YPD medium containing 1.5 M NaCl (incubated at 25°C) as indicated. Segregants from four tetrads are shown. In each tetrad, the *ts* defect also was shown to be linked to the originally selected *vpt* phenotype, secretion of the CPY-Inv hybrid protein (46).

Unlike the class A *vpt*s, the class B mutants exhibited gross aberrations in vacuolar structure. The many small vacuole-like compartments observed in class B *vpt* mutants may represent the accumulation of an intermediate in vacuolar biogenesis or alternatively, fragmentation of a larger vacuole. These mutants might lack a vacuolar surface molecule which promotes fusion of small "prevacuolar" compartments to form a large vacuole. Alternatively, the gene products defined by the mutants could encode cellular constituents required to maintain the structural integrity of the organelle. These mutants also raise the issue of what exactly constitutes a vacuole. The organelles observed in the class B *vpt* mutants accumulated the dyes used to stain wild-type vacuoles and apparently had a similar acidic pH, as determined by the pH-dependent quinacrine staining (Fig. 2). However, the vacuole structures that accumulated in these mutants presumably are not recognized as valid destinations for certain vacuolar proteins, since the vast majority of PrA and CPY expressed in these mutants remains unprocessed and much is secreted (46).

The class C *vpt* mutants exhibited the most extreme defects in vacuole assembly among the *vpt* mutants isolated thus far. Many of these cells appeared to be essentially devoid of any organelles that resembled a vacuole, based on the criteria of size, shape, and histochemical-staining properties of normal vacuoles (Fig. 1 *D*). The observation that these cells are viable despite the absence of a vacuole indicates that many vacuolar functions may not be necessary under optimal growth conditions. Many of the class C mutants, however, are temperature sensitive for growth (46). The block in growth at 37°C exhibited by these mutants may indicate a requirement for a specific vacuolar function or, more likely, the cumulative effect of the loss of several vacuolar functions combined with the stress of growth at a temperature significantly above the preferred growth temperature of the organism.

The class C *vpt* mutants also exhibited an exaggeration of other organelles including Bbs, which are presumably related to Golgi structures and represent an intermediate com-

partment of the secretory pathway (36). The accumulation of Golgi-like structures or Bbs observed in these and certain other *vpt* mutants is consistent with a backup of vacuole proteins at the Golgi, the site of segregation for these proteins (54). In addition, the cytoplasm in these cells was filled with vesicles and complex lamellar arrays that might represent remnant vacuolar material or intermediates in vacuole biogenesis (Fig. 8). Several scenarios could account for the accumulation of organelles in these mutants. The gene products defined by the class C *vpt* mutants might correspond to essential components of a vacuolar protein sorting apparatus or to structural proteins of the vacuole itself. Alternatively, the class C *VPT* gene products might be involved in the regulation of organelle biogenesis, a process about which very little is known. Like its mammalian counterpart, the yeast vacuole may play an important role in the intracellular turnover of macromolecules. Perhaps in the class C mutants, organelles and membrane fragments accumulate because the cells lack a vacuole to perform this digestive function. The vesicles observed in these *vpt* mutants might represent intermediates in endocytic traffic that, in the absence of a vacuole, have no suitable target destination. On the other hand, the structures which accumulate in the class C mutants might represent actual intermediates in vacuole biogenesis. However, because these organelles are present at both permissive and nonpermissive growth temperatures, we are at present unable to test whether they correspond to actual reversible intermediates in the vacuole assembly pathway or are dead-end, nonreversible compartments. Additional experiments, such as immunoelectron microscopy or purification of the vesicles, will be required to address the nature of the content of these structures and their likely origin.

In plant cells, the large central vacuole plays an important role in regulating cell turgor pressure (3, 18, 61). Although not as extensively studied, it is possible that the vacuole may have a similar osmoregulatory function in yeast. High concentrations of solutes such as polyphosphates are stored in the yeast vacuole as osmotically inactive aggregates or polymers that could be converted into osmotically active forms by enzymatic digestion of the polymers. Large pools of sugars and basic amino acids (especially arginine) may also be present (29). Mutants in the four class C complementation groups were sensitive to osmotic stress (Fig. 3 *B*). Perhaps these cells are unable to accumulate the compounds normally used to generate high internal osmotic pressures. Alternatively, the osmotic-sensitive phenotype may not be directly related to vacuolar function. A number of seemingly diverse mutants, including nonsense suppressors, plasma membrane ATPase mutants, and actin mutants are sensitive to osmotic stress (31, 35, 51). Singh and Sherman (52) have suggested that, like *ts* mutations, alterations in a variety of essential proteins may make cells unusually sensitive to stressful growth conditions, in this case hypertonicity. Two class A *vpt* mutants (*vpt* 15 and 29) were partially inhibited for growth in the presence of hypertonic stress. The vacuoles in these strains were abnormally large and occasionally appeared to contain inclusions. Like class C *vpt* mutants, cells in these groups accumulated vesicles and Bbs (Fig. 6), and many of these strains are *ts* for growth. Furthermore, these mutants are severely defective in the processing and sorting of CPY and PrA (46). On the basis of the class C–like phenotypes exhibited by *vpt* 15 and 29, we propose that these mutants may represent an intermediate between the class A and C morphologies.

The class C *vpt* mutants are among the most defective in targeting CPY, PrA, and PrB to the vacuole (46). Unlike other *vpt* mutants, however, the class C mutants secrete 30–50% of the vacuolar membrane marker enzyme, α-mannosidase (46), indicating that the sorting defect in these mutants extends to membrane, as well as lumenal, proteins. Taken together, the data suggest that different recognition systems may participate in the sorting of soluble vacuole proteins, such as CPY and PrA, and vacuole membrane proteins (e.g., α-mannosidase). However, both sets of proteins may transit via common carrier vesicles or other intermediate compartments en route to the vacuole. According to such a model, the class C *vpt* mutants might affect protein sorting at the level of the common compartment. In contrast, the defects observed in many of the class A mutants, which mislocalize only a subset of the vacuole proteins, are likely to affect more specific components in the pathway, such as protein receptors.

A set of vacuolar protein localization (*vpl*) mutants similar to those described here has been isolated by selecting for the presence of active CPY in the periplasm (47). Some of these mutants also exhibit aberrant organelles such as Bbs and multivesicular bodies in addition to a normal vacuole. Complementation analysis has revealed that several of the *vpl* mutations fail to complement various *vpt* mutations. However, none of the *vpl* mutants exhibits the extreme defects in vacuole morphology seen in the class C *vpt* mutants (46, 47; Rothman, J., and T. Stevens, personal communication).

Two mutants which are defective in the accumulation of an endocytic marker, lucifer yellow carbohydrazide, and in pheromone response have been described by Chvatchko et al. (7). One of these mutants, *end* 1, has a morphology similar to that of the class C *vpt* mutants in that it lacks a vacuole and accumulates many small vesicles in the cytoplasm. This mutant is also defective in CPY processing (44) and sorting (46). Like the class C *vpt* mutants, *end* 1 grows poorly on glycerol (Dulic, V., and H. Riezman, personal communication) and is unable to grow under conditions of osmotic stress or high temperature. Crosses between *end* 1 and *vpt* 11 mutants have demonstrated that these two mutations define a single complementation group (46; Dulic, V., and H. Riezman, personal communication). This finding suggests that the vacuolar protein sorting and endocytic pathways may converge and that some gene functions may be common to both pathways. Geuze et al. have suggested that lysosomal enzymes are directed to the lysosome via a prelysosomal compartment which is also the site of uncoupling of endocytosed ligands and receptors (12). More recently, Griffiths et al. have identified a compartment in rat kidney cells which, by immunolocalization studies, appears to be shared by the lysosomal targeting and endocytic pathways (14).

The extreme defects in vacuole biogenesis, aberrations in vacuolar and cellular morphology, and increased sensitivity to suboptimal growth conditions observed in certain of the *vpt* mutants suggest strongly that vacuole structure and/or function are impaired in these strains. However, functions such as secretion and microtubule assembly appear to be normal in all of the *vpt* mutants (46). Although the class C *vpt* mutants, as well as *vpt* 15 and 29, accumulate organelles resembling those seen in certain *sec* mutants, complementa-

tion analysis has indicated that these *vpt* mutants and the *sec* mutants are not allelic (46). Other yeast mutations have been described which result in *vpt*-like morphological defects. A *ts* mutation in the single yeast actin gene (*act*1) leads to an accumulation of Bbs and vesicles similar to that seen in *vpt* 15 and 29 (35); however, complementation analysis indicates that none of the *ts vpt* mutants is allelic to *act* 1 (unpublished results). Likewise, although a deletion of the clathrin heavy chain gene in yeast causes severe morphological and growth defects, these cells continue to sort vacuolar proteins properly (39). A recently isolated mutant, *slp*1, exhibits a *vpt*-like morphology in that it lacks a central vacuole but contains many small vesicles throughout the cytoplasm (24a). We have not yet been able to test the allelism of this mutation with any of the *vpt* mutations.

The class B and C *vpt* mutants, which exhibit altered vacuolar morphologies, may define functions required for specific stages of vacuole biogenesis. These mutants may be especially useful for in vitro studies directed at reconstituting different steps in vacuole assembly. Molecular cloning of the *VPT* genes and characterization of the encoded gene products, coupled with the development of an in vitro system in which these gene products can be assayed, should help elucidate the roles these proteins play in vacuolar function, protein targeting, and organelle biogenesis.

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References

1. Allison, A. C., and M. R. Young. 1969. Vital staining and fluorescence microscopy of lysosomes. *Lysosomes Biol. Pathol.* 2:600–628.
2. Bankaitis, V. A., L. M. Johnson, and S. D. Emr. 1986. Isolation of yeast mutants defective in protein targeting to the vacuole. *Proc. Natl. Acad. Sci. USA.* 83:9075–9079.
3. Boller, T., and A. Wiemken. 1986. Dynamics of vacuolar compartmentation. *Annu. Rev. Plant Physiol.* 37:137–164.
4. Bowman, E. J., A. Siebers, and K. Altendorf. Bafilomycins: a new class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc. Natl. Acad. Sci. USA.* In press.
5. Burgess, T. L., and R. B. Kelly. 1987. Constitutive and regulated secretion of proteins. *Annu. Rev. Cell Biol.* 3:243–293.
6. Byers, B., and L. Goetsch. 1975. The behavior of spindles and spindle plaques in the cell cycle and conjugation of *Saccharomyces cerevisiae*. *J. Bacteriol.* 124:511–523.
7. Chvatchko, Y., I. Howald, and H. Riezman. 1986. Two yeast mutants defective in endocytosis are defective in pheromone response. *Cell.* 46: 355–364.
8. Cramer, C. L., and R. H. Davis. 1979. Screening for amino acid pool mutants of *Neurospora* and yeasts: replica-printing technique. *J. Bacteriol.* 137:1437–1438.
9. DeDuve, C., T. DeBarsy, B. Poole, A. Trouet, P. Tulkens, and F. van Hoof. 1974. Lysosomotropic agents. *Biochem. Pharmacol.* 23:2495–2531.
10. Ericsson, J. L. E. 1969. Mechanism of cellular autophagy. *Lysosomes Biol. Pathol.* 2:345–394.
11. Farquhar, M. G. 1985. Progression in unraveling pathways of Golgi traffic. *Annu. Rev. Cell Biol.* 1:447–488.
12. Geuze, H. J., J. W. Slot, G. J. A. M. Strous, A. Hasilik, and K. von Figura. 1985. Possible pathways for lysosomal enzyme delivery. *J. Cell Biol.* 101:2253–2262.
13. Gonzalez-Noriega, A., J. H. Grubb, V. Talkad, and W. S. Sly. 1980. Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. *J. Cell Biol.* 85: 839–852.
14. Griffiths, G., B. Hoflack, K. Simons, I. Mellman, and S. Kornfeld. 1988. The mannose-6-phosphate receptor and the biogenesis of lysosomes. *Cell.* 52:329–341.
15. Guthrie, B., and W. Wickner. 1988. Yeast vacuoles fragment when microtubules are disrupted. *J. Cell Biol.* 107:115–120.
16. Hasilik, A., and W. Tanner. 1978. Biosynthesis of the vacuolar yeast glycoprotein carboxypeptidase Y. Conversion of precursor into the enzyme. *Eur. J. Biochem.* 85:599–608.
17. Hay, R., P. Böhni, and S. Gasser. 1984. How mitochondria import proteins. *Biochim. Biophys. Acta.* 779:65–87.
18. Hellebust, J. A. 1976. Osmoregulation. *Annu. Rev. Plant Physiol.* 27: 485–505.
19. Hemmings, B. A., G. S. Zubenko, A. Hasilik, and E. W. Jones. 1981. Mutant defective in processing of an enzyme located in the lysosome-like vacuole of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 78: 435–439.
20. Indge, K. J. 1968. The isolation and properties of the yeast cell vacuole. *J. Gen. Microbiol.* 51:441–446.
21. Johnson, L. M., V. A. Bankaitis, and S. D. Emr. 1987. Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. *Cell.* 48:875–885.
22. Jones, E. W. 1984. The synthesis and function of proteases in *Saccharomyces*: genetic approaches. *Annu. Rev. Genet.* 18:233–270.
23. Jones, E. W., and G. R. Fink. 1982. Regulation of amino acid and nucleotide biosynthesis in yeast. *In* The Molecular Biology of the Yeast *Saccharomyces cerevisiae*. Metabolism and Gene Expression. J. N. Strathern, E. W. Jones, and J. R. Broach, editors. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York. 181–299.
24. Kakinuma, Y., Y. Ohsumi, and Y. Anraku. 1981. Properties of H^+-translocating adenosine triphosphatase in vacuolar membranes of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 256:10859–10863.
24a. Kitamoto, K., K. Yoshizawa, Y. Ohsumi, and Y. Anraku. 1988. Mutants of *Saccharomyces cerevisiae* with defective vacuolar function. *J. Bacteriol.* 170:2687–2691.
25. Klionsky, D. J., L. M. Banta, and S. D. Emr. 1988. Intracellular sorting and processing of a yeast vacuolar hydrolase: the proteinase A propeptide contains vacuolar targeting information. *Mol. Cell. Biol.* 8:2105–2116.
26. Lenz, A.-G., and H. Holzer. 1984. Effects of chloroquine on proteolytic processes and energy metabolism in yeast. *Arch. Microbiol.* 137:104–108.
27. Makarow, M. 1985. Endocytosis in *Saccharomyces cerevisiae*: internalization of α-amylase and fluorescent dextran into cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1861–1866.
28. Matile, P. 1969. Plant lysosomes. *Lysosomes Biol. Pathol.* 1:406–430.
29. Matile, P. 1978. Biochemistry and function of vacuoles. *Annu. Rev. Plant Physiol.* 29:193–213.
30. Matile, P., H. Moor, and C. F. Robinow. 1969. Yeast cytology. *In* The Yeasts. A. H. Rose, and J. S. Harrison, editors. Academic Press, London. 219–302.
31. McCusker, J. H., D. S. Perlin, and J. E. Haber. 1987. Pleiotropic plasma membrane ATPase mutations of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 7:4082–4088.
32. Mechler, B., M. Müller, H. Müller, M. Meussdoerffer, and D. H. Wolf. 1982. *In vivo* biosynthesis of the vacuolar proteinases A and B in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* 257:11203–11206.
33. Navon, G., R. G. Shulman, T. Yamane, T. R. Eccleshall, K.-B. Lam, J. J. Baronofsky, and J. Marmur. 1979. Phosphorus-31 nuclear magnetic resonance studies of wild-type and glycolytic pathway mutants of *Saccharomyces cerevisiae*. *Biochemistry.* 18:4487–4499.
34. Nishimura, M. 1982. pH in vacuoles isolated from castor bean endosperm. *Plant Physiol. (Bethesda).* 70:742–744.
35. Novick, P., and D. Botstein. 1985. Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell.* 40:405–416.
36. Novick, P., S. Ferro, and R. Schekman. 1981. Order of events in the yeast secretory pathway. *Cell.* 25:461–469.
37. Novick, P., C. Field, and R. Schekman. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell.* 21:205–215.
38. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA.* 75:3327–3331.
39. Payne, G. S., T. B. Hasson, M. S. Hasson, and R. Schekman. 1987. Genetic and biochemical characterization of clathrin-deficient *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 7:3888–3898.

40. Poole, B., and S. Ohkuma. 1981. Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. *J. Cell Biol.* 90:665–669.
41. Preston, R. A., R. F. Murphy, and E. W. Jones. 1987. Apparent endocytosis of fluorescein isothiocyanate–conjugated dextran by *Saccharomyces cerevisiae* reflects uptake of low molecular weight impurities, not dextran. *J. Cell Biol.* 105:1981–1987.
42. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17:208–212.
43. Riezman, H. 1985. Endocytosis in yeast: several of the yeast secretory mutants are defective in endocytosis. *Cell.* 40:1001–1009.
44. Riezman, H., Y. Chvatchko, and V. Dulic. 1986. Endocytosis in yeast. *Trends Biochem. Sci.* 11:325–328.
45. Robbins, A. R., S. S. Peng, and J. L. Marshall. 1983. Mutant Chinese hamster ovary cells pleiotropically defective in receptor-mediated endocytosis. *J. Cell Biol.* 96:1064–1071.
46. Robinson, J. S., D. J. Klionsky, L. M. Banta, and S. D. Emr. Protein sorting in yeast: isolation of mutants defective in the processing and sorting of multiple vacuolar hydrolases. *Mol. Cell. Biol.* In press.
47. Rothman, J. H., and T. H. Stevens. 1986. Protein sorting in yeast: mutants defective in vacuole biogenesis mislocalize vacuolar proteins into the late secretory pathway. *Cell.* 47:1041–1051.
48. Schwencke, J. 1988. The vacuole, internal membranous systems and vesicles. *In* The Yeasts. A. H. Rose, and J. S. Harrison, editors. Academic Press, London. In Press.
49. Sherman, F., G. R. Fink, and C. W. Lawrence. 1979. Methods in Yeast Genetics: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 1–98.
50. Silver, J. M., and N. R. Eaton. 1969. Functional blocks of the *ad*1 and *ad*2 mutants of *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* 34:301–305.
51. Singh, A. 1977. Nonsense suppressors of yeast cause osmotic-sensitive growth. *Proc. Natl. Acad. Sci. USA.* 74:305–309.
52. Singh, A., and F. Sherman. 1978. Deletions of the iso-1-cytochrome c and adjacent genes of yeast: discovery of the *osm*1 gene controlling osmotic sensitivity. *Genetics.* 89:653–665.
53. Sly, W. S., and H. D. Fischer. 1982. The phosphomannosyl recognition system for intracellular and intercellular transport of lysosomal enzymes. *J. Cell. Biochem.* 18:67–85.
54. Stevens, T., B. Esmon, and R. Schekman. 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell.* 30:439–448.
55. Stevens, T. H., J. H. Rothman, G. S. Payne, and R. Schekman. 1986. Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y. *J. Cell Biol.* 102:1551–1557.
56. Valls, L. A., C. P. Hunter, J. H. Rothman, and T. H. Stevens. 1987. Protein sorting in yeast: the localization determinant of yeast vacuolar carboxypeptidase Y resides in the propeptide. *Cell.* 48:887–897.
57. Weisman, L. S., R. Bacallao, and W. Wickner. 1987. Multiple methods of visualizing the yeast vacuole permit evaluation of its morphology and inheritance during the cell cycle. *J. Cell Biol.* 105:1539–1547.
58. Wieland, F. T., M. L. Gleason, T. A. Serafini, and J. E. Rothman. 1987. The rate of bulk flow from the endoplasmic reticulum to the cell surface. *Cell.* 50:289–300.
59. Wiemken, A., M. Schellenberg, and K. Urech. 1979. Vacuoles: the sole compartments of digestive enzymes in yeast (*Saccharomyces cerevisiae*)? *Arch. Microbiol.* 123:23–35.
60. Willingham, M. C., and A. V. Rutherford. 1984. The use of osmium-thiocarbohydrazide-osmium (OTO) and ferrocyanide-reduced osmium methods to enhance membrane contrast and preservation in cultured cells. *J. Histochem. Cytochem.* 32:455–460.
61. Zimmermann, U. 1978. Physics of turgor- and osmoregulation. *Annu. Rev. Plant Physiol.* 29:121–148.

Chapter 4

The *Saccharomyces cerevisiae* Vps18 protein has a functional, cysteine-rich, C-terminal domain and facilitates the late Golgi processes of protein sorting to the vacuole and α-factor processing.

ABSTRACT

Saccharomyces cerevisiae strains carrying *vps18* mutations have defects in the sorting and transport of vacuolar enzymes such as Carboxypeptidase Y and Proteinase A. The inactive-precursor forms of these proteins are mislocalized to the periplasm and growth medium. Most of the alleles also have a defect in vacuole biogenesis that leaves them without a morphologically identifiable vacuole. Several mutants are unable to divide at elevated growth temperatures or at high salt concentrations. Mutants in the *pep3* complementation group (Jones (1977) *Genetics* **85**: 23-33) have similar phenotypes and define the same locus as *vps18*. A plasmid capable of complementing the temperature-sensitive growth defects of strains carrying the *vps18-4*^{a} allele was isolated from a centromere-based yeast genomic library. Integrative mapping showed that the 26 kb insert in this plasmid derives from the *VPS18* locus. Strains in which the *VPS18* gene has been deleted are viable and have the same phenotypes as spontaneous temperature-sensitive *vps18* alleles. In addition, *vps18Δ1::TRP1*, *MATα* strains secrete a large portion of their α-factor in precursor, rather than mature, form. Therefore the *trans* Golgi compartment in which Kex2p resides is somehow altered in these mutants. The 4kb minimum complementing fragment can encode a 918aa protein. The predicted Vps18p sequence revealed a cysteine-rich, zinc finger like motif close to the carboxyl terminus. A mutant was made in which the first cysteine of the motif was altered to serine. This allele showed a temperature-conditional, CPY sorting defect with very rapid onset when introduced on a *CEN*-vector into the *vps18Δ1::TRP1* strain. A similar cysteine-rich motif is found near the C-terminus of the protein encoded by another gene involved in vacuole biogenesis (*PEP5/END1*). Mutant alleles of this gene were also isolated in our screen for vacuolar protein sorting mutants (*vps11*). Both *vps11* and *vps18* mutants have the Class C vacuole-deficient phenotype. This similarity between the products of two related*VPS* gene products raises the possibility of interaction via disulphide bonds between the cysteine-rich regions of these proteins.

INTRODUCTION

Eukaryotes have cells that contain many membrane-bound compartments, all having a different form and function. The question of how these diverse membrane-enclosed organelles are constructed and maintained, each with its own specific structural components, enzymes and substrates, is interesting and complex.

Proteins destined for the plasma membrane and for the lysosome are initially targeted to the endoplasmic reticulum (ER) and from there travel through a pathway consisting of a series of membrane-enclosed organelles and vesicles. This is known as the secretory pathway. Nascent peptides are translocated into the ER where they receive some carbohydrate additions and some hydrolytic modifications. The proteins then pass in small vesicles to the Golgi apparatus where they travel from one cisterna to the next, probably also in vesicle intermediates, receiving further appropriate modifications. Upon exit from the trans Golgi, the lysosomal proteins enter one pathway directed to the lysosome, and the secretory proteins enter another pathway that will deliver them to the plasma membrane. Plasma membrane delivery and probably also lysosomal delivery are accomplished by the movement of specific vesicles. Where the pathways diverge, a large scale protein sorting operation must continuously be taking place.

The vacuole of the yeast *Saccharomyces cerevisiae* is analogous to the lysosome of mammalian cells in many respects. The lumen of the vacuole contains many of the hydrolytic enzymes of the cell. The soluble vacuolar enzymes of yeast include Carboxypeptidase Y (CPY), Proteinase A (PrA) and Proteinase B (PrB) (Ammerer et al. 1986; Hasilik and Tanner 1978; Jones 1984; Klionsky et al. 1990; Moehle et al. 1987). These digestive enzymes are transported to the vacuole in inactive, precursor form, apparently to prevent degradation of cell components they might come in contact with on the way to the vacuole. As in other eukaryotes, soluble proteins en-route to the yeast vacuole pass through early stages of the yeast secretory pathway. The main evidence for this is genetic; secretion-defective (*sec*$^-$) mutants that block secretion

at early stages of that transport pathway such as in the ER or Golgi compartments, also block the transport of soluble proteins to the vacuole (Stevens et al. 1982). In addition, mutants that have defects in biosynthetic functions known to take place in the secretion pathway, for example the glycosylation of secretory proteins, also affect the biosynthesis of vacuolar proteins (Moehle et al. 1989). There is evidence that when the yeast cell is unable to localize its vacuolar proteins correctly, they instead continue along the final stages of the secretion pathway to the surface of the cell. Mutations in the vacuolar sorting domain of CPY result in the secretion of this protein (Valls et al. 1990). Overproduction of CPY from a multicopy plasmid results in the secretion of the excess CPY, suggesting secretion is the default and that the vacuole delivery pathway or some component of the sorting apparatus for CPY had become saturated (Stevens et al. 1986). When a fusion protein was examined (Johnson et al. 1987, see below) the vacuolar localization signals of CPY appeared to have precedence over the plasma membrane delivery signals of Invertase (Inv).

Many spontaneous mutants with defects in vacuolar protein sorting were recently isolated in this laboratory (Bankaitis et al. 1986; Robinson et al. 1988). This was done with the aid of a selection scheme that made use of CPY-Inv fusion proteins that possess vacuolar targeting signals and invertase activity (Johnson et al. 1987). In wild-type cells, the CPY-Inv fusion protein is accurately localized to the vacuole by virtue of the localization signals contained in the CPY portion. Since the invertase activity of the fusion protein is useful only for purposes of sucrose utilization if it is at the cell surface, it was possible to select for mutants that mistarget the CPY-Inv fusion protein to the cell surface. This screen identified more than 33 complementation groups with defects in *v*acuolar *p*rotein *s*orting (*vps*) (Robinson et al. 1988). Other laboratories also isolated mutants with similar phenotypes using various different genetic selections and screens (Chvatchko et al. 1986; Jones 1977; Kitamoto et al. 1981; Ohya et al. 1986; Rothman and Stevens 1986). Genetic comparisons led to the

estimation that more that 50 gene products have some part to play in the biogenesis and maintenance of the yeast vacuole (Klionsky et al. 1990; Robinson et al. 1988; Rothman et al. 1989).

Among the *vps* mutants isolated as described above, several complementation groups showed interesting plieotropic phenotypes. A set of four genes gave rise to what were termed "Class C" *vps* mutants. The most striking feature of the Class C phenotype is the abnormal morphology of the cells; they possess dramatically reduced vacuoles and accumulate aberrant membrane-enclosed structures within the cytoplasm (Banta et al. 1988). In addition, each of these genes has at least two alleles that exhibit a genetically linked temperature-sensitive (Ts) growth phenotype (Robinson et al. 1988). These mutants, *vps11*, *vps16*, *vps18*, and *vps33*, have several additional vacuole-associated defects in common. These defects include mislocalization of high percentages of soluble vacuolar enzymes such as CPY, slow growth at all incubation temperatures and especially on synthetic media, inability of homozygous diploids to sporulate, lack of the characteristic red color of the endogenous fluorophor normally accumulated in the vacuole of *ade2* mutants and osmotic sensitivity of certain alleles. We reasoned that if mutation of a single genetic locus could cause such major defects in vacuolar protein delivery and vacuole biogenesis, it was likely that such a gene would encode a product that had a central role in direction of the vacuole delivery pathway or in sorting. For this reason one of these genes, *VPS18*, has been the focus of this study.

Eight spontaneous alleles of *vps18* were isolated in our screen for vacuolar protein sorting defective mutants outlined above. Depending on the allele, as much as 85% of p2 CPY is delivered to the cell surface. Most of the alleles also have the Class C defect in vacuole biogenesis that leaves them without a morphologically identifiable vacuole as described above. Four of the alleles (*vps18-1*, *vps18-3*, *vps18-4*^{a} and *vps18-5*) exhibit a Ts growth phenotype, rendering the yeast unable to divide sufficiently to form single colonies on rich medium (YPD) at 37°C. Strains carrying the

same alleles were unable to grow at room temperature on YPD containing 1.5M NaCl, a high salt medium on which the wild-type strains SEY6210 and SEY6211 grow. For the soluble hydrolases tested, the vacuolar protein sorting defect appeared to be as severe at 26°C as at 37°C in the spontaneous Ts alleles of *vps18* . These Ts and osmotic-sensitive (Os) growth defects were genetically linked to the *vps* phenotype in each case (Robinson et al. 1988).

During the course of this work, it became clear (by comparison of restriction maps and sequences) that the *vps18* and *pep3* alleles define the same locus. The *pep3* alleles were isolated in a screen for mutants with reduced CPY activity, and mapped to Chromosome XII R (Jones 1977). The phenotypes described for strains bearing mutant alleles of *pep3* are similar to those of *vps18* and also include the following, not assessed for *vps18*: hypersensitivity to amino acid and pyrimidine analogues, reduced amino acid pools, genetic suppression of alleles at the *CAN1* (arginine permease) locus and inability to utilize glycerol as a carbon source (Jones 1983). The *PEP3* gene has been cloned and sequenced (R. Preston and E. Jones, personal communication).

The isolation of the *VPS18* gene from a yeast genomic library and its DNA sequence are described in this paper. As a result of these studies, a functional region of Vps18p has been identified.

MATERIALS AND METHODS

Strains, growth media, genetic methods and gene cloning. Standard genetic methods were used throughout. The yeast and *E.coli* strains used in this study are shown in Table1. Standard yeast rich (YPD), minimal (SM) and sporulation media were prepared as described previously and supplemented with the appropriate amino acids (Sherman et al. 1979).

Analysis of revertants of the strain SEY18-4 that had recovered the ability to grow at 37°C indicated that the allele, *vps18-4^a*, carries an amber mutation. Four independant revertants were crossed with strain SEY6211, and these diploids were genetically analyzed to determine if extragenic suppressors of *vps18* had been obtained. These diploids were heterozygous for *lys2-801^a*, an allele of the *LYS2* gene carrying an amber mutation. The extragenic suppressors found responsible for reversion of the Ts and *vps* phenotypes of *vps18-4^a* were also found to suppress the auxotrophic Lys$^-$ phenotype that should have segregated 2:2 in the cross, implying that they are all suppressors of amber mutations.

Isolation of the *VPS18* gene was as follows. Approximately 12000 Ura$^+$ transformants of the *vps18-4^a*, *ura3-52* strain (SEY18-4) were selected on minimal medium without uracil (SM-Ura) after introduction of a yeast genomic library carried on the YCp50 shuttle vector (Rose et al. 1987). These colonies were screened for temperature-resistant (Tr) transformants by replica plating to rich medium (YPD) and incubating at 37°C for 2 days. One of the Tr strains identified was found to carry a plasmid (pJSR1) responsible for complementing the defects of *vps18-4^a*.

For integrative mapping of the cloned DNA, a plasmid (pJSR2) was constructed in which the approximately 5kb BamHI to PstI fragment of pJSR1 was inserted next to the yeast TRP1 gene on an integrative vector (pPHY10) (Herman and Emr 1990). After digestion with ClaI (this site maps 3' to the *VPS18* open reading frame), pJSR2 DNA was transformed into SEY6211. Two separate Trp$^+$

transformants, in which the *TRP1* gene should have recombined into the chromosome next to the ClaI site of the *vps18*-complementing DNA, were crossed with a *vps18*$^-$ strain; SEY18-4. Analysis of twelve tetrads dissected from each diploid gave 2:2 segregation of *TRP*$^+$:*trp*$^-$, and of *VPS*$^+$:*vps*$^-$; *TRP*$^+$ cosegregated with *VPS*$^+$ in every case.

Mating types of strains were determined by the standard mating factor halo test on lawns of yeast cells supersensitive to mating pheremone(s). Bioassays for α-factor were on lawns of *sst1*, *MATa* cells suspended in top YPD/agarose for even spreading as described (Julius et al. 1983).

Plasmids and recombinant DNA. Standard methods were used for recombinant DNA manipulations (Ausubel et al. 1987; Maniatis et al. 1982). The YcP50 library (Rose et al. 1987) was the kind gift of M. Rose. Introduction of shuttle vectors into yeast was by the lithium acetate method (Ito et al. 1983).

The original *vps18*-complementing clone; pJSR1, comprised a 26 kilobase (26kb) insert in YcP50 (a centromere carrying shuttle vector for yeast and *E. coli).* Preliminary restriction mapping indicated that a 20kb ClaI fragment could be deleted from pJSR1 to leave 6kb of the insert in the slightly shortened (Sau3A-ClaI deleted), but still functional, YCp50 vector. This plasmid (pJSR3) complemented the Ts-growth and the vacuolar protein sorting defects of SEY18-4. Other subclones of pJSR1 for complementation analysis were constructed in centromeric yeast-*E. coli* shuttle vector pPHYc18 (Herman and Emr 1990). These subclones were as follows: the 4kb EcoRV fragment (Fig. 1A) inserted in the SmaI site of pPHYc18 (pJSR4), which gave partial complementation of the phenotypes of JR*Sc*184; the 2kb XbaI-to-ClaI fragment (pJSR5), which did not complement *vps18-4*^{a} and the 3.5kb SacI-to-KpnI fragment (pJSR6), which complemented all the phenotypes of SEY18-4 tested and therefore defined the smallest *vps18*-complementing subclone of the original plasmid.

The plasmid pJSR2 for integrative mapping was as described in genetic methods. The construct used for gene disruption of *VPS18* was as shown in Figure 1. This was cut with SphI and ClaI to expose recombinagenic ends and then transformed into haploid and diploid *VPS18* yeast strains; SEY6211 and SEY6210·5 (the diploid resulting from crossing SEY6211 and SEY6210, the haploid genotypes are given in Table1), respectively. Trp+ transformants were obtained from both. One haploid Trp$^+$ transformant was tested and found to have the phenotypes of *vps18-4*^{a} ie.; Ts, Os, *vps*, and morphologically abnormal vacuoles. A Southern blot confirmed that this strain, JSR18Δ1, contained the null mutation (*vps18-Δ1::TRP1*).

DNA sequencing and mutagenesis. The 4kb, EcoRV fragment (Fig 1A) was inserted into Bluescript vectors at the SmaI site in both orientations, and two series of nested ExoIII deletions were made. When the long open reading frame was found to extend beyond the 3' EcoRV site, further ExoIII deletions were prepared from another Bluescript construct carrying a large insert extending 10kb 3' from the BglII site shown in Figure 1. All DNA was sequenced using the chain-termination method (Sanger et al. 1977).

Site-directed mutagenesis of the *VPS18* gene was carried out as described (Kunkel 1985). The 3.5 kb KpnI-to-SacI fragment was inserted into the appropriate sites of M13mp18 RF DNA to produce M13JR1. Single-stranded, uracil-containing M13JR1 DNA was isolated from the *dut ung E. coli* strain, BW313. The oligonucleotides 5'-GAAAATTCAATGCATGCGACAAGTCC-3' and 5'-CACATTCATCAGAGCTCTTA CCTGG-3' were used to mutagenize the uracil-containing M13JR1 sequence; each oligonucleotide introduced a restriction site for identification of mutagenized RF M13JR1 carrying the desired alteration. Each mutagenized *VPS18* KpnI-to-SacI (minimum-complementing) fragment was moved back from M13 into a yeast-*E. coli*, centromeric shuttle vector (pPHYc18) to produce pJSR8 ($\Delta FGEI_{400\text{-}403}$) and pJSR9 ($C_{826}$ to S). It was necessary to use partial

digestion to construct pJSR9 as a second SacI site had been introduced by the mutagenesis. Using this newly introduced SacI site in pJSR9, we also generated a construct (pJSR10, $\Delta S_{825\text{-end}}$) in the pPHYc18 vector. These plasmids were transformed into strains JR*Sc*184 and JR*Sc*18Δ1 to test their ability to complement the *vps18* mutant phenotypes.

Immunoprecipitations. For immunoprecipitation of α-factor, cells were labeled with Tran-^{35}S label as described by (Graham and Emr 1991) and in the figure legends where appropriate. The α-factor antisera were the generous gift of R.Sheckman. For carboxypeptidase Y (CPY) immunoprecipitations, cells were labeled as above except that labeling was for 30', and chase was with cold methionine alone for 30' except where noted in the figure legends. The preparation of CPY antisera was described previously (Klionsky et al. 1988).

Materials. Agar and other growth media components were from Difco (Detroit, Mi.). 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal), isopropyl-β-D-thiogalactopyranoside (IPTG), and several DNA-modifying enzymes were from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Other DNA-modifying enzymes were from New England Biolabs (Beverly, Mass.). Sequenase DNA sequencing kit and enzyme were from United States Biochemicals (Cleveland, Ohio). The α-^{35}S(dATP) was from Amersham (Arlington Heights, Ill.) and tran-^{35}S label was from ICN Radiochemicals (Irvine, Calif.). 5(6)-carboxy-2'-7'-dichlorofluorescein diacetate (CDCFDA) was from Molecular Probes (Eugene, Ore.). 5-Fluororotic acid (5FO) was from PCR (Gainesville, Fla.). Other reagents were from Sigma (St. Louis, Mo.) and additional standard sources.

RESULTS

Isolation and analysis of a plasmid carrying the *VPS18* gene. Eight spontaneous alleles of *vps18* that missort CPY and deliver it to the cell surface in precursor (p2) form were isolated in our screen for mutants defective in vacuolar protein targeting (Robinson et al. 1988). For each allele tested all of the CPY is in precursor, p2, form and depending on the allele, as much as 85% of p2 CPY is delivered to the cell surface. Strains with *vps18* mutations showed abnormal cell morphology. Four of the alleles (*vps18-1*, *vps18-3*, *vps18-4*^{a} and *vps18-5*) exhibit a Ts growth phenotype. One allele, *vps18-4*^{a}, was found to carry an amber mutation when we isolated revertants of the strain SEY18-4 that had recovered the ability to grow at 37°C (methods).

We made use of the recessive Ts growth defect of strains carrying the *vps18-4*^{a} allele to isolate the *VPS18* gene from a *CEN*-vector based yeast genomic library (see methods). A complementing plasmid, pJSR1, was isolated. After recovery in *E. coli*, purification and retransformation into SEY18-4, pJSR1 rescued all the recessive defects tested, including the Ts growth defects (Fig. 1B), vacuolar protein sorting defects (CPY and CPY-Inv hybrid protein), and abnormal cell morphology.

To determine if the complementing plasmid contained the *VPS18* gene, we investigated linkage of the cloned DNA to the *VPS18* locus by testing to see whether it could direct integration of a plasmid carrying the yeast *TRP1* gene at the *VPS18* chromosomal locus. The appropriate construct (pJSR2, methods) was transformed into SEY6211. Two independent Trp$^+$ transformants were crossed with SEY18-4. Every tetrad analyzed (12 from each diploid) was a parental ditype with respect to *trp1* and *vps18* (i.e., each segregant was either *vps18 trp1* or *VPS18 TRP1*) indicating that the DNA insert in pJSR2 was indeed derived from the *VPS18* chromosomal locus.

Initial restriction mapping showed that the original complementing plasmid, pJSR1, carries an insert of approximately 26kb. In order to ascertain the size of the

VPS18 gene, smaller fragments were subcloned into a *CEN*-vector (see methods). Transformation of SEY18-4 gave complementation information that placed the putative Vps18p-coding region within about 4kb of DNA. A restriction map for this DNA fragment is shown in Figure 1A.

The novel protein encoded by *VPS18* has a zinc-finger motif and a consensus cAMP binding site. The nucleotide sequence of *VPS18* was determined by sequencing a series of exonuclease III-generated deletion templates using the dideoxy nucleotide chain termination method. The sequence contained a continuous open reading frame with the potential to encode 918 amino acids. The DNA sequence is presented in Figure 2, and the deduced amino acid sequence is also shown. The predicted protein sequence is fairly uniformly hydrophillic in nature as determined from a Kyte and Doolittle analysis (Kyte and Doolittle 1982) (not shown). No obvious signal sequence or trans membrane domains were identified. There are seven potential glycosylation sites present in the sequence. A Chou Fastman prediction of the peptide structure revealed no structural features of known function.

Sequence comparisons with the NBRF (National Biomedical Research Foundation) protein data base and the GenBank nucleic acid data base using homology comparison programs TFASTA and FASTA (Pearson and Lipman 1988) identified a short stretch of sequence identity with the cAMP binding sites of cAMP dependent protein kinases. This seven amino acid region of identity comprises the amino acids FGEIAL and corresponds to the consensus cAMP binding site also found in CAP (or CRP) protein from *E. coli* (underline with dashed line in Figure 2). Although a conserved sequence was missing close to the central motif, the first reasonable match being 49 amino acids distant, the complete identity to the FGEIAL sequence element led us to question its possible involvement in the functioning of Vps18p.

Another sequence comparison (Altschul et al. 1990) performed at NCBI on the same databases using the BLAST network service identified a cysteine-rich

sequence close to the C-terminus of Vps18p with sequence similarity to the 43 kDa postsynaptic protein (Froehner 1989) and certain "zinc finger" proteins (Berg 1990). The pattern of cysteine residues is $CX_2CX_{13}CX_2CX_4CX_{38}CX_2C$ as shown underlined in Figure 2. Some of the Carboxy terminal region of the Vps18p sequence is shown in Figure 3 compared with sequences from the Postsynaptic 43 kDa protein of rat, and Vps11p (=Pep5p/End1p), Rad18p and Pdr1p, all from *S. cerevisiae*.

By visual inspection, we noted that the previously published sequence of another gene involved in the biogenesis or maintenance of yeast vacuoles also shows a cysteine-rich, C-terminal region with an arrangement of cysteines very similar to that of Vps18p (Figure 3). This gene is known in the literature as *PEP5* (Woolford et al. 1990) and as *END1* (Dulic and Riezman 1989). The sequence similarity between Vps18 and Pep5/End1 encompases more amino acids than just the cysteines and has a symmetrical arrangement of histidines and cysteines. It is interesting to note that in our original screen for vacuolar protein sorting defective mutants (Robinson et al. 1988), we had also obtained alleles of this gene; *vps11-1* to *vps11-8* . These *vps11* mutants, like *vps18*, have the unique Class C vacuolar morphology, and some alleles also have defects in growth at high temperatures (Banta et al. 1988; Robinson et al. 1988).

Deletion of the entire *VPS18* open reading frame gave a viable yeast strain that has growth defects at 37°C. To discover the phenotypic consequences of deleting the *VPS18* gene, a plasmid was constructed in which the entire open reading frame deduced from the DNA sequence was replaced with the yeast *TRP1* gene and additional (*E. coli*-derived) DNA. This plasmid was digested with SphI and ClaI to produce recombinogenic ends homologous to the *VPS18* locus (Figure 1A) and transformed into wild-type strains. Among several haploid Trp^+ transformants defective for growth at 37°C, one was crossed with the strain SEY18-4 that has a Ts allele of *vps18*. The resulting diploid was also Ts, indicating that the recessive Ts mutation obtained after *TRP1* integration is in the *vps18* complementation group. This

haploid strain, JSR18Δ1 (with the *vps18-Δ1::TRP1* mutation) was also crossed with SEY6211; the diploid was not Ts, indicating that the *vps18-Δ1::TRP1* mutation is recessive. Tetrads were analyzed; the segregants were tested for Ts and *vps* phenotypes which cosegregated with *TRP1*$^+$ in each case (12 tetrads, all segregants viable), indicating that the integration of *TRP1* and the simultaneous acquisition of *vps18* phenotypes resulted from the same event and that no genetically unlinked suppressor of lethality was segregating in the cross. A southern blot of DNA from the *vps18-Δ1::TRP1*-containing strain confirmed that the *VPS18* locus had been altered in size and DNA sequence (not shown). The growth phenotypes of JSR18Δ1 at different temperatures are shown in Figure 2B. Also shown are wild-type, *vps18-4*^{a} and the *vps18* null mutant transformed with the smallest complementing fragment of the Vps18-encoding DNA clone, under the same growth conditions. That the *VPS18* gene deletion strain was viable, although Ts for growth at 37°C was not unexpected. This is because of the previous observations that a viable, but Ts nonsense allele of *VPS18* exists (methods) and that no known *VPS* gene is essential for growth (discussion).

***In vitro* mutagenesis of *VPS18*.** The role of the putative cAMP-binding site in Vps18 was tested by carrying out *in vitro* mutagenesis of this site. A mutant was made in which four amino acids of the motif were deleted ($\Delta FGEI_{400\text{-}403}$) (methods). This was reintroduced into *vps18Δ::TRP1* yeast on a *CEN*-vector as described (methods). No phenotypic differences from wild-type controls could be detected in any of our assays (not shown). These data indicate that the FGEIAL motif (Figure 2) does not play a major role in the sorting of vacuolar proteins and is probably not functional in the Vps18 protein.

We have mutated the cysteine-rich motif at the carboxy terminus of Vps18p (Figures 2 & 3) to test the requirement of this region for Vps18 function. Using *dut, ung* mutagenesis (methods), the first cysteine of the motif (C_{826}) was changed to the small, neutral amino acid, serine. This mutant allele, C_{826} to S, was inserted into a

CEN-vector and introduced into *vps18Δ::TRP1* yeast by transformation. This plasmid was unable to complement the growth and vacuolar protein sorting defects of strain JSR18Δ1. The control plasmid having the wild-type *VPS18* gene in the *CEN*-vector was capable of complementing all the mutant phenotypes of strain JSR18Δ1. These data suggest that the cysteine-rich motif found in Vps18 is indeed functional and involved in the process of biogenesis or maintenance of the yeast vacuole.

The C_{826} to S mutant allele of *VPS18* has a temperature-conditional CPY sorting defect. In order to determine the extent of the CPY sorting defect in the C_{826} to S mutant allele, a pulse-chase cell labeling with subsequent immunoprecipitation of CPY was carried out,and the proteins were separated by SDS PAGE and visualized by autoradiography (methods). It became apparent that although the CPY sorting defect of the C_{826} to S mutant allele was not very severe at room temperature, the same mutant was extremely defective when tested at 37°C (Figure 4). We found that the onset of this Ts phenotype occurred very rapidy. Mutant cells were labeled at 23°C and then chased at 37°C as described (methods). After the chase, the sorting defect was that of the restrictive temperature, indicating that the conditional defect takes effect within a few minutes at 37°C (not shown).

The vacuolar morphology of the strain carrying the C_{826} to S mutation was examined at both temperatures. The phenotype at 23°C was novel upon staining with the fluorescent dye CDCFDA. We observed vacuoles of wild-type appearance interspersed with the small, bright dots characteristic of the Class C mutants of which *vps18* alleles are an example. Surprisingly, upon shift of cells to 37°C and examination of aliqouts taken every hour for five hours, there was no detectable change in the phenotype observed at room temperature. This is in contrast to the dramatic change in sorting behavior of CPY in response to 37°C incubation, which takes place over a much shorter time period as described above.

***vps18* and other class C *vps* mutants have reduced α-factor halo size.** A routine test for determining the mating type of yeast strains is to replica plate patches of cells onto a YPD plate spread with a lawn of yeast cells that carry a mutation (*sst1* or *sst2*), rendering them supersensitive to mating pheremones. The release of mating factor by the strain tested is indicated by the presence of a zone of inhibition of growth of supersensitive lawns of the opposite mating type. This clear zone is known as a mating factor halo. While testing the mating type of segregants from the JSR18Δ1 by SEY6211 cross (methods), we noticed that each *vps18-Δ1::TRP1*, *MATα* segregant had a smaller α–factor halo on *sst2 MATa* lawns than every *VPS18*, *trp1*, *MATα* segregant. In contrast, there was not a regular difference in the size of *a*-factor halos on *sst1 MATα* lawns, dependent on the segregation of *Δvps18*. This observation led us to check the α–factor halos of several different *vps* mutants on *sst2*, *MATa* lawns. Strains with defects in *vps11*, *vps16* and *vps33* showed small halos like those of *vps18* strains, whereas strains carrying *vps5* and *vps35* mutations had halo sizes not very different from the halos of wild-type, SEY6210 yeast. The size differences described here were seen after incubating the plates at 30°C; the differences were somewhat less pronounced but still visible when incubation was at room temperature (22°C-26°C). Figure 5 shows the α-factor halos produced by isogenic *vps18* and *vps5* mutants and their parental *MATα* strain.

We reasoned that such a defect could be due to defects in the biosynthesis, sorting or processing of α-factor, or to the rapid degradation of mature α-factor by vacuolar hydrolases released outside the mutant cells. A slower growth of the mutant cells could also have led to smaller halo sizes, but if this were the case, one would have also expected to observe smaller *a*-factor halos.

A *MATα* strain with the *VPS18* gene deleted, secretes α-factor in highly glycosylated precursor form. In order to investigate further the small α–factor halo phenotype associated with *vps18-Δ1::TRP1* strains, we examined the α–

factor protein made in the strain, JSR18Δ1. Protein was immunoprecipitated with α–factor antisera from cells pulse-labeled with ^{35}S-methionine (methods) and the different forms were electrophoretically separated on SDS-polyacrylamide gels and examined by autoradiography (Figure 6). This experiment showed that the *vps18-Δ1::TRP1* mutant cells rapidly secrete a highly glycosylated form of α–factor of approximately 95-140kd into the growth medium (Figure 6). Other partial products of Kex2 cleavage are also secreted by the mutant.

This is in sharp contrast with wild-type cells, which secrete only the small 13 amino acid α–factor peptide. This mature α–factor peptide secreted by wild-type cells has been fully processed by Kex2 (Fuller et al. 1989), Kex1 (Dmochowska et al. 1987) and dipeptidyl-aminopeptidaseA (DPAPaseA) (Julius et al. 1983) before exiting the cell. The highly glycosylated form of α–factor secreted by *vps18* mutants appears similar to that secreted by *kex2* mutants and also resembles the form of α–factor secreted from clathrin heavy chain minus mutants (*chc1*) (Payne et al. 1987). However, more experiments will be necessary to determine if the α-factor precursor secreted from *vps18* cells is indeed the *kex2* mutant form of α-factor.

Other *vps* mutants that do not have the Class C vacuole morphology were also examined. Two severely defective *vps* mutants, *vps5* (Class B vacuole morphology) and *vps35* (wild-type vacuole morphology), are almost like wild-type strains with respect to α-factor processing. This is in marked contrast to the Class C mutants of which *vps16* and *vps33* were examined (data not shown) in addition to *vps18*. The small amount of precursor α-factor (approximately 5%) secreted from the *vps5* (Figure 6) and *vps35* (data not shown) mutants could be explained by minor environmental perturbations in the secretory pathway brought about by the lingering presence of incorrectly localized vacuolar proteins.

Like the *vps18* mutants, *vps5* mutants are severely defective in the targeting and processing of soluble vacuolar hydrolases (Robinson et al. 1988). Thus, the secretion

of most of the α-factor in normal, mature form from *vps5-Δ1::HIS3* strains suggests that the defect in α-factor maturation observed for *vps18* mutants is not just a secondary consequence of the defect in vacuolar hydrolase activity of *vps18-Δ1::TRP1* strains.

DISCUSSION

Phenotypes of yeast strains in which the*VPS18* gene has been deleted. The *VPS18* gene of *S. cerevisiae* has been cloned and sequenced. This gene is necessary for vacuolar protein sorting and for forming vacuoles of normal morphological appearance. Disruption of the *VPS18* gene results in a viable strain with vacuolar protein sorting defects and the Class C vacuole-defective morphology. This null mutant shows a Ts growth phenotype at 37°C, indicating that the *VPS18* gene is essential for growth only at elevated temperatures.

The finding that a null mutant or gene deletion in yeast leads to a conditional lethal (Ts) growth defect is not very common. Some examples of null mutants of *S. cerevisiae* leading to Ts growth phenotypes include: deletion of *UBI4*, the yeast polyubiquitin gene (Finley et al. 1987); disruption of the gene for profilin (Haarer et al. 1990), and disruption of the following *VPS* genes: *VPS1* (Rothman et al. 1990); *VPS3* (the *Δvps3* strains exhibit very slow growth at 37°C) (Raymond et al. 1990); *PEP5/END1* (Dulic and Riezman 1989; Woolford et al. 1990); *VPS15* (Herman et al. 1991); *VPS16* (B. Horazdovsky, unpublished observations); *VPS33* (Banta et al. 1990) and *VPS34* (Herman and Emr 1990). It is striking that most of the above are *VPS* genes involved in the biogenesis or maintenance of the yeast vacuole. One explanation for this "conditional" phenotype is that under stressful conditions a fully functional yeast vacuole is necessary for survival, whereas the cell can manage to live and divide with an impaired vacuole under more optimal growth conditions. The Ts

phenotype of *Δubi4* is most probably due to the inability of this strain to respond to stress (Finley et al. 1987). It could also be argued that the strain deleted for profilin might be more sensitive to stress as the cells grow abnormally large (Haarer et al. 1990) and may be physically more unstable as a result. In no case so far described has the disruption or deletion of a *VPS* or related gene led to lethality under all growth conditions. This may indicate that the vacuole is a dispensable organelle except under adverse conditions. But it remains a possibility that some remnant of a vacuole may be necessary for cell survival under even the most favorable of conditions. In this case, it should be possible to find mutants that completely abolish the vacuole and result in cell death under all growth conditions. However, one might expect that the numerous recent genetic studies of the vacuole would already have identified such a gene if it does indeed exist.

At permissive temperatures the strains carrying the *vps18-Δ1::TRP1* deletion have a slight growth defect in comparison with isogenic wild-type strains; see Figure 1B. In liquid culture the null mutant doubles at approximately half the rate of an isogenic wild-type strain. Interestingly, in spite of the lower growth rate, *vps18* strains consistently incorporate a higher level of Tran^{35}S-label than wild-type strains. This may indicate that the vacuolar pool of cold amino acids in *vps18* strains is reduced, in agreement with the small amino acid pools observed in *pep3* alleles (Jones 1983), and would not be surprising, given the other vacuolar defects observed in *vps18* mutant strains. In addition, the colonies rapidly become brown in colour and the cells quickly lose viability upon storage of *vps18-Δ1::TRP1* single colonies on solid media (both rich and minimal) at 4°C or room temperature. A similar phenotype has been observed for protease deficient yeast strains (Teichert et al. 1989).

At the permissive temperatures for growth (23°C and 30°C), the *vps18-Δ1::TRP1* null mutant is extremely defective in the targeting of soluble proteins such as CPY to the vacuole. The strain secretes up to 85% of its CPY from the cell in

precursor, p2, form. Any CPY that does remain inside the cell is also in p2 form, indicating that it has not reached a functional vacuolar compartment (Figure 5). In addition, the null mutant has a very severe morphological defect in the formation of a vacuole at any temperature, just as described for spontaneous mutant alleles of this complementation group (Banta et al. 1988). A spontaneous nonsense allele, *vps18-4*^{a}, confers phenotypes very similar to those of the null mutant; the location of the nonsense mutation within the gene is not yet known.

Diploids homozygous for *vps18-4*^{a} or for *vps18-Δ1::TRP1* show a defect in meiosis that prevents any recognizable spores or tetrads from being formed. A defect in meiosis at the first division stage has been seen in diploids homozygous for *pep4*, which lack Proteinase A and are thus unable to activate the other vacuolar hydrolases (Zubenko and Jones 1981). Apparently, the process of sporulation requires the action of vacuolar hydrolases. It is likely that the impaired meiotic ability of homozygous *vps18* mutants is a secondary result of the highly defective vacuole found in such strains. Further work will be necessary to determine at what stage of meiosis diploids homozygous for *vps18-Δ1::TRP1* are blocked.

Several other severely defective *pep* and *vps* mutants also show defects in sporulation when homozygous in a diploid (Jones 1983; Robinson et al. 1988). In addition, it has been postulated that the sporulation defective, *spoT7* allele is a member of the *pep5/vps11/end1* complementation group (Woolford et al. 1990). The recent finding that *VPS1* (Rothman et al. 1990), and *SPO15* (the *spo15* mutants were identified on the basis of their inability to sporulate) define the same locus and encode a protein with 45% identity to the microtubule bundling protein, dynamin, raises the argument that the meiotic defect in the *vps1* mutant is not necessarily the secondary result of a severely impaired vacuole (Yeh et al. 1991).

Identification of a functional region in Vps18p by sequence analysis and mutagenesis. Two different motifs in the predicted amino acid

sequence of *VPS18* were identified. Their biological significance was tested by making mutations in the appropriate coding regions. A deletion of 4 amino acids in the consensus cAMP-binding site did not lead to a defect in sorting of vacuolar proteins or to any defect in growth under the stressful conditions of high temperature and high salt. Thus, we conclude that this motif was not biologically active in the known functions of Vps18p, although the possibility remains that it functions in some other process carried out by Vps18p that is not yet known to us.

In contrast, a point mutation in the DNA encoding the cysteine-rich carboxy terminal region of Vps18p was sufficient to lead to a conditional lethal growth defect and a conditional sorting defect for the soluble vacuolar protein, CPY. This mutation alters the first cysteine of the motif to a serine residue. This implies that the cysteine-rich, zinc finger like domain is indeed biologically active in the Vps18 protein. The binding of zinc by such a domain may fold the protein in such a way as to make it suitable for interactions with other macromolecules. In some cases the binding of zinc is thought to facilitate complex formation between related zinc finger containing proteins. The sharing of one Zn between the cysteines of two separate proteins may serve to hold them together in a complex. There is some evidence that this role in complex formation might be the case for the cysteine-rich regions of Adenovirus E1A, bacteriophage T4 gene 32 (Berg 1989), *E. coli*, aspartyl transcarbamoylase (Ladjimi and Kantrowitz 1987), RNA polymerase I of *S. cerevisiae* (Yano and Nomura 1991) and possibly members of the Gal4 family of transcription factors such as Pdr1 (*pdr1* is a pleiotropic drug-resistance mutation in yeast) (Balzi et al. 1987). For a brief review of zinc fingers and their functions, see Berg 1990.

The cysteine-rich C-terminal sequence of Vps18 shows 30% identity over 66 amino acids with a similar cysteine-rich region of the mouse muscle postsynaptic 43 kDa protein. The 43 kDa protein is closely associated with nicotinic acetylcholine receptors (AChR) at postsynaptic membranes and is thought to play a role in anchoring

or stabilizing AChR at synapses by forming a complex or by clinging to the membrane, or both. It has been suggested that the cysteine-rich region of 43 kDa postsynaptic protein functions in interactions of this protein with the lipid bilayer of the synaptic membrane (Froehner 1989). However this hypothesis is based merely on the resemblence of the cysteine pattern of 43 kDa protein to that seen in the regulatory domain of protein kinase C and postulated to function in interactions with phospholipid (Maraganore 1987).

It is unknown at present whether the cysteine-rich motif of Vps18p functions in binding zinc. However, this is a likely hypothesis based on the conserved positioning of the cysteines and on the presence of several glycine residues that may facilitate the binding of zinc. Although it is not clear at the present time if the biologically functional zinc finger like motif of Vps18p is responsible for binding the protein to DNA or to a similar zinc finger protein or to membranes, there are several indications that make the hypotheses of function via protein-protein, or perhaps protein-membrane, interaction attractive. The first is the rapid onset of the conditional sorting defect of yeast cells carrying the zinc finger point mutant (C_{826} to S) upon temperature shift to 37°C. The CPY sorting process is completely defective within a few minutes, indicating that if Vps18p functions as a transcription factor, the putative protein, whose expression Vps18p affects, has an extremely rapid turnover time in the cell. This is possible, but for the *VPS* gene-products examined to date such extremely rapid turnover has not been the rule. If Vps18p were to control the expression of just one gene, one would expect it to be a Class C *VPS* gene on the basis of the phenotype observed for *vps18* mutants. Of the Class C *VPS* gene products characterized, Vps33p has a relatively slow turnover time, being stable over chases of 30 to 90 minutes (Banta et al. 1990); in the case of Vps16p, a loss of 50% of the protein is observed after 30 minutes (Horazdovsky, submitted); no published data are available for Pep5/End1p (*pep5* and *end1* are allelic to the fourth Class C complementation group of our collection; *vps11*). However, if

Vps18p were in control of a group of *VPS* genes, they need not necessarily have Class C defects as single mutants. All of the Class A and B genes characterized to date in our laboratory encode relatively stable proteins, perhaps the least stable being Vps15, which has a half-life in the cell of more than 20 minutes (Herman and Emr 1990; Herman et al. 1991; Paravicini, submitted; Horazdovsky, unpublished; Kohrer, unpublished).

The second argument against a role for Vps18p in the binding of DNA is that the C-terminal region of another protein involved in vacuole targeting and biogenesis, Pep5 or End1 (also known as Vps11), contains a cysteine-rich motif strikingly similar to that found at the carboxy terminus of Vps18 (Figure 3). Of course, these two proteins may be members of a new family of transcription factors involved in regulating the expression of proteins involved in vacuole biogenesis or targeting. The experimental evidence that the Pep5 protein may be localized at the yeast vacuole (Woolford et al. 1990) lends support to the hypothesis that proteins with this particular motif need not necessarily be transcription factors acting in the nucleus. Thus far, there has been little evidence that protein targeting to the yeast vacuole is a regulated, (i.e., nonconstitutive) process. More attractive is the hypothesis that the two Vps proteins might interact via zinc molecules to form a protein complex functional in the transport of vacuolar proteins. Another reasonable hypothesis is that Vps11 and Vps18 both interact with similar substrates via their cysteine tails. Such substrates could be *trans* Golgi or vacuolar membranes, or proteins attached to these membranes. The observation that mutations in these two genes lead to practically identical growth, sorting and morphological defects suggests that the two gene products may act in a similar manner, or interact with each other, or be part of the same complex or process. We anticipate that future investigations on the mode of action of the cysteine-rich motif of Vps18p will provide an answer to some of these interesting questions.

Secretion of immature α-factor from *vps18* mutants. The observation that α-factor is secreted from *vps18-Δ1::TRP1* cells in its highly glycosylated precursor form may help us to identify the site of action of the *VPS18* gene product or the site at which protein sorting to the vacuole occurs. The other Class C *vps* mutants also display this pleiotropy, whereas severely defective *vps* mutants belonging to other categories (e.g., the *vps5* mutant that exhibits fragmented, Class B, vacuole morphology) do not have an appreciable defect in the maturation of α-factor (Figure 6).

In order to attain its final, mature form, the highly glycosylated precursor of α-factor must be cleaved by three enzymes: Kex2p, Kex1p and the product of the *STE13* gene, reviewed in Bussey 1988. Kex2p is the protein that clips the tandem α-factor subunits from the large prosegment; *vps18-Δ1::TRP1* strains are clearly defective in this process (Figure 6). Since pro-α-factor is found in the growth medium with kinetics almost as fast as mature α-factor is secreted from wild-type cells (J. Robinson, unpublished observations), there is not a major defect in the actual secretion of α-factor.

There are several possible explanations for such a defect, including the following. First, Kex2p may not reside in its proper location in the *vps18* mutant cell. It appears that Kex2p is not secreted from the *vps18* mutants (G. Payne, personal communication) but at this time we cannot rule out the possibility that Kex2p is sequestered at some other inappropriate location within the cell. The second explanation is that α-factor bypasses the Kex2p-containing compartment in the null mutant. This would be possible if all proteins, including vacuolar proenzymes, were directed to the cell surface from an earlier Golgi compartment than is customary in wild-type cells. A third alternative is that both Kex2p and pro α-factor travel to the correct organelle, probably a late Golgi compartment, but Kex2p is unable to function properly because of an altered environment within the organelle, such as a change in pH. The phenotype would also be expected if this late Golgi compartment was more severely impaired or

did not exist at all, forcing its specific proteins to reside at other Golgi compartments where they would be less active. It has been suggested that vacuolar protein sorting may take place in just such a late Golgi compartment that contains Kex2 (Graham and Emr 1991). The hypothesis that the primary defect of *vps18* mutants is at this location and leads to all the other pleiotropic phenotypes observed, is an attractive one.

In the original mutant screens, we selected for mutants that mislocalized vacuolar proteins and secreted them from the cell. It was expected that some of these mutants would have their primary defect in the sorting event, where and when vacuolar proteins are separated from those destined for the cell surface. Since several lines of evidence have led to the supposition that this sorting event occurs in a late compartment of the Golgi apparatus, one might have expected several of the *vps* mutants to act at this point in the secretory / vacuolar localization pathway, in other words, at the decision point. Surprisingly, the early studies by us and others did not reveal any Golgi-associated defect in the mutants, such as a defect in the processing of some secretory protein in addition to the vacuolar protein processing and localization defects. In view of the recent observations presented here that a subset of the *vps* mutants do indeed exhibit a Golgi associated defect, in that a precursor form of α-factor is secreted from the cells, it is clear that the original studies looked at too restricted a range of secreted proteins. Our finding that the Class C *vps* mutants in general, and *vps18* in particular exhibit a serious defect in the processing of α-factor, secreting at least 50% of it from the cell in precursor form, leads to the hypothesis that the *vps18* complementation group identifies a gene product that acts, directly or indirectly, at the vacuolar protein sorting decision point in the *trans* Golgi compartment..

REFERENCES

Altschul, S., W. Gish, W. Miller, E. Myers and D. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**: 403-410.

Ammerer, G., C. Hunter, J. H. Rothman, G. C. Saari, L. A. Valls and T. H. Stevens. 1986. *PEP4* gene of *Saccharomyces cerevisiae* encodes proteinase A, a vacuolar enzyme required for processing of vacuolar precursors. *Mol. Cell. Biol.* **6**: 2490-2499.

Ausubel, F., R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith and K. Struhl. (1987). Current Protocols in Molecular Biology. New York, John Wiley & Sons.

Balzi, E., W. Chen, S. Ulaszewski, E. Capieaux and A. Goffeau. 1987. The multidrug resistence gene *PDR1* from *Saccharomyces cerevisiae. J. Biol Chem.* **262**: 16871-16879.

Bankaitis, V. A., L. M. Johnson and S. D. Emr. 1986. Isolation of yeast mutants defective in protein targeting to the vacuole. *Proc. Natl. Acad. Sci. USA.* **83**: 9075-9079.

Banta, L. M., J. S. Robinson, D. J. Klionsky and S. D. Emr. 1988. Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting. *J. Cell Biol.* **107**: 1369-1383.

Banta, L. M., T. A. Vida, P. K. Herman and S. D. Emr. 1990. Characterization of yeast Vps33p, a protein required for vacuolar protein sorting and vacuole biogenesis. *Mol. Cell. Biol.* **1990**: 4638-4649.

Berg, J. 1989. Metal binding domains in nucleic acid binding and gene regulatory proteins. *Prog. Inorg. Chem.* **37**: 143-185.

Berg, J. 1990. Zinc fingers and other metal-binding domains. *J. Biol. Chem.* **265**: 6513-6516.

Bussey, H. 1988. Proteases and the processing of precursors to secreted proteins in yeast. *Yeast.* **4**: 17-26.

Chvatchko, Y., I. Howald and H. Riezman. 1986. Two yeast mutants defective in endocytosis are defective in pheromone response. *Cell.* **46**: 355-364.

Dmochowska, A., D. Dignard, D. Henning, D. Thomas and H. Bussey. 1987. Yeast *KEX1* gene encodes a putative protease with a carboxypeptidase B like function involved in killer toxin and a-factor precursor processing. *Cell.* **50**: 573-584.

Dulic, V. and H. Riezman. 1989. Characterization of the *END1* gene required for vacuole biogenesis and gluconeogenic growth of budding yeast. *EMBO J.* **8**(5): 1349-1359.

Finley, D., E. Ozkaynak and A. Varshavsky. 1987. The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation and other stresses. *Cell.* **48**: 1035-1046.

Froehner, S. 1989. Expression of RNA transcripts for the postsynaptic 43 kDa protein in innervated and denervated rat skeletal muscle. *FEBS let.* **249**: 229-233.

Fuller, R. S., A. J. Brake and J. Thorner. 1989. Intracellular targeting and structural conservation of a prohormone-processing endoprotease. *Science.* **246**: 482-486.

Graham, T. and S. Emr. 1991. Sec18/NSF protein is required for multiple steps in the secretory pathway: evidence for the compartmental organization. *J. Cell Biol.* (In press).

Haarer, B., S. Lillie, A. Adams, V. Magdolen, W.Bandlow and S. Brown. 1990. Purification of profilin from *Saccharomyces cerevisiae* and analysis of profilin deficient cells. *J. Cell Biol.* **110**: 105-114.

Hasilik, A. and W. Tanner. 1978. Biosynthesis of the vacuolar yeast glycoprotein carboxypeptidase Y. Conversion of precursor into the enzyme. *Eur. J. Biochem.* **85**: 599-608.

Herman, P. K. and S. D. Emr. 1990. Characterization of *VPS34*, a gene required for vacuolar protein sorting and vacuole segregation in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **10**: 6742-6754.

Herman, P. K., J. H. Stack, J. A. DeModena and S. D. Emr. 1991. A novel protein kinase homolog essential for protein sorting to the yeast lysosome-like vacoule. *Cell.* **64**: ooo-ooo.

Ito, H., Y. Fukada, K. Murata and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bact.* **153**: 163-168.

Johnson, L. M., V. A. Bankaitis and S. D. Emr. 1987. Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protein. *Cell.* **48**: 875-885.

Jones, E. (1983). Genetic approaches to the study of protease function and proteolysis in *Saccharomyces cerevisiae*. Yeast Genetics. Fundamental and Applied Aspects. Springer-Verlag, New York.

Jones, E. W. 1977. Proteinase mutants of *Saccharomyces cerevisiae*. *Genetics*. **85**: 23-33.

Jones, E. W. 1984. The synthesis and function of proteases in *Saccharomyces*: genetic approaches. *Ann. Rev. Genet.* **18**: 233-270.

Jones, J. S., S. Weber and L. Prakash. 1988. The *Saccharomyces cerevisiae RAD18* gene encodes a protein that contains potential zinc finger domains for nucleic acid binding and a putative nucleotide binding sequence. *N. Acids Res.* **16**: 7119-7131.

Julius, D., L. Blair, A. Brake, G. Sprague and J. Thorner. 1983. Yeast α-factor is processed from a larger precursor polypeptide: the essential role of a membrane-bound, dipeptidyl aminopeptidase. *Cell.* **32**: 839-852.

Kitamoto, K., K. Yoshizawa, Y. Ohsumi and Y. Anraku. 1981. Mutants of *Saccharomyces cerevisiae* with defective vacuolar function. *J. Bacteriol.* **170**: 2687-2691.

Klionsky, D. J., L. M. Banta, J. S. Robinson and S. D. Emr. (1988). Vacuolar protein targeting and organelle biogenesis in yeast. Molecular Biology of Intracellular Protein Sorting and Organelle Assembly. Alan R. Liss, New York, NY.

Klionsky, D. J., P. K. Herman and S. D. Emr. 1990. The fungal vacuole: composition, function, and biogenesis. *Microbiol. Rev.* **54**: 266-292.

Kunkel, T. 1985. Rapid and efficient site specific mutagenesis without phenotypic selection. *PNAS.* **82**: 488-492.

Kyte, J. and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**: 105-132.

Ladjimi, M. and E. Kantrowitz. 1987. Catalytic regulatory subunit interactions and allosteric effects in aspartate transcarbamylase. *J. Biol. Chem.* **262**: 312-318.

Maniatis, T., E. F. Fritsch and J. Sambrook. (1982). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Maraganore, J. 1987. Structural elements for protein phospholipid interactions may be shared in protein kinase C and phospholipases A2. *TIBS.* **12**: 176-177.

Moehle, C. M., C. K. Dixon and E. W. Jones. 1989. Processing pathway for protease B of *Saccharomyces cerevisiae*. *J. Cell Biol.* **108**: 309-324.

Moehle, C. M., R. Tizard, S. K. Lemmon, J. Smart and E. W. Jones. 1987. Protease B of the lysosomelike vacuole of the yeast *Saccharomyces cerevisiae* is homologous to the subtilisin family of serine proteases. *Mol. Cell. Biol.* **7**: 4390-4399.

Ohya, Y., Y. Ohsumi and Y. Anraku. 1986. Isolation and characterization of Ca2+-sensitive mutants of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **132**: 979-988.

Payne, G. S., T. B. Hasson, M. S. Hasson and R. Schekman. 1987. Genetic and biochemical characterization of clathrin-deficient *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **7**: 3888-3898.

Pearson, W. and D. Lipman. 1988. Improved tools for biological sequence analysis. *Proc. Natl. Acad. Sci., USA.* **85**: 2444-2448.

Raymond, C. K., P. J. O'Hara, G. Eichinger, J. H. Rothman and T. H. Stevens. 1990. Molecular analysis of the yeast *VPS3* gene and the role of its product in vacuolar protein sorting and vacuolar segregation during the cell cycle. *J. Cell Biol.* **111**: 877-892.

Robinson, J. S., D. J. Klionsky, L. M. Banta and S. D. Emr. 1988. Protein sorting in *Saccharomyces cerevisiae*: Isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol. Cell. Biol.* **8**: 4936-4948.

Rose, M., P. Novick, J. Thomas, D. Botstein and G. Fink. 1987. A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere containing shuttle vector. *Gene*. **60**: 237-243.

Rothman, J. H., I. Howald and T. H. Stevens. 1989. Characterization of genes required for protein sorting and vacuolar function in the yeast *Saccharomyces cerevisiae*. *EMBO J*. **8**: 2057-2065.

Rothman, J. H., C. K. Raymond, T. Gilbert, P. J. O'Hara and T. H. Stevens. 1990. A putative GTP binding protein homologous to interferon-inducible Mx proteins performs an essential function in yeast protein sorting. *Cell*. **61**: 1063-1074.

Rothman, J. H. and T. H. Stevens. 1986. Protein sorting in yeast: mutants defective in vacuole biogenesis mislocalize vacuolar proteins into the late secretory pathway. *Cell*. **47**: 1041-1051.

Sanger, F., F. Nicklen and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*. **74**: 5463-5467.

Sherman, F., G. R. Fink and C. W. Lawrence. (1979). Methods in Yeast Genetics: a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Stevens, T., B. Esmon and R. Schekman. 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell*. **30**: 439-448.

Stevens, T. H., J. H. Rothman, G. S. Payne and R. Schekman. 1986. Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y. *J. Cell Biol.* **102**: 1551-1557.

Teichert, U., B. Mechler, H. Muller and D. H. Wolf. 1989. Lysosomal (vacuolar) proteinases of yeast are essential catalysts for protein degradation, differentiation, and cell survival. *J. Biol. Chem.* **264**: 16037-16045.

Valls, L., J. Winther and T. Stevens. 1990. Yeast carboxypeptidase Y vacuolar targeting signal is defined by four propeptide amino acids. *J. Cell Biol.* **111**: 361-368.

Woolford, C. A., C. K. Dixon, M. F. Manolson, R. Wright and E. W. Jones. 1990. Isolation and characterization of *PEP5*, a gene essential for vacuolar biogenesis in *Saccharomyces cerevisiae*. *Genetics*. **125**: 739-752.

Yano, R. and M. Nomura. 1991. Suppressor analysis of temperature-sensitive mutations of the largest subunit of RNA polymerase I in *Saccharomyces cerevisiae*: a suppressor gene encodes the second largest subunit of RNA polymerase I. *Mol. Cell. Biol.* **11**: 754-764.

Yeh, E., R. Driscoll, M. Coltrera, A. Olins and K. Bloom. 1991. A dynamin-like protein encoded by the yeast sporulation gene *SPO15*. *Nature*. **349**: 713-715.

Zubenko, G. S. and E. W. Jones. 1981. Protein degradation, meiosis and sporulation in proteinase deficient mutants of *Saccharomyces cerevisiae*. *Genetics*. **97**: 45-64.

TABLE 1 Table of strains

Table of strains used in this study.

Saccharomyces cerevisiae strains:

SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801^{a}suc2-Δ9*	(Robinson, 1988)
SEY6211	*MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101^{o}suc2-Δ9*	(Robinson, 1988)
SEY18-4	*MATα vps18-4^a Ts leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801^{a}suc2-Δ9*	(Robinson, 1988)
SEY18-5	*MATa vps18-5 Ts leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101^o suc2-Δ9*	(Robinson, 1988)
SEY18-7	*MATa vps18-7 leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101^o suc2-Δ9*	(Robinson, 1988)
SEY11-1	*MATα vps11-1 Ts leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801^{a}suc2-Δ9*	(Robinson, 1988)
JSR18Δ1	*MATα vps18-Δ1::TRP1 leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801^{a}suc2-Δ9*	(This Study)
BHY151	*MATα vps5-Δ1::HIS3 leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801^{a}suc2-Δ9*	(Horazdovsky, unpub.)
RC634	*MATa sst1-3 rme ade2 his6 met1 ura1*	(Chan, 1983)

Escherichia coli strains:

MC1061	*araD139 (araABOIC-leu)7679 Δ(lac)X74 galU galK hsdR rpsL*	(Casadaban, 1980)
JM101	*F' traD36 lacIq ZΔM15 proAB/supE thiDlac (lac-pro)*	(Miller, 1972)
BW313	*F' lysA/dut ung thi-1 relA spoT1*	(Kunkel, 1985)

FIGURE 1 *VPS18* gene cloning and characterization

A. Restriction map of a 5kb DNA fragment that contains the *VPS18* open reading frame as indicated by the empty arrow. Below is the *vps18* disruption in which the entire open reading frame from KpnI to SacI was replaced with yeast *TRP1* and *E. coli* vector sequences (methods). **C.** Growth phenotypes on YPD at 30° and 37° of SEY6210, JSR*Sc*184, JSR*Sc*189 and JSR*Sc*189 after being transformed with the *vps18*-complementing plasmid, pJSR6 (methods). The plates were incubated at the appropriate temperatures for three days.

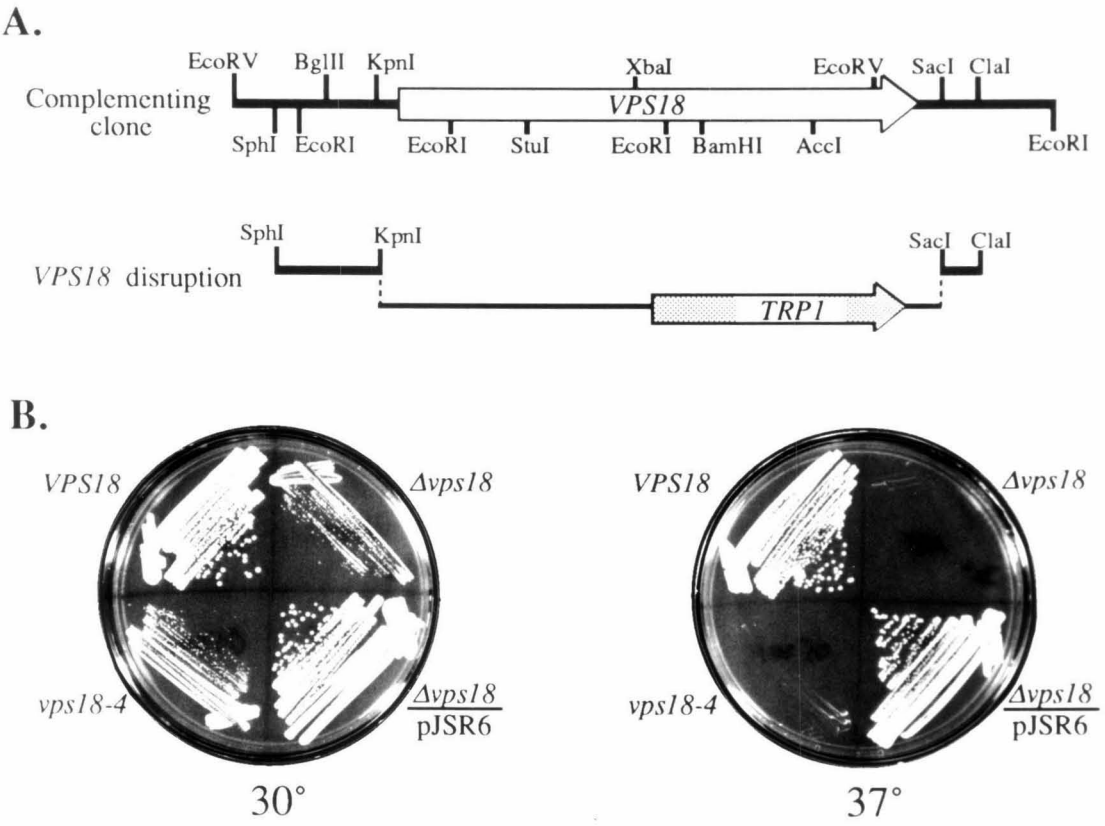

A.
EcoRV
BglII
KpnI
XbaI
EcoRV
SacI
ClaI
Complementing clone
VPS18
SphI
EcoRI
EcoRI
StuI
EcoRI
BamHI
AccI
EcoRI
SphI
KpnI
SacI
ClaI
VPS18 disruption
TRP1
B.
VPS18
Δvps18
vps18-4
Δvps18
pJSR6
30°
VPS18
Δvps18
vps18-4
Δvps18
pJSR6
37°

FIGURE 2 Nucleotide sequencing of the *VPS18* gene

Nucleotide sequence of the *VPS18* gene and the deduced amino acid sequence. Nucleotide residues are numbered relative to the ATG that initiates the long open reading frame. The cAMP-binding motif is underlined with a dashed line and the cysteine-rich region is indicated by a solid line.

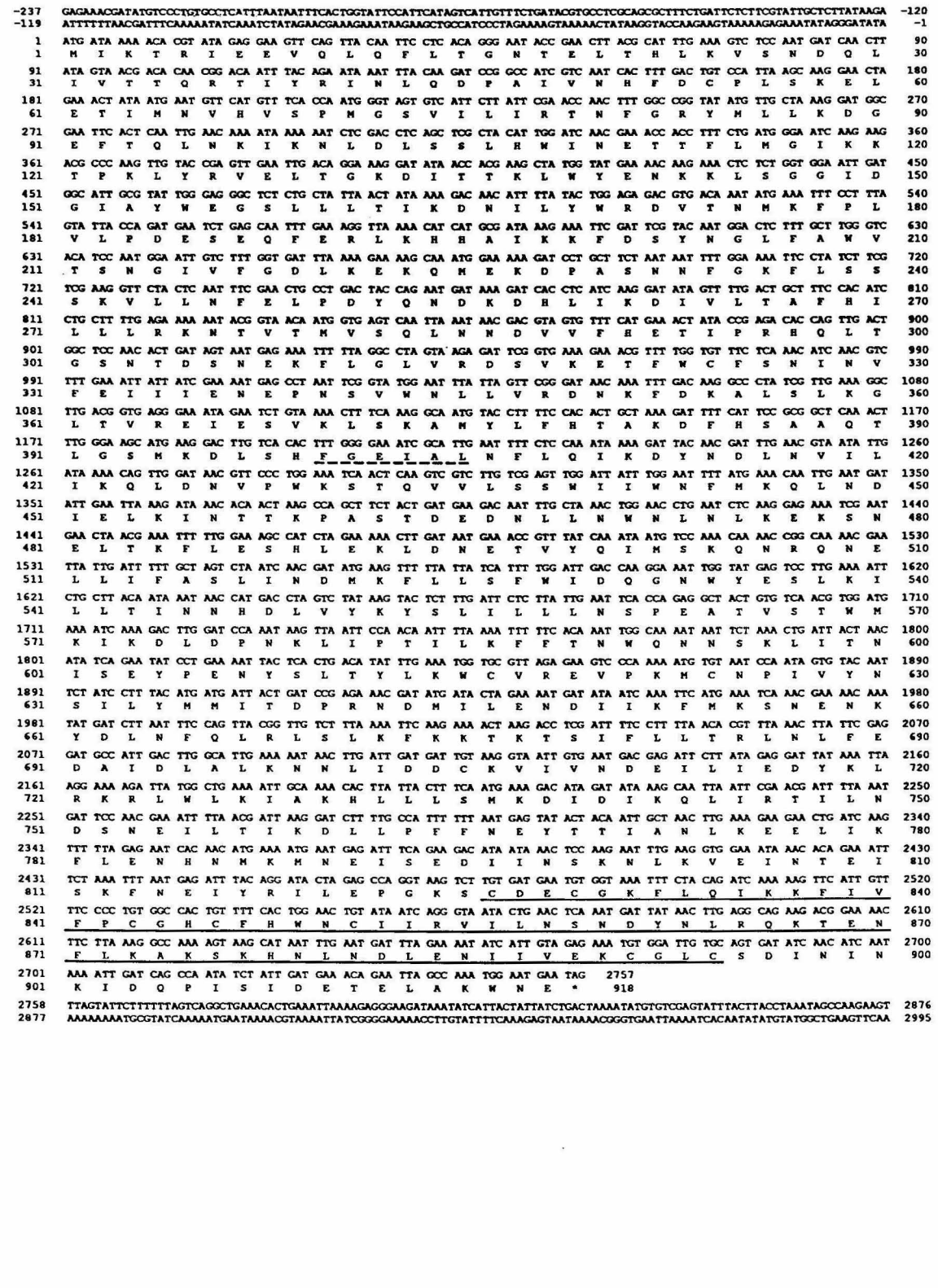


FIGURE 3 Cysteine rich C-terminus, sequence comparison

Alignment of the Vps18 Carboxy terminal cysteine-rich motif with zinc fingers and other cysteine motifs; 43 kDa postsynaptic protein from mouse (Froehner 1989), Pep5/End1 (Wolford et al. 1990; Dulic and Riezman 1989), Rad18 (Jones et al. 1988) and Pdr1 (Balzi et al.1987).

```
                                                                C  C                C       C
Vps18 protein      787 MKMNEISEDIINSKNLKVEINTEISKFNEIYRILEPGKSCDECGKFLQIKKFIVF--PCGHCFHWNCI 842
Postsynaptic 43Kp  326 LKLHCLSESIYRSKGLQRDVRTHVVRFHECVEETE--LYCGLCGESIGERNSRLQALPCSHIFHLRCL 391


                     C  C              C       C
Vps18      812 PGK--SCDECGKFLQIKKFIVFPCGHCFHWNCIIRVILNSNDYNLRQKTENFLKAKSKHLNDLENIIVEKCGLC 883
Pep5/End1  920 PLKNQTCFMCRIILDIP-VVFFKCGHIYHQHCL-------NEEEDTLESERKLFKCPKCLVDLETSNKLFEAQH 987
Rad18       26 LRCHICKDFLKVPVLT--PCGHTFCSLCI 52
Pdr1        46 ACDNCRKRK-IKCNGKFPCASCEIYSC 71
```

FIGURE 4 CPY sorting phenotype of *vps18* C⇒S point mutant

Immunoprecipitation of CPY from *vps18-Δ1::TRP1*, *vps18-Δ1::TRP1* carrying a plasmid encoding the C_{826} to S alteration in the Vps18 protein, and the isogenic wild-type strain, SEY6210.

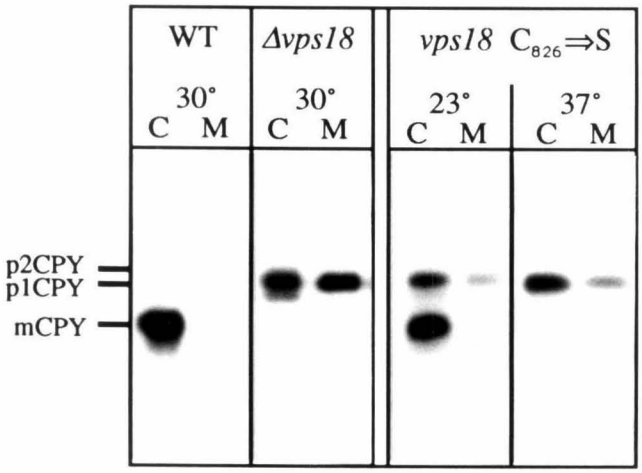

WT
Δvps18
vps18 C826⇒S
30°
C M
30°
C M
23°
C M
37°
C M
p2CPY
p1CPY
mCPY

FIGURE 5 *vps18* mutant, α-factor halo defect

α-factor halos of *vps18-Δ1::TRP1*, *vps5-Δ1::HIS3* and the isogenic wild-type strain, SEY6210, on a lawn of *sst2 MATa* cells.

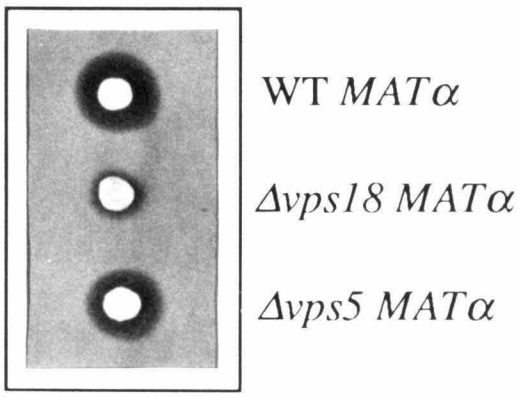
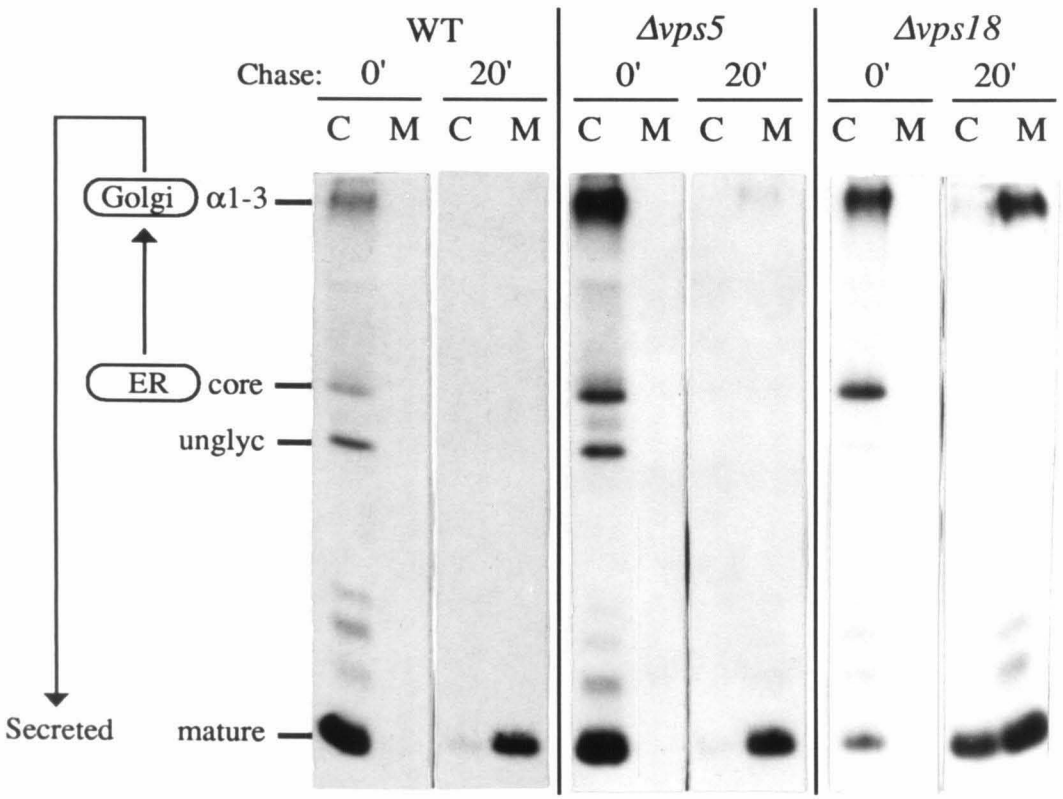

WT *MATα*
Δvps18 MATα
Δvps5 MATα

FIGURE 6 Maturation of α-factor in the *vps18* mutant

Immunoprecipitation of alpha factor from *vps18-Δ1::TRP1*, *vps5-Δ1::HIS3* and the isogenic wild type strain, SEY6210.


WT
Δvps5
Δvps18
Chase:
0'
20'
C
M
Golgi
α1-3
ER
core
unglyc
Secreted
mature