Protein Sorting in the Eukaryotic Secretory Pathway: An Essential Role for a Novel Yeast Protein Kinase.

Thesis by

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Dedication

This thesis is dedicated to the memory of my father, Edgar Herman. Thank you for the love, guidance and support that you were always only too willing to give to me and thank you for sparking my initial curiosity in the sciences and the world about us. I will always miss you.

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Finally, and most importantly, I would like to thank my wife Carol for her unending love and constant support throughout these years. May our love continue to grow and hold us together.

ABSTRACT

The yeast *vps* mutants are defective for the intracellular sorting of proteins to the vacuolar compartment. Mutants from two particular *vps* complementation groups, *vps*15 and *vps*34, share a common set of phenotypes that suggested that the *VPS*15 and *VPS*34 gene products might be functioning at a similar step of the vacuolar protein sorting pathway. *vps*15 and *vps*34 mutants exhibit specific defects in the sorting of soluble hydrolases to the vacuolar compartment. Whereas soluble hydrolases such as carboxypeptidase Y are almost quantitatively mislocalized to the cell surface, vacuolar membrane proteins appear to be properly localized to the vacuole.

The wild-type *VPS*15 and *VPS*34 genes were both cloned from yeast genomic DNA libraries by complementation of temperature-sensitive growth defects associated with mutations in these genes. Haploid yeast strains carrying a disruption of either locus were viable but exhibited a severe *ts* growth defect indicating that both genes are essential for vegetative growth at elevated temperatures. The *vps*34 null mutant was also found to exhibit a defect in the segregation of the vacuolar compartment upon cell division.

The predicted sequence of the *VPS*15 gene product exhibits significant similarity to the catalytic domains of the serine/threonine family of protein kinases. Point mutations altering specific amino acid residues of Vps15p that are highly conserved in all protein kinases result in the biological inactivation of Vps15p. The kinase domain mutants exhibit severe vacuolar protein sorting and *ts* growth defects. In addition, Vps15p is phosphorylated *in vivo* in a reaction that requires a wild-type Vps15p kinase domain. Subcellular fractionation experiments indicate that Vps15p is peripherally associated with the cytoplasmic face of a late Golgi or vesicle compartment. A *vps*15 mutant that encodes a protein lacking 30 carboxy-terminal amino acids exhibits a severe *ts* defect in vacuolar

protein delivery. At the restrictive temperature, carboxpeptidase Y accumulates in a specific intracellular compartment that may represent a normal transport intermediate between the Golgi and vacuolar compartments. The vacuolar delivery defect in this mutant has an extremely rapid rate of onset suggesting that Vps15p is directly involved in the sorting of soluble proteins to the vacuole. Altogether, these data suggest that Vps15p regulates specific protein phosphorylation reactions *in vivo* that are required for the delivery of soluble hydrolases to the vacuole.

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Chapter 1:

Introduction

The eukaryotic cell is a highly compartmentalized structure that is subdivided into a number of functionally distinct membrane-enclosed organelles. The structural and functional characteristics of each of these organelles is defined largely by the unique subset of proteins resident within that particular compartment. Since the great majority of proteins are synthesized upon cytoplasmic ribosomes, the eukaryotic cell must possess specific mechanisms that allow it to sort and deliver proteins from their common site of synthesis to their appropriate final intracellular and extracellular destinations.

The eukaryotic secretory pathway is responsible for mediating the proper modification and delivery of proteins to the cell-surface and to a variety of intracellular compartments (Pfeffer and Rothman, 1987). This pathway is itself composed of a series of distinct membrane-enclosed organelles including the endoplasmic reticulum, the Golgi complex and secretory vesicles. Proteins destined for secretion initiate their synthesis upon cytoplasmic ribosomes. These proteins are synthesized with short, hydrophobic N-terminal extensions (signal peptides) that are responsible for mediating their entry into the secretory pathway. The secretory proteins are co-translationally translocated across the membrane of the endoplasmic reticulum and are then delivered to the Golgi complex and finally to the cell-surface via specific transport vesicles. This delivery of proteins to the cell-surface appears to occur by a default mechanism; proteins lacking any additional sorting information are passively carried to the cell-surface. In contrast, proteins destined for delivery to the vacuolar/lyosomal compartment, or for retention within the intermediate organelles of the secretory pathway, possess specific sorting signals that are required for their proper localization (Pfeffer and Rothman, 1987; Kornfeld and Mellman, 1989).

The sorting of proteins to the vacuolar and lysosomal compartments of eukaryotic cells has been extensively studied and serves as one of the best-understood paradigms of an intracellular protein sorting process (Kornfeld and Mellman, 1987; Klionsky et al.,

1990). Vacuolar proteins are translocated across the membrane of the endoplasmic reticulum and then travel from the endoplasmic reticulum to the Golgi complex together with proteins destined for secretion or assembly into the plasma membrane. However, within a late Golgi compartment, vacuolar proteins are sorted away from the bulk of the secretory protein traffic and targeted to the vacuole. The delivery of proteins to the vacuole appears to be a complex process involving a number of distinct steps whose execution must be precisely controlled both spatially and temporally. These steps presumably include the specific recognition and segregation of vacuolar proteins within the Golgi complex, packaging of the recognized vacuolar proteins into transport intermediates, delivery of these intermediates to the vacuole and fusion with the vacuolar membrane. In addition, many of the cellular components required for the delivery of proteins from the Golgi complex to the vacuole may have to be recycled back to the Golgi to be used in subsequent rounds of protein transport.

Biochemical and genetic studies have identified cis-acting sorting signals required for the initial recognition of vacuolar and lysosomal proteins in the Golgi compartment. In many types of mammalian cells, the N-linked carbohydrate chains of soluble lysosomal proteins are modified with mannose-6-phosphate residues that are recognized by specific integral membrane receptors that mediate lysosomal delivery (Kaplan et al., 1977; reviewed in Kornfeld and Mellman, 1989). However, many lysosomal proteins are not modified by the addition of mannose-6-phosphate residues yet are efficiently sorted to the lysosomal compartment (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989). In yeast, the targeting signals of vacuolar proteins do not involve a particular carbohydrate modification and instead appear to reside directly within the polypeptide backbone of these proteins (Johnson et al., 1987; Valls et al., 1987; Klionsky et al., 1988; Klionsky and Emr, 1990). The recognition of yeast vacuolar proteins in the Golgi complex may therefore serve as a model for the study of this initial

event in the targeting of lysosomal proteins whose delivery occurs independent of the mannose-6-phosphate system.

Beyond this initial recognition event, very little is presently known about the basic cellular mechanisms responsible for the subsequent delivery of proteins to the vacuolar or lysosomal compartments of eukaryotic cells. In the yeast, *Saccharomyces cerevisiae*, an extensive genetic analysis of vacuolar protein sorting has been undertaken in an attempt to develop a better understanding of the trans-acting cellular machinery functioning to deliver proteins to the vacuole (reviewed in Klionsky et al., 1990). Two independent genetic selections have identified a large number of yeast mutants defective in the localization of several soluble vacuolar proteins, including carboxypeptidase Y (CPY), proteinase A and proteinase B (Bankaitis et al., 1986; Rothman and Stevens, 1986; Robinson et al., 1988; Rothman et al., 1989a). Rather than delivering these hydrolases to the vacuole, *vps* mutants (for vacuolar protein sorting defective) mis-sort these enzymes to the cell-surface as Golgi-modified precursors. In addition, a second set of mutants, the *pep* mutants, also appear to be defective for the localization of soluble vacuole hydrolases (Rothman et al., 1989a). The *pep* mutants were originally identified in genetic screens for yeast mutants with decreased enzymatic levels of a particular vacuolar protease, CPY (Jones, 1977). Complementation analyses between the *vps*, *pep* and other related sets of mutants, have demonstrated that these mutants define more than 46 different complementation groups (Klionsky et al., 1990; S. Emr, personal communication). This therefore suggests that vacuolar protein delivery is a relatively complex process requiring the coordinated participation of a large number of gene products.

Many of the *vps* mutants also exhibit defects in the localization of vacuolar membrane proteins in addition to the defects observed with soluble hydrolases (Klionsky et al., 1989). The severe vacuolar protein sorting defects associated with the *vps* mutants

might be expected to compromise other vacuolar functions or to affect the biogenesis of this organelle. To assess the structural integrity of the vacuolar compartment in the *vps* mutants, an extensive morphological analysis was carried out using both light and electron microscopic techniques (Banta et al., 1988). Although most of the *vps* mutants possessed a morphologically normal vacuolar compartment (class A mutants), many of the mutants contained abnormal vacuolar structures. In several mutants (class B), the cells exhibited a fragmented vacuole morphology where multiple small vacuole-like structures were observed within each cell. Several *vps* mutants exhibited a more extreme defect in vacuolar biogenesis. In these class C mutants, the cells possessed no detectable vacuolar compartment by either microscopic analysis. Instead, class C cells accumulated small membrane-bound vesicles and a variety of abnormal membranous structures within their cytoplasm. The gene products defined by the class B and C mutants might be specifically involved in the regulation of vacuolar biogenesis *in vivo*. In addition to the above defects in vacuolar protein sorting and vacuole biogenesis, the *vps* mutants also exhibit defects in a wide variety of cellular functions including organellar acidification, osmoregulation, sporulation, cell growth and vacuole segregation (Banta et al., 1988; Robinson et al., 1988; Rothman et al., 1989b; Herman and Emr, 1990; Raymond et al., 1990).

Mutants in two particular *vps* complementation groups, *vps*15 and *vps*34, share a distinct set of terminal mutant phenotypes. *vps*15 and *vps*34 mutants exhibit severe defects in the localization of soluble vacuolar hydrolases, such as CPY. These mutants secrete >95% of the newly-synthesized CPY as an inactive precursor molecule (Robinson et al., 1988; Herman and Emr, 1990; Herman et al., 1991). Despite these extreme vacuolar protein sorting defects, *vps*15 and *vps*34 cells possess a morphologically normal vacuolar compartment (Banta et al., 1988; Herman and Emr, 1990). These cells are therefore competent for vacuole assembly *in vivo*. In addition, two vacuolar

membrane proteins, alkaline phosphatase and α-mannosidase, appear to be properly localized to the vacuolar compartment in *vps*15 and *vps*34 mutants (Robinson et al., 1988; Klionsky et al., 1989). These data suggest that the *vps*15 and *vps*34 vacuolar protein sorting defects are specific for soluble constituents of the vacuolar compartment. The gene products of the two corresponding wild-type genes, *VPS*15 and *VPS*34, are therefore good candidates for cellular components that may be specifically involved in the sorting and/or delivery of soluble proteins to the yeast vacuole.

In addition to the above mutant phenotypes, *vps*15 and *vps*34 mutants also exhibit severe temperature-sensitive growth defects and a hypersensitivity to osmotic stress (Banta et al., 1988; Robinson et al., 1988). Although these mutants possess a morphologically normal vacuole, *vps*15 and *vps*34 cells also accumulate abnormal membranous structures, including 80 nm membrane vesicles and Golgi-derived Berkeley bodies, within their cytoplasm. The similar set of phenotypes exhibited by mutnats within this pair of *vps* complementation groups suggested that the *VPS*15 and *VPS*34 gene products might be acting at a similar step, or carrying out related functions, within the vacuolar protein sorting pathway.

The following chapters discuss the cloning of the yeast *VPS*15 and *VPS*34 genes and the characterization of their respective gene products. Interestingly, the predicted sequence of the Vps15 protein (Vps15p) exhibits significant similarity to the catalytic domains of serine/threonine protein kinases. A mutational analysis of the Vps15p kinase domain suggests that specific Vps15p-mediated protein phosphorylation reactions are required for the efficient sorting of multiple soluble hydrolases to the vacuolar compartment. In all, the following studies suggest that protein phosphorylation reactions might act as a general regulator at potential branch points within the eukaryotic secretory pathway by actively diverting proteins from a default transport route and into an alternative transit pathway.

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Chapter 2 :

The Fungal Vacuole:
Composition, Function, and Biogenesis.

This chapter was a collaborative effort between Dr. Dan Klionsky, Dr. Scott Emr and myself. I wrote the sections on yeast vacuolar protein sorting mutants and fungal vacuole biogenesis.

The Fungal Vacuole: Composition, Function, and Biogenesis

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INTRODUCTION

The fungal vacuole is often described as an organelle that is analogous to the mammalian lysosome. Although this viewpoint is largely correct, it is also misleading, even though both the lysosome and the vacuole are acidic compartments which contain a variety of hydrolytic enzymes (1, 64, 87). Although macromolecular degradation is one obvious function of the fungal vacuole, it is also involved in a variety of additional cellular processes. The importance of the fungal vacuole in metabolite storage and in cytosolic ion and pH homeostasis, for example, is well documented (3, 31). In this regard, the fungal vacuole has greater similarity to the vacuole of plant cells (102). A number of reviews have focused on individual properties of the vacuole, including proteolysis (1, 64), metabolite transport (31), and acidification (3, 122). The purpose of this review is not only to discuss the current work on the fungal vacuole, but also to

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TABLE 1. Protein constituents of the vacuole

Name or abbreviation used in text	Protein designation	Gene designation
Soluble		
CPY	Carboxypeptidase Y, carboxypeptidase yscY	*PRC1*
PrA	Proteinase A, proteinase yscA	*PEP4*, *PRA1*, *PHO9*
PrB	Proteinase B, proteinase yscB	*PRB1*
API	Aminopeptidase I, aminopeptidase yscI, leucine aminopeptidase IV, aminopeptidase V, aminopolypeptidase, aminopeptidase III	*LAP4*, *APE1*
Trehalase	Trehalase	
Membrane associated		
α-Mannosidase	α-Mannosidase	*AMS1*
DPAP B	Dipeptidyl aminopeptidase B, dipeptidyl aminopeptidase yscV, X-prolyldipeptidyl aminopeptidase	*DAP2*, *DPP2*
ALP	Alkaline phosphatase	*PHO8*
ATPase	H^+-translocating ATPase	*vma*, *VMA*, *VAT*

provide a more comprehensive picture of this complex organelle and reveal how it is integrally involved in a variety of cellular processes.

VACUOLAR HYDROLASES

Synthesis and Processing

The yeast vacuole has been viewed primarily as a degradative organelle because of the variety of hydrolase activities that have been localized to this compartment. Well-characterized vacuolar hydrolases include proteinase A, proteinase B, carboxypeptidase Y, carboxypeptidase S, aminopeptidase I, aminopeptidase Co, dipeptidyl aminopeptidase B, repressible alkaline phosphatase, RNase, and α-mannosidase (see references 1, 64, and 153 for review). Abbreviations for these hydrolases, which will be used throughout this review, are noted in Table 1. The levels of many vacuolar hydrolases vary with the growth stage and nutrient supplies (50, 94, 111, 113, 149). These enzymes are derepressed under conditions of limiting glucose or nitrogen and tend to reach maximal levels as the cells approach the stationary phase. Vacuolar hydrolases have been implicated in several processes that can be viewed as long-term adaptations to changing nutritional conditions. These mostly involve general proteolysis, the degradation and reutilization of small peptides, and sporulation-associated protein degradation (1, 64, 163). Interestingly, there is little direct evidence that the vacuole is required for the turnover of aberrant and nonfunctional proteins (64, 163). Recent applications of molecular biological, genetic, and biochemical techniques to the study of vacuolar proteins have provided information on the biosynthesis, processing, and localization of many of these proteins.

Transit through the secretory pathway: carboxypeptidase Y. The most thoroughly studied vacuolar protein is carboxypeptidase Y (CPY). CPY has frequently been viewed as the typical model vacuolar protein. Although recent studies on a variety of hydrolases now suggest that there may be no such thing as a typical vacuolar protein, an analysis of CPY serves as a useful introduction to the biosynthesis (i.e., translation, transport, and proteolytic and/or glycosyl modifications) of many vacuolar proteins.

CPY is synthesized as an inactive precursor protein that, characteristic of proteins that transit through the secretory pathway (Fig. 1), translocates into the endoplasmic reticu-

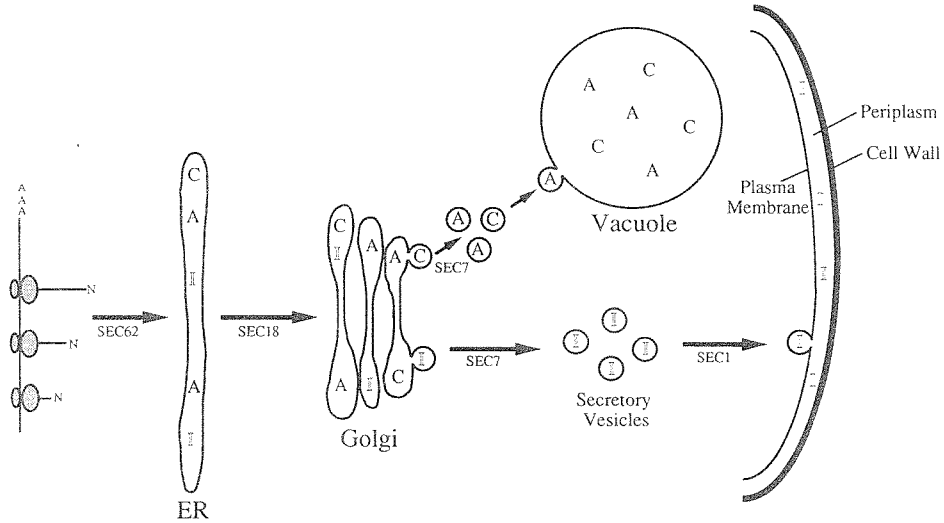

FIG. 1. The secretory enzyme invertase (I) and the vacuolar hydrolases CPY (C) and PrA (A) are synthesized in the cytoplasm and sequestered into the lumen of the ER, where they are modified with *n*-glycosidically linked core oligosaccharides. The proteins then transit to the Golgi complex, where further glycosyl modification takes place. Invertase is then packaged in secretory vesicles that deliver this enzyme to the cell surface, and CPY and PrA are targeted to the vacuole by a secretory vesicle-independent route. The *SEC* gene products are required for transport through the secretory pathway: *SEC62*, translocation into the ER (33, 34); *SEC18*, transit beyond the ER (123, 156); *SEC7*, transit beyond the Golgi complex (43, 123, 156); *SEC1*, secretion via secretory vesicles (123).

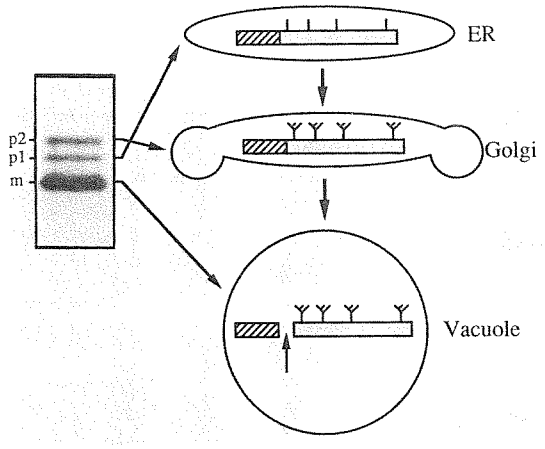

FIG. 2. Carbohydrate and proteolytic processing forms of CPY. As the precursor protein translocates into the ER, the signal peptide is proteolytically removed and the protein is core glycosylated (p1 form). During transit through the Golgi complex, additional carbohydrate modification takes place (p2 form). The protein is then targeted to the vacuole, where a propeptide segment is proteolytically removed, before or upon arrival, to generate the mature active enzyme (m form). Symbols: ▨, propeptide; ▭, mature enzyme. The approximate locations of the four N-linked oligosaccharide addition sites are indicated above the enzyme. Reprinted from UCLA Symp. Mol. Cell. Biol. (83a) with permission of the publisher.

lum (ER) as a result of the presence of an N-terminal signal sequence (11, 62). Temperature-sensitive mutations in the secretory pathway that block ER translocation, *sec61* and *sec62* (33, 34) (Fig. 1), cause the unglycosylated, signal sequence-containing form of CPY, termed preproCPY (54), to accumulate in the cytoplasm. In a wild-type strain, the 20-amino-acid signal peptide is proteolytically removed during or immediately after translocation (11, 62). Translocation into the ER is accompanied by an increase in molecular mass as the protein undergoes dolichol-mediated core glycosylation (54). The addition of core oligosaccharides, having the structure $(GlcNac)_2(Man)_9(Glc)_3$ (93), to the four sites in proCPY having the tripartite recognition sequence Asn-X-Thr, produces the 67-kilodalton (kDa) (54) form called p1 CPY (156) (Fig. 2). The addition of phosphate via phosphomonoester and -diester groups to the carbohydrate side chains also takes place in the ER (53, 152, 156, 166). The precise mechanism of transit from the ER to the Golgi complex is not known but is believed to occur via vesicular carriers. The dependence of transit on the *sec18* gene product (156) (Fig. 1) supports this prediction, since *sec18* has been shown to be homologous to the mammalian *N*-ethylmaleimide-sensitive fusion protein, a protein that appears to be involved in ER-to-Golgi transit and intra-Golgi vesicle fusion (10, 193). Passage of proteins through the yeast Golgi complex is not as well defined as it is in mammalian cells, because of the lack of good fractionation procedures for the yeast Golgi complex and the relatively simple carbohydrate modifications that take place in yeasts. A distinction can be made between early and late Golgi compartments by analyzing proteins that accumulate in the Golgi-blocked *sec7* mutant strain (43, 156) (Fig. 1). The proCPY that accumulates in a *sec7* mutant at the restrictive temperature is modified with α-1,6-mannose-linked carbohydrates, but does not contain the α1,3-mannose carbohydrate linkages that are present on the mature protein (43). In contrast to the long mannose outer chains characteristic of secreted yeast proteins, CPY and other vacuolar proteins contain oligosaccharide side chains that undergo limited elongation (see Sorting Signals). During transit through the Golgi complex, three of the four core oligosaccharides on CPY are elongated to produce carbohydrate side chains containing an average of 11 to 18 mannoses (166). The fully glycosylated Golgi precursor form, p2 CPY (156), has a molecular mass of 69 kDa (56). The sorting of proCPY from nonvacuolar proteins which also utilize the secretory pathway is believed to take place in the *trans*-Golgi (48). The final transport step, delivery to the vacuole, is again not well defined but is presumed to occur through the use of specific vesicular intermediates. These vesicles, however, are distinct from secretory vesicles involved in cell surface transport of secreted and plasma membrane proteins, since transit to the vacuole is not blocked in *sec1* mutant yeast cells which accumulate secretory vesicles at the nonpermissive temperature (156) (Fig. 1). Just before or upon arrival in the vacuole, the N-terminal propeptide segment of proCPY (56) is proteolytically removed, generating the active 61-kDa mature form (54). The half-time for the maturation process has been shown to be approximately 6 min (54).

Processing pathway. Many of the vacuolar hydrolases, including CPY, proteinase A (PrA), and proteinase B (PrB), are synthesized as inactive zymogens that contain propeptide segments (56) (Fig. 3). Maturation of proCPY, and other vacuolar proenzymes, is dependent on a functional *PEP4* (PrA) gene product (56). These precursor enzymes, including proCPY and proPrA, are likely to transit the same compartments in the early secretory pathway and utilize the same vesicle carriers for delivery to the vacuole, yet no processing of the precursors appears to occur until arrival of each of these proteins in the vacuole. The central role of PrA in the maturation of several vacuolar proenzymes led to the proposal of an activation mechanism that is triggered by the pH-dependent autoactivation of PrA (2, 195). This model is supported by the observation that processing of proCPY and proPrB in vitro has a pH optimum of 5.0 (103, 105). Recent determinations of the vacuolar pH and an analysis of processing in mutants with an altered vacuolar pH (see Vacuolar ATPase and Vacuole Acidification), however, do not presently support this model. The requirement of an additional vacuolar constituent for PrA-dependent processing, such as polyphosphate, may in part be the trigger that initiates vacuolar processing (105). Both genetic and biochemical evidence point to a role for PrA in the processing pathway (56, 105, 200). Strains with *pep4* mutations have extremely reduced levels of CPY activity and accumulate the p2 precursor form of the enzyme. In addition, purified PrA can activate proCPY in vitro in the absence of other known vacuolar hydrolases. Interestingly, the CPY that is processed by PrA alone migrates on sodium dodecyl sulfate-polyacrylamide gels with an apparent molecular mass slightly greater than that of the in vivo form (105). When this form is incubated in vitro in the presence of PrB, the authentic mature form of CPY is generated, suggesting a role for PrB in the processing pathway in vivo. This is further supported by the observation that the propeptide cleavage site between amino acids Asn-111 and Lys-112 is not a preferred substrate for PrA (105). The initial cleavage of proCPY by PrA is not required for the PrB-dependent processing in vitro (54, 105), and it is not clear whether both

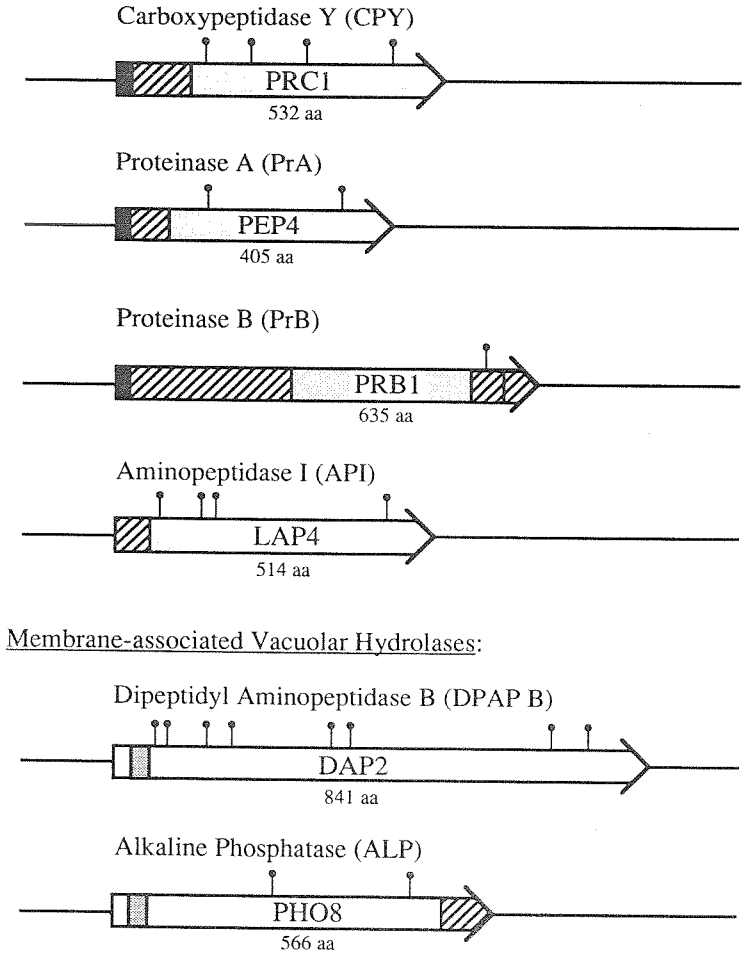

FIG. 3. Biosynthesis of vacuolar hydrolases. The genes are divided into the coding regions for the presumed signal peptide ( ), the proteolytically cleaved propeptide sequences ( ), and the mature enzyme ( ). Transmembrane domains within the mature enzyme are also shown ( ). The approximate positions of the asparagine-linked core oligosaccharides are indicated. The number of amino acids (aa) encoded by each gene is indicated below the gene designation. The schematic diagrams are based upon the nucleotide sequence information from the cloned *PRC1* (157, 171), *PEP4* (2, 195), *PRB1* (111, 114), *LAP4* (23, 30), *DAP2* (142) and *PHO8* (71, 72) genes.

normally occur in vivo. Mature PrB, when overproduced, is in fact capable of processing vacuolar proCPY in the absence of PrA (65). Conversely, CPY is still activated in PrB-defective strains, indicating that PrA can activate proCPY directly in vivo (56). Finally, the facts that PrB can activate other vacuolar hydrolases and its specific activity in crude extracts shows a gene dosage dependence on the *PEP4* gene may indicate a complexity within the processing pathway that allows for considerable regulation (66).

Proteinase A. PrA is similar to CPY in that it is a soluble vacuolar glycoprotein hydrolase that is initially synthesized as a larger inactive precursor (Fig. 3). The extreme N terminus of preproPrA contains a sequence that fits with the consensus for signal sequences and is predicted from the rules of von Heijne (174) to be cleaved by signal peptidase between amino acids 22 and 23. In agreement with this prediction, the first 23 amino acids of preproPrA are able to functionally replace the signal sequence of the normally secreted enzyme invertase (80). Full-length, unglycosylated preproPrA accumulates in *sec62* mutant yeast cells which are defective in translocation across the ER membrane. The signal peptide is cleaved at the level of the ER, and the cleaved form of the protein accumulates in *sec18* (ER-to-Golgi transit defective) yeast cells at the restrictive temperature. N-linked core oligosaccharides are added in the ER at two sites (106) corresponding to preproPrA residues Asn-144 and Asn-345 (2, 195). As with CPY, the core glycosylated species is referred to as p1 PrA. During transit through the ER and Golgi complex, the core oligosaccharides are trimmed and extended, resulting in an increased molecular

mass of 48 to 52 kDa. The size increase of this p2 form is consistent with its undergoing the same type of limited carbohydrate modification seen with CPY. During or upon delivery to the vacuole, the propeptide is cleaved between amino acids Glu-76 and Gly-77 of preproPrA (2, 145) to generate the 42-kDa mature active enzyme (Fig. 3). The half time for the maturation process, approximately 6 min, is identical to that seen for CPY, suggesting that both precursors utilize the same set of transit intermediates en route to the vacuole. As stated above, proPrA may mature autocatalytically. PrA activation is not dependent on CPY, PrB, or carboxypeptidases (105) but the participation of another, as yet unidentified protein, cannot be ruled out.

Proteinase B. The analyses of the biosynthesis of CPY and PrA provided a deceptively simple and cohesive model for the modification, processing, and transport of soluble vacuolar hydrolases. Initial studies of PrB added to this common model; a precursor form of PrB of approximately 40 kDa was identified that underwent a *PEP4*-dependent maturation step to generate a mature enzyme of approximately 30 kDa (104, 106). This maturation occurred with the same kinetics as seen for proCPY (112), suggesting that PrB traversed the secretory pathway in much the same way as CPY and PrA did. One difference was that whereas the PrB precursor showed a tunicamycin-sensitive molecular mass shift, the mature protein did not (106). Since it was known that mature PrB was a glycoprotein (85), this suggested the presence of hydroxyl-linked (O-linked) carbohydrate groups. A major insight into the biosynthesis of PrB was afforded by the cloning of the PrB structural gene, *PRB1* (111). Sequencing of the *PRB1* gene revealed an open reading frame of 635 amino acids encoding a protein of approximately 70 kDa, substantially larger than the previously identified precursor (114). The first 19 residues of the deduced amino acid sequence have the characteristics of a hydrophobic signal sequence, supporting the prediction that PrB travels through the secretory pathway. In addition, the N-terminal 20 amino acids of precursor PrB can functionally replace the invertase signal sequence, allowing translocation of invertase into the ER and subsequent secretion from the cell (D. Klionsky and S. Emr, unpublished observations). Surprisingly, a comparison of the amino acid sequence from purified mature PrB with that of the deduced amino acid sequence from the *PRB1* gene indicated that the mature protein begins at amino acid 281 (starting from the initiation codon) (114). The segment between the end of the signal peptide and the start of the mature protein, an additional 261 amino acids, must therefore correspond to a propeptide domain. The large size of this propeptide and the fact that it is highly charged make it unclear whether the propeptide translocates across the ER membrane. The predicted molecular mass shift resulting from the proteolytic removal of this N-terminal propeptide would not yield a protein the size of mature PrB, but, rather, one the size of the 40-kDa precursor that accumulates in *pep4* cells. This led to the conclusion that a second processing event must occur during the maturation of PrB that involves the removal of a C-terminal polypeptide segment. Since this part of the protein contains a site for N-linked glycosylation, removal of this segment by PrA cleavage would explain the difference in tunicamycin sensitivity seen between the precursor and mature forms of PrB. A detailed analysis of the different forms of PrB present in conditional secretion-deficient mutants confirmed the predictions of a more complicated processing pathway than that seen for CPY or PrA (103, 112) (Fig. 3). In vitro (103) and in vivo experiments involving a rapid pulse-chase analysis (112) revealed a PrB precursor of >70 kDa. This precursor could be accumulated in *sec61* and *sec62* mutant yeast cells and was unaffected by tunicamycin, indicating that it represented preproPrB which had not translocated into the ER (103, 112). Shortly after translocation of all or part of preproPrB into the ER, the N-terminal 280-amino-acid propeptide is removed to generate an intermediate form of approximately 39 kDa (41.5 kDa in reference 103). Since this cleavage still takes place in *sec18* cells at the nonpermissive temperature, it is presumed to occur in the ER (103, 112). This initial cleavage (it is not known whether the signal peptide is removed independently) also occurs in a *pep4* strain, which demonstrates that it is not PrA dependent. During transit of the peptide through the Golgi complex, elongation of the N-linked (but probably not the O-linked) oligosaccharide side chain results in a small increase in molecular mass (103, 112). A detailed analysis of strains containing mutations that affect glycosyl modification, *alg6*, *gls1*, and *mnn1*, confirmed that proPrB has only one N-linked chain (112). The Golgi form of PrB identified in these experiments is identical to the previously identified (106) 40-kDa precursor found in *pep4* mutant cells (42 kDa in reference 103). Although mature vacuolar PrB has a molecular mass of 31 kDa (33 kDa in reference 103), it appears that this form of the enzyme results from two successive cleavage reactions. The penultimate proteolytic step is carried out by PrA and results in the conversion of the 40-kDa Golgi intermediate to a 37-kDa form (112). An unidentified protease then converts the 37-kDa species to the final 31-kDa mature PrB. Interestingly, there is evidence that PrB itself is involved in the final activation step(s), since inhibitors of PrB block this reaction (103). This processing scheme involves the action of at least one and possibly two uncharacterized proteases for the eventual maturation of a vacuolar protease. The presence of O-linked sugars and the removal of a C-terminal polypeptide also reveal that the processing pathway of PrB is quite different from that of CPY and PrA. Since the large N-terminal propeptide is proteolytically removed early in the secretory pathway, its role is unclear. It is possible that it remains associated with PrB after cleavage and plays some role in sorting or in inhibition of PrB activity (103, 112).

PrB from the yeast *Candida albicans* has been purified and shown to be very similar to that from *Saccharomyces cerevisiae* on the basis of physical and enzymatic properties (41). This enzyme appears to be located in the vacuole; a specific protein inhibitor resides in the cytoplasm, as is seen with CPY, PrA, and PrB from *S. cerevisiae* (42, 94).

Aminopeptidase I. Yeast cells contain a variety of aminopeptidases, but only one of these, aminopeptidase I (API), is known to be localized in the vacuole (44). API is a soluble metalloexopeptidase that is strongly activated toward leucine substrates by Zn^{2+} (108). The native enzyme is an approximately 569- to 640-kDa multimeric glycoprotein composed of identical subunits of 50 to 53 kDa (23, 108, 167). API activity is *PEP4* dependent (167) and is derepressed by growth under conditions of limiting glucose or nitrogen (44), typical of other soluble vacuolar hydrolases. The structural gene coding for API, *LAP4*, was recently cloned and sequenced (23, 30). The open reading frame encodes a 514-amino-acid protein. Analysis of the N-terminal amino acid sequence of the mature protein indicates the presence of a 45-residue prosequence (23) (Fig. 3). API is synthesized as a 57-kDa precursor (23) that contains four potential sites for N-linked glycosylation (23, 30). The calculated molecular mass of the mature protein lacking carbohydrate is 44.8 kDa (108), making it unlikely that all four sites are glycosylated or

that they have the standard oligosaccharide structure found on vacuolar proteins. Since two of the potential glycosylation sites have the less frequently used Asn-X-Ser sequence (23, 30, 114), it seems likely that these sites are not glycosylated in vivo. Resolution of this question will require more definitive experiments. Interestingly, the extreme N terminus of the API precursor lacks a sequence that fits with the consensus hydrophobic signal sequence. A 16-residue stretch of the prosequence, however, contains both hydrophobic and hydrophilic residues that can be arranged in an amphiphilic α-helix (23, 30). The function of this unusual prosequence and the means by which API translocates into the ER have yet to be ascertained.

Trehalase. Trehalase is the only enzyme known to be involved in the catabolism of trehalose, a neutral disaccharide that is used as a storage carbohydrate in a variety of fungi (reviewed in reference 164). *S. cerevisiae* possesses two distinct trehalase activities, a cyclic AMP-dependent cytosolic enzyme and a vacuolar enzyme (75, 98, 190). Since the cytosolic trehalase is active only when phosphorylated, compartmentalization plays an important role in trehalase metabolism; trehalose is located primarily in the cytosol (75), whereas the constitutively active trehalase is sequestered within the vacuole (190). The vacuolar enzyme is a glycoprotein with a pH optimum of 4 to 5 and has an apparent molecular mass of 215 kDa based on gel filtration (99). Vacuolar trehalase appears to transit through the early stages of the secretory pathway. Delivery to the vacuole is blocked in *sec18* and *sec7* mutant yeast cells at the nonpermissive temperature but, like in other vacuolar hydrolases, is unaffected in a *sec5* (accumulates secretory vesicles) mutant (52). The activity of vacuolar trehalase is also *PEP4* dependent, suggesting that it is synthesized as a precursor form that is processed in a PrA-dependent manner (51). The vacuolar trehalase is probably not essential, since a *pep4* strain is able to grow as well as a wild-type strain on trehalose (51).

α-Mannosidase. At present the biosynthesis of α-mannosidase is not fully understood, even though it is the classic marker enzyme of the vacuole membrane. α-Mannosidase is localized in a particulate fraction and displays the same type of carbon catabolite repression and sporulation-induced increase in activity seen with other vacuolar enzymes (132). Recently, α-mannosidase was purified from yeast vacuoles (197). Although the enzyme activity appears to be membrane associated, its solubilization characteristics suggest that it may be a peripheral membrane protein or an ecto-type integral membrane protein with a small hydrophobic region that is attached to the inner surface of the vacuole membrane (197). This may also be true for the vacuolar α-mannosidase from *Neurospora crassa*, since only 22% of the activity remains associated with purified vacuolar membranes (172). The molecular mass of the native enzyme from *S. cerevisiae* was determined to be 560 kDa, which represents active isoforms composed of three polypeptides of 107, 73, and 31 kDa. The 107- and 73-kDa polypeptides are closely related, as determined by peptide mapping and cross-reactivity with specific antisera. The appearance of the 73-kDa species relative to the 107-kDa polypeptide increases with time in the stationary phase of growth, suggesting that the former is produced as the result of a specific proteolytic conversion (197). The same is presumably true of the 31-kDa polypeptide, since it is present in approximately equimolar amounts with the 73-kDa polypeptide. The structural gene for yeast α-mannosidase, *AMS1*, has been cloned and sequenced (89, 196) and was shown to encode the 107-kDa polypeptide (196). A strain carrying a chromosomal disruption of *AMS1* does not synthesize either the 107- or 73-kDa polypeptides, confirming that the smaller polypeptide is proteolytically derived from the 107-kDa primary gene product (196). An analysis of the deduced amino acid sequence does not reveal the presence of an N-terminal signal sequence or any transmembrane domains that may function as internal signal sequences (196). The lack of a large hydrophobic region fits with the observation that α-mannosidase activity is extractable by Na_2CO_3 at high pH (197). The apparent absence of a signal sequence raises an intriguing question about the biosynthesis of α-mannosidase. Since the enzyme activity is present within the vacuolar lumen, it must translocate across some membrane during its biosynthesis. α-Mannosidase, however, is not mannosylated even though it has seven potential sites for N-linked glycosylation, suggesting that it may not transit through the secretory pathway (196). This would explain the insensitivity of α-mannosidase to the sorting defects exhibited by most of the vacuolar protein-sorting mutants (see Mutants Defective in Vacuolar Protein Sorting). α-Mannosidase may be delivered to the vacuole and translocated across the vacuolar membrane directly from the cytoplasm (196). This would be similar to the direct translocation of proteins targeted for degradation into the lumen of lysosomes in mammalian cells (24).

Dipeptidyl aminopeptidase B. Yeast express at least two dipeptidyl aminopeptidases (DPAPs) that are associated with a particulate fraction (158, 159). One of these activities represents the product of the *STE13* gene, dipeptidyl aminopeptidase yscIV (DPAP A), which is involved in α-factor pheromone processing (67, 159) and is presumably localized to a late Golgi compartment. The other enzyme, dipeptidyl aminopeptidase yscV (DPAP B), is encoded by the *DAP2* gene (159) and is associated with the vacuolar membrane (12). DPAP A and DPAP B activities are easily distinguished because of the thermolability of the latter. DPAP B is the first vacuolar membrane protein for which detailed biosynthetic data were determined. In contrast to the soluble vacuolar hydrolases, DPAP B activity is not *PEP4* dependent or enhanced by incubation of a crude extract at pH 5.0 (158), suggesting that it does not contain a propeptide domain (Fig. 3). This lack of *PEP4* dependence is similar to that seen with α-mannosidase. The nucleotide sequence of the *DAP2* gene was recently determined (142) and found to encode an 841-amino-acid polypeptide of predicted molecular mass 96,429 Da. Although DPAP B and α-mannosidase activities cofractionate (12), DPAP B is not removed from the vacuole membrane by treatment with Na_2CO_3 at pH 11.5 (142), indicating that it is an integral membrane protein. A pulse-chase analysis with antiserum specific to DPAP B reveals that the protein is initially made as 110- and 113-kDa species in the ER (142). The difference in size between the two ER forms is due to heterogeneous core glycosylation, since both species migrate with a molecular mass of 96 kDa after treatment with endoglycosidase F. Titration with endoglycosidase F indicates that at least five of the eight potential N-linked glycosylation sites are used. This type of heterogeneous glycosylation is similar to that seen with core-glycosylated invertase (40, 156). The ER forms of DPAP B chase into a 120-kDa form in the Golgi complex (142). This molecular mass shift suggests that the oligosaccharide chains on DPAP B undergo the same type of limited extension seen with other vacuolar hydrolases. As expected from earlier studies (158), DPAP B does not undergo a detectable *PEP4*-dependent cleavage and the enzyme is in fact active in the ER (142). Localization by immunofluorescence confirms that

the protein is associated with the vacuolar membrane in wild-type yeast cells (142). In contrast, *sec18* and *sec7* mutant strains accumulate nonvacuolar forms of DPAP B as revealed by both immunofluorescence and sodium dodecyl sulfate-polyacrylamide gel analysis. Like the soluble vacuolar hydrolases, DPAP B is transported through the early stages of the secretory pathway. A hydropathy analysis of the deduced amino acid sequence of DPAP B showed the presence of a single hydrophobic domain near the amino terminus (142). This is predicted to result in the orientation of DPAP B as a type II integral membrane protein, with the C terminus inside the lumen and an N-terminal cytoplasmic domain of approximately 29 amino acids.

Alkaline phosphatase. The nonspecific alkaline phosphatase (ALP) is the best-characterized yeast alkaline phosphatase. It is a repressible enzyme regulated in response to phosphate levels through a system of genes including *PHO4*, *PHO80*, *PHO81*, and *PHO85* (see reference 133 for a review). Its activity is also modulated posttranslationally by the *PEP4* gene product. ALP has a molecular mass of 130 kDa and is composed of two identical subunits of molecular mass 66 kDa (131). It is a glycoprotein containing N-linked oligosaccharides which constitute 8% of its total weight (131), suggesting the presence of two glycosidic side chains. ALP is encoded by the *PHO8* gene (73, 165) which has been cloned (72) and sequenced (71). The deduced amino acid sequence predicts a protein of 566 amino acids, with two potential sites for N-linked glycosylation at Asn-268 and Asn-401 (71). ALP activity has been localized to the vacuole (9, 26, 110, 191), but there have been differing reports concerning its association with the vacuolar membrane. Cytochemical studies have localized ALP to the inner side of the vacuolar membrane (9, 26), and a particulate alkaline phosphatase activity was found to cofractionate with vacuolar membranes (110). This enzyme, however, was characterized as being different from the previously identified soluble ALP (131). Subsequent studies suggest that the two enzymes are identical (26). Recently, a detailed biochemical characterization of ALP has been carried out (82). ALP is initially made as a larger precursor that is matured in a PrA-dependent manner with a half-time of approximately 6 min. The ALP precursor transits through the same part of the secretory pathway as many other vacuolar proteins and can be accumulated in *sec18* and *sec7* mutant yeast cells at the restrictive temperature. Both the precursor and mature forms of ALP are membrane associated. Analysis of ALP solubility in the presence of saponin and Na_2CO_3 indicate that it transits through the secretory pathway and resides in the vacuole as an integral membrane protein. A hydropathy analysis of the deduced amino acid sequence of ALP reveals the presence of a single hydrophobic domain near the N terminus, preceded by a stretch of basic amino acids (71, 82). Since ALP lacks a standard signal sequence at its extreme N terminus, this hydrophobic domain presumably functions as both an ER translocation signal and a membrane anchor. This would predict an orientation of ALP as a type II integral membrane protein (Fig. 3). A type II orientation has been confirmed by protease protection experiments, which indicate the presence of an N-terminal cytoplasmic tail on ALP (82). ALP is unusual in that it is the only characterized vacuolar membrane protein that undergoes a PrA-dependent cleavage. The ALP propeptide was shown to be removed from the C terminus of the precursor protein. This is consistent with earlier observations that a mutated ALP with a C-terminal truncation expressed activity independently of the *PEP4* gene (71, 72).

ATPase. A vacuolar membrane ATPase has been identified and partially purified in yeasts and *Neurospora crassa* Because of the substantial amount of data available on this enzyme, and its importance in vacuole function, it is discussed separately below (see Vacuolar ATPase and Vacuole Acidification).

Sorting Signals

All of the vacuolar proteins that have been characterized with regard to their transport properties, with the possible exception of α-mannosidase (see above), travel to the vacuole via the secretory pathway (80, 82, 103, 112, 142, 156) (Fig. 1). They transit from the ER to the Golgi complex, undergoing both proteolytic and glycosyl modifications, and can be accumulated in these organelles along with normally secreted proteins in *sec* mutants that, at the nonpermissive temperature, are transport defective. The *sec* mutants that block the movement of secretory proteins after the Golgi complex do not affect the delivery of vacuolar proteins to the vacuole (142, 156). This suggests that secretory and vacuolar proteins travel together through the ER and Golgi complex before being sorted from one another for final delivery to their distinct subcellular destinations. Since all of the proteins that use the secretory pathway also use the same set of transit organelle intermediates, there must be signals within the proteins themselves that allow them to be sorted and targeted in a precise and efficient manner. One of the major modifications made to proteins upon translocation into the ER is the addition of core oligosaccharides, which are further modified in the Golgi complex, resulting in a structure for vacuolar proteins of the form $(GlcNAc)_2(Man)_{11-18}$. These oligosaccharide side chains are often also modified with phosphodiester groups (53). Secretory proteins such as invertase receive the same type of core oligosaccharides in the ER, but undergo a more extensive elongation of these side chains in the Golgi complex, where 50 to 100 mannose residues may be added to each side chain (6, 162). One explanation for this differential modification would be that secretory and vacuolar proteins travel separately through the secretory pathway and are accessible to different mannosyl transferases in the Golgi complex. The specific type of glycosyl modification might mark each protein for delivery to the vacuole or the cell surface. This would be analogous to the mechanism used for the sorting of some mammalian lysosomal proteins that are modified with mannose 6-phosphate, allowing recognition and lysosomal delivery through interactions with the mannose 6-phosphate receptor (reviewed in references 87 and 173). Four lines of evidence, however, argue against a direct role for glycosyl modifications in vacuolar protein sorting. First, it has been demonstrated that CPY, PrA, and ALP can be delivered to the vacuole and matured in the presence of tunicamycin, a drug that blocks the addition of N-linked oligosaccharides (26, 80, 152, 156). Second, hybrid proteins consisting of segments of vacuolar proteins fused to invertase are efficiently delivered to the vacuole, even though they undergo the same type of extensive carbohydrate elongation as is seen with wild-type invertase (62, 80). Third, certain hybrid proteins that lack any oligosaccharide addition sites on the vacuolar part of the hybrid are delivered to the vacuole (62, 80, 83). Fourth, overproduction of CPY (157) leads to secretion of this protein without the addition of long mannose outer chains (see Overproduction-Induced Mislocalization). These observations suggest that (i) vacuolar proteins are accessible to the same compartments of the secretory pathway as are

secreted proteins, (ii) the type of glycosylation received does not specify the ultimate subcellular destination, and (iii) the elimination of glycosylation, and hence phosphorylation, does not prevent proper sorting. This is supported by recent data showing the correct vacuolar localization of CPY which has been altered to remove one or more of the N-linked glycosylation sites by site-specific mutagenesis (J. R. Winther, T. H. Stevens, and M. C. Kielland-Brandt, personal communication). This apparent lack of a role for glycosylation in the sorting process suggests that any targeting information expressed by the vacuolar proteins must be contained within the polypeptide chains themselves.

Carboxypeptidase Y. Two independent approaches were undertaken simultaneously to characterize the sorting information in CPY. The first of these relies on the use of gene fusions to the *SUC2* gene. The *SUC2* gene codes for invertase, a glycoprotein enzyme that is easily assayed and is competent for delivery through the secretory pathway. Invertase normally resides in the periplasm, where it catalyzes the hydrolysis of extracellular sucrose. Secretion of invertase is believed to occur by a default pathway; there is no evidence that active signals beyond the N-terminal signal peptide are required for its localization in the periplasm (62, 76, 171, 186). These properties make invertase a useful marker enzyme with which to monitor vacuolar protein sorting. Plasmid vectors have been constructed which contain a truncated *SUC2* gene missing the N-terminal signal sequence and the first two amino acids of the mature protein (62, 83). Portions of the gene of interest, in this case *PRC1*, were cloned in front of and in frame with the *SUC2* gene, so that hybrid proteins are produced that retain invertase activity. When the N-terminal 20 amino acids of preproCPY are fused to invertase, the hybrid protein is secreted from the cell (62). This indicates that the first 20 amino acids of preproCPY can functionally replace the invertase signal sequence. This hybrid construct is competent to translocate into the ER and transit through the secretory pathway. In contrast, larger hybrid proteins containing 50 to 433 amino acids of preproCPY are efficiently retained within the cell, and subcellular fractionation studies demonstrate that they cofractionate with isolated vacuoles (62). This shows that a vacuolar targeting signal in preproCPY resides within the N-terminal 50 amino acids.

The N-terminal 50 amino acids of preproCPY are sufficient to direct invertase to the vacuole. To determine whether this region is also required for vacuolar delivery of the wild-type protein and to precisely define the location of the sorting information, a second approach was taken that used mutations in the wild-type *PRC1* gene (62). This approach was also used to independently map a vacuolar sorting signal in CPY (171). A deletion of amino acids 21 to 50 (62) or smaller deletions in the vicinity of amino acids 25 to 31 (171) of preproCPY result in missorting and secretion of an otherwise wild-type precursor protein. In addition, random chemical and site-directed mutagenesis of the *PRC1* gene identified a single amino acid change, Gln-24-to-Lys, that was sufficient to cause the same missorting phenotype (171). Subsequent mutational analyses of a region spanning the vacuolar sorting information, residues 18 to 34, confirmed the importance of Gln-24 and identified three additional residues that may contribute to the sorting signal (147). The mutant CPY that was secreted from yeast cells was present as the p2 form, indicating that it transits through the ER and Golgi compartments of the secretory pathway similar to the wild-type CPY protein. This fact, along with the observation that secretion is blocked in *sec1* (accumulates secretory vesicles) mutant yeast cells under restrictive conditions, supports the hypothesis that sorting occurs at a late stage in the Golgi complex (171). The proCPY that is secreted can be matured and activated, indicating that missorting is not due to gross structural changes or misfolding (171). These mutational analyses suggest that amino acids 24 to 31 of preproCPY are critical for efficient sorting. Taken together with the gene fusion studies, these results indicate that the N terminus of proCPY is both sufficient and necessary for vacuolar delivery.

Proteinase A. A similar analysis was carried out with PrA to determine the location and important features of its vacuolar-sorting determinant (80). Hybrid proteins containing 76 amino acids from the N terminus of preproPrA fused to invertase were efficiently delivered to the vacuole. In contrast, a hybrid protein with 61 N-terminal amino acids of preproPrA was inefficiently delivered to the vacuole, with the majority of the protein being secreted from the cell. The N-terminal 76 amino acids of PrA define the signal sequence and propeptide (2, 145), indicating that vacuolar-sorting information in PrA is located within the propeptide domain, similar to CPY, and suggesting that this may be a common theme for soluble vacuolar hydrolases. The N-terminal prosequence of API (23), which is presumably cleaved in a PrA-dependent reaction (167), may also contain sorting information. Deletions within the PrA propeptide cause dramatic instability of the mutated protein, suggesting that an additional role of the propeptide is to allow the precursor protein to fold into its normally protease-resistant form (80). This instability, however, has made it difficult to assess the effects of these mutations on PrA sorting or to further define the PrA sorting signal. A comparison of the amino acid sequences of proPrA and proCPY does not reveal any strong sorting signal consensus sequence that is shared by these two proteins. The most likely explanations for this are (i) the two proteins contain distinct vacuolar-targeting information and use different sorting components even though they share a common delivery pathway and (ii) the vacuolar-sorting signal may contain secondary and/or tertiary structural information. Recent data indicate that glycosylation of PrA may be required for efficient vacuolar sorting (J. R. Winther and M. C. Kielland-Brandt, personal communication). The implications of this observation are not clear, however, since PrA-invertase hybrid proteins lacking the oligosaccharide addition sites on PrA are efficiently delivered to the vacuole (80).

Alkaline phosphatase. ALP is the first vacuolar membrane protein for which detailed sorting information has been determined (83). Gene fusions between *PHO8* and *SUC2* indicate that the N-terminal 52 amino acids of ALP are sufficient to direct the vacuolar delivery of invertase. This segment contains just the cytoplasmic tail and most of the transmembrane domain of ALP. A shorter hybrid protein lacking the N-terminal hydrophobic domain remains in the cytoplasm. This confirms that one role of the transmembrane domain is to act as an internal uncleaved signal sequence, allowing translocation into the ER. Replacement of the first 53 amino acids from wild-type ALP with a functional signal sequence results in missorting and secretion of approximately 50% of the protein (83), confirming the requirement of the N-terminal domain for vacuolar sorting. The remainder of the protein is not delivered to the vacuole but instead accumulates in the ER, possibly because some of the ALP folds improperly and aggregates. The propeptide of ALP maps to the C terminus of the precursor protein (see Synthesis and Processing: Alkaline Phosphatase) (Fig. 3). ALP

is therefore different from CPY and PrA in that its sorting information is not contained in a lumenal propeptide segment that is removed from the protein after it reaches its final destination, the vacuole.

An analysis of ALP sorting has also provided some insight into potential differences between targeting information in soluble and membrane-associated vacuolar proteins. Although both types of proteins transit through the same stages of the secretory pathway and are delivered to the vacuole with similar kinetics (see Synthesis and Processing), ALP exhibits some differences in the characteristics of the sorting process. The most notable of these is an apparent pH independence for vacuolar delivery. Precursor ALP is matured with normal kinetics in the presence of bafilomycin A_1 (83), a drug that inhibits the vacuolar ATPase and causes missorting of soluble vacuolar hydrolases (see Vacuolar ATPase and Vacuole Acidification). This is most easily explained by the location of the ALP sorting signal in the cytoplasmic and/or transmembrane domains of the protein; changes in the lumenal pH of the vacuole or Golgi complex are less likely to affect interactions with these sequences. The importance of this observation is that it points out the likely interaction of ALP with a different sorting component(s), such as a receptor, than is used by the soluble hydrolases. This reliance on unique sorting components is also suggested by the relative insensitivity of ALP to the missorting defects exhibited by certain of the vacuolar protein-sorting mutants (see Mutants Defective in Vacuolar Protein Sorting; Vacuolar Protein-Targeting (*vpt*) Mutants).

Dipeptidyl aminopeptidase B. Preliminary evidence indicates that a short N-terminal region of DPAP B is sufficient to direct invertase to the vacuole (C. Roberts and T. Stevens, unpublished observation). This would indicate that, similar to ALP, the vacuolar sorting information in DPAP B is contained within the cytoplasmic tail and/or transmembrane region (Fig. 3).

Overproduction-induced mislocalization. Some insight into the mechanism of vacuolar protein sorting is afforded by the observation that overproduction of CPY-invertase hybrid proteins or, more importantly, wild-type CPY, leads to missorting and secretion of these proteins (7, 157). The missorted CPY is secreted as the p2 form, and secretion is blocked in *sec1* mutant yeast cells at the nonpermissive temperature, indicating that secreted proCPY must transit through the late secretory pathway (157). These results could be explained by the saturation of a limiting component that is required either for recognizing and sorting CPY directly or for modifying CPY such that it can be subsequently sorted and delivered. This type of result is indicative of a saturable receptor-mediated sorting process; production of CPY above normal physiological levels leads to secretion of much of the excess precursor. Since glycosylation of the secreted precursor is normal (157), the machinery responsible for glycosyl modifications is able to handle the higher levels of substrate. This further shows that glycosylation does not determine the subcellular location of vacuolar proteins. A similar result is seen with overproduction of PrA-invertase hybrid proteins (Klionsky and Emr, unpublished observation) and wild-type PrA (145). Interestingly, overexpression of PrA does not significantly affect the sorting of CPY (145) and, similarly, overproduction of CPY does not cause secretion of PrA (157). This may indicate the use of different receptors, consistent with the lack of homology in the vacuolar sorting signals. Alternatively, overproduction-induced secretion may result from some other effect of overexpression, such as the production of sorting-deficient aggregates. At present, no definitive genetic or biochemical evidence exists for the presence of a receptor for vacuolar proteins. It is interesting that overexpression of *AMS1*, the gene encoding α-mannosidase, does not lead to the presence of enzymatic activity at the cell surface (89).

Mutants Defective in Vacuolar Protein Sorting

The highly compartmentalized nature of the eucaryotic cell suggests that mechanisms exist to effectively target and deliver cellular proteins from their site of synthesis in the cytoplasm to their appropriate destination. Analyses of the delivery of several vacuolar proteins, including CPY, PrA, and ALP, have indicated that specific structural determinants present within the proteins themselves are responsible for their observed vacuolar localization (see Sorting Signals). The loss of this sorting information, through mutational alteration, results in the missorting of the mutated vacuolar protein to the cell surface (62, 80, 83, 171). These observations indicate that the delivery of proteins to the yeast vacuole is an active process. Since vacuolar and other secretory-pathway proteins transit through the same compartments, specific components within the cell must function to distinguish vacuolar proteins from the rest of the secretory traffic and to ultimately deliver these proteins to the vacuole. As discussed above (see Synthesis and Processing; CPY), vacuolar proteins enter into the secretory pathway at the ER and follow an intracellular path similar to that of lysosomal proteins in mammalian cells (86, 87). The genetic data are consistent with the conclusion that the vacuolar protein sorting reaction occurs at a late stage within the Golgi complex and suggest that vacuolar proteins transit from the ER to the Golgi together with proteins destined for secretion or assembly into the plasma membrane. At some point within the Golgi, or shortly after exit from this organelle, the vacuolar proteins are sorted away from the rest of the secretory traffic and are targeted to the vacuole.

Recent genetic studies of vacuolar protein localization in *S. cerevisiae* have led to the isolation of a large number of yeast mutants that exhibit defects in vacuolar protein sorting (7, 143, 144, 146). Studies of these mutants, and the genes affected in them, have indicated that the delivery of proteins to the vacuole is a rather complex process, which requires the coordinated participation of a large number of cellular functions. Two independent genetic approaches have been used in efforts to obtain yeast mutants defective for vacuolar protein targeting (7, 143, 144, 146). Both selections are based upon the assumption that defects in the vacuolar protein-sorting machinery will result in the mislocalization of vacuolar proteins to the cell surface. This was a logical extension of earlier genetic studies which had demonstrated that alteration of the *cis*-acting sorting signals within CPY resulted in its secretion from yeast cells (62, 171). In addition, the overproduction of CPY in wild-type yeast cells results in the appearance of precursor CPY at the cell surface (157). These data suggest that a failure to properly sort vacuolar proteins would result in their secretion from the yeast cells. Both of these genetic approaches have been successful, resulting in the isolation of a large number of mutants which secrete vacuolar proteins. The *vpt* mutants (for vacuolar protein targeting defective) were isolated by a gene fusion approach which took advantage of the efficient vacuolar localization of a CPY-invertase hybrid protein (7, 143). In the second scheme, yeast *vpl* mutants (for vacuolar protein localization defective) were identified by directly selecting for the presence of CPY enzymatic activity at the cell surface (144, 146).

TABLE 2. Genetic overlap between the *vps*, *pep*, and other related sets of mutants

vps mutant	*vpt* mutant	*vpl* mutant	*pep* mutant	Others
vps1	*vpt26*	*vpl1*		
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*
vps12	*vpt12*			
vps13	*vpt2*			
vps14	*vpt14*			
vps15	*vpt15*			
vps16	*vpt16*			*vam9*
vps17	*vpt3*		*pep21*	
vps18	*vpt18*		*pep3*[a]	*vam8*
vps19	*vpt19*			
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*[a]	*slp1*, *vam5*, *cls14*
vps34	*vpt29*			
vps35	*vpt7*			
vps36		*vpl11*		
vps37		*vpl16*		
vps38		*vpl17*		
vps39		*vpl18*		
vps40		*vpl19*		

[a] Recent data have indicated that, in contrast to a previous report (144), the *pep* mutants marked with an asterisk exhibit the indicated overlaps (J. Robinson, S. Emr, R.Preston, J. Zhang and E. Jones, unpublished observations). No overlap has been detected between *pep1*, *pep2*, *pep4*, *pep7*, *pep9*, *pep11*, *pep13*, *pep16*, and the 40 *vps* complementation groups (143, 144). The relationship between the remaining *vam* mutants and the *vps* and *pep* sets of mutants has not yet been determined.

Not surprisingly, the *vpt* and *vpl* mutants obtained from these schemes exhibit very similar mutant phenotypes. Considerable genetic overlap exists between these two sets of mutants and, in all, the *vpt* and *vpl* mutants define 40 unique complementation groups (143, 144). The *vpt* and *vpl* complementation groups have recently been consolidated and are now collectively referred to as *vps*, for vacuolar protein sorting defective (Table 2). A third set of mutants, *pep*, originally identified as defective for CPY enzymatic activity (63), have recently been shown to exhibit defects in the localization of several soluble vacuolar hydrolases, including CPY (144). Complementation analysis with the *vps* and *pep* sets of mutants has again demonstrated extensive genetic overlap. Presently, all of these mutations affecting vacuolar protein sorting have been placed into 47 unique complementation groups (Table 2). The following sections discuss the identification and characterization of these three sets of mutants.

***vpt* mutants.** Early gene fusion studies demonstrated that specific N-terminal sequences of CPY were sufficient to direct the normally secreted enzyme, invertase, to the yeast vacuole (7, 62). These fusions retained invertase activity, but this normally periplasmic activity was now intracellular, sequestered within the vacuolar compartment (7, 62). Emr and colleagues have taken advantage of these observations to develop a genetic selection for yeast mutants defective in the localization of these fusion proteins (7, 143). Briefly, yeast Δ*suc2* strains, which lack secreted invertase activity, are unable to grow on media containing sucrose as the sole fermentable carbon source. Δ*suc2* yeast strains which harbor a single-copy plasmid encoding a vacuolar CPY-invertase fusion protein continue to exhibit a sucrose-negative (Suc^-) growth phenotype, because yeast cells are unable to transport sucrose across the plasma membrane and into the vacuole, where the invertase activity is now sequestered (7, 62). Such Suc^- yeast strains were placed on media containing sucrose as the sole fermentable carbon source, and mutants which could grow on sucrose (Suc^+) were selected (7, 143). Subsequent analyses of these Suc^+ mutants demonstrated that all of the mutations perturb the localization of the CPY-invertase hybrid protein and result in secretion of invertase activity. More importantly, the intracellular sorting and delivery of several wild-type vacuolar proteins are also defective in each of these Suc^+ mutants (see below). More than 600 *vpt* mutants were isolated and characterized. The recessive *vpt* mutations have been assigned to at least 33 *vpt* complementation groups (143).

Invertase assays with the *vpt* mutants demonstrate that all of the mutations result in the secretion, to various extents, of the gene fusion-encoded invertase activity (7, 143). This extracellular activity is not due to cell lysis as there is no increase in the periplasmic levels of two cytoplasmic proteins, α-glucosidase and glyceraldehyde-3-phosphate dehydrogenase, in the *vpt* mutants. In addition, many of the *vpt* mutants secrete more than 80% of this CPY-invertase fusion protein, a level much too high to be consistent with cell lysis, as these *vpt* cells exhibited near wild-type growth rates (7, 143). Since the *vpt* mutants display normal levels and rates of protein secretion, their defects appear to be specific for vacuolar sorting. If the CPY-invertase fusion protein was using normal host functions during its transit to the vacuolar compartment, *vpt* mutations might also be expected to affect the localization of wild-type CPY. In all *vpt* mutants examined, CPY accumulates as a Golgi-modified (p2) precursor form. The majority of this p2 CPY was secreted by mutant cells into the extracellular media fraction. The extent of the CPY mislocalization closely mirrored the CPY-invertase fusion defect (143). In at least one case, this appearance of CPY at the cell surface was shown to be dependent upon *SEC1* gene function (7). This result, together with the apparently normal ER and Golgi modification of the mislocalized CPY, suggests that the normal secretion pathway is being used during *vpt*-dependent secretion of CPY.

The *vpt* sorting defects extend beyond CPY to other soluble vacuolar hydrolases. In most *vpt* mutants, the processing and localization of both PrA and PrB are abnormal (143). In general, the accumulation of proPrA and proPrB can be correlated with the processing defects seen with CPY. However, the extent of secretion of the precursor forms of PrA and PrB is not as great as that observed for proCPY in the same mutants (143). The site of accumulation of the cell-associated PrA and PrB precursor molecules is not known. The *vpt* mutations therefore appear to be pleio-

tropic, affecting the delivery of several different soluble vacuolar hydrolases.

Another important question concerning the *vpt* mutants was whether they affected the localization of vacuolar membrane proteins in addition to soluble proteins. In mammalian cells, lysosomal membrane proteins are not modified with mannose 6-phosphate and do not utilize the mannose 6-phosphate receptor, indicating that at least some components of the sorting machinery are different (reviewed in reference 87). The targeting of yeast vacuolar membrane proteins was initially examined by assaying for α-mannosidase activity in *vpt* mutant cells (7, 143). α-Mannosidase is a classic marker enzyme for the yeast vacuolar membrane (132). The α-mannosidase active domain is thought to reside within the vacuolar lumen, and therefore mislocalization to the cell surface should result in the appearance of detectable cell surface activity. The majority of *vpt* mutants do not show a significant increase in external α-mannosidase activity, suggesting that the localization of this membrane protein is not significantly perturbed in these mutants (7, 143). Recent studies suggesting that α-mannosidase may be directed to the vacuole via a mechanism that is independent of the secretory pathway (see Synthesis and Processing) could provide an explanation for the relative insensitivity of this protein to the *vpt* defects. However, mutants with mutations in four different *vpt* complementation groups (*vpt11*, *vpt16*, *vpt18*, and *vpt33*) do exhibit significant α-mannosidase activity at the cell surface (143) (see below). Recently, the sorting of another vacuolar membrane protein, ALP, has been analyzed in the *vpt* mutants (82). ALP is an integral constituent of the vacuolar membrane and appears to transit through the same early stages of the secretory pathway as CPY (see Synthesis and Processing). In this study, the processing of proALP to a mature species was used as an indicator of vacuolar delivery. In most *vpt* mutants analyzed, there was some accumulation of proALP, although the extent of the ALP processing defect was relatively minor when compared with the processing and localization defects observed with CPY (82). In addition, since *vpt* mutants missort PrA, an enzyme involved in ALP maturation, defects resulting in reduced processing of ALP may not result from missorting of this membrane protein. *vpt11*, *vpt16*, *vpt18*, and *vpt33* cells, however, exhibited a complete block in ALP maturation; this was the same subset of *vpt* mutants which was found to have cell surface α-mannosidase activity (143). This subset of *vpt* mutants constitutes a distinct class, in which the mutant cells appear to lack a normal vacuolar compartment (8) (see below). This analysis suggests that ALP is less sensitive to the sorting defects of most *vpt* mutations than are the soluble vacuolar hydrolases. This might indicate the presence within yeast cells of sorting components which are specific for vacuolar membrane protein localization. However, it should be pointed out that ALP maturation, and not specifically sorting, was assessed in this study. It must still be demonstrated that the processed ALP is indeed in the vacuole in these *vpt* mutants.

Many of the *vpt* mutants exhibit extreme defects in the sorting and/or processing of multiple vacuolar hydrolases. Such severe sorting defects might be expected to compromise other vacuolar functions or to affect the biogenesis of this organelle. To assess the structural integrity of the vacuolar compartment in the *vpt* mutants, an extensive morphological analysis, using both light and electron microscopy techniques, was carried out (8). The morphology of the vacuole in yeast cells can be easily visualized by using fluorescent dyes which accumulate specifically within this compartment (100, 136, 179). Both fluorescein isothiocyanate and an endogenous fluorophore which accumulates within the vacuoles of *ade2* yeast cells were used in this study. Wild-type yeast cells, when visualized with either of these vacuole-specific dyes, are observed to possess one to three relatively large vacuoles per cell (8, 179). The majority of *vpt* mutants, representing 26 complementation groups, exhibit this wild-type staining pattern and have been designated class A mutants (8). Class B mutants, representing three other complementation groups, exhibited a fragmented vacuole morphology. Multiple small vacuolelike structures are present within each cell. Identical results were obtained when each mutant was analyzed by electron microscopy (Fig. 4). The vacuoles present within class B mutants might represent intermediates in vacuole biogenesis or, alternatively, may be by-products of the fragmentation of a larger vacuolar structure. Members of the final four *vpt* complementation groups, class C mutants, exhibited the most severe morphological defects. No significant intracellular staining was observed with either fluorophore, suggesting that class C cells may lack a vacuolar compartment (8). Instead, class C mutants accumulated small vesicles and a variety of abnormal membranous organelles within their cytoplasm (8) (Fig. 4). The precise origin or function of these structures is not presently known. It is possible that some of these structures represent remnants of a vacuolar compartment. The lack of a normal vacuole in these cells may suggest that the gene products defined by the class C mutants are involved in the regulation of the biogenesis of this organelle. In this case, the severe sorting defects might be due to the lack of an appropriate target structure. However, the lack of a vacuolar compartment might, instead, be a consequence of the extreme sorting defects associated with class C mutations. To choose the correct alternative, we require a better understanding of the primary lesion in the class C mutants. It is interesting that class C mutants, which lack a normal vacuolar compartment, are viable but exhibit a temperature-sensitive growth phenotype even though the severe morphological and sorting defects are seen at both temperatures (143). This might suggest that most vacuolar functions are nonessential for growth, at least at the lower temperatures. Alternatively, class C cells might retain residual levels of vacuolar activities, possibly in the accumulated membrane vesicles, which are sufficient for vegetative growth.

The CPY-invertase fusion selection scheme has been successful in identifying many gene functions which are required, directly or indirectly, for the proper sorting of proteins to the yeast vacuole (7, 143). Two other genetic approaches (see below) have also identified components of the cell necessary for this sorting process.

***vpl* mutants.** When CPY is overproduced in wild-type yeast cells, precursor CPY is detected at the cell surface (157). A fraction of this extracellular proCPY is processed to an active form by an unknown protease in a *PEP4*-independent reaction (157). This observation has formed the basis for a genetic selection used by Rothman et al. to obtain yeast mutants defective in the vacuolar localization of CPY (144, 146). This genetic selection also takes advantage of the observation that CPY possesses the major yeast proteolytic activity capable of cleaving the dipeptide *N*-carbobenzoxyl-L-phenylalanine-L-leucine (CBZ-PheLeu) to liberate free leucine (88, 194). Leucine auxotrophs are able to grow on medium which contains CBZ-PheLeu as the sole source of leucine as long as CPY activity is present (64, 146). *pep4* yeast cells, which possess no CPY activity (66), are unable

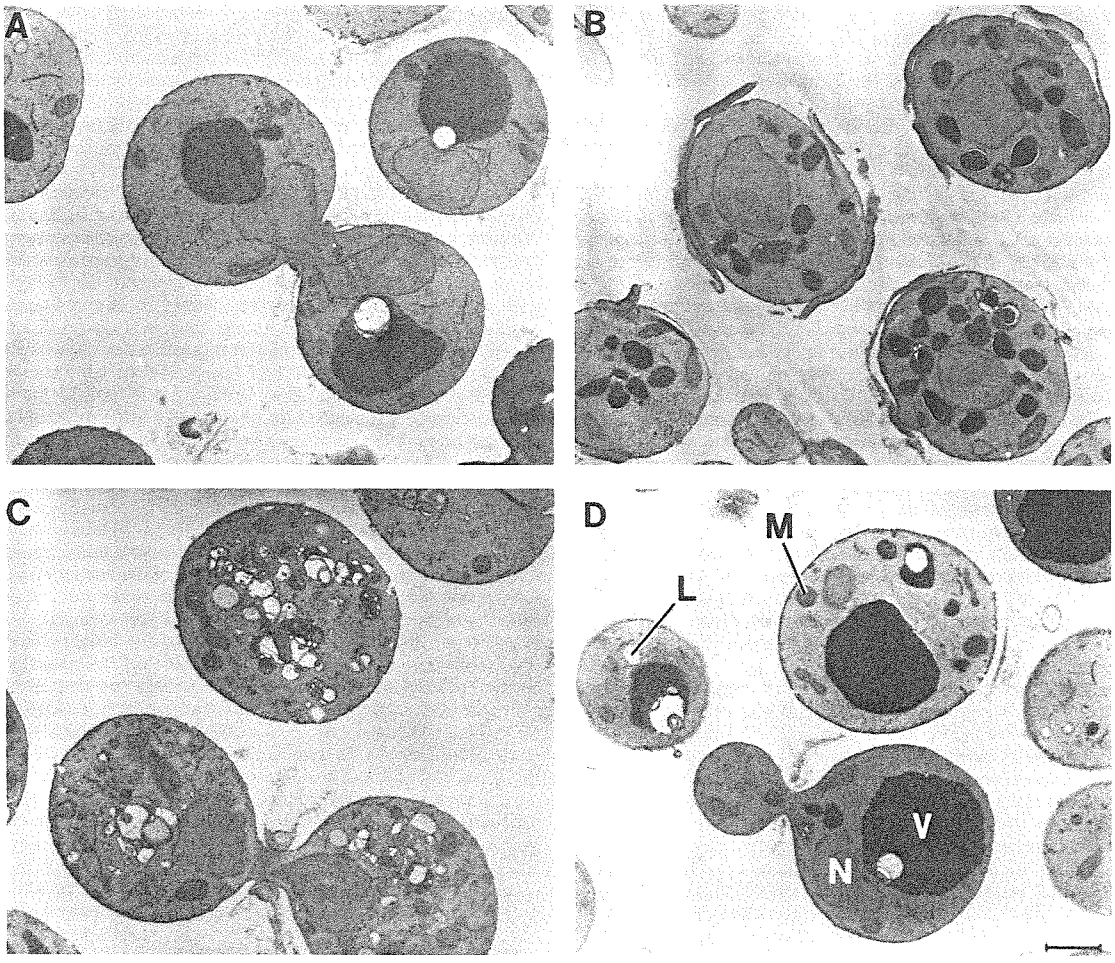

FIG. 4. Morphology of *vpt* mutant strains. *vpt* mutant strains with class A (A), class B (B) or class C (C) morphology and the isogenic wild-type strain SEY6210 (D) were prepared for electron microscopy (21) and processed by the reduced osmium-thiocarbohydrazide-reduced osmium procedure (192). Cells were then washed, dehydrated with ethanol, and embedded in LR white. Thin sections were stained with lead citrate (140). Abbreviations: V, vacuole; M, mitochondria; N, nucleus; L, lipid droplet. Bar, 1 μm.

to utilize the CBZ-PheLeu dipeptide molecule, and, hence, *pep4 leu2* cells are unable to grow on the above medium (146). Therefore, by plating *pep4 leu2* yeast cells (expressing normal levels of CPY) onto medium containing CBZ-PheLeu as the sole leucine source, it was possible to select for yeast mutants which aberrantly localize CPY to the cell surface. A large number of *vpl* mutants, both spontaneously arising (144) and appearing after ethyl methanesulfonate-induced mutagenesis (146), were isolated in two separate studies. It is interesting that very different allele distributions were obtained in the two studies (144, 146). The recessive *vpl* mutations define at least 19 *vpl* complementation groups.

All of the *vpl* mutants, by definition, possess extracellular CPY activity (146). Therefore, it was not surprising that CPY was detected in an extracellular fraction of all *vpl* mutants examined (144, 146). Many of the *vpl* mutants secrete as much as 80 to 90% of their total CPY, and this was shown not to result from cell lysis or CPY overproduction. The fact that *vpl*-mediated secretion of CPY is dependent upon *SEC1* gene function further supports this notion (146). This dependence on *SEC1* gene function also suggests that the route followed by proCPY to the cell surface is the same as that used by normally secreted proteins, such as invertase. In fact, normal protein secretion appears to be unaffected in the *vpl* mutants. Invertase glycosylation and delivery to the cell surface occur with near-wild-type kinetics in *vpl* mutants (146).

The *vpl* mutations also affect the delivery and/or maturation of other soluble vacuolar proteins (146). A significant proportion of PrA (up to 60%) is secreted from *vpl* cells, but, as is also observed with the *vpt* mutants (143), some of the proPrA remains cell associated (146). In addition, PrB enzymatic activity is reduced in *vpl* mutants, suggesting that its delivery is also defective in these cells. Therefore, *vpl*

tivity can be blocked or enhanced, while the ATPase activity is relatively unaffected (115).

Precursor maturation. The reduced pH of the vacuole has been implicated in the processing of precursor proteins by triggering the autocatalytic maturation of proPrA (195). This proposal was based on the low pH values predicted for the vacuole and on the observation that proPrA is homologous to pepsinogen, a zymogen that has been shown to undergo pH-induced conformational changes (60). Recent reports on the vacuolar pH, however, suggest that it is not acidified to the same extent as the lysosome; the lysosomal pH may be as low as 4.5 to 5.0, whereas that of the vacuole is closer to 6.0 (90, 107, 118, 137). It is not clear whether the relatively moderate difference between the vacuolar pH and that of a prevacuolar compartment is sufficient to promote a change in protein conformation. Mutants that fail to acidify the vacuole, however, do accumulate precursor forms of vacuolar hydrolases (8, 148). It is necessary to determine whether these precursor proteins are located in the vacuole before assessing the role of pH in precursor processing.

Sorting. The acidification of intracellular compartments plays a role in a variety of intracellular processes (reviewed in reference 107) including protein sorting. One of the best characterized of these is the low-pH-induced dissociation of ligands from their receptors during receptor-mediated endocytosis and lysosomal protein targeting. The observation that some mutants which missort vacuolar proteins are defective in maintaining the proper pH of the vacuole suggested a role for acidification in vacuolar protein sorting. The effect of vacuole acidification on protein sorting has been examined by (i) inhibiting the vacuolar ATPase, the enzyme primarily responsible for generating the proton gradient and electrochemical potential; (ii) dissipating or neutralizing the proton gradient; and (iii) deleting structural genes which encode subunits of the vacuolar ATPase.

Bafilomycin A_1 is a specific and potent inhibitor of the vacuolar ATPase (19, 70, 148). Treatment of yeast cells with bafilomycin A_1 causes an increase in vacuolar pH which can be demonstrated by the abolition of quinacrine accumulation in the vacuole (8). Inhibition of the vacuolar ATPase by bafilomycin A_1 causes precursor accumulation and missorting of CPY, PrA, and PrB (8; D. Klionsky and S. Emr, unpublished observations). Although the reduced vacuolar pH may play a role in promoting the processing of precursor proteins, the precursor accumulation seen in the presence of bafilomycin A_1 is not due simply to the inability to proteolytically process zymogens. First, a substantial fraction of the vacuolar hydrolases are secreted from the cell, similar to the situation with vacuolar protein-sorting mutants. Second, the increase in vacuolar pH caused by bafilomycin A_1 had no effect on the processing of ALP (82), suggesting that the proteolytic maturation capacity of the vacuole was intact. Proper sorting of ALP in the presence of bafilomycin A_1 is similar to the result seen for the sorting of DPAP B in acidification-defective mutants. DPAP B is correctly localized to the vacuole in strains that have extremely reduced levels of vacuolar ATPase activity (69).

Lysosomotropic or acidotropic weak bases can be used to raise the vacuolar pH owing to the neutralization of protons (107). Protonophores can also be used to eliminate the pH gradient by permitting the equilibration of protons across the vacuolar membrane. Treatment of yeast cells with ammonium acetate, ammonium chloride, or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) results in precursor accumulation and missorting of vacuolar proteins (8, 148). It should be noted that these experiments do not differentiate between effects of acidification of the vacuole and that of other components of the vacuolar system including the ER, Golgi complex, and endocytic or other prevacuolar compartments. Weak bases will accumulate in and neutralize any acidic compartment. CCCP will also act in a nonspecific manner. Although bafilomycin A_1 is relatively specific for vacuolar ATPase, this type of ATPase may be involved in acidification of the entire vacuolar system. A Golgi ATPase from rat liver was recently purified and shown to be related to the vacuolar-type ATPases (117). This Golgi ATPase is inhibited by bafilomycin A_1 although its activity accounts for only a small fraction of the total ATPase in the Golgi complex. Since the *trans* Golgi is presumed to be the site of vacuolar protein sorting, acidification of this organelle may be important for proper localization of vacuolar proteins.

One model for the role of acidification in vacuolar protein sorting is that the lower pH encountered in the vacuole or prevacuolar compartment allows a receptor to dissociate from vacuolar proteins and recycle back to the Golgi complex, analogous to the receptor recycling involved in mannose 6-phosphate receptor-mediated sorting of lysosomal enzymes. Although the presence of receptors for vacuolar proteins has not been clearly established, one feature of this proposal is that it could explain the differential sorting of soluble and membrane-associated proteins under conditions of elevated vacuolar pH. Since membrane proteins appear to utilize nonlumenal sorting components (see Sorting Signals), they presumably do not rely on low pH-induced changes in receptor affinity. Soluble proteins which rely on lumenal sorting components, however, would be affected by changes in the lumenal pH. If the ligand-receptor complexes are unable to dissociate, the available receptors will become saturated, resulting in secretion of any additional soluble vacuolar proteins by the default pathway. Even though the vacuolar pH may be much higher than that determined for the lysosome, acid-releasable ligands can dissociate at a pH of approximately 6.0 (107), similar to the predicted vacuolar pH (90, 118, 137). A definitive role of the vacuolar pH in protein sorting will be best addressed by using mutants that specifically affect the generation and maintenance of the vacuolar pH. Current work involving the disruption of genes encoding vacuolar ATPase subunits should provide definitive answers specifically regarding the role of the vacuolar ATPase in vacuolar protein sorting (3, 147). Initial studies indicate that a disruption of the genes encoding either the 16-kDa (121) or 57-kDa (121; T. Stevens, personal communication) ATPase subunits prevents vacuolar accumulation of quinacrine and results in the accumulation of precursor CPY, presumably reflecting a vacuolar protein-sorting defect. These results indicate a role for the vacuolar ATPase in vacuole acidification and protein sorting.

Assembly of ATPase

All of the vacuolar ATPases that have been purified have at least three subunits and probably four or five additional polypeptides associated with the final enzyme (15, 16, 69, 70, 96, 116). Radiation inactivation studies suggest that the ATPase complex capable of steady-state ATP hydrolysis has a molecular mass of 410 to 530 kDa (16, 57, 169). Kinetic analyses also indicate catalytic-site cooperativity similar to that of the F_0F_1 ATPases (57, 74, 169). For cooperative interactions to occur, steady-state hydrolysis must involve an integrated multisubunit structure (57). At present, there are few data available on the stoichiometry of all the subunits in the complex or the minimal active domain. The

N. crassa 67- and 57-kDa polypeptides appear to be purified in a 2:1 ratio (16). Analysis of a complex released by KNO_3 (discussed below) led to a proposed stoichiometry of $(67)_3$:$(57)_3$:30:16 (15). A complex of the *S. cerevisiae* enzyme that is capable of single-site hydrolysis has a molecular mass of approximately 100 kDa (57, 169). Taking into account the apparent molecular masses of the purified subunits, this value suggests that the two major subunits may be sufficient for single-site ATP hydrolysis. This would be similar to the situation with the F_1F_0 ATPases, for which a minimal complex of ATPase subunits α_1:β:$_1\gamma_1$ is sufficient for hydrolytic activity (45).

The catalytic site of the enzyme is accessible to a membrane-impermeable substrate (68), suggesting that it is on the cytoplasmic surface of the vacuolar membrane. This orientation is also inferred from the function of the ATPase in acidifying the interior of the vacuole and establishing an electrochemical gradient across the vacuolar membrane with the lumen positive in relation to the cytoplasm. In addition, the large subunits from *S. cerevisiae* and *N. crassa* do not appear to have signal sequences or hydrophobic membrane-spanning domains and are extractable by alkaline Na_2CO_3 (13, 20, 69, 70, 119), suggesting that they are peripheral membrane proteins. Finally, indirect immunofluorescence with monoclonal antibodies to the 69-kDa subunit confirms the cytoplasmic location of the catalytic sector (3). Recent experiments in several laboratories have also presented evidence that the vacuolar ATPase may be assembled in a bipartite structure similar to the F_0F_1 ATPases, having an integral membrane sector forming the proton channel and a peripheral membrane (F_1-like)-catalytic domain (15, 70, 116). The vacuolar-type ATPase from chromaffin granules undergoes an ATP-dependent cold inactivation, resulting in the release of a 400- to 500-kDa complex composed of five different polypeptides (116). Similarly, an ATP-dependent removal of peripheral membrane subunits by KNO_3 was demonstrated with *N. crassa* (15) and *S. cerevisiae* (69, 70). In *N. crassa*, treatment with KNO_3 results in the coordinate release of four to six subunits which behave as an aggregate of 440 kDa. Additional evidence for an F_1-like structure is provided by electron microscopy, which reveals the presence of ball-and-stalk structures typical of F_0F_1 ATPases, which were removed with KNO_3 (15). In *S. cerevisiae*, KNO_3 stripping results in complexes of 60 to 240 kDa, suggesting a greater degree of dissociation (70). The presence of large complexes released from the membrane by cold inactivation or KNO_3 indicates that the peripheral membrane subunits may be arranged in an F_1-like structure. Unlike the F_1 of F_0F_1 ATPases, none of these complexes from the vacuolar ATPases retain hydrolytic activity (15, 70, 116).

An obvious question arises concerning the assembly of the ATPase and, in particular, the cytoplasmic domain. The available nucleotide sequence data fail to reveal the presence of signal sequences or transmembrane domains on the major subunits (13, 20, 69, 119), and the 69- and 57-kDa polypeptides do not undergo N-linked glycosylation (69). These observations suggest that the subunits which make up the cytoplasmic domain of the ATPase may not travel through the secretory pathway. In contrast, the DCCD-binding protein behaves as a proteolipid and is an integral membrane protein (69). This subunit, which is involved in forming the proton channel, and any other unidentified components of the membrane sector are likely to utilize the early stages of the secretory pathway. If the peripheral and integral membrane subunits of the ATPase arrive at the vacuole via separate mechanisms, assembly becomes a more complex issue. Do the peripheral membrane proteins, including the catalytic segments, assemble into a complex as soon as they are synthesized (69)? If such a complex forms, it may be capable of ATP hydrolysis. This may imply the presence of an inhibitory subunit similar to that found in *Escherichia coli*, for which it has been demonstrated that the F_1 can assemble into a functional complex in the absence of the membrane domain (81, 84). Evidence for some type of regulated assembly is seen with certain mutants that show greatly reduced levels of ATPase activity and a decrease in the association of the 69- and 57-kDa subunits with the vacuolar membrane, even though these subunits are present at wild-type levels (148). The same questions arise concerning the assembly of the proton channel. Can the integral membrane subunits associate with each other to form a functional proton channel in the absence of the peripheral membrane polypeptides? Again, this type of independent assembly has been demonstrated in *E. coli* (5). Since the two functional domains may arrive at the vacuole by separate pathways, are there temporal controls to coordinate the synthesis of the different subunits? Are the levels of synthesis controlled to ensure the appropriate stoichiometric production of the various polypeptides? If the peripheral membrane subunits do not transit through the secretory pathway, how are they targeted to the vacuole? Finally, since there are single copies of the ATPase genes, and the same gene products may be localized in different compartments of the vacuolar system, including the Golgi complex, how are they targeted to the correct locations? It is clear that many questions remain concerning the biogenesis of the ATPase complex. A coordinated genetic, biochemical, and molecular biological approach is being applied to this problem and should begin to provide many of the answers.

Mutations Affecting Vacuole Acidification

Acidification of the vacuolar system clearly plays a role in protein sorting (see Functions of Vacuole Acidification; Sorting). A logical corollary is that some mutants which missort vacuolar proteins may be defective in establishing or maintaining the correct vacuolar pH. A genetic analysis of the mechanism(s) involved in regulating the vacuolar pH should prove useful in further defining the in vivo roles of vacuole acidification. Two major approaches have been followed in initiating this type of analysis. The first relies on the screening of existing missorting mutants to identify those which are acidification defective, and the second involves the isolation of new mutants which are isolated on this basis directly.

The relative acidification of the vacuole can be assessed by a variety of methods (reviewed in references 107 and 179). One of the most frequently used techniques involves labeling the vacuole with the weak base quinacrine (179). Quinacrine is able to diffuse across the vacuolar membrane because of its lipophilic nature, but once exposed to the low pH of the vacuolar lumen it becomes protonated and is unable to leave the organelle. In wild-type cells, the vacuole is clearly labeled with quinacrine (179). The selection of mutants that missort vacuolar proteins is described above (see Mutants Defective in Vacuolar Protein Sorting). These mutants were analyzed with regard to quinacrine labeling, and several were identified that were unable to concentrate the dye within the vacuole, indicating a defect in vacuole acidification. The *vpt10*, *vpt13*, and *vpt24* (8), *vpl3* and *vpl6* (148), and *pep12* (137) mutants all show little or no staining

mutations affect the localization and/or processing of at least three different luminal vacuolar hydrolases, CPY, PrA, and PrB. The localization of one vacuolar membrane protein, α-mannosidase, did not appear to be affected in the *vpl* mutants (146).

The intracellular morphology of the *vpl* mutants was analyzed by both Nomarski interference optics and electron microscopy (144, 146). Mutants with mutations in four different *vpl* complementation groups, *vpl1*, *vpl5*, *vpl9*, and *vpl19*, exhibit aberrant vacuolar morphologies; the mutant cells possess multiple, small vacuolelike organelles in place of the normal, large vacuolar compartments usually observed in wild-type cells (144). These structures were stained with the fluorescent dye quinacrine, indicating that they are acidic organelles and are most probably related to the vacuole. Therefore, these *vpl* mutants exhibit morphological defects similar to those seen with class B *vpt* mutants (8). In fact, complementation analyses indicate that significant overlaps exist between these two groups of mutants (143, 144) (see below). In addition to fragmented vacuoles, *vpl1*, *vpl9*, and *vpl19* mutant cells accumulate a variety of abnormal membrane-enclosed organelles within their cytoplasm (144, 146). Unlike the class C *vpt* mutants, however, each still possesses a vacuole or vacuolelike organelle.

***pep* mutants.** The *pep* mutants were originally identified in a genetic screen for yeast mutants with reduced levels of CPY activity (63). Many of the *pep* mutants also exhibit decreased levels of PrA and PrB enzymatic activities. Seventeen *pep* complementation groups have been reported (63, 64), and one gene, *PEP4*, has been cloned and shown to encode PrA (2, 195). The *vpl* and *vpt* mutants display a phenotype that is very similar to that of the *pep* mutants; the *vas* mutant cells exhibit greatly reduced levels of cell-associated CPY, PrA, and PrB enzymatic activities (7, 143, 144, 146). In these mutants, the decreased enzymatic activities result from the mislocalization of the protease zymogens to the cell surface, where proteolytic activation occurs very inefficiently. This phenotypic similarity suggested that some of the *pep* mutants might be deficient in vacuolar enzyme activities because of the mislocalization of these enzymes to the cell surface. All of the *pep* mutants, except *pep4*, display vacuolar protein-sorting defects (144). A significant fraction of the total CPY and PrA protein is detected in an extracellular fraction of these *pep* mutants. Therefore, the *VPT*, *VPL*, and *PEP* gene products all appear to define a similar set of intracellular protein-sorting functions.

Genetic complexity of the sorting pathway. The delivery of proteins to the vacuole presumably involves a number of distinct reactions, which must be precisely regulated both spatially and temporally. Specific cellular components must recognize the vacuolar proteins, sort them away from the rest of the secretory protein traffic, and package them into specific transport vesicles that ultimately must recognize and fuse with the vacuole. If the sorting components, such as a putative receptor protein, are to be reused for multiple sorting cycles, additional cellular functions would be required for the recycling process. The genetic complexity would again increase if vacuolar proteins are delivered via a prevacuolar compartment such as an endosome, which might also receive endocytic traffic from the cell surface. Therefore, the list of potential activities and structures required for transport between the Golgi complex and the vacuole can easily accommodate the large number of gene products presently implicated by the genetic studies as having a role in vacuolar protein sorting and delivery.

In addition to the vacuolar protein-sorting defects, *vps* mutations appear to affect vacuole assembly, organellar acidification, cell growth, sporulation, and osmoregulation (8, 143, 144, 146, 148). Therefore, *vps* mutations affect a wide variety of cellular functions. At least in the instances of vacuole biogenesis and acidification, it seems important to ask whether these phenotypes are a secondary consequence of the sorting defects or whether they correspond to the primary defects in *vps* cells. The extreme vacuole morphological defects seen in several *vps* mutants, for example, might suggest a role for these *VPS* gene products in the biogenesis or maintenance of the wild-type vacuolar structure. Therefore, *vps* mutations might also define gene functions necessary for a variety of vacuole-related processes. An understanding of this sorting pathway and the individual *VPS* gene functions will be greatly facilitated by the molecular isolation and characterization of the different *VPS* genes and their respective gene products. The cloning and sequencing of one of the *VPS* genes, *VPS15*, has revealed that the predicted protein product exhibits significant sequence similarity to the serine-threonine family of protein kinases (P. Herman and S. Emr, unpublished observations). This might suggest that protein phosphorylation-dephosphorylation reactions are responsible for controlling specific steps in this sorting pathway. The cloning, sequencing, and localization of other *VPS* and *PEP* gene products should permit additional insights into their possible role(s) in the vacuolar protein-sorting reaction. An in vitro system which reconstitutes Golgi-to-vacuole transit will also be essential for determining the biochemical function of each gene product in the sorting process. Recent observations with semi-intact perforated yeast cells suggest that this type of in vitro reconstitution assay may be possible (T. Vida and S. Emr, unpublished observations).

VACUOLAR ATPase AND VACUOLE ACIDIFICATION

The fungal vacuole is considered to be analogous to the mammalian lysosome mainly because of two similar features: they both contain a variety of hydrolytic enzymes, and they are acidic organelles. It is only recently, however, that the functions associated with compartment acidification, and the components responsible for establishing and maintaining the vacuolar pH, have been elucidated.

Vacuolar ATPase

A vacuolar ATPase has been identified and partially purified from *S. cerevisiae* (68, 69, 168, 191), *Saccharomyces carlsbergensis* (96, 128), and *N. crassa* (16, 18). The vacuolar ATPases from these different organisms have certain common features and are differentiated from the mitochondrial F_0F_1 and plasma membrane ATPases on the basis of pH optima, subunit composition and structure, and sensitivity to inhibitors (reviewed in references 14, 18, 69, and 122). (i) Vacuolar ATPases have pH optima of approximately 7.0 to 7.5 (18, 68, 128). (ii) The enzyme complex has a molecular mass of approximately 400 to 500 kDa (16, 57, 169) and consists of at least three different types of subunits: two major polypeptides of approximately 70 and 60 kDa and a *N,N'*-dicyclohexylcarbodiimide (DCCD)-binding protein of 15 to 20 kDa (16, 96, 168), all of which are highly conserved. Minor polypeptides that may also be structural subunits have been observed in all three organisms (16, 69, 96). (iii) The vacuolar ATPases are insensitive to oligomycin, azide, and vanadate and are inhibited by *N*-ethylmaleimide, KNO_3, KSCN, and bafilomycin A_1 (17–19, 68, 95, 96, 168).

Recent sequencing data and improved purification of the vacuolar ATPase have led to several revisions in subunit designations. The designations that are used in this review, along with previous or alternative designations, indicated in parentheses are as follows: *S. cerevisiae* 69 kDa (70) (subunit A [119], subunit *a*, 89 kDa, 67 kDa [57, 168], *S. carlsbergensis* 75 kDa [96]), *N. crassa* 67 kDa (20) (70 kDa [20]), *S. cerevisiae* 57 kDa (119) (subunit B [119], subunit *b*, 64 kDa [168], 60 kDa [70, 147], *S. carlsbergensis* 62 kDa [96]), *N. crassa* 57 kDa (13) (62 kDa, 60 kDa [13, 16]), *S. cerevisiae* 16 kDa (120) (subunit c, 19.5 kDa [168], 20 kDa [57], 17 kDa [70], *S. carlsbergensis* 9 kDa [96]), *N. crassa* 16 kDa (16, 20). The functions of the different ATPase subunits are not well characterized. The 69-kDa *S. cerevisiae* and 67-kDa *N. crassa* polypeptides are proposed to contain the catalytic site for ATP hydrolysis. These subunits bind radioactive ATP analogs such as 8-azido-ATP and are labeled by inhibitors of ATPase activity such as 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole and *N*-ethylmaleimide (16, 169). The gene encoding this subunit from *N. crassa*, *vma-1*, and *S. cerevisiae*, *VMA1*, has been cloned, and sequence analysis reveals that it is particularly homologous to the β subunit of F_0F_1 ATPases, which is known to contain the enzyme active site (3, 20). The *S. cerevisiae VMA1* gene contains an internal coding region for a nonhomologous peptide insert that is presumably removed by an unidentified splicing mechanism (3). The 57-kDa polypeptide is inferred to be a regulatory nucleotide-binding protein by analogy to the homologous beet tonoplast 57-kDa polypeptide (69, 101). The gene encoding this subunit has been cloned from both *N. crassa* (designated *vma-2* [13]) and *S. cerevisiae* (designated *VAT2* [119, 147] and *VMA2* [3]). This subunit shows homology to the α subunit of F_0F_1 ATPases, also a regulatory nucleotide-binding protein, and the plasma membrane ATPase of the archaebacterium *Sulfolobus acidocaldarius*. These relationships have evolutionary implications for the origin of the vacuolar ATPase (46, 120, 122). The 16-kDa polypeptide is likely to be involved in forming the proton channel. This polypeptide binds DCCD (16, 69, 96, 168), resulting in a block in proton translocation. In addition, the analogous subunit from the coated vesicle ATPase has been purified and reconstituted into a functional proton channel (161). The 16-kDa polypeptide is an integral membrane protein (69, 120) and is presumably present in multiple copies analogous to the DCCD-binding proteins in other vacuolar-type ATPases and in F_0F_1 ATPases (4, 122). The gene encoding the yeast proteolipid has been cloned and found to code for a 160-amino-acid protein with a calculated molecular mass of 16,352 Da (3, 120). The protein is predicted to have four transmembrane segments. The molecular masses and functions of additional subunits which are likely to form part of the ATPase enzyme have not been reliably determined (15, 69, 70). Although the available nucleotide sequence information for the vacuolar ATPase subunits reveals significant homology to F_0F_1 ATPases, there is a much stronger degree of conservation among vacuolar-type ATPases even from widely divergent organisms (20, 120, 122).

Functions of Vacuole Acidification

Amino acid and ion transport. The vacuolar ATPase utilizes the energy generated by hydrolysis of ATP to pump protons into the vacuole lumen. This results in a calculated electrochemical potential difference of protons on the order of 180 mV contributing to both a decreased pH and a membrane potential of approximately 75 mV for *S. cerevisiae* and 25 to 40 mV for *N. crassa* (14, 68). The electrochemical potential is able to drive amino acid and ion transport (35, 68, 124) (Fig. 5). Since protonophores block transport into the vacuole, ATP hydrolysis itself is not sufficient for the transport process, but must be coupled to the generation of a proton gradient (14). The primary mechanism for transport of storage molecules into the vacuole appears to rely on a proton antiport system (see Compartmentalization of Metabolites). Although the vacuolar ATPase is the major energy donor for these transport systems, there is some evidence that a pyrophosphatase activity is associated with the vacuole membrane in *S. carlsbergensis*, which may be responsible for a PP_i-dependent formation of a pH gradient (97). This would be similar to the function of vacuolar pyrophosphatase of sugar beet taproots (55). Pyrophosphatase activity in *S. cerevisiae* is not associated with the vacuole, but a substantial portion of the endo- and exopolyphosphatase activity is recovered in a vacuole membrane fraction (191).

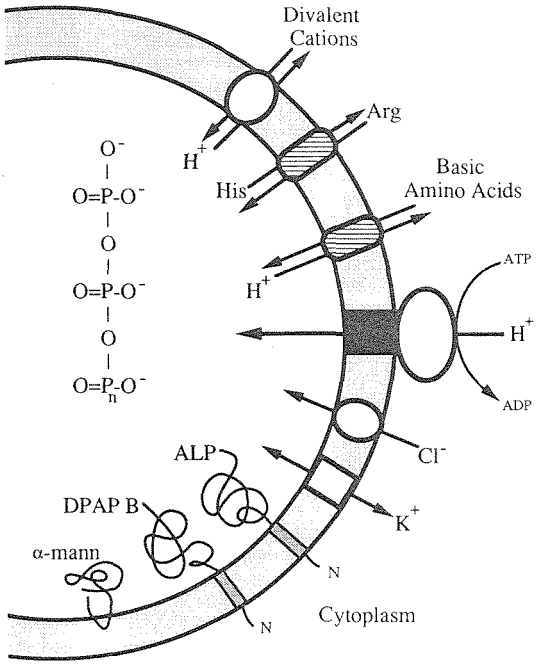

FIG. 5. Overview of the vacuolar membrane and vacuolar transport systems. The H^+-ATPase functions as the primary proton pump and generates an electrochemical potential that is used to drive other transport systems. The activity of the proton pump may be regulated through specific ion channels in the vacuole membrane. The role of polyphosphate and details of the various transport systems are described in the text.

The pH and electrical potential differences across the vacuolar membrane may be regulated through the interactions of a membrane potential-dependent cation channel (178), chloride transport systems (176), and the vacuolar ATPase (3). The ΔpH may be regulated by modulating the membrane potential; a positive membrane potential inside the vacuole inhibits proton uptake (115). By altering the ion conductivity of the vacuolar membrane, proton uptake ac-

with quinacrine. Although these mutants were isolated independently and through different selection procedures, there is evidence that *vpt13*, *vpl6*, and *pep12* are allelic. Further analyses of these mutants has provided additional insights into the function of vacuole acidification. The *vpt13* mutant displays increased sensitivity to low-pH media relative to a wild-type strain or other *vpt* mutants (8). This may suggest a role of the vacuole in regulating the intracellular pH. The *vpl3* and *vpl6* mutants are deficient in vacuolar ATPase activity and exhibit reduced levels of at least two of the ATPase subunits in isolated vacuole membranes (148). Since these subunits are present at wild-type levels in total-cell extracts from these mutants, the mutations may affect assembly and/or sorting of these proteins. Although the vacuole in *pep12* mutant cells does not accumulate quinacrine, there appears to be only a modest change in the vacuolar pH (approximately 0.1 pH unit) relative to the wild-type vacuole (137). Although the exact nature of these different mutations has not yet been determined, it is clear that they all affect vacuolar protein sorting. With regard to vacuole acidification, then, it is not known whether the primary defect is in acidification which leads to missorting, or in sorting which leads to defective acidification.

Two direct approaches are being used to generate new mutants that affect vacuole acidification. One method relies on a fluorescence ratio assay for measuring vacuolar pH to identify mutants after labeling cells with 6-carboxyfluorescein. By using this technique, a recessive mutant was identified (designated *vph1-1*) that is defective in vacuole acidification and maintains the vacuolar pH at 6.9 (137). To directly analyze the function of the vacuolar ATPase, workers in the laboratories of Stevens, Nelson, and Anraku have begun to clone and disrupt the genes encoding the ATPase subunits. These genes have been designated *VAT* (147) and *VMA* (3). Strains carrying a disruption of the gene encoding either the 16- or 57-kDa subunit of the ATPase are viable, indicating that these genes are nonessential (121, 147). This is in agreement with the observation that the *vph1* mutant has a near-normal growth rate. Proper vacuole acidification is apparently not required for vegetative growth. This is not surprising, since no vacuolar functions essential for vegetative growth have been identified. Interestingly, recent evidence suggests that deletions of ATPase subunits result in conditional lethality that may be influenced by the pH of the medium (121). This may be similar to the temperature-dependent conditional lethality seen with strains having deletions of certain *VPS* genes (see Mutants Defective in Vacuolar Protein Sorting: *vpt* Mutants). These results may indicate a requirement for maintenance of the pH of the vacuolar system under stress conditions.

COMPARTMENTALIZATION OF METABOLITES

One of the most prominent features of eucaryotic cells is the reliance on subcellular compartmentalization. The presence of distinct membrane-enclosed organelles allows the cell to spatially separate otherwise competing reactions. The regulation of various catabolic and anabolic processes may also be mediated simply by compartmentalizing and restricting the appropriate substrates. In addition, fine levels of control may be exerted by modulating the concentrations of physiologically important ions. The role of the vacuole in sequestering many of the major hydrolases of the cell is well appreciated. No less important, however, are its roles as the main storage organelle for a variety of metabolically important compounds and ions. Even a brief examination of the function of the vacuole in this regard, however, makes it clear that its role as a storage organelle is not a passive one (Fig. 5). The vacuole is involved in the active and precise homeostatic control over the cytosolic access to, and concentration of, many different constituents.

Role of the Vacuolar ATPase in Metabolite Transport

The vacuolar ATPase is the primary enzyme involved in generating an electrochemical potential difference of protons across the vacuolar membrane. The role of the ATPase in providing the energy for transport processes is demonstrated by the sensitivity of the transport reactions to inhibitors of the vacuolar ATPase such as DCCD, KNO_3, and KSCN (27, 125, 185, 198) and the requirement of ATP for the reaction to proceed (124, 125, 185, 198). In addition, reductions in vacuolar ATPase activity are correlated with decreased vacuolar storage capability (27). The ATPase uses the energy derived from ATP hydrolysis to generate both a proton gradient (ΔpH) and an electrical potential (E_m) across the vacuolar membrane. The electrical potential does not appear to play an obligate role in most transport processes, since they are not inhibited by valinomycin (124, 125). Valinomycin can in fact be stimulatory, most probably because of an alleviation of inhibition resulting from the increased charge separation generated by an electrogenic transport process (125). The proton gradient is the primary driving force for the transport of most metabolites. Protonophore uncouplers such as CCCP and SF6847 and the ionophore nigericin block many transport processes (124, 125, 150, 185, 198). Accordingly, transport of arginine and other amino acids, Ca^{2+}, P_i, Zn^{2+}, Mg^{2+}, and other ions is proposed to occur via H^+ antiporters (124, 125, 129, 150, 198).

Amino Acid Transport and Storage

The presence of two distinguishable amino acid pools, a large pool with a low metabolic turnover and a small pool with a high turnover, has been noted for some time. The large pool was identified as being vacuolar and contains primarily basic amino acids such as arginine (160, 182, 187, 189). The metabolism and compartmentalization of arginine have been thoroughly reviewed by Davis (31), and transport of *S*-adenosyl-L-methionine has been covered by Schwencke and de Robichon-Szulmajster (153). In this review, we will highlight the main points of amino acid transport and storage, including the most recent research in these areas.

Amino acid transport into the vacuole is mediated by a number of transport systems in the vacuolar membrane. Since the transport reactions show saturable kinetics, they presumably reflect interactions with specific protein channels or carriers (126, 151). The differing specificities and kinetic properties have allowed the identification of eight independent transporters in vacuolar vesicles from *S. cerevisiae* (150). H^+/amino acid antiport systems are present for arginine, arginine-lysine, histidine, phenylalanine-tryptophan, tyrosine, glutamine-asparagine, and isoleucine-leucine. An additional arginine-histidine exchange mechanism that utilizes the chemical potential of the histidine concentration gradient was also detected (150, 151). The presence of these transport systems is in agreement with earlier findings that vacuoles and vacuolar vesicles accumulated primarily these amino acids (124, 172), as well as more recent analyses of vacuolar amino acid pools in Cu^{2+}-permeabilized cells (79). The utilization of three separate systems for sequestering arginine, the amino acid with the

highest nitrogen content, points to the importance of the vacuole as a nitrogen reserve. Research on amino acid transport in *N. crassa* and *S. carlsbergensis* has focused largely on arginine uptake, which also occurs by H^+/arginine antiport (129, 198). The carrier in *N. crassa* appears to be arginine specific, and there is no evidence for an arginine-lysine transporter as seen in *S. cerevisiae* (198). A protein that is likely to be the arginine carrier has been identified in *N. crassa* by labeling with a reactive arginine derivative (134). The putative carrier protein has a molecular mass of approximately 40 kDa and appears to be membrane associated. Acidic amino acids are not accumulated in the vacuole but, instead, are located almost exclusively in the cytosol, confirming that the presence of particular amino acids in the vacuole is due to specific uptake processes (58, 79, 124, 187). Amino acids are taken up against a concentration gradient, and most are accumulated in the vacuole at levels 5- to 40-fold higher than the corresponding cytosolic concentration (124, 150). Although the size and composition of the cytosolic amino acid pool stay relatively constant, the size and composition of the vacuolar pool vary widely depending on the available nutrients and growth conditions (58, 79). Arginine normally makes up 25 to 30% of the basic amino acid pool in *N. crassa* and *S. cerevisiae* (28, 38). When arginine is the sole nitrogen source, however, it can account for 85% of the basic amino acids in the vacuole (28). Similar effects on the vacuolar, but not the cytosolic, concentrations of ornithine, citrulline, lysine, and histidine are seen when these compounds are added to the growth medium (58, 79).

Although it has been well documented that amino acid uptake is an energy-requiring process (35, 124, 129, 198), the means by which amino acids are retained against a concentration gradient are not as well understood. It has been proposed that polyphosphate serves as a cation trap and forms complexes that are involved in metabolite retention (38). These types of complexes are likely to exist and can be demonstrated to occur in vitro (28, 109). In *S. cerevisiae*, there is generally a stoichiometric correlation between the amounts of arginine and polyphosphates that are accumulated in the vacuole (38). Although polyphosphate may allow larger concentrations of arginine to be accumulated (38, 198), the two pools are, or can be, independently regulated in both *N. crassa* and yeasts (29, 38). Experiments in which the polyphosphate level is reduced by phosphate starvation show that polyphosphates are not required for vacuolar amino acid uptake or retention in either organism (28, 38). The simplest explanation for retention is that the vacuolar membrane is essentially impermeable to cations (28). Once taken up by an active transport process, cations are retained without further expenditure of energy until they are needed.

Since vacuoles serve as stores for numerous metabolites, there must be specific mechanisms for triggering the release of these substances into the cytoplasm under conditions where they become limiting. Basic amino acids, especially arginine, serve as nitrogen reserves. As expected, nitrogen starvation causes mobilization of the vacuolar arginine pool, resulting in increased levels of cytosolic arginine (79, 91, 92). Similarly, limitation of glutamine also results in the release of vacuolar arginine (92). In this case, arginine release is not a general response to amino acid starvation, since it is not elicited by limiting the proline concentration, even though proline is a breakdown product of arginine degradation (31). In fact, arginine is seen to accumulate in the vacuole during proline starvation. Inhibitors of glycolysis also lead to arginine mobilization (35), but, as is the case with glutamine and nitrogen starvation, the actual effector has not been identified. The observation that respiratory inhibitors or uncouplers block vacuolar release of arginine (35) suggests an energy requirement for efflux.

Inorganic Ion Transport and Storage

It is essential for the cell to regulate the cytosolic ion concentration for several reasons: (i) some ions, such as Sr^{2+}, Co^{2+} and Pb^{2+}, are potentially toxic and must be removed from the cytosol; (ii) physiologically useful ions including Ca^{2+}, Mg^{2+}, and Zn^{2+} may become harmful at excess concentrations; and (iii) precise controls of ion concentrations must be maintained if the ions are to be useful in regulatory processes (27, 139, 184). As with amino acids, the vacuole displays selective uptake and storage of particular cations. K^+ and Na^+, for example, are minor constituents of the vacuolar pools of most organisms even though they are major cytosolic cations (28, 58). The vacuolar uptake of many ions is proposed to occur by H^+ antiport. The activity of the vacuolar ATPase is stimulated by the presence of several ions, and these ions can inhibit amino acid uptake, presumably as the result of transport-induced reductions in the proton gradient (125, 129). There is evidence that Ca^{2+} transport in *S. cerevisiae*, *S. carlsbergensis*, and *N. crassa* is driven by the ATP-dependent formation of a proton gradient (27, 125, 129). The same is true of Zn^{2+} uptake in yeasts (129, 185). A variety of other ions including iron, Mn^{2+}, Co^{2+}, Ni^{2+}, and P_i may also be accumulated in the vacuole, but their uptake properties have not been as well characterized (129, 130, 184). There is substantial evidence that vacuolar cations interact with polyphosphate both in vitro and in vivo (39, 109, 125, 139). In *S. cerevisiae*, the levels of vacuolar inclusions which are presumably due to Ca^{2+}-polyphosphate complexes vary with the level of available polyphosphate (126). Retention of Ca^{2+} is not due solely to trapping by polyphosphate, however, as seen by the release of vacuolar Ca^{2+} upon the addition of proton or Ca^{2+} ionophores (125).

K^+ plays a role in maintaining the ionic and osmotic environment in the cytoplasm, but it may also be involved in the formation of vacuolar ion pools (126). The addition of KCl to the medium results in Ca^{2+} efflux from the vacuole and an increase in vacuolar K^+ (39). A similar coupling of arginine efflux with K^+ influx is also observed (79). There is some evidence that in *S. carlsbergensis*, K^+ is accumulated in the vacuole against a concentration gradient (130). A vacuolar K^+ concentration gradient can also be attained in Cu^{2+}-treated yeast cells and may be able to supply the driving force for transport processes (126). These observations are interesting considering the identification of a membrane potential-dependent cation channel capable of conducting K^+ and other monovalent cations (178).

Polyphosphates

Polyphosphates are the only macromolecular anion in the vacuole (28), and their roles in basic amino acid and cation retention (see above) and osmoregulation (see below) are discussed elsewhere in this review. Polyphosphate serves in a storage capacity for P_i and is located only in the vacuole (29, 38, 59, 170). The polyphosphate chains range in size from 3 to 260 units, with most being 3 to 45 or 7 to 20 units in *N. crassa* and *S. cerevisiae*, respectively (47, 170, 172). These sizes must be treated with some caution, however, because of the potential action of phosphatases during purification. The chain length may be an important factor in

the stability of polyphosphate-cation complexes (109) and may also be related to the growth stage (47). The addition of ammonium salts to the medium causes polyphosphate hydrolysis and a subsequent increase in cytosolic phosphate (47). This may reflect a nitrogen-induced efflux of metabolically useful phosphate reserves.

pH and Osmoregulation

The vacuole is an acidic organelle, and maintenance of the vacuolar pH is important for a number of cellular functions (see Functions of Vacuole Acidification). The vacuolar pH in *S. cerevisiae* and *C. albicans* has been shown to vary with the growth stage (22, 47). The vacuolar and intracellular pH values undergo relatively small changes in response to substantial alterations in the extracellular pH when cells are in the stationary phase of growth (47, 118). The observation that some yeast mutants which lack a normal vacuole are pH sensitive suggests that vacuoles may play a role in homeostasis of the intracellular pH (8). These mutants also show some degree of osmosensitivity, indicating an additional role in osmoregulation. In addition, other mutants defective in vacuolar protein sorting have extremely large vacuoles (8), which may reflect a defect in osmoregulatory capabilities. Polyphosphate formation plays some role in osmoregulation by reducing the osmotic pressure of P_i, and interaction with polyphosphate reduces the osmotic activity of vacuolar amino acids (28, 38). Certainly some of the ions and amino acids in the vacuole are in an osmotically active form (124, 125); however, vacuole size does not change during nitrogen starvation in *S. cerevisiae*, even though there is a rapid decrease in the vacuolar arginine pool (79). This may be due to the approximately stoichiometric increase in vacuolar K^+ which accompanies the arginine efflux. Although regulation of osmotic or electrical potential differences is not well understood, some control may be afforded by the membrane potential-dependent cation channel (178). Since the vacuole and cytosol are isotonic (28, 37), this channel may be an osmotic regulator which acts to balance the osmotic potential difference resulting from the uptake of cations into the vacuole (178). Growth of *N. crassa* on arginine as the sole carbon source or under conditions of phosphate starvation results in large vacuoles (28). In this case, the excess cationic charge results in increased osmotic pressure due to small ions, which are needed for charge neutralization.

Regulation of Transport

Regulation of the transport systems is not well understood, but kinetic analyses provide some insight into the control of metabolite uptake. In *S. cerevisiae*, *S. carlsbergensis*, and *N. crassa*, the cation antiporters have K_m values similar to the concentrations of those cations in the cytosol (124, 129, 150, 198). This suggests that transport occurs until the cytosolic level reaches the K_m value. In addition, the *S. carlsbergensis* plasma membrane ATPase is inhibited by Mg^{2+} concentrations that activate the vacuolar ATPase and are within the K_m range for the vacuolar Mg^{2+}/H^+ antiporter (129). Similarly, the various H^+/amino acid antiporters in *S. cerevisiae* have K_m values 10 to 100 times higher than the corresponding values for uptake systems in the plasma membrane (124, 150). This reflects the need to remove these metabolites from the cytosol when their concentrations become too high. One important aspect of the transport systems that is not well understood is the way in which efflux from the vacuole is controlled. Release of arginine, for example, is triggered by nitrogen or glutamine limitation, but the means by which they exert their effect is not known (91, 92). As with other vacuolar functions, it is necessary to examine the effects of specific mutations to fully understand the physiological roles of metabolite compartmentalization. Recent advances along these lines should provide useful information.

Vacuolar Storage Mutants

Although amino acids and certain ions are critical for various cellular processes, excess levels of these substances can be toxic to the cell. For this reason, homeostatic control of the cytoplasmic concentration of amino acids and ions, carried out by the vacuole, is extremely important. Accordingly, it was reasonable to predict that any mutations which affected the ability of the vacuole to store a particular substance might lead to impaired growth in the presence of a high concentration of that substance. These mutations could exert their effect through a number of possible ways, including (i) inability to transport into or out of the vacuole, (ii) loss of storage capacity, and (iii) impaired regulatory control and loss of homeostasis. In fact, it is likely that there would be considerable overlap among these effects. A defect in the vacuolar ATPase, for example, could prevent the accumulation of amino acids and ions, a transport defect, as well as resulting in the loss of homeostatic control of these metabolites. This type of pleiotropic mutation is demonstrated by the Ca^{2+}-sensitive mutants of *N. crassa* isolated by Cornelius and Nakashima (27). Although these mutants were selected on the basis of their growth sensitivity to high levels of Ca^{2+}, they are not defective in Ca^{2+}-specific transport or storage. Uptake of Ca^{2+} into the vacuoles of these mutants occurred more slowly, consistent with the finding that lower Ca^{2+} concentrations were required for optimal growth. The vacuoles also had reduced levels of arginine and showed a substantial decrease in ATPase activity (27). Because of their pleiotropic nature, it is unlikely that the mutations affect the Ca^{2+}/H^+ antiporter. One possibility would be that they are mutations in the vacuolar ATPase, since an impaired ability to generate a proton gradient would affect the transport of both Ca^{2+} and arginine. Calcium-sensitive mutants of *S. cerevisiae* have also been isolated (127). Analyses of Ca^{2+} content and uptake activity indicate that some of these mutants may be defective in sequestering the intracellular Ca^{2+} pool, suggesting an impaired vacuole. These mutants, designated *cls* type III, also show increased sensitivity to trifluoperazine (TFP). Recent findings indicate that an allele of the yeast gene encoding the 69-kDa subunit of the vacuolar ATPase confers TFP resistance (155; T. Stevens and N. Neff, personal communication). This suggests that mutations leading to TFP sensitivity could simultaneously cause a defect in the function of the vacuolar ATPase. The resulting decrease in the electrochemical potential across the vacuolar membrane, and the corresponding reduction in vacuolar Ca^{2+} uptake activity, may be one explanation for the Ca^{2+}-sensitive phenotype of *cls* mutants. In support of this, a mutation in the TFP resistance gene conferred a calcium-sensitive growth phenotype, although in this case the mutation caused TFP resistance (155).

A similar rationale was used by Kitamoto et al. to identify mutants that were defective in the storage of basic amino acids (78). The catabolic enzymes involved in arginine metabolism are located in the cytosol and have been well characterized (reviewed in reference 31). This cytosolic degradation is an additional way in which arginine is pre-

vented from reaching toxic levels. In contrast, no mechanisms appear to exist for degrading histidine or lysine. This may be the reason why yeast cells grown in the presence of lysine or histidine increase the levels of these amino acids in the vacuole 27- and 42-fold, respectively, compared with a 7-fold increase for arginine (79). If the ability to concentrate these amino acids in the vacuole were impaired as a result of a mutation, the mutants would presumably grow poorly in the presence of high concentrations of lysine or histidine. Mutants displaying a lysine-sensitive growth phenotype were isolated and found to have small vacuolar pools of lysine, histidine, and arginine (175). These mutants are designated *slp1* (for small lysine pool) and had vacuolar levels of basic amino acids that were reduced 30 to 90% compared with the wild type. Since the mutants were defective in the storage of more than one amino acid and the phenotypes result from a single mutation, they are not likely to be specific transport mutants. In addition, the mutants show increased sensitivity to Ca^{2+} and heavy-metal ions, suggesting a more general defect in homeostatic control. A morphological examination reveals the absence of a typical large vacuole in these mutants, accompanied by an increase in vesicular structures. Since the available lysine pool was utilizable, it was suggested that the vesicles could be vacuole related and still retain some normal vacuolar functions (78). Similarly, analysis of another vacuole-deficient mutant, the *end1* mutant (25), revealed an inability to accumulate arginine and polyphosphate (183). This lack of nitrogen and phosphate reserves results in decreased growth rates during starvation conditions compared with a wild-type strain. The gene complementing the *slp1* mutation was recently cloned and sequenced (175). The deduced amino acid sequence predicts a protein of 691 residues with an estimated molecular mass of approximately 79 kDa, and it does not appear to have any transmembrane domains. *SLP1* is a nonessential gene, but loss of *SLP1* function causes a two- to threefold decrease in the growth rate. The morphological defect exhibited by the *slp1* mutant is similar to the defect seen in certain *vps* mutants (8). The class C mutants isolated by Robinson et al. (143) also lack a single large vacuole and contain numerous vesicular structures (see Mutants Defective in Vacuolar Protein Sorting; *vpt* Mutants). Comparison of the nucleotide sequence of *SLP1* with that of cloned *VPS* genes reveals that *SLP1* and *VPS33* are identical (175; L. Banta and S. Emr, unpublished results). This explains the observation that *slp1* mutants accumulate precursor forms of vacuolar proteases (78). Interestingly, *SLP1* had previously been identified as being allelic to *VAM5*, a vacuole morphology mutant (77). In addition, *vam5* is allelic to *cls14*, a calcium-sensitive mutant (127, 177). Similarly, the vacuole-deficient *end1* mutant described above is allelic to *vps11* (143), *vam1* (177), and *cls13* (127, 177). The same genes have been identified by screening for defects in protein sorting, amino acid and Ca^{2+} storage and homeostasis, and vacuole morphology. This allows us to pose a question which is a general one when dealing with vacuolar mutants and arises because of the many overlapping functions carried out by the vacuole. What is the nature of the primary defect; and does this mutation affect vacuole morphology, resulting in decreased storage capability and vacuolar protein missorting owing to the absence of a proper target, or is it a sorting defect which prevents normal vacuole formation because of the missorting of a protein critical in morphogenesis? An answer to this question may be provided by a detailed analysis of strains with mutations that affect vacuole morphology. Certain alleles of the *vps33* gene, for example, lead to the absence of a normal vacuole (class C; see Mutants Defective in Vacuolar Protein Sorting), whereas other *vps33* alleles contain morphologically normal vacuoles. Vacuolar hydrolases, however, are still missorted by each of the *vps33* mutants (Banta and Emr, unpublished). This observation suggests that, at least in this case, the primary defect is due to missorting.

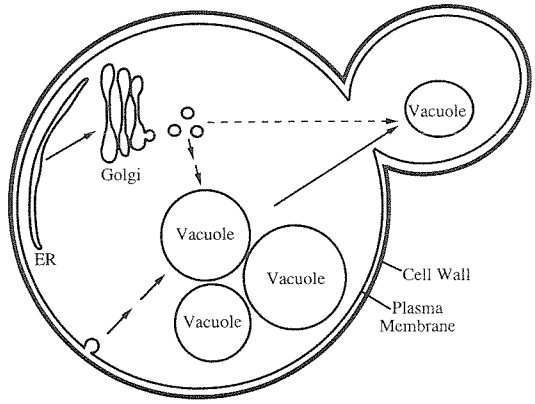

FIG. 6. Biogenesis of the fungal vacuole. The daughter cell or bud inherits a substantial portion of its vacuolar contents from the mother cell. The role of the Golgi complex and endocytosis in the development and maintenance of the vacuolar structure is discussed in the text.

VACUOLE BIOGENESIS

Studies of the vacuolar compartment in the budding yeast *S. cerevisiae* have provided some insight into the mechanisms regulating the assembly and inheritance of this organelle (Fig. 6). The wild-type vacuolar compartment is a very dynamic structure, which is capable of undergoing rapid changes in its morphological appearance. Studies of the biogenesis of this organelle have been complicated by observations which suggest that vacuolar structure is influenced by the preparative techniques used during the analysis (138). The yeast cell vacuole is easily detected at a very early stage in the growth cycle. When the daughter bud is only a fraction of the size of the mother cell, a vacuole is already present within the bud (179). Experiments with stable fluorophore labeling of yeast vacuoles suggest that the daughter cell inherits a substantial portion of its vacuolar contents from the mother cell (179, 181). Such vacuolar inheritance had been suggested by the pronounced phenotypic lag observed in the expression of the *Pep4* mutant phenotype in newly sporulated cells (199). However, the cellular mechanisms responsible for faithful vacuole partitioning during cell division are poorly understood. Early experiments with synchronized yeast cultures had indicated that the vacuole undergoes a cyclic pattern of fragmentation and coalescence during the yeast cell cycle (154, 188). Upon bud emergence, a rapid transition from large to multiple, small vacuoles was observed. It was suggested that the small vacuolar structures were then distributed between the mother and daughter cells. However, a more recent study with asynchronous cultures has suggested a very different mechanism of partitioning (179). In this study, the morphology of the vacuolar compartment was observed to be relatively constant

throughout the cell cycle, and most mother and daughter cells possessed a single vacuole. It was proposed that in the absence of vacuole fragmentation, traffic between the mother and daughter cell vacuoles, mediated by either vesicles or tubular connections, is responsible for the observed partitioning of the vacuolar contents (179). The isolation of a *vac1* yeast mutant, which may be defective in this vacuolar partitioning process, has recently been reported (180). It seems unlikely that the cyclic vacuole fragmentation pattern observed in the synchronous-culture experiments was an artifact of the synchronization procedure used, as each study used a different technique to achieve synchrony (154, 179, 188). It is interesting that a fragmented vacuole morphology is observed in several of the *vps* mutants (8, 144) and in yeast cells which have been treated with microtubule-disrupting agents (49). However, it is not known whether the fragmented vacuoles observed under these conditions represent true physiological intermediates in vacuole biogenesis. Although the precise mechanisms of vacuolar segregation are not yet understood, these studies clearly demonstrate that the mother cell vacuole contributes significantly to the vacuolar contents of the newly forming bud.

The vacuolar compartment continues to grow in volume following its initial appearance in the newly emerging bud. The intracellular mechanisms responsible for this observed growth are not well understood. An analysis of yeast mutants defective in the localization of vacuolar proteins has identified mutants which also appear to be defective in vacuole assembly (see Mutants Defective in Vacuolar Protein Sorting). These observations suggest that the biosynthetic pathway delivering vacuolar proteins from the Golgi complex may be contributing to the growth and/or maintenance of the vacuolar structure. Endocytosis may also participate in this process, as is seen in mammalian cells in which endocytic traffic is routed to the lysosomal compartment via specific endosomal intermediates (107). Endocytosis in yeast cells has been examined through an analysis of the internalization of enveloped viruses (99), α-amylase (100), lucifer yellow CH (25, 141), and α-factor (25, 61) by whole cells or spheroplasts. Fluorescein isothiocyanate-dextran has also been used as an endocytic marker, but conflicting reports have appeared regarding the ability of yeast cells to endocytose this macromolecule (32, 99, 136). Genetic and biochemical evidence from these studies indicates that this endocytic traffic contributes to vacuolar content. The dye lucifer yellow CH accumulates within the vacuolar compartment as a result of fluid-phase endocytosis (25, 141). The mating pheromone, α-factor, is bound by a specific cell surface receptor and is subsequently internalized and degraded (61, 141). This degradation of α-factor has been shown to be *PEP4* dependent, suggesting that this breakdown occurs within the lumen of the vacuole (36). Therefore, the growth of the vacuole may involve the coordinate regulation of at least these two different pathways of de novo biosynthetic traffic and endocytosis. An assessment of the contributions of either pathway would be greatly facilitated by the identification of mutants defective in only one component. Thus far, however, no yeast mutants specifically defective in endocytosis have been identified. The *end1* mutant (*end1* is allelic to *vps11*; see Mutants Defective in Vacuolar Protein Sorting), originally identified as being defective in the receptor-mediated endocytosis of α-factor (141), has subsequently been shown to be competent for the uptake of the α-factor pheromone but defective in its degradation (36). Finally, an analysis of clathrin-deficient yeast cells has indicated that the clathrin heavy chain is not essential either for α-factor uptake or for vacuolar protein delivery (135). Continued analysis of vacuolar protein sorting-defective mutants might provide some insight into the mechanisms regulating vacuole biogenesis (8, 144).

Several different genetic approaches have been successful in identifying mutants which may be defective in specific aspects of vacuole biogenesis (see Mutants Defective in Vacuolar Protein Sorting). A preliminary report has described the isolation of yeast mutants defective in vacuole assembly (77, 177). These vacuole morphology (*vam*) mutants were isolated by visually screening for cells possessing abnormal vacuolar structures (177). Many of the *vam* mutations are allelic to previously isolated vacuolar storage and protein-sorting mutations (175, 177; S. Emr, unpublished observations) (Table 2). Many mutants originally identified as being defective for specific vacuolar functions, such as Ca^{2+} (127) or lysine (78) storage and vacuolar protein sorting (8, 144), have subsequently been shown to possess an abnormal vacuolar compartment. These mutants may therefore define gene functions required for the assembly and/or maintenance of the wild-type vacuolar structure.

CONCLUSION

Perhaps the single most important point that we have tried to convey in this review is that the fungal vacuole is an extremely complex organelle that is involved in a wide variety of functions. The vacuole not only carries out degradative processes, the role most often ascribed to it, but also is the primary storage site for important metabolites such as basic amino acids and polyphosphate, plays a role in osmoregulation, and is involved in the precise homeostatic regulation of cytosolic ion concentration and pH. These many functions necessitate an intricate interaction between the vacuole and the rest of the cell; the vacuole is part of both the secretory and endocytic pathways and is also directly accessible from the cytosol. Although models have been proposed to describe general features of the vacuole and its protein constituents, it is probably not generally useful to think of the vacuole in terms of unifying themes or prototypical proteins. This point is illustrated by examining the diverse ways in which proteins arrive at the vacuole. The secretory pathway is the major route used for the delivery of most hydrolases. This mechanism of delivery is best illustrated by CPY. Even when dealing with delivery through the secretory pathway, however, distinctions must be made between proteins that are soluble and those that transit as membrane-bound forms, such as ALP and DPAP B. Although both of these classes of proteins most probably travel to the vacuole via vesicular carriers, this is probably not true for all of the vacuolar constituents. An interesting alternative may be seen with α-mannosidase, a lumenal protein that may be translocated directly across the vacuolar membrane. Other proteins which may not use the secretory pathway include the peripheral membrane subunits of the vacuolar ATPase. Since the integral membrane components of this enzyme are likely to utilize the secretory pathway, an additional complexity is added, as there must be some coordination between delivery and/or assembly of proteins that arrive at the vacuole through separate mechanisms. Similarly, the processing pathways are not as simple as first believed. CPY again provides the classic model of signal peptide removal at the ER followed by cleavage of an N-terminal propeptide upon, or just prior to, arrival in the vacuole. The classic model, however, may not be widely

applicable, even among proteins that undergo PrA-dependent maturation. API, for example, has an N-terminal propeptide but apparently lacks a hydrophobic signal sequence. ALP has an internal uncleaved signal sequence that causes it to remain membrane bound, and its propeptide is removed from the C terminus. PrB has an even more complicated processing scheme involving the cleavage of a large N-terminal segment in the ER followed by two successive proteolytic events later in the delivery process.

Other vacuolar functions besides those involving the hydrolases also show considerable variation. Although the vacuole functions as a storage compartment, this term is too simplistic. An examination of different metabolites reveals tremendous diversity in compartmentalization. The major cellular pool of arginine is kept metabolically inactive, since it is sequestered within the vacuole and is inaccessible to the cytosolic biosynthetic and catabolic enzymes. The reverse situation occurs for trehalose, with the substrate and the degradative enzyme being located in the cytosol and in the vacuole, respectively. Still another example is demonstrated by the storage of polyphosphate, which is localized to the vacuole along with at least some of the enzymes responsible for its degradation. The means by which entry or release of substrates and regulation of degradation are achieved are not well understood. Because of the various roles and properties of the vacuole, it has been possible to isolate mutants which are defective in various vacuolar functions including the storage and uptake of metabolites, regulation of pH, sorting and processing of vacuolar proteins, and vacuole biogenesis. Interestingly, these mutants show a remarkable degree of overlap, suggesting that these functions are not individual, discrete properties of the vacuole but, rather, are closely interrelated.

Many questions remain to be answered about the vacuole and its constituent proteins. The precise characterization of sorting signals used to target proteins to the vacuole has not been achieved. Identification of components of the sorting apparatus, including potential receptors, will rely on continued analyses of missorting mutants. At present, almost all of the data available on vacuolar metabolite transport systems concern their kinetic properties. The purification of vacuolar permeases, analyses of their biosynthesis, and elucidation of control mechanisms await future efforts. Although rapid progress has been made in understanding the vacuolar ATPase, basic questions still remain; these address the subunit composition and stoichiometry as well as more intriguing problems concerning assembly. The role of the vacuole in endocytosis remains largely undefined. Similarly, little is known about vacuolar biogenesis and inheritance. Continued work on the vacuole will further reveal the complex nature of this organelle and the ways in which it is integrally involved in a variety of cellular processes. Clearly, the vacuole will remain a rich and exciting area of research for many years to come. We can also expect that the insights gained from these studies of the fungal vacuole will influence our view of related processes in mammalian and plant cells, which are far less tractable to genetic and molecular dissection.

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Chapter 3:

Characterization of *VPS*34, a Gene Required for Vacuolar Protein Sorting and Vacuole Segregation in *Saccharomyces cerevisiae.*

Characterization of *VPS34*, a Gene Required for Vacuolar Protein Sorting and Vacuole Segregation in *Saccharomyces cerevisiae*†

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***VPS34* gene function is required for the efficient localization of a variety of vacuolar proteins. We have cloned and sequenced the wild-type *VPS34* gene in order to gain a better understanding of the role of its protein product in this intracellular sorting pathway. Interestingly, disruption of the *VPS34* locus resulted in a temperature-sensitive growth defect, indicating that the *VPS34* gene is essential for vegetative growth only at elevated growth temperatures. As with the original *vps34* alleles, *vps34* null mutants exhibited severe vacuolar protein sorting defects and possessed a morphologically normal vacuolar structure. The *VPS34* gene DNA sequence identifies an open reading frame that could encode a hydrophilic protein of 875 amino acids. The predicted protein sequence lacks any apparent signal sequence or membrane-spanning domains, suggesting that Vps34p does not enter the secretory pathway. Results from immunoprecipitation experiments with antiserum prepared against a TrpE-Vps34 fusion protein were consistent with this prediction: a rare, unglycosylated protein of ~95,000 Da was detected in extracts of wild-type *Saccharomyces cerevisiae* cells. Cell fractionation studies indicated that a significant portion of the Vps34p is found associated with a particulate fraction of yeast cells. This particulate Vps34p was readily solubilized by treatment with 2 M urea but not with Triton X-100, suggesting that the presence of Vps34p in this pelletable structure is mediated by protein-protein interactions. *vps34* mutant cells also exhibited a defect in the normal partitioning of the vacuolar compartment between mother and daughter cells during cell division. In more than 80% of the Δ*vps34* dividing cells examined, no vacuolar structures were observed in the newly emerging bud, whereas in wild-type dividing cells, more than 95% of the buds had a detectable vacuolar compartment. Our results suggest that the Vps34p may act as a component of a relatively large intracellular structure that functions to facilitate specific steps of the vacuolar protein delivery and inheritance pathways.**

In eucaryotic cells, the secretory pathway is responsible for the modification and delivery of proteins to a wide variety of intracellular and extracellular compartments. Entry into this pathway is mediated by the presence of an amino-terminal signal sequence and results in translocation across the endoplasmic reticulum (ER) membrane. Proteins destined for secretion are subsequently delivered from the ER to the Golgi complex and then from the Golgi complex to the cell surface via specific membrane-enclosed transport vesicles. This interorganellar flow of proteins from the ER to the cell surface appears to occur by a default mechanism; proteins that lack an intracellular "address" are passively carried to the cell surface (32). However, proteins resident within the ER, Golgi, or lysosomal compartment are characterized by the presence of specific retention or sorting signals which are responsible for their accurate subcellular localizations (22, 32). Therefore, mechanisms must exist to allow the cell to recognize and distinguish these various sorting signals and to ultimately deliver the marked proteins to their appropriate intracellular compartments.

Genetic and biochemical studies of protein secretion in the yeast *Saccharomyces cerevisiae* have demonstrated that many of the essential features of the secretory pathway have been conserved in all eucaryotes, from yeasts to mammals (28, 29). In particular, the delivery of proteins to the yeast cell surface also appears to proceed through the ER and Golgi compartments by a bulk flow, or default, mechanism. As with mammalian lysosomal enzymes, proteins destined for the yeast lysosome-like vacuole depend on the presence of additional sorting information (15, 19, 20, 52). Genetic studies indicate that vacuolar proteins transit through the early stages of the secretory pathway together with proteins destined for secretion or assembly into the plasma membrane (15, 48, 52). Within the Golgi compartment, the vacuolar proteins are segregated away from proteins destined for secretion and targeted to the yeast vacuole.

In an attempt to identify cellular components involved in the specific segregation, packaging, and delivery of proteins to the vacuole, we used a gene fusion-based selection scheme to isolate a large number of yeast mutants defective in vacuolar protein localization or processing (2, 36). More than 600 *vpt* (for vacuolar protein targeting defective) mutants have been isolated, and the recessive mutations have been assigned to at least 33 complementation groups. The *vpt* mutations have been demonstrated to affect the intracellular sorting of a variety of vacuolar proteins, including the soluble hydrolases carboxypeptidase Y (CPY), proteinase A (PrA), and proteinase B (PrB), and an integral membrane protein, repressible alkaline phosphatase (2, 20, 36). In addition to the observed sorting defects, analysis by both light and electron microscopy techniques has demonstrated that several of the *vpt* mutants possess morphologically abnormal vacuolar structures (3). These observations have allowed us to assign the *vpt* mutants to three distinct classes based on their vacuole morphology. The majority of mutants, class A, possess one to three large vacuoles per cell, similar to what is seen in wild-type cells. In other *vpt* complementation groups, the cells are characterized either by the presence of multiple, small vacuolelike organelles (class B mutants) or by the apparent lack of any normal

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† Dedicated to the memory of E. W. Herman.

TABLE 1. Strains used

Strain	Genotype	Reference or source
S. cerevisiae		
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	36
SEY6211	*MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9*	36
SEY34-2	SEY6210 *vps34-2*	36
SEY34-6	SEY6211 *vps34-6*	36
PHY102	SEY6210 *vps34Δ1::TRP1*	This study
PHY103	SEY6211 *vps34Δ1::TRP1*	This study
DKY6224	MATa, *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 Δpep4::LEU2*	19
SEY6210.5	*MATα/MATa leu2-3,112/leu2-3,112 ura3-52/ura3-52 his3-Δ200/his3-Δ200 trp1-Δ901/trp1-Δ901 suc2-Δ9/suc2-Δ9 ADE2/ade2-101 lys2-801/LYS2*	This study
PHY120	Same as SEY6210.5 except *VPS34/vps34Δ1::TRP1*	This study
E. coli		
MC1061	F^- *hsdR hsdM*$^+$ *araD139* (*araABOIC-leu*)*7679* (*lac*)X74 *galU galK rpsL*	5a
JM101	F′ *traD36 lacI*^{q} ΔM15 *proAB* Δ*lac*(*lac-pro*) *supE thi*	27

vacuolar structures (class C mutants). In addition to the *vpt* mutants, two other sets of mutants (*pep* and *vpl*) that affect vacuolar protein localization or processing have been described (16, 21, 38, 40). The *vpl* mutants were also identified by selecting for mutants defective in vacuolar protein localization (38, 40). Genetic analysis has demonstrated that significant overlap exists between the *VPT* and *VPL* complementation groups, and the *vpt* and *vpl* mutants are now collectively referred to as *vps*, for vacuolar protein sorting defective (36, 38). The *pep* mutants were originally identified in genetic screens for yeast mutants exhibiting decreased levels of CPY activity (16), and subsequent analysis has shown that most of the *pep* mutants also exhibit vacuolar protein sorting defects (38). In all, the mutants in these different collections define more than 47 complementation groups which appear to be required for the delivery of soluble vacuolar hydrolases such as CPY (21). These initial genetic studies, therefore, indicate that vacuolar protein delivery is a complex process that involves the direct or indirect participation of a relatively large number of gene functions. To functionally dissect this pathway and gain an understanding of the underlying molecular mechanisms, we have initiated efforts to clone specific *VPS* genes and to characterize their respective gene products.

Eight independent mutant alleles of the *VPS34* locus were originally isolated in a genetic selection for yeast vacuolar protein targeting mutants (36). *vps34* mutant cells were observed to exhibit severe defects in the sorting of the soluble vacuolar hydrolases, CPY, PrA, and PrB. However, light microscopy analyses revealed that *vps34* cells contain morphologically normal vacuoles (class A; 3). This latter observation indicated that *vps34* cells are competent for vacuole assembly; in combination, these observations suggested that the wild-type *VPS34* gene product might be specifically involved in the intracellular sorting and delivery of soluble vacuolar proteins. However, an electron microscopy analysis of *vps34* mutants has demonstrated that in addition to a morphologically normal vacuole, *vps34* cells accumulate a variety of abnormal membranous structures within their cytoplasm, including Berkeley bodies and 80-nm vesicles (3). Similar membrane-enclosed material was also observed in class C *vps* mutants, which lack a detectable normal vacuolar compartment. *vps34* cells have several other phenotypes in common with the class C *vps* mutants, including sensitivity to osmotic stress and extreme temperature-sensitive growth defects (3, 36). This phenotypic similarity to yeast mutants that are defective in the assembly of the vacuolar compartment might also suggest a role for the *VPS34* gene product in vacuole biogenesis.

Toward a better understanding of the role of the *VPS34* gene product in vacuolar protein sorting and biogenesis, we report here on the cloning and sequencing of the *VPS34* gene, the phenotypic consequences of a *VPS34* null allele (*Δvps34*), and the identification and localization of the *VPS34* gene product. Our results suggest that Vps34p is a relatively rare yeast polypeptide that may function as a component of a large intracellular multiprotein structure to facilitate vacuolar protein delivery.

MATERIALS AND METHODS

Strains and media. The *S. cerevisiae* and *Escherichia coli* strains used are listed in Table 1. The yeast strains were constructed by standard genetic techniques (46). Standard yeast (46) and *E. coli* (27) media were used and supplemented as needed.

Reagents. DNA restriction and modifying enzymes were from either New England BioLabs, Inc. (Beverley, Mass.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Zymolyase-100T (Kirin Brewery Co.) was obtained from Seikagako Kogyo Co. (Tokyo, Japan), lyticase was from Enzogenetics, and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal), phenylmethanesulfonyl fluoride, and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Boehringer Mannheim Biochemicals. Tran ^{35}S label was from ICN Radiochemicals (Irvine, Calif.), [α-^{32}P]CTP and [α-^{35}S]dATP were from Amersham (Arlington Heights, Ill.), Autofluor was from Dupont, NEN Research Products (Boston, Mass.), 5(6)-carboxy-2′-7′-dichlorofluorescein diacetate (CDCFDA) was from Molecular Probes, Inc. (Eugene, Ore.), and RNasin was from Promega Biotec (Madison, Wis.). Sequenase enzyme and the Sequenase DNA sequencing kit were from United States Biochemicals (Cleveland, Ohio), the T3/T7 RNA transcription kit was from Stratagene (La Jolla, Calif.), and the polymerase chain reaction (PCR) Gene Amp DNA amplification kit was from Perkin Elmer Cetus (Norwalk, Conn.). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Antiserum to phosphoglycerokinase was a gift from Jeremy Thorner, and the antiserum to CPY was described previously (19).

Recombinant DNA methods and plasmid constructions. All

recombinant DNA manipulations were done as previously described (25).

The yeast *E. coli* shuttle vector, pPHYC18, was constructed from pSEYC68 in two steps. pSEYC68 is a derivative of plasmid pSEYC58 (11) in which the pUC8 polylinker sequences have been replaced with those of pUC18. First, pSEYC68 DNA was digested with *Xho*I and *Hpa*I, and the *Xho*I 3′-recessed ends were filled in with Klenow polymerase. These two blunt ends were subsequently ligated to remove ~600 bp from the *CEN4* region of pSEYC68. This deletion removed the only *Kpn*I site in pSEYC68 and did not affect the mitotic stability of the resultant yeast plasmid, pPHYC16 (data not shown; see reference 26). The polylinker sequences of pPHYC16 were then replaced with the polylinker from pBluescript KS(+) (pBP; Stratagene) by replacing the ~400-bp *Pvu*II fragment of pPHYC16 with that from the pBP plasmid to yield pPHYC18. The ~5.0-kb *Cla*I-*Sac*I fragment of pPHY34 was subcloned into pBP (KS+) to yield plasmid pPHY46. This *VPS34*-containing fragment was then removed as a *Sal*I-*Sac*I fragment and subcloned into pSEY18 (a derivative of pSEY8 with pUC18 polylinker sequences; 11) to make pPHY52, a multicopy *VPS34* plasmid.

The yeast integrating vector, pPHYI10, was constructed by inserting the ~800-bp *Eco*RI-*Pst*I fragment of YRp7, which contains the yeast *TRP1* gene (51), into the *Nde*I site of pUC18. All restriction enzyme ends were filled in or digested with Klenow polymerase to create blunt ends. This plasmid contains a yeast selectable marker, *TRP1*, but no yeast origin of replication.

Plasmid pPHY40 was used to construct exonuclease III-mung bean nuclease deletions of *VPS34* sequences in an attempt to define the minimum complementing fragment and was constructed as follows. The original YCp50 complementing plasmid, pPHY34, was digested with *Kpn*I, and the 3′ overhang ends were removed by treatment with mung bean nuclease. The plasmid was then digested with *Cla*I, and this 4.1-kb *ClaI-Kpn*I (blunt) fragment was subcloned into *Cla*I-*Sma*I-digested pPHYC18 to yield pPHY40. Plasmids pPHY42 and pPHY43 were constructed by subcloning the *VPS34* locus, as a ~4.1-kb *Kpn*I-*Sac*I fragment from pPHY40, into pBP (−) and pBP (+), respectively (both pBP plasmids were of the KS series).

The integrative mapping plasmid, pPHY35, was constructed by cloning the 3.4-kb *Bam*HI-*Sac*I fragment of pPHY34 into the pPHYI10 vector. Plasmid pPHY38 was used to make a gene disruption of the *VPS34* locus and was constructed in multiple steps, as follows. First, the 4.1-kb *Cla*I-*Kpn*I fragment of pPHY34 was subcloned into the pPHYC18 yeast vector. This *VPS34*-containing DNA fragment was subsequently removed as a *Pst*I-*Kpn*I fragment and subcloned into pUC18, to construct pPHY36. pPHY36 was digested with *Xho*I, the *Xho*I 3′-recessed ends were filled in, and the plasmid was then cut with *Bam*HI. The yeast *TRP1* gene was subcloned into this plasmid as an *Eco*RI (blunt)-*Bgl*II fragment from YRp7, where the *Eco*RI 3′-recessed ends were filled in with Klenow polymerase. Plasmid pPHY34.17 was made by cloning the 1.0-kb *Bam*HI-*Xho*I fragment of pPHY34 into pPHYC18.

Northern (RNA) analysis. Yeast RNA was prepared as previously described (10). Poly(A)$^+$ RNA was isolated by binding total yeast to oligo(dT)-cellulose in the presence of 0.5 M LiCl, extensive washing with the high-salt buffer, and then batch elution with 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA. The RNA was subsequently electrophoresed on formaldehyde–1% agarose gels and transferred to GeneScreen membranes (1). Single-stranded [^{32}P]RNA probes were prepared by transcription from pPHY34.17 with either T3 or T7 RNA polymerase and were hybridized to the RNA blot as described previously (1).

Yeast genetics. Standard yeast genetics techniques were used throughout (46). Yeast transformation was achieved by the method of alkali cation treatment (14), and transformants were selected on SD medium.

The *VPS34* gene was cloned by complementing the severe temperature-sensitive growth defect associated with the *vps34-2* allele. SEY34-2 cells (*vps34-2 ura3-52*) were transformed with a yeast genomic DNA library constructed in plasmid YCp50 (37), and Ura$^+$ transformants were selected at 26°C. Transformant colonies were subsequently replicated to 37°C YPD plates, and plasmids conferring a temperature-resistant phenotype upon the cells were isolated and analyzed. Plasmid DNA was isolated from yeast cells by resuspension of a moderately large colony in 0.2 ml of 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA and extraction two times with a 50:50 phenol-chloroform mixture. The DNA was precipitated from the aqueous phase and used to transform competent *E. coli* cells to ampicillin resistance.

To construct a gene disruption of the *VPS34* locus, the 2.7-kb *Hind*III-*Kpn*I fragment of pPHY38 was gel isolated (from a partial digestion) and used to transform the diploid yeast strain, SEY6210.5, and the haploid strains, SEY6210 and SEY6211, to tryptophan prototrophy. For integrative mapping, plasmid pPHY35 was digested with *Xho*I and transformed into SEY6211. Trp$^+$ transformants were selected and subsequently crossed to the yeast strain SEY34-2 for genetic analysis.

PCR analysis of yeast genomic DNA. Approximately 0.2 OD_{600} (optical density at 600 nm) equivalents of a yeast culture were pelleted and resuspended in 200 μl of H_2O. Glass beads (0.55 mm) were added, and the cells were broken by vigorous vortexing for 60 s. The lysates were centrifuged for 2 min at 13,000 × *g* to remove cell debris, and 10 μl of this cleared lysate was used as the genomic DNA template. The PCR reactions were performed as described in the Perkin Elmer Cetus Gene Amp DNA amplification kit. The nucleotide sequences of the primers are as follows: 1, ATAACATCTCCGTGAAGCATTGAGG; 2, TACGTGAT TAAGCACACAAAGGCAG; and 3, TTTAATGTCCGGC TTCACTTGCTTG (5′ to 3′). Before addition of the *Taq*I polymerase, the reaction mixes were heated to 94°C for 10 min. *Taq*I enzyme was added, and 30 cycles of amplification were carried out, with the typical cycle consisting of 2 min at 55°C (annealing), 3 min at 72°C (extension), and 1 min at 94°C (denaturation). The reaction products were analyzed on 1.2% agarose gels stained with ethidium bromide. Since these PCR primers are contained within the transforming DNA fragment, we analyzed the genomic DNA from haploid progeny of independent PHY120 meioses to ensure that integration had occurred at the *VPS34* locus. We have also disrupted the *VPS34* gene with the 2.1-kb *Alw*NI fragment of pPHY38 (primer 1 is upstream of the 5′-most *Alw*NI site) and have obtained identical results in the subsequent PCR analysis.

Preparation of antisera against Vps34p. A gene fusion between *E. coli trpE* gene and *VPS34* was constructed by using the pATH vector system (8). We subcloned the 1.0-kb *Xba*I-*Xho*I fragment of pPHY34 into *Xba*I-*Sal*I-digested pATH2 DNA to produce plasmid pPHY51.1, which encodes a TrpE-Vps34p fusion protein containing 338 amino acids of Vps34p. This TrpE fusion protein was induced and prepared as previously described (18) except that 2% Triton X-100

was used instead of 0.2% Nonidet P-40. Protein induction was examined by analyzing whole-cell lysates on sodium dodecyl sulfate (SDS)-polyacrylamide gels stained with Coomassie blue. A large-scale preparation of the fusion protein was electrophoresed and eluted from a 9% preparative SDS-polyacrylamide gel. The protein eluant was mixed with Freund adjuvant and injected into New Zealand White male rabbits (~150 μg per rabbit). Antiserum was collected after multiple secondary injections.

DNA sequencing and sequence analysis. Exonuclease III-mung bean nuclease deletions were performed on plasmids pPHY42 and pPHY43 as described in the Stratagene Bluescript manual except that following nuclease digestion the treated DNA was eluted from a 1% agarose gel. Single-stranded phagemid DNA was purified following M13 superinfection, and the single-stranded DNA templates were sequenced by using standard dideoxy-chain termination techniques (44).

The predicted protein sequence of Vps34p was compared with the contents of the National Biomedical Research Foundation (NBRF) protein data base (release 21.0, June 1989) with the FASTA program and the contents of the GenBank data base (release 60.0, June 1989) with the TFASTA program (31). The comparisons were performed with the University of Wisconsin Genetics Computer Group sequence analysis package for VAX/VMS computers (6).

Cell labeling and immunoprecipitation. Immunoprecipitations from whole cells were performed as previously described (19), with the following modifications. Yeast cells were grown to mid-logarithmic phase in yeast nitrogen base (YNB) minimal medium supplemented with the appropriate amino acids. Two units of cells at an OD_{600} of 1.0 was centrifuged and resuspended in 0.5 ml of the same medium. Bovine serum albumin (final concentration, 1 mg/ml) and 150 μCi of Tran ^{35}S label were added to this culture, and the cells were incubated for 20 to 30 min at the appropriate temperature. A chase, if necessary, was initiated by adding cold methionine to a final concentration of 2 mM. The labeling or chase reaction was terminated by the addition of trichloroacetic acid (TCA) to a final concentration of 5%. The remaining steps were as described (19) except that only two immunoprecipitation washes were performed, one with IP buffer (0.5% Tween 20, 50 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 0.1 mM EDTA) and one with IP buffer 2 (50 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 0.1 mM EDTA). The samples were electrophoresed on 8% SDS-polyacrylamide gels. To assess N-linked oligosaccharide modification, the cells were incubated in the presence of tunicamycin (20 μg/ml) for 15 min prior to labeling. The CPY fractionation immunoprecipitations were done as previously described (36).

Fractionation of Vps34p. Strain DKY6224 (*Δpep4*) harboring plasmid pPHY52 was grown at 30°C to an OD_{600} of ~0.7 in YNB-glucose minimal medium. Cells were pelleted and incubated with 100 mM Tris-sulfate (pH 9.4)–10 mM dithiothreitol for 15 min at 30°C. The cells were pelleted and resuspended in YNB-glucose medium (pH 7.5) supplemented with 1.3 M sorbitol. Oxylyticase was added to 40 U per OD_{600} equivalent, and the cells were spheroplasted for 20 min at 30°C. The spheroplasts were pelleted and labeled in YNB–glucose–1.3 M sorbitol medium with Trans ^{35}S label (300 μCi/ml) for 30 min at 30°C. NaF and NaN_3 were added to 10 mM, and the cultures were immediately put on ice. The labeled spheroplasts were gently pelleted at 2,000 × *g* and then resuspended in 1.2 M sorbitol–100 mM Tris hydrochloride (pH 7.5)–10 mM EDTA containing phenylmethylsulfonyl fluoride (1 mg/ml), leupeptin (50 μg/ml), and pepstatin A (14 μg/ml). The cells were osmotically lysed by the rapid addition of 10 volumes of lysis buffer (0.25 M sucrose, 100 mM Tris hydrochloride [pH 7.5], 10 mM EDTA, 1 mg of bovine serum albumin per ml; 10). The unlysed cells were removed by centrifugation at 500 × *g* for 2 min. The resulting supernatant was separated into four equal aliquots, and 0.2 volume of one of the following was added: distilled water, 5% Triton X-100, 10 M urea, or 5 M NaCl. The lysate solutions were incubated at 0°C for 10 min and then centrifuged at 100,000 × *g* for 30 min in a 70.1 Ti rotor (Beckman Instruments, Inc.). The supernatant was carefully removed and made 6% in TCA, and the centrifugation pellet was resuspended in 6% TCA. Both TCA precipitations were held on ice for 20 min and then centrifuged at 13,000 × *g* for 2 min. The TCA pellets were washed twice with acetone, dried, and resuspended in 150 μl of boiling buffer (50 mM Tris hydrochloride [pH 7.5], 1 mM EDTA, 1% SDS). The samples were then immunoprecipitated as described above.

Labeling of cells with fluorescent dyes. Yeast cells were labeled with fluorescein isothiocyanate (FITC) and CDCFDA as previously described (3, 34) except that when FITC was used, the cells were washed with solutions containing 2% glucose. The *ade2* endogenous fluorophore was visualized in stationary-phase yeast cells grown either in YPD medium or in adenine-limiting medium, as previously described (53). Microscopy and photography were done as previously described (3).

Nucleotide sequence accession number. The GenBank accession number for the sequence reported is X53531.

RESULTS

Cloning and characterization of the *VPS34* locus. The *VPS34* gene was cloned by complementation of the recessive temperature-sensitive growth defect associated with the *vps34-2* allele. SEY34-2 cells (*vps34-2 ura3-52*; Table 1) were transformed with a yeast genomic library constructed in the YCp50 centromere-containing plasmid (37). Approximately 20,000 Ura^+ transformants were selected at 26°C on minimal plates lacking uracil. After 3 days at 26°C, the transformants were replicated onto prewarmed YPD plates and incubated at 37°C. Temperature-resistant colonies were picked, and the plasmid DNA was isolated. After amplification in *E. coli*, the plasmids were reintroduced into SEY34-2 to retest their complementing activity. Only one plasmid, pPHY34, was able to confer a temperature-resistant phenotype upon the cells (Fig. 1). This plasmid also complements the temperature-sensitive defect associated with the *vps34-6* allele (data not shown).

We also tested the ability of pPHY34 to complement the other phenotypes associated with *vps34* mutants. *vps34* cells have previously been shown to be sensitive to osmotic stress (3), as demonstrated by their inability to grow on solid media containing 1.5 M NaCl (Fig. 1). The cloned DNA was able to fully complement the osmotic sensitivity phenotype of *vps34* mutants (Fig. 1).

The cloned DNA was also able to complement the vacuolar protein sorting defects associated with *vps34* mutations. We directly analyzed the localization of CPY in yeast cells by labeling intact spheroplasts with [^{35}S]methionine and [^{35}S]cysteine, fractionating the cultures into supernatant (extracellular) and pellet (intracellular) fractions, and then immunoprecipitating the cultures with antiserum to CPY. The spheroplasts were labeled for 20 min and then chased for an additional 30 min. In wild-type yeast cells, >95% of the

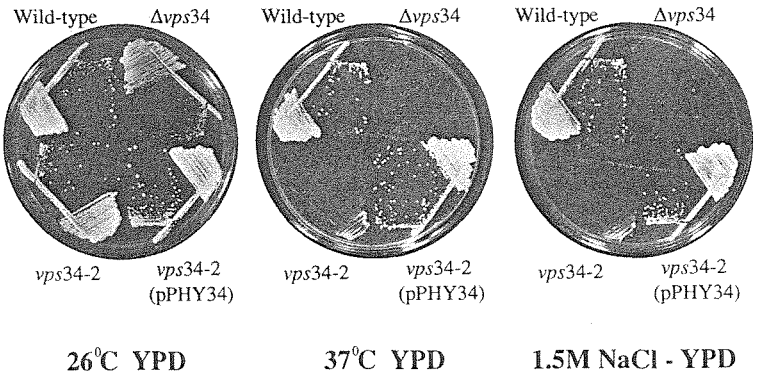

FIG. 1. Ability of plasmid pPHY34 to fully complement the growth defects associated with the *vps34-2* allele. The following strains were streaked out onto either 26°C YPD, 37°C YPD, or 26°C YPD–1.5 M NaCl plates and incubated for 2 to 6 days: SEY6210 (wild type), PHY102 (Δ*vps34*), SEY34-2 (*vps34-2*), and SEY34-2 harboring plasmid pPHY34 [*vps34-2*(pPHY34)].

newly synthesized CPY was present as a 61-kDa mature species in an intracellular fraction (Fig. 2). In contrast, in *vps34-2* cells, <5% of the CPY was present as a mature species. The majority of the CPY was present as the Golgi-modified 69-kDa (p2) precursor molecule, and more than 90% of this p2 CPY was secreted by the mutant cells (similar to results for the Δ*vps34* mutant in Fig. 2). Therefore, *vps34-2* mutants exhibited severe defects in the localization of CPY. The introduction of plasmid pPHY34 into *vps34-2* mutants corrected this sorting defect (data not shown; see Fig. 2). The genomic DNA present within plasmid pPHY34 was therefore capable of complementing all *vps34* mutant phenotypes examined.

Restriction enzyme mapping demonstrated that this complementing plasmid, pPHY34, contained a genomic DNA insert of ~8 kb (Fig. 3A). Various restriction fragments were subcloned into the yeast single-copy vector, pPHYC18, and were subsequently tested for complementing activity (Fig. 3A). This analysis localized the complementing activity to a 4.1-kb *Cla*I-*Kpn*I fragment. To more precisely define the limits of the *VPS34* functional unit, we performed exonuclease III-mung bean nuclease deletions upon this *Cla*I-*Kpn*I

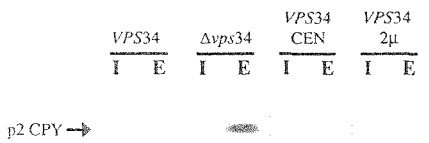

FIG. 2. Intracellular sorting of CPY. Yeast spheroplasts were radiolabeled with Tran ^{35}S label for 20 min at 30°C and then chased for 30 min at 30°C following the addition of cold methionine and cysteine to 2 mM. The labeled cultures were centrifuged for 2 min at 13,000 × *g* and separated into a pellet (I, intracellular) and a supernatant (E, external) fraction. The level of CPY in each fraction was assessed by quantitative immunoprecipitation with antiserum to CPY. The strains examined were SEY6210 (*VPS34*), PHY102 (Δ*vps34*), PHY102 harboring the pPHY34 plasmid (*VPS34* CEN), and SEY6210 harboring the multicopy *VPS34* plasmid pPHY52 (*VPS34* 2μ). The positions of mature CPY (mCPY; 61 kDa) and p2 CPY (69 kDa) are indicated.

fragment. Deletions of as little as 200 bp from the *Kpn*I side resulted in a loss of complementing activity. However, we found that as much as 1,200 bp could be removed from the *Cla*I side without any detectable loss of function. The final fragment shown in Fig. 3A represents the smallest DNA fragment found to possess *VPS34* complementing activity.

To determine whether the pPHY34 complementing activity represented the authentic *VPS34* locus, we assessed the genetic linkage between the cloned genomic DNA and the *vps34-2* allele. The 3.4-kb *Bam*HI-*Sac*I fragment of pPHY34 was subcloned into a *TRP1* yeast integrating plasmid, pPHYI10. The resultant plasmid, pPHY35, was digested with *Xho*I to direct its integration to the chromosomal homolog of the cloned DNA (41). This digested DNA was transformed into the yeast strain SEY6211, and Trp$^+$ transformants were selected and mated to SEY34-2. The resultant diploid was induced to sporulate, and the meiotic progeny were analyzed by standard tetrad analysis. If the cloned DNA represented the true *VPS34* locus, then the Trp$^+$ and Ts$^+$ phenotypes would be expected to cosegregate. In all 20 tetrads analyzed, a 2 Trp$^+$ Ts$^+$:2 Trp$^-$ Ts$^-$ segregation pattern was observed. These results indicate that the complementing DNA within pPHY34 originated from a region of the yeast genome that is homologous to the *VPS34* gene.

RNA transcripts from the *VPS34* locus were identified by probing Northern blots of yeast RNA with single-stranded RNA probes prepared from either strand of the *VPS34* clone. A single poly(A)$^+$ RNA of approximately 2,800 nucleotides was detected from this locus (Fig. 3B). The direction of transcription was determined to be from the *Bam*HI site toward the *Xho*I site (Fig. 3).

DNA sequencing and sequence analysis. The *VPS34* minimum complementing fragment (Fig. 3A) was sequenced by using standard dideoxy-chain termination methods as described in Materials and Methods. The DNA sequence identifies an open reading frame of 2,625 bp which has the potential to encode a protein of 875 amino acids (Fig. 4A). Upstream of the initiation codon, at position −120, there is a TATA-like sequence element, TATAT, which closely resembles the consensus TATAAA yeast sequence (Fig. 4A; 49). A sequence, TAGT. . .TAG. . .TTT, which closely approximates the proposed yeast transcription termination consensus signal, was identified 86 nucleotides downstream

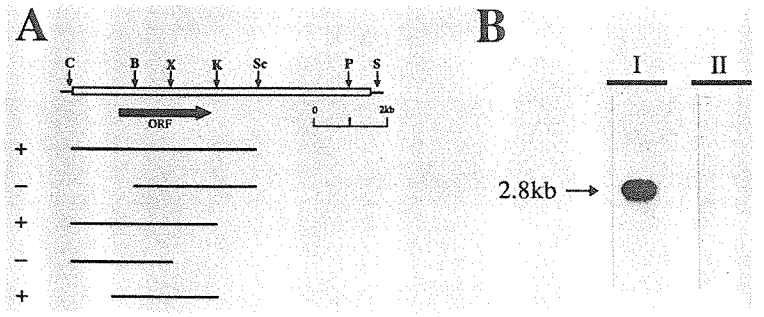

FIG. 3. (A) Restriction enzyme map of the ~8-kb genomic DNA insert in plasmid pPHY34. The *VPS34* coding region and direction of transcription are indicated by the heavy arrow. Restriction enzyme abbreviations: *Cla*I (C), *Bam*HI (B), *Xho*I (X), *Kpn*I (K), *Sac*I (Sc), *Pvu*II (P), and *Sal*I (S). Specific restriction fragments or exonuclease III-mung bean deletions were subcloned into pPHYC18 and transformed into SEY34-2. A plus sign indicates that the fragment was able to complement the *vps34-2* temperature-sensitive growth defect. ORF, open reading frame. (B) Identification of the *VPS34* RNA transcript by Northern blot analysis. Poly(A)$^+$ RNA (5 μg) was run out on formaldehyde–1% agarose gels, transferred to GeneScreen membranes, and probed with antisense (I) or sense (II) *VPS34* [^{34}P]RNA made with either T7 or T3 RNA polymerase from plasmid pPHY34.17.

from the stop codon (Fig. 4A; 56). The predicted open reading frame is terminated by a single UGA codon, and its size is in good agreement with that of the single 2.8-kb poly(A)$^+$ RNA detected from this region. The deduced protein sequence indicates that Vps34p is relatively hydrophilic and possesses 10 potential sites for N-linked glycosyl modification. A hydropathy analysis of this sequence suggests the absence of an N-terminal signal sequence or any potential membrane-spanning domains within this protein (Fig. 4B). A comparison of the predicted protein sequence with those in the GenBank and NBRF data bases failed to reveal any sequence similarities of obvious significance (24, 31).

Deletion-disruption of the *VPS34* gene results in a temperature-sensitive growth defect. To examine the phenotypic consequences of a null allele of *VPS34*, we constructed a gene deletion-disruption of this locus by using plasmid pPHY38. This plasmid contains a copy of the *VPS34* gene in which the 1.0-kb *Bam*HI-*Xho*I fragment has been replaced with the yeast *TRP1* gene (Fig. 5A). Following linearization, this DNA was used to transform the diploid strain SEY6210.5 to tryptophan prototrophy by replacing one wild-type copy of *VPS34* with the disrupted allele through homologous recombination. Two independent Trp$^+$ transformants were placed on sporulation medium, and their resultant progeny were subjected to tetrad analysis. In all 19 tetrads examined, the Trp phenotype was observed to segregate 2 Trp$^+$:2 Trp$^-$, as expected for a single replacement event. As this result implies, all four haploid progeny, including the disrupted (or Trp$^+$) haploid strains, were viable at 26°C, and therefore *VPS34* is not an essential yeast locus. Because we had previously isolated recessive temperature-sensitive alleles of the *VPS34* locus (36), we examined the growth of the aforementioned meiotic progeny at 37°C. We found that all tetrads displayed a 2 Trp$^+$ Ts$^-$:2 Trp$^-$ Ts$^+$ segregation pattern, indicating that our gene disruption of the *VPS34* locus results in a temperature-sensitive growth defect. This finding implies that the *VPS34* gene product is essential for growth only at elevated growth temperatures. The construction of a *VPS34* gene disruption in the haploid yeast strains SEY6210 and SEY6211 also resulted in a temperature-sensitive growth defect (Fig. 1). The Δ*vps34* strains were observed to arrest at the one- to two-cell stage following a shift to 37°C. Δ*vps34* cells that had been arrested for 3 h at 37°C did not assume a uniform arrest morphology (data not shown). At 26°C, the generation time of Δ*vps34* mutants is approximately 1.5 to 2 times that of an isogenic wild-type strain.

We verified the genomic structure of the *vps34Δ1::TRP1* alleles with an application of PCR DNA amplification methods (42, 43). Yeast cells were lysed with glass beads and centrifuged for 2 min at 13,000 × *g*. An aliquot of this clarified lysate corresponding to 0.01 OD_{600} cell equivalents was used for the PCR analysis. Each DNA template was mixed with a set of three 25-mer oligonucleotide primers that hybridize to specific regions of either the *VPS34* gene or the *TRP1* locus (Fig. 5A). The primers were chosen such that the wild-type *VPS34* gene would produce a single PCR product of 900 bp from primers 1 and 2 and the *vps34Δ1::TRP1* allele would result in a single 1,100-bp PCR product from primers 1 and 3 (Fig. 5A). When SEY6210 lysates, containing the wild-type *VPS34* gene, were used as the template, the predicted 900-bp PCR product was observed (Fig. 5B). The heterozygous diploid, PHY120 (*VPS34/vps34Δ1::TRP1*), produced the expected doublet of 900 and 1,100 bp (Fig. 5B). Genomic DNA from four haploid progeny of a single PHY120 meiosis were also analyzed, and the results obtained are consistent with the genetic analysis of these strains (Fig. 5B). Further confirmation of the identity of the *VPS34* allele(s) present was obtained by restriction of the PCR products with either *Bam*HI or *Hin*dIII (Fig. 5A). The restriction enzyme *Bam*HI specifically cut the 900-bp PCR product of the wild-type gene into 600- and 300-bp fragments and did not digest the products from the disruption allele (data not shown). Conversely, *Hin*dIII restriction resulted in the production of a 600- and a 500-bp fragment from the 1,100-bp disruption allele product without affecting the 900-bp fragment (data not shown). Therefore, this PCR technique allows for a rapid (less than 6 h), nonisotopic, and precise analysis of the genomic DNA at the *VPS34* locus.

As expected, the Δ*vps34* cells exhibited an extreme vacuolar protein sorting defect, mislocalizing >95% of its CPY to the cell surface in a p2 precursor form (Fig. 2). The presence of the wild-type *VPS34* gene on a CEN-containing plasmid completely corrected this sorting defect (Fig. 2). As with the original temperature-sensitive alleles (36), the CPY

A

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                                                                                                                                1   AAA  AGGTATTCTT      13
  14  TGAGTGAATT  GTAGATGTCT  GAGCGTTTGC  GCTGAGGGTT  TTATAGGATG  TGTCATTTCC  TTGAGAGCAG  AGGCATAAGT  GCTAAAGCAT  TGTCGCCTAC  AGACATTGAA  GTTTGTGCAT  133
 134  ACGGACTGGC  GAATACACCT  CGACATGGGG  CTGAAGCTTT  CGACAAATCT  TAAATTGTGA  CTTTGTGCAT  GAAATTTTTA  TATGAACGTT  TTAAATAAGT  GGACATAGTC  CTTCATTCGG  253
 254  TATATGCAGA  TCCGGCATCA  AACACTTTTT  CAAGAAAGTT  TGAGTCAAAA  GGATTAAAGG  ATAAAGTAAA  ACAACAATAA  CATCTCCGTG  AAGCATTGAG  GGAAGGGTTT  AACTCCAACA  373
      *****

 374  ATG TCA CTG AAC AAC ATA ACA TTC TGT GTC TCA CAG GAT CTG GAT GTT CCC CTG AAA GTG AAA ATC AAG TCA TTG GAA GGA CAT AAG CCA CTG TTG AAG CCA TCT CAA  481
   1  Met Ser Leu Asn Asn Ile Thr Phe Cys Val Ser Gln Asp Leu Asp Val Pro Leu Lys Val Lys Ile Lys Ser Leu Glu Gly His Lys Pro Leu Leu Lys Pro Ser Gln   36

 482  AAA ATC CTG AAC CCT GAA TTA ATG CTG ATA GGG TCA AAT GTA TTC CCT TCT AGT GAT CTA ATT GTA TCC TTA CAG GTA TTT GAT AAA GAG AGA AAC AGA AAT TTA ACT  589
                                                  XbaI
  37  Lys Ile Leu Asn Pro Glu Leu Met Leu Ile Gly Ser Asn Val Phe Pro Ser Ser Asp Leu Ile Val Ser Leu Gln Val Phe Asp Lys Glu Arg Asn Arg Asn Leu Thr   72

 590  CTT CCG ATA TAC ACG CCG TAT ATT CCT TTC AGA AAC TCT AGA ACC TGG GAT TAT TGG TTG ACT TTG CCC ATA CGT ATC AAA CAG CTT ACC TTT AGC AGT CAT TTA CGC  697
  73  Leu Pro Ile Tyr Thr Pro Tyr Ile Pro Phe Arg Asn Ser Arg Thr Trp Asp Tyr Trp Leu Thr Leu Pro Ile Arg Ile Lys Gln Leu Thr Phe Ser Ser His Leu Arg  108
                          BamHI                                                XbaI
 698  ATT ATT TTG TGG GAA TAC AAT GGA TCC AAG CAA ATT CCC TTT TTC AAT CTA GAA ACG AGC ATC TTT AAC TTA AAA GAC TGT ACT TTA AAA AGA GGG TTT GAA TCC TTA  805
 109  Ile Ile Leu Trp Glu Tyr Asn Gly Ser Lys Gln Ile Pro Phe Phe Asn Leu Glu Thr Ser Ile Phe Asn Leu Lys Asp Cys Thr Leu Lys Arg Gly Phe Glu Ser Leu  144

 806  AAA TTT CGT TAT GAT GTG ATT GAC CAC TGT GAA GTG GTC ACT GAT AAC AAA GAT CAA GAA AAT TTG AAT AAA TAT TTT CAG GGT GAA TTC ACG AGA CTC CCT TGG CTT  913
 145  Lys Phe Arg Tyr Asp Val Ile Asp His Cys Glu Val Val Thr Asp Asn Lys Asp Gln Glu Asn Leu Asn Lys Tyr Phe Gln Gly Glu Phe Thr Arg Leu Pro Trp Leu  180

 914  GAC GAA ATC ACT ATA AGC AAA TTA AGG AAA CAA CCG GAA AAT AGG ACT TGG CCT CAG GGC ACC TTT GTC TTA AAC TTA GAA TTT CCA ATG TTA GAG CTT CCT GTT GTG 1021
 181  Asp Glu Ile Thr Ile Ser Lys Leu Arg Lys Gln Arg Glu Asn Arg Thr Trp Pro Gln Gly Thr Phe Val Leu Asn Leu Glu Phe Pro Met Leu Glu Leu Pro Val Val  216

1022  TTC ATC GAA AGG GAA ATT ATG AAT ACT CAA ATG AAT ATT CCA ACT TTG AAG AAC AAT CCT GGA CTA AGC ACA GAC TTA CGA GAA CCG AAT AGG AAT GAT CCT CAA ATC 1129
 217  Phe Ile Glu Arg Glu Ile Met Asn Thr Gln Met Asn Ile Pro Thr Leu Lys Asn Asn Pro Gly Leu Ser Thr Asp Leu Arg Glu Pro Asn Arg Asn Asp Pro Gln Ile  252

1130  AAA ATT TCT TTG GGG GAC AAA TAT CAC TCC ACA TTG AAG TTT TAC GAT CCT GAC CAA CCA AAC AAT GAT CCA ATA GAG GAA AAG TAT AGG AGA TTG GAA AGA GCA TCT 1237
 253  Lys Ile Ser Leu Gly Asp Lys Tyr His Ser Thr Leu Lys Phe Tyr Asp Pro Asp Gln Pro Asn Asn Asp Pro Ile Glu Glu Lys Tyr Arg Arg Leu Glu Arg Ala Ser  288

1238  AAA AAT GCA AAC TTG GAC AAG CAA GTG AAG CCG GAC ATT AAA AAG AGG GAC TAC TTG AAT AAA ATC ATC AAC TAC CCC CCT GGC ACT AAA TTA ACA GCA CAT GAA AAG 1345
 289  Lys Asn Ala Asn Leu Asp Lys Gln Val Lys Pro Asp Ile Lys Lys Arg Asp Tyr Leu Asn Lys Ile Ile Asn Tyr Pro Pro Gly Thr Lys Leu Thr Ala His Glu Lys  324

1346  GGT TCA ATA TGG AAA TAT AGG TAT TAT TTG ATG AAC AAT AAA AAA GCT CTT ACA AAG TTA TTA CAG AGT ACA AAT TTG AGA GAA GAA TCG GAA AGA GTA GAG GTT TTA 1453
 325  Gly Ser Ile Trp Lys Tyr Arg Tyr Tyr Leu Met Asn Asn Lys Lys Ala Leu Thr Lys Leu Leu Gln Ser Thr Asn Leu Arg Glu Glu Ser Glu Arg Val Glu Val Leu  360

1454  GAA CTA ATG GAC TCA TGG GCA GAA ATT GAT ATA GAC GAC GCA TTA GAA CTA TTA GGC TCA ACT TTT AAA AAC CTT TCA GTG AGA TCG TAC GCC GTA AAT AGG CTG AAG 1561
 361  Glu Leu Met Asp Ser Trp Ala Glu Ile Asp Ile Asp Asp Ala Leu Glu Leu Leu Gly Ser Thr Phe Lys Asn Leu Ser Val Arg Ser Tyr Ala Val Asn Arg Leu Lys  396

1562  AAG GCA TCA GAC AAG GAG CTG GAA TTG TAC TTA TTA CAG TTG GTA GAA GCT GTC TGT TTT GAG AAC CTG TCC ACT TTT TCC GAC AAA TCT AAC AGT GAA TTC ACT ATT 1669
 397  Lys Ala Ser Asp Lys Glu Leu Glu Leu Tyr Leu Leu Gln Leu Val Glu Ala Val Cys Phe Glu Asn Leu Ser Thr Phe Ser Asp Lys Ser Asn Ser Glu Phe Thr Ile  432
                                                                                                                  XhoI
1670  GTG GAT GCC GTA TCT TCG CAA AAG CTT TCG GGT GAT TCT ATG TTA CTA TCT ACA TCG CAT GCC AAC CAA AAA TTA TTA AAG TCC ATC TCG AGT GAA TCG GAA ACT TCC 1777
 433  Val Asp Ala Val Ser Ser Gln Lys Leu Ser Gly Asp Ser Met Leu Leu Ser Thr Ser His Ala Asn Gln Lys Leu Leu Lys Ser Ile Ser Ser Glu Ser Glu Thr Ser  468

1778  GGG ACA GAA TCG CTA CCA ATC GTC ATT TCT CCC TTG GCT GAG TTT TTG ATT AGG AGA GCA CTG GTC AAC CCA AGG TTA GGA AGT TTT TTC TAT TGG TAT TTA AAG TCC 1885
 469  Gly Thr Glu Ser Leu Pro Ile Val Ile Ser Pro Leu Ala Glu Phe Leu Ile Arg Arg Ala Leu Val Asn Pro Arg Leu Gly Ser Phe Phe Tyr Trp Tyr Leu Lys Ser  504

1886  GAG TCT GAG GAC AAG CCA TAT TTA GAC CAG ATT TTA AGT TCC TTT TGG AGT AGA CTG GAT AAA AAA TCT CGG AAT ATA CTG AAC GAT CAA GTC AGG CTA ATA AAT GTG 1993
 505  Glu Ser Glu Asp Lys Pro Tyr Leu Asp Gln Ile Leu Ser Ser Phe Trp Ser Arg Leu Asp Lys Lys Ser Arg Asn Ile Leu Asn Asp Gln Val Arg Leu Ile Asn Val  540
                                                                                                                  StuI
1994  CTC AGG GAA TGT TGT GAA ACA ATT AAG AGG CTG AAA GAT ACT ACA GCC AAA AAG ATG GAA TTA CTA GTG CAT TTA TTG GAG ACG AAA GTC AGG CCT CTT GTC AAA GTA 2101
 541  Leu Arg Glu Cys Cys Glu Thr Ile Lys Arg Leu Lys Asp Thr Thr Ala Lys Lys Met Glu Leu Leu Val His Leu Leu Glu Thr Lys Val Arg Pro Leu Val Lys Val  576

2102  CGA CCA ATT GCT TTA CCA CTA GAC CCT GAT GTG TTG ATA TGC GAC GTT TGT CCT GAA ACT TCG AAG GTA TTT AAA AGC TCT TTA TCG CCA CTA AAA ATA ACA TTC AAA 2209
 577  Arg Pro Ile Ala Leu Pro Leu Asp Pro Asp Val Leu Ile Cys Asp Val Cys Pro Glu Thr Ser Lys Val Phe Lys Ser Ser Leu Ser Pro Leu Lys Ile Thr Phe Lys  612

2210  ACG ACC TTA AAC CAA CCA TAT CAC TTA ATG TTT AAG GGT GGC GAT GAT TTG AGA CAA GAT CAA CTG GTA GTA CAG ATC ATA AGT TTA ATG AAT GAG TTA TTA AAA AAT 2317
 613  Thr Thr Leu Asn Gln Pro Tyr His Leu Met Phe Lys Val Gly Asp Asp Leu Arg Gln Asp Gln Leu Val Val Gln Ile Ile Ser Leu Met Asn Glu Leu Leu Lys Asn  648

2318  GAA AAC GTG GAC TTG AAA TTG ACA CCA TAC AAA ATT TTG GCA ACA GGA CCA CAA GAA GGT GCC ATT GAA TTT ATC CCT AAT GAC ACA TTA GCT AGC ATA CTA AGC AAA 2425
 649  Glu Asn Val Asp Leu Lys Leu Thr Pro Tyr Lys Ile Leu Ala Thr Gly Pro Gln Glu Gly Ala Ile Glu Phe Ile Pro Asn Asp Thr Leu Ala Ser Ile Leu Ser Lys  684

2426  TAT CAC GGT ATT CTT GGC TAC CTT AAA CTC CAC TAT CCT GAT GAG AAC GCG ACA TTA GGC GTA CAA GGC TGG GTT TTA GAT AAT TTT GTC AAA TCT TGT GCT GGC TAT 2533
 685  Tyr His Gly Ile Leu Gly Tyr Leu Lys Leu His Tyr Pro Asp Glu Asn Ala Thr Leu Gly Val Gln Gly Trp Val Leu Asp Asn Phe Val Lys Ser Cys Ala Gly Tyr  720

2534  TGT GTT ATT ACA TAC ATC TTA GGT GTC GGC GAT AGG CAT TTA GAC AAC TTA CTA GTC ACG CCA GAT GGG CAC TTT TTT CAC GCA GAC TTT GGT TAT ATC TTG GGT CAG 2641
 721  Cys Val Ile Thr Tyr Ile Leu Gly Val Gly Asp Arg His Leu Asp Asn Leu Leu Val Thr Pro Asp Gly His Phe Phe His Ala Asp Phe Gly Tyr Ile Leu Gly Gln  756

2642  GAC CCC AAA CCT TTT CCG CCA TTA ATG AAA TTG CCC CCT CAA ATT ATA GAG GCG TTT GGG GGT GCA GAA TCA TCA AAT TAT GAT AAA TTT CGC AGC TAC TGT TTT GTG 2749
 757  Asp Pro Lys Pro Phe Pro Pro Leu Met Lys Leu Pro Pro Gln Ile Ile Glu Ala Phe Gly Gly Ala Glu Ser Ser Asn Tyr Asp Lys Phe Arg Ser Tyr Cys Phe Val  792

2750  GCA TAT TCG ATT TTA AGA AGG AAT GCA GGC TTA ATC TTA AAC GTG TTT GAA TTA ATG AAG ACT TCG AAC ATA CCT GAT ATC AGA ATA GAT CCC AAT GGT GCT ATA TTA 2857
 793  Ala Tyr Ser Ile Leu Arg Arg Asn Ala Gly Leu Ile Leu Asn Leu Phe Glu Leu Met Lys Thr Ser Asn Ile Pro Asp Ile Arg Ile Asp Pro Asn Gly Ala Ile Leu  828

2858  CGT GTA AGA GAG AGA TTT AAT TTG AAT ATG TCC GAA GAA GAT GCC ACA GTG CAT TTT CAG AAT CTA ATC AAT GAT AGT GTA AAT GCT TTG CTG CCT ATC GTG ATT GAT 2965
 829  Arg Val Arg Glu Arg Phe Asn Leu Asn Met Ser Glu Glu Asp Ala Thr Val His Phe Gln Asn Leu Ile Asn Asp Ser Val Asn Ala Leu Leu Pro Ile Val Ile Asp  864

2966  CAT TTA CAT AAT CTG GCA CAA TAC TGG CGG ACC TGA   3001
 865  His Leu His Asn Leu Ala Gln Tyr Trp Arg Thr End    875

                                                                                                                                  KpnI                                                                                                                                
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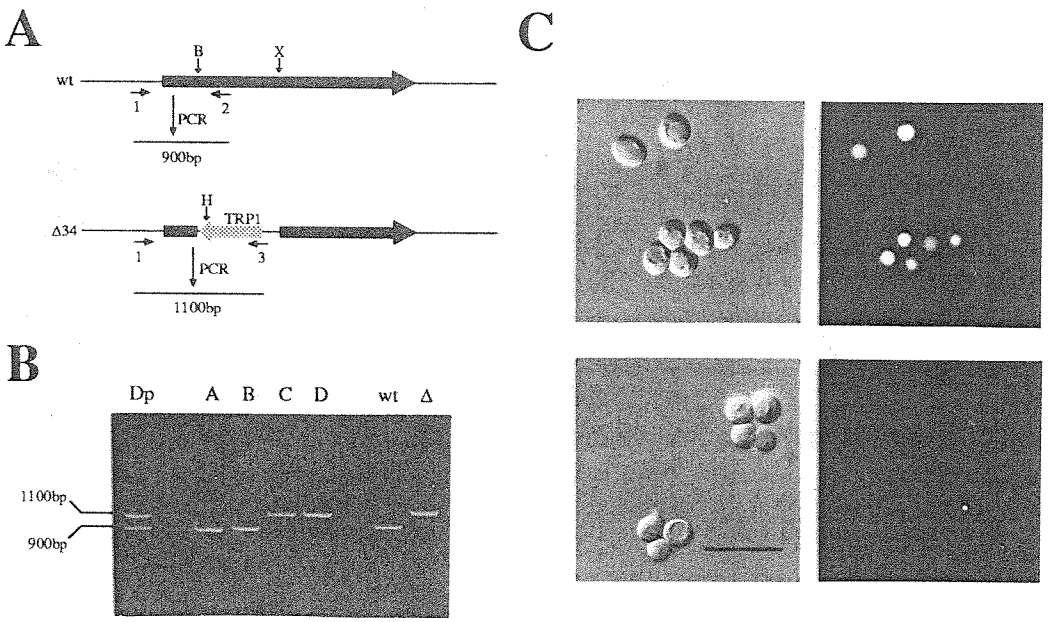

FIG. 5. (A) Construction of a gene disruption at the *VPS34* locus and verification of the resulting genomic structures. The 1.0-kb *Bam*HI-*Xho*I fragment of the *VPS34* gene was replaced by the yeast *TRP1* gene as described in Materials and Methods. The genomic structure at the *VPS34* genetic locus was analyzed by PCR DNA amplification. Three 25-mer primers were added to the genomic DNA template, and 30 cycles of amplification with *Taq*I polymerase were performed. If the wild-type (wt) *VPS34* gene is present, an amplification product of ~900 bp is produced with primers 1 and 2. The presence of the *vps34Δ1::TRP1* allele results in the appearance of a DNA fragment of ~1,100 bp from primers 1 and 3. The heavy black arrows indicate *VPS34* coding sequence, and the large stippled arrow represents the *TRP1* coding region. (B) Analysis of the PCR products by agarose gel electrophoresis. The PCR products were run out on 1.2% agarose gels, stained with ethidium bromide, and then photographed. The genomic DNA analyzed was obtained from yeast as follows: Dp indicates the diploid strain PHY120; A, B, C, and D indicate the four haploid progeny from a single PHY120 meiosis; and wt refers to SEY6210. The Δ reaction mix contained ~0.5 ng of pPHY38 plasmid DNA. Genetic analysis of the PHY120 progeny indicated that A and B were wild type at the *VPS34* locus and C and D were mutant (growth at 37°C and CPY localization were assessed; data not shown). The positions of the 900- and 1,100-bp products are indicated. (C) Analysis by Nomarski optics and epifluorescence. No intracellular *ade2* fluorescence is detected within PHY103 cells (*vps34Δ1::TRP1 ade2*). Yeast cells were grown to stationary phase in YPD medium, mounted for microscopy, and then examined by either Nomarski interference optics (left) or epifluorescence (right). The top panels show SEY6210 (*VPS34*) cells, and the bottom panels show PHY103 (*vps34Δ1::TRP1*). Bar, 20 μm.

missorting phenotype was equivalent at both the permissive and nonpermissive growth temperatures in the Δ*vps34* strains (data not shown). As well, Δ*vps34* yeast cells were observed to be osmotically sensitive, as illustrated by their lack of growth on medium supplemented with 1.5 M NaCl (Fig. 1).

The disruption allele of *VPS34* resulted in one additional phenotype not seen with any other *vps34* allele. Normally, *ade2* yeast colonies turn red on adenine-limiting media. This coloring is apparently due to the accumulation of a naturally fluorescent metabolic intermediate of adenine biosynthesis in the vacuolar compartment (17, 47, 53). Therefore, the vacuoles of *ade2* yeast cells are fluorescent and easily visualized by light microscopy (53). When Δ*vps34 ade2* double mutants were placed on adenine-limiting medium, the colonies remained white (as do *ADE2* colonies). By introducing the wild-type *VPS34* gene back into these mutants, they regained the red *ade2* phenotype, demonstrating that the *ade2*-white phenotype was a direct consequence of the *vps34Δ1::TRP1* allele. This defect was also apparent at the cellular level. Whereas the vacuoles of SEY6211 (*ade2 VPS34*) were strongly fluorescent, Δ*vps34 ade2* cells exhibited no significant intracellular fluorescence (Fig. 5C). A similar *ade2*-white phenotype was observed with the class C *vps* mutants (3). These mutants apparently lack a normal vacuolar compartment, and the absence of *ade2* fluorescence may be due to the lack of a compartment in which to sequester this metabolic intermediate of adenine biosynthesis. In contrast, Δ*vps34* cells do possess a detectable vacuolelike intracellular organelle (see Nomarski photographs, Fig. 5C). These organelles, like wild-type vacuoles, are able to accumulate fluorescent dyes specific for the vacuole, such as FITC and CDCFDA (see below). It is interesting that the two spontaneously isolated temperature-sensitive alleles of *VPS34* result in an intermediate *ade2* phenotype (pink), suggesting that these two alleles retain residual *VPS34* gene function.

Diploid strains homozygous for the *vps34Δ1::TRP1* allele sporulated with a much lower efficiency than did wild-type diploids or those that were heterozygous at the *VPS34* locus (data not shown). A similar sporulation-deficient phenotype has been observed with *pep4*/*pep4* diploid strains (50, 57), suggesting that the sporulation defects of Δ*vps34* diploids may be due to the decreased levels of PrA enzymatic activity associated with these mutants (data not shown; 36).

Identification and characterization of Vps34p. To charac-

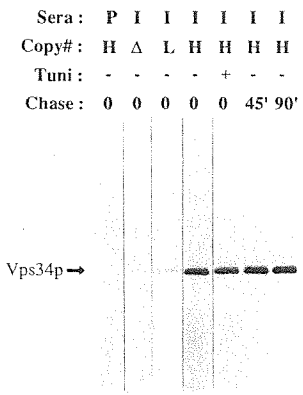

FIG. 6. Identification and characterization of Vps34p. Yeast strains were labeled with Tran ^{35}S label in YNB-glucose minimal medium for 30 min at 30°C. The cells were broken, and immunoprecipitations were performed from the clarified cell lysates with either the preimmune control serum (P) or the Vps34p immune serum (I). Copy number refers to the gene dosage of *VPS34*; Δ is PHY102 (no copies), L is SEY6210 (one copy), and H is SEY6210 with plasmid pPHY52 (multiple copies). The PHY102 and SEY6210 immunoprecipitations were performed from eight OD_{600} equivalents of cells instead of two (see Materials and Methods). Where indicated (+), the cells were treated with tunicamycin (20 μg/ml) for 15 min prior to labeling. The labeled cultures were chased with cold methionine and cysteine (final concentration, 2 mM) for the indicated times. The position of Vps34p is indicated (95,000 Da).

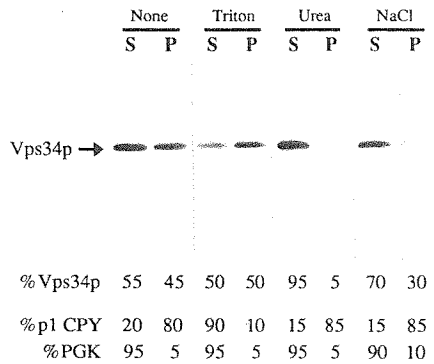

FIG. 7. Subcellular fractionation of Vps34p. DKY6224 (Δ*pep4*) cells harboring the VPS34 multicopy plasmid pPHY52 were spheroplasted, labeled with Tran ^{35}S label, and osmotically lysed. The cell lysates were then centrifuged at 100,000 × *g* for 30 min following extraction with either 1% Triton X-100, 2 M urea, or 1 M NaCl for 10 min at 0°C. The relative levels of Vps34p in the supernatant and pellet fractions were assessed by quantitative immunoprecipitation with antiserum to Vps34p. The values beneath the gel represent the averages of three different experiments that examined the solubilization properties of Vps34p, p1 CPY, and phosphoglycerokinase (PGK); the relative levels of each were determined by immunoprecipitation with the appropriate antisera. p1 CPY is a marker for the ER and early Golgi compartments, and phosphoglycerokinase is a cytoplasmic protein.

terize the *VPS34* gene product, we prepared polyclonal antisera against a TrpE-Vps34 fusion protein isolated from *E. coli* (see Materials and Methods). This fusion protein consisted of amino acids 125 to 462 of Vps34p fused in frame to the C terminus of the *E. coli trpE* protein. Upon induction with indoleacrylic acid, an artificial inducer of the *trp* operon, *E. coli* cells carrying this *trpE-VPS34* fusion gene produced a novel protein of 74-kDa at a relatively high level (~10% of total cell protein). This hybrid protein was purified and used to immunize rabbits. The resulting polyclonal antiserum was used in quantitative immunoprecipitations from radiolabeled yeast extracts. This antiserum detected a unique polypeptide of an apparent molecular weight of ~95,000 from wild-type yeast cells (Fig. 6). This relatively rare yeast protein was not immunoprecipitated by the preimmune control serum and was not detected in Δ*vps34* cell extracts (Fig. 6). In addition the presence of a multicopy *VPS34* plasmid resulted in an approximately 30 to 50-fold increase in the level of this 95-kDa polypeptide (Fig. 6). These data indicate that the polyclonal antiserum specifically recognizes the protein product of the *VPS34* gene. Densitometric analysis of the levels of Vps34p relative to CPY (~0.1% of total cell protein) suggested that Vps34p comprises <0.01% of total cell protein in logarithmically growing yeast cultures. It is interesting that the overproduction of Vps34p did not result in a vacuolar protein sorting defect (Fig. 3).

The predicted sequence of Vps34p indicates that this protein contains 10 potential sites for N-linked glycosyl modification. However, the apparent absence of any potential N-terminal signal sequence, or other membrane-spanning domains, suggests that this protein does not enter the secretory pathway. To directly test this prediction, we treated yeast cells with the drug tunicamycin, a potent inhibitor of N-linked glycosylation, prior to labeling and immunoprecipitation. The Vps34p detected from tunicamycin-treated cells was indistinguishable from the wild-type protein on SDS-polyacrylamide gels, suggesting the absence of asparagine-linked oligosaccharide modification (Fig. 6). Pulse-chase experiments indicated that Vps34p was a relatively stable protein species, with a half-life of at least 90 min (Fig. 6). Therefore, Vps34p is a relatively rare, unglycosylated protein of approximately 95,000 Da in molecular mass.

Subcellular fractionation of Vps34p suggests an association with a multiprotein complex. The nucleotide sequence data indicated that Vps34p is a relatively hydrophilic protein lacking any potential transmembrane domains and therefore might have a cytoplasmic localization. We used differential centrifugation techniques to directly analyze the intracellular location and associations of Vps34p. Yeast spheroplasts were labeled for 30 min with [^{35}S]methionine and [^{35}S] cysteine, osmotically lysed, and subjected to differential centrifugations prior to quantitative immunoprecipitation with antiserum to Vps34p. The osmotic lysis conditions were chosen so that the integrity of specific internal organelles, including the ER and Golgi compartments, was not disturbed. Approximately 80% of the p1 and p2 CPY, markers for the ER and Golgi compartments (12, 48), was detected in a 13,000 × *g* pellet fraction (Fig. 7; 10). In addition, more than 90% of the mature CPY was released into a soluble fraction, indicating that the vacuolar compartment was disrupted by this lysis procedure (data not shown; 10).

The lysates of DKY6224 cells harboring the multicopy *VPS34* plasmid pPHY52 were centrifuged at 100,000 × *g* for 30 min, and the distribution of Vps34p between the supernatant and pellet fractions was assessed. Approximately 50% of the Vps34p was present in the pellet fraction (Fig. 7). The nature of the association of Vps34p with this particulate fraction was analyzed by using several different extraction

procedures. Following osmotic lysis, the cell lysates were incubated with either 1% Triton X-100, 2 M urea, or 1 M NaCl for 10 min at 4°C. The lysates were then centrifuged at 100,000 × *g* for 30 min, and the supernatant and pellet fractions were analyzed for the presence of Vps34p by immunoprecipitation. Extraction with 1% Triton X-100 did not significantly alter the distribution of Vps34p between the two fractions; ~50% of the Vps34p was still present in the pellet fraction (Fig. 7). This Triton X-100 treatment was sufficient to solubilize p1 CPY (Fig. 7) and the integral vacuolar membrane protein, alkaline phosphatase (data not shown; 20). Treatment with 2 M urea readily solubilized Vps34p from the particulate fraction (Fig. 7), suggesting that Vps34p is associated with this pelletable structure through protein-protein interactions. This urea extraction failed to solubilize either p1 CPY (Fig. 7) or alkaline phosphatase (data not shown). The partial solubilization by a 1 M NaCl extraction further suggests that the presence of Vps34p in this complex is mediated by ionic interactions. We have observed that ~30% of this particulate Vps34p is present in a 13,000 × *g* lower-speed pellet. The presence of Vps34p in the particulate fraction was not the result of its overexpression in these experiments, as ~40 to 50% of Vps34p was detected in 100,000 × *g* pellet of DKY6224 cells expressing wild-type levels of this protein (data not shown). This particulate Vps34p was also solubilized by an extraction with 2 M urea but not with 1% Triton X-100. Indirect immunofluorescence experiments were performed with the Vps34p-specific antiserum to localize the Vps34p within the yeast cell. In wild-type cells, we observed a weak punctate staining evenly distributed throughout the entire cytoplasm; this signal was absent in Δ*vps34* cells (data not shown).

Vacuole formation is delayed in Δ*vps34* cells. A large vacuolelike organelle was detected within Δ*vps34* cells by Nomarski optics, but this compartment did not accumulate the endogenous *ade2* fluorophore (Fig. 5C). To determine the nature of this compartment, we labeled PHY103 yeast cells (*vps34Δ1*::*TRP1 ade2*) with two fluorescent dyes, FITC and CDCFDA, which have been shown to accumulate specifically within the vacuoles of wild-type yeast cells (3, 34, 53). The intracellular compartments within Δ*vps34* cells were specifically stained by both of these two fluorophores, indicating that these structures are similar to wild-type vacuoles (Fig. 8A and data not shown). However, within a population, the vacuolar compartment visualized in Δ*vps34* strains was a much more heterogeneous structure than that observed in wild-type cells. Generally one to three large FITC-staining compartments are detected within wild-type cells (3, 33, 53). In Δ*vps34* cells, the size of the FITC-staining compartment varied greatly, with approximately 50 to 60% of the population resembling wild-type cells (data not shown). Approximately 5 to 10% of the Δ*vps34* cells in a logarithmically growing culture did not exhibit any FITC fluorescence, a phenotype similar to that observed with the class C *vps* mutants (3). The remaining ~30% of the Δ*vps34* cells exhibited an intermediate morphology in which the cells possessed multiple small staining compartments.

The most striking observation made with the Δ*vps34* strains was that in general, no vacuolar compartment was observed in newly forming cells. In wild-type cells, a vacuole is detected in the newly forming bud when it is only a fraction of the size of the mother cell (53). In Δ*vps34* cells, no vacuole, or only a very small compartment, was observed in the newly forming bud by both FITC fluorescence and Nomarski microscopy (Fig. 8A). A total of 45 budding cells were observed at a time when the bud was greater than 50%

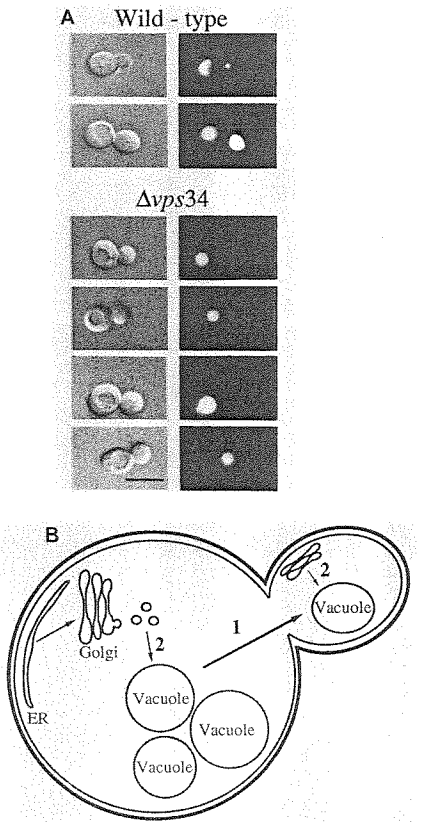

FIG. 8. (A) Delay of vacuole formation in PHY103 cells (*vps34Δ1*::*TRP1*). Yeast cells were grown in YPD medium to early log phase and were stained with the fluorescent dye FITC for 15 min at room temperature. The cells were then mounted for microscopy and examined by either Nomarski interference optics (left) or epifluorescence (right). The top two rows show SEY6210 dividing pairs (wild type), and the bottom four show PHY103 pairs (Δ*vps34*). No FITC staining is detected in the buds of PHY103 cells. Bar, 10 μm. (B) Vacuolar assembly in yeast cells. A simple model depicting two intracellular pathways contributing to vacuolar growth is shown. Pathway 1 (inheritance pathway) represents the contribution of the mother cell vacuole to the contents of the bud vacuole. Pathway 2 (biosynthetic pathway) depicts the transport of newly synthesized vacuolar constituents from the Golgi complex to the vacuolar compartment(s). The contribution of endocytosis to vacuolar content and the potential delivery of vacuolar constituents from the mother cell Golgi complex to the bud vacuole are not shown. See text for further discussion.

the size of the mother cell. In 38 cases no FITC staining was observed in the daughter bud, and in the 7 remaining dividing pairs only a very small vacuole was detected in the bud. In all cases but one, the mother cell possessed a normal FITC-staining compartment (Fig. 8A). At a similar point in the cell cycle, 28 of 30 wild-type buds examined were observed to possess a relatively large vacuolar compartment (Fig. 8A; 53). Identical results were obtained by staining the dividing yeast cells with a second fluorescent dye, CDCFDA (data not shown). In a logarithmically growing Δ*vps34* yeast

culture, the majority of the unbudded cells possessed a vacuolar structure, as demonstrated by FITC fluorescence microscopy. Therefore, these cells are competent for vacuole assembly, but the data indicate that the formation of this vacuolar compartment is delayed relative to that in wild-type cells. In agreement with this view, in stationary-phase cultures, in which cells arrest in G_1 as unbudded cells, almost all of the Δ*vps34* cells possessed wild-type vacuolar structures (data not shown).

DISCUSSION

Mutations in the *VPS34* gene result in severe defects in the localization and processing of a number of vacuolar proteins. To better understand the physiological role of the *VPS34* gene product in this protein sorting process, we have cloned and sequenced the wild-type *VPS34* gene. In all, our studies indicate that the *VPS34* gene product is a hydrophilic protein that may act as a component of a relatively large intracellular complex to facilitate the delivery of vacuolar proteins. The wild-type *VPS34* gene was cloned by complementation of a *vps34* temperature-sensitive growth defect, and the *VPS34* gene sequence identifies an open reading frame that could encode a protein of 875 amino acids. The size of this open reading frame is in good agreement with the RNA Northern blot analysis and the protein data. The predicted protein sequence indicates that Vps34p lacks any apparent N-terminal signal sequence or other membrane-spanning domains, suggesting that Vps34p is not a passenger of the secretory pathway. Consistent with this prediction, immunoprecipitation of cells labeled in the presence of tunicamycin indicates that Vps34p contains no N-linked carbohydrate, even though the predicted sequence possesses 10 potential sites for such oligosaccharide modification. A comparison of the Vps34p sequence with those in the GenBank and NBRF data bases failed to reveal any significant homologies which might have provided some clues into the biochemical nature of Vps34p function.

Some insight into the possible function of Vps34p may be provided by subcellular fractionation studies which indicate that approximately 50% of the radiolabeled Vps34p associates with a particulate fraction of yeast cells. The solubility properties of this particulate Vps34p suggest that protein-protein interactions are responsible for the presence of Vps34p in the pellet fraction. The failure of Triton X-100 to solubilize Vps34p from this structure further suggests that this protein is not simply associating as a peripheral protein with a specific intracellular membrane. Instead, the fractionation data are consistent with Vps34p being a component of a relatively large multiprotein complex. This complex is presumably functioning to facilitate a specific step(s) in the vacuolar protein sorting process. Since only about 50% of the Vps34p was detected within the pellet fraction, Vps34p may exist in, and perhaps cycle between, two separate intracellular pools. One interesting possibility, suggested by the Triton X-100 fractionation data, is that Vps34p is associated with the cytoskeletal network of yeast cells. In higher eucaryotic cells, the cytoskeleton has been operationally defined as the interconnected proteins that remain following an extraction of the cells with nonionic detergents such as Triton X-100 (5). Further biochemical studies are necessary to determine the precise nature of the association of Vps34p with this particulate fraction. We have initiated an analysis of temperature-resistant suppressors of *vps34*(Ts) mutants in an attempt to identify the specific cellular components which interact with the Vps34p in vivo. The genetic analysis, in combination with the biochemical studies, should lead to a better understanding of the role of Vps34p in the delivery of vacuolar proteins.

We constructed a gene disruption of *VPS34* to assess the phenotypic consequences of a null allele of this locus. The structure of the disrupted allele in the genomic DNA was verified by a rapid, nonisotopic PCR analysis (Fig. 5A and B). Haploid yeast strains possessing the *vps34Δ1::TRP1* allele produced no detectable Vps34p and exhibited a severe temperature-sensitive growth defect. Therefore, the *VPS34* gene appears to be essential for vegetative growth only at elevated growth temperatures. To our knowledge, the observation of a null allele exhibiting such a conditionally lethal growth phenotype has been made only rarely in the literature. In *S. cerevisiae*, disruptions of two different genes, both encoding products that might interact with actin, result in conditional lethal phenotypes (13, 30). The biological basis for the temperature-sensitive phenotype that we observe with the *vps34* null mutants is not known. The severity of the vacuolar protein sorting defect was observed to be equivalent at the permissive and nonpermissive growth temperatures, at least for vacuolar delivery of CPY. It is possible that more severe sorting defects, perhaps affecting additional proteins, become apparent at the elevated growth temperatures and result in the observed temperature-sensitive phenotype. The secretion of invertase, as well as other secreted proteins, appears to be normal at the nonpermissive growth temperature in *vps34*(Ts) mutants (36), suggesting that the cessation of growth is not due to a block in general protein secretion. It is possible that the *vps34* sorting defects result in a decrease in specific vacuolar activities that are required for vegetative growth at this elevated temperature. The proteins responsible for these activities may themselves be mislocalized in *vps34* mutant cells. Alternatively, the cumulative stress of decreased vacuolar function plus the elevated growth temperature may, in an additive fashion, result in the observed inviability. In light of the rarity of conditionally lethal null alleles, it is interesting that a similar temperature-sensitive null phenotype has been observed for a number of *VPS* genes, including *VPS1* (39), *END1/VPS11 (9)*, *VPS15* (P. Herman et al., submitted for publication), *VPS33* (4), and *VPS16* (B. Horazdovsky, unpublished observations). Interestingly, mutations in each of these genes result in defects in vacuole assembly and an extreme sensitivity to osmotic stress (3, 9). Altogether, these data might suggest a role for the yeast vacuole in cellular responses to situations of environmental stress, such as elevated growth temperatures or osmotic stress. In cultured mammalian cells, the rate of lysosomal degradation of certain cytoplasmic proteins has been shown to specifically increase in response to cell starvation (7). Clearly, more experimentation is necessary to define the precise role of the vacuole in these stress responses.

The majority of the *vps34* mutant cells possess a morphologically normal vacuolar structure when analyzed with the fluorescent dyes FITC and CDCFDA, both of which specifically accumulate within the vacuoles of wild-type yeast cells (3, 33, 34). It is interesting that although *vps34* mutants appear to be competent for vacuole assembly, they are grossly defective in the delivery of several vacuolar hydrolases, including CPY, PrA, and PrB (Fig. 3; 36). Several different models may be proposed to explain this apparent paradox. Multiple, possibly overlapping, pathways may exist for the delivery of vacuolar constituents from the Golgi complex. This appears to be the case for mammalian lysosomal proteins, as only a subset of all lysosomal constituents

utilize the mannose-6-phosphate targeting system (22). A second possibility is that the *VPS34* gene product acts relatively early in the vacuolar protein sorting process, perhaps in the packaging of the vacuolar proteins into their appropriate vesicular carriers. In this event, vesicular traffic to the vacuole may proceed almost normally except that the vesicles would lack much of their usual protein content. In both models, vacuole assembly could proceed in the absence of *VPS34* gene function but vacuolar functions would be compromised since many vacuolar proteins would now be mislocalized.

During our analysis of the vacuolar compartment in Δ*vps34* mutants, we observed that more than 80% of the newly forming buds lacked a detectable vacuole. In contrast, a normal vacuolar structure was generally observed in the mother cell and in the majority of the unbudded cells in the Δ*vps34* population. Therefore, the inheritance and assembly of the vacuolar structure in dividing Δ*vps34* cells appear to be delayed relative to the processes in wild-type yeast cells. In wild-type cells, a vacuole is detected in the bud when it is only a fraction of the size of the mother cell (Fig. 8A; 53). Analyses of vacuole segregation during cell division have indicated that the daughter bud inherits a substantial portion of its vacuolar contents from the mother cell vacuole (45, 53, 5, 58; this inheritance pathway is shown as pathway 1 in Fig. 8B). The absence of a vacuole in the newly emerging bud in Δ*vps34* cells could reflect a defect in vacuolar inheritance. Similar defects in vacuolar segregation have been observed in two other yeast mutants, *vac1* (54) and *vps3* (35). Complementation tests have demonstrated that the *vps34*, *vac1*, and *vps3* mutations define different yeast genes (21; L. Weisman and W. Wickner, personal communication). At least two different models could be proposed to explain this apparent defect in vacuole segregation in Δ*vps34* cells. Vacuolar segregation and Golgi-to-vacuole protein transport may be mechanistically similar, and Vps34p may be required to execute a common event along both pathways. Alternatively, the severe missorting defects associated with *vps34* mutations could result in a decrease in specific vacuolar activities that are required for vacuolar inheritance. Further genetic and biochemical studies are required to determine the precise role of Vps34p in the partitioning of vacuolar contents between mother and daughter yeast cells.

We believe that the data presented here are most consistent with Vps34p being directly involved in the sorting of proteins to the yeast vacuolar compartment. In a population of *vps34* cells, the majority of the cells exhibit a normal vacuole morphology, yet CPY is almost quantitatively mislocalized to the cell surface. The observation that *vps34* cells are competent for vacuole assembly indicates that at least some vacuolar constituents are properly delivered to this organelle. The severe *vps34* missorting defects would be expected to result in a significant decrease in a variety of normal vacuolar activities. Such a decrease in vacuolar function may be responsible for the phenotypes associated with *vps34* alleles, including temperature-sensitive growth, osmosensitivity, and the vacuolar segregation defect. On the basis of our observations, we propose that Vps34p may be a component of a relatively large multiprotein structure that functions to facilitate specific steps of the vacuolar protein sorting pathway.

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Chapter 4:

A Novel Protein Kinase Homolog Essential for Protein Sorting to the Yeast Lysosome-like Vacuole.

A Novel Protein Kinase Homolog Essential for Protein Sorting to the Yeast Lysosome-like Vacuole

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Summary

The *VPS15* gene encodes a novel protein kinase homolog that is essential for the efficient delivery of soluble hydrolases to the yeast vacuole. Point mutations altering highly conserved residues within the Vps15p kinase domain result in the secretion of multiple vacuolar proteases. In addition, the in vivo phosphorylation of Vps15p is defective in these kinase domain mutants, suggesting that Vps15p may regulate specific protein phosphorylation reactions required for protein sorting to the yeast vacuole. Subcellular fractionation studies further demonstrate that the 1455 amino acid Vps15p is peripherally associated with the cytoplasmic face of a late Golgi or vesicle compartment. This association may be mediated by myristate as Vps15p contains a consensus signal for N-terminal myristoylation. We propose that protein phosphorylation may act as a molecular "switch" within intracellular protein sorting pathways by actively diverting proteins from a default transit pathway (e.g., secretion) to an alternative pathway (e.g., to the vacuole).

Introduction

The highly compartmentalized nature of the eukaryotic cell requires that specific mechanisms exist to sort and deliver proteins efficiently from their site of synthesis in the cytoplasm to their final intracellular or extracellular destinations. The general pathway followed by proteins destined for the lysosomal/vacuolar compartment of eukaryotic cells has been extensively studied and serves as one of the best-understood paradigms of an intracellular protein sorting process (Kornfeld and Mellman, 1989; Klionsky et al., 1990). Like those proteins destined for secretion, lysosomal proteins are translocated across the membrane of the endoplasmic reticulum (ER) and then travel from the ER to the Golgi complex. However, the transit pathway of lysosomal proteins diverges within the Golgi apparatus, where these proteins are sorted away from secretory protein traffic and are targeted toward the lysosome.

The delivery of proteins to the lysosome appears to be a complex process involving a relatively large number of distinct steps whose execution must be precisely controlled both spatially and temporally. These steps include the specific recognition of lysosomal proteins, their packaging into transport vesicles, delivery and fusion of these vesicles with the correct target organelle, release of the vesicular contents, and the subsequent recycling of transport components for further rounds of protein sorting. Biochemical and genetic studies have identified *cis*-acting targeting signals required for the initial recognition event in lysosomal and vacuolar protein delivery. In many types of mammalian cells, the N-linked carbohydrate chains of soluble lysosomal proteins are modified with mannose-6-phosphate residues, which are recognized by specific integral membrane receptors that mediate lysosomal delivery (Kaplan et al., 1977; reviewed in Kornfeld and Mellman, 1989). In contrast, the targeting signals of yeast vacuolar proteins do not involve a specific carbohydrate modification and instead appear to reside directly within the polypeptide backbone of these proteins (Johnson et al., 1987; Valls et al., 1987; Klionsky et al., 1988; Klionsky and Emr, 1990). However, beyond this initial recognition event, very little is currently known about the basic cellular mechanisms underlying the lysosomal or vacuolar protein delivery pathways.

In the yeast Saccharomyces cerevisiae, the application of several genetic selections has resulted in the isolation of a large number of mutants that exhibit defects in vacuolar protein localization and/or processing (Jones, 1977; Bankaitis et al., 1986; Robinson et al., 1988; Rothman and Stevens, 1986; Rothman et al., 1989; reviewed in Klionsky et al., 1990). Instead of delivering vacuolar hydrolases to the vacuole, these *vps* (for "vacuolar protein sorting defective") mutants missort vacuolar enzyme precursors to the yeast cell surface (Bankaitis et al., 1986; Rothman and Stevens, 1986; Rothman et al., 1989; Robinson et al., 1988). Protein secretion and protein glycosylation appear to be normal in most of the *vps* mutants, indicating that the defects in these mutants are specific for the targeting of vacuolar proteins (Rothman and Stevens, 1986; Robinson et al., 1988). Genetic comparisons among these mutants have demonstrated that they collectively define more than 47 unique complementation groups (see Klionsky et al., 1990). These results suggest that the delivery of proteins to the yeast vacuole is a complex process requiring the coordinated participation of a relatively large number of gene products. In addition to identifying cellular components directly involved in the specific segregation, packaging, and delivery of vacuolar proteins, these yeast sorting mutants should also define genes whose products are involved in the regulation, or control, of these processes.

The *VPS15* gene product is an essential component of the yeast vacuolar protein sorting apparatus. Mutations in the *VPS15* gene result in severe defects in the localization of several soluble vacuolar hydrolases, including carboxypeptidase Y (CPY), proteinase A, and proteinase B (Robinson et al., 1988). In contrast, two vacuolar membrane proteins, α-mannosidase and alkaline phosphatase, appear to be properly localized to the vacuole in *vps15* mutants (Robinson et al., 1988; Klionsky and Emr, 1990; C. Raymond and T. Stevens, personal communication). In addition, *vps15* mutant cells appear to possess a somewhat enlarged but morphologically normal vacuolar com-

Table 1. Strains Used in This Study

Strain	Genotype	Reference
S. cerevisiae		
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson et al. (1988)
SEY6211	*MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9*	Robinson et al. (1988)
SEY15-2	SEY6210 *vps15-2*	Robinson et al. (1988)
SEY15-9	SEY6211 *vps15-9*	Robinson et al. (1988)
PHY112	SEY6210 *vps15Δ1::HIS3*	This study
PHY113	SEY6211 *vps15Δ1::HIS3*	This study
SEY6210.5	*MATα/MATa leu2-3,112/leu2-3,112 ura3-52/ura3-52 his3-Δ200/ his3-Δ200 trp1-Δ901/trp1-Δ901 suc2-Δ9/suc2-Δ9 ADE2/ade2-101 lys2-801/LYS2*	This study
E. coli		
MC1061	*araD139 (araABOIC-leu)7679 Δ(lac)X74 galU galK hsdR rpsL*	Casadaban and Cohen (1980)
JM101	*F' traD36 lacI^q ZΔM15 proAB/supE thiΔlac(lac-pro)*	Miller (1972)
BW313	*F' lysA/dut ung thi-1 relA spoT1*	Kunkel (1985)

partment (Banta et al., 1988). These observations indicate that *vps15* mutants are competent for vacuole assembly and suggest that the wild-type *VPS15* gene product might be specifically involved in the intracellular sorting and delivery of soluble vacuolar proteins.

Here we report on the cloning and sequencing of the wild-type *VPS15* gene and on the identification and characterization of its protein product. The *VPS15* gene encodes a protein that exhibits significant sequence similarity to the catalytic domains of the serine/threonine family of protein kinases. We demonstrate that the mutational inactivation of the Vps15 protein (Vps15p) kinase domain results in a severe vacuolar protein sorting defect. In addition, in vivo phosphate labeling experiments demonstrate that Vps15p is a phosphoprotein and that its phosphorylation is dependent on the presence of a wild-type Vps15p kinase domain. In all, our results suggest that Vps15p regulates specific protein phosphorylation reactions that are required for the efficient delivery of proteins to the yeast vacuole.

Results

Cloning and Characterization of the *VPS15* Locus

All of the 14 originally identified *vps15* alleles result in a severe vacuolar protein sorting defect, which is generally characterized by the secretion of >90% of the newly synthesized CPY as a Golgi-modified precursor molecule (Robinson et al., 1988; P. H., unpublished data). In addition, eight of the *vps15* alleles result in a severe temperature-sensitive (ts) growth defect; however, the extent of the vacuolar protein sorting defect is the same at both the permissive and nonpermissive growth temperatures (Robinson et al., 1988). The wild-type *VPS15* gene was cloned by complementation of the ts growth phenotype associated with the *vps15-2* allele. SEY15-2 cells (*vps15-2, ura3-52*; see Table 1) were transformed with a yeast genomic DNA library constructed in the multicopy plasmid YEp24 (Carlson and Botstein, 1982). Of approximately 10,000 Ura$^+$ transformants analyzed, 2 were observed to be temperature resistant. Plasmid DNA was isolated from these two transformants, amplified in Escherichia coli, and reintroduced into both SEY15-2 and SEY15-9 cells. Both plasmids, pPHY15-1 and pPHY15-2, were found to correct the ts growth defects associated with these *vps15* yeast strains. Restriction enzyme mapping demonstrated that the two complementing plasmids contained overlapping genomic DNA inserts. Because the ~9 kb genomic insert of pPHY15-1 (Figure 1A) was contained entirely within that of

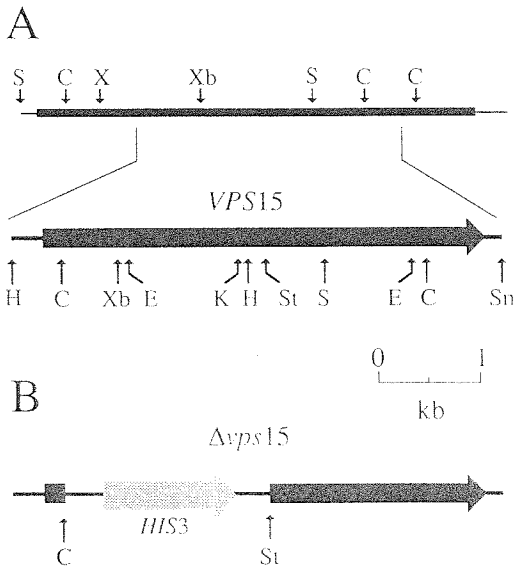

Figure 1. Characterization and Disruption of the *VPS15* Locus

(A) Restriction enzyme map of the ~9 kb genomic DNA insert in plasmid pPHY15-1. The bold line represents the yeast genomic DNA insert and the thin line the YEp24 vector sequences. The lower map is an enlargement of the 4.8 kb HindIII–SnaBI *VPS15* minimum complementing fragment with important restriction enzyme positions shown. Restriction enzymes are as follows: ClaI (C), EcoRI (E), HindIII (H), KpnI (K), SalI (S), SnaBI (Sn), StuI (St), XhoI (X), and XbaI (Xb). The large arrow indicates the *VPS15* coding region and the direction of transcription.

(B) A *VPS15* gene deletion/disruption is shown where the 2.2 kb ClaI–StuI fragment from within the *VPS15* coding region was replaced with the yeast *HIS3* gene. The large stippled arrow repesents the *HIS3* coding region.

pPHY15-2, only pPHY15-1 was analyzed further. Integrative mapping techniques demonstrated that the cloned DNA originated from a region of the yeast genome corresponding to the *vps15* mutant locus (see Experimental Procedures).

The *VPS15*-complementing activity was localized to a 4.8 kb HindIII–SnaBI fragment (Figure 1A) that complemented the *vps15* growth defect when present on a single copy (pPHY15C) as well as a multicopy (pPHY15E) plasmid. The ability of the pPHY15C plasmid to complement the other mutant phenotypes associated with the *vps15-2* allele was also tested. We directly analyzed the localization of CPY in yeast cells by labeling intact spheroplasts with Tran^{35}S-label, fractionating the cultures into supernatant (extracellular media) and pellet (cell-associated) fractions, and then immunoprecipitating with antisera to CPY. In wild-type yeast cells, >95% of the newly synthesized CPY was present as a 61 kd mature species in an intracellular fraction, indicative of correct delivery to the vacuole. In contrast, *vps15-2* cells secreted >95% of the CPY into the extracellular media fraction as a Golgi-modified 69 kd (p2) precursor form (similar to Δ*vps15* in Figure 6B). When the pPHY15C plasmid was introduced into the *vps15-2* mutant, this severe CPY sorting defect was completely corrected (data not shown). *vps15* mutants have also been found to be extremely sensitive to osmotic stress, as demonstrated by their inability to grow on media containing 1.5 M NaCl (Banta et al., 1988). The pPHY15C plasmid was able to complement fully the osmotic sensitivity of the *vps15-2* mutant (data not shown). Therefore, the cloned DNA present within the pPHY15C plasmid is capable of complementing all of the phenotypes associated with *vps15* cells.

Disruption of the *VPS15* Locus Results in a ts Growth Defect

To assess the phenotypic consequences of a *VPS15* null allele, we constructed a deletion/disruption of the *VPS15* gene. A linear fragment of the *VPS15* gene in which the 2.2 kb ClaI–StuI internal fragment had been replaced with the yeast *HIS3* gene (Figure 1B) was used to transform SEY6210.5 diploid cells (*his3-Δ200/his3-Δ200*) to histidine prototrophy by replacing one wild-type copy of the *VPS15* gene with the disrupted allele through homologous recombination (Rothstein, 1983). The structure of the disrupted allele was verified with a polymerase chain reaction (PCR) DNA amplification analysis (data not shown; see Herman and Emr, 1990). Two independent His$^+$ transformants were sporulated and their progeny were subjected to tetrad analysis. In the 27 tetrads analyzed, all haploid progeny were viable, indicating that the *VPS15* gene is not essential for vegetative growth at 26°C. Because our original selection had identified eight *vps15* alleles that exhibited a recessive ts growth defect, we examined the growth of these haploid progeny at 37°C. In all tetrads, the two His$^+$ progeny were unable to grow at 37°C, indicating that the *VPS15* gene product is required for growth at this elevated temperature. The ts progeny were observed to arrest growth after one or two cell divisions at 37°C. A second disruption of the *VPS15* gene confirmed these results (see Experimental Procedures). Therefore, the *VPS15* gene is required for vegetative growth only at elevated growth temperatures.

The Δ*vps15* strains exhibited the same spectrum of phenotypes as the original *vps15* mutants. As expected, Δ*vps15* cells mislocalized >95% of their CPY to the cell surface as the p2 precursor form (Figure 6B). Introduction of the wild-type *VPS15* gene (plasmid pPHY15C) completely corrected this sorting defect (Figure 6B). The Δ*vps15* yeast strains were also unable to grow on media supplemented with 1.5 M NaCl, and this osmotic sensitivity was complemented by the wild-type *VPS15* gene (data not shown). Δ*vps15* cells also possess a morphologically normal vacuole compartment.

The *VPS15* Gene Encodes a Protein That Has Significant Sequence Similarity to Protein Kinases

The 5.6 kb XhoI–ClaI fragment of pPHY15-1 (see Figure 1A) was sequenced using standard dideoxy chain termination methods as described in Experimental Procedures. The nucleotide sequence (Figure 2) identifies a single long open reading frame of 4365 bp contained entirely within the 4.8 kb HindIII–SnaBI *VPS15*-complementing fragment. This open reading frame has the potential to encode a protein of 1455 amino acids with a predicted molecular weight of 166,000. A hydrophobicity analysis of the predicted protein sequence (Kyte and Doolittle, 1982) indicates that Vps15p is relatively hydrophilic and appears to possess no N-terminal signal sequence or transmembrane domains (data not shown). Therefore, this protein would not be expected to enter the secretory pathway.

A comparison of the predicted amino acid sequence of Vps15p to other known protein sequences revealed that the N-terminal 300 amino acids of this protein share a significant degree of sequence similarity with the catalytic domains of the serine/threonine family of protein kinases (Figure 3). The most striking similarities were seen with the γ (catalytic) subunit of phosphorylase b kinase from rabbit skeletal muscle (Reimann et al., 1984), the protein product of the Schizosaccharomyces pombe *wee1*$^+$ gene (Russell and Nurse, 1987), and the ribosomal protein S6 kinase from Xenopus laevis (Jones et al., 1988). A lesser degree of sequence conservation was seen with the tyrosine protein kinases, such as pp60^{src} (Takeya and Hanafusa, 1983). Over the entire protein kinase catalytic domain, Vps15p shares 25% sequence identity with phosphorylase b kinase and 23% with the Wee1 protein. These values are consistent with the level of sequence conservation generally observed between unrelated protein kinases. In fact, phosphorylase b kinase and Wee1p share 25% sequence identity with each other over this region.

Protein kinases are generally classified into two relatively broad groups based on their substrate specificity: those that phosphorylate serine or threonine residues and those that modify tyrosine (see Hanks et al., 1988). The catalytic domains of both the serine/threonine and tyrosine protein kinases are modular structures in which regions of very high sequence conservation are interspersed with regions of little similarity (Hunter, 1987;

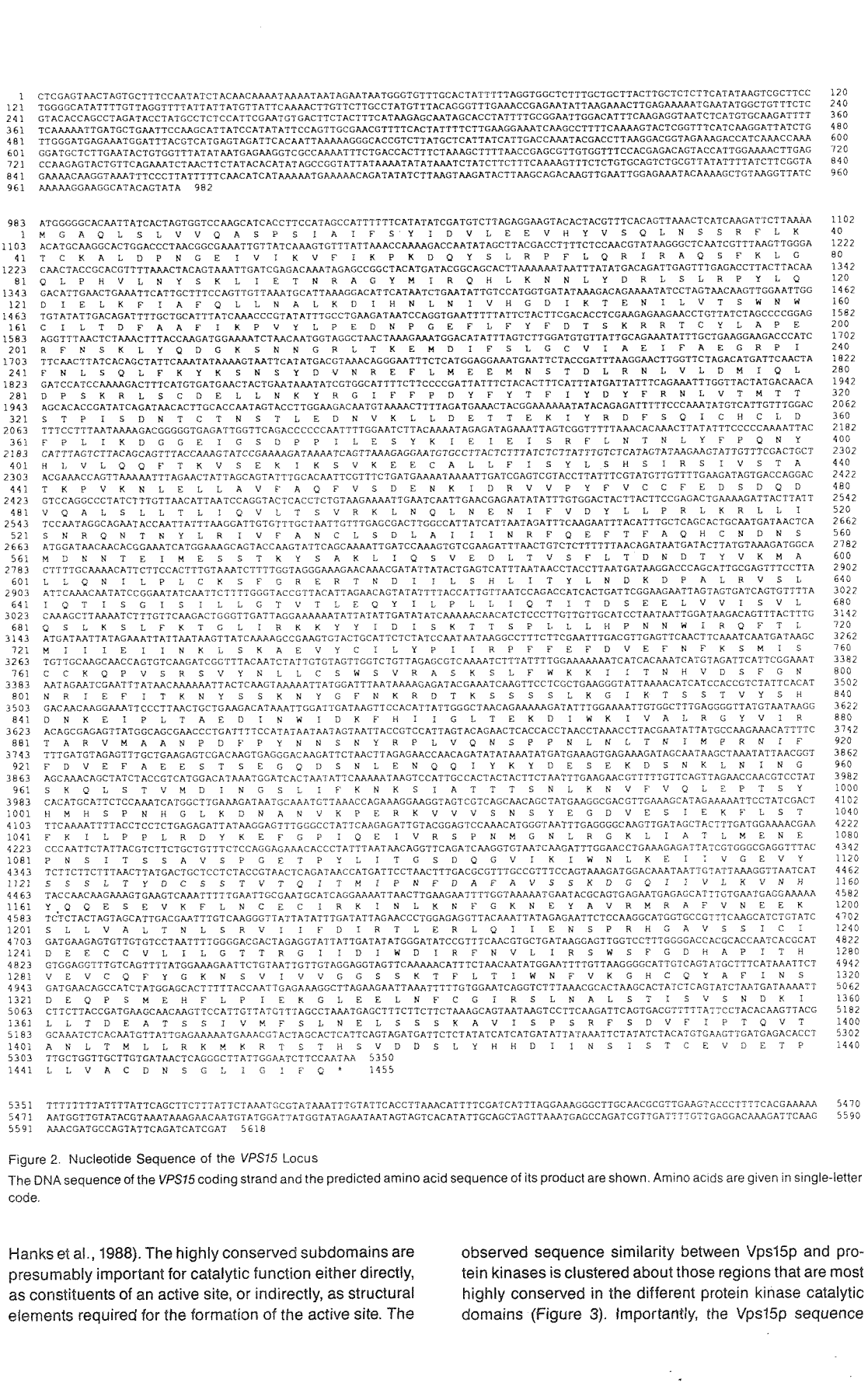

Figure 2. Nucleotide Sequence of the *VPS15* Locus

The DNA sequence of the *VPS15* coding strand and the predicted amino acid sequence of its product are shown. Amino acids are given in single-letter code.

Hanks et al., 1988). The highly conserved subdomains are presumably important for catalytic function either directly, as constituents of an active site, or indirectly, as structural elements required for the formation of the active site. The observed sequence similarity between Vps15p and protein kinases is clustered about those regions that are most highly conserved in the different protein kinase catalytic domains (Figure 3). Importantly, the Vps15p sequence

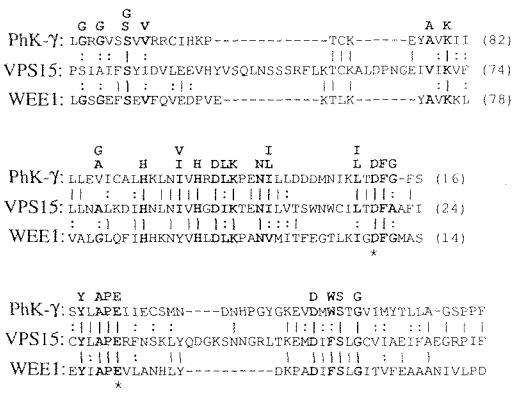

Figure 3. Comparison of the Vps15p Sequence with the Kinase Domains of Phosphorylase b Kinase and the Product of the *wee1*$^+$ Gene

The predicted sequence of Vps15p is compared with the catalytic domains of the two serine/threonine-specific protein kinases, phosphorylase b kinase from rabbit skeletal muscle (PhK-γ; Reimann et al., 1984) and the product of the S. pombe *wee1*$^+$ gene (Wee1p; Russell and Nurse, 1987). In the comparisons, identical amino acids are designated with a bar, and conservative changes are indicated with two dots. Gaps in the amino acid sequence are represented by dashed lines. The numbers given in parentheses indicate the number of amino acids separating the sequences shown. Residues that are very highly conserved in protein kinase catalytic domains are indicated in boldface above the sequence comparisons (see Hanks et al., 1988). The asterisks indicate the two amino acids altered in this study.

contains the consensus triplet A-P-E (see Figure 3), a conserved sequence element that is often referred to as an indicator of protein kinase catalytic domains (Hunter and Cooper, 1986).

An especially high degree of sequence conservation is seen in the central core of the protein kinase domain (shown in the bottom two comparisons of Figure 3), where Vps15p and phosphorylase b kinase share 42% sequence identity over a stretch of 96 amino acids. Included within this region is a short stretch of amino acids that has been used as an indicator of protein kinase substrate specificity (Hanks, 1987). Generally, serine/threonine-specific kinases possess a sequence resembling the consensus element DLKPEN, in which the lysine residue is absolutely conserved, while the tyrosine-specific kinases have either DLRAAN or DLAARN. The Vps15p sequence of DIKTEN closely resembles the serine/threonine consensus element, suggesting that Vps15p belongs to the serine/threonine family of protein kinases. A lower degree of sequence conservation with other protein kinases was seen in the most N-terminal regions of the Vps15p kinase-like domain (Figure 3; see Discussion). The observation that Vps15p possesses sequence similarity to the catalytic domain of protein kinases suggests that Vps15p may be a protein kinase and that protein phosphorylation may play an important role in controlling protein delivery to the yeast vacuole.

A visual examination of the predicted amino acid sequence of Vps15p identified a potential site for the attachment of myristic acid at the N-terminus of this protein. Myristic acid is a 14 carbon fatty acid that is cotranslationally added to an N-terminal glycine residue of specific eukaryotic proteins via an amide linkage (reviewed in Towler et al., 1988a). An extensive analysis of the substrate specificity of the yeast myristoyl CoA:protein N-myristoyltransferase (NMT) has suggested a consensus sequence for N-terminal myristoylation of $Gly_1-X_2-Z_3-Z_4-Ser_5-Z_6$ (Towler et al., 1987, 1988b). The yeast NMT enzyme exhibits an absolute specificity for a glycine residue at position 1 (following removal of the initiating methionine); the primary amino group of this glycine residue appears to be critical for the binding of substrate by yeast NMT (Towler et al., 1988a). In addition, a serine residue at position 5 favors a high affinity interaction of the protein/peptide substrate with the yeast NMT enzyme (Towler et al., 1988b). Finally, position 2 should be a small, uncharged amino acid (indicated by an X), and neutral residues (indicated by a Z) are preferred at the somewhat more permissive positions 3, 4, and 6. The Vps15p sequence of (Met)-$Gly_1-Ala_2-Gln_3-Leu_4-Ser_5-Leu_6$ fits this consensus, suggesting that Vps15p may be myristoylated in vivo (see below). The large C-terminal domain of Vps15p (>1000 amino acids) exhibits no significant sequence conservation with any other known protein sequence.

Vps15p Associates with a Yeast Membrane Fraction

Polyclonal antisera were raised against a trpE–Vps15 fusion protein and used in immunoprecipitation experiments from radiolabeled yeast cell extracts. These antisera detected a single ~170 kd protein in wild-type extracts that was not recognized by the preimmune control sera and was absent from Δ*vps15* yeast cell extracts (Figure 4). In addition, in yeast cells harboring a multicopy *VPS15* plasmid, the level of this 170 kd protein increased 30- to 50-fold (Figure 4). Therefore, these polyclonal antisera specifically recognize the product of the *VPS15* gene. An examination of the synthesis levels of Vps15p relative to CPY (~0.1% of total cell protein) by densitometric methods indicates that Vps15p constitutes less than 0.002% of the total yeast cell protein in logarithmically growing cultures (data not shown). Pulse-chase experiments indicate that Vps15p is a stable protein with a half-life of at least 60 min (Figure 4). It is interesting to note that a relatively high level of Vps15p overproduction does not result in a vacuolar protein sorting defect (see Figure 6B).

The absence of a signal sequence and any potential transmembrane domains in the predicted Vps15p sequence suggests that this protein does not enter the secretory pathway. Immunoprecipitation experiments with yeast cells treated with tunicamycin, a potent inhibitor of N-linked glycosylation, are consistent with this prediction. The Vps15p detected in tunicamycin-treated cells migrates with the same apparent molecular weight as the wild-type protein on SDS–polyacrylamide gels, suggesting that none of the 15 potential N-linked glycosylation sites within the Vps15p sequence are utilized (Figure 4). Two additional observations indicate that Vps15p is in contact with the cytosol and not sequestered within the lumen of an intracellular organelle. First, protease protection experiments with osmotically lysed spheroplasts indicate that Vps15p is exposed to the yeast cytoplasm. In these

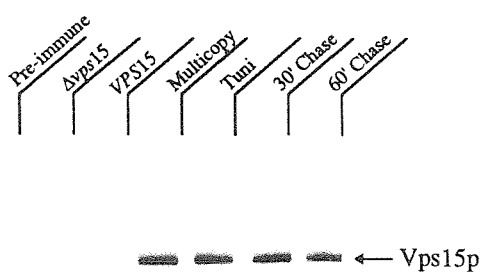

Figure 4. Identification and Characterization of the Vps15 Protein

Either eight (Δ*vps15* and *VPS15*) or two (all other lanes) OD_{600} units of yeast cells were labeled with Tran^{35}S-label in YNB glucose minimal medium for 20 min at 30°C. The radiolabeled cells were broken with glass beads and immunoprecipitations were performed from the clarified cell extracts as described in Experimental Procedures. In the first lane (Pre-immune), SEY6210 cells harboring the *VPS15* multicopy plasmid pPHY15E were immunoprecipitated with the preimmune control sera. In all other lanes, the Vps15p immune sera were used. The next three lanes show the effect of *VPS15* gene dosage on the synthesis levels of the ~170 kd Vps15p. No protein is detected in PHY112 cell extracts (Δ*vps15*). This protein is overexpressed 30-to 50-fold in SEY6210 cells possessing the pPHY15E plasmid (Multicopy) relative to the level seen in SEY6210 cells (*VPS15*). To assess the N-linked carbohydrate modification, SEY6210 cells with the plasmid pPHY15E were pretreated with 20 μg/ml tunicamycin for 15 min prior to labeling (Tuni). In the last two lanes, methionine and cysteine were added to a final concentration of 2 mM and the cultures were chased for 30 min or 60 min prior to cell lysis (30' Chase and 60' Chase). The position of Vps15p (~170 kd) is indicated.

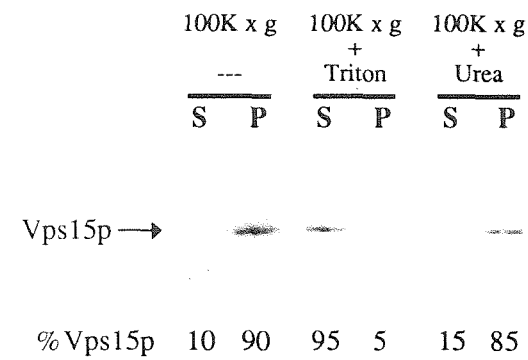

Figure 5. Subcellular Fractionation of the Vps15 Protein

SEY6210 cells harboring the *VPS15* multicopy plasmid pPHY15E were spheroplasted, labeled with Tran^{35}S-label, and osmotically lysed. The clarified cell lysates were incubated for 10 min at 0°C with either 2% Triton X-100, 2 M urea, or no additions and then centrifuged at 100,000 × g for 30 min at 4°C. Quantitative immunoprecipitations were performed from the supernatant and pellet fractions with antisera specific for Vps15p. A comparison of the relative levels of Vps15p detected following centrifugation relative to the level found in the total cell extract indicated that Vps15p recovery was greater than 80%.

experiments, spheroplasts were labeled with Tran^{35}S-label and gently lysed by the addition of DEAE–dextran under conditions that disrupt the yeast plasma membrane but maintain the structural integrity of internal organelles (see Klionsky and Emr, 1990). Following this lysis, Vps15p was found to be completely degraded by exogenously added proteinase K, whereas lumenal constituents of the ER, Golgi, and vacuolar compartments were resistant to this proteolysis (data not shown). In addition, biochemical labeling of yeast cells with tritiated myristic acid has demonstrated that Vps15p is myristoylated in vivo (P. Herman, unpublished data). Since the yeast NMT enzyme activity is cytoplasmic (see Towler et al., 1988a), the N-terminus of Vps15p must be exposed to the yeast cytosol. These data clearly demonstrate that Vps15p is in contact with the yeast cytosol.

We used differential centrifugation techniques to determine more precisely the intracellular location of Vps15p in vivo. In our initial experiments, yeast spheroplasts were radiolabeled and osmotically lysed, and the clarified lysates were immediately centrifuged at 100,000 × g for 30 min. Vps15p was then immunoprecipitated from the resulting supernatant and pellet fractions. Greater than 90% of Vps15p was detected in the pellet fraction, indicating that this protein is associated with a particulate fraction of yeast cell extracts (Figure 5). The nature of this Vps15p association was investigated by treating the clarified yeast lysates with either 2 M urea, 2% Triton X-100, or 1 M NaCl prior to centrifugation. Although treatment with 2 M urea and 1 M NaCl had very little effect, extraction with 2% Triton X-100 resulted in the solubilization of >95% of the particulate Vps15p (Figure 5 and data not shown). These data therefore suggest that Vps15p is associating with a membrane fraction of yeast cell extracts. This is especially interesting since the N-terminus of Vps15p possesses a consensus site for the addition of myristic acid and appears to be myristoylated in vivo. The N-terminal myristoylation of several proteins, including the product of the c-*src* proto-oncogene and the α subunits of G proteins, has been demonstrated to be essential for their membrane association (Buss et al., 1986; Jones et al., 1990).

In an attempt to characterize further the membrane association of Vps15p, radiolabeled yeast spheroplasts were osmotically lysed and subjected to a set of differential centrifugations, and the relative level of Vps15p within each fraction was assessed by immunoprecipitation. The majority, ~90%, of Vps15p was detected in the P100 fraction (Table 2). In contrast, p1 CPY (a marker for ER and early Golgi; Franzusoff and Schekman, 1989), p2 CPY (marker for Golgi; Stevens et al., 1986), and mature alkaline phosphatase (vacuolar membrane protein; Klionsky and Emr, 1990) were all found predominantly in the P13 fraction (Table 2). Interestingly, Kex2p, which is probably a resident of a late Golgi compartment (Julius et al., 1984), is also found largely within the P100 fraction (Table 2). Therefore, Vps15p exhibits a fractionation profile very similar to that of Kex2p. In addition to this late Golgi compartment, the P100 fraction probably includes the vesicular intermediates that transit between secretory pathway organelles (Walworth et al., 1989), presumably including those vesi-

Table 2. Differential Centrifugation with the Vps15 Protein

	P13	P100	S100
Vps15p	5%	90%	5%
p1 CPY	90%	5%	5%
p2 CPY	80%	15%	5%
ALP	90%	10%	<2%
Kex2p	25%	75%	<2%
PGK	5%	5%	90%

The relative levels of Vps15p and relevant protein markers in the 13,000 × g pellet (P13), 100,000 × g pellet (P100), and 100,000 × g supernatant (S100) fractions of yeast cell extracts were assessed by quantitative immunoprecipitation with the appropriate antisera as described in Experimental Procedures. PGK is a marker for the cytoplasmic fraction, p1 CPY for the ER and early Golgi compartments, p2 CPY for more distal Golgi compartments, alkaline phosphatase for the vacuolar membrane, and Kex2p for a late Golgi compartment.

cles trafficking between the Golgi and vacuolar compartments. Therefore, these experiments suggest that Vps15p is associating with a late Golgi compartment and/or membrane vesicles in yeast cells.

The Vps15p Kinase Domain Is Required for Vacuolar Protein Localization

To assess the functional relevance of the observed sequence similarity between Vps15p and protein kinases, we altered specific residues within the Vps15p kinase domain and examined the phenotypic consequences of these changes. Two different kinase domain mutations, each in a separate protein kinase subdomain, were constructed and analyzed. We based our *VPS15* mutations on previous mutational analyses performed on other known protein kinases. In the tyrosine kinase pp60^{src}, a series of single amino acid alterations was incorporated into the highly conserved A-P-E sequence triplet (Bryant and Parsons, 1984). All of the changes, including a glutamic acid to lysine substitution, resulted in dramatically lowered protein kinase activity and in the loss of pp60^{src} transforming ability. Based on this study, we constructed a mutant *vps15* allele that encoded a protein with an arginine residue replacing the glutamic acid normally present at amino acid 200 within the A-P-E sequence motif of Vps15p (E_{200}→R; Figure 6).

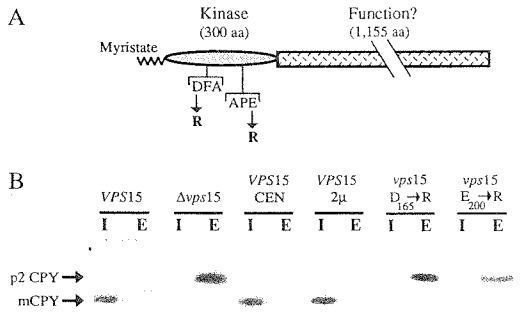

Figure 6. Intracellular Sorting of CPY

(A) A schematic representation of Vps15p showing the potential N-terminal myristic acid modification, the 300 amino acid protein kinase domain, and the large C-terminal domain of unknown function. The two kinase domain alterations used throughout this study are shown: D_{165}→R and E_{200}→R.

(B) Yeast spheroplasts were labeled with Tran^{35}S-label for 20 min at 30°C and then chased for 30 min following the addition of methionine and cysteine to 2 mM. The labeled cultures were centrifuged for 2 min at 13,000 × g and separated into pellet (I, intracellular) and supernatant (E, extracellular) fractions. The level of CPY in each fraction was assessed by quantitative immunoprecipitation with antisera to CPY. The strains examined were SEY6210 (*VPS15*), PHY112 (Δ*vps15*), PHY112 with the pPHY15C plasmid (*VPS15* CEN), PHY112 with the pPHY15E plasmid (*VPS15* 2μ), PHY112 with the pPHY15C-K1 plasmid (*vps15* D_{165}→R), and PHY112 with the pPHY15C-K2 plasmid (*vps15* E_{200}→R). The positions of mature and p2 CPY are indicated. Neither of the kinase mutant alleles is able to complement the Δ*vps15* CPY sorting defect.

For the second kinase domain mutant, a *vps15* allele that encoded a protein with an arginine substituted for aspartic acid at position 165 was constructed (D_{165}→R; Figure 6). An alteration of the corresponding aspartic acid in the product of the *CDC28* gene of S. cerevisiae results in a loss of in vitro protein kinase activity and *CDC28* gene function (Mendenhall et al., 1988). Plasmids encoding either the wild-type or one of the kinase domain mutant forms of Vps15p were introduced into Δ*vps15* cells (PHY112; see Table 1). The ability of the mutant *vps15* alleles to complement the vacuolar protein sorting defects associated with the Δ*vps15* mutant was examined. In Δ*vps15* cells, greater than 95% of the newly synthesized CPY is secreted from the cell as a Golgi-modified p2 precursor (Figure 6B; see above). Introduction of the wild-type *VPS15* gene into the null mutant completely corrects this CPY sorting defect (Figure 6B). In contrast, neither of the *VPS15* kinase domain mutants is able to even partially complement the Δ*vps15* protein sorting defect (Figure 6B), indicating that these mutations result in the biological inactivation of the *VPS15* gene product. These *vps15* mutant alleles are also unable to complement the ts growth defects and osmotic sensitivity associated with the Δ*vps15* yeast strains (data not shown).

Pulse-chase immunoprecipitation experiments demonstrated that the defects associated with these altered forms of Vps15p are not due to decreased synthesis rates or decreased stability of the mutant proteins (see below). A wild-type Vps15p kinase domain therefore is required for the delivery of proteins to the yeast vacuole, suggesting that protein phosphorylation may be involved in the regulation of this protein sorting process.

Phosphorylation of Vps15p Requires a Wild-Type Vps15p Kinase Domain

Many protein kinases appear to catalyze specific autophosphorylation reactions (Krebs, 1986), although the biological significance of this phosphorylation in most cases is not known. We attempted to analyze whether Vps15p participated in a similar autophosphorylation reaction by first determining if this protein is phosphorylated in vivo and then whether or not this phosphorylation reaction is affected by mutations in the Vps15p kinase domain. To test if Vps15p is a phosphoprotein, we radiolabeled yeast cells with $^{32}PO_4$, lysed the cells with glass

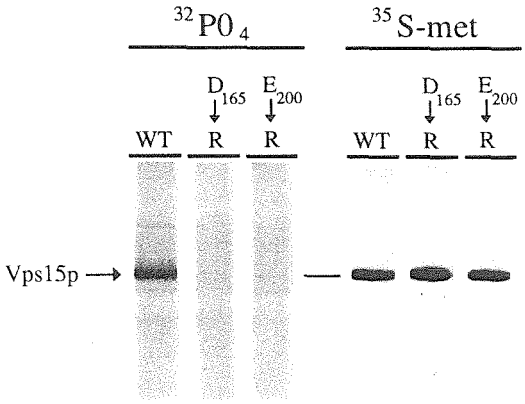

Figure 7. In Vivo Phosphorylation of the Vps15 Protein

Yeast cells were labeled with $^{32}PO_4$ for 20 min at 30°C and immunoprecipitated with antisera specific for Vps15p as described in Experimental Procedures. PHY112 (Δ*vps15*) cells harboring the following plasmids were examined: pPHY15E (WT), pPHY15E-K1 (D_{165}→R), and pPHY15E-K2 (E_{200}→R). The same strains were also labeled with Tran^{35}S-label for 20 min at 30°C, chased with methionine and cysteine (2 mM) for either 0, 30, 60 or 90 min, and then immunoprecipitated with the Vps15p antisera to assess the relative synthesis rates and stability of the three forms of Vps15p. Similar amounts of the three proteins were detected at all chase times; the 30 min chase point is shown. The position of Vps15p (~170 kd) is indicated.

beads, and then performed immunoprecipitations with antisera specific for Vps15p. The antisera detected a single labeled protein of ~170 kd in the extracts of yeast cells harboring a multicopy *VPS15* plasmid, indicating that Vps15p is phosphorylated in vivo (Figure 7). The dependence of this phosphorylation on the presence of a wild-type Vps15p kinase domain was examined by analyzing the phosphorylation of the proteins encoded by the two kinase domain mutant alleles in identical labeling experiments. Both of these mutant proteins were very poorly phosphorylated; the signal was decreased greater than 20-fold relative to that of the wild-type protein (Figure 7). However, both mutant proteins exhibit wild-type synthesis rates and stability (Figure 7), indicating that the low levels of phosphate incorporation are not due to reduced levels of these proteins in the cell. Therefore, the Vps15p kinase domain appears to be required for the in vivo phosphorylation of this protein.

Discussion

VPS15 gene function is required for the efficient delivery of soluble proteins to the yeast vacuole. With the aim of understanding the molecular role of the *VPS15* gene product in this protein sorting process, we have cloned and sequenced the wild-type *VPS15* gene and initiated a characterization of its protein product. A comparison of the predicted amino acid sequence of Vps15p with other known protein sequences revealed that Vps15p exhibits significant sequence similarity to the catalytic domains of the serine/threonine family of protein kinases (Figure 3). The observed sequence conservation is clustered about regions of the kinase domain that are very highly conserved among all known protein kinases (Hunter, 1987; Hanks et al., 1988). These sequence elements have been implicated in the ATP-binding and phosphotransferase activities associated with protein kinases (Brenner, 1987; Hanks et al., 1988). This sequence similarity therefore raises the interesting possibility that Vps15p is a protein kinase and that protein phosphorylation reactions regulate specific steps of the yeast vacuolar protein delivery pathway.

We constructed mutations within two separate regions of the Vps15p kinase domain (D_{165}FA→RFA and APE_{200}→APR) and assessed their effects on *VPS15* gene function and Vps15p phosphorylation. Phosphate labeling experiments demonstrated that Vps15p is phosphorylated in vivo (Figure 7). Furthermore, this phosphorylation was dependent on the presence of a wild-type Vps15p kinase domain, since both kinase domain mutant forms of Vps15p are very poorly phosphorylated in vivo (Figure 7). Alterations at the corresponding amino acid residues of other known protein kinases have been demonstrated to result in a dramatic decrease in associated kinase activity (Bryant and Parsons, 1984; Mendenhall et al., 1988). These data are therefore consistent with this kinase-like domain of Vps15p catalyzing a specific autophosphorylation reaction.

An alternative explanation is that the introduced amino acid changes alter Vps15p structure such that it can no longer serve as a substrate for another yeast protein kinase. However, several observations suggest that this latter possibility is unlikely. The Vps15 kinase domain mutant proteins are as stable as wild-type Vps15p in vivo, and, like the wild-type protein, these mutant proteins are also associated with the P100 fraction of yeast cell extracts (P. H., unpublished data). In addition, we have recently constructed a third mutation in a separate kinase subdomain of Vps15p (D_{147}→T of the DIKTEN sequence motif) and have found that this change also results in a significant decrease in the Vps15p phosphorylation signal (J. H. S. and P. H., unpublished data). Therefore, the alteration of three distinct Vps15p kinase domain motifs results in a dramatic loss of Vps15p phosphorylation in vivo. Altogether, these data suggest a direct role for the Vps15p kinase domain in the regulation of specific protein phosphorylation reactions. The two aspartic acid residues that were altered in Vps15p, D_{147} and D_{165}, correspond to amino acids that are conserved in a variety of phosphotransferase systems, including protein kinases and aminoglycoside phosphotransferases (Brenner, 1987). It has been suggested that these two aspartic acid residues may bind the ATP phosphate groups through an intermediate Mg^{2+} ion (Brenner, 1987), and it is therefore very interesting that changes in either of these residues result in a decrease in Vps15p activity.

We have demonstrated a functional role for the Vps15p kinase domain by analyzing vacuolar protein sorting in yeast cells possessing only the kinase-deficient forms of Vps15p. In these cells, as with Δ*vps15* cells, greater than 95% of the newly synthesized CPY is secreted in a Golgi-

modified precursor form (Figure 6B). Therefore, mutational alteration of the Vps15p kinase domain results in the biological inactivation of this protein. This, together with our observations concerning the dependence of Vps15p phosphorylation on the Vps15p kinase domain, suggests a role for protein phosphorylation in the vacuolar protein delivery pathway.

Although a high degree of sequence conservation is observed between protein kinases and Vps15p, relatively weak similarity is detected in the most N-terminal regions of the kinase domain (Figure 3). These sequence differences suggest that this protein may be a novel kinase homolog. This region of the kinase catalytic domain usually contains a Gly-X-Gly-X-X-Gly/Ser sequence element (where X refers to any amino acid), which has been termed a "nucleotide-fold" (see Walker et al., 1982). A similar sequence motif has been observed in both ATP- and GTP-binding proteins (Walker et al., 1982; McCormick et al., 1985; Dever et al., 1987), and X-ray crystallographic data of E. coli elongation factor Tu and the human c-H-*ras* protein have suggested that this subdomain interacts with the phosphate groups of the bound nucleotide (la Cour et al., 1985; de Vos et al., 1988). In Vps15p, the first and second glycine residues of this motif are replaced with a serine and an alanine residue, respectively (Figure 3). Since it has been suggested that an essential feature of the conserved glycines in this region is their small size (de Vos et al., 1988), it is possible that the conservative sequence substitutions observed in Vps15p may not alter the function of this kinase subdomain. A recently identified protein kinase homolog encoded by the Drosophila melanogaster *ninaC* locus has the sequence Ala-X-Gly-X-X-Ala, where alanines replace two of the glycines usually found in this sequence motif (Montell and Rubin, 1988).

Another interesting possibility is that Vps15p constitutes only a partial protein kinase domain. Vps15p may be part of a hetero-oligomeric complex in which Vps15p contributes the majority of the kinase catalytic domain and a second protein in the complex contributes the nucleotide-fold region and nearby sequences. Different kinase molecules already display some level of structural diversity with respect to the arrangement of their regulatory and catalytic domains. In some protein kinases, both domains are present on the same polypeptide chain (e.g., EGF receptor; see Ullrich and Schlessinger, 1990), while in others, these domains are present on distinct proteins (e.g., cAMP-dependent protein kinase; see Edelman et al., 1987). If the Vps15p kinase catalytic domain does possess such a novel structural arrangement, then it may be possible to identify a candidate gene that encodes this second component by examining the in vivo phosphorylation of Vps15p in other *vps* mutant backgrounds.

A more complete understanding of the role of Vps15p in vacuolar protein sorting will require the identification of other cellular components that functionally interact with this protein. The previous identification of a large number of *vps* mutants provides us with a starting point for this analysis. Potential regulators of Vps15p activity might be identified by assessing the extent of Vps15p phosphorylation in other *vps* mutant backgrounds. In addition, an analysis of the in vivo phosphorylation of other *VPS* gene products in *VPS15* and *vps15* yeast strains could identify potential substrates of the Vps15p kinase. Interestingly, two lines of evidence suggest that the products of the *VPS15* and *VPS34* genes may functionally interact in vivo (J. H. S. and P. H., unpublished data; see Herman and Emr, 1990). The overproduction of Vps34p in *vps15* kinase domain mutants partially suppresses both the growth defects and the vacuolar protein missorting defects associated with these mutants. In addition, chemical cross-linking experiments in total yeast cell extracts have indicated that Vps15p and Vps34p physically interact. Preliminary experiments also have demonstrated that Vps34p is a phosphoprotein in vivo, and we are currently analyzing this phosphorylation in kinase-deficient *vps15* yeast strains.

Subcellular fractionation and protease protection experiments (Figure 5 and Table 2) indicate that Vps15p is associated with the cytoplasmic face of an intracellular membrane, probably that of a late Golgi compartment or of a transport intermediate between the Golgi complex and the vacuole (e.g., vesicles). We are therefore presented with the problem of understanding how a protein kinase present on the cytoplasmic side of a secretory pathway organelle(s) is able to influence the delivery of specific lumenal constituents within this compartment. Some insight into this problem might be provided by observations from other systems that implicate protein phosphorylation as a key regulator of protein sorting. Specific protein phosphorylation reactions appear to act within protein sorting pathways at branch positions where proteins must choose between two or more different transport fates. The proper sorting of two transmembrane receptors within an early endosomal compartment appears to be dependent on specific protein phosphorylation reactions. Wild-type EGF receptors are targeted for lysosomal degradation, whereas kinase-inactive EGF receptors are observed to recycle back to the cell surface (Felder et al., 1990; Honegger et al., 1990). In addition, the phosphorylation of a specific serine residue within the cytoplasmic tail of the polymeric immunoglobulin receptor is required for its efficient transcytosis across polarized epithelial cells (Casanova et al., 1990). In the absence of this phosphorylation, these receptors appear to recycle back to the cell surface. In this study, we observed that a wild-type Vps15p kinase domain is required for the localization of proteins to the yeast vacuole. In each of these examples, the diversion of proteins from what is likely to be a default transport pathway into an auxiliary route appears to require specific protein phosphorylation reactions. Therefore, protein phosphorylation may act as a molecular "switch" within intracellular protein sorting pathways by actively diverting proteins from a default route into an alternative delivery pathway.

Other studies of eukaryotic protein secretion have suggested that GTP hydrolysis is an essential step for the vesicular transport of proteins between all secretory compartments (Balch, 1989) and that each interorganellar transfer event appears to involve a unique GTP-binding protein (see, for example, Salminen and Novick, 1987;

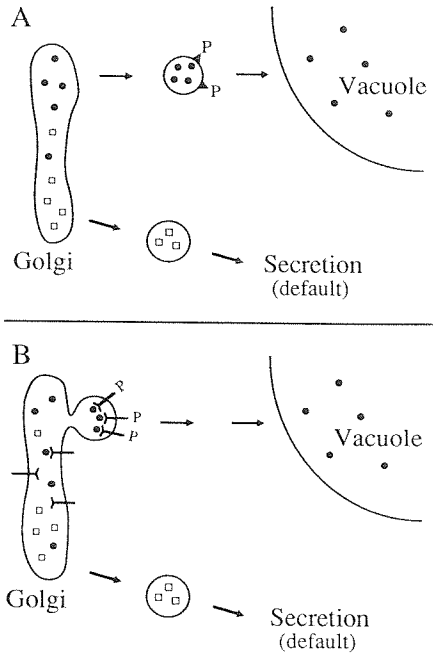

Figure 8. Possible Roles for Vps15p-Mediated Protein Phosphorylation in Vacuolar Protein Sorting

In (A), the phosphorylation of a specific vesicle surface protein designates that vesicle for delivery to, or fusion with, the vacuole. Alternatively, in (B), Vps15p-mediated phosphorylation might be required for the packaging of vacuolar proteins into the appropriate transport vesicles or for the formation of these transport vesicles. The phosphorylation of the cytoplasmic tails of transmembrane receptors specific for soluble vacuolar proteins may direct their proper packaging into transport vesicles destined for the yeast vacuole. The sorting of proteins to the yeast vacuolar compartment has been shown to be an active process that requires the recognition of specific sorting determinants present within the polypeptide backbone of vacuolar proteins. In the absence of this recognition, vacuolar proteins are secreted from yeast cells (reviewed in Klionsky et al., 1990; shown as the default pathway). See the text for further discussion.

Segev et al., 1988). It has been proposed that these GTP-binding proteins may be responsible for the unidirectionality observed in secretory protein traffic (Bourne, 1988) and may possibly designate a transport vesicle's final destination (Goud et al., 1988). Protein phosphorylation could then be thought of as a second, or an alternative, level of control superimposed upon this basic regulatory circuitry at specific branchpoints within a protein delivery pathway. In vacuolar protein sorting, Vps15p might function as such a switch in at least two different ways (Figure 8). The Vps15p-mediated phosphorylation of a specific vesicle surface protein (possibly Vps15p itself) could serve to direct the delivery, or fusion, of this transport carrier to the vacuolar membrane.

Alternatively, Vps15p might function in earlier steps of this sorting pathway, possibly in the selective packaging of vacuolar proteins into their appropriate vesicular carriers or in the formation of these vesicles. The observation that *vps15* cells are competent for vacuole assembly is most consistent with the latter packaging model, in which vesicular traffic to the vacuole could proceed almost normally except that the vesicles would lack much of their usual lumenal content. Vps15p might facilitate the selective packaging of appropriate vacuolar proteins by phosphorylating the cytoplasmic tails of transmembrane receptors specific for these soluble vacuolar hydrolases. Through the application of genetics together with a recently developed in vitro reconstitution assay for vacuolar protein sorting (Vida et al., 1990), we hope to be able to develop an understanding of the precise role Vps15p plays in the vacuolar protein sorting pathway of yeast. This understanding should provide insights into the more general role of protein phosphorylation in intracellular protein sorting processes.

Experimental Procedures

Strains and Media

The S. cerevisiae and E. coli strains used in this study are listed in Table 1. Standard genetic techniques were used to construct the yeast strains (Sherman et al., 1979). Standard yeast (Sherman et al., 1979) and E. coli (Miller, 1972) media were used and supplemented as needed.

Recombinant DNA Methods

All recombinant DNA manipulations were performed as described previously (Maniatis et al., 1982; Ausubel et al., 1987). The 4.8 kb *VPS15*-containing fragment from the upstream HindIII site to the SnaBI site in pPHY15-1 (see Figure 1A) was subcloned into either pSEYC58 or pSEY8 (Emr et al., 1986) to produce pPHY15C or pPHY15E, respectively. The plasmid pPHY15Δ1 was constructed by first subcloning the 3.8 kb XhoI–SalI fragment of pPHY15-1 (Figure 1A) into pUC8 and then replacing the 2.2 kb ClaI–StuI fragment within *VPS15* with the yeast *HIS3* gene (Figure 1B). The second *VPS15* disruption plasmid, pPHY15Δ2, was made by subcloning the 1.1 kb EcoRI–KpnI fragment of pPHY15-1 into the yeast *TRP1*-integrating vector pPHYI10 (Herman and Emr, 1990). The 3.8 kb XhoI–SalI fragment of pPHY15-1 was subcloned into pPHYI10 to produce the integrative mapping plasmid, pPHY122. The plasmid pPHY135 was constructed by subcloning the 1.4 kb EcoRI fragment of pPHY15-1 into pBluescript II KS$^+$ (Stratagene). The 1.2 kb ClaI fragment of pPHY135 was then subcloned into the ClaI site of pATH2 to construct a gene fusion of the E. coli *trpE* gene and *VPS15*.

Site-directed mutagenesis of the *VPS15* gene was performed using *dut*$^-$ *ung*$^-$ E. coli as previously described (Kunkel, 1985; Ausubel et al., 1987). The 1.8 kb XhoI–XbaI fragment of pPHY15-1 was subcloned into SalI- and XbaI-digested M13mp18 RF DNA to produce M13V15. Single-stranded uracil-containing M13V15 DNA was isolated from the E. coli strain BW313 (*dut*$^-$ *ung*$^-$), and the oligonucleotides 5′-GTATATTGACGCGTTTTGCTGCA-3′ and 5′-TCTAGCCCCGCGGAGGTTTAAC-3′ were used to mutagenize the *VPS15* sequences to produce M13V15-K1 and M13V15-K2, respectively. The mutagenized *VPS15* sequences were moved back into the full-length *VPS15* gene by exchanging the 2.4 kb HindIII fragment of pPHY15C with the mutated HindIII fragment from M13V15-K1 or M13V15-K2 to produce pPHY15C-K1 and pPHY15C-K2, respectively. Equivalent exchanges were performed with the pPHY15E plasmid to produce pPHY15E-K1 and pPHY15E-K2, respectively. K1 corresponds to a change of D_{165} to R and K2 to a change of E_{200} to R (see Figure 6A).

Yeast Genetic Methods

Standard yeast genetic methods were used throughout this study (Sherman et al., 1979). Yeast transformation was achieved by the method of alkali cation treatment (Ito et al., 1983) and transformants were selected on SD media.

The *VPS15* gene was cloned by complementation of the severe ts growth defect associated with the *vps15-2* allele. SEY15-2 cells (*vps15-2*, *ura3-52*) were transformed with a yeast genomic DNA library constructed in the YEp24 plasmid (DNA kindly provided by M. Carlson;

Carlson and Botstein, 1982). Ura$^+$ transformants were selected at 26°C and were subsequently replicated to 37°C YPD plates. Plasmids conferring a temperature-resistant phenotype upon the cells were isolated and analyzed.

Two different schemes were used to generate gene disruption/deletions of the *VPS15* locus. In the first, the plasmid pPHY15Δ1 was digested with HindIII and SalI and the 2.3 kb fragment was gel purified. This linearized DNA was then used to transform either the diploid strain SEY6210.5 or the haploid strains SEY6210 and SEY6211 (see Table 1) to histidine prototrophy. With the second method, an integrative disruption of the *VPS15* gene was constructed by digesting pPHY15Δ2 DNA with EcoRV (the EcoRV site is internal to the *VPS15* EcoRI–KpnI fragment) and transforming the diploid yeast strain SEY6210.5 to tryptophan prototrophy. Integration would result in the replacement of the wild-type *VPS15* locus with two noncomplementing, but overlapping, fragments of the *VPS15* gene. In 23 tetrads examined the Trp$^+$ phenotype segregated 2:2 and absolutely cosegregated with a ts growth defect. In both constructions, the genomic DNA structure about the *VPS15* locus was verified with a PCR amplification method previously described (Herman and Emr, 1990).

For integrative mapping, the plasmid pPHY122 was digested with EcoRV to direct its integration to the chromosomal homolog of the cloned DNA (Rothstein, 1983). SEY15-2 cells were transformed with this linearized DNA, and four independent Trp$^+$ transformants were analyzed and found to be temperature resistant. (The XhoI–SalI fragment encodes a truncated form of Vps15p that complements the *vps15-2* ts growth defect.) Two of these transformants were crossed to SEY6211, and the resultant diploids were sporulated and their meiotic progeny were analyzed by tetrad analysis. A 4 Ts$^+$:0 Ts$^-$ segregation pattern was observed for all 21 tetrads analyzed, indicating that the identified cloned DNA represented the authentic *VPS15* locus.

DNA Sequencing and Sequence Analysis

Restriction fragments encompassing the region from the XhoI site to the rightmost ClaI site in Figure 1A were subcloned into the appropriate pBluescript vectors and sequenced using standard dideoxy chain termination methods (Sanger et al., 1977) as previously described (Herman and Emr, 1990).

The predicted protein sequence of the *VPS15* gene product was compared with the contents of the National Biomedical Research Foundation (NBRF) protein data base and the GenBank DNA data base with the FASTA and TFASTA programs (Pearson and Lipman, 1988), respectively. The comparisons were performed with the University of Wisconsin Genetics Computer Group sequence analysis package for VAX/VMS computers (Devereux et al., 1984).

Preparation of Vps15p Antisera

A gene fusion was constructed between the E. coli *trpE* gene and *VPS15* using the pATH vector system (Dieckmann and Tzagoloff, 1985). The 1.2 kb ClaI–EcoRI fragment of *VPS15* was subcloned into the pATH2 polylinker (see above for details) to produce the plasmid pPHY139. This plasmid encodes a trpE–Vps15 fusion protein containing 246 amino acids of Vps15p, from amino acid number 21 to 266. The trpE–Vps15p fusion was induced, prepared, and used to immunize New Zealand White male rabbits as described previously (Herman and Emr, 1990).

Cell Labeling and Immunoprecipitation

Immunoprecipitations from whole yeast cells labeled with Tran^{35}S-label (ICN Radiochemicals) were performed as described previously (Herman and Emr, 1990) except that urea-cracking buffer (10 mM sodium phosphate [pH 7.2], 6 M urea, 1% SDS, 1% β-mercaptoethanol) was substituted for the boiling buffer and the final sample buffer. If Vps15p is boiled in normal SDS-containing buffers, in the absence of urea, it forms an aggregate that is unable to enter the SDS–polyacrylamide resolving gel. This molecular aggregate is efficiently dissociated by the addition of urea. To assess N-linked oligosaccharide modification, yeast cells were incubated in the presence of 20 μg/ml tunicamycin for 15 min prior to labeling. The CPY fractionation immunoprecipitations were performed as previously described (Robinson et al., 1988). Radiolabeled proteins were electrophoresed on 8% SDS–polyacrylamide gels. Following electrophoresis the gels were fixed in 50% methanol, 10% acetic acid, and 10% trichloroacetic acid (TCA) and treated with Autofluor (National Diagnostics).

The subcellular fractionation experiments were performed as described previously (Herman and Emr, 1990) with the following modifications. The yeast strain SEY6210, harboring the plasmid pPHY15E, was grown to mid-logarithmic phase, spheroplasted, and labeled with Tran^{35}S-label for 15 min at 30°C. The cells were then osmotically lysed and the resulting clarified lysate was centrifuged at 13,000 × g for 15 min at 4°C. The 13,000 × g supernatant was carefully removed and centrifuged at 100,000 × g for 30 min at 4°C. The 100,000 × g supernatant fraction (S100) was made 5% with respect to TCA and precipitated for 20 min on ice. The 13,000 × g pellet (P13) and 100,000 × g pellet (P100) fractions were resuspended in 5% TCA and also held on ice for 20 min. Immunoprecipitations were performed as described above with the appropriate antiserum. The antiserum to PGK was a gift from Dr. Jeremy Thorner and to Kex2p from Dr. William Wickner. The antisera to CPY (Robinson et al., 1988) and alkaline phosphatase (Klionsky and Emr, 1990) were described previously. The extraction studies with Vps15p were performed as previously described (Herman and Emr, 1990).

In Vivo Phosphorylation Assays

Yeast cells were grown to mid-logarithmic phase in Wickerham's minimal medium supplemented with 0.2% yeast extract (Wickerham, 1946; Robinson et al., 1988). Five OD_{600} units of cells were collected by centrifugation, resuspended in 1 ml of LPSM medium (Reneke et al., 1988) supplemented with 5 mM $MgSO_4$, and incubated for 30 min at 30°C. Bovine serum albumin (to a final concentration of 1 mg/ml) and 500 μCi of $^{32}PO_4$ (Amersham) were then added, and the cells were incubated for 20 min at 30°C. The labeling was terminated by the addition of TCA to a final concentration of 5%. The TCA pellet was washed twice with acetone, dried, and resuspended in 100 μl of the urea-cracking buffer. One milliliter of Tween-20 immunoprecipitation buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.1 mM EDTA, 0.5% Tween-20) and 10 μl of a 100 mg/ml bovine serum albumin solution were added, and immunoprecipitations were performed with antisera to Vps15p as described above except that the protein A–Sepharose beads were washed as follows: twice with Tween-20 IP buffer; once with Tween–urea buffer (100 mM Tris–HCl [pH 7.5], 200 mM NaCl, 2 M urea, 0.5% Tween-20); once with IP salts buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.1 mM EDTA); once with 1% β-mercaptoethanol; and once with 0.5 M LiCl, 0.1 M Tris–HCl (pH 7.5).

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Chapter 5:

A Genetic and Structural Analysis of the Yeast Vps15 Protein Kinase: Evidence of a Direct Role for Vps15p in Vacuolar Protein Delivery

INTRODUCTION

The cytoplasmic environment of eukaryotic cells is subdivided into a number of functionally distinct membrane-enclosed organelles. In order to maintain the functional integrity of these subcellular organelles, specific mechanisms must exist to allow the cell to efficiently sort and deliver proteins from their common site of synthesis in the cytoplasm to their appropriate final destinations. In particular, the secretory pathway of eukaryotic cells is responsible for the proper modification and delivery of proteins to the cell-surface and to a variety of intracellular compartments and is itself composed of a series of membrane-enclosed organelles (Pfeffer and Rothman, 1987). The delivery of proteins to the lysosomal, or vacuolar, compartment of eukaryotic cells is mediated by the secretory pathway and is one of the best characterized examples of an intracellular protein sorting process (Kornfeld and Mellman, 1989; Klionsky et al., 1990). Following translocation across the membrane of the endoplasmic reticulum, lysosomal proteins transit on to the Golgi complex together with proteins destined for secretion from the cell. Within a late Golgi compartment, lysosomal proteins are sorted away from the secretory protein traffic and are targeted to the lysosome. This rerouting of proteins from the default secretion path is an active process requiring specific sorting information present within lysosomal proteins. In mammalian cells, the best characterized lysosomal sorting system involves the addition of a specific carbohydrate moiety, mannose-6-phosphate, to a subset of soluble lysosomal proteins (Kaplan et al., 1977; reviewed in Kornfeld and Mellman, 1989). This carbohydrate modification is recognized in the Golgi apparatus by specific membrane receptors that mediate delivery of the modified proteins to the lysosome. In contrast, the cis-acting sorting information present in several yeast vacuolar proteins is not associated with any specific carbohydrate modification and instead appears to reside within the polypeptide backbone of these proteins (Johnson et al., 1987; Valls et al., 1987; Klionsky et al., 1988; Klionsky and Emr, 1990). It is possible that these yeast

vacuolar proteins are sorted by a mechanism similar to that used by lysosomal proteins not processed by the mannose-6-phosphate system. Despite the gains made in our understanding of the initial recognition of lysosomal and vacuolar proteins, very little is presently known about the cellular components that function subsequently to bring about the proper packaging and delivery of these proteins to the lysosomal and vacuolar compartments.

In the yeast, *Saccharomyces cerevisiae*, an extensive analysis of vacuolar protein localization has been undertaken in an attempt to develop a better understanding of the trans-acting factors responsible for mediating protein delivery to the vacuole (reviewed in Klionsky et al., 1990). Two independent genetic selections have been successful in identifying a large number of yeast mutants defective in the localization of multiple soluble vacuolar hydrolases, including carboxypeptidase Y (CPY), proteinase A (PrA) and proteinase B (PrB) (Bankaitis et al. 1986; Rothman and Stevens, 1986; Robinson et al., 1988; Rothman et al., 1989). Rather than delivering these proteins to the vacuole, *vps* mutants (for vacuolar protein sorting defective) missort these enzymes to the cell-surface as Golgi-modified precursors. In addition to these localization defects, many of the *vps* mutants also exhibit additional phenotypes that could provide insights into the precise role played by a particular *VPS* gene product *in vivo*. These phenotypes include defects in vacuole biogenesis and segregation, severe temperature-sensitive (*ts*) growth defects and vacuolar membrane protein mislocalization (Banta et al., 1988; Robinson et al., 1988; Herman and Emr, 1990; Raymond et al., 1990). Complementation analyses between the *vps* mutants, and other related sets of mutants, have demonstrated that there are at least 47 complementation groups required for the efficient targeting of vacuolar proteins in yeast (Klionsky et al., 1990). This high level of genetic complexity indicates that vacuolar protein delivery is a complex process requiring the direct, or indirect, participation of a relatively large number of gene functions. In order to gain an

understanding of the basic cellular processes underlying vacuolar, and lysosomal, protein delivery, we have initiated efforts to identify specific *VPS* gene products and to characterize, genetically and biochemically, the step(s) of the sorting pathway at which these products are acting (Banta et al., 1990; Herman and Emr, 1990; Herman et al., 1991).

Several experimental observations indicate that the yeast *VPS*15 gene product may play a central role in regulating the sorting of soluble hydrolases to the vacuole (Herman et al., 1991). First, mutations in the *VPS*15 gene result in severe defects in the localization of several vacuolar hydrolases, including CPY, PrA and PrB (Robinson et al., 1988; Herman et al., 1991). However, these defects appear to be highly specific for soluble constituents of the yeast vacuole as the localization of two vacuolar membrane proteins, alkaline phosphatase (ALP) and α-mannosidase, appears to be relatively unperturbed in *vps*15 mutants (Robinson et al., 1988; Klionsky and Emr, 1989). Second, the cloning and sequencing of the *VPS*15 gene has indicated that the amino acid sequence of the predicted Vps15 protein (Vps15p) shares significant sequence similarity to the catalytic domains of the serine/threonine family of protein kinases. Mutations altering highly conserved residues within the Vps15 protein kinase domain result in the biological inactivation of this protein. In addition, an analysis of the *in vivo* phosphorylation of the wild-type Vps15p and the kinase domain mutants demonstrated that this phosphorylation of Vps15p was dependent upon a wild-type Vps15p kinase domain. Finally, subcellular fractionation studies indicated that Vps15p may be associated with the cytoplasmic face of a late Golgi or vesicle compartment. Since vacuolar protein sorting appears to occur within a late Golgi compartment, this intracellular location is consistent with Vps15p having a direct influence upon vacuolar protein sorting. On the basis of these observations, we proposed that Vps15p-mediated phosphorylations may play a key role in regulating protein delivery to the yeast vacuole

and that protein phosphorylation reactions, in general, might act as a molecular "switch" within the eukaryotic secretory pathway to divert proteins from a default transit pathway (e.g., secretion) to an alternative pathway (e.g., to the vacuole).

In this study, we extend our mutational analysis of the Vps15p kinase domain and also examine the phenotypic consequences of alterations within the myristic acid attachment site and the large carboxy-terminal (C-terminal) domain of Vps15p. Interestingly, we find that relatively short C-terminal deletions of Vps15p result in a severe, but highly specific, temperature-conditional defect in vacuolar protein delivery. Our analysis of these *ts* defects indicates that Vps15p directly influences the delivery of soluble hydrolases to the vacuolar compartment of yeast.

MATERIALS AND METHODS

Strains and media. *Escherichia coli* strains BW313 (*F' lysA/dut ung thi-1 relA spoT1*; Kunkel, 1985) and CJ236 (*dut1 ung1 thi-1 relA1*/pCJ105 [cam^r F1]; Kunkel et al., 1987) were used for the oligonucleotide-directed mutagenesis experiments and JM101 (*F' traD36 lacI^{q}ZΔm15 proAB/supE thiDlac Δlac-pro*; Miller, 1972) was used for all other purposes. The *S. cerevisiae* strains PHY112 (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Δvps15::HIS3*; Herman et al., 1991) and SEY6210 (*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*; Robinson et al., 1988) were used for all experiments described in this study. Standard yeast and *E. coli* media were used and supplemented as needed (Miller, 1972; Sherman, 1979) .

Yeast methods. Standard yeast genetic methods were used throughout this study (Sherman, 1979). Yeast cells were transformed by the method of alkali cation treatment (Ito et al., 1983) and transformants were selected on the appropriate SD media.

Plasmid construction. Recombinant DNA manipulations were performed as described previously (Ausubel et al., 1987; Maniatis et al., 1982). Two yeast shuttle

vectors, pPHYC18 (*CEN*, *URA3*; Herman and Emr, 1990) and pJS324 (2 micron, *TRP1*) were used for most of the plasmid constructions described within this study. The yeast vector pJS324 was constructed by subcloning the 1.6 kb HpaI-HindIII fragment of YEP13, containing the yeast 2 micron plasmid origin of replication, into the AatII site of pRS304 (Sikorski and Hieter, 1989). The HindIII 3' recessed ends were filled in with Klenow polymerase and the appropriate deoxynucleotides prior to ligation. The yeast *VPS15* plasmids pPHY15-1, pPHY15C and pPHY15E were described previously (Herman et al., 1991).

Oligonucleotide-directed mutagenesis of the *VPS15* gene was performed using *dut⁻ ung⁻ E. coli,* as described previously (Kunkel, 1985; Ausubel et al., 1987). Two different mutagenesis procedures were used to generate the mutants described in Figure 1. The first scheme was used to produce the $G_2 \rightarrow A$ and $E_{151} \rightarrow R$ alterations and was described previously (Herman et al., 1991). For the second, the *VPS15* gene was subcloned as a 4.8 kb ScaI-SnaBI fragment from pPHY15-1 into the SmaI site of both pJS324 and pBluescriptII KS$^+$ (Stratagene) to produce pJS324.15 and pBP.15, respectively. These *VPS15*-containing plasmids were introduced into the *dut⁻ ung⁻ E. coli* strain CJ236 and single-stranded plasmid DNA was isolated and mutagenized as described in the BioRad MutaGene kit manual. The mutagenized *VPS15* DNA was subsequently subcloned as a BamHI-XhoI fragment into pPHYC18 and pJS324 as required. The wild-type ScaI-SnaBI fragment was also cloned into the SmaI site of pPHYC18 to generate pPHY15.1 and into pJS324 to generate pPHY15.2.

The *VPS15* C-terminal deletion plasmids were constructed by subcloning the appropriate *VPS15* restriction fragments from pPHY15-1 into the SmaI site of pPHYC18. The following restriction fragments were gel isolated and, if required, were treated with either T4 DNA polymerase to remove 3'-overhangs, or Klenow polymerase to fill in 3'-recessed ends: ScaI-BspHI (ΔC30); ScaI-HgiAI (ΔC128); and ScaI-XmnI

(ΔC167). For the double mutants combining either the $G_2 \rightarrow A$ or $E_{151} \rightarrow R$ alteration with a specific C-terminal deletion, the above fragments were excised from either pPHYC15-G_2A ($G_2 \rightarrow A$) or pPHYC15-$E_{151}R$ ($E_{151} \rightarrow R$), respectively.

The largest C-terminal deletion, removing 214 amino acids of the Vps15p, was constructed from the M13V15 mutagenesis products as follows. First, the 3.6 kb ClaI fragment of pPHY15-1 was subcloned into the ClaI site of pPHYC18 to produce the plasmid pPHY127. The 1.8 kb XbaI fragment from M13V15 RF DNA (see Herman et al., 1991), wild-type or mutagenized, was gel isolated and used to replace the XbaI fragment of pPHY127, thereby reconstructing a *VPS15* gene truncated at the 3'-internal ClaI site.

Cell labeling and immunoprecipitation. Immunoprecipitations from whole yeast cells labeled with Tran ^{35}S-label (ICN Radiochemicals) were performed as described previously (Herman and Emr, 1990; Herman et al., 1991). For the analysis of the temperature-conditional CPY processing defect in the *VPS15* C-terminal deletion mutants, yeast cells were pre-incubated at either 26° or 38°C for 5 min prior to labeling. The cells were then labeled with Tran ^{35}S-label for 10 min and chased for 30 min at the same temperature. The chase was initiated by the addition of methionine and cysteine to 25 mM. CPY fractionation immunoprecipitations from radiolabeled yeast spheroplasts were performed as described previously (Robinson et al., 1988).

For the *ts* shift experiments, typically 20 OD_{600} cell equivalents of yeast spheroplasts were labeled for 3 or 4 min at 26°C with Tran ^{35}S-label in 2.5 mls of spheroplast labeling media (Wickerham's minimal proline media supplemented with 1.3 M sorbitol; Wickerham, 1946). The culture was then divided into two aliquots of 1.5 and 1.0 mls. To the first aliquot an equal volume of 47-48°C chase solution (spheroplast labeling media plus 50 mM methionine, 50 mM cysteine, and 0.4% yeast extract) was added to rapidly bring up the culture temperature. The chase was then continued for 30

min at 38°C. To the second aliquot, an equal volume of 26°C chase solution was added and 1.0 mls were immediately removed and fractionated for subsequent immunoprecipitation as follows. The spheroplast cultures were centrifuged at 13,000 xg for 10 secs and the supernatant was carefully removed and TCA was added to a final concentration of 5%. The spheroplast pellet was resuspended in 1.0 mls of 5% TCA. The TCA precipitations were held on ice for at least 20 min. The remaining one-half of the second aliquot was chased for 30 min at 26°C. After 30 min of chase, 1.0 mls of both the 38°C and 26°C cultures were fractionated as described above. One-half, or 1.0 mls, of the 38°C culture was shifted to 26°C and the other one-half was kept at 38°C for an additional 20 min. After this additional chase, the samples were processed as described above.

For the whole cell temperature-shift experiments, a similar experimental protocol was followed except that the labeled aliquots removed for analysis were added to an equal volume of ice-cold stop solution (50 mM Tris hydrochloride, pH 7.5, 2 M sorbitol, 40 mM NaN_3, 40 mM NaF, 20 mM dithiotreitol) and held on ice for 5 min. Zymolyase-100T (Seikagako Kogyo Co.) was added to 20 $\mu g/OD_{600}$ cell equivalents and spheroplasting was carried out for 30 min at 30°C. The cultures were then fractionated as described above for the spheroplast labelings.

The *in vivo* phosphate labeling of yeast cells and the subsequent immunoprecipitation of Vps15p were performed as described previously (Herman et al., 1991) except that the protein A-Sepharose beads were washed as follows: once with Tween-20 IP buffer (0.5% Tween-20, 50 mM Tris hydrochloride, pH 7.5, 150 mM NaCl, 0.1 mM EDTA) and once with IP buffer 2 (50 mM Tris hydrochloride, pH 7.5, 150 mM NaCl, 0.1 mM EDTA).

For the analysis of Vps15p myristoylation, yeast cells were grown in Wickerham's minimal media supplemented with 0.2% yeast extract (Wickerham, 1946;

Robinson et al., 1988). Five OD_{600} units of cells were collected by centrifugation and resuspended in 1 ml of the same media. Cerulenin was added to a final concentration of 20 μg/ml and the cells were incubated for 20 min at 30°C. Following this pre-incubation, 1.0 mCi of [9,10(n)-^{3}H] myristic acid (New England Nuclear) was added and the cells were labeled for 60 min at 30°C. The labeling was terminated by the addition of TCA to a final concentration of 5%. The protein A-Sepharose beads were washed as described above for the *in vivo* phosphorylations.

RESULTS

Mutational analysis of the Vps15p kinase domain. The wild-type *VPS15* gene was cloned from a yeast genomic DNA plasmid library by complementation of the extreme *ts* growth defects associated with many of the original *vps15* alleles (Herman et al., 1991). Our initial analysis of the predicted Vps15p amino acid sequence suggested that this protein could be divided into three separate sequence domains (Fig. 1). The immediate N-terminal sequences of Vps15p form a potential attachment site for myristic acid, a rare 14-carbon fatty acid (see below). The next ~300 amino acids of Vps15p exhibit significant sequence similarity to the catalytic domains of protein kinases. Our analysis of mutations within this second domain suggest that Vps15p may function as a protein kinase *in vivo* and that specific protein phosphorylation reactions may be required for the efficient delivery of proteins to the yeast vacuole (Herman et al., 1991). The third domain includes the remaining C-terminal sequences of Vps15p. This region of Vps15p exhibited no significant similarities to any other known protein sequence. The tripartite nature of the 1455 amino acid Vps15p is shown schematically in Fig. 1.

The catalytic domains of protein kinases are modular structures with short stretches of highly conserved sequences interspersed with regions of little similarity (Hanks et al., 1988). The sequence similarities between Vps15p and other known

serine/threonine kinases are clustered within these regions of high conservation. In a previous study, we assessed the functional significance of the Vps15p kinase domain sequences by altering two of the most conserved kinase motifs of Vps15p and analyzing the phenotypic consequences of these changes. Both of the alterations, D165R (i.e., $D_{165} \rightarrow R$) and E200R, resulted in the biological inactivation of Vps15p (Herman et al., 1991; see Fig. 1). For all mutants described in this study, we assayed the biological activities of the engineered *vps15* mutant alleles by introducing these alleles into a *Δvps15* yeast strain (PHY112) on either a centromere-containing, or multicopy, yeast plasmid. The single copy plasmids were tested for their ability to complement the severe *ts* growth and CPY localization defects of the *Δvps15* yeast strains. To analyze CPY localization, we labeled yeast spheroplasts with Tran ^{35}S-label, fractionated the cultures into supernatant (extracellular) and pellet (intracellular) fractions and then immunoprecipitated with antisera specific for the vacuolar hydrolase, CPY. The *in vivo* phosphorylation of the mutant VPS15p proteins was assayed by labeling *Δvps15* yeast strains, carrying the appropriate *vps15* alleles on a 2μ multicopy plasmid, with ^{32}P-orthophosphate and then immunoprecipitating with antisera specific for Vps15p.

The first kinase motif examined includes the Vps15p sequence DIKTEN and corresponds to the kinase domain region that exhibits the highest degree of sequence similarity, in general (Hanks et al., 1988). In addition, the sequence conservation observed in this region of protein kinases also serves as an indicator of kinase substrate specificity (Hanks, 1987). The lysine residue within this sequence motif (K_{149} in the Vps15p sequence) is strictly conserved in all known serine/threonine protein kinase sequences. In tyrosine-specific protein kinases, this lysine residue is replaced with either an alanine or an arginine residue. By an oligonucleotide mutagenesis, we constructed a *VPS15* allele that encoded a protein with aspartic acid replacing this lysine at position 149 (K149D). This alteration resulted in the biological inactivation of Vps15p in each of the

three different assays. The K149D mutant protein was unable to even partially complement the *Δvps15* defects and was therefore similar to the originally described kinase domain mutants, D165R and E200R (Figs. 1 and 2).

Two additional alterations, D147R and E151R, were constructed within this kinase domain motif. The aspartic acid at position 147 of Vps15 is very highly conserved and is found in a corresponding position of all known protein kinases, both serine/threonine and tyrosine-specific (Hanks et al., 1988). In addition, the aspartic acids D_{147} and D_{165} correspond to residues that appear to be conserved in a variety of phosphotransferase systems, including protein kinases and aminoglycoside phosphotransferases (Brenner, 1987). It was suggested that this common pair of aspartic acids could be involved in the binding of the ATP phosphate groups. The substitution of an arginine residue for this aspartic acid at position 147 resulted in a severe reduction in Vps15p activities. The D147R mutant exhibits a severe *ts* growth defect and mislocalizes ~95% of the newly-synthesized CPY to the cell surface in a Golgi-modified precursor form (Fig. 1, 2). In addition, the D147R protein is very poorly phosphorylated *in vivo*. The D147R protein incorporates only about 5-10% of the $^{32}PO_4$ incorporated by the wild-type Vps15p in a similar labeling experiment.

The final alteration within this motif, E151R, was constructed on the basis of observations made with the cAMP-dependent protein kinase, and other serine/threonine-specific kinases, that suggested that this residue was critical for kinase interaction with substrate (see Taylor et al., 1990). In general, protein kinases having a preference for basic amino acids preceding the substrate phosphorylation site have an acidic residue conserved at this position. Conversely, a basic amino acid tends to be present in kinases with a preference for acidic groups near the target phosphorylation site. We therefore assessed the phenotypic effects of substituting a basic residue, arginine, for the glutamic acid normally present at this position in Vps15p. As seen in Fig. 1, this alteration had

only a modest effect on Vps15p activity as judged by our three assays. While 38°C growth and CPY sorting appeared normal with the E151R mutant (Fig. 1, 2), the level of $^{32}PO_4$ incorporation into this altered Vps15p was only approximately 35-50% of that seen with the wild-type Vps15p *in vivo* (Fig. 1).

The final Vps15p kinase domain alteration changes a lysine residue, K_{54}, that corresponds to an invariant lysine observed in both serine/threonine and tyrosine protein kinases (Hanks et al., 1988; Taylor et al., 1990). This lysine residue has been suggested to be within, or near, the ATP-binding domain of protein kinases as it can be specifically labeled with ATP analogs such as p-fluorosulfonyl 5'-benzoyl adenosine (Zoller et al., 1981; Kamps et al., 1984). We constructed a mutant *VPS15* allele that encodes a protein with an aspartic acid replacing this lysine at position 54, K54D, and have found that this alteration greatly reduces Vps15p activity *in vivo*. The K54D mutant exhibits severe *ts* growth and CPY mislocalization defects (Fig. 1). Approximately 95% of the newly-synthesized CPY is secreted as p2CPY by the K54D mutant (Fig. 2). In addition, this mutant is also very defective for the *in vivo* phosphorylation of Vps15p (Fig. 1).

Vps15p is N-terminal myristoylated *in vivo*. The 14-carbon fatty acid, myristate, is added cotranslationally to the N-terminal glycine residue of many cellular proteins following the removal of the initiating methionine (reviewed in Schultz et al., 1988 and Towler et al., 1988a). The yeast myristoyl CoA:protein N-myristoyl transferase (NMT) enzyme has been purified to apparent homogeneity and an extensive analysis of its substrate specificity has been carried out *in vitro* using synthetic peptides (Towler et al., 1987; Towler et al., 1988b). From these studies, a general consensus sequence for N-terminal myristoylation has been suggested. Briefly, a glycine at position 2 appears to be absolutely critical for myristic acid addition and a serine at position 6 promotes a high affinity interaction between the yeast NMT enzyme and its protein/peptide substrate. Finally, position 3 should be a small, uncharged residue and

neutral amino acids are preferred at the somewhat more permissive positions 4, 5 and 7. The N-terminal sequence of Vps15p, (Met_1)-Gly_2-Ala_3-Gln_4-Leu_5-Ser_6-Leu_7, fits this consensus very well suggesting that Vps15p may be modified by the addition of myristic acid *in vivo*.

To directly analyze whether Vps15p was myristoylated *in vivo*, we radiolabeled yeast cells with tritiated myristic acid for 60 min and then performed immunoprecipitations from the clarified cell lysates with antisera specific to Vps15p. The relatively short labeling period was chosen to minimize the conversion of the labeled myristic acid to other fatty acids, such as palmitate. Vps15p was observed to be specifically labeled in these experiments suggesting that it is modified by the addition of myristic acid *in vivo* (Fig. 3). Further support for this assertion was obtained from our analysis of two different *vps15* mutants that possessed alterations in the myristic acid attachment site. In these mutants, the critical glycine residue at position 2 was changed to either an alanine, G2A, or a tryptophan, G2W (Fig. 1). In order to assess the N-terminal myristoylation of these two altered forms of Vps15p, we introduced either mutant allele, on a multicopy plasmid, into a *Δvps15* yeast strain. We then labeled with ^{3}H-myristic acid and immunoprecipitated with Vps15p antisera as described above. Consistent with results from previous studies on the yeast NMT substrate specificity, neither of the mutant Vps15 proteins were labeled by the ^{3}H-myristic acid in these experiments (Fig. 3). Therefore, the N-terminal sequences of Vps15p appear to act as a site for N-terminal myristoylation in yeast cells.

Although these two alterations abolished the myristoylation of Vps15p, the nonmyristoylated Vps15 proteins appeared to possess near wild-type levels of biological activity *in vivo*. The G2A and G2W mutants both exhibited near wild-type growth rates at 38°C (Fig. 1, see below) and neither mutant was defective for CPY delivery to the vacuole at 26°C (Fig. 1, 2). In addition, since the N-terminal myristoylation of many

proteins results in their association with a particular intracellular membrane, we examined whether the nonmyristoylated Vps15 proteins were associated with a similar membrane fraction of yeast cell extracts as wild-type Vps15p (Herman et al., 1991). These differential centrifugation experiments demonstrated that, like wild-type Vps15p, the G2A mutant protein was associated with a 100,000 xg pellet fraction and could be extracted from this fraction with 1% Triton X-100 (data not shown). Therefore, the membrane association of Vps15p does not appear to be mediated solely by the N-terminal myristic acid moiety. As with the "silent" kinase domain mutant, E151R, the nonmyristoylated Vps15 proteins were observed to be defective in the *in vivo* Vps15p phosphorylation reaction, as the phosphorylation signal was only ~35-50% of that observed with the wild-type protein (Fig. 1).

C-terminal Vps15p truncations exacerbate the effects of alterations in the Vps15p myristoylation site and kinase domain. During our initial search for a *VPS15* minimum complementing fragment, we identified C-terminal Vps15p truncations that were able to fully complement the *ts* growth defects associated with the *vps15-2* allele (Herman et al., 1991). One particular genomic DNA fragment included all *VPS15* sequences up to an internal ClaI site near the 3' end of the gene and encoded a truncated Vps15p that was lacking 214 C-terminal amino acids (ΔC214; see Fig. 1). This truncated *VPS15* fragment was subcloned into a single copy yeast plasmid, pPHYC18, and introduced into a *Δvps15* strain. The ΔC214 mutant exhibits a near wild-type growth rate at 38°C but is slightly defective for CPY sorting at 26°C, mislocalizing ~15% of the newly-synthesized CPY to the cell-surface (Fig. 1, 2). However, when we examined Vps15p phosphorylation, we found that the ΔC214 protein was not phosphorylated *in vivo* (Fig. 1; see below). Immunoblotting experiments with antisera specific for Vps15p demonstrated that the steady state level of this truncated protein is very similar to that of the wild-type Vps15p (data not shown). Therefore, the C-terminal 214 amino acids of

Vps15p appear to either contain the site for Vps15p phosphorylation or be required for this phosphorylation reaction *in vivo*.

In order to further investigate the role of this C-terminal domain in Vps15p function, we introduced the G2A and E151R alterations into the ΔC214 truncated version of Vps15p (Fig. 4A). These double mutant alleles of *VPS15*, G2A/ΔC214 and E151R/ΔC214, were introduced into a *Δvps15* strain on a single copy yeast plasmid (see Materials and Methods) and both were found to be unable to even partially complement the *Δvps15 ts* growth defects or severe CPY missorting phenotype (Fig. 4). Therefore, the ΔC214 truncation greatly exaggerates the effects of both the G2A and E151R alterations. The defects associated with the double mutants are not due to lower levels of the mutant Vps15p as immunoprecipitation experiments have demonstrated that the synthesis rates and *in vivo* half lives of the double mutant proteins are essentially identical to that of the wild-type Vps15p (data not shown). The relative importance of the ΔC214 truncation in these double mutant interactions is illustrated by an analysis of a G2A/E151R double mutant. This double mutant exhibits a temperature-resistant growth phenotype and no CPY sorting defect (data not shown). In addition, the double mutant Vps15 proteins described above were all demonstrated to be associated with the P100 membrane fraction of yeast cell extracts indicating that their phenotypic effects were not due to the disruption of the Vp15p membrane association (data not shown).

These experiments therefore suggest that the deletion of a specific C-terminal domain of Vps15p results in an exacerbation of the effects of other *vps15* mutations. Using endogenous restriction enzyme sites, we constructed a series of deletions at the 3' end of the *VPS15* gene in order to more precisely map the C-terminal domain responsible for the observed synergy (see Materials and Methods). These *vps15* deletion alleles encode truncated Vps15 proteins lacking either 167, 128, or 30 C-terminal amino acids of the wild-type Vps15p. The deletions were constructed in both a wild-type *VPS15*

background and in the G2A mutant and were introduced into a *Δvps15* strain on a single copy plasmid. The ability of these plasmids to complement the *ts* growth defect associated with the null mutant was then tested. Interestingly, each of the double mutants, including G2A/ΔC30, exhibited an extreme *ts* growth defect (Fig. 5A). As expected, each of the single ΔC deletion mutants were able to fully complement the *Δvps15 ts* growth phenotype (Fig. 5A). These results suggest that a C-terminal deletion of as little as 30 amino acids is sufficient for the synergistic interaction observed with the G2A alteration.

C-terminal deletion mutants of Vps15p exhibit a rapid and specific block in vacuolar protein delivery *in vivo*. The extreme defects observed with the G2A/ΔC214 and E151R/ΔC214 double mutants suggested that a C-terminal domain of Vps15p might play a significant role in Vps15p function *in vivo*. Although the C-terminal deletion mutants exhibited only a minor CPY sorting defect at 26°C, we found that this vacuolar protein sorting defect was greatly exaggerated at elevated growth temperatures. To assay CPY sorting and/or processing in these mutants, yeast whole cells were pre-incubated for 5 min at 26° or 38°C and then were radiolabeled with Tran ^{35}S-label for 10 min at the same temperature. Methionine and cysteine were added to a final concentration of 25 mM and the cultures were then chased for an additional 30 min at the same temperature. The labeled cultures were then processed for subsequent immunoprecipitation with antisera specific for CPY. In wild-type cells, all of the radiolabeled CPY was present as the 61 kd mature species, indicative of efficient vacuolar delivery (Fig. 5B). In contrast, in *Δvps15* cells all of the CPY is present as the 69 kd Golgi-modified p2 precursor. At 26°C, all of the C-terminal deletion mutants exhibited only a slight CPY processing defect, varying from ~5% p2 CPY with the ΔC30 mutant to ~15% with ΔC214 (Fig. 5B). However, at 38°C, all of the C-terminal deletions were extremely defective for p2 CPY maturation, accumulating greater than 95% of the newly-

synthesized CPY in a precursor form (Fig. 5B). These results suggest that each of these mutants exhibits an extreme *ts* defect in vacuolar protein delivery (see below). We have classified these types of alleles as "*tsf*," or temperature-sensitive for function, alleles to distinguish them from other *vps15* alleles (including null alleles) that exhibit *ts* growth defects and a severe CPY missorting phenotype at both the permissive and nonpermissive growth temperatures.

We analyzed the fate of the p2 CPY in these 38°C-blocked cells in a series of temperature shift experiments. In these experiments, yeast spheroplasts were briefly labeled with Tran ^{35}S-label at 26°C and a chase was then initiated by the addition of methionine and cysteine to a final concentration of 25 mM. One aliquot of the labeled culture was rapidly shifted to 38°C and chased for 30 min at that temperature. The remainder of the culture was kept at 26°C and chased for an additional 30 min. The cultures were then fractionated into an intracellular and an extracellular fraction and immunoprecipitations were performed with antisera specific for CPY. Following the short labeling period, the majority of the CPY was present as p1 CPY in both the wild-type and ΔC30 mutant (Fig. 6). Following the 30 min chase at either 26° or 38°C, all of the radiolabeled CPY was present in the intracellular fraction in a 61 kd mature form in the wild-type cells, indicative of correct delivery to the vacuolar compartment. In the ΔC30 mutant, after 30 min of chase at 26°C, ~95% of the CPY was processed to its mature form and was found in the intracellular fraction (Fig. 6). However, after 30 min of chase at 38°C, >95% of the radiolabeled CPY was present as the 69 kd p2 precursor. Surprisingly, this p2 CPY was not secreted from the cell but was instead found associated with the spheroplast pellet (Fig. 6). In all previously examined *vps15* mutants, p2 CPY was not retained within the cell pellet, but was instead efficiently secreted from the cells (see for e.g., Fig. 2 and Herman et al., 1991). One possible explanation for the observed lack of p2 CPY secretion is that the ΔC30 mutant may

exhibit a general block in protein secretion at 38°C. The observation that the ΔC30 mutant is able to grow at 38°C with a near wild-type growth rate is not consistent with this explanation (see Fig. 5A). Nonetheless, we directly tested this possibility by examining the secretion of a 33 kd cell wall protein, CWP33, in ΔC30 and wild-type yeast cells at 38°C. The CWP33 protein is a major constituent of the yeast cell wall and is released from the wall upon treatment with zymolase (Sanz et al., 1987). The passage of this protein through the secretory pathway has been extensively studied (Sanz et al., 1987; Toyn et al., 1988) and it serves as a good marker of secretory protein flow to the cell surface. Using antibody directed specifically against this cell wall protein, we were able to show that ΔC30 mutant spheroplasts secrete the CWP33 protein into the media as efficiently as wild-type yeast at both 26° and 38°C (Fig. 6). Therefore, the intracellular retention of p2 CPY in ΔC30 cells at 38°C is not due to a general block in protein secretion in these mutants.

It is important to point out that the onset of the CPY sorting and/or processing defect is extremely rapid following the shift to the nonpermissive temperature in these experiments. In the whole cell experiments, we have completely eliminated the 38°C pre-incubation and have instead just shifted the cells to 38°C when labeling is initiated. Under these experimental conditions, the p2 CPY processing block is also complete (data not shown; see below). The above temperature shift experiments are even more dramatic as they suggest that an almost complete block in CPY processing is established in 1 to 2 min after a shift up to 38°C. To ensure that the observed block in p2 CPY secretion at 38°C was not specific to spheroplasts or the experimental procedure used, we have performed similar temperature shift experiments with whole yeast cells and have obtained identical results (data not shown). In addition, if whole cells, or spheroplasts, are labeled and chased at 38°C, CPY is once again observed to accumulate intracellularly in its Golgi-modified p2 form (data not shown). This latter experiment indicates that the ΔC30 block

was not due to the rapid increase in culture temperature performed in the above temperature shift experiments (see Materials and Methods).

Altogether, our results suggest that the 38°C induced block is relatively specific for vacuolar protein traffic as protein secretion appears to continue unabated in the ΔC30 mutant at the nonpermissive temperature. In order to examine the specificity of the vacuolar delivery block more carefully, we analyzed the fate of two additional vacuolar proteins, PrA and ALP, in ΔC30 mutant spheroplasts at 38°C. In identical temperature shift experiments, we observed that the soluble hydrolase PrA behaved much like CPY; the majority of the radiolabeled PrA was associated with the cell pellet in a p2 precursor form (data not shown). In contrast, precursor ALP was processed to its mature form with near wild-type kinetics in the ΔC30 mutant blocked at 38°C (Fig. 6). Therefore, the temperature-conditional block in vacuolar protein processing appears to be relatively specific for soluble vacuolar hydrolases, as at least one vacuolar membrane protein, ALP, is processed normally at 38°C. The efficient processing of ALP in ΔC30 cells at 38°C suggests that ALP is delivered to the vacuolar compartment since this protealytic processing event is mediated by the vacuolar enzyme, PrA (Klionsky et al., 1990). This assertion is further supported by indirect immunofluorescence experiments and biochemical analyses of ALP processing that indicate that ALP is efficiently localized to the vacuolar compartment in *vps15* mutants that display severe CPY mislocalization defects (Klionsky and Emr, 1989; C. Raymond and T. Stevens, personal communication). In addition, as both CPY and ALP are processed in the vacuole in a PrA-dependent manner, our results suggest that CPY is not present in the same intracellular compartment as ALP and is therefore not likely in the vacuolar compartment in 38°C-blocked ΔC30 cells.

The onset of p2 CPY secretion is delayed in the ΔC30 mutant at 38°C. In a *Δvps15* mutant essentially all of the CPY is present as the p2 precursor form

and the great majority of this p2 CPY is secreted from the cell at both 26° and 30°C (Fig. 2 and Herman et al., 1991). We therefore decided to analyze p2 CPY secretion in a *vps15* null mutant at 38°C to test whether *vps15* mutants, in general, were competent for the secretion of vacuolar protein precursors at this elevated temperature. PHY112 (Δ*vps15* ::*HIS3*) and wild-type yeast spheroplasts were labeled briefly at 26°C and then were chased at 38°C for 30 min as described above. Under these conditions, all of the CPY in wild-type cells was processed to mature CPY and was associated with the spheroplast pellet (Fig. 7). In the Δ*vps15* yeast strain, >95% of the radiolabeled CPY was in its p2 precursor form and most of this was secreted from the cells and into the media (Fig. 7). Therefore, *vps15* mutants appear to be competent for p2 CPY secretion at 38°C.

In contrast to the above results with a Δ*vps15* yeast strain, in the ΔC30 *tsf* sorting mutant, ~95% of the radiolabeled CPY was retained within the spheroplast pellet as p2 CPY after a 30 min chase at 38°C (see above). The fundamental difference between the Δ*vps15* and ΔC30 mutants in the above temperature shift experiments is that prior to the imposition of the 38°C block Δ*vps15* mutants are completely defective for vacuolar protein sorting while ΔC30 cells exhibit only a very slight CPY sorting defect (Fig. 5B, 7). We reasoned that if ΔC30 cells were blocked at 38°C for extended periods of time they might begin to mimic Δ*vps15* cells and missort newly-synthesized CPY to the cell surface at 38°C. Since the ΔC30 mutant is able to grow at this elevated temperature with a near wild-type growth rate, the 38°C block could be imposed for any desired length of time. To analyze the effects of a 38°C pre-incubation upon p2 CPY secretion in the ΔC30 mutant, we labeled ΔC30 and wild-type whole cells for 5 min at 38°C after 0, 30 or 60 min of pre-incubation at the same temperature. The cultures were then chased for 30 min at 38°C, converted to spheroplasts and separated into an extracellular and an intracellular fraction. Immunoprecipitations were then performed with antisera specific to CPY.

In wild-type cells, essentially all of the radiolabeled CPY was present in the intracellular fraction as the 61kd mature species for all three times of pre-incubation (data not shown). With the ΔC30 mutant, we observed that varying the length of the 38°C pre-incubation had a significant effect upon the fate of CPY trafficking through the secretory pathway. When no pre-incubation was performed, >95% of the radiolabeled CPY was blocked in an intracellular fraction as p2 CPY (Fig. 7). However, when ΔC30 cells were pre-incubated for either 30 or 60 min prior to labeling, we observed that the majority of the newly-synthesized CPY was secreted from the cells as the Golgi-modified p2 precursor (Fig. 7). After 60 min of pre-incubation at 38°C, essentially all of the p2 CPY was detected in the extracellular fraction. Therefore, the ΔC30 mutant is competent for and will secrete p2 CPY under the appropriate experimental conditions.

The ΔC30 temperature-conditional block in CPY delivery is reversible. When the ΔC30 mutant is rapidly shifted to 38°C, p2 CPY and other soluble vacuolar precursors accumulate within a specific intracellular compartment. We examined the reversibility of this 38°C block in ΔC30 cells in order to gain some insight into the nature of this compartment. As above, we briefly labeled ΔC30 spheroplasts at 26°C and then chased with cold methionine and cysteine for 30 min at 38°C. After this chase period, ~95% of the radiolabeled CPY is cell-associated and its p2 precursor form (Fig. 8). This culture was subsequently split in half, with one aliquot remaining at 38°C for an additional 20 min and the other placed at 26°C for 20 min. The additional 20 min chase at 38°C did not significantly alter the CPY distribution in the ΔC30 cells (Fig. 8). The p2 CPY that was blocked after 30 min at 38°C remained blocked after an additional 20 min at 38°C. In contrast, we found that during the 20 min incubation at 26°C that > 90% of the originally blocked CPY was processed to its mature form, indicating that this CPY had been efficiently delivered to the yeast vacuolar compartment (Fig. 8). Vacuolar delivery appears to occur very rapidly upon a shift back down to 26°C, as we have

observed that most of the p2 CPY is processed to mature within 10 min of the temperature shift (data not shown). This rapid reversal of the 38°C block suggests that the observed vacuolar delivery of p2 CPY is due to the renaturation of pre-existing heat-denatured Vps15p rather than new protein synthesis. Consistent with this, we have found that the p2 CPY processing observed at 26°C is insensitive to the addition of cycloheximide; processing of p2 CPY to its mature form occurs at the same rate in the presence or absence of protein synthesis (T. Vida and S. Emr, unpublished observations). Our results therefore indicate that the great majority of the p2 CPY that accumulates within 38°C-blocked ΔC30 cells remains competent for subsequent vacuolar delivery during the time that the block is imposed. Moreover, the efficient reversal of this temperature block suggests that the compartment housing the p2 CPY may in fact represent a normal functional intermediate in the vacuolar protein delivery pathway.

A short C-terminal domain of VPS15p is required for its phosphorylation *in vivo*. Vps15p has been demonstrated to be a phosphoprotein *in vivo* and mutational analyses of the Vps15p kinase domain have suggested that this phosphate incorporation may be due to a specific autophosphorylation reaction (see Fig. 1 and Herman et al. 1991). Our analysis of the ΔC214 protein demonstrated that this truncated Vps15 protein was very defective in this *in vivo* phosphorylation reaction (Fig. 1). Since the ΔC214 mutant also exhibits an extreme *ts* CPY sorting defect, it is possible that the phosphorylation of Vps15p is important for it to achieve, or maintain, an active conformation at elevated growth temperatures. In order to examine this possibility more carefully, we analyzed the *in vivo* phosphorylation of each of the C-terminal Vps15p truncation proteins. Each of the C-terminal *VPS15* deletion alleles were subcloned into a multicopy plasmid and introduced into a Δ*vps15* yeast strain. The cells were then labeled for 30 min with ^{32}P-orthophosphate and immunoprecipitated with antisera specific for Vps15p. All of the C-terminal deletion mutants, including ΔC30, were completely

defective for the *in vivo* phosphorylation of Vps15p (Fig. 9). In order to determine if the observed lack of Vps15p phosphorylation was specifically due to the deletion of sequences within the C-terminal 30 amino acids of Vps15p, we constructed an internal deletion of amino acids 1412 to 1427, ΔI16, and analyzed the phosphorylation of this Vps15p deletion. The ΔI16 protein was observed to be phosphorylated to a wild-type level *in vivo* (Fig. 9). This result indicates that the lack of Vps15p phosphorylation in the C-terminal deletion mutants is not due to a general misfolding of Vps15 proteins that possess deletions in their C-termini. Rather, our results suggest that a short C-terminal domain of Vps15p is specifically required for its phosphorylation *in vivo*. Furthermore, since deletions of this domain result in a *ts* vacuolar protein sorting defect (see above), the phosphorylation of Vps15p may be essential for its biological activity at elevated temperatures.

DISCUSSION

The sorting of proteins to the vacuolar compartment of yeast cells is a complex process requiring the coordinated participation of a relatively large number of cellular components. In a previous study, we identified a cytoplasmic protein kinase, encoded by the *VPS15* gene, that is essential for the efficient vacuolar delivery of multiple soluble hydrolases. The severe phenotypic consequences of mutations within the *VPS15* protein kinase domain, together with the specificity of *vps15* defects for soluble constituents of the vacuole, suggested that Vps15p might regulate specific protein phosphorylation reactions required for the delivery of soluble proteins to the yeast vacuolar compartment. In addition to the N-terminal 300 amino acid protein kinase domain, Vps15p also possesses a myristic acid addition site at its extreme N-terminus and a large C-terminal domain of greater than 1100 amino acids that exhibited no significant similarity to any known protein sequence (see below). In this study, we have extended our previous

mutational analysis of Vps15p and have now examined multiple alterations in each of these three Vps15p domains.

The Vps15p sequence exhibits a significant degree of similarity to the catalytic domains of the serine/threonine family of protein kinases. With this present study, we have now constructed mutations in four distinct Vps15p kinase subdomains and have, in each case, altered specific amino acid residues that are highly conserved among all protein kinase molecules. This high level of conservation, together with observations from chemical modification experiments, suggests that these amino acids are directly involved in catalytic function (Hanks et al., 1988; Taylor et al., 1990). Alterations within each of the four Vps15p kinase motifs result in severe defects in the *in vivo* phosphorylation of Vps15p and the delivery of soluble hydrolases to the vacuole (see Fig. 1). Therefore, these mutational studies suggest that the Vps15p kinase domain regulates Vps15p phosphorylation *in vivo* and that it may do so directly, by catalyzing a specific autophosphorylation reaction. We are currently attempting to demonstrate Vps15p kinase activity *in vitro* in order to determine if the *in vivo* phosphorylation of Vps15p is, indeed, an autophosphorylation reaction. The Vps15p kinase domain may regulate other protein phosphorylation reactions *in vivo*, since the phenotypes of kinase domain mutations (e.g., D165R in Fig. 1) are generally more extreme than those associated with a loss of Vps15p phosphorylation (e.g., ΔC214 in Fig. 1). The severe vacuolar protein sorting defects associated with Vps15p kinase domain alterations may therefore be due to defects in multiple Vps15p-mediated protein phosphorylation reactions involving, as yet unknown, cellular substrates of the Vps15p kinase.

We have recently noted that sequences within the middle third of Vps15p exhibit sequence similarity to the 65 kd regulatory subunit of the mammalian protein phosphatase 2A (P. Herman, J. Stack and S. Emr, unpublished observations). This 65 kd protein is generally found associated with the 36 kd protein phosphatase 2A catalytic subunit and

has been demonstrated to regulate the activity of the catalytic subunit in a substrate-specific manner (Usui et al., 1988; Chen et al., 1989). The protein sequence of this regulatory subunit is highly unusual in that it consists of 15 imperfect repeats of approximately 39 amino acids (Hemmings et al., 1990). Vps15p possesses three of these 39 amino acid repeats and we have recently demonstrated that the deletion of these sequences renders Vps15p biologically inactive (J. Stack and S. Emr, unpublished observations). We are currently examining the potential role of the yeast protein phosphatase 2A in vacuolar protein sorting and whether Vps15p in some way regulates the activity of this protein phophatase *in vivo*.

Biochemical labeling experiments with ^{3}H-myristic acid indicated that Vps15p is modified *in vivo* by the addition of the 14-carbon fatty acid, myristate, at its N-terminus. However, this lipophilic moiety does not appear to mediate the observed membrane association of Vps15p as non-myristoylated forms of this protein remain associated with a similar membrane fraction of yeast cell extracts. Although, the majority of myristoylated proteins are associated with specific intracellular membranes, there are several examples of proteins that are myristoylated but soluble, including the catalytic domain of the cAMP-dependent protein kinase (Towler et al., 1988a). The biological significance of the myristic acid in these soluble proteins is for the most part unknown. In the case of the cAMP-dependent protein kinase, the myristate does not appear to be important for catalytic activity as the myristolated and non-myristoylated forms of the catalytic subunit have identical kinase activities *in vitro* (Slice and Taylor et al., 1989). In contrast, the non-myristoylated form of Vps15p is phophorylated to a lesser extent than the wild-type protein *in vivo*. If this *in vivo* phosphorylation of Vps15p is, in fact, due to a specific autophosphorylation reaction, it will be interesting to determine if the myristate does directly influence Vps15p catalytic activity.

The analysis of conditional alleles of a given genetic locus generally allows investigators to develop a more complete understanding of the precise physiological role played by the product of that locus in the developmental or biochemical pathway under study. For example, in the analysis of the yeast *sec* mutants, the accumulation of specific intermediates at the nonpermissive temperature allowed Schekman and his co-workers to assign particular gene functions to different positions throughout the secretory pathway (Schekman and Novick, 1982). Our initial genetic selection uncovered multiple *vps15* alleles that were *ts* for growth, however, all of these alleles resulted in an equally severe CPY sorting defect at both the permissive and nonpermissive growth temperatures (Robinson et al., 1988). During our present analysis of a series of C-terminal Vps15p deletion mutants, we found that relatively short C-terminal truncations of Vps15p, removing as little as 30 amino acids, resulted in a severe *ts* defect in the delivery of CPY to the vacuole (see below). This *ts* defect is especially dramatic in the ΔC30 mutant where CPY delivery is essentially wild-type at the permissive temperature but almost completely blocked when cells are shifted to the restrictive temperature of 38°C. Upon imposition of the temperature block, ΔC30 cells accumulate ~95% of the newly-synthesized CPY within a specific intracellular compartment as a p2 precursor molecule. This intracellular retention of p2 CPY was a somewhat unexpected result as all of the previously characterized *vps15* mutants efficiently secreted p2 CPY. The majority of the cell-associated CPY in these mutants was detected in its mature form suggesting that it was properly delivered to the vacuolar compartment (see Fig. 2). These data indicate that *vps15* mutants are not defective for the processing of CPY but are instead defective in CPY localization to the vacuole. This would therefore suggest that the p2 CPY detected within ΔC30 cells at 38°C is not in the vacuolar compartment. Furthermore, the *ts* vacuolar delivery defects associated with the ΔC30 mutant appear to be specific for soluble vacuolar hydrolases. ALP, a vacuolar membrane protein, is efficiently processed

to its mature form at the nonpermissive temperature in ΔC30 mutants. Since ALP and CPY are both processed in the vacuole by a PrA-dependent mechanism (Klionsky et al., 1990), the effective maturation of ALP suggests that ΔC30 cells are processing-proficient and that CPY is apparently sequestered within an intracellular compartment distinct from the vacuole. Therefore the *ts* defects observed in the C-terminal deletion mutants are apparently due to a failure to properly delivery CPY to the vacuole where it could be processed to its active form.

The *ts* vacuolar protein delivery block in ΔC30 mutants exhibits an extremely rapid rate of onset as an essentially complete block in CPY processing can be established in less than 1 to 2 min of incubation at the nonpermissive temperature. The rapid onset of the mutant phenotype suggests that the *VPS15* gene product is quickly inactivated at 38°C and that this inactivation almost immediately manifests itself as a defect in CPY delivery to the vacuole. This, in turn, suggests that Vps15p is directly involved in the delivery of proteins to the vacuole and that the observed vacuolar protein sorting defects in *vps15* mutants are not due to secondary effects of the loss of Vps15p activity. Athough the exact manner in which the C-terminal domain influences Vps15p activity is unclear, the observation that all of the C-terminal truncated forms of Vps15p, including ΔC30, are not phosphorylated *in vivo* suggests that the phosphorylation of Vps15p is important for its biological activity at elevated temperatures. The C-terminal 30 amino acids of Vps15p may either include the site of Vps15p phosphorylation or else constitute a specific domain required for phosphorylation elsewhere within this protein. We are presently attempting to map the specific site(s) of protein phosphorylation in Vps15p in order to determine how this phosphorylation, and the C-terminal domain in general, contribute towards Vps15p function *in vivo*.

The immediate phenotypic consequence of a loss of Vps15p activity therefore appears to be the intracellular accumulation of Golgi-modified precursor forms of soluble

vacuolar hydrolases, such as p2 CPY. These precursors appear to be present within a specific intracellular compartment that is distinct from the vacuole. Moreover, this compartment does not appear to represent an aberrant, dead-end structure since the resident p2 CPY can be efficiently processed to its mature form following a shift back to the permissive temperature. This rapid and efficient reversal of the 38°C delivery block in ΔC30 cells suggests that p2 CPY may have been present within a normal intermediate of the vacuolar protein transport pathway. Since very little is presently known about the transport intermediates functioning between the Golgi complex and the vacuole, the identification and characterization of the p2 CPY compartment from 38°C-blocked ΔC30 mutants might provide some fundamental insights into the vacuolar protein delivery pathway. It is interesting to note that an electron microscopic analysis of *vps15* mutants indicated that the cytoplasm of *vps15* cells accumulated 80 nm vesicles and abnormal membranous structures that resembled Golgi-derived Berkeley bodies (Banta et al., 1988). A similar analysis of ΔC30 cells following a shift to 38°C could be performed in an attempt to identify any membranous intermediates that may rapidly accumulate at this temperature.

Our results indicate that there is a delay in the onset of p2 CPY secretion in ΔC30 cells at 38°C. CPY synthesized in ΔC30 cells immediately following a shift to 38°C is observed to accumulate intracellularly in a p2 precursor form. This p2 CPY remains cell-associated during at least 50 min of subsequent incubation at 38°C. These data suggest that this p2 CPY may be sequestered within a compartment that is unable to communicate with the cell surface. One possibility is that this p2 CPY is packaged into a specific transport intermediate, possibly a membrane vesicle, destined for the vacuolar compartment. If Vps15p activity is required for either the delivery or fusion of these vesicles with the vacuole then a rapid inactivation of Vps15p may result in an accumulation of p2 CPY within this intracellular compartment. Note that our data

indicate that these vesicles would not be competent for transport and/or fusion to the plasma membrane. In this model, p2 CPY would be packaged into vesicles until a cellular component essential for either this packaging, or the formation of the transport intermediates, became limiting. At this time, p2 CPY would presumably enter the default secretion pathway and be delivered to the cell surface.

The above model represents one of the two we presented in our initial characterization of the *VPS15* locus (Herman et al., 1991). The second model proposed that Vps15p activity was required for the packaging of specific receptor-ligand (i.e., p2 CPY) complexes into transport vesicles destined for the vacuole. A loss of Vps15p activity in this case would lead to an accumulation of p2 CPY, bound by its specific transmembrane receptor, in the Golgi. Secretion would then result from the saturation of these p2 CPY-specific receptors. In its simplest version, this latter model seems unlikely as exchange between the receptor-bound radiolabeled p2 CPY and the newly-synthesized unlabeled molecules would be expected to occur during the 38°C chase period resulting in the secretion of the labeled precursor. However, we observe almost no p2 CPY secretion from ΔC30 cells incubated at 38°C.

Although *vps15* mutants exhibit severe defects in the localization of multiple soluble vacuolar hydrolases, vacuolar membrane proteins, such as ALP, appear to be efficiently delivered to the vacuole in *vps15* cells. This observation has several interesting implications for Vps15p function within the vacuolar protein delivery pathway. ALP and CPY may transit together from the late Golgi to the vacuole within the same vesicular transport intermediate. Since ALP is efficiently delivered to the vacuole in *vps15* mutants, the cellular machinery required for the formation and delivery of these transport vesicles must be present and functional. Therefore, Vps15p would presumably function prior to these transport events and could possibly be required for the packaging of p2 CPY into the vacuole-bound transport intermediates. On the other hand,

CPY and ALP might be delivered to the vacuole by two independent pathways involving separate and distinct transport intermediates. In this model, Vps15p could act at any step along the CPY delivery pathway but would not be required for transit along the ALP route. The intracellular compartment that accumulates p2 CPY in the ΔC30 *vps15* mutant is a candidate for the transport intermediate between the Golgi and vacuolar compartments in the CPY-specific pathway of the latter model.

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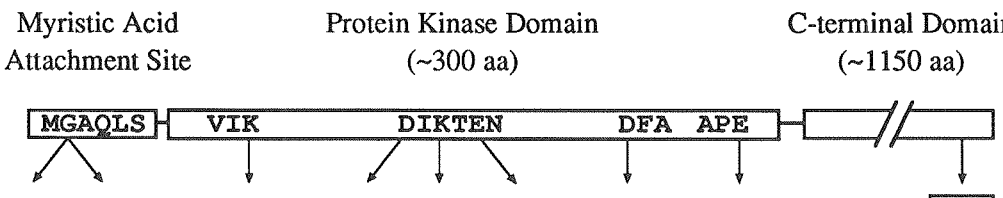
Figure 1. Summary of the mutational analyses of *VPS15*.

A schematic representation of the tripartite nature of the Vps15p sequence is shown. The alterations made at each residue are indicated below the bar diagram of Vps15p along with the amino acid position of this residue in the Vps15p sequence. The growth phenotype (Growth Phen) indicates the ability of the different *vps15* mutants to grow at 38°C on YPD plates. The next row indicates the extent of the CPY sorting defect in the different mutants. The values indicate the percentage of the total CPY that is found as p2 CPY in the extracellular fraction. The final row (Vps15p Phos) is a relative measure of the extent of ^{32}P-incorporation into Vps15p in the different mutants (see Materials and Methods for all experimental details). "++" indicates a signal approximately 30-50% of wild-type; "+/-" approximately 5% of wild-type; and "-" indicates no detectable phosphorylation was observed.



Mutation:	WT	A	W	D	R	D	R	R	R	
Position:	-	2	2	54	147	149	151	165	200	ΔC214
Growth Phen:	TR	TR	TR	ts	ts	ts	TR	ts	ts	TR
CPY Sorting: (% secreted)	<5%	<5%	<5%	95%	90%	>95%	<5%	>95%	>95%	15%
Vps15p Phos:	+++	++	++	+/-	+/-	-	++	-	-	-

Figure 2. Intracellular sorting of CPY in yeast *vps15* mutants.

Yeast spheroplasts were labeled with Tran ^{35}S-label for 20 min at 26°C and then chased for 30 min following the addition of methionine and cysteine to 25 mM. The labeled cultures were centrifuged for 2 min at 13,000 xg and separated into a pellet (I, intracellular) and supernatant (E, extracellular) fraction. The level of CPY in each fraction was assessed by quantitative immunoprecipitation with antisera to CPY. In each sample, the strain examined was PHY112 (Δ*vps15*::*HIS3*) carrying a particular *VPS15* allele on a single copy plasmid. WT refers to the wild-type allele (pPHY15.1) and Δ*vps15* to PHY112 harboring the vector plasmid (pPHYC18). For all others the particular *vps15* allele present would encode the Vps15 proteins with the indicated alterations (see Figure 1). The positions of mature (61 kd) and p2 CPY (69 kd) are indicated.

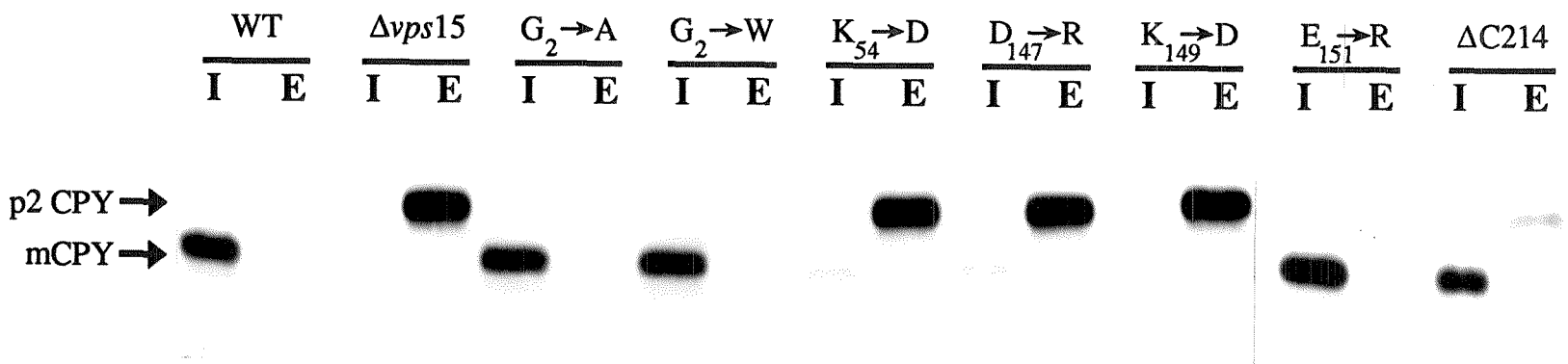

WT
Δvps15
G2→A
G2→W
K54→D
D147→R
K149→D
E151→R
ΔC214
I E I E I E I E I E I E I E I E I E
p2 CPY
mCPY

Figure 3. *In vivo* myristoylation of the Vps15 protein.

Yeast cells were labeled with [^{3}H]-myristic acid for 60 min at 30°C and immunoprecipitated with antisera specific for Vps15p as described in Materials and Methods. The strains examined were PHY112 harboring the single copy plasmids with the indicated *VPS15* alleles. WT refers to the wild-type allele (pPHY15.1) and Δ15 to the vector (pPHYC18). The position of Vps15p (~170 kd) is shown.

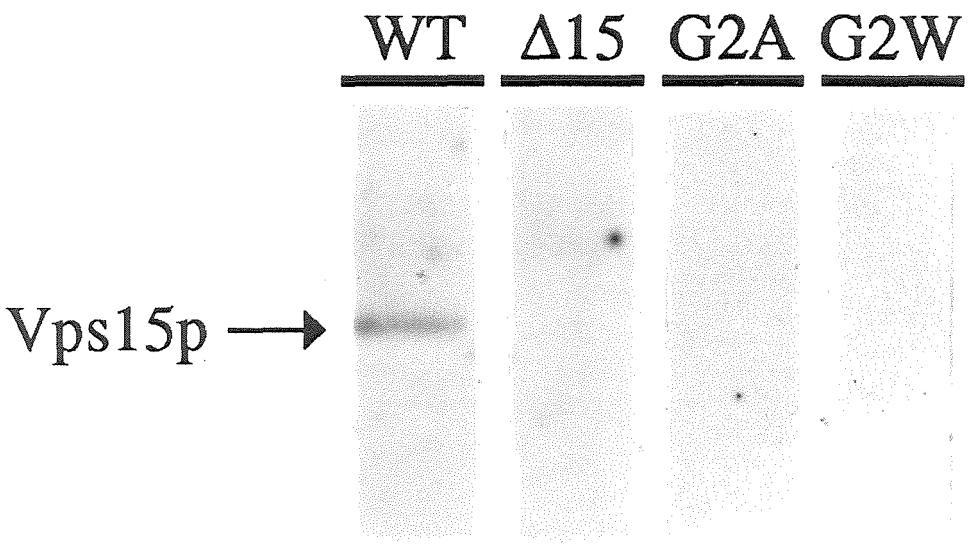

WT
Δ15
G2A
G2W
Vps15p

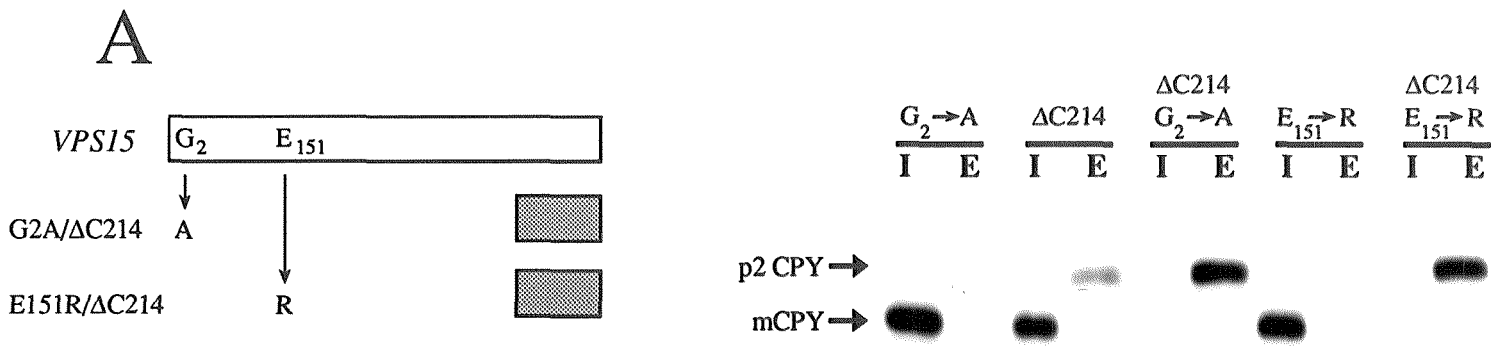
Figure 4. Analysis of *vps15* double mutants.

(A) Intracellular sorting of CPY in *vps15* double mutants.

A schematic drawing illustrating the two *vps15* double mutants analyzed. A CPY sorting analysis is shown on the right-hand side. Yeast spheroplasts were labeled and processed as described in the legend to Figure 2. The strains were PHY112 harboring different *vps15* alleles on a single copy plasmid (pPHYC18). The alleles encoded Vps15 proteins with the single or double alterations as indicated.

(B) Analysis of *ts* growth defect of *vps15* double mutants.

PHY112 cells harboring the indicated *vps15* alleles on a single copy yeast plasmid were streaked onto YPD plates and incubated at either 26° or 38°C. WT refers to the wild-type allele (pPHY15C) and Δ*vps15* refers to the vector control (pPHYC18).


A
VPS15
G2
E151
A
R
G2A/ΔC214
E151R/ΔC214
ΔC214
G2→A
ΔC214
ΔC214
G2→A
E151→R
ΔC214
E151→R
I E I E I E I E I E
p2 CPY
mCPY

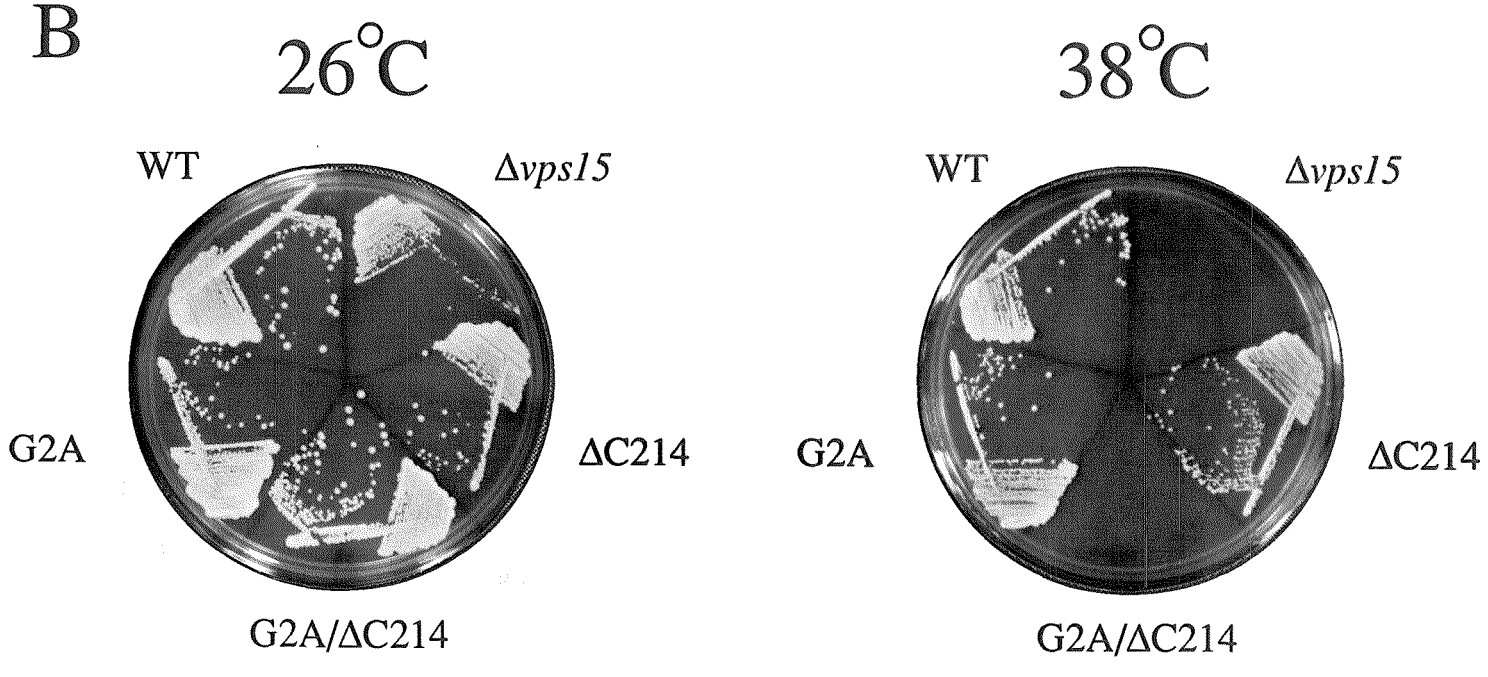

B
26°C
38°C
WT
Δvps15
G2A
ΔC214
G2A/ΔC214
WT
Δvps15
G2A
ΔC214
G2A/ΔC214

Figure 5. Analysis of C-terminal deletion mutants of *VPS15*.

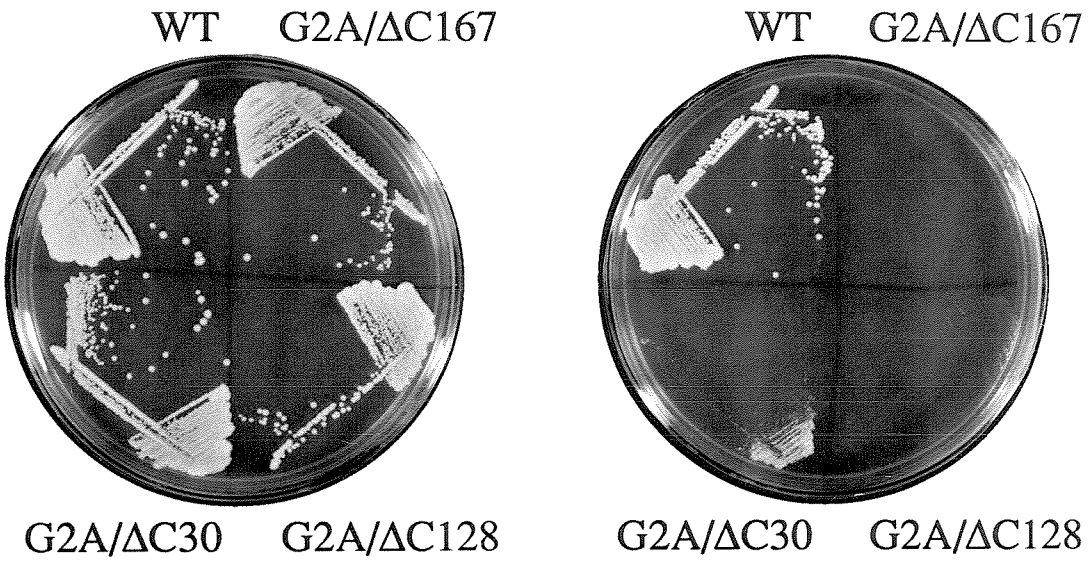
(A) Analysis of the *ts* defects of C-terminal deletion / G2A double mutants.

PHY112 cells harboring the indicated *vps15* alleles on a single copy plasmid were streaked out onto YPD plates and incubated at either 26° or 38°C. WT refers to the wild-type allele (pPHY15.1). The other alleles encode proteins with C-terminal deletions of 167, 128 or 30 amino acids as indicated.

(B) Temperature-sensitive CPY processing in *vps15* C-terminal deletion mutants.

Yeast cells were pre-incubated at either 26° or 38°C for 5 min prior to label addition. The cells were labeled with Tran ^{35}S-label for 5 min, methionine and cysteine were added to a final concentration of 25 mM and the cells were chased for an additional 30 min. The labeling and chase were performed at the same temperature as the pre-incubation. The cells were then processed for immunoprecipitation with antisera specific for CPY as described in Materials and Methods. All strains analyzed were PHY112 containing a single copy yeast plasmid with the specific *vps15* allele indicated. WT refers to the wild-type allele (pPHY15.1) and Δ*vps15* to the vector control (pPHYC18). The positions of mature and p2 CPY are indicated.

A


WT
G2A/ΔC167
WT
G2A/ΔC167
G2A/ΔC30
G2A/ΔC128
G2A/ΔC30
G2A/ΔC128
26°C
38°C

B

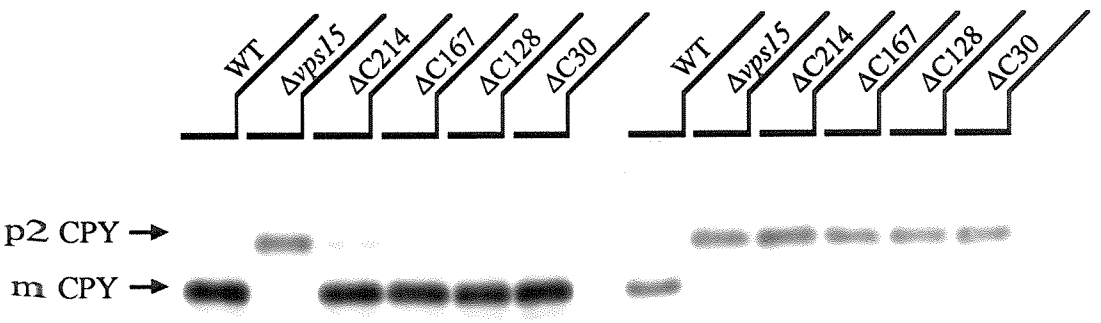

WT
Δvps15
ΔC214
ΔC167
ΔC128
ΔC30
WT
Δvps15
ΔC214
ΔC167
ΔC128
ΔC30
p2 CPY
m CPY

Figure 6. Temperature shift analysis of the *ts* defects associated with the ΔC30 mutant. Yeast spheroplasts were labeled with Tran ^{35}S-label for 3 min at 26°C and methionine and cysteine were then added to 25 mM to initiate the chase period. One aliquot was chased at 26°C for 30 min and the other at 38°C for 30 min. The labeled cultures were then centrifuged for 2 min at 13,000 xg and separated into a pellet (I, intracellular) and supernatant (E, extracellular) fraction. The level of either CPY, ALP or CWP33 in each fraction was assessed by quantitative immunoprecipitation with antisera specific for the appropriate protein. WT refers to PHY112 cells harboring the pPHY15.1 plasmid (wild-type) and ΔC30 to PHY112 cells with a single copy plasmid carrying the indicated C-terminal deletion allele.

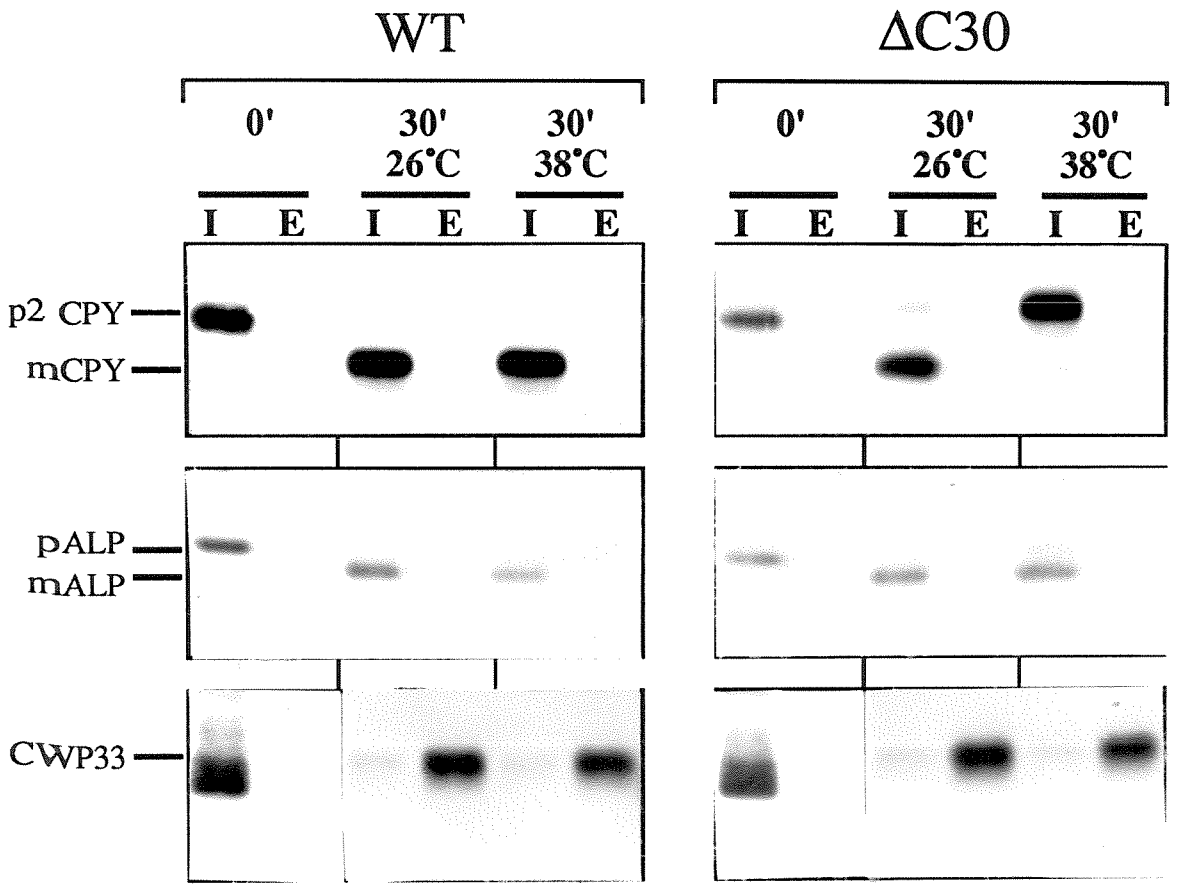

WT
ΔC30
0'
30' 26°C
30' 38°C
I E I E I E
p2 CPY
mCPY
pALP
mALP
CWP33

Figure 7. p2 CPY secretion in the ΔC30 mutant.

For the first two pairs of lanes, PHY112 spheroplasts harboring either pPHY15.1 (WT, wild-type allele) or pPHYC18 (Δ*vps15*, vector) were labeled with Tran ^{35}S-label for 3 min at 26°C. The cultures were then shifted to 38°C and chased for 30 min at this temperature following the addition of methionine and cysteine to 25 mM. The spheroplasts were processed as described below. For the final three pairs of lanes, PHY112 cells harboring a single copy yeast plasmid with the ΔC30 allele were pre-incubated for 0, 30, or 60 min at 38°C. The cells were then labeled with Tran ^{35}S-label for 5 min at 38°C and chased for an additional 30 min at 38°C. The chase was initiated by the addition of methionine and cysteine to a final concentration of 25 mM. The whole cells were converted to spheroplasts, separated into a pellet (I, iintracellular) and supernatant (E, extracellular) fraction by centifugation at 13,00xg for 2 min and immunoprecipitated with antisera specific to CPY. The positions of mature and p2 CPY are indicated.

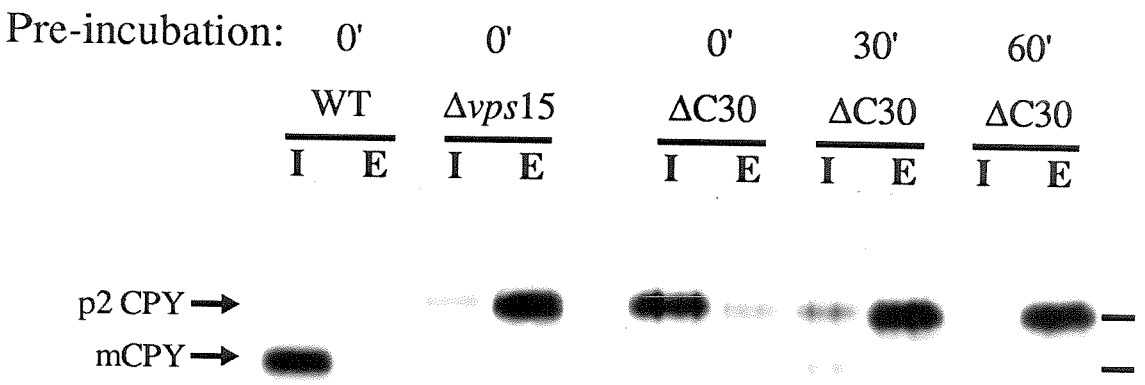

Pre-incubation:
0'
0'
0'
30'
60'
WT
Δvps15
ΔC30
ΔC30
ΔC30
I E
I E
I E
I E
I E
p2 CPY
mCPY

Figure 8. Reversibility of *ts* vacuolar protein delivery defect of ΔC30 mutants. PHY112 spheroplasts harboring a single copy yeast plasmid with the ΔC30 allele were labeled for 3 min at 26°C and chased for 30 min at 38°C following the addition of methionine and cysteine to 25 mM. The labeled culture was then split into three and processed as follows. One aliquot was centrifuged for 2 min at 13,000 xg and separated into a pellet (I, intracellular) and supernatant (E, extracellular) fraction. The level of CPY in each fraction was assessed by quantitative immunoprecipitation with antisera to CPY. The second aliquot was chased for an additional 20 min at 38°C and the third for 20 min at 26°C. These latter two aliquots were then processed as the first was above. The positions of mature and p2 CPY are indicated.

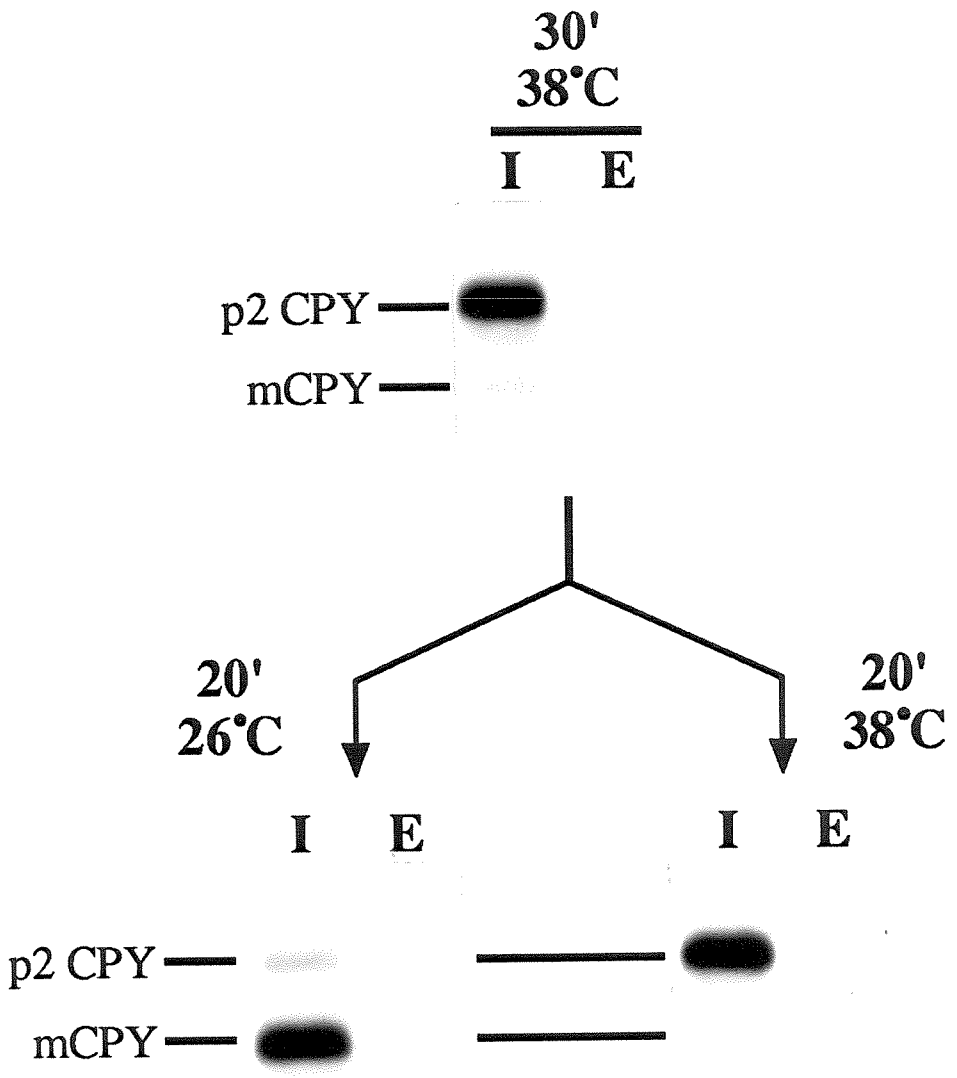
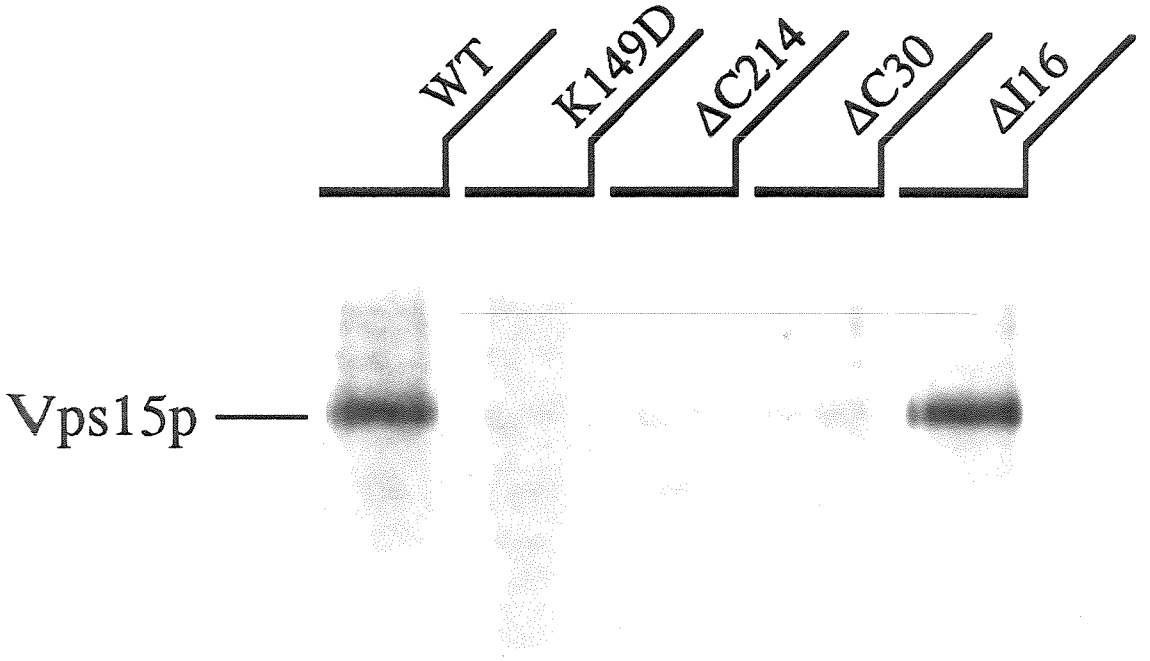

30'
38°C
I E
p2 CPY
mCPY
20'
26°C
20'
38°C
I E
I E
p2 CPY
mCPY

Figure 9. *In vivo* phosphorylation of the *VPS15* C-terminal deletion mutants.
Yeast cells were labeled with $^{32}P0_4$ for 30 min at 30°C and immunoprecipitated with antisera specific for Vps15p as described in Materials and Methods. PHY112 cells harboring the indicated *vps15* alleles on a multicopy plasmid were analyzed. WT refers to plasmid pPHY15.2 (wild-type allele; see Materials and Methods). The position of Vps15p (~170 kd) is shown.


WT
K149D
ΔC214
ΔC30
ΔI16
Vps15p

Chapter 6:

Discussion

A large number of yeast mutants defective for the localization of proteins to the vacuole have been identified and characterized (Bankaitis et al., 1986; Rothman and Stevens, 1986; Banta et al., 1988; Robinson et al., 1988; Rothman et al., 1989). Genetic complementation analyses between these *vps* mutants (for vacuolar protein sorting defective) have identified at least 46 yeast complementation groups required for the efficient sorting of vacuolar proteins. This genetic complexity indicates that this protein delivery process involves the direct or indirect participation of a relatively large number of cellular components. The molecular cloning and analysis of the cellular genes defined by the *vps* mutations should provide some basic insights into the underlying cellular machinery operating to control protein sorting decisions, and subsequent protein delivery, within the eukaryotic secretory pathway. In the preceding chapters, we have discussed the initial genetic, molecular, and biochemical characterizations of the yeast *VPS15* and *VPS34* genes and their respective gene products. Our data indicate that the Vps15 and Vps34 proteins may be functionally interacting within the yeast cell and that the Vps15 protein (Vps15p) may regulate specific protein phosphorylation reactions that are required for the efficient delivery of soluble vacuolar hydrolases.

Mutations within the yeast *VPS15* and *VPS34* genes result in a similar set of terminal mutant phenotypes that was distinct from that observed with any of the other *vps* mutants (Banta et al., 1988; Robinson et al., 1988; Herman and Emr, 1990; Herman et al., 1991). This phenotypic similarity suggested that the *VPS15* and *VPS34* gene products might be functioning at a similar step within the vacuolar protein delivery pathway. Furthermore, the protein sorting defects associated with *vps15* and *vps34* mutants appear to be highly specific for soluble constituents of the yeast vacuole. These mutants exhibit the most severe defects in the sorting and/or processing of multiple soluble vacuolar hydrolases including carboxypeptidase Y (CPY), proteinase A (PrA)

and proteinase B (Robinson et al., 1988; Herman and Emr, 1990; Herman et al., 1991). In contrast, the delivery of vacuolar membrane proteins appears to proceed rather efficiently in *vps15* and *vps34* mutants. This latter assertion is supported by several experimental observations that are summarized below. In spite of the extreme defects in the localization of soluble vacuolar proteins, *vps15* and *vps34* cells possess a morphologically normal vacuolar compartment. This indicates that these mutants are competent for vacuole assembly *in vivo* and suggests that membrane constituents are delivered to the vacuolar compartment in *vps15* and *vps34* cells. In addition, the localization of two vacuolar membrane proteins, alkaline phosphatase (ALP) and α-mannosidase, appears to be unperturbed in *vps15* and *vps34* mutants (Robinson et al., 1988; Klionsky and Emr, 1989; P. K. Herman, unpublished observations). ALP is initially synthesized as an inactive precursor that is delivered to the vacuole via secretory pathway organelles (reviewed in Klionsky et al., 1990). Within the vacuole, this precursor is proteolytically activated by the action of the soluble vacuolar hydrolase, PrA. In *vps15* and *vps34* mutants, ALP is efficiently processed to its mature form indicating that it is properly delivered to the vacuolar compartment in these mutants (Klionsky and Emr, 1989; P. K. Herman, unpublished observations). Furthermore, indirect immunofluorescence experiments with antisera specific for ALP have demonstrated that this membrane protein is indeed localized to the vacuolar compartment in *vps15* and *vps34* mutants (C. Raymond and T. Stevens, personal communication). Experiments with a *ts* allele of *VPS15* support these observations (see below and Chapter 5). Therefore the Vps15 and Vps34 proteins appear to be specifically involved in the sorting of soluble vacuolar proteins.

The predicted sequence of the *VPS15* gene product exhibits significant similarity to the catalytic domains of protein kinases (Herman et al., 1991). In general, the Vps15p sequence is most similar to the catalytic domains of the serine/threonine-specific

protein kinases such as the γ-subunit of phosphorylase b kinase. Many protein kinase molecules have been identified and sequenced. Comparisons of the catalytic domains of these protein kinases have indicated that this domain is a modular structure where short stretches of highly conserved amino acids are interspersed with sequences of relatively little identity (Hanks et al., 1988). These highly conserved amino acids are likely important for catalytic function either directly, as constituents of the active site, or indirectly, as structural elements required for the formation of the active site. The observed sequence conservation between Vps15p and other known protein kinases was clustered within these regions of the kinase catalytic domain that are highly conserved among all known protein kinases (Herman et al., 1991). This sequence similarity therefore raised the interesting possibility that Vps15p is a protein kinase and that protein phosphorylation reactions regulate specific steps of the yeast vacolar protein sorting pathway.

Mutations have been constructed in four distinct Vps15p kinase subdomains that are highly conserved among all protein kinase molecules. Alterations within each of these four Vps15p kinase motifs result in the biological inactivation of this protein. These kinase domain mutants exhibit severe defects in the delivery of soluble hydrolases to the vacuole and in the *in vivo* phosphorylation of Vps15p. Moreover, alterations of amino acids that correspond to residues that are less conserved among protein kinases have less severe phenotypic consequences for Vps15p function *in vivo* (see Chapter 5). Therefore this mutational analysis suggests that the Vps15p kinase domain is responsible for regulating Vps15p phosphorylation *in vivo* and the data are consistent with Vps15p catalyzing a specific autophosphorylation reaction. These mutational studies also suggest that a wild-type Vps15p kinase domain is required for the efficient sorting of soluble proteins to the vacuolar compartment. Specific Vps15p-mediated protein phosphorylation reactions may then be required for vacuolar protein delivery in

yeast cells. The identification of physiologically relevant *in vivo* substrates for the Vps15 protein kinase would therefore provide insights into the potential role played by protein phosphorylation in this protein sorting pathway. These substrates could either be identified as second-site suppressors of *vps15* kinase domain mutants or as *VPS* gene products that are phosphorylated in a Vps15p-dependent manner *in vivo*.

An analysis of a *tsf* (temperature-sensitive for function; see Chapter 5) allele of *vps15* has provided strong genetic evidence that Vps15p is directly involved in the sorting of soluble hydrolases to the yeast vacuole. Short C-terminal deletions of Vps15p, removing as little as 30 amino acids (ΔC30), result in a severe *ts* vacuolar protein sorting defect. At the permissive sorting temperature, CPY is efficiently delivered to the vacuolar compartment where it is processed to its mature form. In contrast, at the nonpermissive temperature, CPY accumulates within a specific intracellular compartment in a p2, Golgi-modified precursor form. This *ts* vacuolar protein delivery block exhibits an extremely rapid rate of onset suggesting that the ΔC30 *vps15* gene product is quickly inactivated at the nonpermissive sorting temperature and that this inactivation almost immediately manifests itself as a defect in CPY delivery to the vacuole. Moreover, this *ts* sorting defect is specific for soluble vacuolar proteins as the vacuolar delivery of ALP, a vacuolar membrane protein, continues unabated at the nonpermissive sorting temperature in the ΔC30 mutant. The extremely rapid rate of onset of this *ts* defect, together with the high degree of specificity for soluble consitituents of the vacuole, strongly suggest that the observed vacuolar protein sorting defects in *vps15* mutants do not result from a secondary consequence of the loss of Vps15p function. Rather, the primary *in vivo* role of Vps15p appears to be in the sorting of proteins to the vacuolar compartment of yeast cells.

The immediate phenotypic consequence of a loss of Vps15p activity therefore appears to be the accumulation of Golgi-modified precursor forms of soluble vacuolar

hydrolases within a specific intracellular compartment distinct from the vacuole. The p2 CPY that accumulates within this compartment remains competent for vacuolar delivery as upon a shift back down to the permissive sorting temperature this CPY is observed to be efficiently processed to its mature form. The intracellular compartment housing p2 CPY at the nonpermissive sorting temperature may represent a normal transport intermediate functioning between the Golgi apparatus and the vacuole. The identification and characterization of this p2 CPY compartment could therefore provide some fundamental insights into the vacuolar protein transport process.

In order to assess the phenotypic consequences of null alleles of the *VPS15* and *VPS34* loci, we constructed haploid yeast strains that carried a gene deletion-disruption of either locus. In each case, we replaced a large portion of the *VPS15* or *VPS34* coding region with a yeast selectable marker such as the *TRP1* or *HIS3* gene (see Chapters 3 and 4). In both cases, a null allele resulted in a temperature-sensitive (*ts*) growth defect indicating that the *VPS15* and *VPS34* genes were essential for vegetative growth only at elevated growth temperatures. The biological basis for the observed temperature-conditional growth phenotypes associated with the Δ*vps15* and Δ*vps34* mutants is not known. Both Δ*vps15* and Δ*vps34* mutants exhibited an equally severe CPY sorting defect at the permissive and nonpermissive growth temperatures. More severe protein sorting defects, perhaps affecting additional proteins, might become apparent at elevated growth temperatures and result in the observed *ts* growth defects. In another model, it is possible that the severe *vps15* and *vps34* protein sorting defects result in a decrease in specific vacuolar activities that are required for vegetative growth at elevated, but not lower, growth temperatures. A similar *ts* growth defect has been observed with a number of *VPS* null mutants including null mutants of the *VPS1* (Rothman et al., 1990), *VPS11/END1* (Dulic and Reizman, 1989), *VPS16* (B. Horazdovsky and S. Emr, unpublished observations), *VPS18* (J. Robinson and S.

Emr, unpublished observations), and *VPS33* (Banta et al., 1990) genes. Interestingly, mutations in each of these genes result in defects in vacuole assembly and/or segregation (see below) and a hyper-sensitivity to osmotic stress. These data therefore suggest that the yeast vacuole may play a critical role in cellular responses to situations of environmental stress, such as elevated growth temperatures or osmotic stress. In cultured mammalian cells, the lysosomal degradation of specific cytosolic proteins appears to increase in response to cell starvation suggesting that the mammalian lysosome may also play a role in cellular responses to environmental stress conditions (Dice, 1987). A study of these *ts* growth defects associated with the *vps15* and *vps34* null mutants may prove to be especially interesting since, in contrast to the other null mutants mentioned above, *vps15* and *vps34* cells generally possess a morphologically normal vacuolar compartment.

Although *vps15* and *vps34* mutants are competent for the assembly of the vacuolar compartment *in vivo*, these mutants exhibit defects in the segregation of this organelle upon cell division. The appearance of the vacuole in the newly-forming buds of *vps15* and *vps34* cells is greatly delayed relative to its time of appearance in wild-type cells (Herman and Emr, 1990; P. Herman and S. Emr, unpublished observations; C. Raymond and T. Stevens, personal communication). In general, no vacuolar structures are observed in the bud of *vps15* and *vps34* cells. In contrast, a normal vacuolar structure was generally observed in the mother cell and in the majority of the unbudded cells in populations of *vps15* and *vps34* cells. Since the bud vacuole inherits a substantial portion of its content from the mother cell vacuole (Wiemken et al., 1970; Weisman et al., 1987), the absence of a vacuolar structure in the bud of *vps15* and *vps34* mutants could be due to a defect in vacuolar inheritance. The vacuole segregation defects of *vps15* and *vps34* mutants could be a secondary consequence of the severe vacuolar protein sorting defects associated with these mutants. The sorting defects

could result in a decrease in specific vacuolar activities required for proper vacuole partitioning during cell division. In contrast, the Vps15 and Vps34 proteins may be required to execute a common step in the vacuole segregation and protein sorting pathways. It may be possible to distinguish between these two models by identifying a cellular component that when over-produced suppresses the vacuole segregation defect but not the missorting of soluble vacuolar hydrolases. This component would therefore correspond to the missing vacuolar activity in the former model. A better understanding of the precise role of the Vps15 and Vps34 proteins in the vacuolar protein delivery pathway and of the basic molecular mechanisms governing vacuolar segregation in yeast would provide some insight into the potential role of these proteins in vacuole inheritance *in vivo*.

Subcellular fractionation experiments suggested that Vps15p was peripherally associated with the cytoplasmic face of either a late Golgi or membrane vesicle compartment of yeast cells (Herman et al., 1991). In contrast, Vps34p was present in approximately equivalent proportions in two different intracellular pools (Herman and Emr, 1990). Approximately one-half of the total Vps34p was detected in a particulate fraction of yeast cell extracts. Attempts to solubilize this particulate Vps34p with a variety of reagents, including Triton X-100, urea and sodium chloride, indicate that this particulate Vps34p was likely a component of a relatively large multiprotein complex *in vivo*. The remaining ~50% of the total Vps34p was found in the soluble fraction of yeast cell extracts. It is possible that Vps34p may cycle between these two intracellular pools in a manner essential for vacuolar protein sorting. These fractionation studies therefore suggest that the Vps15 and Vps34 proteins exist within biochemically distinct intracellular fractions. However, recent immunological studies of these proteins have indicated that the Vps15 and Vps34 proteins exist as components of a hetero-oligomeric

complex *in vivo* and that this interaction may be essential for the efficient sorting of proteins to the vacuolar compartment of yeast cells.

Two separate studies have indicated that Vps15p and Vps34p either directly interact *in vivo* or interact indirectly through a common subunit in a multiprotein complex (J. Stack, P. Herman and S. Emr, unpublished observations). Nondenaturing immunoprecipitations from radiolabeled yeast cell extracts with antibodies specific for Vps15p result in the co-precipitation of Vps34p. Conversely, antibodies specific for Vps34p are able to co-precipitate Vps15p. A similar result was obtained from chemical cross-linking experiments with radiolabeled yeast cell extracts; the Vps15 and Vps34 proteins were detected within the same hetero-oligomeric complex. Genetic experiments analyzing the effects of the over-production of Vps34p in *vps15* mutants further suggest that this interaction is functionally relevant to the vacuolar protein sorting pathway of yeast cells. The over-production of Vps34p in *vps15* kinase domain mutants results in the partial suppression of the *ts* growth and CPY missorting defects associated with these mutants (P. Herman, J. Stack and S. Emr, unpublished observations). This suppression appears to require the presence of the Vps15 protein, even a nonfunctional one, as the over-production of Vps34p does not suppress these phenotypes of *vps15* null mutants. In addition this suppression apparently only works in one direction as the over-production of Vps15p in *vps34 ts* yeast strains does not suppress the growth or protein sorting phenotypes of these mutants.

Quantitation of the relative levels of these two proteins in this hetero-oligomeric complex suggest that approximately 1 to 2 molecules of Vps15p are present for each molecule of Vps34p in the observed protein complex. Only about 10% of the total radiolabeled Vps34p is found in a complex with Vps15p in these experiments suggesting that the majority of the particulate Vps34p in the fractionation experiments was present in a multiprotein complex that did not contain Vps15p. In one possible

model, Vps34p could be directly associated with the yeast cytoskeletal network. Vps34p could then transiently, and perhaps reversibly, interact with Vps15p either to facilitate packaging of soluble vacuolar hydrolases into specific transport intermediates trafficking between the Golgi and the vacuole or to mediate the delivery of these intermediates to the vacuole. In this model the Vps15 and Vps34 proteins would be the cellular components linking together the yeast cytoskeleton and the membranous intermediates of the secretory pathway.

These studies therefore indicate that the Vps15p kinase is present on the cytoplasmic side of a secretory pathway organelle(s) and is able to influence the delivery of specific lumenal proteins present within this organelle. The two models presented in Figure 1 attempt to illustrate how Vps15p might function in the vacuolar protein sorting pathway to specifically mediate the delivery of soluble hydrolases to the vacuolar compartment. In the upper pathway, ALP and CPY transit to the vacuole within the same transport intermediates. Since ALP appears to be efficiently delivered to the vacuole in *vps15* mutants, the cellular machinery required for the formation and delivery of these transport vesicles must be present and functional. Therefore Vps15p would presumably function prior to these transport events in the vacuolar protein delivery pathway. Vps15p activity might be required for the proper packaging of soluble vacuolar proteins into their appropriate vesicular carriers. Vps15p might facilitate the selective packaging of appropriate vacuolar proteins by phosphorylating the cytoplasmic tails of transmembrane receptors specific for these soluble vacuolar hydrolases in the Golgi complex.

Alternatively, ALP and CPY could traffic to the vacuole by two independent pathways involving distinct vesicular carriers. As shown in the second model in Figure 1, Vps15p might then act at a later step of the vacuolar protein sorting pathway, possibly in the formation of these transport intermediates, or in their delivery to the

vacuole. Vps15p activity would not be required for the delivery of vacuolar membrane proteins like ALP (see Figure 1). The Vps15p-mediated phosphorylation of a specific vesicle surface protein could serve to direct the delivery of the appropriate transport carrier to the vacuolar compartment. In this latter model, the rapid loss of Vps15p activity would result in the accumulation of p2 CPY within the transport intermediates trafficking between the Golgi complex and the vacuole. In the above packaging model, p2 CPY would instead be expected to accumulate within a late Golgi compartment upon loss of Vps15p activity.

Rather than being specific for regulating protein delivery to the yeast vacuolar compartment, protein phosphorylation reactions may act as a general regulatory mechanism to control protein sorting decisions throughout the eukaryotic secretory pathway. This general usage of protein phosphorylation is suggested by observations concerning the sorting of two transmembrane receptors within an early endosomal compartment. The correct and efficient sorting of these two receptors in the endosome appears to depend upon specific protein phsophorylation reactions *in vivo*. The phosphorylation of a specific serine residue within the cytoplasmic tail of the polymeric immunoglobulin receptor is required for its efficient transcytosis across polarized epithelial cells (Casanova et al., 1990). In the absence of this phosphorylation, these receptors appear to recycle back to the cell-surface. In addition, whereas wild-type EGF-receptors are targeted for lysosomal degradation from this early endosome, kinase-inactive EGF-receptors are instead observed to recycle back to the cell-surface (Felder et al., 1990; Honegger et al., 1990). Therefore, the diversion of proteins from what is likely to be a default transport pathway into an auxiliary route appears to require specific protein phosphorylation reactions. Protein phosphorylation may then be acting as a molecular "switch" within intracellular protein sorting pathways by actively diverting proteins from a default route and into an alternative delivery pathway.

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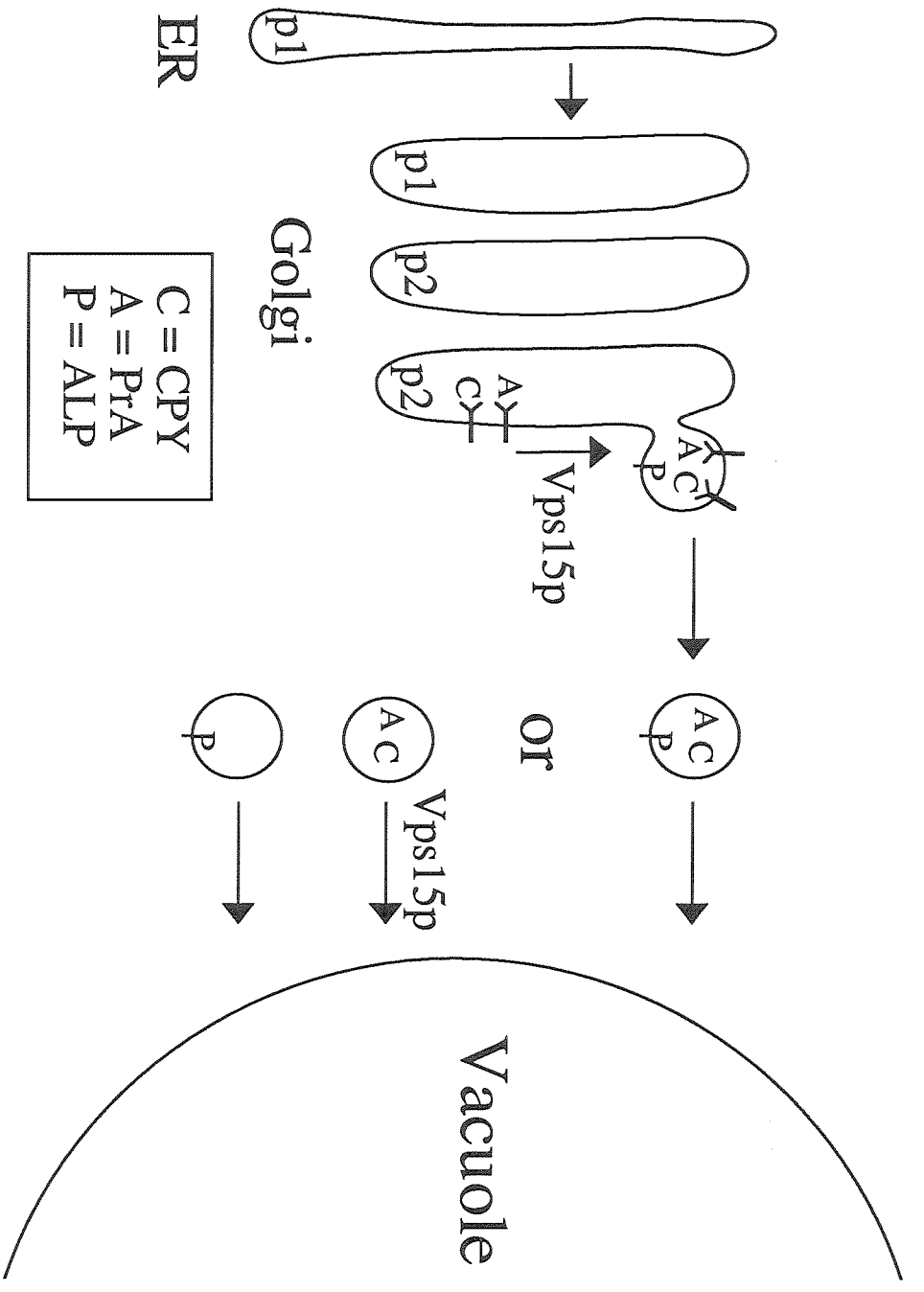
Figure 1. Possible roles for Vps15p in vacuolar protein sorting.

Two possible models for the action of Vps15p in the vacuolar protein delivery pathway are shown. In the top model, soluble vacuolar hydrolases, such as CPY and PrA, are shown trafficking to the vacuole within the same transport vesicle as vacuolar membrane proteins such as ALP. Vps15p is shown as functioning in the specific packaging of soluble vacuolar proteins into their appropriate transport carriers. In the bottom model, soluble and membrane proteins are shown to be transiting to the vacuole in distinct transport intermediates. In this model, Vps15p is required for the delivery of the soluble hydrolase-specific vesicles to the vacuolar compartment.


ER
p1
Golgi
p1
p2
p2
A
C
P
Vps15p
C = CPY
A = PrA
P = ALP
A C P
or
A C
Vps15p
P
Vacuole