The EMBO Journal vol.8 no.8 pp.2241 - 2250, 1989

# Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase

**Daniel J.Klionsky and Scott D.Emr**

California Institute of Technology, Pasadena, CA 91125, USA

Communicated by D.Meyer

**The *Saccharomyces cerevisiae PHO8* gene product, repressible alkaline phosphatase (ALP), is a glycoprotein enzyme that is localized to the yeast vacuole (lysosome). Using antibodies raised against synthetic peptides corresponding to two distinct hydrophilic sequences in ALP, we have been able to examine the biosynthesis, sorting and processing of this protein. ALP is synthesized as an inactive precursor containing a C-terminal propeptide that is cleaved from the protein in a PEP4-dependent manner. The precursor and mature protein are anchored in the membrane by an N-terminal hydrophobic domain that also appears to function as an uncleaved internal signal sequence. ALP has the topology of a type-II integral membrane protein containing a short basic N-terminal cytoplasmic tail that is accessible to exogenous protease when associated both with the endoplasmic reticulum and the vacuole. Similar to the soluble vacuolar hydrolases carboxypeptidase Y (CPY) and proteinase A (PrA), ALP transits through the early stages of the secretory pathway prior to vacuolar delivery. Two observations indicate, however, that ALP is localized to the vacuole by a mechanism which is in part different from that used by CPY and PrA: (i) maturation of proALP, which is indicative of vacuolar delivery, is less sensitive than CPY and PrA to the defects exhibited by certain of the vacuolar protein sorting (*vps*) mutants; and (ii) maturation of proALP proceeds normally in the presence of a potent vacuolar ATPase inhibitor, bafilomycin A1, which is known to block vacuole acidification and leads to the mis-sorting and secretion of precursor forms of CPY and PrA. These results indicate that ALP will be a useful model protein for studies of membrane protein sorting in yeast.**

*Key words:* membrane protein/*PHO8*/sorting/vacuoles/yeast

## Introduction

The vacuole (lysosome) of the yeast *Saccharomyces cerevisiae* is an acidic compartment that is bounded by a lipid bilayer. The vacuolar lumen or tonoplasm contains a variety of soluble degradative enzymes including carboxypeptidase Y (CPY), proteinase A (PrA) and proteinase B (PrB) (Lenney *et al.*, 1974; Wiemken *et al.*, 1979). These enzymes have been well characterized in terms of their biosynthesis (Hasilik and Tanner, 1978; Mechler *et al.*, 1982, 1988; Ammerer *et al.*, 1986; Woolford *et al.*, 1986; Klionsky *et al.*, 1988; Moehle *et al.*, 1989). All of these hydrolases are synthesized as inactive precursors with N-terminal signal peptides that are proteolytically removed during translocation into the endoplasmic reticulum (ER). They are core glycosylated in the ER and undergo additional carbohydrate modification in the Golgi complex. Sorting of vacuolar enzymes and other proteins utilizing the secretory pathway is believed to occur in a late Golgi compartment (Stevens *et al.*, 1982; Griffiths and Simons, 1986; Johnson *et al.*, 1987). This is supported by the observation that vacuolar protein transport is blocked only in *sec* mutants that affect transport out of the ER or Golgi complex. Just before or upon arrival in the vacuole, proCPY, proPrA and proPrB are activated by the proteolytic removal of a propeptide segment (Hemmings *et al.*, 1981; Ammerer *et al.*, 1986; Mechler *et al.*, 1987, 1988). This maturation process is dependent on PrA which is thought to activate itself autocatalytically (Woolford *et al.*, 1986). The half-time for maturation is ~6 min for all three proteins (Hasilik and Tanner, 1978; Klionsky *et al.*, 1988; Mechler *et al.*, 1988). The sorting of these soluble vacuolar hydrolases has also been examined in mutant strains of yeast that are defective in vacuolar protein sorting (Bankaitis *et al.*, 1986; Robinson *et al.*, 1988; Rothman and Stevens, 1986). These *vps* mutants are pleiotropic in their targeting defects; precursor forms of each of these three soluble vacuolar proteins accumulate to similar extents in each mutant strain. As judged by these criteria, soluble vacuolar proteins appear to be targeted and delivered to the vacuole by a common mechanism.

The vacuole membrane contains a number of proteins and protein complexes including α-mannosidase, dipeptidyl aminopeptidase B, a proton-translocating ATPase and several permeases (Wiemken *et al.*, 1979; Kakinuma *et al.*, 1981; Ohsumi and Anraku, 1983; Sato *et al.*, 1984; Garcia Alvarez *et al.*, 1985; Roberts *et al.*, 1989). Unlike the soluble enzymes, most of the vacuolar membrane proteins have not been well characterized and many are only defined with regard to their biochemical activities. Accordingly, little is known about the mechanism(s) of membrane protein sorting. The same situation exists for the mammalian lysosome; soluble lysosomal proteins have been much more extensively characterized than lysosomal membrane proteins (von Figura and Hasilik, 1986). Interestingly, for some time there has been evidence suggesting that these two classes of proteins may be delivered to the lysosome by different mechanisms (Gabel *et al.*, 1983; Owada and Neufeld, 1982). While many soluble lysosomal proteins utilize the mannose-6-phosphate receptor pathway, some, e.g. certain lysosomal integral membrane proteins, appear to be transported by a mannose-6-phosphate-independent mechanism (Waheed *et al.*, 1982, 1988; Barriocanal *et al.*, 1986; Green *et al.*, 1987). Unlike the soluble hydrolases, these membrane proteins also appear to be modified with complex asparagine-linked carbohydrates (Lewis *et al.*, 1985). Consistent with these observations, several lysosomal membrane proteins are correctly sorted in mutant I-cells which are deficient for the mannose-6-phosphate modification (Kornfeld, 1987).

Similarly, the yeast vacuolar protein sorting (*vps*) mutants

also appear to be much less defective in the localization of at least one vacuolar membrane protein (Bankaitis *et al.*, 1986; Robinson *et al.*, 1988). Most of the mutant strains that were extremely defective in sorting the soluble hydrolases had very little effect on α-mannosidase. This is consistent with the presence of separate mechanisms for the delivery of soluble and membrane-bound vacuolar proteins. In order to address the possibility of an alternative sorting pathway for vacuolar membrane proteins in yeast, we decided to characterize the biosynthesis, sorting and processing of the repressible alkaline phosphatase (ALP), the product of the *PHO8* gene. While several lines of evidence have shown that ALP is located in the vacuole, there was some discrepancy as to the association of ALP with the vacuolar membrane (Wiemken *et al.*, 1979). By some reports the mature enzyme does not appear to be membrane bound (Ohsumi and Anraku, 1981; Uchida *et al.*, 1985). Cytochemical analysis, however, revealed that the enzyme is located along the innerside of the vacuolar membrane (Bauer and Sigarlakie, 1973, 1975; Clark *et al.*, 1982). Interestingly, unlike other vacuolar membrane proteins, ALP activity is dependent on the presence of active PrA, the product of the *PEP4* gene, suggesting that it has a cleavable propeptide (Hemmings *et al.*, 1981). Based on this observation it seemed possible that the membrane and soluble forms share a precursor−product relationship (Mitchell *et al.*, 1981). This would be similar to the case with certain lysosomal proteins in *Dictyostelium discoideum* (Cardelli *et al.*, 1986) and in mammalian BHK cells (Waheed *et al.*, 1988).

Recently, the *PHO8* gene was cloned and its nucleotide sequence was determined (Kaneko *et al.*, 1987). To analyze the nature of ALP's association with the membrane, we prepared antiserum to ALP and used it to examine the biosynthesis and sorting of this enzyme. ALP appears to be an integral membrane protein that is anchored by a hydrophobic domain near the N terminus. It is synthesized as a zymogen containing a C-terminal propeptide segment that is proteolytically removed to generate the mature active enzyme. We also compared the vacuolar transport of ALP to that of CPY, PrA and PrB. Vacuolar delivery of ALP shows some characteristics that suggest it may be sorted by a mechanism which is at least partly different from that used by at least some of the soluble vacuolar hydrolases.

## Results

### *Biosynthesis and processing of alkaline phosphatase*

The *PHO8* gene product, the repressible alkaline phosphatase (ALP), is one of many hydrolytic glycoprotein enzymes which are known to reside within the vacuole. It was determined that ALP is synthesized as a zymogen based upon characterization of its activity in wild-type and *pep4-3* mutant yeast (Jones *et al.*, 1982). This finding suggested that the ALP precursor contains a propeptide that is responsible for maintaining the enzyme in an inactive state prior to its arrival in the vacuole. Since antisera to ALP have not been available, studies on its biosynthesis have been limited to approaches that employ biochemical or cytochemical techniques to monitor its activity and/or subcellular localization. The absence of an immunological analysis, coupled with the presence of additional phosphatase activities in *S.cerevisiae* have resulted in conflicting reports concerning the biosynthesis of ALP. To examine more carefully the biosynthesis of this enzyme, we decided to generate antisera specific for the repressible alkaline phosphatase. To this end, we prepared two synthetic peptides based upon the deduced amino acid sequence of ALP, corresponding to amino acids 222−244 and 281−301. These sequences were chosen from the 566 amino acid sequence of precursor ALP based upon their highly charged hydrophilic nature. The synthetic peptides were prepared for injection into rabbits as described in Materials and methods. For a careful characterization of the antisera, it was necessary to construct a yeast strain that was deleted for the chromosomal *PHO8* gene. A strain deleted and disrupted at the *PHO8* locus, DKY6280 (*Δpho8::LEU2*), was constructed by standard techniques as described in Materials and methods. To characterize the antisera, wild-type, *Δpho8* and *Δpep4* yeast strains were labeled and used for immunoprecipitation. Based on the findings that the antisera (i) immunoprecipitated from a wild-type strain a specific protein of mol. wt ~72 000 that the preimmune serum did not recognize; (ii) did not immunoprecipitate any proteins from a *Δpho8* strain; and (iii) detected a larger, 76 000 mol. wt protein in a *Δpep4* strain consistent with the requirement of PrA for ALP activity, we concluded that the antisera were able to immunoprecipitate specifically the product of the *PHO8* gene (data not shown). Based on previous studies and the nucleotide sequence of *PHO8* (Onishi *et al.*, 1979; Kaneko *et al.*, 1987), the mature glycosylated protein should have a mol. wt of 66 000−68 000. The difference between the apparent and predicted mol. wts was probably due to a slightly aberrant gel migration.

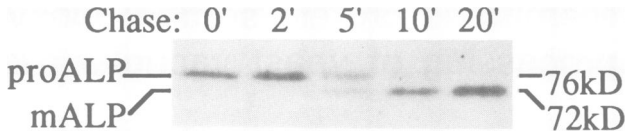


**Fig. 1.** Processing kinetics of alkaline phosphatase. Strain SEY2108 was labeled at 30°C for 5 min and chased for the indicated times. Double immunoprecipitations were performed with ALP antiserum. The approximate molecular sizes of the different forms of ALP are indicated (kD, kilodalton).

The ALP immunoprecipitated from a wild-type strain migrated more rapidly than that from a *Δpep4* strain when analyzed by SDS−PAGE. This suggested a precursor−product relationship consistent with the findings that ALP is activated in a proteinase-A-dependent manner. To examine this relationship in greater detail, we analyzed the processing kinetics of the wild-type ALP protein. Pulse−chase labeling of yeast cells followed by immunoprecipitation with the ALP-specific antiserum showed a half-time of ~6 min for the processing of the precursor to the mature enzyme (Figure 1). This is similar to the previously observed rates of maturation seen for procarboxypeptidase Y and proproteinase A (Hasilik and Tanner, 1978; Klionsky *et al.*, 1988).

The vacuolar glycoproteins that have been analyzed are synthesized in the ER as core glycosylated (p1) forms. After transit to the Golgi complex they undergo further carbohydrate modification to generate the larger p2 intermediate. ALP is believed to have two glycosidic side chains (Onishi *et al.*, 1979). This is consistent with the *PHO8* nucleotide sequence which reveals two potential N-linked carbohydrate addition sites at amino acid residues 268 and

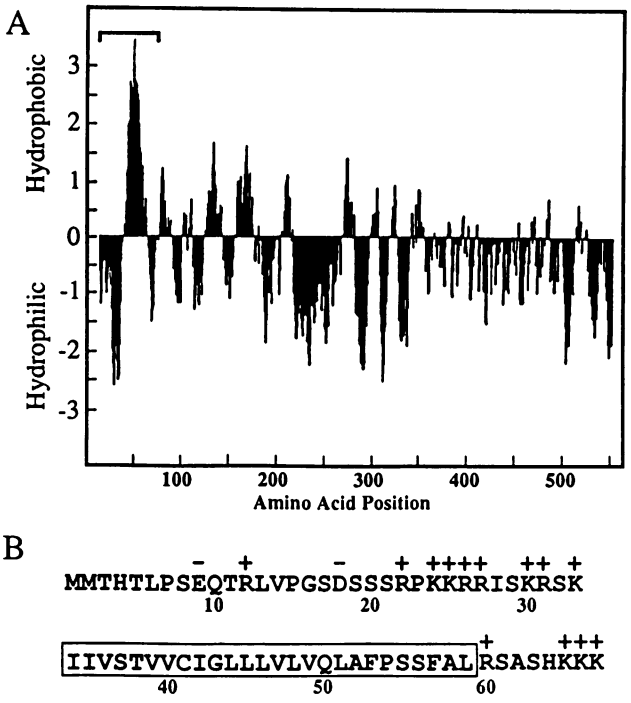


**Fig. 2. Panel A**: computer-generated hydrophobicity analysis of proALP. The deduced amino acid sequence of ALP was analyzed by the method of Kyte and Doolittle (1982) averaging over a range of 11 amino acids. The amino acid sequence is provided in panel B for the region delineated by the bracket. **Panel B**: deduced amino acid sequence of the first 67 residues of proALP. The boxed sequence indicates the putative transmembrane domain.

401 of the precursor protein (Kaneko *et al.*, 1987). To analyze more carefully the nature of the glycosylation on ALP we immunoprecipitated the protein from *sec* mutant yeast strains (Novick *et al.*, 1980). Comparison of ALP immunoprecipitated from *sec18* (ER blocked) and *sec7* (Golgi blocked) strains labeled at the non-permissive temperature and separated by SDS−PAGE did not reveal any detectable size difference. The p1 to p2 conversion would be difficult to detect for ALP, however, due to the relatively small size difference expected between the ER and Golgi forms. The *sec18* mutation resulted in a complete block in transport of ALP from the ER, while in the *sec7* mutant there was only a kinetic delay in movement through the Golgi complex as seen by the eventual maturation of ALP at the non-permissive temperature. ALP probably does undergo a p1 to p2 type conversion, however, since the Golgi (p2) and mature forms are immunoprecipitable with antisera specific to α-1,3-mannose linkages (our unpublished results), a Golgi-specific modification also observed on PrA and CPY (Hasselbeck and Schekman, 1986; Klionsky *et al.*, 1988). These results showed that ALP was subject to the *sec*-induced ER and Golgi transit blocks similar to PrA and CPY (Stevens *et al.*, 1982; Klionsky *et al.*, 1988). In agreement with this observation, proALP was matured with normal kinetics in a *sec1* mutant strain at the non-permissive temperature indicating that delivery to the vacuole does not depend on late secretory pathway functions. Finally, we determined the size difference between the ALP species synthesized with or without glycosyl modification. The mature enzyme has an apparent mol. wt of 66 and 72 kd in the presence and absence of tunicamycin, respectively. This would be

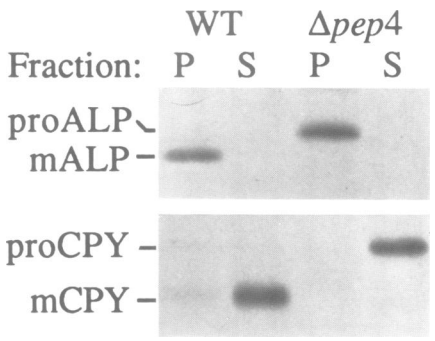


**Fig. 3.** Precursor and mature ALP associated with the vacuolar membrane. Spheroplasts were prepared from strains SEY2101 and SEY2101-Δ*pep4*, labeled, lysed and separated into pellet (membrane-associated) and supernatant (soluble) fractions as described in Materials and methods. Double immunoprecipitations were carried out with ALP and CPY antiserum. The positions of the different processed forms of the proteins are indicated. P, pellet; S, supernatant.

consistent with the addition of two N-linked glycosyl side chains of ~2500 mol. wt each. In agreement with this, ALP migrates as a discrete band after SDS−PAGE indicating that it is modified like CPY and not like the secreted enzyme invertase (Trimble and Maley, 1977). ALP apparently undergoes the same type of limited glycosyl modification seen for soluble vacuolar proteins.

### *Membrane association of alkaline phosphatase*

An analysis of the deduced amino acid sequence of ALP indicated that the extreme N terminus of the protein did not contain a sequence which fits with the normal hydrophobic consensus for signal peptides or a signal sequence cleavage site based upon the rules established by von Heijne (1986). Accordingly, we analyzed the protein for the presence of an internal signal sequence which could function in allowing the protein to translocate across the ER. A hydropathy analysis was carried out according to the rules of Kyte and Doolittle (1982). This analysis revealed a very hydrophilic region at the extreme N terminus, which contains several basic amino acid residues (Figure 2). The absence of a hydrophobic core in this region correlates with the apparent lack of a signal peptide. The clustered set of basic amino acids at the N terminus is followed by a long stretch of ~20 hydrophobic amino acids. No other areas of the protein display this level of hydrophobicity. This hydrophobic region is followed by a second, smaller cluster of basic residues, suggesting that the N terminus of ALP may function as a transmembrane anchor (Eisenberg, 1984).

While it has not been clear whether ALP is a soluble or membrane protein, it was suggested that a precursor−product relationship may exist between soluble and membrane-associated forms (Mitchell *et al.*, 1981). The hydropathy analysis suggested that at least the precursor form of ALP was capable of assuming the configuration of a type-II integral membrane protein (Lipp and Dobberstein, 1986). Since the location of the ALP propeptide has not been determined previously, it was not known whether the N-terminal hydrophobic domain would be proteolytically removed upon maturation in the vacuole. To examine this, we first determined whether ALP was associated with the vacuolar membrane in both wild-type and Δ*pep4* mutant yeast. Yeast cells were enzymatically converted to

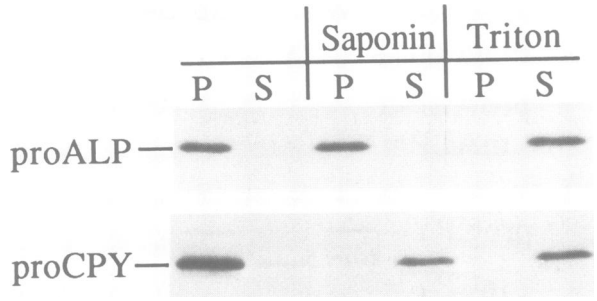


**Fig. 4.** ALP is not released from the ER by saponin. Spheroplasts from strain SEY5186 were labeled at the non-permissive temperature, lysed and treated with saponin (0.2%) or Triton X-100 (0.1%) as described in Materials and methods. Following centrifugation the pellet and supernatant fractions were immunoprecipitated with ALP and CPY antiserum. P, pellet; S, supernatant.

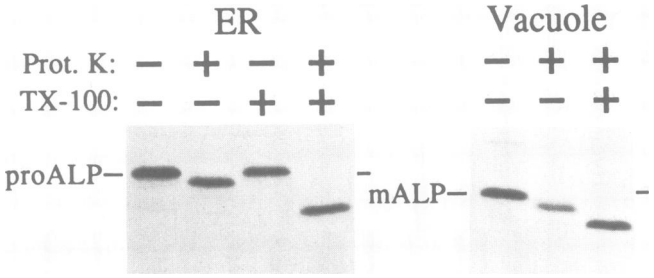


**Fig. 5.** ER and vacuolar ALP contain protease sensitive cytoplasmic domains. The conditions used for the lysis of labeled wild-type and *sec18* mutant spheroplasts and treatment with proteinase K were as described in Materials and methods.

spheroplasts prior to labeling and chase (Robinson *et al.*, 1988) and then were subjected to osmotic lysis. The samples were centrifuged at 100 000 *g* to pellet membranes and the pellet (membrane-associated) and supernatant (soluble) fractions were immunoprecipitated with antisera to ALP and CPY (Figure 3). The osmotic lysis conditions used disrupt the vacuolar membrane resulting in release of soluble vacuolar CPY from both strains. In contrast, both the precursor and mature forms of ALP were found exclusively in the pellet fractions. This indicated that ALP associates with the vacuolar membrane.

To address more carefully the nature of ALP's association with the membrane we used additional methods to assess its interaction with the lipid bilayer. The ALP precursor appeared to be membrane-associated when it was accumulated in the vacuole in a Δ*pep4* strain. Accordingly, we predicted that the precursor protein would also be membrane associated in the ER and we examined this through the use of detergents. Spheroplasts were prepared from *sec18* mutant yeast and labeled at the non-permissive temperature to accumulate proALP in the ER. After labeling and chase, the spheroplasts were gently lysed so as to retain the integrity of the ER (Eakle *et al.*, 1988). The lysed cultures were treated with saponin (0.2%) or Triton X-100 (0.1%) and centrifuged at 13 000 *g* to pellet the ER membrane. The soluble (supernatant) and membrane-associated (pellet) fractions were immunoprecipitated with antisera to ALP and CPY (Figure 4). When nothing is added to the lysed spheroplasts, essentially all of proALP and proCPY are located in the pellet fraction indicating that the lysis conditions did not disrupt the integrity of the ER. Treatment with saponin, a detergent that permeabilizes membranes, but does not solubilize membrane proteins, had no effect on the localization of proALP, similar to the result seen for the ER integral membrane protein NADPH cytochrome *c* reductase (Schauer *et al.*, 1985). In contrast, CPY was released into the supernatant fraction in the presence of saponin indicating that it did not bind to the ER membrane but rather was soluble within the lumen of the ER. ALP was solubilized, however, by the addition of 0.1% Triton X-100 indicating that its association with the pellet fraction did not result simply from its forming an insoluble aggregate. Precursor ALP appears to be an integral membrane protein at least in the ER.

To determine whether mature ALP is also tightly associated with the membrane, we examined its partitioning during extraction with alkali buffer. Spheroplasts from a wild-type yeast strain were labeled (15 min) and chased for a sufficient time (20 min) so that all of the labeled ALP was present as the mature form in the vacuole. The spheroplasts were pelleted and osmotically lysed by resuspension in 10 mM sodium carbonate, pH 11.0. The samples were centrifuged to separate the extractable and precipitable fractions and immunoprecipitated with antisera to ALP and PrB. The immunoprecipitated proteins were resolved on an SDS−polyacrylamide gel and quantitated by densitometry. Alkali buffers extract soluble and peripheral membrane proteins (Fujiki *et al.*, 1982). As expected, all of the PrB (>95%) was found in the extracted fraction. Most of the alkaline phosphatase, however, remained in the pellet fraction (70%) after extraction with sodium carbonate. Based on this result, mALP in the vacuole is also tightly associated with the membrane. It is interesting to note that 73% of the activity of the vacuolar membrane protein α-mannosidase could be extracted with sodium carbonate under similar conditions (Yoshihisa *et al.*, 1988).

### *Presence of a cytoplasmic domain on proALP*

Our analysis of the membrane association of ALP suggested that it was an integral membrane protein. Given that the ALP enzyme activity is present on the lumenal side of the vacuole and taking into consideration the deduced amino acid sequence, hydropathy plot, and location of the N-linked oligosaccharide addition sites, proALP may be predicted to be oriented in the membrane as a type-II integral membrane protein. The cluster of basic amino acids preceding the hydrophobic domain may prevent the extreme N terminus from translocating across the ER membrane. This would result in the presence of a cytoplasmic domain of ~30 amino acids. To determine if proALP possesses such a cytoplasmic tail segment in the ER, *sec18* mutant yeast cells were converted to spheroplasts, labeled and subjected to gentle osmotic lysis to retain the integrity of the ER. Samples were then treated with proteinase K in the absence and presence of detergent followed by immunoprecipitation with antisera to ALP and PrA (Figure 5). There was essentially no degradation of proteinase A in the absence of detergent, indicating that the ER remained intact (data not shown). In contrast, proteinase K present on the cytoplasmic surface of the ER was able to cleave proALP as seen by a shift in size during SDS−PAGE. This cleavage was not due to the presence of protease within the ER since PrA immuno-

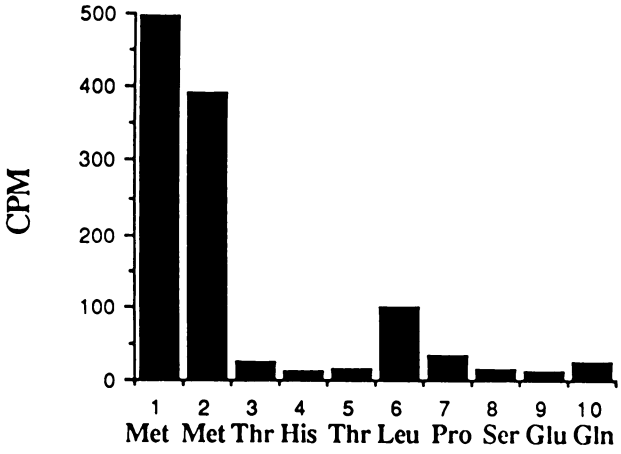


**Fig. 6.** The propeptide of precursor ALP is removed from the C terminus. Spheroplasts from strain SEY2108 were labeled with [$^{35}$S]methionine. The mALP recovered after a double immunoprecipitation was subjected to automated sequential Edman degradation. The c.p.m. released at each cycle are shown. The primary amino acid sequence of precursor ALP is shown beneath the graph.

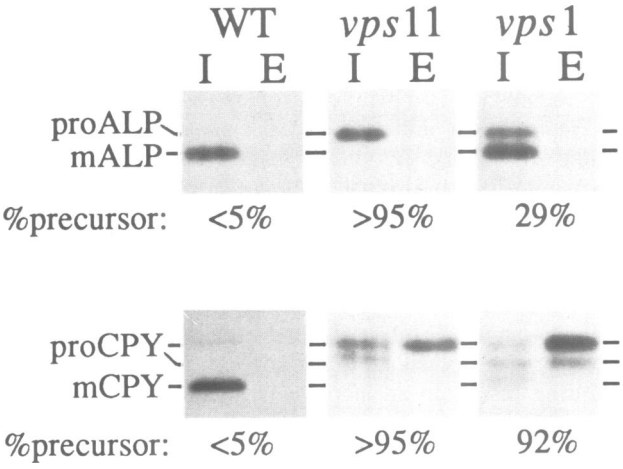


**Fig. 7.** Immunoprecipitation of ALP and CPY in wild-type and *vps* mutant yeast strains. Spheroplasts were labeled for 20 min and chased for 30 min in the presence of BSA and $\alpha_2$-macroglobulin to reduce nonspecific proteolysis. The cultures were separated into spheroplast (I) and extracellular (E) fractions. Radiolabeled proteins were immunoprecipitated with antiserum to ALP and CPY.

precipitated from the same sample showed no evidence of proteolysis, and ALP was subject to additional, but not complete, proteolysis when detergent was used to disrupt the ER membrane. The sensitivity to exogenously added proteinase K in the absence of detergent indicates that ALP does possess a cytoplasmic domain and shows that the precursor is a transmembrane protein. This result also supports our prediction about the orientation of proALP in the membrane. Analysis of the migration after SDS−PAGE indicated that ~22−26 amino acids were removed from the portion of ALP exposed to the cytoplasm. This is in close agreement with the predicted size of the putative cytoplasmic tail. It is interesting to note that the addition of the non-ionic detergent Triton X-100 along with proteinase K resulted in additional but not complete degradation of proALP. This limited susceptibility to proteolysis is similar to that seen with PrA, CPY and PrB (unpublished observations). The resistance to proteolysis shown by mature vacuolar enzymes presumably reflects their normal residence in a compartment that contains a variety of hydrolytic enzymes.

### *Location of the ALP propeptide*

It is known that activation of proALP is *PEP4*-dependent (Hemmings *et al.*, 1981; Figure 3, lanes 1 and 3). Based on the size difference between precursor and mature ALP (Figure 1), ~50 amino acids are removed during the propeptide cleavage reaction. The terminus at which the propeptide was located, however, was not known. An ALP polypeptide that is truncated at the C terminus shows significant levels of activity in a *pep4* strain, suggesting cleavage of a C-terminal propeptide from the wild-type protein (Kaneko *et al.*, 1987). The finding that mALP was membrane-associated supports this prediction since removal of an ~50-amino-acid propeptide from the N terminus would remove the hydrophobic domain. To address this question directly, we examined the presence of a cytoplasmic domain on mALP in the vacuole. Spheroplasts were prepared from a wild-type yeast strain, labeled and chased. The labeled spheroplasts were subjected to gentle lysis through the use of DEAE−dextran to allow access to, and preserve the integrity of, the vacuole (Bankaitis *et al.*, 1986). The lysed spheroplasts were incubated on ice for 30 min in the presence or absence or proteinase K and immunoprecipitated (Figure 5). We found that proteinase K present on the cytoplasmic surface of the vacuole caused the same shift in molecular weight as observed with proALP in the ER. Since mature ALP also has a cytoplasmic domain it seemed likely that propeptide cleavage occurred at the C terminus. In support of this, we have recently found that an ALP protein mutated to remove the N-terminal hydrophobic domain no longer associates with the membrane (unpublished observations).

At this point, however, we had not directly shown that the cytoplasmic domain is derived from the N terminus. To address this, we decided to obtain amino acid sequence data from mALP. Spheroplasts were prepared, labeled with [$^{35}$S]methionine and chased for a sufficient time to ensure that only mature ALP was present in the vacuole. The radiolabeled mALP was purified by successive immunoprecipitations with ALP antiserum until it was radiochemically pure as judged by SDS−PAGE. The use of two successive immunoprecipitations also allowed for the complete removal of unincorporated [$^{35}$S]methionine. The purified protein was subjected to sequential Edman degradation and the radioactivity released per cycle was determined by counting each fraction in a scintillation counter. These data were plotted and compared with the sequence of the precursor protein (Figure 6). Sequential cleavage from the N terminus of mALP yielded two peaks of radioactivity corresponding to the first two cycles of the Edman degradation. The only place in proALP where there are two methionine residues next to each other is at amino acid positions 1 and 2. Precursor and mature ALP apparently have identical N termini indicating that the propeptide must be cleaved from the C terminus of the precursor protein. This result also confirms that the cytoplasmic domain of mature ALP corresponds to the N terminus. Since precursor and mature ALP have N-terminal cytoplasmic domains they must have the topology of type-II integral membrane proteins.

### *Vacuolar sorting of ALP in vps mutants*

Since ALP is an integral vacuolar membrane protein, unlike the previously characterized vacuolar proteins CPY, PrA and PrB, we decided to examine its intracellular localization in the vacuolar protein sorting (*vps*) mutants (Robinson *et al.*, 1988). Previous data suggested that α-mannosidase was much less sensitive than the soluble vacuolar hydrolases to the sorting defects present in many of the *vps* mutant strains. To examine the effect of thse mutations on the sorting of ALP, *vps* mutant strains were converted to spheroplasts, labeled for 20 min followed by a 30 min chase, separated into intracellular and extracellular fractions and sequentially immunoprecipitated with antisera to ALP and CPY (Figure 7). Under these conditions, the wild-type parent strain has only mature forms of these proteins in the intracellular (I) fraction. Class C *vps* mutants (*vps11, 16, 18* and *33*), which mislocalize as much as 50% of α-mannosidase and lack any obvious vacuolar structures, accumulate only precursor forms of CPY, PrA and PrB. Similarly, only proALP was seen in these strains, indicating that it is possible to accumulate this form of the protein as a result of mis-sorting defects. Interestingly, other strains that are also extremely defective for the sorting of the soluble vacuolar hydrolases have relatively minor effects on ALP and α-mannosidase. The strain *vps1*, for example, accumulates ~92% of its CPY as precursor forms, most of which is secreted into the extracellular (E) fraction. In contrast, only 29% of ALP is accumulated as precursor in this strain. Similarly, only 15% of α-mannosidase activity is present at the cell surface in *vps1* (Robinson *et al.*, 1988). Several other mutant strains including *vps5, 8* and *26*, show strong defects in CPY sorting and processing (75–95% precursor accumulation) (Robinson *et al.*, 1988), but have relatively minor defects (<30% precursor) in the sorting of ALP. The remaining *vps* mutants that were examined (*vps3, 6, 9, 15, 19, 34* and *35*) showed intermediate levels of precursor accumulation for ALP. In almost all cases, the observed defects were more pronounced with CPY than with ALP. It should be noted that proALP is never secreted into the extracellular fraction consistent with its retention as a membrane-associated protein. In our analysis of the *vps* mutants we have relied on propeptide cleavage as a means of assessing vacuolar delivery and hence, proper sorting. It should be pointed out that we cannot differentiate between sorting and/or processing defects as the cause of precursor accumulation in strains that accumulate all of their ALP as the precursor form, e.g. *vps11* (Figure 7) and other class C mutants. Since these mutants accumulate and/or secrete all of their CPY, PrA and PrB as inactive precursors (Robinson *et al.*, 1988), they are likely to be processing defective. Other *vps* mutants, however, are clearly able to mature PrA and CPY (Robinson *et al.*, 1988). In addition, very low levels of PrA are sufficient for efficient processing of vacuolar precursors (Mechler and Wolf, 1981; Klionsky *et al.*, 1988). Since most of the mutants are processing competent, the blocks in ALP maturation are most likely due to defects in sorting. Similar to the results seen for the membrane protein α-mannosidase which we could only follow by enzyme activity (Robinson *et al.*, 1988), ALP is less sensitive to most of the *vps* mis-sorting defects than are the soluble vacuolar hydrolases.

### Mechanism of ALP transport to the vacuole

Since ALP transits to the vacuole in a membrane-associated form it serves as a useful model to analyze the transit of membrane proteins. In addition, the proteolytic maturation of ALP provides a simple way to assess vacuolar delivery. The vacuole is a low pH compartment and this feature may be important in initiating the mechanism of maturation of the proenzyme (Woolford *et al.*, 1986). By analogy to mammalian lysosomal protein sorting, compartment acidification may also play a role in the sorting of at least some vacuolar enzymes (Sly and Fischer, 1982). We have examined the importance of a vacuolar pH gradient in the sorting of vacuolar proteins through the use of the drug bafilomycin A1. This compound is a potent and specific inhibitor of the vacuolar ATPase and brief treatment with the drug results in an increase in vacuolar pH (Banta *et al.*, 1988; Bowman *et al.*, 1988). To examine the effect of bafilomycin on the sorting of ALP, yeast spheroplasts were treated with the drug prior to the addition of label. After labeling, cultures were separated into intracellular (spheroplast) and extracellular (media and periplasm) fractions and immunoprecipitated with antisera to various vacuolar proteins (Figure 8). Bafilomycin resulted in the mis-sorting and secretion of significant levels of the precursor forms of CPY and PrA (Banta *et al.*, 1988). In contrast, ALP did not appear to be affected by bafilomycin treatment. Wild-type levels of the mature protein were present in the intracellular fraction consistent with proper sorting and processing of the protein. Since proALP that has been mis-sorted is very stable as the precursor form (e.g. *vps11* in Figure 7) we do not think it likely that maturation of ALP is due to a non-vacuolar processing event. In addition, the maturation of ALP seen in the presence of bafilomycin occurs with kinetics that are indistinguishable from those seen in the absence of the drug (Figure 1). This implies that treatment with bafilomycin does not alter the transport pathway normally used by ALP. The differential effect of bafilomycin on the sorting of ALP compared with PrA and CPY suggests that membrane proteins may transit to the vacuole by a mechanism that is less dependent on compartmental pH and at least partly different from that used for soluble proteins.

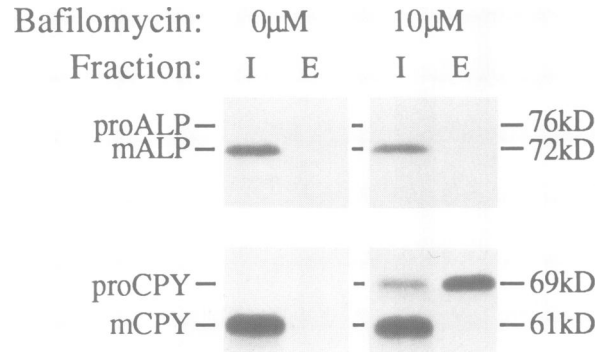


**Fig. 8.** Vacuolar delivery of alkaline phosphatase occurs in the presence of the vacuolar ATPase inhibitor bafilomycin A1. Spheroplasts from strain SEY2101 were treated with bafilomycin A1 (10 μM final concentration) in dimethylsulfoxide (DMSO) (1.0% final concentration) or DMSO alone, for 10 min prior to the addition of label. After labeling for 20 min at 30°C, chase was initiated and continued for 30 min. The cultures were separated into intracellular and extracellular fractions and immunoprecipitated with antiserum to ALP and CPY.

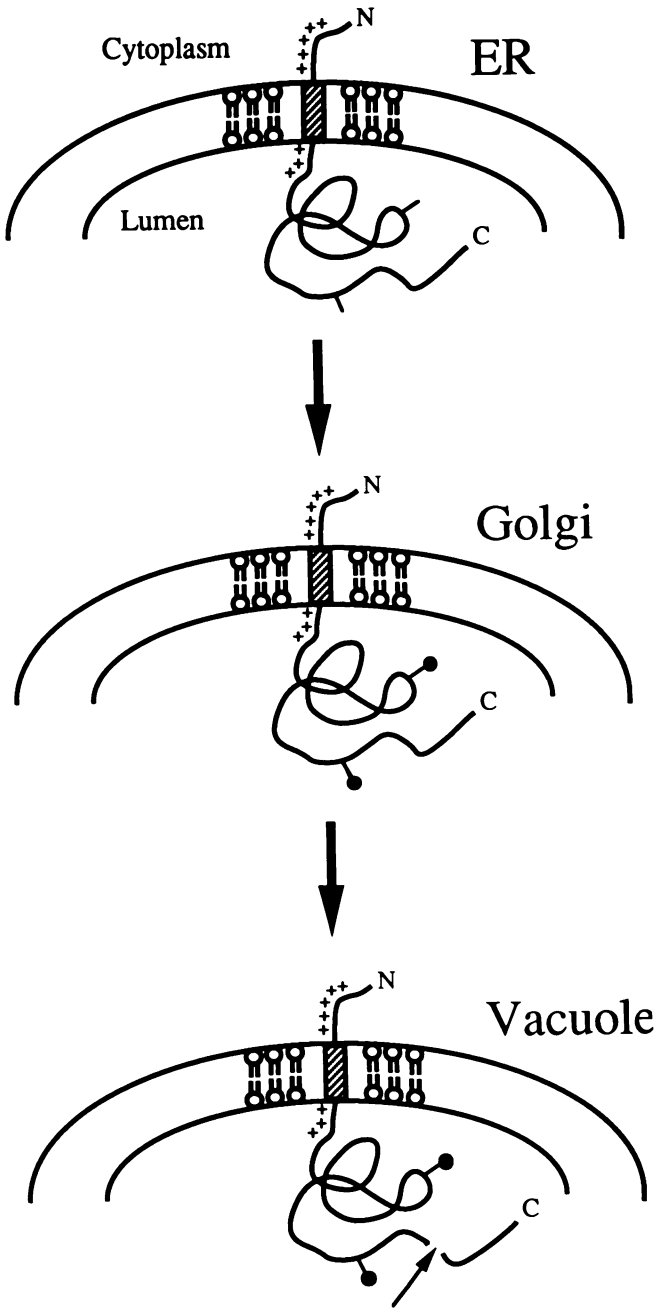


**Fig. 9.** Model for the biosynthesis of ALP. The location of basic amino acid residues (+), the transmembrane domain (▨) and oligosaccharide side chains (✎) are approximate. The arrow at the bottom indicates the approximate position of the *PEP4*-dependent propeptide cleavage site.

## Discussion

Considerable attention has focused on the mechanisms by which proteins are correctly sorted and delivered to their appropriate intracellular destinations. Recently, vacuolar protein sorting in yeast has been shown to be a useful model system to address many questions about protein sorting (Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Johnson *et al.*, 1987; Klionsky *et al.*, 1988; Robinson *et al.*, 1988). While vacuolar proteins utilize a portion of the secretory pathway en route to the vacuole, delivery of proteins to this organelle is in some ways a unique process. Secretion of many proteins from the cell is generally believed to occur by a default mechanism (Johnson *et al.*, 1987; Wieland *et al.*, 1987). Accordingly, sorting signals are not needed for movement of proteins from the ER to the Golgi complex or to the cell surface. In this sense, many secreted proteins are not subject to an active sorting process. ER resident proteins, in contrast, may be retained in the ER due to the presence of unique sequence determinants at their C termini (Pelham *et al.*, 1988). All other proteins that are competent to translocate into the ER will move on to the Golgi complex in what may amount to a bulk flow type of movement. A similar retention mechanism presumably exists for proteins that remain in the Golgi complex. For proteins that proceed beyond the *trans* Golgi, however, some additional type of active sorting must take place; since plasma membrane and secreted proteins are not found in the vacuole and similarly vacuolar proteins are not found at the cell surface, delivery of proteins to at least one of these destinations must involve specific recognition, sorting and transport mechanisms. Signals present within vacuolar proteins that contain targeting information have recently been identified (Johnson *et al.*, 1987; Klionsky *et al.*, 1988; Valls *et al.*, 1987). Delivery of proteins to the vacuole then appears to rely on an active mechanism(s) of protein sorting.

Vacuolar protein delivery involves processes that are similar to those used for the transport of lysosomal proteins in higher eukaryotes. In both systems, however, many of the important components of the sorting apparatus have yet to be identified. Interestingly, some evidence exists which suggests that soluble and membrane-associated proteins may be delivered to the vacuole by overlapping but at least partly independent mechanisms. In order to define more carefully the presence of an alternative sorting mechanism, and to add to our knowledge about protein sorting in general, we decided to examine the biosynthesis and transport of a vacuolar membrane protein.

While it is well established that the nonspecific repressible alkaline phosphatase is a vacuolar enzyme, little is known about its biosynthesis. In particular, a seemingly fundamental point—whether or not it was a membrane protein—was not definitively established. We have undertaken a rigorous immunological and biochemical analysis of the biosynthesis of ALP in order to resolve the question of its association with the membrane. Our results indicate that ALP is an integral membrane protein by several criteria: (i) it was not released from the ER membrane by treatment with saponin (Figure 4), which has the property of permeabilizing membranes while not solubilizing membrane proteins; (ii) ALP was also shown to be non-extractable by alkali buffer, another characteristic of integral membrane proteins; and (iii) while alkaline phosphatase activity is present within the lumen of the vacuole, ALP appears to have a cytoplasmic domain that is accessible to externally added protease (Figure 5). Obviously, it must be a transmembrane protein if it has domains on both sides of the lipid bilayer.

ALP is synthesized as an inactive precursor protein and enzyme activation is dependent on the removal of a propeptide segment. Our data show that the ALP propeptide is cleaved from the C terminus of the precursor protein. A C-terminal location of the ALP propeptide could also be predicted based on the glycosylation of the protein. ALP is known to have two N-linked oligosaccharide side chains (Onishi *et al.*, 1979). Since ALP has only one hydrophobic domain, at the N terminus, and since the only consensus

N-linked carbohydrate addition sites are C-terminal to the hydrophobic domain, the C terminus must be inside the vacuole. This means that ALP must be oriented as a type-II integral membrane protein. Finally, since mature ALP remains membrane associated and retains its cytoplasmic domain, the propeptide must be cleaved from the C terminus. This is different from PrA and CPY but similar to PrB, which also appears to have a C-terminal propeptide segment (Moehle *et al.*, 1989). Since mALP retains its original N terminus, the mature protein remains associated with the vacuolar membrane. This is in contrast to the *D.discoideum* lysosomal proteins β-glucosidase and α-mannosidase and the human lysosomal acid phosphatase which transit to the lysosome as membrane-associated forms but are proteolytically released into the lumen as a result of lysosomal processing (Cardelli *et al.*, 1986; Waheed *et al.*, 1988).

Based on the observations presented in this report, we have proposed a model for the biosynthesis of ALP (Figure 9). According to this model, the protein translocates into the ER by means of an uncleaved internal signal sequence. This is similar to other membrane proteins possessing hydrophobic domains that function both in ER targeting and membrane anchoring (Bos *et al.*, 1984; Holland *et al.*, 1984; Spiess and Lodish, 1986; Hull *et al.*, 1988; Lipp and Dobberstein, 1988). ALP is synthesized as an inactive precursor and is core glycosylated in the ER. The precursor is oriented as a type-II integral membrane protein and possesses a cytoplasmic domain of ~30 amino acids. The protein transits to the Golgi complex where it undergoes further carbohydrate modification. It is finally sorted and delivered to the vacuole where it is activated by the removal of a C-terminal propeptide by a PrA-dependent cleavage step. This PrA-dependent activation makes ALP unique among the yeast vacuolar membrane proteins that have been characterized. DPAP B and α-mannosidase activities, for example, are not dependent on PrA (Hemmings *et al.*, 1981; Roberts *et al.*, 1989). Recent studies have shown that DPAP B does not undergo any proteolytic processing upon arrival in the vacuole (Roberts *et al.*, 1989). For this reason, ALP should prove to be a useful vacuolar membrane marker since the removal of its propeptide makes it relatively easy to assess vacuolar delivery.

Since an analysis of ALP maturation provided a simple way to follow sorting, we used ALP to examine the effects of the previously isolated *vps* mutants (Robinson *et al.*, 1988) on the targeting of vacuolar membrane proteins. We found that delivery of ALP to the vacuole is less sensitive to certain of the *vps* mis-sorting defects than is the delivery of the soluble vacuolar hydrolases (Figure 7). The simplest explanation for this involves the presence of sorting components that are specific for the delivery of membrane versus soluble proteins. Soluble secreted proteins have been shown to transit together with certain integral plasma membrane proteins (Brada and Schekman, 1988). It seems likely that this economical transport scheme will also be used for membrane and soluble protein delivery from the Golgi complex to the vacuole. Therefore, it is not surprising that some sorting functions will be shared by both soluble and membrane vacuolar enzymes. This is supported by the observation that CPY, PrA and ALP are all processed to mature forms with similar kinetics ($t_{1/2}$ ~6 min). Other functions, however, such as the selective packing of these proteins into the appropriate vesicular carriers may require components that are unique for one or another class of vacuolar enzymes. One implication of this is that it may be possible to obtain mutants that are specific for mis-sorting vacuolar membrane proteins. Efforts are currently under way to isolate these types of mutants. Interestingly, delivery of ALP to the vacuole appears not to be dependent on vacuole acidification (Figure 8). We have preliminary data from *SUC2* gene fusion studies which indicate that the vacuolar sorting information in ALP is contained within its N-terminal cytoplasmic tail and/or hydrophobic domain (unpublished results). It is tempting to speculate on the significance of this result with regard to the apparent pH insensitivity of ALP sorting. If ALP contains vacuolar sorting information in its cytoplasmic or hydrophobic domains, it may interact with sorting components. e.g. a receptor, that are not in contact with the vacuolar lumen. It would be easy to see why interactions with non-lumenal sorting components might be insensitive to changes in the vacuolar pH. We are in the process of further defining the location of the vacuolar sorting information in ALP. A continued analysis of ALP and other vacuolar membrane proteins should provide useful information pertaining to the mechanism of vacuolar membrane protein sorting as well as the biogenesis of this organelle.

## Materials and methods

### Strains and media

Yeast strains used were SEY2109 (*MATa ura3-52 leu2-3,112 suc2-Δ9 Δprc1::leu2* (Bankaitis *et al.*, 1986), SEY2101 *MATa ura3-52 leu2-3,112 suc2-Δ9 ade2-1* (Emr *et al.*, 1983), SEY2101-*Δpep4 MATa ura3-52 leu2-3,112 suc2-Δ9 ade2-1 Δpep4::LEU2* (Klionsky *et al.*, 1988), SEY5186 *MATα sec18-1 leu2-3,112 ura3-52, GAL* (Banta *et al.*, 1988), SEY6210 *MATα leu2-3,112, ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 GAL*, SEY6211 *MATa leu2-3,112 ura3-52 his3-Δ200 trp-Δ901 ade2-101 suc1-Δ9 GAL*, SEY6210 *vps3-24, 5-7, 35-17* and SEY6211 *vps1-3, 6-12, 8-30, 9-5, 11-2, 15-14, 16-3, 18-2, 19-7, 26-8, 33-7, 34-4* (Robinson *et al.*, 1988). Standard methods (Sherman *et al.*, 1979) were used to construct yeast strain DKY6280 *MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 Δpho8::TRP1*.

YPD medium (Sherman *et al.*, 1979) and modified Wickerham's minimal medium (Johnson *et al.*, 1987) were prepared as described previously.

### Materials

Lyticase was obtained from Enzogenetics (Corvallis, OR), Tran $^{35}$S-label was from ICN Radiochemicals (Irvine, CA), DNA restriction and modifying enzymes were from New England Biolabs, Inc. (Beverly, MA), $\alpha_2$-macroglobulin was from Boehringer Mannheim Biochemicals, Autofluor was from National Diagnostics and all other chemicals were from Sigma Chemical Co. (St Louis, MO). Antisera to PrA and CPY were prepared as described previously (Klionsky *et al.*, 1988). PrB antiserum was a gift from Charles Moehle and Elizabeth Jones (Moehle *et al.*, 1989). Bafilomycin A1 was generously provided by Karlheinz Altendorf (Universität Osnabruck, Osnabruck, FRG).

### Antiserum to ALP

To produce antiserum to ALP we had two synthetic peptides synthesized based on the deduced amino acid sequence. Peptides corresponding to amino acid residues 222–244 and 281–301 were made on an Applied Biosystems Model 430A peptide synthesizer. Both peptides separately were conjugated at their C termini to ovalbumin and keyhole limpet hemocyanin. The peptide–carrier conjugates (0.5 mg) were mixed with Freund's adjuvant and injected into male New Zealand White rabbits. Antiserum was collected after multiple secondary injections (100 μg).

### Plasmid constructions

A 4-kb *Bam*HI fragment containing the entire *PHO8* gene cloned into the plasmid YEp13 (YEp13::PHO8) was supplied by Yoshinobu Kaneko and Yasuji Oshima (Kaneko *et al.*, 1987). This plasmid was restricted with the

enzymes *Eco*RI, *Bam*HI and *Pst*I. The 4-kb *Eco*RI−*Bam*HI fragment was isolated and cloned into the corresponding restriction sites in the low copy number vector pSEYC58 (*CEN4 ARS1*) and the multicopy number vector PSEY8 (2 μ circle) (Emr *et al.*, 1986).

The 3-kb *Eco*RI fragment from YEp13::PHO8-322 encoding the truncated *PHO8* gene (Kaneko *et al.*, 1987) was cloned into the *Eco*RI site of pUC8 to generate the plasmid pUC8-PHO8(322). This plasmid was restricted with *Xho*I and *Spe*I to delete part of the *PHO8* coding region and the ends were filled in using the Klenow fragment of DNA polymerase I. The TRP1 gene was prepared from the plasmids YRp7 (Tschumper and Carbon, 1980) by restricting with *Eco*RI and *Bgl*II. The 0.8-kb fragment was isolated, filled in with the Klenow fragment and cloned into the modified *Xho*I−*Spe*I sites of pUC8-PHO8(322). The resulting plasmid was linearized with *Eco*RI and transformed into strain SEY6211 to disrupt the chromosomal PHO8 locus.

### *Labeling and immunoprecipitation*

The procedures used for the preparation, labeling, fractionation and immunoprecipitation of yeast spheroplasts were as described previously (Klionsky *et al.*, 1988; Robinson *et al.*, 1988). To examine the association of proteins with the vacuolar membrane, spheroplasts were labeled for 20 min followed by a 30 min chase. Labeled spheroplasts were pelleted, resuspended in lysis buffer (50 mM KPi, pH 7.5, 10 mM $NaN_3$) and centrifuged at 38 000 r.p.m. in a Beckman 70.1 Ti rotor for 30 min. Supernatant and pellet fractions were TCA precipitated and immunoprecipitated.

To test the solubility of ER-accumulated vacuolar proteins, spheroplasts were prepared from a *sec18* mutant strain, shifted to 37°C for 15 min and labeled with Tran $^{32}$S-label (0.2 mCi/m) for 15 min followed by a 5 min chase. The spheroplasts were collected by centrifugation at 3000 r.p.m. for 1.5 min in a Savant microcentrifuge. The spheroplast pellet was resuspended in 1.5 ml of lysis buffer containing bovine serum albumin (BSA) (2 mg/ml) and $\alpha_2$-macroglobulin (100 μg/ml) and divided into three aliquots. Saponin (0.2%) or Triton X-100 (0.1%) were each added to one aliquot and the samples were gently rocked at 4°C for 30 min followed by centrifugation in a Fisher microcentrifuge for 5 min. The supernatant and pellet fractions were separated, precipitated with TCA (5% final concentration) and prepared for immunoprecipitation.

To analyze the partitioning of vacuolar proteins in alkali buffer, yeast spheroplasts were prepared, resuspended in 500 μl, labeled for 15 min at 30°C and chased for 20 min. An aliquot (125 μl) was removed and TCA precipitated as a control. The remaining spheroplasts were pelleted in a microcentrifuge and the spheroplast pellet was lysed by resuspension in 10 mM $Na_2CO_3$, pH 11.0, followed by vigorous mixing on a VWR vortex. The sample was centrifuged at 38 000 r.p.m. in a Beckman 70.1 Ti rotor for 30 min. The supernatant fraction was removed and TCA precipitated. The $Na_2CO_3$ extraction and centrifugation steps were repeated on the resuspended pellet fraction. After the second centrifugation, the supernatant and pellet fractions were recovered and TCA precipitated. The control (total), supernatant (extractable) and pellet (precipitable) fractions were immunoprecipitated with antisera to ALP, CPY and PrA.

Accessibility of ALP to exogenous protease was examined in both wild-type and *sec18* mutant yeast spheroplasts. Labeled precursor ALP was accumulated in the ER of *sec18* mutant yeast spheroplasts at the non-permissive temperature which were subsequently subjected to gentle osmotic lysis as described above. The sample was divided into four aliquots, three of which received either proteinase K (200 μg/ml), Triton X-100 (0.5%) or both proteinase K and Triton X-100. The samples were incubated on ice for 30 min, phenylmethylsulfonyl fluoride (PMSF) (1 mM) was added and the samples were TCA precipitated and analyzed for immunoprecipitation. To examine the protease sensitivity of mALP, strain SEY2101 was converted to spheroplasts and labeled for 20 min followed by a 30 min chase. The spheroplasts were pelleted and resuspended in 0.2 M sorbitol, 0.2 M imidazole, pH 6.5 (3 ml). DEAE−dextran (8.8 μg) was added and the sample was held on ice for 1 min followed by a 5 min incubation at 30°C. The sample was divided into four aliquots, treated with proteinase K and immunoprecipitated as above.

Following immunoprecipitation, samples were suspended in SDS sample buffer, boiled for 4 min and loaded onto an 8 or 9% polyacrylamide−SDS gel. After electrophoresis, gels were fixed and treated with Autofluor. An LKB 2202 ultrascan laser densitometer was used for quantitation of autoradiograms.

### N-terminal amino acid analysis of mature ALP

For N-terminal analysis of mature vacuolar ALP, 12 $OD_{600}$ units of SEY2108 were converted to spheroplasts and labeled with Tran $^{35}$S-label (0.7 mCi/ml) for 1 h, followed by a 30 min chase. The culture was precipitated with acetone and subjected to two immunoprecipitations with antiserum to ALP. The final immunoprecipitate was eluted from the antiserum by boiling for 2 min in the presence of 0.1% SDS, 1.0% β-mercaptoethanol (240 μl). An aliquot (5 μl) was analyzed by SDS−PAGE and a second aliquot was counted in a scintillation counter. The remainder (15 000 c.p.m.) was placed onto a glass fiber filter, washed to remove unbound labeled material and then subjected to sequential Edman degradation using an Applied Biosystems Model 477A protein sequencer. Material released in each of the first 15 cycles was measured for $^{35}$S c.p.m. in a Beckman scintillation counter.

## Acknowledgements

We thank Chris Acklin, Jill Jenik and Suzanna Horvath for producing the ALP synthetic peptides, Haul Wong and David Teplow for performing the automated Edman degradation, and Yoshinobu Kaneko and Yasuji Oshima for providing the *PHO8* gene and nucleotide sequence information prior to publication. We also thank Lois Banta and Editte Gharakhanian for critically reading the manuscript. This study was supported by a National Science Foundation Presidential Young Investigator Award (to S.D.E.). D.J.K. was supported by a research fellowship from the Helen Hay Whitney Foundation.

## References

Ammerer,G., Hunter,C., Rothman,J.H., Saari,G.C., Valls,L.A. and Stevens,T.H. (1986) *Mol. Cell. Biol.*, **6**, 2490−2499.

Bankaitis,V.A., Johnson,L.M. and Emr,S.D. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9075−9079.

Banta,L.M., Robinson,J.S., Klionsky,D.J. and Emr,S.D. (1988) *J. Cell Biol.*, **107**, 1369−1383.

Barriocanal,J.G., Bonifacino,J.S., Yuan,L. and Sandoval,I.V. (1986) *J. Biol. Chem.*, **261**, 16755−16763.

Bauer,H. and Sigarlakie,E. (1973) *J. Microsc. (Oxford)*, **99**, 205−218.

Bauer,H. and Sigarlakie,E. (1975) *J. Ultrastruct. Res.*, **50**, 208−215.

Bos,T.J., Davies,A.R. and Nayak,D.P. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 2327−2331.

Bowman,E.J., Siebers,A. and Altendorf,K. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7972−7976.

Brada,D. and Schekman,R. (1988) *J. Bacteriol.*, **170**, 2775−2783.

Cardelli,J.A., Golumbeski,G.S. and Dimond,R.L. (1986) *J. Cell Biol.*, **102**, 1264−1270.

Clark,D.W., Tkacz,J.S. and Lampen,J.O. (1982) *J. Bacteriol.*, **152**, 865−873.

Eakle,K.A., Bernstein,M. and Emr,S.D. (1988) *Mol. Cell. Biol.*, **8**, 4098−4109.

Eisenberg,D. (1984) *Annu. Rev. Biochem.*, **53**, 595−623.

Emr,S.D., Schekman,R., Flessel,M.C. and Thorner,J. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7080−7084.

Emr,S.D., Vassorotti,A., Garrett,J., Geller,B.L., Takeda,M. and Douglas,M.G. (1986) *J. Cell Biol.*, **102**, 523−533.

Fujiki,Y., Hubbard,A.L., Fowler,S. and Lazarow,P.B. (1982) *J. Cell Biol.*, **93**, 97−102.

Gabel,C.A., Goldberg,D.A. and Kornfeld,S. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 775−779.

Garcia Alvarez,N., Bordallo,C, Gascon,S. and Suarez Rendueles,P. (1985) *Biochim. Biophys. Acta*, **832**, 119−125.

Green,S.A., Zimmer,K.-P., Griffiths,G. and Mellman,I. (1987) *J. Cell Biol.*, **105**, 1227−1240.

Griffiths,S. and Simons,K. (1986) *Science*, **234**, 438−443.

Hasilik,A. and Tanner,W. (1978) *Eur. J. Biochem.*, **85**, 599−608.

Hasselbeck,A. and Schekman,R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2017−2021.

Hemmings,B.A., Zubenko,G.S., Hasilik,A. and Jones,E.W. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 435−439.

Holland,E.C., Leung,J.O. and Drickamer,K. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7338−7342.

Hull,J.D., Gilmore,R. and Lamb,R.A. (1988) *J. Cell Biol.*, **106**, 1489−1498.

Johnson,L.M., Bankaitis,V.A. and Emr,S.D. (1987) *Cell*, **48**, 875−885.

Jones,E.W., Zubenko,G.S. and Parker,R.R. (1982) *Genetics*, **102**, 665−667.

Kakinuma,Y., Ohsumi,Y. and Anraku,Y. (1981) *J. Biol. Chem.*, **256**, 10859−10863.

Kaneko,Y., Hayashi,N., Toh-e,A., Banno,I. and Oshima,Y. (1987) *Gene*, **58**, 137−148.

Klionsky,D.J., Banta,L.M. and Emr,S.D. (1988) *Mol. Cell. Biol.*, **8**, 2105−2116.

Kornfeld,S. (1987) *FASEB J.*, **1**, 462–468.
Kyte,J. and Doolittle,R.F. (1982) *J. Mol. Biol.*, **157**, 105–132.
Lenney,J., Matile,P., Wiemken,A., Schellenberg,M. and Meyer,J. (1974) *Biochem. Biophys. Res. Commun.*, **60**, 1378–1383.
Lewis,V., Green,S.A., Marsh,M., Vihko,P., Helenius,A. and Mellman,I. (1985) *J. Cell Biol.*, **100**, 1839–1847.
Lipp,J. and Dobberstein,B. (1986) *J. Cell Biol.*, **102**, 2169–2175.
Lipp,J. and Dobberstein,B. (1988) *J. Cell Biol.*, **106**, 1813–1820.
Mechler,B. and Wolf,D.H. (1981) *Eur. J. Biochem.*, **121**, 47–52.
Mechler,B., Muller,M., Muller,H., Meussdoerffer,F. and Wolf,D.F. (1982) *J. Biol. Chem.*, **257**, 11203–11206.
Mechler,B., Muller,H. and Wolf,D.H. (1987) *EMBO J.*, **6**, 2157–2163.
Mechler,B., Hirsch,H., Muller,H. and Wolf,D.H. (1988) *EMBO J.*, **7**, 1705–1710.
Mitchell,J.K., Fonzi,W.A., Wilkerson,J. and Opheim,D.J. (1981) *Biochim. Biophys. Acta*, **657**, 482–494.
Moehle,C.M., Dixon,C.K. and Jones,E.W. (1989) *J. Cell Biol.*, **108**, 309–324.
Novick,P., Field,F. and Schekman,R. (1980) *Cell*, **21**, 205–215.
Ohsumi,Y. and Anraku,Y. (1981) *J. Biol. Chem.*, **256**, 2079–2082.
Ohsumi,Y. and Anraku,Y. (1983) *J. Biol. Chem.*, **258**, 5614–5617.
Onishi,H.R., Tkacz,J.S. and Lampen,J.O.(1979) *J. Biol. Chem.*, **254**, 11943–11952.
Owada,M. and Neufeld,E.F. (1982) *Biochem. Biophys. Res. Commun.*, **105**, 814–820.
Pelham,H.R.B., Hardwick,K.G. and Lewis,M.J. (1988) *EMBO J.*, **7**, 1757–1762.
Roberts,C.J., Pohlig,G., Rothman,J.H. and Stevens,T.H. (1989) *J. Cell Biol.*, **108**, 1363–1373.
Robinson,J.S., Klionsky,D.J., Banta,L.M. and Emr,S.D. (1988) *Mol. Cell. Biol.*, **8**, 4936–4948.
Rothman,J.H. and Stevens,T.H. (1986) *Cell*, **47**, 1041–1051.
Sato,T., Ohsumi,Y. and Anraku,Y. (1984) *J. Biol. Chem.*, **259**, 11505–11508.
Schauer,I., Emr,S., Gross,C. and Schekman,R. (1985) *J. Cell Biol.*, **100**, 1664–1675.
Sherman,F., Fink,G.R. and Lawrence,L.W. (1979) *Methods in Yeast Genetics: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sly,W.S. and Fischer,H.D. (1982) *J. Cell. Biochem.*, **18**, 67–85.
Spiess,M. and Lodish,H.F. (1986) *Cell*, **44**, 177–185.
Stevens,T., Esmon,B. and Schekman,R. (1982) *Cell*, **30**, 439–448.
Trimble,R.B. and Maley,F. (1977) *Biochem. Biophys. Res. Commun.*, **78**, 935–944.
Tschumper,G. and Carbon,J. (1980) *Gene*, **10**, 157–166.
Uchida,E., Ohsumi,Y. and Anraku,Y. (1985) *J. Biol. Chem.*, **260**, 1090–1095.
Valls,L.A., Hunter,C.P., Rothman,J.H. and Stevens,T.H. (1987) *Cell*, **48**, 887–897.
von Figura,K. and Hasilik,A. (1986) *Annu. Rev. Biochem.*, **55**, 167–193.
von Heijne,G. (1986) *Nucleic Acids Res.*, **14**, 4683–4690.
Waheed.,A., Pohlman,R., Hasilik,A., von Figura,K., Van Elsen,A. and Leroy,J.G. (1982) *Biochem. Biophys. Res. Commun.*, **105**, 1052–1058.
Waheed,A., Gottschalk,S., Hille,A., Krentler,C., Pohlmann,A., Braulke,T., Hauser,H., Geuze,H. and von Figura,K. (1988) *EMBO J.*, **7**, 2351–2358.
Wieland,F.T., Gleason,M.L., Serafini,T.A. and Rothman,J.E. (1987) *Cell*, **50**, 289–300.
Wiemken,A., Schellenberg,M. and Urech,K. (1979) *Arch. Microbiol.*, **123**, 23–35.
Woolford,C.A., Daniels,L.G., Park,F.J., Jones,E.W., van Arsdell,J.N. and Innis,M.A. (1986) *Mol. Cell. Biol.*, **6**, 2500–2510.
Yoshihisa,T., Ohsumi,Y. and Anraku,Y. (1988) *J. Biol. Chem.*, **263**, 5158–5163.

*Received on March 28, 1989; revised on May 11, 1989*