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Novel Golgi to vacuole delivery pathway in yeast: identification of a sorting determinant and required transport component

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More than 40 vacuolar protein sorting (*vps*) mutants have been identified which secrete proenzyme forms of soluble vacuolar hydrolases to the cell surface. A subset of these mutants has been found to show selective defects in the sorting of two vacuolar membrane proteins. Under non-permissive conditions, *vps45*^{tsf} (*SEC1* homolog) and *pep12/vps6*^{tsf} (endosomal t-SNARE) mutants efficiently sort alkaline phosphatase (ALP) to the vacuole while multiple soluble vacuolar proteins and the membrane protein carboxypeptidase yscS (CPS) are no longer delivered to the vacuole. Vacuolar localization of ALP in these mutants does not require transport to the plasma membrane followed by endocytic uptake, as double mutants of *pep12*^{tsf} and *vps45*^{tsf} with *sec1* and *end3* sort and mature ALP at the non-permissive temperature. Given the demonstrated role of t-SNAREs such as Pep12p in transport vesicle recognition, our results indicate that ALP and CPS are packaged into distinct transport intermediates. Consistent with ALP following an alternative route to the vacuole, isolation of a *vps41*^{tsf} mutant revealed that at non-permissive temperature ALP is mislocalized while vacuolar delivery of CPS and CPY is maintained. A series of domain-swapping experiments was used to define the sorting signal that directs selective packaging and transport of ALP. Our data demonstrate that the amino-terminal 16 amino acid portion of the ALP cytoplasmic tail domain contains a vacuolar sorting signal which is responsible for the active recognition, packaging and transport of ALP from the Golgi to the vacuole via a novel delivery pathway.

Keywords: ALP/*PEP12*/protein sorting/vacuole/*VPS41*

Introduction

Correct delivery of newly synthesized proteins to their resident organelles is required for maintenance of a eukaryotic cell's compartmentalized structure. For a protein transiting the secretory pathway, numerous potential destinations exist, including the endoplasmic reticulum (ER), the multiple distinct regions of the Golgi apparatus, the plasma membrane and the lysosome/vacuole. Sorting signals have been identified for many resident proteins of the ER, Golgi and lysosome/vacuole, suggesting that several distinct sorting machineries function to sort and deliver proteins within the secretory pathway.

One of the best-characterized sorting systems is that of the mammalian mannose 6-phosphate receptor. This *trans*-Golgi-localized receptor binds to mannose 6-phosphate on newly synthesized lysosomal proteins and directs their inclusion in vesicles destined for the endosome, an intermediate compartment en route to the lysosome (Kornfeld and Mellman, 1989; Kornfeld, 1992). In *Saccharomyces cerevisiae*, a similar receptor-mediated machinery exists for sorting of the soluble hydrolase carboxypeptidase Y (CPY) to the lysosome/vacuole. CPY transits the early secretory pathway in precursor form. Upon arrival in the late Golgi, a sorting signal in precursor CPY is recognized by a transmembrane receptor, Vps10p, resulting in diversion of CPY from bulk protein flow to the cell surface via packaging of precursor CPY into vesicles bound for the endosome (Marcusson *et al.*, 1994).

Mutations in the vacuolar sorting signal of precursor CPY result in secretion of mutant CPY to the cell surface (Johnson *et al.*, 1987; Valls *et al.*, 1987), and several genetic approaches have been employed to detect vacuolar protein sorting (*vps*) mutants which missort and secrete soluble vacuolar hydrolases (Bankaitis *et al.*, 1986; Robinson *et al.*, 1988). To date, >40 *vps* mutant complementation groups have been identified; in each of these mutants, delivery of CPY to the vacuole is compromised. The majority of *vps* mutants also mislocalize the soluble hydrolases proteinase A (PrA) and proteinase B (PrB).

Not all vacuolar proteins are affected by mutations in *VPS* genes. For example, the type II integral vacuolar membrane protein-repressible alkaline phosphatase (ALP) is localized to the vacuole in *vps1* (Nothwehr *et al.*, 1995), *vps15* (Herman *et al.*, 1991; Raymond *et al.*, 1992), *vps34* (Stack *et al.*, 1995), *chc1* (Seeger and Payne, 1992), *vps8* (Horazdovsky *et al.*, 1996), *vps21* (B.F.Horazdovsky, unpublished data) and *vps45* (Raymond *et al.*, 1992) mutant cells, suggesting that ALP may use a vacuolar targeting machinery that is distinct from that required by CPY and other vacuolar proteases. The gene products mutated in each of these strains are believed to function in vesicle-mediated transport from the late Golgi to the endosome (Horazdovsky *et al.*, 1995). Observations of ALP vacuolar localization in the *vps45* mutant strain are of particular interest due to the purported role of Vps45p (a Sec1p homolog) in endosomal targeting and/or fusion of Golgi-derived transport vesicles (Cowles *et al.*, 1994; Piper *et al.*, 1994). Vacuolar delivery of ALP in this mutant suggests that ALP trafficking might bypass the endosome and invokes the possibility of post-Golgi ALP transit involving a transport intermediate and delivery route distinct from that of CPY, PrA and PrB.

Little is known regarding the mechanism that localizes ALP to the vacuole. Initial studies of ALP localization

that used an ALP–invertase fusion protein implicated a vacuolar sorting determinant within the amino-terminal 52 amino acid residues of ALP (Klionsky and Emr, 1990), yet more recent studies of a *vps1* mutant strain suggest that ALP is capable of reaching the vacuole by a mechanism which involves transit via the plasma membrane (Nothwehr *et al.*, 1995). Here we present evidence that ALP reaches the vacuole in *vps45* mutant cells, as well as in cells harboring mutations in the gene encoding the endosomal t-SNARE, *PEP12* (Becherer *et al.*, 1996), via a route which does not involve the late secretory pathway. This Pep12p-independent pathway requires the function of Vps41p for the vacuolar delivery of ALP. Entry into this pathway requires a sorting signal present in the amino-terminal cytoplasmic tail of ALP. Carboxypeptidase yscS (CPS) (Spormann *et al.*, 1991; Spormann *et al.*, 1992), another type II membrane protein that is also delivered to the vacuole, lacks this signal and follows a Pep12p-dependent, Vps41p-independent route to the vacuole.

Results

Differential sorting of alkaline phosphatase and carboxypeptidase yscS is observed in pep12^{tsf} and vps45^{tsf} mutants

More than 40 genes are required for the proper localization of soluble vacuolar hydrolases such as CPY (Stack and Emr, 1993). A subset of *vps* mutants, the class D *vps* mutants, affect transport of CPY between the late Golgi and the pre-vacuolar endosome and are thus required for one of the earliest steps in the *VPS* pathway (Stack and Emr, 1993). We generated temperature-conditional mutations in two class D *VPS* genes (see Materials and methods): *PEP12*, encoding an endosomal vesicle receptor (t-SNARE), and *VPS45*, a *SEC1* family member required for Golgi to endosome transport (Cowles *et al.*, 1994; Piper *et al.*, 1994). When grown at permissive temperatures, *pep12^{tsf}* and *vps45^{tsf}* mutant cells contained vacuoles that were competent to mature CPY, ALP and CPS (Figures 1B and 3). However, when *pep12^{tsf}* or *vps45^{tsf}* cells were incubated at non-permissive temperature, CPY accumulated in Golgi-modified precursor form (p2CPY) and was secreted from these cells (Figure 3C), indicating that these mutations resulted in a temperature-conditional missorting of CPY. In order to examine processing of the two vacuolar type II membrane hydrolases ALP and CPS in these *tsf* mutants, haploid strains harboring *pep12^{tsf}* (Figure 1B, lanes 1–4) and *vps45^{tsf}* (Figure 1B, lanes 5–8) alleles were incubated for 10 min at permissive (26 or 30°C) or non-permissive (37°C) temperature, pulse-labeled with [^{35}S]methionine/[^{35}S]cysteine (Tran^{35}S-label) for 10 min and chased with unlabeled methionine and cysteine for 0 (Figure 1B, lanes 1, 3, 5 and 7) or 45 min (Figure 1B, lanes 2, 4, 6 and 8). Labeled cells were lysed and subjected to immunoprecipitation with antisera raised against CPS (see Materials and methods; Figure 1B, top panels) and ALP (Figure 1B, bottom panels). Following synthesis, CPS transits the early secretory pathway and is differentially glycosylated, receiving *N*-linked oligosaccharide modifications at either two or three distinct sites within the protein (Spormann *et al.*, 1992). These modifications produce both 81 and 78 kDa transmembrane precursor forms of the enzyme (Spormann *et al.*, 1992).

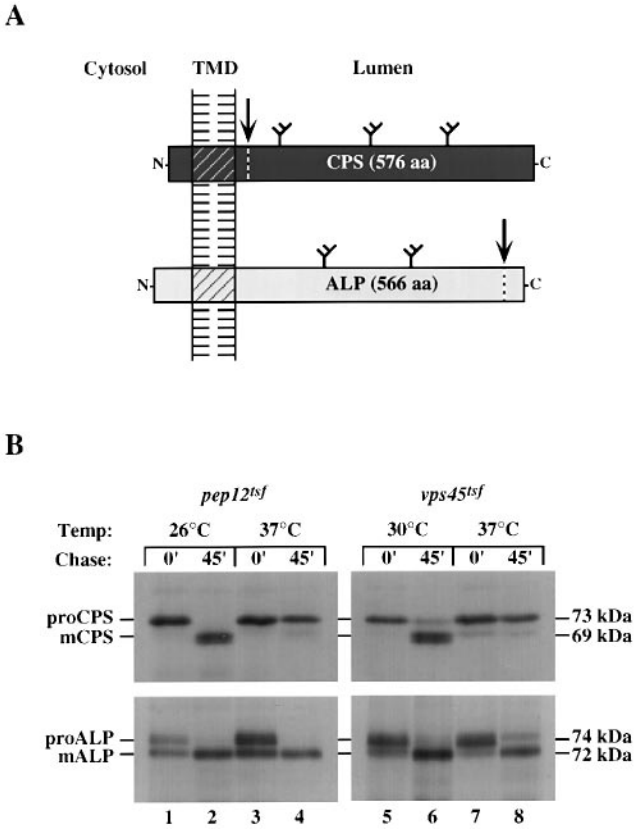

Fig. 1. Temperature-conditional sorting of CPS and ALP in *pep12^{tsf}* and *vps45^{tsf}* mutant cells. (**A**) Topology of the ALP and CPS proteins, with *PEP4*-dependent cleavage sites indicated by arrows, and branched chains illustrating glycosylation sites. (**B**) Mutant cells harboring the *pep12^{tsf}* or *vps45^{tsf}* alleles were incubated at permissive (26 or 30°C) or non-permissive (37°C) temperature for 10 min, labeled for 10 min with Tran^{35}S-label, then chase periods were initiated via addition of unlabeled methionine and cysteine to the incubations. Cells were harvested either immediately upon chase initiation or 45 min after chase initiation. Cell extracts were generated, and CPS and ALP were isolated by immunoprecipitation. Immunoprecipitated proteins were resolved via SDS–PAGE and visualized by fluorography.

Upon delivery to the vacuole, precursor CPS is cleaved in a *PEP4*-dependent fashion adjacent to its transmembrane domain, yielding soluble 77 and 74 kDa mature forms of the enzyme (see Figure 1A). The precursor (proCPS) and mature (mCPS) forms of CPS are collapsed to single bands of 73 and 69 kDa, respectively, when *N*-linked oligosaccharides are removed from immunoprecipitated samples via endoglycosidase H treatment. The *pep12^{tsf}* mutant matured CPS at permissive temperature (26°C) following a 45 min chase period, based on the appearance of a 69 kDa mature form of CPS (Figure 1B, lane 2), but failed to process CPS within the same chase period at non-permissive temperature (37°C; Figure 1B, lane 4).

Like CPS, ALP is a type II membrane protein that is delivered to the vacuole in proenzyme form. Upon arrival at the vacuole, precursor ALP is cleaved at a site near the carboxy-terminus in a *PEP4*-dependent manner to yield a mature, membrane-spanning form of the hydrolase (see Figure 1A). When ALP processing was examined in a *pep12^{tsf}* mutant, the protein was found to mature rapidly from the 74 kDa precursor form (proALP) to a 72 kDa vacuole-localized mature form (mALP) at both permissive and non-permissive temperatures. Both precursor and

mature ALP were observed in the *pep12*^{tsf} mutant following 0 min of chase at either temperature (Figure 1B, lanes 1 and 3), and complete maturation of labeled ALP had occurred at both temperatures within 45 min of chase initiation (Figure 1B, lanes 2 and 4).

Similar results were obtained when the same experiment was performed on a *vps45*^{tsf} mutant (Figure 1, lanes 5–8). At the permissive temperature of 30°C, the vast majority of both CPS and ALP were matured within a 45 min chase period in the *vps45*^{tsf} mutant (Figure1, lane 6). When *vps45*^{tsf} cells were shifted to 37°C for 10 min prior to labeling and chasing at 37°C, the bulk of labeled CPS failed to mature following a 45 min chase. In contrast, most labeled ALP matured in the *vps45*^{tsf} mutant at the elevated temperature, though a very small amount of precursor ALP was still detected at the 45 min chase point (Figure 1, lane 8). These data suggest that vacuolar localization of CPS requires Pep12p and Vps45p function, whereas vacuolar localization of ALP does not.

ALP is delivered to the vacuole in the absence of Pep12p and Vps45p function

Unlike *pep12*^{tsf} and *vps45*^{tsf} mutant cells, the vacuoles of *pep12* and *vps45* deletion mutants are processing incompetent due to an absence of soluble proteases. Even properly vacuole-localized ALP cannot be matured in these deletion mutants. For this reason, in order to examine the effects of *pep12* and *vps45* deletion/disruption mutations on ALP localization, wild-type (SEY6210), *pep12* deletion mutant (*pep12*Δ2) and *vps45* deletion mutant (*vps45*Δ2) (Cowles *et al.*, 1994) cells were visualized via indirect immunofluorescence following treatment with antisera directed against ALP (Pringle *et al.*, 1989; Raymond *et al.*, 1990, 1992). Wild-type cells were observed to contain multiple vacuoles when viewed with Nomarski optics (Figure 2), and ALP immunoreactivity was observed at the vacuolar membrane in these cells (Figure 2). When *pep12*Δ2 and *vps45*Δ2 mutant cells were visualized under identical conditions, Nomarski optics revealed cells containing single, enlarged vacuoles (Figure 2, left panels), as are characteristic of class D *vps* mutants (for a description of *vps* mutant classification, refer to Raymond *et al.*, 1992). As in wild-type cells, ALP immunoreactivity was localized to the vacuolar membrane (Figure 2, right panels). These results are consistent with previous observations of ALP vacuolar localization in a strain containing a *vps45* mutant allele (Raymond *et al.*, 1992) and confirm that vacuolar localization of ALP does not require the *PEP12* and *VPS45* gene products.

Vacuolar delivery of ALP in pep12^{tsf} *and vps45*^{tsf} *mutants does not require late secretory pathway function*

Recent studies of a *vps1* mutant strain have implicated the plasma membrane as an intermediate destination for biosynthetic ALP during transit to the vacuole (Nothwehr *et al.*, 1995). To test whether ALP follows such a route in *pep12*^{tsf} or *vps45*^{tsf} mutants, haploid strains combining either of these alleles with the temperature-sensitive late secretory pathway mutant, *sec1-1*, were generated (Novick *et al.*, 1980). The *sec1-1* mutation results in intracellular accumulation of 100 nm secretory transport vesicles which do not fuse with the plasma membrane (Novick *et al.*, 1980). *pep12*^{tsf}/*sec1-1* and *vps45*^{tsf}/*sec1-1* mutant cells were incubated at permissive (26°C) or non-permissive (37°C) temperature for 10 min prior to labeling for 10 min. Cells were then chased for 0 or 45 min and converted to spheroplasts. Spheroplast pellets (I, internal fraction) were separated from culture media (E, external fraction), and ALP, CPS and CPY in each fraction were visualized after immunoprecipitation and SDS–PAGE. In Figure 3A, mature ALP was detected following a 45 min chase at either permissive or non-permissive temperature in the *pep12*^{tsf}/*sec1-1* and *vps45*^{tsf}/*sec1-1* mutant cells (Figure 3A, lanes 3 and 7). Identical results for ALP maturation were obtained when the *vps45*^{tsf} mutant was combined with the endocytic pathway mutant, *end3-1* (Benedetti *et al.*, 1994; data not shown). Figure 3B depicts immunoprecipitation results for CPS in *pep12*^{tsf}/*sec1-1* and *vps45*^{tsf}/*sec1-1* mutant cells following a 45 min chase. At the permissive temperature of 26°C, the bulk of labeled CPS was processed to its vacuole-localized mature form (Figure 3B, lanes 1 and 5), while >90% of labeled CPS remained in precursor form following a 45 min chase at non-permissive temperature (37°C) in both mutants (Figure 3B, lanes 3 and 7).

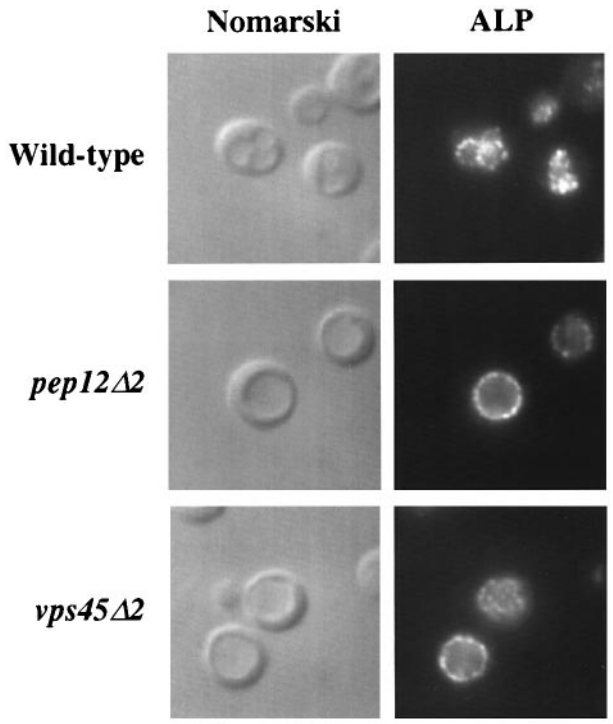

Fig. 2. Immunolocalization of ALP. Wild-type (SEY6210), *pep12*Δ*2* (CBY31) and *vps45*Δ*2* (CCY120) cells were fixed, labeled with antibodies recognizing ALP and viewed using a fluorescent microscope equipped with Nomarski optics and a 546 nm filter (ALP staining).

Following synthesis in wild-type cells, CPY is observed initially in ER-modified p1 precursor form (67 kDa), and then is converted rapidly to p2 precursor form (69 kDa) via extension of core oligosaccharides in the Golgi apparatus. Upon delivery to the vacuole, p2CPY is converted to mature CPY (61 kDa) via a *PEP4*-dependent cleavage event. When the processing state of labeled CPY was examined in *pep12*^{tsf}/*sec1-1* and *vps45*^{tsf}/*sec1-1* mutant cells following 45 min of chase, mature CPY was observed

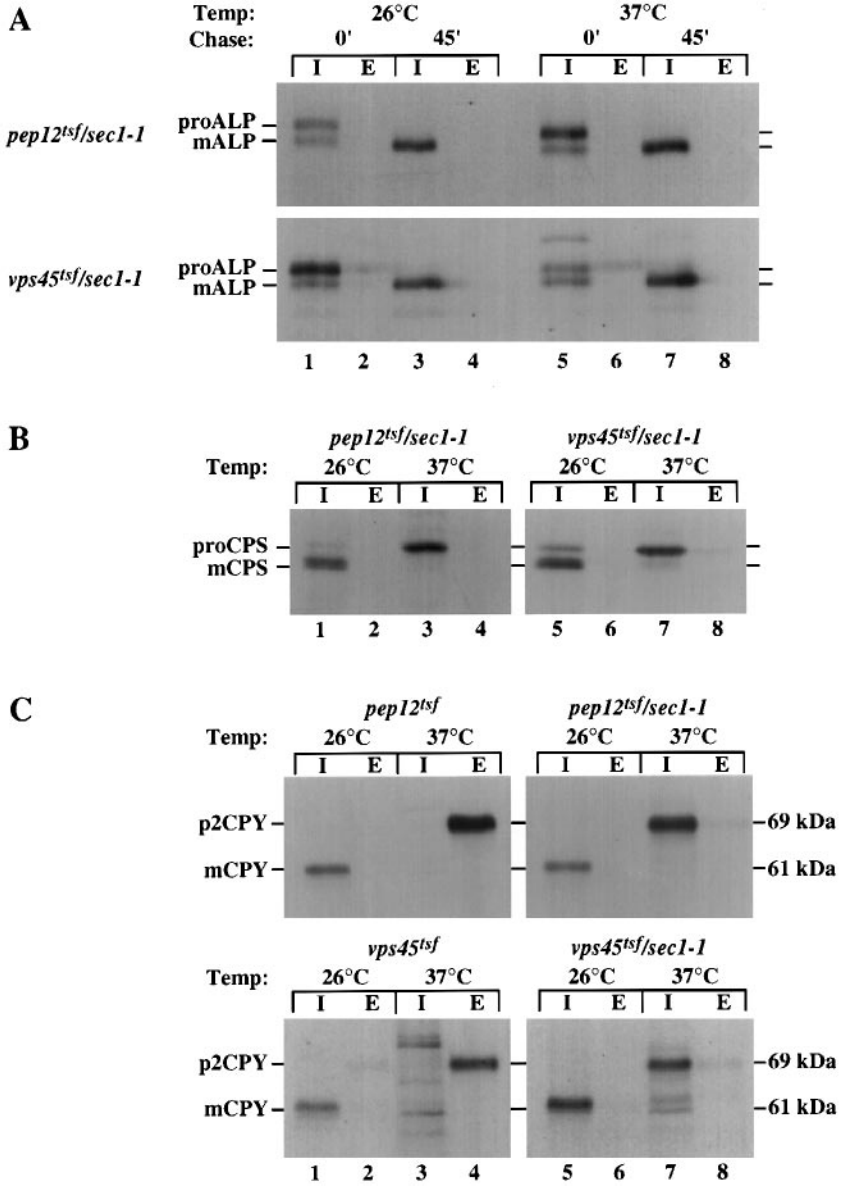

Fig. 3. Intracellular sorting of vacuolar hydrolases in *pep12^{tsf}/sec1-1* and *vps45^{tsf}/sec1-1* double mutants. Yeast cells were incubated for 10 min at permissive (26°C) or non-permissive (37°C) temperature prior to labeling for 10 min with Tran^{35}S-label. Unlabeled methionine and cysteine were added, and cells were harvested immediately or following a 45 min chase period. Harvested cells were converted to spheroplasts and separated into pellet (I, internal) and supernatant (E, external) fractions. (**A**) ALP, (**B**) CPS and (**C**) CPY were immunoprecipitated from these fractions, resolved via SDS–PAGE, and viewed by fluorography. The yeast strains used in this analysis included *pep12^{tsf}* (CBY9), *pep12Δ2/sec1-1* (CBY34) transformed with a single copy (*CEN*) plasmid containing the *pep12^{tsf}* allele and strains *vps45Δ2* (CCY130) and *vps45Δ2/sec1-1* (CCY143) harboring the *vps45^{tsf}* allele on a *CEN* plasmid.

in cell pellet fractions of both mutants grown at permissive temperature (Figure 3C, lane 5), yet predominately Golgi-modfied precursor CPY (p2CPY) was present in cell pellet fractions of both mutants incubated at non-permissive temperature (Figure 3C, lane 7). These results verify the following: first, a temperature-dependent block in CPY processing had been induced in both double mutants; and second, a secretory block had occurred at non-permissive temperature in both *pep12^{tsf}/sec1-1* and *vps45^{tsf}/sec1-1* mutant cells, as little precursor CPY was observed in media fractions generated from either double mutant at non-permissive temperature (Figure 3C, lane 8). This intracellular retention of precursor CPY in the double mutant strains contrasts with the predominant extracellular localization of precursor CPY seen in identically treated *pep12^{tsf}* and *vps45^{tsf}* single mutants at non-permissive temperature (Figure 3C, lanes 3 and 4). These results demonstrate that vacuolar localization of ALP does not involve transport via the plasma membrane in either *pep12^{tsf}* or *vps45^{tsf}* mutants.

A vps41^{tsf} mutant mislocalizes ALP but not CPY or CPS

The differential processing of ALP and CPS observed in *vps45^{tsf}* and *pep12^{tsf}* mutant cells is consistent with ALP and CPS transiting distinct pathways from the Golgi to the vacuole. Just as mutations in *vps45* and *pep12* preferentially affect vacuolar delivery of CPS and CPY relative to ALP, mutations in genes which act primarily in an ALP vacuolar delivery pathway would be expected

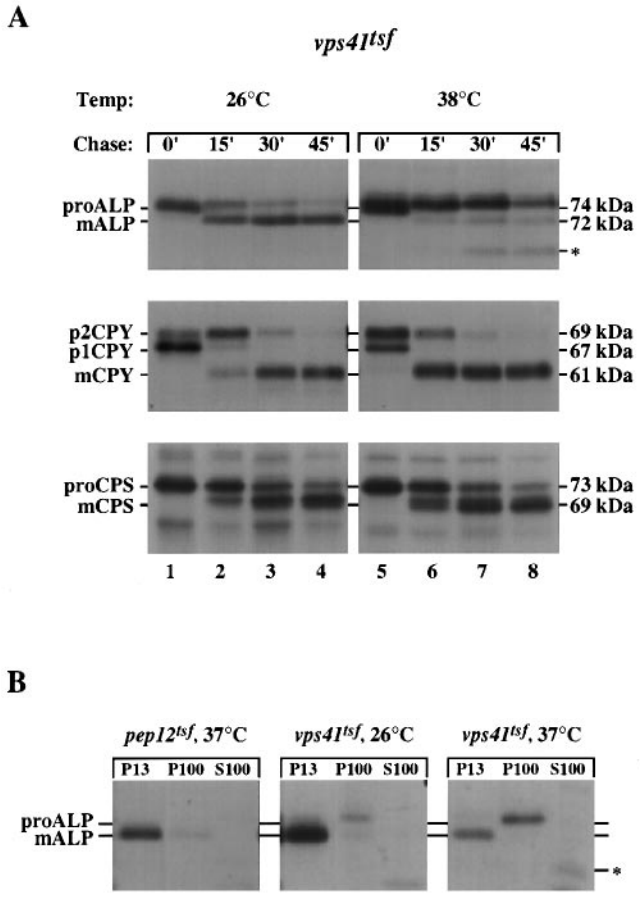

Fig. 4. Temperature-conditional missorting and mislocalization of ALP in *vps41^{tsf}* cells. (**A**) Mutant cells harboring the *vps41^{tsf}* allele were incubated at permissive (26°C) or non-permissive (37°C) temperature for 10 min, labeled for 10 min with Tran^{35}S-label, then chase periods of the indicated durations were initiated via addition of unlabeled methionine and cysteine to the incubations. Cells were harvested and extracts were generated. ALP, CPY and CPS were isolated by immunoprecipitation, resolved via SDS–PAGE and visualized by fluorography. An aberrantly processed form of ALP is indicated with an asterisk (*). (**B**) Mutant cells harboring the *pep12^{tsf}* and *vps41^{tsf}* alleles were converted to spheroplasts and incubated for 10 (*pep12^{tsf}*) or 0 min (*vps41^{tsf}*) prior to addition of Tran^{35}S-label for 15 min. Labeling was halted via addition of unlabeled methionine and cysteine, and chase periods of 30 min were implemented. Labeled spheroplasts were then lysed and subjected to sequential differential centrifugation. Equivalent amounts of the 13 000 *g* pellet (P13), 100 000 *g* pellet (P100) and 100 000 *g* supernatant (S100) were subjected to quantitative immunoprecipitations with antisera directed against ALP. The immunoprecipitates were resolved by SDS–PAGE, and labeled ALP was visualized via fluorography.

to affect ALP localization more severely than localization of CPS and CPY.

Deletion mutants of *vps41* exhibit a strong inhibition of ALP processing and vacuolar localization, in addition to secretion of p2CPY (Radisky *et al.*, submitted). The *VPS41* gene encodes a 992 amino acid protein with no significant homology to any other protein of known function (YPD:YDR080W; SwissProt:D4446; Radisky *et al.*, 1997). To examine the primary effects of loss of Vps41p function, a functionally temperature-conditional allele of *vps41*, *vps41^{tsf}*, was generated (see Materials and methods). In Figure 4A, haploid strains harboring the *vps41^{tsf}* mutation were incubated at the permissive temperature of 26°C or at the non-permissive temperature of 38°C for 10 min prior to a 10 min labeling followed by a chase period. Labeled cells were harvested at 0, 15, 30 and 45 min of chase, lysed and subjected to immunoprecipitation with antibodies directed against ALP, CPY and CPS. Following a 45 min chase period at the permissive temperature of 26°C, *vps41^{tsf}* mutant cells were observed to process 80–90% of labeled ALP, CPY and CPS to mature form, consistent with proper vacuolar localization of all three hydrolases (though ALP transport kinetics were ~2-fold slower than observed for wild-type cells). In contrast, ALP was observed predominately in a Golgi-modified precursor form following a 45 min chase period in *vps41^{tsf}* cells incubated at non-permissive temperature (38°C; Figure 4A, lanes 5–8). A small amount of mature ALP, as well as a pool of aberrantly processed ALP (Figure 4A, lanes 7 and 8; asterisk), was also observed in the *vps41^{tsf}* mutant at high temperature. While ALP maturation was largely compromised in *vps41^{tsf}* cells at non-permissive temperature, the maturation kinetics of CPY and CPS were similar at both permissive and non-permissive temperatures in this mutant (Figure 4A, lanes 5–8). These results suggest that the primary defect in *vps41^{tsf}* mutant cells is missorting of ALP. CPY and CPS sorting defects are secondary: only upon extended incubations (3 h or greater) at non-permissive temperature do *vps41^{tsf}* cells begin to mislocalize CPY to the cell surface (data not shown).

In order to investigate the intracellular localization of the misprocessed pool of ALP observed in *vps41^{tsf}* cells at non-permissive temperature, ALP localization was inspected via subcellular fractionation of *vps41^{tsf}* cells incubated at both permissive and non-permissive temperatures. Labeled spheroplasts were lysed and subjected to differential centrifugation, resulting in a P13 (13 000 *g*) pellet fraction, a P100 (100 000 *g*) pellet fraction and an S100 (100 000 *g*) supernatant fraction. Membranes of the vacuole, ER and plasma membrane are highly enriched in the P13 fraction (Marcusson *et al.*, 1994), while the P100 fraction is enriched for membranes of the Golgi apparatus, transport vesicles and endosome (Vida *et al.*, 1993; Marcusson *et al.*, 1994). Consistent with labeled ALP reaching the vacuole in *pep12^{tsf}* cells at non-permissive temperature, mature ALP was observed almost exclusively in a P13 pellet fraction in these cells (Figure 4B, left). A similar localization and processing state of labeled ALP was observed for *vps41^{tsf}* cells at permissive temperature: ALP was found predominately in mature form in a P13 pellet fraction (Figure 4B, middle). In contrast, subcellular fractionation of *vps41^{tsf}* cells labeled and chased at non-permissive temperature revealed that labeled precursor ALP accumulated in a P100 fraction while the mature form of ALP that escaped the *vps41^{tsf}* block was found in a P13 fraction (Figure 4B, right). The presence of the aberrantly processed form of ALP in an S100 fraction (Figure 4B, right, asterisk) indicated that this form of ALP had been cleaved off the membrane and released from an osmotically fragile compartment.

The vps41^{tsf} mutant accumulates novel membrane-enclosed structures

The mislocalization of ALP observed in *vps41^{tsf}* cells at non-permissive temperature suggested that these cells accumulate ALP in non-vacuolar structures which might

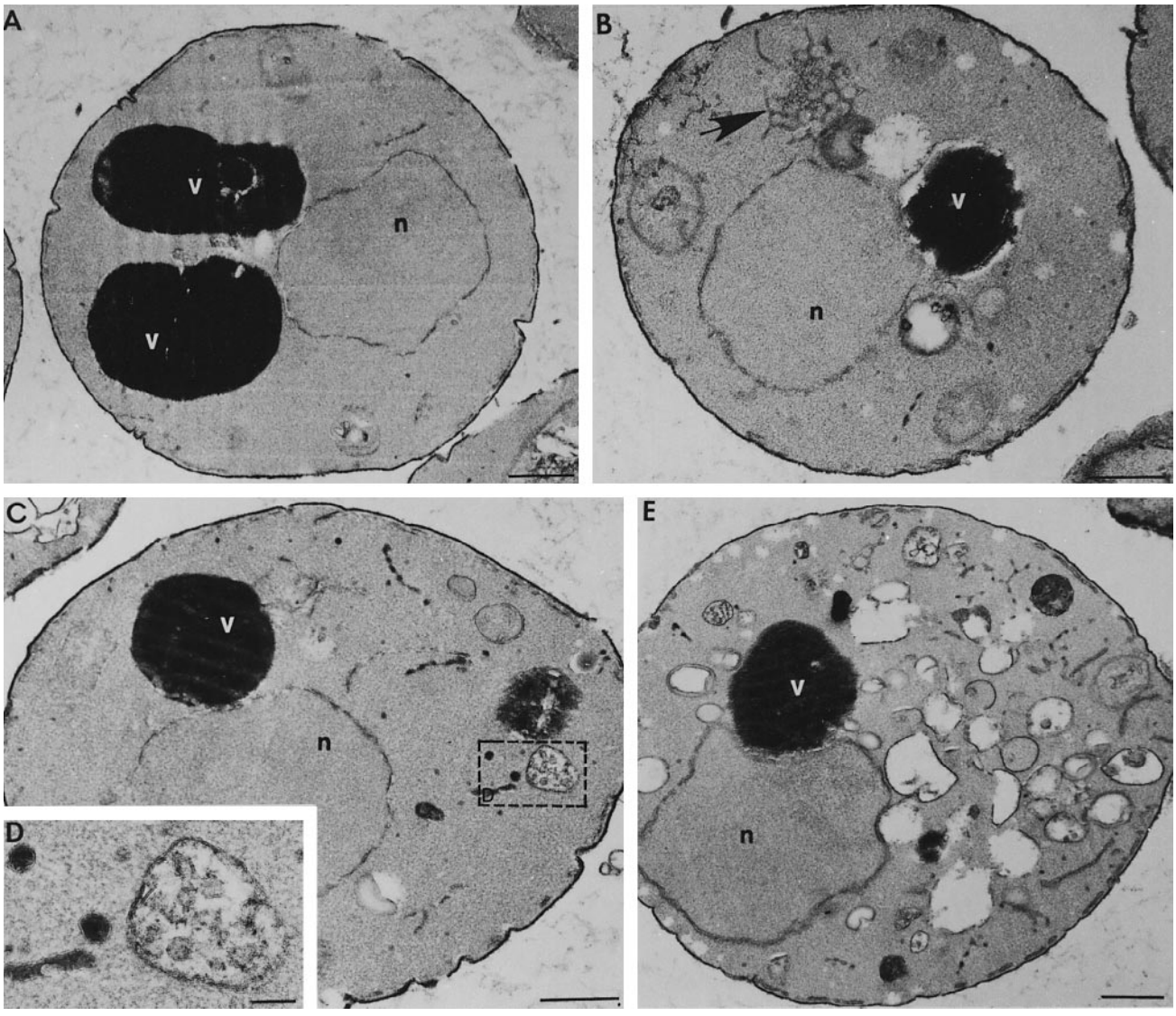

Fig. 5. Morphology of the *vps41*^{tsf} mutant strain. Mutant cells harboring the *vps41*^{tsf} allele were incubated at permissive (26°C; **A**) or non-permissive (38°C) temperature for 1 (**B**) or 3 (**C–E**) h prior to preparation for electron microscopic analysis. The arrow points to an aberrant tubular membrane structure. In (A), (B), (C) and (E), n identifies the nucleus; v, vacuole. Bars in (A), (B), (C) and (E): 0.5 μM. The broken line box region in (C) is enlarged in (D) to show a multivesicular body-like structure. Bar in (D), 0.1 μM.

correspond to intermediates in the pathway responsible for delivering ALP to the vacuole. A deletion mutant of *vps41* exhibits severe vacuolar fragmentation (Radisky *et al.*, 1997), consistent with identification of the *vps41* mutant as a class B *vps* mutant (Raymond *et al.*, 1992) and a vacuolar morphology mutant (*vam2*) (Wada *et al.*, 1992). In order to examine the initial effects of loss of Vps41p function on cellular morphology, *vps41*^{tsf} cells were incubated at permissive (26°C) or non-permissive (38°C) temperature for 1 or 3 h, then examined by electron microscopy. *vps41*^{tsf} cells incubated at permissive temperature resembled wild-type cells: nuclei and intact vacuoles were observed to be the most prominent structures present in these cells (Figure 5A) (Cowles *et al.*, 1994). When *vps41*^{tsf} cells incubated at non-permissive temperature were examined, many cells were found to accumulate aberrant membrane structures (Figure 5B, arrow; D), yet maintain an intact vacuole (Figure 5B–E). Membrane-enclosed vesicles reminiscent of mammalian multivesicular bodies (Figure 5C–E) (Hopkins, 1983) accumulated in a portion of cells. In addition, tubular membrane networks were observed in several cells following both 1 and 3 h shifts to non-permissive temperature (Figure 5B and E). Many of the *vps41*^{tsf} cells incubated at non-permissive temperature for 3 h exhibited a more exaggerated accumulation of membranous structures, while near normal vacuoles were still present (Figure 5E). Indeed, aberrant membrane structures appeared rapidly upon loss of Vps41p function, but structural integrity of the vacuole was largely maintained in these cells (Figure 5B–E).

A portion of overexpressed ALP shifts into a PEP12-dependent vacuolar sorting pathway

To address whether the machinery responsible for *PEP12*-independent vacuolar delivery of ALP is saturable, processing of ALP was examined in a *pep12*^{tsf} strain harboring the *PHO8* gene (encoding ALP) on a 2μ-based (high copy) plasmid. These mutant cells, which overexpressed ALP at ~20-fold wild-type levels (data not shown), were incubated for 15 min at either permissive (30°C) or non-

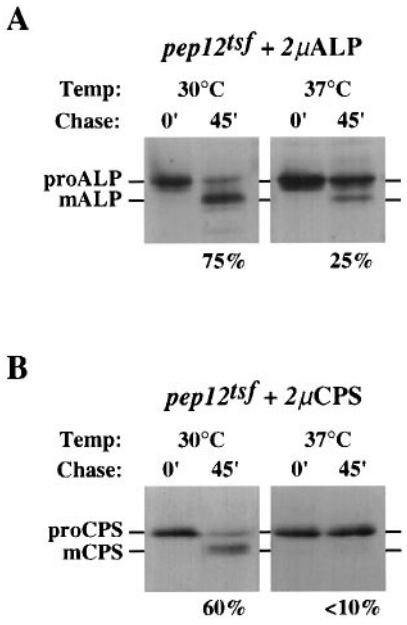

Fig. 6. Processing of overexpressed ALP and CPS in *pep12*^{tsf} mutant cells. *pep12*^{tsf} cells transformed with high copy (2μ) plasmids containing (**A**) the *PHO8* gene or (**B**) the *CPS1* gene were incubated for 15 min at permissive (30°C) or non-permissive temperature (37°C) prior to initiation of a 5 min labeling period with addition of Tran^{35}S-label. Unlabeled methionine and cysteine were then added to the incubations, and cells were harvested immediately (0 min chase) or following a 45 min chase period. Cell extracts were generated and ALP and CPS were isolated via immunoprecipitation. The immunoprecipitates were resolved by SDS–PAGE and visualized via fluorography. Percentages of (A) mature ALP and (B) mature CPS observed following 45 min of chase are notated.

permissive (37°C) temperature prior to labeling for 5 min. Cells were then chased for 0 or 45 min, lysed and subjected to immunoprecipitation with ALP antisera. At permissive temperature, only 75% of ALP matured following a 45 min chase period (Figure 6A, left panel), consistent with overexpressed ALP having saturated its delivery and/or processing machinery. Surprisingly, only 25% of overexpressed ALP was found in its vacuole-localized mature form at non-permissive temperature in this mutant strain (Figure 6A, right panel), suggesting that a significant portion of overexpressed ALP in this mutant had been rendered *PEP12* dependent for processing. This result contrasts the completely *PEP12*-independent maturation observed for endogenously expressed ALP (Figure 1B).

When a similar experiment was performed to assess CPS processing in a *pep12*^{tsf} strain overexpressing CPS at ~20-fold wild-type levels, a defect in CPS maturation was observed at permissive temperature, with only 60% of CPS found in its vacuole-localized mature form following a 45 min chase period in these cells (Figure 6B, left panel). This defect in CPS processing implicates a saturation of the CPS delivery and/or processing machinery in this mutant. Consistent with prior observations of a CPS processing block at non-permissive temperature in the *pep12*^{tsf} mutant (Figure 1B, chromosomal CPS expression), no mature CPS was observed at non-permissive temperature in a *pep12*^{tsf} mutant overexpressing CPS (Figure 6B, right panel). This finding suggests that overexpressed CPS does not enter a Pep12p-independent vacuolar delivery route, whereas some overexpressed ALP does enter the Pep12p-dependent vacuolar delivery pathway.

Differential sorting of ALP and CPS is mediated by a cytosolic tail determinant in ALP

The differential sorting of ALP and CPS apparent in *pep12* and *vps45* mutants implicates the presence of distinct vacuolar sorting signals in one or both of these hydrolases. In order to map such a signal, a PCR-mediated approach was used to create two gene fusions, termed AAC and CCA (see Figure 7 and Materials and methods). The AAC gene fusion consists of the *PHO8* promoter and regions of the *PHO8* gene encoding the cytosolic tail and transmembrane domain of ALP fused in-frame with *CPS1* sequences encoding the lumenal domain of CPS. Likewise, the CCA fusion consists of the *CPS1* promoter and cytosolic tail- and transmembrane domain-encoding sequences fused in-frame with the region of *PHO8* encoding the lumenal domain of ALP. Single copy (*CEN*) plasmids harboring these two gene fusions, AAC and CCA, as well as plasmids containing wild-type *CPS1* and *PHO8* genes, were transformed into *pep4*Δ mutant cells (TVY1) or *pep12*^{tsf} mutant cells containing genomic deletions of either *PHO8* or *CPS1*. Immunoreactive proteins of the predicted molecular weights were produced in strains harboring each of these plasmids, and the processing of CPS, ALP and AAC proteins occurred as predicted in *pep12*^{tsf} cells at permissive temperature (Figure 8). Surprisingly, the CCA fusion protein underwent two distinct processing events at permissive temperature in the *pep12*^{tsf} mutant (Figure 8). One processing event was the expected cleavage of the ALP lumenal domain at a site near the carboxy-terminus of the protein, while the other cleavage event appeared to occur just lumenal to the transmembrane domain of the CCA fusion protein. Neither AAC nor CCA proteins were processed in the *pep4*Δ mutant (data not shown), verifying that processing of each fusion protein could be used as a direct indication of vacuolar delivery. When processing of the AAC fusion protein was examined in *pep12*^{tsf} mutant cells, the fusion protein exhibited processing at both permissive and non-permissive temperatures (Figure 8). Conversely, when sorting of the CCA fusion protein was examined, the fusion protein showed processing only at permissive temperature in the *pep12*^{tsf} strain (Figure 8), akin to the processing of wild-type CPS in this mutant (Figure 8). These results indicate that the vacuolar sorting determinant(s) of ALP and/or CPS map to the tail and transmembrane domains of these two proteins.

In order to assess the relative importance of cytosolic tail versus transmembrane domain regions in mediating alternative sorting of CPS and ALP, the cytosolic tail sequences of the CCA and AAC gene fusions were swapped with one another to generate the ACA and CAC gene fusions, respectively (Figure 7). As before, *CEN*-based plasmids containing these gene fusions were transformed into *pep12*^{tsf} cells, and vacuolar processing of the two fusion proteins, ACA and CAC, was examined at permissive and non-permissive temperatures. The ACA fusion protein was processed rapidly to mature form at both permissive and non-permissive temperatures (Figure 8). In contrast, processing of the CAC fusion protein was almost entirely *PEP12* dependent. More than 80% of the CAC protein was processed to mature form following

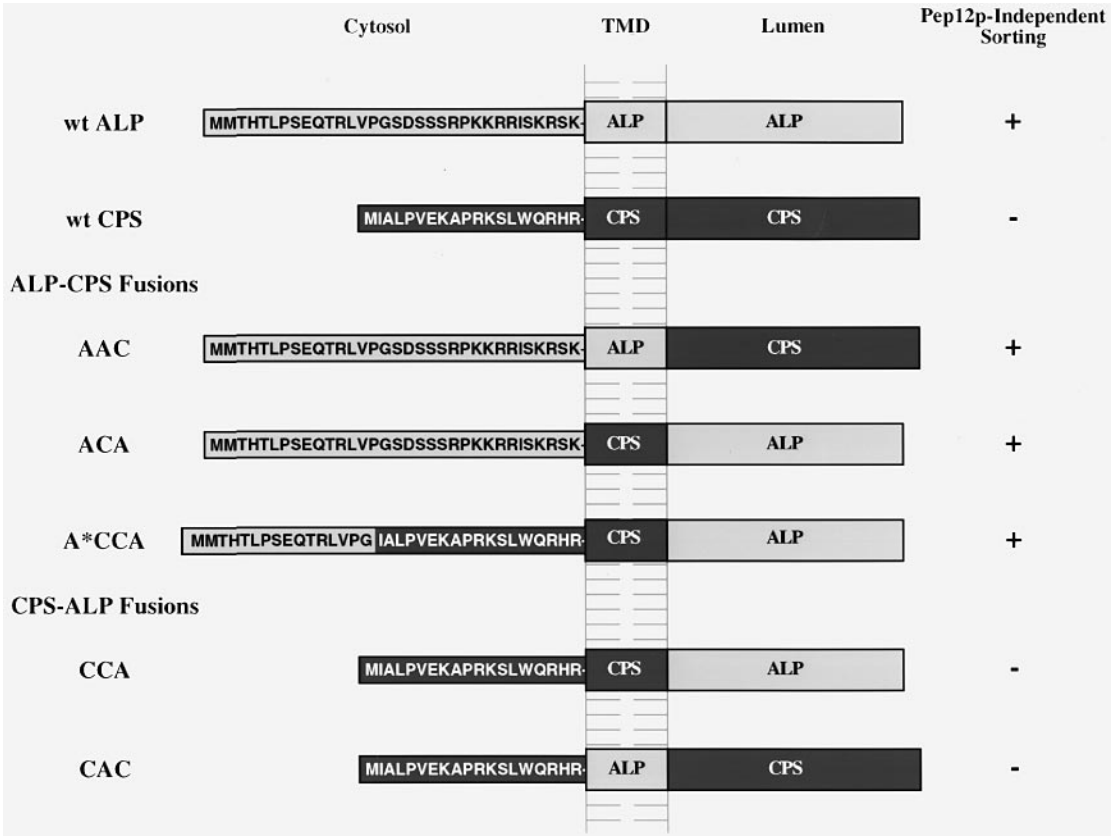

Fig. 7. Schematic diagram of ALP–CPS fusion proteins. Regions of fusion proteins corresponding to CPS fragments are depicted in dark gray, while ALP fragments contained within fusion proteins are featured in light gray. The precise amino acid residue composition of cytosolic tail regions is depicted for all fusion proteins.

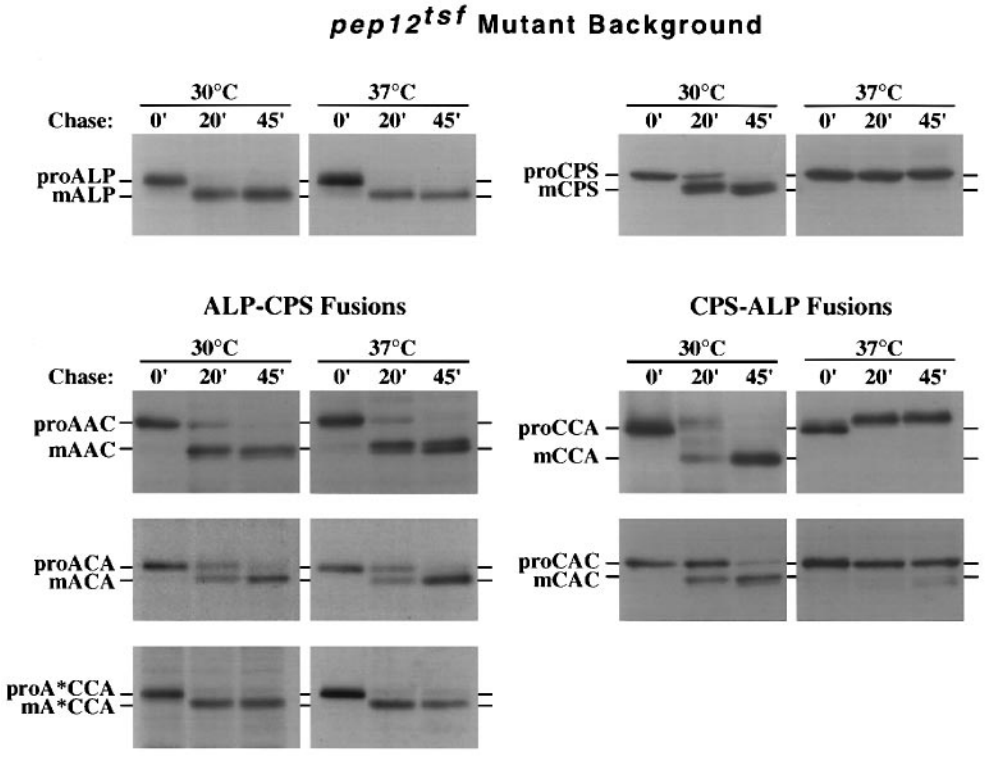

Fig. 8. Processing of fusion proteins in *pep12*^{tsf} mutant cells. *pep12*^{tsf} cells expressing the indicated fusion proteins were incubated for 15 min at permissive (30°C) or non-permissive (37°C) temperature, then labeled for 5 min with Tran^{35}S-label. Chase periods of the indicated duration were initiated via addition of unlabeled methionine and cysteine. Labeled cells were harvested, cell extracts were generated and fusion proteins were immunoprecipitated with either ALP (fusions containing the ALP lumenal domain) or CPS (fusions containing the CPS lumenal domain) antisera. Immunoprecipitates were resolved via SDS–PAGE and visualized by fluorography.

45 min of chase in the *pep12^{tsf}* mutant at permissive temperature, while the vast majority of CAC protein was blocked in precursor form following a 45 min chase at non-permissive temperature (Figure 8). When combined with processing results obtained for the AAC and CCA gene fusions, these ACA and CAC gene fusion results implicate the cytosolic tail regions of ALP and CPS in conferring differential sorting of the two proteins.

The preceding fusion results, together with evidence that overexpressed ALP shifted into a Pep12p-dependent delivery pathway, indicate that an active sorting signal exists in the cytosolic tail of ALP, and a final gene fusion was constructed to test for such a determinant. The A*CCA gene fusion combines the *PHO8* promoter and sequences encoding the first 16 amino acid residues of ALP with the CCA gene fusion (starting at the second amino acid residue of CCA; see Figure 7). When tested for *PEP12*-dependent vacuolar processing, the vast majority of the A*CCA fusion protein was processed to mature form at both permissive and non-permissive temperatures in the *pep12^{tsf}* mutant (Figure 8).

Discussion

This study has uncovered distinct genetic requirements for the trafficking of the vacuolar hydrolases ALP and CPS. CPS follows a Pep12p- and Vps45p-dependent pathway to the vacuole, whereas ALP transits to the vacuole via a Pep12p- and Vps45p-independent route. The Pep12p- and Vps45p-independent route that delivers ALP to the vacuole functions in the presence of a late secretory pathway (*sec1-1*) or endocytic pathway (*end3-1*) block, suggesting that newly synthesized ALP does not reach the vacuole via a plasma membrane/endocytosis route in the *pep12^{tsf}* and *vps45^{tsf}* mutants. In agreement with ALP transiting a vacuolar delivery pathway which is distinct from the one followed by CPS, a *vps41^{tsf}* mutant shows a preferential block in ALP delivery and processing relative to CPS, suggesting that Vps41p functions primarily in the pathway responsible for vacuolar delivery of ALP. Fusion protein studies have revealed that the cytosolic amino-terminal 16 amino acids of ALP contain a sorting signal which directs inclusion of ALP as cargo in this alternative vacuolar delivery route.

Previous studies have implicated Pep12p (endosomal t-SNARE) and Vps45p (Sec1p homolog) to function in targeting and/or fusion of Golgi-derived vesicles with the endosome (Cowles *et al.*, 1994; Piper *et al.*, 1994; Becherer *et al.*, 1996). If ALP, CPY and CPS were packaged at the late Golgi into a common vesicle carrier, mutations in *PEP12* and *VPS45* would be expected to affect the vacuolar delivery of all three of these hydrolases, not just that of CPY and CPS. Since ALP delivery is unaffected in *pep12* and *vps45* mutant cells, the existence of two distinct Golgi-derived transport intermediates, one containing ALP and the other carrying CPY and CPS, is the most plausible explanation for the vacuolar delivery of ALP observed in these mutants (see Figure 9).

Prior studies of ALP localization in *vps1* mutant cells have indicated that functional secretory and endocytic pathways are required for vacuolar delivery of ALP in the *vps1* mutant (Nothwehr *et al.*, 1995). These observations suggest that in *vps1* mutants, ALP reaches the vacuole by an indirect route via the plasma membrane. When *vps45^{tsf}*, *pep12^{tsf}* and *vps8* (Horazdovsky *et al.*, 1996) mutant alleles were combined with the late secretory pathway mutant, *sec1*, or the endocytic pathway mutant, *end3*, ALP was localized to the vacuole at non-permissive temperature, indicating that the exocytic and endocytic pathways are not required for vacuolar localization of ALP in these mutants. Thus, our results demonstrate the existence of a new Golgi to vacuole pathway that is functional in the presence of *pep12* and *vps45* mutations, but that may be dependent on Vps1p. The role of this alternative pathway is not yet clear; however, it may be advantageous for certain cargoes to bypass the Pep12p-dependent endosomal intermediate.

Formation of distinct Golgi to vacuole transport intermediates probably occurs at the late Golgi, since both CPY and ALP receive α1,3-mannose linkages (Esmon *et al.*, 1981; Klionsky and Emr, 1989; Graham and Emr, 1991). One issue yet to be resolved is whether newly synthesized ALP transits the Kex2p-containing compartment of the Golgi, which is where CPS and CPY are thought to be packaged into vesicles destined for the endosome. It is possible that ALP-containing transport intermediates bud from an earlier Golgi compartment than CPS-containing transport intermediates because (i) the ALP delivery pathway is incapable of carrying CPS as cargo, even under conditions of CPS overexpression and Pep12p inactivation (Figure 6B) and (ii) a portion of overexpressed ALP may be transported via the 'more distal' CPS transport pathway (Figure 6A).

Regardless of the specific Golgi compartment that gives rise to ALP-containing transport intermediates, delivery of ALP and CPY to the vacuole via disparate pathways might occur in a manner similar to the parallel trafficking of invertase and Pma1p from the Golgi to the plasma membrane (Harsay and Bretscher, 1995). Generation of CPY- and ALP-containing transport intermediates could resemble budding of separable secretory vesicle populations from the late Golgi. Furthermore, the machinery responsible for the eventual targeting and consumption of Golgi to vacuole transport intermediates at the vacuole might be shared by both ALP- and CPY-trafficking pathways, just as the late secretory pathway proteins, Sec1p, Sec4p and Sec6p, are required for fusion of both populations of Golgi-derived secretory vesicles at the plasma membrane (Harsay and Bretscher, 1995). Future studies of both parallel pathways (Golgi to vacuole and Golgi to plasma membrane) should clarify whether such comparisons remain valid.

The presence of a delivery pathway to the vacuole that is distinct from the one followed by CPY and CPS predicts the existence of transport components which function directly in that pathway. Mutations in genes encoding components of the trafficking route that delivers ALP to the vacuole could result in compromised vacuole function due to mislocalization of other cargoes (e.g. a vacuolar t-SNARE) transported by the same pathway. Such mutations could cause secondary defects in soluble vacuolar protein sorting. Indeed, this appears to be the case, as *vps41* mutants secrete p2CPY (Raymond *et al.*, 1992; data not shown), yet characterization of a *vps41^{tsf}* mutant strain revealed dramatic mislocalization of ALP at non-permissive temperature in the presence of rapid

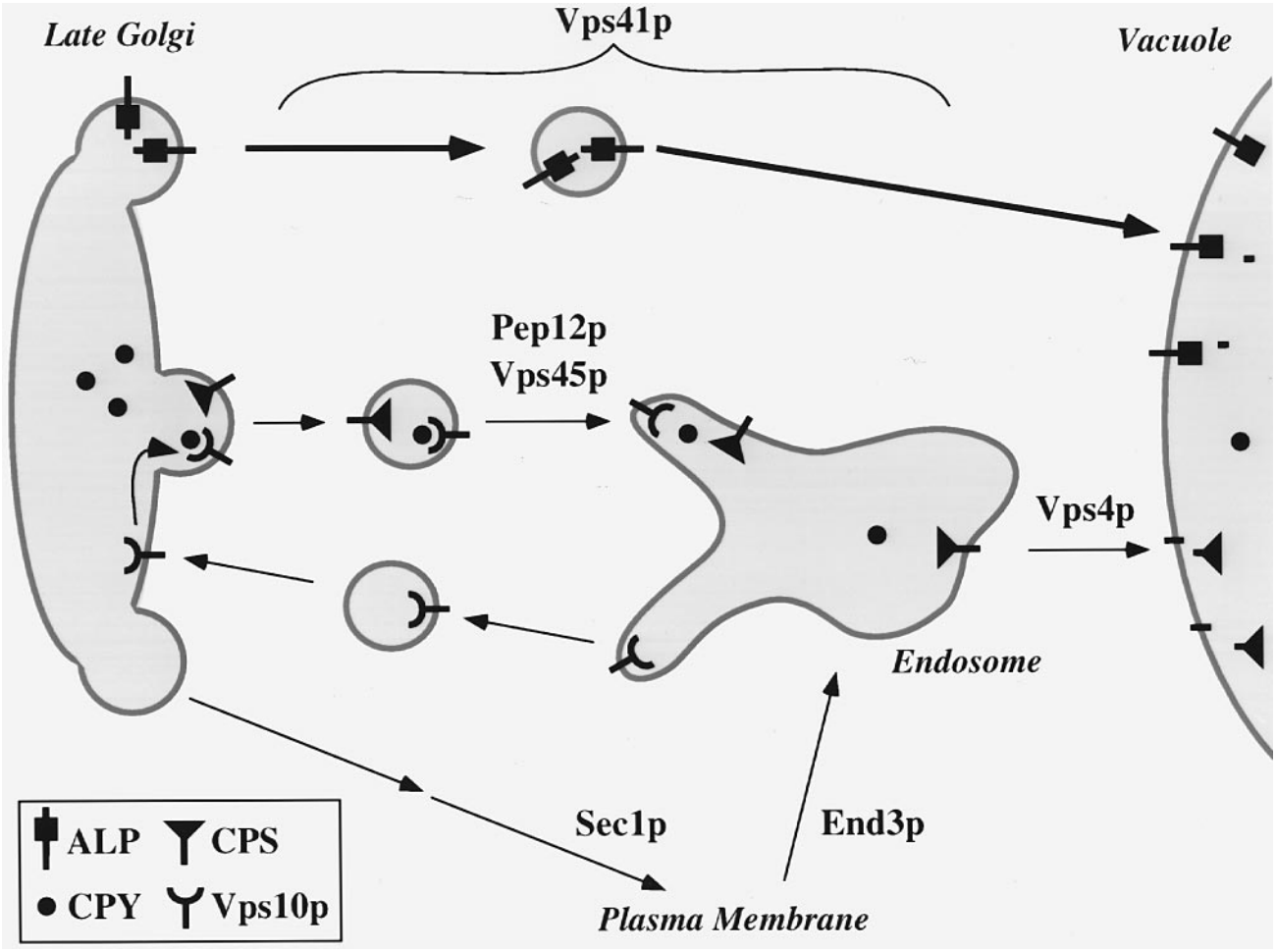

Fig. 9. Model of ALP sorting. An unidentified cytosolic factor recognizes a signal contained within the first 16 amino acid residues of pALP, directing post-Golgi transport of ALP to the vacuole via a Vps41p-dependent pathway. Trafficking of ALP is distinct from trafficking of CPS and CPY due to this ALP sorting determinant, as CPY and CPS are packaged at the late Golgi into vesicles destined for the early endosome. Endosomal targeting and/or fusion of Golgi-derived vesicles carrying CPY and CPS requires the function of Vps45p and Pep12p proteins, whereas vacuolar targeting of ALP-containing intermediates probably bypasses this proposed site of Pep12p/Vps45p activity.

CPY and CPS maturation (Figure 4). Soluble hydrolase secretion is not manifested in the *vps41*^{tsf} mutant until several hours after incubation at non-permissive temperature (data not shown). Although no precise site of action may be assigned to Vps41p at present, electron micrographs of *vps41*^{tsf} cells at non-permissive temperature revealed an accumulation of structures that resemble mammalian multivesicular bodies (Figure 5C and D) (Hopkins, 1983), as well as various aberrant tubular network structures (Figure 5B–E). Such structures might correspond to intermediates in the pathway that delivers ALP to the vacuole. Studies are presently underway to elucidate the function of Vps41p in membrane protein traffic, as well as to determine whether any other known *VPS* genes are involved directly in trafficking of ALP (i.e. other *vps* mutants that exhibit morphological defects similar to *vps41*: *vps39*, *vps43*, *ypt7*, *vam3*; Raymond *et al.*, 1992; Wada *et al.*, 1992; Wichmann *et al.*, 1992).

Prior studies have implicated the vacuole as a default destination for membrane protein traffic in yeast (Cooper and Bussey, 1992; Roberts *et al.*, 1992; Wilcox *et al.*, 1992). However, the Pep12p- and Vps45p-independent vacuolar localization of ALP observed in *pep12*^{tsf} and *vps45*^{tsf} mutants involves an active sorting signal. When we examined processing of the CCA fusion protein, which is comprised of the cytosolic tail and transmembrane domains of CPS fused with the lumenal domain of ALP, we found that vacuolar delivery of this fusion protein was entirely Pep12p dependent (Figure 8). In contrast, addition of the amino-terminal 16 amino acids of ALP to the CCA fusion protein yielded the A*CCA fusion protein, which now localized to the vacuole in an entirely Pep12p-independent manner (Figure 8). These data identify an active vacuolar sorting determinant within the amino-terminal 16 amino acids of ALP. Several other membrane proteins contain cytoplasmic targeting sequences that interact with sorting/trafficking machinery, including the dilysine ER retention/retrieval motif of Wbp1p with coatomer (Cosson and Letourneur, 1994), and the cytosolic tail of the cation-dependent mannose 6-phosphate receptor, which interacts with clathrin adaptor proteins (Glickman *et al.*, 1989; Johnson and Kornfeld, 1992; Leborgne *et al.*, 1993). By analogy, it is likely that the tail of ALP directly associates with transport components (e.g. vesicle coat proteins) that package ALP (and other proteins) into a novel class of Golgi to vacuole transport intermediates (see Figure 9). Although these transport intermediates could target first to the pre-vacuolar endosome and then

be delivered to the vacuole, recent evidence indicates that the ALP carrier targets directly from the Golgi to the vacuole. In a temperature-conditional class E *vps* mutant, *vps4^{tsf}*, that blocks CPY transport out of the pre-vacuolar endosome at high temperature, ALP transport to the vacuole is not affected (Babst *et al.*, 1997). Future studies will be directed towards isolating the ALP carrier, defining its cargo and identifying other transport components specific to this novel pathway. Similar studies in mammalian cells may uncover an analogous alternative Golgi to lysosome transport pathway.

Materials and methods

Materials

Escherichia coli were grown in LB supplemented with ampicillin (Miller, 1972). *Saccharomyces cerevisiae* were propagated in yeast extract–peptone–dextrose (YPD), yeast extract–peptone–fructose (YPF) or synthetic dextrose (SD) augmented with amino acids as required (Sherman *et al.*, 1979). Restriction and modifying enzymes were purchased from Boehringer Mannheim, New England Biolabs and Stratagene. TA cloning kits were supplied by Invitrogen. Zymolyase 100-T (Kirin Brewery Co.) was from Seikagaku Kogyo Co. (Tokyo, Japan). Glusulase was purchased from DuPont Co. 5-Bromo-4-chloro-3-indoyl-β-D-galactoside and α_2-macroglobulin were supplied by Boehringer Mannheim. Tran^{35}S-label was obtained from ICN Biochemicals. [α-^{35}S]dATP was from Amersham Corp. A fusion protein containing TrpE and lumenal Cps1p residues was expressed, purified and used to immunize New Zealand White rabbits as previously described (Horazdovsky and Emr, 1993). Collected antiserum was screened and titrated by immunoprecipitation of labeled yeast cell extracts. Production of antisera to vacuolar zymogens CPY and ALP has been detailed previously (Klionsky *et al.*, 1988; Klionsky and Emr, 1989). All other reagents were purchased from Sigma.

Plasmid constructions and nucleic acid manipulations

Construction of plasmids utilized recombinant DNA techniques described previously (Maniatis *et al.*, 1989), with the exception of DNA fragment isolations performed by the glass bead method of Vogelstein and Gillespie (1979). Bacterial DNA transformations employed the protocols of Hanahan (1983). Temperature-conditional alleles of *VPS45*, *PEP12* and *VPS41* were generated by PCR mutagenesis (Muhlrad *et al.*, 1992; Stack *et al.*, 1995). PCR amplification of *VPS45* utilized oligonucleotides CC451, which anneals 215 nucleotide 5′ of the ATG encoding the start methionine of Vps45p, and CC452, which anneals 220 nucleotides 3′ of the *VPS45* stop codon. PCR reactions were performed using *Taq* polymerase under standard reaction conditions (Perkin-Elmer Cetus Corp., Norwalk, CT) modified by the presence of 0.1 mM $MnCl_2$ and 500 μM dATP. The 2.2 kb PCR product resulting from these reactions was co-transformed with equimolar amounts of gel-purified *Hpa*I–*Bgl*II-digested pVPS45-10 (Cowles *et al.*, 1994) (Trp$^+$) into CCY130 (*vps45Δ2* CPY-Inv), and cells were plated to selective media. Selections for temperature-conditional secretion of CPY–invertase were performed as previously described (Stack *et al.*, 1995). One plasmid which conferred temperature-conditional secretion of CPY–invertase in CCY130, pVPS45-28, was isolated and digested with *Apa*I and *Sac*I. The resulting *Apa*I–*Sac*I fragment containing *vps45^{tsf}* coding sequence was ligated with *Apa*I–*Sac*I-digested pRS415 (Sikorski and Hieter, 1989) to generate pVPS45-37. pVPS45-37 was then transformed into CCY120 (*vps45Δ2*) cells to generate the *vps45^{tsf}* strain utilized in this study. *VPS41* was cloned from a yeast centromeric library (*LEU2*, *CEN*; Philip Hieter) as described elsewhere (Radisky *et al.*, 1997). A 4.25 kb *Nhe*I–*Pvu*II fragment containing *VPS41* (YPD:YDR080W; SwissProt:D4446) was subcloned into pRS414 (*CEN TRP1*) (Sikorski and Hieter, 1989) that had been digested with *Xho*I, filled in with Klenow polymerase, and then digested with *Xba*I. The resulting plasmid, pWS24, was digested with *Kpn*I and *Sac*I to release the *VPS41*-containing fragment from the multiple cloning site and ligated into *Kpn*I–*Sac*I-digested pBluescriptSK$^+$ to generate pBS41. A deletion of *VPS41* was then generated by replacing the *Pst*I–*Hin*dIII fragment of pBS41 with *LEU2*. This pBS41Δ plasmid was digested with *Sca*I and *Bgl*II to release a fragment containing *vps41Δ1::LEU2* which was used to transform SEY6210 to Leu$^+$, thus generating the *vps41* deletion mutant strain WSY41 (see Table I). The deletion mutant was confirmed by PCR amplification of the mutant locus. To generate the *vps41^{tsf}* allele, *VPS41* was amplified by PCR with oligonucleotides VPS41UP, which anneals 154 nucleotides 5′ of the ATG encoding the start methionine of Vps41p, and VPS41DOWN, which anneals 218 nucleotides 3′ of the *VPS41* stop codon. PCR amplification was performed by standard reaction conditions with *Taq* polymerase and *Taq* Extender™ (Stratagene). The resulting 3.3 kb product was then co-transformed into WSY41 at equimolar amounts with gel-purified *Pst*I-digested pWS24, and the cells were plated to selective media at 26°C. Transformants were screened for temperature-conditional secretion of CPY–invertase following a 6 h incubation at 38°C by previously described methods (Stack *et al.*, 1995). One such mutant plasmid containing the *vps41-85* allele (pVPS41-85) was used to generate the *vps41^{tsf}* strain characterized in this study. Generation of pCB49 (containing the *pep12^{tsf}* allele) and CBY9, the genomically integrated *pep12^{tsf}* strain employed in this study, will be described elsewhere (Burd *et al.*, 1997).

Creation of *PHO8*-containing plasmids was achieved via *Stu*I–*Kpn*I digestion of a pSEYC58 (*CEN ARS1*) plasmid containing the *PHO8* gene (Klionsky and Emr, 1989), gel isolation of the resulting 3.0 kb fragment, and ligation of this *PHO8*-containing fragment into *Hin*dIII (blunted by a Klenow fill-in reaction)–*Kpn*I-digested pRS416 (*CEN URA3*) (Sikorski and Hieter, 1989). The resulting plasmid, pALP1, was then digested with *Xba*I and *Kpn*I, and the excised *PHO8*-containing 3.0 kb fragment was gel isolated and ligated into *Xba*I–*Kpn*I-digested pRS426 (2μ *URA3*) (Sikorski and Hieter, 1989) to yield pALP4. *CEN*- and 2μ-based *CPS1*-containing plasmids were fashioned in the following manner: a plasmid harboring the *CPS1* gene, pDP83 (Spormann *et al.*, 1991), was used as template in PCR amplification reactions involving oligonucleotides CPSPRIC (5′-AACCTCTGAATTCCCA-3′) and CPS3′ (5′-GCAAAACGGTGTATGG-3′); reactions were performed in duplicate using *Taq* polymerase under standard PCR conditions (Perkin-Elmer Cetus Corp., Norwalk, CT), and the resulting 2.4 kb product was ligated into the TA cloning kit vector, pCR^{TM}II (Stratagene); the *CPS1*-containing insert was then excised via digestion with *Eco*RI and ligated into *Eco*RI-digested pRS414 (*CEN TRP1*) and pRS424 (2μ *TRP1*) (Sikorski and Hieter, 1989) to yield pCPS1 and pCPS3, respectively.

Generation of gene fusions employed the gene Splicing by Overlap Extension ('gene SOEing') technique (Yon and Fried, 1989; Horton *et al.*, 1990). All PCRs occurred in the presence of *Taq* polymerase and *Taq* Extender™ (Stratagene). Gene fusion AAC employed oligonucleotides ALP5′ (5′-CTTGCTGTGCAGAAACAG-3′), ACPS3′ (5′-GAGGTGGTGCTGGGTGTGATGCAGAACGTATAGCAAAACTG-3′), ACPS5′ (5′-CAGTTTTGCTATACGTTCTGCATCACACCCAGCACCACCTC-3′) and CPS3′ in amplifications of templates pALP1 and pDP83. Synthesis of the CCA gene fusion utilized oligonucleotides CPSPRIC, CSAPATHN (5′-GTGTGATGCAGAACGGAGACCCGAAGTGAGG-3′), CSAPATHC (5′-CCTCACTTCGGGTCTCCGTTCTGCATCACAC-3′) and ALP3NSIN (5′-GCGGTTATTCTTTCG-3′) in amplifications of pALP1 and pDP83. Creation of the CAC gene fusion employed PCR primers CPSPRIC, CA23′ (5′-GACCACAGTGGATACTATTATTCTGTGCCTTTGCCATAGGG-3′), CA25′ (5′-CCCTATGGCAAAGGCACAGAATAATAGTATCCACTGTGGTC-3′) and CPS3′ in amplifications of pDP83 and the AAC gene fusion. Generation of the ACA fusion utilized oligonucleotides ALP5′, AC23′ (5′-GGCAACTATTCCACTAATAAAGGCCTTCGATCTCTTCGAGATCC-3′), AC25′ (5′-GGATCTCGAAGAGATCGAAGGCCTTTATTAGTGGAATAGTTGCC-3′) and ALP3NSIN in amplifications of pALP1 and the CCA gene fusion. Creation of gene fusion A*CCA involved use of PCR primers ALP5′, A1-16C3′ (5′-CTACTGGTAAGGCCATTCCAGGAACAAGACGTGTC-3′), A1-16C5′ (5′-CGTCTTGTTCCTGGAATCGCCTTACCAGTAGAGAAG-3′) and ALP3NSIN in amplifications of pALP1 and the CCA gene fusion.

Gene fusions containing CPS lumenal domain sequence (AAC, CAC) were initially ligated into pCR™II (Stratagene), then excised from pCR™II via *Eco*RI digestion and ligated into *Eco*RI-digested pRS414 (Sikorski and Hieter, 1989) to generate pAAC and pCAC, respectively. Fusions containing the ALP lumenal domain sequence (CCA, ACA, A*CCA) were ligated into pCR™II, then excised from pCR™II via digestion with *Eco*RI and *Nar*I and ligated into gel-isolated *Eco*RI–*Nar*I-digested pALP1 vector to generate pCCA, pACA and pA*CCA, respectively. Each gene SOE product was generated in duplicate from parallel, independent reactions. All gene fusion plasmids were denatured and purified over 2 ml of Sephacryl spun columns using the protocol of the Pharmacia MiniprepKit Plus manual. Resultant denatured single-stranded templates were hybridized to either CPSSEQ (5′-CTAATCCTGCATCATCAC-3′) or ALPSEQ (5′-GCCAGCAAGTGGCTAC-3′) and then subjected to dideoxy chain termination sequence analysis

Table I. Strains used in this study

Strain	Genotype	Reference or source
S.cerevisiae		
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al.* (1988)
BHY10	SEY6210; *leu2-3,112*::pBHY11 (CPY-Inv *LEU2*)	Horazdovsky *et al.* (1994)
EGY111-2	*MAT***a** *leu2 ura3 suc2-Δ9 his3 sec1-1*	Gaynor and Emr (1997)
SEY5017	*MAT***a** *leu2-3,112 ura3-52 suc2-Δ9 sec1-1*	lab strain
TVY1	SEY6210; *pep4Δ::LEU2*	Tom Vida
CCY120	SEY6210; *vps45Δ2::HIS3*	Cowles *et al.* (1994)
CCY130	BHY10; *vps45Δ2::HIS3*	Cowles *et al.* (1994)
CCY143	*MAT***a** *leu2-3,112 ura3-52 suc2-Δ9 vps45Δ2::HIS3 sec1-1*	this study
CBY9	BHY10; *pep12^{tsf}*	Burd *et al.* (1997)
CBY31	SEY6210; *pep12Δ2::HIS3*	Burd *et al.* (1997)
CBY34	*MAT***a** *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 pep12Δ2::HIS3 sec1-1*	this study
DKY6280	*MAT***a** *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 pho8Δ::TRP1*	Klionsky and Emr (1989)
CCY236	CBY9; *pho8Δ::TRP1*	this study
YD19	*MATα his3-11 his3-15 ura3Δ5 leu2::ura3ΔSE can^R* [*cir°*] *cps1Δ::URA3*	Spormann *et al.* (1992)
CCY239	CBY9; *cps1Δ::URA3*	this study
WSY41	SEY6210; *vps41Δ1::LEU2*	this study
E.coli		
XL1-Blue	*supE44 thi-1 lac endA1 gyrA96 hsdR17 relA1* [*F′ proAB lacI^q ZΔM15* Tn*10*]	Bullock *et al.* (1987)

(Sanger *et al.*, 1977) using the Sequenase sequencing protocol (US Biochemical Corp.). All gene fusion regions were verified, and no *Taq*-induced mutations were detected in readable sequence (~300 nucleotides) of any gene fusion.

Genetic manipulations

Standard yeast genetic procedures were followed as previously described (Miller, 1972; Sherman *et al.*, 1979). Yeast transformations utilized a LiAc treatment protocol (Ito *et al.*, 1983). A *pep12Δ2::HIS3* disruption construct was generated as follows: pBSPEP12, containing a *Cla*I–*Nsi*I yeast genomic DNA fragment encoding *PEP12* (Becherer *et al.*, 1996), was cut with *Pst*I and *Hin*dII, and a *Pst*I–*Sma*I-cut DNA fragment containing *HIS3* was cloned into this vector, resulting in pCB34. PCR was used to amplify a DNA fragment of pCB34 with primers complementary to ends of the *PEP12* ORF, and this DNA was used to disrupt the wild-type *PEP12* locus by homologous recombination-mediated transformation of SEY6210. To confirm that the *PEP12* locus has been disrupted, several His$^+$ colonies were screened by PCR with primers designed to amplify the *PEP12* locus. A strain derived from one colony that yielded a PCR product of the size corresponding to the size predicted for the disrupted *pep12Δ2::HIS3* locus was renamed CBY31 (*pep12Δ2*). A *vps45Δ2/sec1-1* double mutant, CCY143, was obtained by crossing CCY120 (*vps45Δ2*) (Cowles *et al.*, 1994) with SEY5017 (*sec1-1*). Diploid colonies were selected, sporulated and resulting asci were dissected. Double mutants were selected from tetrads showing 2:2 segregation of *TR/ts* at 38°C. Spores harboring the *sec1-1* mutation were selected based on their inability to grow at 37°C, while spores containing a *vps45* deletion were selected on the basis of secretion of p2CPY at 30°C (Roberts *et al.*, 1991). CCY143 was transformed with pVPS45-37 to generate the *vps45^{tsf}/sec1-1* double mutant used for experiments shown in Figure 3. To construct a double mutant *sec1-1*/*pep12Δ2::HIS3* strain, EGY111-2 (Gaynor and Emr, 1997) was transformed with the *pep12Δ2::HIS3* disruption construct, and *pep12Δ2::HIS3* transformants were confirmed by PCR as described above. This strain, CBY34, was transformed with pCB49, encoding *pep12^{tsf}*. The *pho8* deletion/disruption strain, DKY6280 (Klionsky and Emr, 1989), was employed in creating CCY236, a deletion/disruption of *PHO8* in the *pep12^{tsf}* strain, CBY9. Genomic DNA was isolated from the DKY6280 (*pho8Δ*) strain and used as PCR template for amplification of the *TRP1*-disrupted portion of *PHO8* with primers ALP5′ and ALP3NSIN. The product of this reaction was gel isolated and used to transform CBY9. Trp$^+$ transformants were selected, and *pho8* deletion/disruption mutants were confirmed by both PCR and immunoprecipitation. To create CCY239, a *cps1* deletion/disruption in CBY9, genomic DNA was isolated from YD19 (*cps1Δ*) (Spormann *et al.*, 1991) and used as template in a PCR reaction with CPSPRIC and CPS3′, resulting in amplification of the *URA3*-disrupted *cps1* allele contained in the YD19 strain. This reaction product was gel isolated and used to transform CBY9. Ura$^+$ transformants were selected, and *cps1* deletion/disruption mutants were confirmed by both PCR and immunoprecipitation.

Indirect immunofluorescence

Immunolocalization of ALP was performed as previously described (Redding *et al.*, 1991), with modifications as described (Gaynor and Emr, 1997), using affinity-purified ALP antisera (Raymond *et al.*, 1990, 1992).

Electron microscopy analysis

WSY41 (*vps41Δ1*) mutant cells harboring the *vps41^{tsf}* allele on a low copy (*CEN*) plasmid (pVPS41-85) were grown in SD supplemented with the required amino acids at 26°C to an absorbance at 600 nm (A_{600}) of 0.15, then incubated at either 26 or 38°C for 2 h. Following this 2 h incubation, a portion of cells from the 26°C population were incubated at 38°C for 1 h, while other incubations were allowed to continue for 1 h. Approximately 50 A_{600} units of cells were harvested by centrifugation, fixed at either 26 or 38°C and processed for electron microscopy as described previously (Burd *et al.*, 1996).

Cell labeling and immunoprecipitation

Yeast cells were propagated to an A_{600} of 0.8 in SD supplemented with the required amino acids. For experiments in which ALP, CPS and CPY were immunoprecipitated, 5 A_{600} units of cells per time point were harvested by centrifugation and resuspended in 1 ml per time point of SD medium. A 10 min (Figures 1, 3 and 4) or 15 min (Figures 6 and 8) incubation at either permissive or non-permissive temperature preceded addition of 100 μCi of Tran^{35}S-label per time point. Labeling proceeded for either 10 min (Figures 1, 3 and 4) or 5 min (Figures 6 and 8), and was terminated via addition of methionine, cysteine and yeast extract to final concentrations of 5 mM, 1 mM and 0.2%, respectively. For the experiments whose results are shown in Figure 3, cells were converted to spheroplasts following cell labeling and chase periods (Horazdovsky and Emr, 1993). After spheroplasting, cultures were centrifuged at 13 000 *g* for 1 min to yield an intracellular (I) fraction and an extracellular (E) media fraction. Total protein was recovered from all fractions via trichloroacetic acid precipitation. The presence of CPS, ALP and CPY in each fraction was determined by immunoprecipitation (Klionsky *et al.*, 1988; Robinson *et al.*, 1988). Endoglycosidase H treatment was performed as previously described (Gaynor *et al.*, 1994) on all CPS samples, as well as on ALP samples presented in Figure 6.

Subcellular fractionation

Subcellular fractionations were performed as previously described (Horazdovsky and Emr, 1993; Cowles *et al.*, 1994) with the following modifications. *pep12^{tsf}* spheroplasts were incubated at 37°C for 10 min prior to initiation of a 15 min labeling period. *vps41^{tsf}* spheroplasts were labeled for 15 min at either 26°C or immediately upon incubation at

37°C. Spheroplasts were then subjected to a 30 min chase. Spheroplast lysis was performed in a buffer containing 0.2 M sorbitol, 50 mM KOAc, 2 M EDTA and 20 mM HEPES pH 6.8.

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