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Novel Syntaxin Homologue, Pep12p, Required for the Sorting of Lumenal Hydrolases to the Lysosome-like Vacuole in Yeast

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pep12/vps6 mutants of *Saccharomyces cerevisiae* are defective in delivery of soluble vacuolar hydrolases to the vacuole. Morphological analysis by electron microscopy revealed that *pep12* cells accumulate 40- to 50-nm vesicles. Furthermore, *pep12* cells have enlarged vacuoles characteristic of class D *pep/vps* mutants. *PEP12* encodes a protein of 288 amino acids that has a C-terminal hydrophobic region and shares significant sequence similarity with members of the syntaxin protein family. These proteins appear to participate in the docking and fusion of intracellular transport vesicles. Pep12p is the first member of the syntaxin family to be implicated in transport between the Golgi and the vacuole/lysosome. Pep12p-specific polyclonal antisera detected a 35-kDa protein that fractionated as an integral membrane protein. Subcellular fractionation experiments revealed that Pep12p was associated with membrane fractions of two different densities; the major pool (~90%) of Pep12p may associate with the endosome, while a minor pool (~10%) cofractionated with the late Golgi marker Kex2p. These observations suggest that Pep12p may mediate the docking of Golgi-derived transport vesicles at the endosome.

INTRODUCTION

The vacuole of *Saccharomyces cerevisiae* is an acidic lysosome-like compartment that contains a complement of soluble lumenal hydrolases, including proteases and peptidases, nucleases, phosphatases, and glycosidases (Wiemken *et al.*, 1979). Most of the hydrolases are synthesized as inactive protein precursors that pass through the endoplasmic reticulum and Golgi complex along with secretory proteins (Stevens *et al.*, 1982; Mechler *et al.*, 1988; Klionsky and Emr, 1989; Moehle *et al.*, 1989; for reviews see Jones, 1991; Jones and Murdock, 1994). Sorting of secretory proteins from vacuolar proteins appears to take place in a late Golgi compartment (Graham and Emr, 1991). The vacuolar proteins then transit to the vacuole via an intermediate endosomal compartment (Vida *et al.*, 1993). The final maturation steps, typically proteolytic cleavages that activate the hydrolase precursors, occur upon arrival at the vacuole (Woolford *et al.*, 1986; Mechler *et al.*, 1988; Moehle *et al.*, 1989; Nebes and Jones, 1991; Klionsky *et al.*, 1992; Spormann *et al.*, 1992; van den Hazel *et al.*, 1992; Rupp and Wolf, 1993; Woolford *et al.*, 1993; for reviews see Jones, 1991; Jones and Murdock, 1994).

A large group of mutants have been identified that have defects in the proper transport and processing of several vacuolar hydrolases (Jones, 1977; Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988). One of the class D mutants, *pep12 (vps6)*, is defective in the localization and activation of the lumenal vacuolar hydrolases proteinase A (PrA), proteinase B (PrB), and carboxypeptidase Y (CpY); furthermore, *pep12* cells exhibit decreased vacuolar acidification due to impaired assembly of the vacuolar H^+-ATPase, as well as other defects (Jones, 1977, 1983; Banta *et al.*, 1988; Robinson *et al.*, 1988; Preston *et al.*,

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1989, 1992; Rothman *et al.*, 1989). Here we report the predicted sequence of Pep12p, a number of its properties, and some consequences of loss of Pep12p function.

Pep12p is a cytoplasmically oriented, integral membrane protein that belongs to the syntaxin family. Syntaxin-like proteins and the related synaptobrevin-like proteins, referred to as SNARES, are thought to mediate the specificity required to accurately target transport vesicles to their proper destination: the syntaxin-like target-SNARE (t-SNARE) may act as a receptor for its cognate synaptobrevin-like vesicle-SNARE (v-SNARE) and thus allow transport vesicles to dock and fuse only with the appropriate target membrane (Söllner *et al.*, 1993a,b). The docking and fusion steps also appear to be regulated by a number of other proteins, such as the rab family of small GTP-binding proteins, and the NSF and Sec1p protein families (for reviews see Ferro-Novick and Jahn, 1994; Rothman, 1994; Rothman and Warren, 1994; Südhof, 1995). We discuss a possible role for Pep12p as a t-SNARE for the docking of Golgi-derived transport vesicles at the endosome.

MATERIALS AND METHODS

Materials

Restriction enzymes, T4 DNA ligase, Klenow enzyme, exonuclease III, mung bean nuclease, and *Xho*I synthetic linkers were purchased from New England Biolabs (Beverly, MA) or Boehringer Mannheim Biochemicals (Indianapolis, IN) and were used according to the manufacturer's directions. Goat anti-rabbit horseradish peroxidase conjugate was purchased from Bio-Rad (Hercules, CA), Vectastain kit was purchased from Vector Labs (Burlingame, CA), IgG Sorb was purchased from The Enzyme Center (Malden, MA), and protein A Sepharose CL4B was purchased from Pharmacia (Piscataway, NJ). [α-^{35}S]dATP (1320 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were purchased from New England Nuclear (Boston, MA) and Trans^{35}S was purchased from ICN (Costa Mesa, CA). Nitrocellulose type HA85 paper was obtained from Schleicher and Schuell (Keene, NH), and the Sequenase kit for sequencing was obtained from United States Biochemicals (Cleveland, OH). Zymolyase 100-T (Kirin Brewery) was purchased from Seikagaku (Tokyo, Japan). Antisera to Kar2p and Kex2p were kindly given to us by Mark Rose (Princeton University, NJ) and Robert Fuller (University of Michigan, Ann Arbor, MI), respectively. Other chemicals were from Sigma Chemical (St. Louis, MO), standard sources, or as indicated.

Media and Genetic Methods

YPD and synthetic yeast media (Jones *et al.*, 1982), 2× YT, M9, and LB media (Miller, 1972) were prepared as described previously. Standard genetic methods were used (Hawthorne and Mortimer, 1960). The *pep12*-associated CpY deficiency was scored by the APE test (Jones, 1977).

Strains

Bacterial strains used to propagate plasmids were the following: HB101, RR1 (Maniatis *et al.*, 1982); LM1035 (Cepko *et al.*, 1984); BL21(DE3) (Studier *et al.*, 1989); BW313 (Kunkel, 1985); and JM101 (Yanisch-Perron *et al.*, 1985). Helper phage IR1 was used to generate single-stranded DNA (Yanisch-Perron *et al.*, 1985).

All yeast strains save SEY6210 were derived in our laboratory from strain X2180–1B (*MAT*α *gal2 SUC2*) or from crosses between the strains in our isogenic series and strains congenic to strain X2180–1B that we obtained from D. Botstein (Stanford University, CA), R. Schekman (University of California, Berkeley, CA), and M. Carlson (Columbia University, NY). The derivation of SEY6210 is described by Robinson *et al.* (1988). The strains and their genotypes are given in Table 1.

Nucleic Acid Preparation and Manipulation

Bacterial plasmid DNA was purified by a scaled up version of the alkaline lysis method of Birnboim and Doly (1979) and either used directly or after purification by ethidium bromide-cesium chloride density gradient centrifugation (Petes *et al.*, 1978) or passage through a pZ523 column purchased from 5Prime → 3Prime (Boulder, CO). The $CaCl_2$ method (Maniatis *et al.*, 1982) was used for bacterial transformation.

The $CaCl_2$ (Bruschi *et al.*, 1987) or modified LiAc (Ito *et al.*, 1983) methods were used for transformation of yeast. DNA for probes was labeled in vitro to 10^7 cpm/μg with [α-^{32}P]dCTP by random primer extension (Feinberg and Vogelstein, 1983) using a kit purchased from Amersham (Arlington Heights, IL).

The techniques for preparation and analysis of DNA fragments have been described previously (Maniatis *et al.*, 1982). After electrophoresis in 1% agarose gels run in TBE buffer (Maniatis *et al.*, 1982), staining in ethidium bromide, and visualization by the use of long wave UV light, gel slices containing DNA fragments were excised from agarose gels and DNA was extracted by use of an electroeluter using 10 mM Tris, pH 8, 5 mM NaCl, 0.2 mM EDTA as elution buffer.

Single-stranded plasmid DNA was isolated after 6 h of growth post-infection at 37°C. Intact phages were isolated by polyethylene glycol precipitation and deproteinized by phenol-chloroform extraction (Messing and Vieira, 1982).

DNA sequencing was carried out by the chain termination dideoxy sequencing reactions (Sanger *et al.*, 1977) as described in instructions accompanying the Sequenase kit obtained from United States Biochemicals, using [α-^{35}S]dATP, 1 μg template DNA, and 10 ng primer, or by the method of Maxam and Gilbert (1980). Synthetic oligonucleotide primers were obtained from the University of Pittsburgh DNA Synthesis Facility (Pittsburgh, PA).

Exonuclease III/mung bean nuclease deletions were produced according to the instructions in Stratagene's Bluescript DNA Sequencing System (La Jolla, CA). Plasmids pKB712 and pKB713 were used for exonuclease III digestion. pKB712 contains the 2.6-kb *Xho*I/*Sph*I fragment that contains the *PEP12* complementing sequence subcloned into *Sph*I/*Xho*I-digested pKB711X DNA; pKB713 contains the 4.6-kb *Bam*HI/*Sph*I fragment that contains the same *Xho*I/

Table 1. Strain list

Saccharomyces cerevisiae		
BJ1984	*Mat*α *SUC2 gal2 trp1 pep4-3*	
BJ2948	*MAT***a** *SUC2 gal2*	*(X2180-1A)*
BJ2977	*MAT*α *SUC2 gal2 ura3-52 leu2 trp1*	
BJ4037	*MAT***a** *SUC2 gal2 ura3-52 leu2 his1 pep12-2*	
BJ4227	*MAT*α *SUC2 gal2 ura3-52 leu2 trp1 pep12-2*	
BJ4278	*MAT***a** *SUC2 gal2 ura3-52 leu2 his1*	
BJ4369	Pep$^+$ Ura$^+$ integrant of BJ4227	
BJ4842	*MAT*α *SUC2 gal2 URA3 LEU2 trp1 / MAT***a** *SUC2 gal2 ura3-52 leu2 trp1*	
BJ7666	*MAT***a** *SUC2 gal2 ura3-52 leu2 Δpep12 ::TRP1*	
SEY6210	*MAT*α *suc2-Δ9 ura3-52 leu2-3, 113 his3-Δ200 trp1-Δ901 lys2-801*	

*Sph*I fragment described above and an additional 2-kb *Xho*I/*Bam*HI fragment. For exonuclease III digestion, pKB712 was digested with *Xho*I and *Sst*I, and pKB713 was digested with *Bam*HI and *Sst*I. After exonuclease III digestion and mung bean nuclease treatment, the newly created ends were treated with Klenow fragment and ligated. This treatment causes juxtaposition of the *Eco*RI site of the polylinker to the exonuclease shortened yeast sequences.

Plasmids

The parent plasmids used were YCp50 (Fitzgerald-Hayes *et al.*, 1982; Kuo and Campbell, 1983), YIp5 (Struhl *et al.*, 1979), YEp213 (Broach *et al.*, 1979), *TRP1 CEN3* (Futcher and Carbon, 1986), and pKB711 (Becherer, 1991). pKB711 was constructed as follows: the 2-kb *Pst*I/*Pvu*II fragment of pZ152 (Zagursky and Berman, 1984), containing the M13 single-stranded origin for + strand replication and part of the β-lactamase gene, was ligated into the 4.9-kb *Pst*I/*Pvu*II fragment recovered by partial digestion of *TRP1 CEN3*, restoring ampicillin resistance. The resulting plasmid was then digested with *Pvu*II and *Sma*I and religated to reduce the size of the plasmid by 1.7 kb. One of the *Eco*RI sites flanking the 1.4-kb *TRP1 ARS1* fragment was destroyed by linearizing with *Eco*RI, filling in with the Klenow fragment of DNA polymerase, and ligating the blunt ends. This treatment creates an *Xmn*I site at the destroyed *Eco*RI site. The *Eco*RI to *Hin*dIII polylinker from pBSM13+ (Messing and Vieira, 1982) was then inserted into the 5.2-kb *Hin*dIII/*Eco*RI fragment created by partial digestion of the vector with *Hin*dIII, thus creating a multicloning site. Insertion of a *Xho*I synthetic linker (NEB#1006) at the *Eco*RV site adjacent to the polylinker created pKB711X.

Plasmid pKB714 was constructed by ligation of the 4.6-kb *Bam*HI/*Sph*I fragment from pKB58 into *Bam*HI/*Sph*I-cleaved YIp5 plasmid (Struhl *et al.*, 1979) from which the *Eco*RI site had been deleted, and pkb7125 and pKB718 were created by ligation of the same *Bam*HI/*Sph*I fragment into *Bam*HI/*Sph*I-cleaved YCp50 and YEp24, respectively.

A deletion-disruption plasmid was constructed by combining the *Eco*RI/*Sph*I fragment from pKB713B (a deletion derivative of pKB713) and the *Eco*RI/*Xho*I fragment of pKB712D (a deletion derivative of pKB712) into the previously used sequencing vector pKB711X, which had been digested with *Xho*I and *Sph*I, and inserting the 1.4-kb *Eco*RI fragment containing the *TRP1* gene into the *Eco*RI site created by this construct. For transplacement, the *Bam*HI/*Sph*I fragment bearing *pep12::TRP1* was recovered after digestion and used to transform the recipient strain to Trp^+.

Antisera

Two polyclonal antisera were raised to *trpE-pep12* fusion proteins. Antiserum A was used for immunoblots (Figure 6), and antiserum B for immunoprecipitation (Figures 5, 7, and 8). Antiserum A was created as follows: a *Bgl*II/*Eco*RI fragment was excised from one of the nested deletions, Klenow treated, and inserted into the *Hin*dIII- and *Bam*HI-cleaved Klenow-treated pATH3 fusion vector (Dieckmann and Tzagoloff, 1985), thereby fusing amino acids 74–203 of Pep12p to the *trpE* product in a protein of 48.3 kDa. Protein was produced in strain RR1 and isolated (Kleid *et al.*, 1981). Rabbits were immunized at the Pocono Rabbit farm using pulverized gel slices containing *trpE-pep12* fusion protein. The resulting antiserum was affinity purified against a gene 10-*pep12* fusion protein (Spindler *et al.*, 1984; Studier and Moffatt, 1986; Studier *et al.*, 1989; Becherer, 1991). To generate the gene 10-*pep12* fusion protein, a fusion gene encoding 10 amino acids of the T7 gene 10 protein, an Arg residue, and amino acids 8–260 of Pep12p was constructed by insertion of a fragment from one of the nested deletions into pET3B after cleavage with *Bam*HI and Klenow treatment. The inserted fragment carried one end produced by *Eco*RI cleavage and Klenow treatment; the second end was produced by *Kpn*I cleavage and mung bean nuclease treatment. The fusion protein was recovered in the insoluble pellet after production in BL21(DE3)/pLysS. Antiserum B was created as follows: the *Hin*dIII/*Bgl*II fragment from pKB718 was ligated into *Hin*dIII (blunted)/*Bam*HI sites of pATH3 to generate a fusion protein consisting of 332 residues of *trp*E and 159 residues (74–233) of Pep12p. The fusion protein was produced in JM101 and purified for immunization of rabbits as previously described (Horazdovsky and Emr, 1993). The preparation of antisera to the following vacuolar hydrolases has been previously described: PrB (Moehle *et al.*, 1989), CpY, and PrA (Klionsky *et al.*, 1988), and alkaline phosphatase (AlP) (Klionsky and Emr, 1989).

Electrophoresis and Immunoblotting

SDS-PAGE of proteins was performed on 7.5% or 10% polyacrylamide gels according to the method of Laemmli (1970). Immunoblots were made as described by Burnette (1981) and immunocomplexes were visualized via avidin-biotin-linked peroxidase activity using a Vectastain kit according to the manufacturer's instructions or by use of goat anti-rabbit horseradish peroxidase conjugate activity.

Cell and Spheroplast Labeling and Immunoprecipitation

Cells were grown in appropriately supplemented YNB medium to mid-logarithmic phase (OD_{600} of 0.6–1.0). Cells were either labeled or converted to spheroplasts as previously described (Vida *et al.*, 1990).

To examine the *PEP12* gene product, cells were collected by centrifugation, resuspended in YNB medium (5 OD_{600}/ml), preincubated for 5 min at 30°C, and pulse labeled for 15 min with Tran^{35}S (20 μCi/OD_{600}) at 30°C. A chase period of 0 or 50 min was initiated by addition of methionine, cysteine, yeast extract, and glucose (final concentrations of 5 mM, 1 mM, 0.2%, and 4%, respectively). The chase was terminated by the addition of trichloroacetic acid (TCA) to a final concentration of 8%. Pep12p was analyzed by immunoprecipitation, SDS-PAGE, and fluorography (Klionsky *et al.*, 1988; Robinson *et al.*, 1988; Horazdovsky and Emr, 1993).

For determining the sorting and processing of vacuolar hydrolases, spheroplasts were resuspended in YNB/1 M sorbitol medium containing 1 mg/ml bovine serum albumin and 100 μg/ml α_2-macroglobulin, preincubated for 5 min at 30°C, pulse labeled at 30°C with Tran^{35}S (20 μCi/OD_{600}) for 10 min, and chased as above for either 0 or 30 min. The cultures were then centrifuged after the appropriate chase time for 1 min at 13,000 × *g* to generate intracellular (I) spheroplast and extracellular (E) medium fractions. TCA was added to each fraction to a final concentration of 8%. The presence and size of the vacuolar hydrolases CpY, PrA, PrB, and AlP in each fraction were determined by immunoprecipitation, SDS-PAGE, and fluorography as above.

Differential and Sucrose Gradient Centrifugation

Spheroplasts were radiolabeled as above, except that they were labeled with Tran^{35}S (30 μCi/OD_{600}) for 30 min and chased for 60 min at 30°C. The labeled spheroplasts were resuspended in an ice-cold hypoosmotic lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM sorbitol, 1 mM EDTA, and protease inhibitors: 5 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 50 μg/ml α_2-macroglobulin, and 100 μg/ml phenylmethylsulfonyl fluoride). The resulting suspension was dounce homogenized in an ice-cold tissue homogenizer 5–10 times and then subjected to sequential centrifugation at 500 × *g* (5 min), 13,000 × *g* (15 min), and 100,000 × *g* (60 min). The levels of Pep12p, AlP (vacuole), glucose-6-phosphate dehydrogenase (G6PDH-cytosol), and Kex2p (late Golgi) in each fraction were determined by immunoprecipitation, SDS-PAGE, and fluorography as above.

All sucrose solutions were made wt/wt (%) with ultra-pure sucrose (J.T. Baker) in 10 mM HEPES-KOH, pH 7.6. The gradient was generated using the following steps: 0.5 ml 60%, 1.0 ml 37%, 1.5 ml

34%, 2.0 ml 32%, 2.0 ml 29%, 1.0 ml 27%, and 1.5 ml 22%. Thirty OD_{600} units of labeled spheroplasts were resuspended in 2.0 ml ice-cold lysis buffer. The suspension was homogenized as above and centrifuged at 500 × *g* for 5 min to remove unbroken cells. The resulting crude lysate was subjected to centrifugation at 13,000 × *g* for 15 min to generate P13 (pellet) and S13 (supernatant) fractions. The S13 (1.9 ml) and the P13 (resuspended in 1.9 ml lysis buffer) were loaded on top of separate gradients, which were then subjected to centrifugation in a Beckman SW41 rotor at 170,000 × *g* for 17–18 h at 4°C. Fourteen fractions were collected from the top and the sucrose concentration was determined by refractive index. After adding 300 μg of carrier BSA to each fraction, the proteins were precipitated by adding TCA to 10%. The distribution of Pep12p, mAlP (vacuole), and Kex2p (late Golgi) was analyzed by immunoprecipitation, SDS-PAGE, and fluorography as above. All quantitation of proteins on gels was done with either a Molecular Dynamics Phosphorimager (Sunnyvale, CA) or an LKB Ultrascan XL densitometer.

Protease Accessibility

Spheroplasts were prepared as described by Deshaies and Schekman (1987) except that 11 U of Sigma Lyticase/OD_{600} were used, and gently lysed as described. After twofold dilution with lysis buffer and removal of debris by sedimentation at 700 × *g* for 4 min in the HB4 rotor, supernatants were centrifuged for 10 min at 12,000 × *g* in the Sorvall SS34 rotor at 4°C. One hundred micrograms of low speed pellet per time point was resuspended in lysis buffer and used for protease protection experiments as detailed by Rose *et al.* (1989).

Electron Microscopy

Cells were grown at 30°C to an OD_{600} of 0.5 in YPD medium, harvested by gentle centrifugation, and fixed for 2 h at 30°C in 3% glutaraldehyde, 5 mM $CaCl_2$, 0.1 M cacodylate, pH 6.8. Spheroplasting, staining, and dehydration were performed as described previously (Banta *et al.*, 1988), except that spheroplasts were suspended in 2% ultra-low gelling temperature agarose (Sigma, type IX) before staining. Cells were embedded in low viscosity Spurr plastic resin for 48 h as described by Horazdovsky *et al.* (1994). After sections were stained with Reynold's lead citrate for 2 min and 2% uranyl acetate for 10 min at 25°C, they were viewed at 80 Kv using a JOEL 12 EX transmission electron microscope.

RESULTS

Cloning of the PEP12 Gene

Strain BJ4227 (*MATα trp1 leu2 ura3–52 pep12–2*) was transformed with DNA from each of eight different YCp50 yeast genomic banks (Rose *et al.*, 1987) by the $CaCl_2$ method (Bruschi *et al.*, 1987). Approximately 20,000 Ura^+ transformants were replica plated to YEPD plates and tested for the Pep^+ phenotype after 3 days of growth at 30°C. For the two Pep^+ transformants recovered (both containing identical inserts from the same bank), the loss of the plasmid resulted in reversion to the Ura^- Pep^- phenotype, indicating that the Ura^+ Pep^+ phenotype of the transformed cells was due to the presence of the plasmid.

Restriction analysis indicated that the two complementing plasmids, pKB56 and pKB58, contained identical 12-kb inserts. Subclones of the original 12-kb fragment from pKB58 were constructed in one of two yeast/*Escherichia coli* sequencing vectors, pKB711 or pKB711X, and tested for retention of the ability to complement. The smallest complementing fragment was found to be a 2.6-kb *Xho*I/*Sph*I fragment (Figure 1A).

To determine whether the gene cloned was the *PEP12* gene, homologous recombination was used to

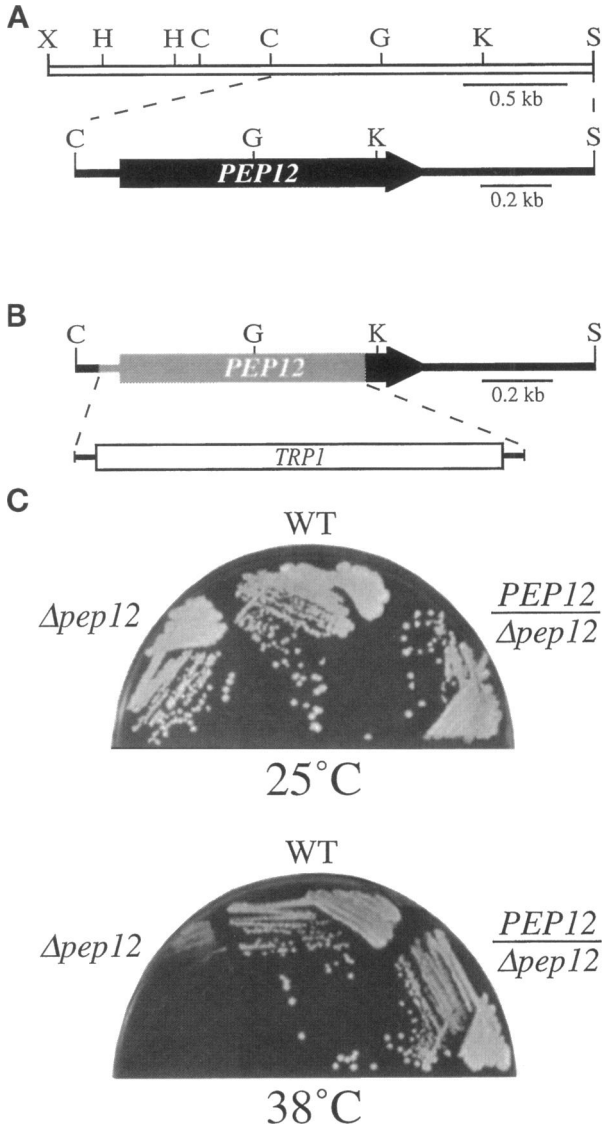

Figure 1. Characterization and disruption of the *PEP12* locus. (A) The smallest subclone that complements the *pep12–2* mutation. The 2.6-kb *Xho*I/*Sph*I fragment contains an open reading frame corresponding to *PEP12*, indicated by an open arrow. The region from nucleotide −91 (ATG is the reference point) to 650 of the ORF was deleted and an *Eco*RI fragment bearing *TRP1* was inserted as indicated. The abbreviations are as follows: B, *Bam*HI; H, *Hin*dIII; X, *Xho*I; C, *Cla*I; G, *Bgl*II; S, *Sph*I; and R, *Eco*RI. (B) Analysis of the *ts* growth defect of the Δ*pep12* strain. Growth phenotypes of BJ2977 (WT), BJ7666 (Δ*pep12*), and BJ7666 carrying the complementing *CEN* plasmid pkb7125 (Δ*pep12/PEP12*) are shown following 3 days of incubation on YPD plates at either 25 or 38°C.

target integration into the chromosomal locus from which the insert originated. The 4.6-kb *Bam*HI/*Sph*I restriction fragment from pKB713 was cloned into YIp5 (Δ*Eco*RI), giving pKB714. The plasmid was linearized at the unique *Xho*I site within the insert and transformed into BJ4227 (*MATα pep12–2 trp1 leu2 ura3–52*). One of the 40 integrants obtained, BJ4369, was mated to two strains: one of *pep12–2* genotype (BJ4037, *MAT***a** *pep12–2 ura3–52 leu2 his1*; cross 1) and the other of *PEP12* genotype (BJ4279, *MAT***a** *PEP12 leu2 his1*; cross 2). After sporulation and dissection, all 52 tetrads from the first cross segregated 2 Ura^+Pep^+: 2 Ura^-Pep^-, confirming that the complementing sequence and *URA3* were tightly linked and integrated. The results of cross 2, in which 26 of 28 tetrads showed 4 Pep^+: 0 Pep^- segregation, indicated that the site of integration was closely linked to the *PEP12* gene and that we had, therefore, cloned the *PEP12* gene.

To facilitate sequencing of the gene, nested sets of unidirectional exonuclease III deletions were constructed in both orientations using the 2.6-kb *Xho*I/*Sph*I fragment as the starting point for deletions. The exonuclease III sets were sequenced using the dideoxy-chain termination (Sanger *et al.*, 1977) and chemical (Maxam and Gilbert, 1980) methods. Computer-aided translation of the 864-nucleotide open reading frame (Figure 1) gave a potential gene product of 288 amino acids, corresponding to a gene product with relative molecular mass of 32,153 daltons (Figure 2A). The amino acid sequence contains three potential N-linked glycosylation sites. Hydropathy analysis by the method of Kyte and Doolittle (1982) revealed an 18-amino acid highly hydrophobic carboxyl-terminus flanked by positively charged residues (Figure 2B). Pep12p is thus predicted to be a cytoplasmically oriented, integral membrane protein.

The region between amino acids 214 and 240 of Pep12p contains a heptad repeat that is predicted to adopt an α-helical coiled-coil conformation (Figure 2C). The probability that regions of Pep12p would form coiled-coils was calculated using the COILS 2.1 program (Lupas *et al.*, 1991), which is based on a protocol proposed by Parry (1982) to predict the formation of α-helical coiled-coils (McLachlan and Stewart, 1975; McLachlan and Karn, 1982; Cohen and Parry, 1986). This putative coiled-coil domain in Pep12p was found in the more conserved region of the syntaxin family (Figure 3). Two amino-terminal regions of Pep12p may also form coiled-coils, although the calculated probability is lower (Figure 2C).

Since we entered the *PEP12* sequence in the data base, several similar proteins have been identified. This protein family, first recognized by Hardwick and Pelham (1992), is the syntaxin family. Proteins in this family have been implicated in vesicular docking and fusion events at various points within the secretory pathway, such as the docking and fusion of synaptic

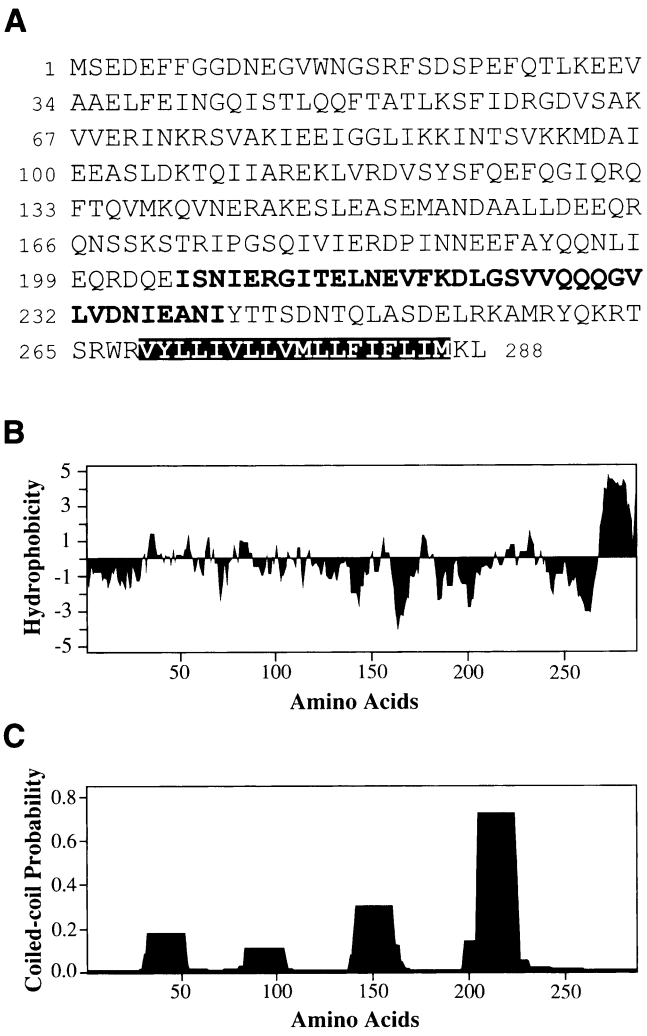

Figure 2. Predicted sequence and structure of the *PEP12* gene product. (A) The predicted amino acid sequence of the 288 residue polypeptide encoded by *PEP12* (GenBank M90395). In this corrected sequence, A replaces G in residue 13 (the corresponding codon change is GGG → GCC). The boxed hydrophobic residues of Pep12p are predicted to form a transmembrane domain. The residues in bold lettering are the region of Pep12p that is predicted to adopt an α-helical coiled-coil confirmation. (B) Hydropathy plot of the predicted Pep12p by the method of Kyte and Doolittle (1982). (C) Coiled-coil formation probability generated using the COILS-2.1 program (MTIK matrix, a and d positions weighted, 21-residue window).

vesicles with the pre-synaptic membrane (for reviews see Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994; Rothman, 1994; Table 2). Pep12p shows the greatest similarity to rat syntaxin 2c (2′) with 25% identity and 49% similarity (Bennett *et al.*, 1993), and an *Arabidopsis* syntaxin homologue (29% identity/44% similarity) (Bassham *et al.*, 1995). The 288-amino acid size of Pep12p is typical of the shorter syntaxins (unlike Sed5p with its 340 residues). The more highly conserved region of Pep12p (amino acids 121–268) is

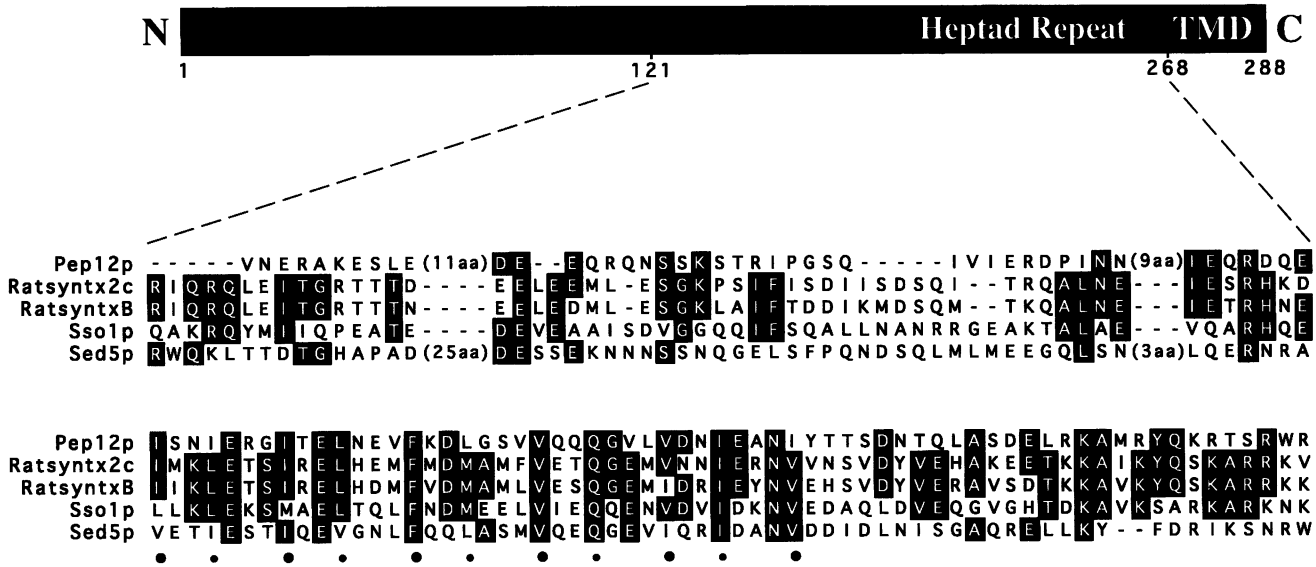

Figure 3. Pep12p belongs to the syntaxin family of proteins. A segment of Pep12p (amino acids 121–268) is aligned and compared with several members of the syntaxin protein family. Members shown are rat syntaxin 2c (2′) (Bennett *et al.*, 1993), rat syntaxin B (Bennett *et al.*, 1993), and two yeast members of the family, Sso1p (Aalto *et al.*, 1993) and Sed5p (Hardwick and Pelham, 1992). The dark boxes indicate residues that are identical in at least three of these proteins. The dots indicate the well-conserved residues at the a and d positions of the predicted coiled-coil domain.

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Phenotypic Consequences of Deletion of the PEP12 Gene

The *PEP12* gene was disrupted and most of it deleted by combining two members of the exonuclease III

Table 2. Yeast homologues of proteins that participate in docking and fusion events in the secretory pathway and its branches

Transport step	Vamp/synaptobrevin (v-SNAREs)	Syntaxin (t-SNAREs)	SNAP-25	Sec1p	NSE	αSNAP	rab
ER→Golgi	Bos1p (Bet1p, Sec22p) Sft1p (intra-Golgi)	Sed5p	?	Sly1p	Sec18p	Sec17p	Ypt1p
Golgi→PM	Snc1p, Snc2p	Sso1p, Sso2p	Sec9p	Sec1p	Sec18p	Sec17p	Sec4p
Golgi→endosome	?	Pep12p	?	Vps45p	Sec18p	?	Vps21p?
endosome→vacuole	?	?	?	Vps33p? (Pep14p, Slp1p)	Sec18p-homologue?	?	Ypt7p?

Bos1p (Shim *et al.*, 1991), Bet1p (Newman *et al.*, 1990), Sec22p (Lian and Ferro-Novick, 1993), Sed5p (Hardwick and Pelham, 1992; Banfield *et al.*, 1994), Sly1p (Ossig *et al.*, 1991) Sec18p (Eakle *et al.*, 1988; Graham and Emr, 1991), Sec17p (Griff *et al.*, 1992), Ypt1p (Becker *et al.*, 1991), Snc1p, Snc2p (Protopopov *et al.*, 1993), Sso1p, SSo2p (Aalto *et al.*, 1993; Jantti *et al.*, 1994), Sec9p (Brennwald *et al.*, 1994), Sec1p (Aalto *et al.*, 1991; Aalto *et al.*, 1992), Sec4p (Goud *et al.*, 1988; Kabcenell *et al.*, 1990), Slp1p/Vps33p/Pep14p (Jones, 1977; Banta *et al.*, 1990; Wada *et al.*, 1990), Vps45p (Cowles *et al.*, 1994), Vps21p (Horazdovsky *et al.*, 1994; Singer-Kruger *et al.*, 1994), Ypt7p (Wichmann *et al.*, 1992), and Sft1p, (Banfield *et al.*, 1995).

sequencing set and inserting a 1.4-kb fragment containing the *TRP1* gene into the internal *Eco*RI site generated at the join (Figure 1B). The deletion removed 91 bp upstream of the ATG and 649 of 864 bp of the ORF. The fragment containing the yeast sequences was then recombined into the chromosome at the *PEP12* locus of BJ4842 (*MAT***a** *trp1 PEP12 ura3–52 leu2/MATα trp1 PEP12 URA3 LEU2*), a diploid recipient strain that is homozygous for *PEP12* and *trp1*. All tetrads obtained from sporulation of one stable Trp^+ transformant gave segregations of 2 Trp^+Pep^-: 2 Trp^-Pep^+, indicating that the *PEP12* gene is not essential but that its absence results in the Pep^- (CpY deficient) phenotype as expected. The Pep^- spore clones showed no obvious growth defect at 25°C or 30°C when glucose was the carbon source, although the cell density at stationary phase was only about 50% of wild type. At 38°C, however, very little growth of the deletion/disruptant was seen; the plasmid-borne *PEP12* gene complemented the growth defect (Figure 1C). Growth on carbon sources like glycerol was impaired even at 25°C and 30°C and diploids homozygous for *pep12* mutations were unable to sporulate (our unpublished observations).

To assess the effects of Pep12p deficiency on vacuolar protein sorting, spheroplasts of wild-type and Δ*pep12::TRP1* strains were examined for their ability to process and target hydrolase precursors to the vacuole. Wild-type cells completely processed the CpY precursor to mature CpY during the 10-min pulse and 30-min chase, indicating that CpY was properly delivered to the vacuole (Figure 4A, lanes 1 and 2). In contrast, in the Δ*pep12* strain most of CpY remained in its Golgi-modified precursor form, proCpY (p2CpY). Moreover, most of the precursor was recovered in the extracellular, secreted fraction (Figure 4A, lanes 3 and 4). The fact that most of the proCpY was secreted during the 30-min chase implies that the secretory pathway is intact in the mutant. The *PEP12* gene, borne on YCp50, was able to complement the sorting and processing defects (Figure 4A, lanes 5 and 6).

The Δ*pep12* mutant was also defective in the maturation of two additional lumenal hydrolases, PrA and PrB. For both enzymes, wild-type cells processed the PrA and PrB precursors to their mature forms during the 10-min pulse and 30-min chase (Figure 4B, lanes 1 and 2). In the Δ*pep12* mutant, most of PrA and PrB were recovered in their Golgi-modified precursor forms: proPrA (p2PrA) and proPrB (p3PrB) (Figure 4B, lanes 3 and 4). In contrast to what was seen for CpY, however, the Golgi-modified PrA and PrB precursors primarily remained within the cell; they were recovered in the intracellular (I) spheroplast fraction rather than in the extracellular (E) medium fraction (Figure 4B, lanes 3 and 4). Our unpublished observations indicated that extending the chase time to 60 min did not increase the level of secretion or maturation of

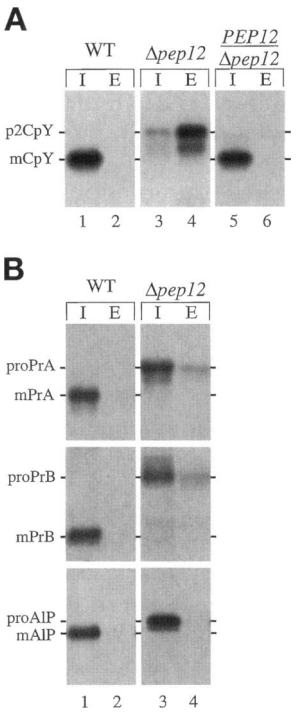

Figure 4. Intracellular sorting of vacuolar hydrolases. (A) Spheroplasts of BJ2977 (WT), BJ7666 (Δ*pep12*), and BJ7666 carrying the complementing plasmid pkb7125 (*PEP12*/Δ*pep12*) were pulse-labeled for 10 min with Tran^{35}S and chased for 30 min at 30°C. The labeled cultures were separated into spheroplast (internal, I) and medium (external, E) fractions. The abundance and sizes of CpY in these fractions were determined by immunoprecipitation, SDS-PAGE, and fluorography. The positions of proCpY (P2CpY) and mature CpY are indicated. (B) Spheroplasts of BJ2977 (WT) and BJ7666 (Δ*pep12*) were pulse-labeled for 10 min with Tran^{35}S and chased for 30 min at 30°C. The labeled cultures were separated into spheroplast (internal, I) and medium (external, E) fractions. The abundance and sizes of PrA, PrB, and AlP in these fractions were determined by immunoprecipitation, SDS-PAGE, and fluorography. The molecular weights of the Golgi-modified precursors [proPrA (P2PrA), proPrB (P3PrB), and proAlP] and mature vacuolar forms (mPrA, mPrB, and mAlP) are indicated.

PrA and PrB. Moreover, even very long term incubation did not allow maturation of the PrA and PrB precursors, even though they remain within the cell; under steady state conditions the *pep12* mutant is defective in PrA and PrB maturation and enzymatic activity (our unpublished results). This loss of PrA and PrB activity in *pep12* cells is sufficient to account for the sporulation defect of the mutant (Zubenko and Jones, 1981; Teichert *et al.*, 1989). In summary, Δ*pep12* cells are defective in delivery of soluble vacuolar hydrolases to the vacuole.

The type II vacuolar membrane protein, AlP, underwent little or no processing during the time course presented here (Figure 4B). Unpublished observations indicate that an extended chase of 60 min did not change the amount of mAlP observed. The precursor must be slowly processed, however, for activity levels for the enzyme are similar between *pep12* and wild-type strains (Becherer, 1991). Furthermore, indirect immunofluorescence experiments indicated that the majority of AlP is properly localized to the vacuole in *pep12* cells (unpublished observations). DPAPB, another type II vacuolar integral membrane protein, was also present at normal specific activities (1.69 units for *pep12* parent; 1.85 for *PEP12* parent; mean and range for four *pep12* segregants, 1.69, 1.30–2.46; mean and range for four *PEP12* segregants, 1.85, 1.40–2.46). These results indicate that vacuolar membrane proteins are transported to the vacuole in *pep12* cells;

however, the processing is probably slowed by the low PrA and PrB levels in *pep12* vacuoles. Thus Pep12p is required for the proper localization of soluble vacuolar hydrolases, but not for the transport of integral-membrane proteins to the vacuole.

α-Mannosidase levels were also normal (our unpublished observations). Because this peripheral membrane protein reaches the vacuolar lumen by an extracellular secretory pathway (Yoshihisa *et al.*, 1989; 1990), we infer that this pathway is intact and does not utilize Pep12p.

Because vacuolar hydrolases traverse the same pathway as the secreted proteins from the endoplasmic reticulum to the trans-Golgi network (Stevens *et al.*, 1982), the transport of secreted proteins was examined in *pep12* and wild-type cells. The extracellular activities of two secreted enzymes, invertase and acid phosphatase, were analyzed in segregants from a diploid heterozygous for the disruption allele. The mean and range of specific activities for acid phosphatase for four *pep12* segregants (3.2, 2.1–4.8) and four *PEP12* segregants (2.9, 2.3–3.3) were very similar as were invertase activities for segregants of both genotypes. The secretion of proCpY by *pep12* mutant cells (Figure 4, lanes 3 and 4) also suggests that secretion is not greatly impaired by the loss of Pep12p. Furthermore, the secretion of multiple secreted proteins was examined in Δ*pep12* and wild-type cells. In unpublished experiments, cells were pulsed with Tran^{35}S at 30°C for 20 min. After a 10- or 40-min chase period, the cells were removed by centrifugation and the secreted proteins in the medium were precipitated with 10% TCA and analyzed by SDS-PAGE (Robinson *et al.*, 1988). The rate of protein secretion in Δ*pep12* and wild-type cells was very similar. Taken together these results suggest that the secretory pathway is functioning normally in the Δ*pep12* mutant.

To assess the morphology of cells lacking the *PEP12* gene product, wild-type and Δ*pep12* mutant cells were examined using electron microscopy (Figure 5). Typically, the vacuole in Δ*pep12* mutant cells was larger and less reticulated than the wild-type vacuole (Figure 5, compare panels A, *PEP12*, and B, Δ*pep12*). Small 40- to 50-nm vesicles were present in larger numbers in the cytoplasm of Δ*pep12* mutant cells compared with wild-type cells (Figure 5, compare panel B, Δ*pep12*, with panel A, *PEP12*). Figure 5C shows an enlargement of the Δ*pep12* vesicles found in the boxed region of Figure 5B.

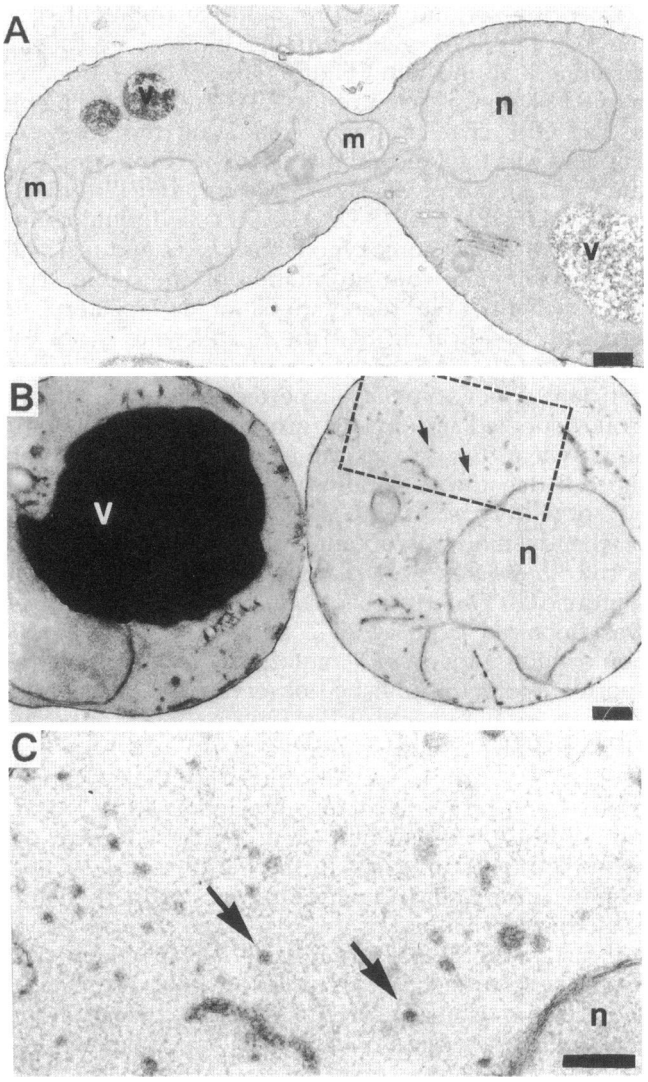

Figure 5. Morphology of the Δ*pep12* mutant strain. Cells were grown at 30°C and prepared for electron microscopy as follows: (A) BJ2977 (*PEP12*); (B) and (C) BJ7666 (Δ*pep12*). The dashed box region in panel B is enlarged in panel C to show the accumulated 40- to 50-nm membrane vesicles. The arrows indicate vesicles seen in Δ*pep12* cells (B and C). N identifies the nucleus; V, the vacuoles; and M, the mitochondria. The bars represent 0.35 μm in the upper two panels (A and B), and 0.25 μm in panel C.

Properties and Intracellular Location of Pep12p

Polyclonal antibodies raised against Pep12p recognized a protein of about 35 kDa (Figure 6, lanes 2–5). The protein was absent in the Δ*pep12* mutant (Figure 6, lane 1) and was present in greater abundance (about 50-fold) when the strain carried *PEP12* on a high copy plasmid (Figure 6, lanes 4 and 5). The Pep12p was stable during a 50-min chase even when overexpressed (Figure 6, lanes 2–5).

Upon subcellular fraction, Pep12p was found in the membrane fraction. Pep12p was extracted from pelleted membranes by Triton X-100, but not by 100 mM Na_2CO_3, pH 11.5 (Becherer, 1991), indicating that Pep12p is an integral membrane protein (Fujiki *et al.*, 1982). To determine whether Pep12p is oriented toward the cytosol like other syntaxins (Bennett *et al.*, 1992, 1993), intact membrane vesicles were subjected

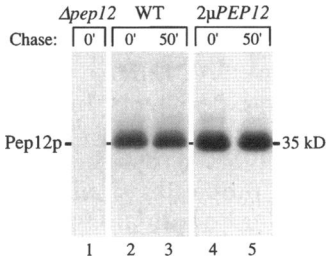

Figure 6. Immunoprecipitation of the *PEP12* gene product. BJ7666 (Δ*pep12*, lane 1), wild-type BJ2977 cells (WT, lanes 2 and 3), and BJ2977 carrying the 2 μ expression plasmid pKB718 (2 μ *PEP12*; lanes 4 and 5) were labeled for 10 min with Tran^{35}S at 30°C. Labeling was terminated after a 0-min (lanes 1, 2, and 4) or a 50-min chase period at 30°C (lanes 3 and 5) by addition of TCA to 8%. Pep12p was analyzed by immunoprecipitation using Pep12p-specific antiserum B, SDS-PAGE, and fluorography. The size of Pep12p was determined by comparison to molecular weight standards. Fifty percent less material was loaded in lanes 4 and 5 than in lanes 1–3; additionally, the exposure time for lanes 4 and 5 was about one-fourth as long as that for lanes 1–3.

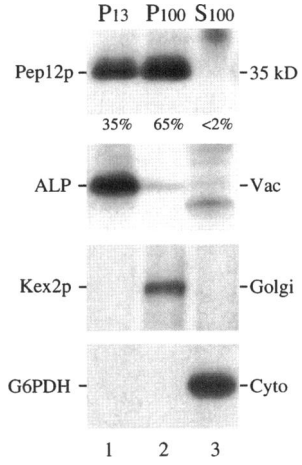

Figure 8. Subcellular fractionation of the *PEP12* gene product. Wild-type SEY6210 cells were converted to spheroplasts, labeled with Tran^{35}S-label for 30 min, and chased for 60 min. The spheroplasts were lysed and fractionated by differential centrifugation. First, the lysate was subjected to centrifugation at 13,000 × *g*, yielding a pellet (P13, lane 1) and a supernatant (S13) fraction. The S13 fraction was then subjected to a second centrifugation at 100,000 × *g*, resulting in a pellet (P100, lane 2) and a supernatant fraction (S100, lane 3). Pep12p was quantitatively immunoprecipitated from each fraction, as were the following organelle marker proteins: Kex2p, late Golgi (Redding *et al.*, 1991); mAlP, vacuole (Klionsky and Emr, 1989); and glucose 6-phosphate dehydrogenase (G6PDH), cytoplasm.

to protease treatment. Immunoblot analysis indicated that Kar2p, a resident of the lumen of the endoplasmic reticulum (Rose *et al.*, 1989), was resistant to proteinase K treatment unless Triton X-100 was present during the incubation, indicating that the vesicles were sealed and in the proper orientation (Figure 7). Pep12p was sensitive to proteinase K whether or not Triton X-100 was present, indicating that Pep12p is oriented cytoplasmically. The absence of N-linked glycosyl side-chains on the protein (our unpublished observations), despite the presence of acceptor sequences at residues 16, 90, and 167, is in accord with a cytoplasmic orientation. Taken together, these data suggest that Pep12p is a cytoplasmically oriented, integral membrane protein.

To determine the intracellular location of Pep12p, wild-type spheroplasts were labeled with Trans^{35}S for 30 min and chased for 60 min. Lysates were fractionated by centrifugation into a 13,000 × *g* pellet (P13), a 100,000 × *g* pellet (P100), and a 100,000 × *g* supernatant solution (S100). Each fraction was examined for the presence of Pep12p and the marker enzymes mAlP (vacuole membrane), Kex2p (late Golgi membrane), and glucose 6-phosphate dehydrogenase (G6PDH-cytosol) by immunoprecipitation and SDS-PAGE (Figure 8). Virtually all (95%) of the mAlP was recovered in the P13 fraction, which primarily contains the vacuolar membrane, as well as plasma membrane, endoplasmic reticulum, mitochondria, and nuclei. Nearly all of Kex2p (95%) was recovered in the P100 fraction, which contains Golgi membranes and transport vesicles. Endosomal membranes seem to fractionate in both the P13 and the P100 (Vida *et al.*, 1993). Neither pellet fraction was contaminated with the cytosolic G6PDH, indicating that little whole cell contamination or membrane aggregation occurred.

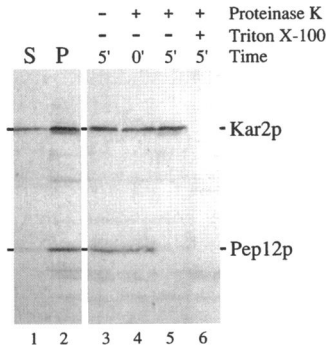

Figure 7. Protease susceptibility of Pep12p. Spheroplasts of BJ2948, a *PEP12* strain, were lysed to produce intact vesicles. The lysate was centrifuged at 12,000 × *g*. Immunoblots were prepared using antisera to Pep12p and Kar2p. Lanes 1 and 2 contain the supernatant and pellet fractions, respectively. Lanes 3–6 contain pellet fractions treated as indicated.

Essentially all of the Pep12p was found distributed between the P13 and P100 fractions. In the experiment shown in Figure 8, 35% of Pep12p was found in the P13 fraction, while the remaining 65% was located in the P100. The average over several experiments was 40% of Pep12p in the P13 fraction and 60% in the P100 fraction. One possible interpretation of the behavior of Pep12p during fractionation is that it resides in a compartment with properties intermediate to the late Golgi and vacuole; the endosome is a candidate for such a compartment. An alternative hypothesis is that Pep12p resides in two different membrane types, such as transport vesicles and endosomal or vacuolar membranes. The endoplasmic reticulum and plasma membrane, which pellet at 13,000 × *g*, are unlikely candidates because Δ*pep12* cells do not exhibit any defect in protein secretion.

To gain additional information about the compartmental location of Pep12p, P13 and S13 fractions were each subjected to equilibrium sedimentation in sucrose gradients (Figure 9). The S13 fraction was sedimented as a source of the Pep12p-containing membranes that would pellet at 100,000 × *g* (Figure 8). In the P13 fraction, both AlP and Pep12p were present in several fractions of the gradient. These Pep12p-con-

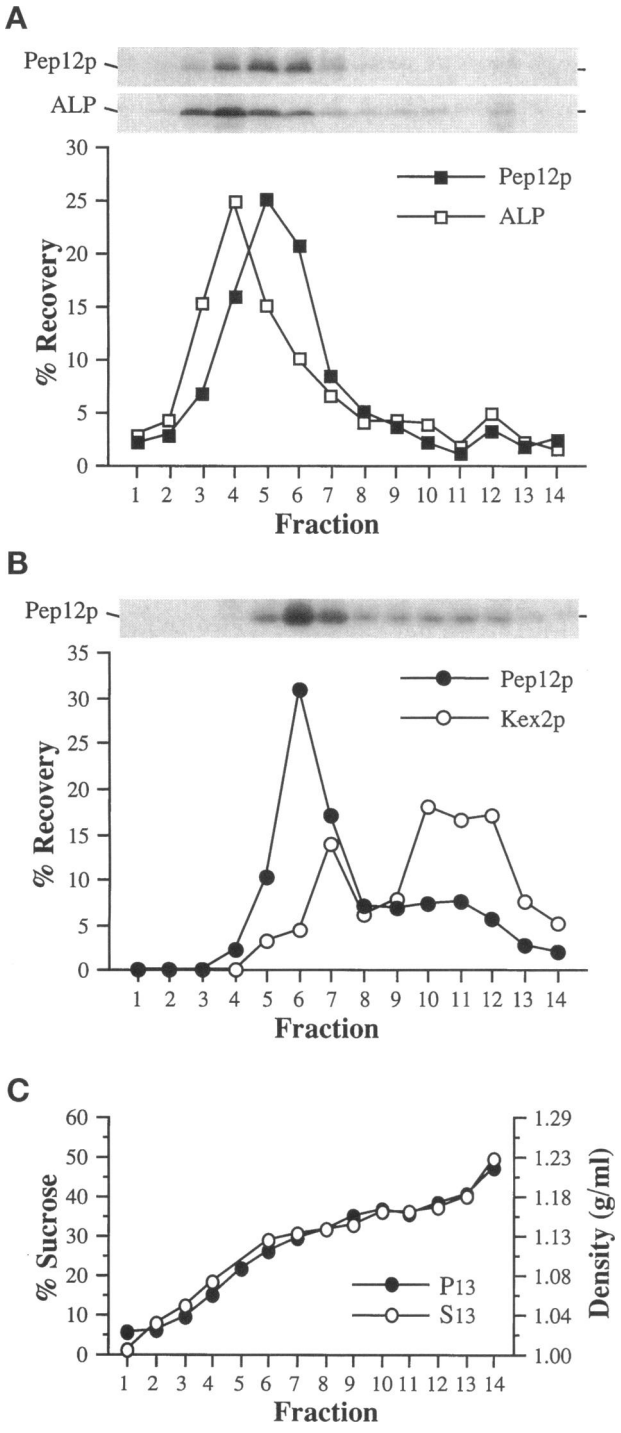

Figure 9. Sucrose gradient fractionation of Pep12p. Wild-type SEY6210 spheroplasts were labeled for 30 min with Tran^{35}S, chased for 60 min, and then lysed. The lysate was subjected to centrifugation at 13,000 × *g* to generate pellet (P13) and supernatant (S13) fractions, which were subsequently fractionated further by sucrose gradient centrifugation. (A) P13 membranes. Pep12p and the vacuolar membrane protein mAlP were immunoprecipitated simultaneously from gradient fractions and resolved by SDS-PAGE. (B) S13 ("P100 membranes"). Pep12p and the late Golgi membrane protein Kex2p were immunoprecipitated simultaneously from gradient fractions and resolved by SDS-PAGE. (C) Sucrose concentration (%) and the corresponding density (g/ml) across the S13 and P13 gradients as determined by refractive index of collected fractions.

taining membranes had a higher density on average than did the vacuolar membranes, marked by mAlP (Figure 9A). Similar results were obtained in several other experiments, including one in which the membranes were loaded at the bottom of the gradient. One possibility is that Pep12p resides in a compartment with properties similar but not identical to vacuoles. The best candidate for such a compartment would be the endosome. The endoplasmic reticulum and plasma membrane are unlikely candidates because Pep12p is not directly involved in secretion; transport vesicles and Golgi membranes are unlikely candidates because they are not significant components of P13 fractions. It is also possible that the apparent single Pep12p peak actually consists of two different membrane components, such as the vacuole and a more dense component that shifts the overall Pep12p distribution toward the denser end of the gradient. Again the best candidate for such a nonvacuolar component is the endosome.

The Pep12p contained within the S13 fraction distributed into two peaks. The major Pep12p peak was found at a density similar to that of the Pep12p peak in the P13 fraction. A minor (<15%) component was distributed in the dense region of the gradient, where the bulk of the the late Golgi protein Kex2p fractionates (Figure 9B). Although Kex2p appears to reside primarily in the late Golgi (Bryant and Boyd, 1993; Whitters *et al.*, 1994), it is thought to cycle between this late Golgi compartment and an endosome-like compartment (Wilcox *et al.*, 1992). Thus the Kex2p found in fractions 5–7 may represent Kex2p in transport vesicles or in endosomal membranes. It is not known how transport vesicles trafficking between the late Golgi and the vacuole would fractionate under these conditions.

Together, the portion of Pep12p found in the lighter membranes of the P13 and the S13 fractions accounts for about 90% of the Pep12p present in the cell (35% plus 55%, respectively). The remaining 10% of Pep12p fractionates with the more dense late Golgi membranes.

DISCUSSION

PEP12/VPS6 encodes a syntaxin homologue that is essential for the transport of lumenal, but not membrane-bound, vacuolar hydrolases from the late Golgi to the vacuole. The fractionation and protease sensitivity characteristics of Pep12p indicate that Pep12p is

a cytoplasmically oriented, integral membrane protein like other syntaxins (Bennett *et al.*, 1992, 1993). Electron microscopy revealed that Δ*pep12* cells accumulate 40- to 50-nm vesicles. Subcellular fractionation data indicated that the majority of Pep12p appeared to associate with a membrane compartment distinct from the vacuole and the late Golgi. The membranes containing Pep12p fractionated at a density very similar to that of the endosome-like prevacuolar compartment previously demonstrated to contain proCpY and α-factor (Singer-Krüger *et al.*, 1993; Vida *et al.*, 1993). Taken together, our data are most consistent with Pep12p functioning as a t-SNARE on the endosome for docking Golgi-derived vesicles containing the precursors to lumenal vacuolar hydrolases.

Deletion/disruption of the *PEP12* gene resulted in missorting of precursors to several soluble vacuolar hydrolases. However, the transport of integral membrane vacuolar proteins and secretory proteins appears to be normal in *pep12* mutant cells. *pep12* mutations greatly retarded the rate of processing of the vacuolar membrane protein AlP (Figure 4; Becherer, 1991), which is most likely due to the low levels of the soluble proteases PrA and PrB needed to catalyze activation of AlP in the vacuole (Jones *et al.*, 1982; Zubenko *et al.*, 1982). Several other vacuolar protein sorting mutants, such as *vps15* and *vps34*, also have a selective defect in the transport of soluble vacuolar hydrolases, suggesting that AlP may reach the vacuole by a different mechanism than the soluble hydrolases (Klionsky and Emr, 1990; Herman *et al.*, 1991; Stack *et al.*, 1995).

It is likely that Pep12p functions like previously studied syntaxins. The presented data on Pep12p do not bear on the mechanism of vesicle docking or fusion; rather they broaden the cellular context in which the syntaxin proteins function. In the current models, syntaxins play a key role in the docking and fusion of transport vesicles. The specificity required to target transport vesicles from a donor organelle to a particular acceptor organelle is postulated to reside in a set of membrane proteins called SNAREs: for each v-SNARE on a donor vesicle there is a corresponding t-SNARE on the target organelle (for reviews see Ferro-Novick and Jahn, 1994; Rothman, 1994; Rothman and Warren, 1994; Südhof, 1995). Syntaxin 1, SNAP-25, and their homologues are t-SNAREs that appear to function together as receptors for docking of vesicles to the proper target membranes. For example, rat syntaxin 1A plays a key role in the docking and fusion of synaptic vesicles at the nerve terminal (Bennett *et al.*, 1992, 1993). However, syntaxin 1 is localized to both synaptic and nonsynaptic regions of neuronal membranes, suggesting that an additional mechanism is needed to define where fusion should occur, and that syntaxins may have another function in addition to their proposed t-SNARE role (Bennett *et al.*, 1992, 1993; Söllner *et al.*, 1993b; Garcia *et al.*, 1995).

Yeast has at least three distinct proteins with similarity to syntaxins: Sed5p, located in the Golgi and required for endoplasmic reticulum to Golgi transport (Banfield *et al.*, 1994); Sso1p and Sso2p, functionally redundant proteins located in the plasma membrane and required for docking of secretory vesicles with the plasma membrane (Aalto *et al.*, 1993; Jäntti *et al.*, 1994); and Pep12p, required for transport between the Golgi and the vacuole. Pep12p is the first member of the syntaxin family to function in Golgi to vacuole/lysosome transport; all other known syntaxins mediate either transport between the endoplasmic reticulum and the Golgi or the docking/exocytosis of secretory or synaptic vesicles. In Table 2 are presented the yeast homologues, where known, for proteins that participate in docking and fusion of transport vesicles within the secretory pathway and its branches (for review see Rothman, 1994).

Transport from the late Golgi to the vacuole in yeast proceeds via an intermediate endosome-like compartment (Vida *et al.*, 1993). The Golgi to endosome transport step appears to be mediated by clathrin-coated transport vesicles (Seeger and Payne, 1992). The endosome to vacuole step may also be vesicle mediated; alternatively, the endosome may mature and fuse with the vacuole. Pep12p is the first syntaxin homologue to be reported for the transport steps leading from the late Golgi to the vacuole. However, two nonredundant Sec1p homologues have been identified: Vps33p/Slp1p/Pep14p (Jones, 1977; Banta *et al.*, 1990; Wada *et al.*, 1990) and Vps45p (Cowles *et al.*, 1994; Piper *et al.*, 1994). Mutation of either gene alone causes vacuolar sorting and vacuolar morphology defects. Like *pep12* mutants, *vps45* mutants (class D) accumulate 40- to 50-nm vesicles and contain enlarged vacuoles (Cowles *et al.*, 1994; Piper *et al.*, 1994), while *vps33/slp1/pep14* mutants (class C) lack any identifiable vacuolar structure (Banta *et al.*, 1988, 1990). Genetic interactions have been detected between *VPS45* and *PEP12* (Burd, Cowles, and Emr, personal communication), indicating that the two gene products may participate in the same transport step. Furthermore, two nonredundant rab protein homologues, Ypt7p and Vps21p, have also been identified (Wichmann *et al.*, 1992; Horazdovsky *et al.*, 1994; Singer-Krüger *et al.*, 1994). The fact that two nonredundant Sec1 and rab protein homologues are required for vacuolar protein sorting suggests that two distinct transport steps are required between the late Golgi and the vacuole. The endosomal t-SNARE (possibly Pep12p) may mediate the docking of Golgi-derived vesicles at the endosome, while a second unidentified SNARE-like complex may be required for transport between the endosome and the vacuole. Loss of function of a t-SNARE would be in accord with the observed accumulation of 40- to 50-nm vesicles in

the cytoplasm of Δ*pep12* mutants (Figure 5), as would the loss of any other component required for vesicle docking or fusion. Sec18p appears to participate in the Golgi to endosome reaction, but not in the endosome to vacuole reaction (Graham and Emr, 1991). Thus there may be additional syntaxin and NSF homologues required for endosome-to-vacoule transport, or an alternative set of proteins may mediate this transport step.

It seems unlikely that the key site of Pep12p function is on the vacuole. The average density of the membranes containing Pep12p was greater than that of vacuolar membranes (Figure 9). The entire distribution of Pep12p was shifted to higher density relative to the vacuolar marker AlP in multiple experiments, including one in which the membranes were loaded at the bottom of the gradients instead of the top. It is the reproducible shift of the entire distribution of Pep12p relative to AlP that suggests that at least a portion of Pep12p is not in the vacuole. Furthermore, over 60% of Pep12p is found in membranes that pellet only at high speed (100,000 × *g*), while only 5% of vacuolar membranes are found in this fraction (Figure 8). Although we cannot rule out that a small pool of Pep12p is in vacuolar membranes, these data indicate that the majority of Pep12p (>75%) resides in a nonvacuolar compartment, suggesting that the primary site of action for Pep12p is unlikely to be the vacuole.

Our data are most consistent with Pep12p residing in endosomal membranes. The vast majority of Pep12p (90%) was found in a membrane fraction that had an average density somewhat greater than that of vacuoles (Figure 9) as expected for an endosome (Singer-Krüger *et al.*, 1993; Vida *et al.*, 1993). The remaining 10% of Pep12p fractionated with the more dense late Golgi membranes containing Kex2p. Although the majority of Kex2p was found in the denser region of the gradient, a minor pool of the Kex2p cofractionated with the less dense Pep12p peak (Figure 9B). Because Kex2p appears to cycle between the endosome and Golgi (Wilcox *et al.*, 1992), the Kex2p that colocalizes with the major Pep12p peak may represent the Kex2p found in transport vesicles or endosomal membranes.

Syntaxin 1, which mediates exocytosis, is primarily found throughout the plasma membrane (Bennett *et al.*, 1992, 1993; Söllner *et al.*, 1993a; Garcia *et al.*, 1995; Walch-Solimena *et al.*, 1995). However, smaller pools of syntaxin 1 have also been found in synaptic vesicles and clathrin-coated vesicles (Walch-Solimena *et al.*, 1995) and in chromaffin granules (Tagaya *et al.*, 1995). The yeast syntaxins Sed5p and Sso1p/Sso2p are primarily localized to the Golgi and the plasma membrane, respectively (Aalto *et al.*, 1993; Banfield *et al.*, 1994; Jantti *et al.*, 1994). Thus although it is a possibility that a portion of Pep12p found in the S13 fraction may reside in transport vesicles, it seems unlikely that this is a major location of the Pep12p within the cell.

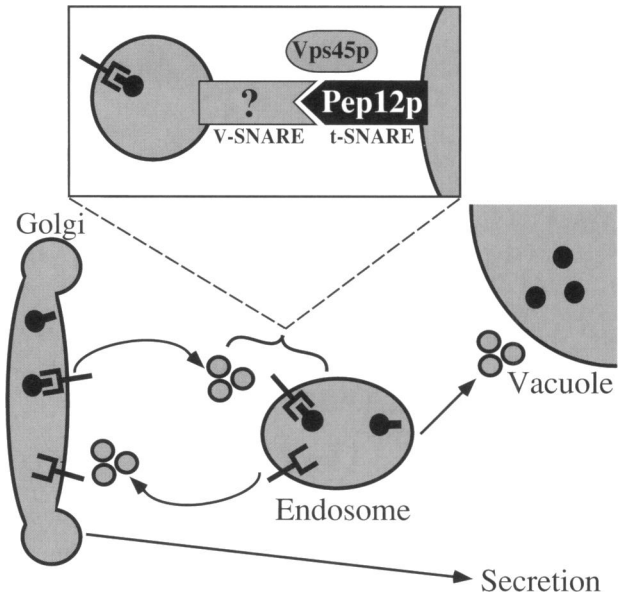

Figure 10. The possible site of Pep12p function in the yeast vacuolar protein sorting pathway. The accumulation of 40- to 50-nm vesicles and vacuolar protein sorting defects observed in *pep12* cells, together with the subcellular localization of Pep12p, suggest that Pep12p functions as a t-SNARE for the docking of Golgi-derived transport vesicles at the endosome.

Taken together, our data are most consistent with Pep12p functioning at the endosome to mediate the docking of Golgi-derived transport vesicles (Figure 10). Additional genetic selections should permit the identification of the cognate v-SNARE, as well as other proteins that interact with Pep12p. Furthermore, future genetic and biochemical experiments with *PEP12* should help clarify the exact function of Pep12p and other SNARE molecules involved in vesicle docking and fusion.

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