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A Sorting Nexin-1 Homologue, Vps5p, Forms a Complex with Vps17p and Is Required for Recycling the Vacuolar Protein-sorting Receptor

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A number of the *Saccharomyces cerevisiae* vacuolar protein-sorting (*vps*) mutants exhibit an altered vacuolar morphology. Unlike wild-type cells that contain 1–3 large vacuolar structures, the class B *vps5* and *vps17* mutant cells contain 10–20 smaller vacuole-like compartments. To explore the role of these *VPS* gene products in vacuole biogenesis, we cloned and sequenced *VPS5* and characterized its protein product. The *VPS5* gene is predicted to encode a very hydrophilic protein of 675 amino acids that shows significant sequence homology with mammalian sorting nexin-1. Polyclonal antiserum directed against the *VPS5* gene product detects a single, cytoplasmic protein that is phosphorylated specifically on a serine residue(s). Subcellular fractionation studies indicate that Vps5p is associated peripherally with a dense membrane fraction distinct from Golgi, endosomal, and vacuolar membranes. This association was found to be dependent on the presence of another class B *VPS* gene product, Vps17p. Biochemical cross-linking studies demonstrated that Vps5p and Vps17p physically interact. Gene disruption experiments show that the *VPS5* gene product is not essential for cell viability; however, cells carrying the null allele contain fragmented vacuoles and exhibit defects in vacuolar protein-sorting similar to *vps17* null mutants. More than 95% of carboxypeptidase Y is secreted from these cells in its Golgi-modified p2 precursor form. Additionally, the Vps10p vacuolar protein-sorting receptor is mislocalized to the vacuole in *vps5* mutant cells. On the basis of these and other observations, we propose that the Vps5p/Vps17p protein complex may participate in the intracellular trafficking of the Vps10p-sorting receptor, as well as other late-Golgi proteins.

INTRODUCTION

Intracellular organelles serve a vital function by compartmentalizing different and often competing biochemical reactions required to establish and maintain cellular homeostasis. Cells utilize specific transport systems to populate many of these organelles with unique sets of protein constituents that carry out these reactions. One of the best characterized is the lysosomal protein transport system (Kornfeld and Mellman, 1989; Kornfeld, 1992). Lysosomes are acidic compartments involved in macromolecular turnover and in the degradation of molecules brought into the cell by endocytosis. The degradative nature of lysosomes stems from the large number of hydrolytic enzymes that reside within them. These enzymes are synthesized on endoplasmic reticulum (ER)-bound ribosomes and translocated into the lumen of the ER where they are modified by the addition of N-linked oligosaccharides (Kornfeld and Mellman, 1989). The proteins are then delivered to and through the Golgi complex compartments, where their oligosaccharides

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are modified selectively by the addition of the mannose 6-phosphate- (M6P) sorting determinant. After delivery to the trans-Golgi network (TGN), lysosomal proteins are actively sorted away from secretory proteins and diverted to the lysosome. The majority of soluble lysosomal proteins are sorted by receptor molecules (MPRs) in the TGN that recognize the M6P found on lysosomal proteins (Kornfeld, 1992). The receptor/ligand complexes are then packaged into clathrin-coated vesicles and transported to the prelysosomal endosome. Here, the receptor releases its ligand, which continues on to the lysosome, whereas the free receptor is either recycled back to the Golgi for another round of sorting, or cycles through the plasma membrane where it binds and internalizes extracellular lysosomal proteins. Although much is known about these sorting receptors and their ligands, little is known about the cellular machinery that regulates trafficking of the receptors.

The vacuole of *Saccharomyces cerevisiae* is the structural and functional analog of the mammalian lysosome (Klionsky *et al.*, 1990). Vacuoles are acidic compartments that contain a variety of hydrolytic enzymes involved in macromolecular degradation (Jones, 1984). The biosynthesis of vacuolar proteins and lysosomal proteins also share many common features. Like lysosomal proteins, vacuolar proteins transit the early stages of the secretory pathway until they reach a late Golgi compartment (Stevens *et al.*, 1982). In this compartment, vacuolar proteins are selectively sorted away from the secretory protein pool and delivered to the vacuole (Graham and Emr, 1991; Vida *et al.*, 1993). For many hydrolases, arrival in the vacuole is accompanied by a proteolytic processing event that activates these enzymes (Klionsky *et al.*, 1990). The delivery of soluble proteins to the yeast vacuole does not involve the recognition of M6P modifications. Instead, the sorting information is contained within the N-terminal amino acid sequences of vacuolar proteins like carboxypeptidase Y (CPY) (Johnson *et al.*, 1987; Valls *et al.*, 1987). This sorting signal is recognized by a sorting receptor, Vps10p, which binds CPY in a late Golgi compartment (Marcusson *et al.*, 1994). The receptor/ligand complexes are packaged into transport vesicles which are delivered to a prevacuolar endosome (Cereghino *et al.*, 1995; Cooper and Stevens, 1996). Here CPY is thought to dissociate from its receptor and moves on to the vacuole, whereas the receptor recycles back to the Golgi for another round of sorting.

Fusion proteins formed between CPY and the normally secreted enzyme invertase (Inv) demonstrated that as little as 50 N-terminal residues of CPY fused to invertase target the CPY-Inv hybrid protein to the vacuole (Bankaitis *et al.*, 1986; Johnson *et al.*, 1987). Alteration of the sorting information contained in the CPY portion of the hybrid protein results in its mislocalization to the cell surface (Johnson *et al.*, 1987). The mislocalization of vacuolar proteins to the cell surface (*vps*) or deficiencies in vacuolar protease activity (*pep*) has served as a basis for several selections and screens for mutants specifically defective in the delivery of proteins to the vacuole (Jones, 1977; Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989). The *vps* mutants together with the *pep* mutants define more than 40 complementation groups (Robinson *et al.*, 1988; Rothman *et al.*, 1989; Raymond *et al.*, 1990). Morphological analyses have revealed that these mutants fall into distinct morphological classes (Banta *et al.*, 1988; Raymond *et al.*, 1990). Most mutants contain a vacuole that is the same or very similar to the vacuole found in wild-type cells. However, several mutant classes exhibit severely altered vacuole morphology. Included in this set are the class B mutants which contain fragmented vacuole-like structures. Two in particular, *vps5* and *vps17*, exhibit a unique phenotype with 10–20 small vacuoles (Banta *et al.*, 1988). To gain further insights into the role these gene products play in vacuolar protein sorting and vacuolar biogenesis, the wild-type gene affected in the *vps5* mutants was cloned and its gene product analyzed. *VPS5* encodes a 76 kDa phosphoprotein that forms a heteromeric complex with another phosphoprotein, Vps17p. This complex is associated with a dense membrane fraction, distinct from Golgi membranes. Cells that completely lack Vps5p function missort and secrete soluble vacuolar proteins, contain fragmented vacuoles and mislocalize the CPY-sorting receptor, Vps10p. Sequence comparisons have shown that Vps5p shares significant similarity to a mammalian protein sorting nexin 1 (SNX1) (Kurten *et al.*, 1996). SNX1 has been implicated in the intracellular trafficking of the epidermal growth factor (EGF) receptor. Together these results indicated that Vps5p may function in a phosphoprotein complex, to facilitate the intracellular trafficking of the CPY-sorting receptor.

MATERIALS AND METHODS

Media and Reagents

Escherichia coli cells were grown in LB and M9 media supplemented with appropriate antibiotics and amino acids (Miller, 1972). *S. cerevisiae* was propagated in yeast extract-peptone-dextrose (YPD), yeast extract-peptone-fructose (YPF), or synthetic dextrose (SD) supplemented with amino acids as required (Sherman *et al.*, 1979). The strains used in this study are listed in Table 1. Restriction and modifying enzymes were purchased from Boehringer Mannheim (Indianapolis, IN), New England Biolabs (Beverly, MA), and Stratagene (La Jolla, CA). Zymolyase 100-T was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). DSP [dithio*bis*(succinimidylpropionate)] was purchased from Pierce (Rockford, IL). Glusulase was obtained from DuPont Co (Boston, MA). 5-Bromo-4-chloro-3-indoyl-β-D-galactoside, phenylmethylsulfonyl fluoride, α_2-macroglobulin, aprotinin, leupeptin, pepstatin, and isopropyl-β-D-thiogalactopyranoside were acquired from Boehringer Mannheim

(Indianapolis, IN). Tran^{35}S-label was supplied by ICN Biochemicals (Costa Mesa, CA). [α-^{35}S]dATP was provided by Amersham Corp (Arlington Heights, IL). Production of antisera to vacuolar hydrolases (CPY, ALP, PrA) has been described previously (Klionsky *et al.*, 1988; Klionsky and Emr, 1989). All other reagents were purchased from Sigma (St. Louis, MO).

Plasmid Constructions

Plasmid constructions were performed using previously described recombinant DNA manipulation methods (Sambrook *et al.*, 1989). The glass bead method of Vogelstein and Gillespie (1979) was used for DNA fragment isolations. The *CEN*-based *VPS5* plasmid pBHY5–41 was generated by subcloning the 3.3-kb *Sma*I-*Xho*I fragment of library plasmid pBHY5–1 (containing *VPS5*, refer to Figure 1A) into the *Sma*I and *Xho*I sites of pPHYC18 (Herman and Emr, 1990). The 2 micron-based plasmid pBHY5–43 was constructed by ligating the *Sma*I-*Xho*I fragment of pBHY5–1 into pJSY324 (Herman *et al.*, 1991). Integrative mapping plasmid pBHY5–39 was made by inserting the *VPS5*-containing *Sma*I-*Xho*I fragment of pBHY5–1 into pRS304 (Sikorski and Hieter, 1989). The *VPS5* deletion/disruption plasmid pBHY5–35 was made by subcloning the 2.3-kb *Sma*I-*Sal*I fragment of pBHY5–1 into pBluescriptKS (Stratagene, La Jolla, CA) and then replacing the 0.7-kb *Xba*I-*Stu*I fragment of the resulting plasmid with the *HIS3* gene (Figure 1C). For preparation of a trpE-Vps5 fusion protein, pBHY5–1 was digested with *Eco*RI yielding a 914 bp fragment which was purified and ligated into *Eco*RI-digested pATH1 fusion vector (Dieckmann and Tzagoloff, 1985; containing *trpE* coding sequences) to generate pBHY5–40.

Nucleic Acid and Genetic Manipulations

Bacterial DNA transformations were accomplished using the method of Hanahan (1983) and yeast transformations employed a LiAc treatment protocol (Ito *et al.*, 1983). All other standard yeast genetic procedures were performed as described previously (Guthrie and Fink, 1991). pBHY11 (CPY-invertase::*LEU2*) (Horazdovsky *et al.*, 1994) was integrated at the *leu2–3,122* locus of SEY5–7 to produce strain BHY156. Integrative mapping studies of cloned *VPS5* were initiated by linearizing pBHY5–39 with *Stu*I and transforming BHY11 cells. Trp$^+$ transformants (BHY155) were mated with BHY156. Diploid colonies were selected, sporulated, and 56 of the resulting ascii were dissected. Trp$^+$/Trp$^-$ and Vps$^+$/Vps$^-$ phenotypes segregated 2:2, with all haploid segregants displaying Vps$^-$ phenotype also being Trp$^-$. Construction of *vps5* chromosomal deletion mutant was initiated by digesting pBHY5–35 with *Sma*I and *Sal*I, to excise the Δ*vps5::HIS3* fragment. This fragment was used to transform SEY6210. His$^+$ transformants were screened for secretion of p2CPY by colony blot (Roberts *et al.*, 1991). Genomic DNA was prepared from a His$^+$ Vps$^-$ isolate and the disruption of the *VPS5* locus was confirmed by polymerase chain reaction as described previously (Herman and Emr, 1990).

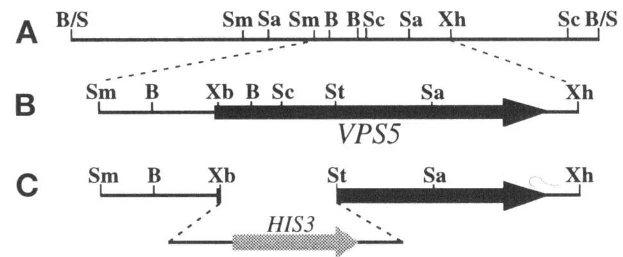

Figure 1. Characterization and disruption of the *VPS5* locus. (A) A restriction map of the ~12-kb genomic DNA fragment containing the *VPS5* locus. (B) The minimum *vps5* complementing fragment. The *VPS5* coding sequence is indicated by a black arrow. (C) A 0.7-kb *Xba*I-*Stu*I fragment of *VPS5* that was removed and replaced with *HIS3* gene (gray arrow) to generate the deletion/disruption strain BHY152 (*vps5Δ1*). The restriction enzymes shown includes; B, *Bam*HI; S, *Sau*3A; Sa, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; St, *Stu*I; Xb, *Xba*I; Xh, *Xho*I.

Isolation of the VPS5 Gene

A plasmid-based yeast genomic DNA library (*LEU2, CEN*; a gift from Philip Hieter) was used to transform SEY5–7 cells harboring a plasmid encoding a CPY-invertase fusion protein (Horazdovsky *et al.*, 1994). Transformed cells were replica-plated to YPF media, incubated at 30°C overnight, then subjected to an assay designed to detect extracellular invertase activity (Horazdovsky *et al.*, 1994). Plasmids were isolated from Vps$^+$ cells and used to transform SEY5–7 to confirm complementing activity. Isolated plasmids were digested and fragments containing genomic sequence were subcloned and tested for their ability to complement the SEY5–7 mutant phenotype for the purpose of identifying the minimum complementing DNA fragment shown in Figure 1A. A 2.3-kb *Sma*I/*Sal*I and a 3.4-kb *Sac*I/*Sac*I fragment from pBHY5–1 were cloned into the *E. coli* plasmid pBluescriptKS(-) to generate pBHY5–30 and pBHY5–31 respectively. Exonuclease III-mung bean nuclease deletions were constructed using these plasmids (pBHY5–30 and pBHY5–31) according to the pBluescript manual supplied by Stratagene, except nuclease digestion products were size fractionated and isolated from an 1% agarose preparative gel. Nested deletion constructs were identified and denatured plasmid DNA was purified over a 2 ml Sephacryl S-400 spun column using the procedure described in the Pharmacia Miniprep Kit Plus manual. The resultant

Table 1. Strains used in this study

Strain	Genotype	Reference
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Johnson *et al.* (1987)
SEY6211	*MAT***a** *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ90 ade2-101 suc2-Δ9*	Johnson *et al.* (1987)
BHY10	SEY6210, *leu2-3, 112*::pBHY11(CPY-Inv *LEU2*)	Horazdovsky *et al.* (1994)
BHY11	SEY6211, *leu2-3, 112*::pBHY11(CPY-Inv *LEU2*)	Horazdovsky *et al.* (1994)
SEY5-7	SEY6210, *vps5-7*	Robinson *et al.* (1988)
BHY155	BHY11, *VPS5-TRP1*	This study
BHY156	SEY5-7, *leu2-3, 112*::pBHY11(CPY-Inv *LEU2*)	This study
BHY152	SEY6210, *vps5Δ1::HIS3*	This study
KKY10	SEY6210, *vps17Δ1::HIS3*	Köhrer and Emr (1993)
TVY614	SEY6210, *Δpep4::LEU2 Δprc1::HIS3 Δprb1::HISG*	Thomas Vida (personal communication)

denatured double stranded templates were hybridized to the T7 or T3 primers and subjected to dideoxy-chain termination sequence analysis using the Sequenase sequencing protocol (United States Biochemical Corp., Cleveland, OH). An open reading frame was uncovered that covered bp 453770–455795 of chromosome XV in the *S. cerevisiae* genome database.

Antiserum Preparation

Bacterial JM101 cells were transformed with *trpE-VPS5* gene fusion plasmid pBHY5–40. Induced production and purification of fusion protein followed the method of Kleid *et al.* (1981), as modified by Herman and Emr (1990). Immunization of New Zealand White rabbits with fusion protein was executed as described previously (Horazdovsky *et al.*, 1994). CNBr-activated Sepharose (Pharmacia, Piscataway, NJ) was coupled to purified trpE-Vps5 fusion protein and used to affinity-purify harvested antiserum according to the manufacturer's instructions. Eluted antiserum was screened and titrated by immunoprecipitation of labeled yeast cell extracts.

Cell Labeling and Immunoprecipitation

Yeast cells were grown in SD containing required amino acids to an OD_{600} of 0.8. For experiments involving immunoprecipitation of vacuolar hydrolases, 5 OD_{600} units of cells were harvested by centrifugation and suspended in 1 ml of SD medium containing 1 mg/ml bovine serum albumin. Cells were preincubated for 10 min at 30°C; labeling was initiated via addition of 100 μCi Tran^{35}S-label and allowed to proceed for 10 to 15 min. Chase periods were then started by adding methionine, cysteine, and yeast extract to final concentrations of 5 mM, 1 mM and 0.2%, respectively. For experiments involving separation of pellet and media fractions, cells were converted to spheroplasts prior to cell labeling and chase periods (Vida *et al.*, 1990; Paravicini *et al.*, 1992). Following the labeling, chase cultures were then centrifuged at 13,000 × *g* for 1 min to yield an intracellular (I) pellet fraction and an extracellular (E) media fraction. The presence of CPY, PrA, and ALP proteins in each fraction was determined by immunoprecipitation (Klionsky *et al.*, 1988; Robinson *et al.*, 1988).

Phosphate Labeling and Phosphoamino Acid Analysis

Wild-type cells (SEY6210), *vps5Δ1* cells (BHY152) or wild-type cells carrying *VPS5* on a 2 μ expression vector (pBHY5–43) were grown to early-log phase in SD media. Cells were collected, resuspended to 5 OD_{600} units/ml in low phosphate media supplemented with 5 mM $MgSO_4$, and incubated for 30 min at 30°C. $^{32}PO_4$ was then added to a final concentration of 1 mCi/ml and incubation was continued for 30 min. Labeling was terminated by the addition of trichloroacetic acid to 10%, cell extracts were generated and Vps5p was immunoprecipitated from the labeled extracts as described previously (Herman *et al.*, 1991). Phosphoamino acid analysis was performed following the methods of Meisenhelder (Meisenhelder and Hunter, 1991). Briefly, ^{32}P-labeled Vps5p was immunoprecipitated and resolved by SDS-PAGE. The gel was fixed washed extensively with H_2O and dried. Labeled Vps5p was visualized by autoradiography and the corresponding band was cut from the dried gel. Vps5p was eluted from the gel slice and hydrolyzed in 5.7 M HCl at 110°C for 60 min. The hydrolysates were lyophilized from water three times and subjected to two-dimensional thin-layer electrophoresis using a 100-μm thin-layer cellulose plate (EM Science, Gibbstown, NJ).

Subcellular Fractionation and Gradient Analyses

SEY6210, KKY10 (*Δvps17Δ1*), or BHY152 (*vps5Δ1*) cells were propagated at 30°C to an OD_{600} of 0.8 in SD supplemented with appropriate amino acids. 30 OD_{600} units of cells were collected by centrifugation (2000 × *g* for 5 min) and spheroplasts were generated as described previously (Vida *et al.*, 1990; Paravicini *et al.*, 1992). Cultures were incubated at 30°C for 10 min prior to addition of 1 mCi Tran^{35}S-label. Labeling proceeded for 15 min at 30°C and was followed by a chase period of 45 min. Spheroplasts were harvested and lysed by douncing six times using a tissue homogenizer in buffer containing 200 mM sorbitol, 50 mM Tris (pH 7.5), 1 mM EDTA, and a protease inhibitor cocktail (2 μg/ml antipain, 2 μg/ml leupeptin, 2 μg/ml chymostatin, 2 μg/ml pepstatin, 0.1 TIU/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml α_2-macroglobulin). The resultant cell suspension was subjected to sequential centrifugation at 500 × *g* (5 min), 13,000 × *g* (10 min), and 100,000 × *g* (60 min) as described previously (Horazdovsky and Emr, 1993). Proteins in supernatant fractions were precipitated by the addition of trichloroacetic acid to 10% and the pellet fractions were washed with a 10% trichloroacetic acid solution. The pelleted proteins were then suspended in 1 ml of immunoprecipitation buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Tween 20, 0.1 mM EDTA, 1 mg/ml BSA] and the levels of Vps5p, Vps10p, ALP, glucose-6-phosphate dehydrogenase, and Kex2p in each subcellular fraction were determined by immunoprecipitation as previously described (Klionsky, *et al.*, 1988). The membranes in the P100 cell fraction were separated on a Accudenz (Nycodenz) gradient. Spheroplasts (30 OD equivalents) generated from TVY614 were labeled and chased as above, lysed and fractionated as above, except the lysis buffer consisted of 20 mM HEPES-KOH (pH 7.5), 50 mM potassium acetate, 1 mM EDTA, and 200 mM sorbitol. P100 membrane fraction was suspended in 1 ml of lysis buffer and loaded onto a 9–37% Accudenz gradient prepared by layering the following Accudenz solutions in a Beckman 14 × 89-mm UltraClear tube; 1 ml 37%, 1.5 ml 31%, 1.5 ml 27%, 1.5 ml 23%, 1.5 ml 20%, 1.5 ml 17%, 1 ml 13%, and 1 ml 9%. Accudenz solutions were prepared in a buffer containing 20 mM HEPES-KOH (pH 7.5), 50 mM potassium acetate, and 1 mM EDTA. The gradient was subjected to centrifugation at 170,000 × *g* for 16 h and the gradient was fractionated (1-ml fractions). Vps5p, Vps17p, Vps10p and Kex2p were immunoprecipitated from each fraction and the immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. Refractive indexes of each column fraction was used to calculate the gradient density profile.

Microscopy

Wild-type cells (SEY6210) or *vps5Δ1* cells (BHY152) were grown in rich media (YPD) to mid-log phase. The cells were harvested and resuspended at 20 OD_{600} U/ml in YPD media. FM 4–64 (Vida and Emr, 1995) was added to 20 μM and the cells were incubated with shaking for 30 min at 30°C. The cells were harvested and resuspended in YPD media, placed on standard slides and viewed with a Nikon Microphot-SA microscope equipped with a 100× CF N Plan DIC achromatic objective and a 546-nm filter for visualizing FM 4–64 fluorescence.

Cross-Linking Labeled Cell Extracts

Cells were grown in SD media to an OD_{600} of 1.0 and harvested by centrifugation. Spheroplasts were generated as described and incubated for 10 min at 30°C (Vida *et al.*, 1990). The spheroplasts were labeled for 10 min at 30°C with 30 μCi of Tran^{35}S per OD_{600} unit of spheroplasts. The spheroplasts were then pelleted for 30 seconds and osmotically lysed in 1 ml of a buffer containing 100 mM potassium phosphate (pH 7.5), 5 mM EDTA, 5 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 50 μg/ml α-2 macroglobulin, and 100 μg/ml phenylmethylsulfonyl fluoride (Stack *et al.*, 1993). To initiate the cross-linking reaction, dithio*bis*(succinimidylpropionate) (DSP) was added to the cell lysates at a final concentration of 200 μg/ml and the lysates were incubated at room temperature for 30 min. The cross-linking agent was then quenched by the addition of hydroxylamine to 20 mM. Proteins in

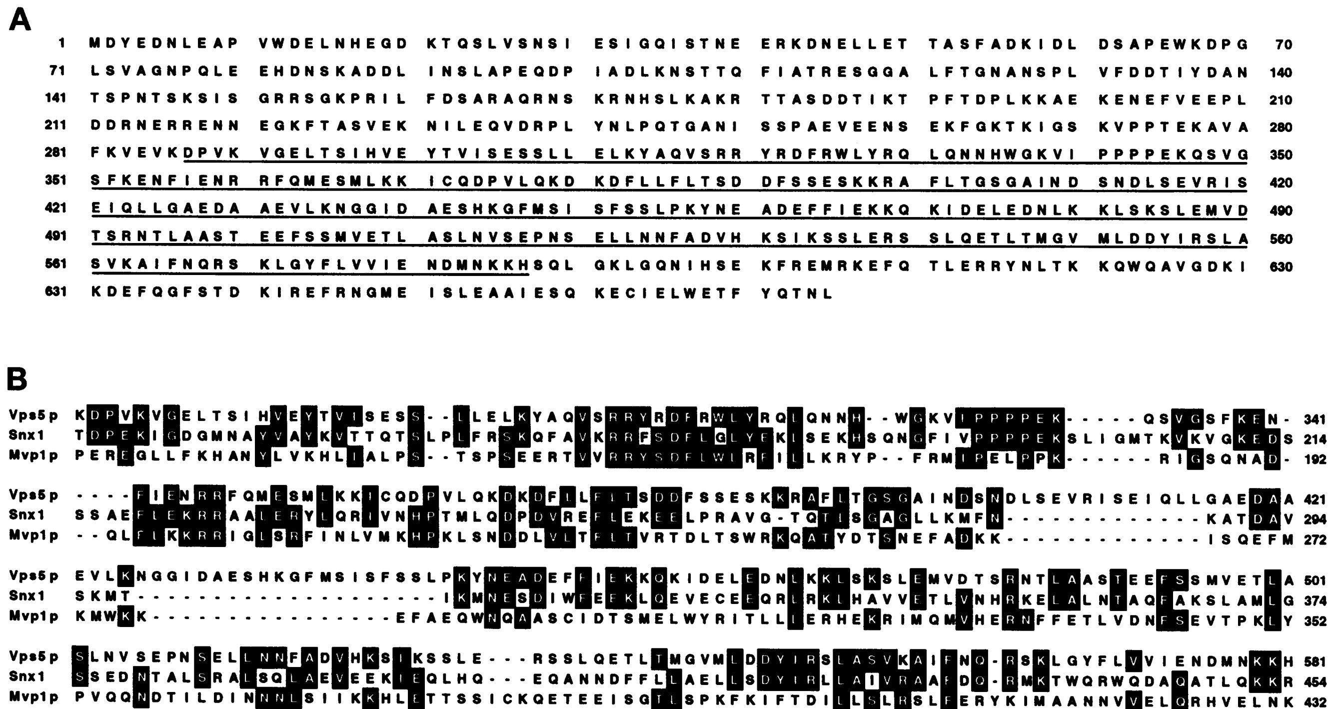

Figure 2. The Vps5p sequence and alignments. (A) The deduced amino acid sequence of Vps5p. The amino acids shown in the sequence alignment in B are underlined. (B) A comparison of Vps5p with mammalian sorting nexin 1 (SNX1) and yeast Mvp1p, the conserved residues are highlighted by black boxes.

the cross-linking reaction were precipitated with trichloroacetic acid and prepared for sequential immunoprecipitations with Vps5p and Vps17p antisera as detailed above.

RESULTS

VPS5 Encodes a Sorting Nexin Homologue

Wild-type cells localize CPY-invertase hybrid proteins to the vacuole due to the sorting information contained in the CPY portion of the molecule (Bankaitis *et al.*, 1986; Johnson *et al.*, 1987). Cells that contain defects in the vacuolar protein localization pathway missort these fusions to the cell surface which leads to a selectable phenotype, the ability of these mutant cells to grow on media that contain sucrose (an invertase substrate) as a sole carbon source. Using this selection, a large number of *vps* (vacuolar protein-sorting defective) mutants have been identified, including *vps5* (Robinson *et al.*, 1988). Like most *vps* mutants, *vps5* mutant cells missort and secrete the vast majority of CPY-invertase fusion proteins and this phenotype was utilized to clone the wild-type *VPS5* gene. *vps5* mutant cells (SEY5–7) expressing a CPY-invertase fusion protein were transformed with a plasmid-based yeast genomic library (*CEN LEU2*). Transformants were selected and those transformants that carried a *vps5* complementing library plasmid were identified using a simple plate assay (see MATERIALS AND METHODS). A single complementing clone was identified with a 12-kb genomic DNA insert (Figure 1A). The complementing activity was further defined and found to be contained on a 3.3-kb *Sma*I-*Xho*I fragment (Figure 1B). Integrative mapping studies demonstrated that *Sma*I-*Xho*I fragment contained the *VPS5* locus (see MATERIALS AND METHODS). The fragment was sequenced and a comparison to sequences in the yeast database revealed that the *Sma*I-*Xho*I fragment corresponded to bases 453770–455795 of chromosome XV (open reading frame YOR069W). A single large open reading frame (*VPS5*) was present in this fragment, capable of encoding a protein of 675 amino acids with a molecular mass of 76,437 daltons (Figure 2A). No hydrophobic stretches that could serve as a signal sequence or transmembrane domains were detected in Vps5p. Overall, Vps5p was predicted to be a very hydrophilic protein with a pI of 5.04. Sequence comparisons with other known proteins revealed that Vps5p shared sequence similarity with the yeast protein Mvp1p (Ekena and Stevens, 1995; 13% over 301 amino acids) and a mammalian protein, SNX1 (Kurten *et al.*, 1996; 29% over 301 amino acids) (Figure 2B). Mvp1p and SNX1 have been implicated in protein trafficking to the vacuole or lysosome, respectively.

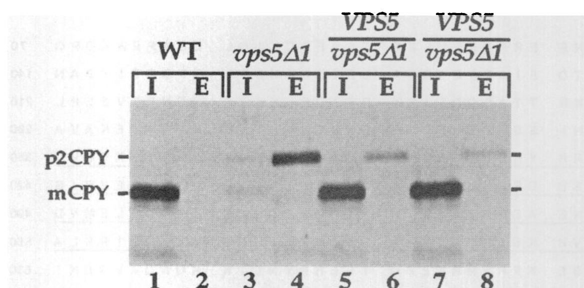

Figure 3. Complementation of the CPY-sorting defect by the cloned *VPS5* gene. Yeast spheroplasts were labeled for 10 min with Tran^{35}S-label at 30°C, and then unlabeled methionine and cysteine were added and the incubation was continued for 30 min. The labeled spheroplasts were separated into pellet (I, internal) and supernatant (E, external) fractions. CPY was immunoprecipitated from these fractions, resolved by SDS-PAGE, and visualized by fluorography. The yeast strains used in this analysis included SEY6210 (WT, lanes 1 and 2), BHY152 (*vps5Δ1*, lanes 3 and 4) and BHY152 transformed with a low-copy (lanes 5 and 6) or high-copy (lanes 7 and 8) plasmid containing *VPS5*. The migration positions of Golgi-modified precursors (p2) and mature (m) CPY are shown.

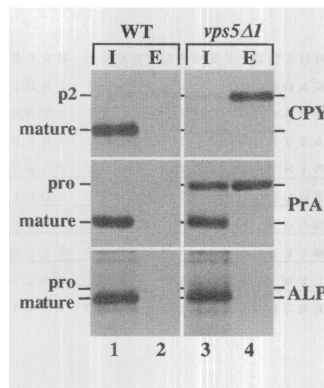

Figure 4. Intracellular sorting of vacuolar hydrolases. Spheroplasts from SEY6120 (WT) and BHY152 (*vps5Δ1*) were generated and labeled as described in Figure 3. The labeled cultures were split into spheroplast (I, internal) and media (E, external) fractions by centrifugation. The presence of CPY, PrA, and ALP in each fraction was determined by immunoprecipitation.

vps5 Mutants Missort Only Soluble Vacuolar Proteins

To determine the phenotypic consequences of a complete loss of Vps5p function, a null allele of *vps5* was generated by removing the sequence that encodes the N-terminal half of Vps5p and replacing it with a *HIS3* gene cassette (Figure 1C). Cells carrying this deletion/insertion were viable, but exhibited a severe defect in sorting of soluble vacuole proteins. The transport of many vacuolar proteins through the secretory pathway can be easily monitored due to compartmental-specific posttranslational modifications. Carboxypeptidase Y (CPY) serves as a particularly good example. Following translocation into the lumen of the ER, CPY is modified by the addition of four core oligosaccharides, generating the p1 precursor. As p1CPY moves through the Golgi complex, the core oligosaccharides are further modified by the addition of mannose residues, generating the Golgi-modified p2 precursor which is then delivered to the vacuole where its prosegment is cleaved, generating the mature vacuolar form of the enzyme. All three forms of CPY can be resolved by SDS-PAGE. In the experiments shown in Figure 3, spheroplasts were generated from wild-type cells, *vps5Δ1* cells or *vps5Δ1* cells carrying the cloned *VPS5* gene on a *CEN*- or 2 μ- based plasmid vector. These spheroplasts were metabolically labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine for 10 min and then chased for 30 min with an excess of unlabeled methionine and cysteine. The cultures where then split into spheroplast (I, internal) and media (E, external) fractions by centrifugation and the presence of CPY in each fraction was determined by immunoprecipitation with antiserum directed against CPY. In wild-type cells, CPY was properly delivered as evidenced by the presence of CPY inside the spheroplasts in its mature vacuolar form (mCPY) (Figure 3, lane 1). By contrast, the vast majority of CPY was missorted and secreted from *vps5Δ1* cells as the Golgi modified precursor (p2CPY) (Figure 3, lane 4). The missorting phenotype associated with *vps5*Δ1 cells was complemented by the presence of cloned *VPS5* in a *CEN*-based (lanes 5 and 6) or 2 μ-based (lanes 7 and 8) plasmid vector.

To determine whether other vacuolar proteins were also mislocalized in *vps5Δ1* cells, the sorting of another soluble vacuolar protease, proteinase A (PrA), was examined, as well as the transmembrane vacuolar protein alkaline phosphatase (ALP). The soluble vacuolar protease, PrA, was also secreted from these mutants in its Golgi-modified proform (Figure 4, lane 4). However, a portion of this protein was retained by the cells and approximately 30% was present in its mature vacuolar form (Figure 4, lane 3), indistinguishable from the mature from of the protein found in wild-type cells (Figure 4, lane 1). In addition, all of the vacuolar membrane protein ALP was retained and converted to its mature form, indicating delivery to a vacuole-like compartment (Figure 4. lane 3). Similar protein-sorting phenotypes were seen in another class B *vps* mutant, *vps17* (Köhrer and Emr, 1993).

The Vacuole Is Fragmented in vps5 Mutant Cells

The vacuole in wild-type cells can easily be visualized using the lipophilic fluorescent membrane dye FM-4–64 (Vida *et al.*, 1995) as one to three large structures per cell (Figure 5). This technique was used to examine vacuole morphology in cells carrying the *vps5* null allele. *vps5Δ1* cells lacked the large staining structures seen in wild-type cells. Instead, smaller staining structures accumulated in these mutant cells (Figure 5). This morphology defect was completely complemented by the presence of the cloned *VPS5* gene (our unpublished results). It is unclear whether the structures present in *vps5* mutant cells are intermediates in vacuole biogenesis or fragmentation of larger vacuolar

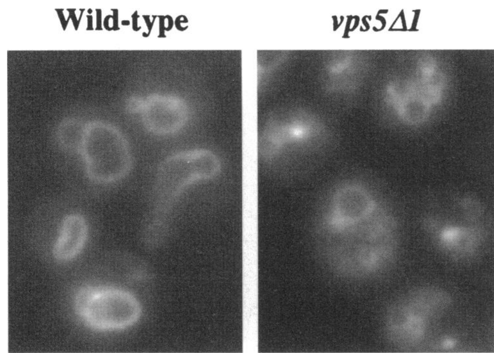

Figure 5. Morphological analysis of wild-type and *vps5Δ1* cells. Wild-type cells (SEY6210) and *vps5Δ1* cells (BHY152) were incubated for 15 min at 30°C in YPD media containing FM4–64. The cells were pelleted, resuspended in fresh YPD media, and incubated for 30 min at 30°C. The cells were then viewed using a fluorescent microscope equipped with a 546-nm filter and Nomarski optics.

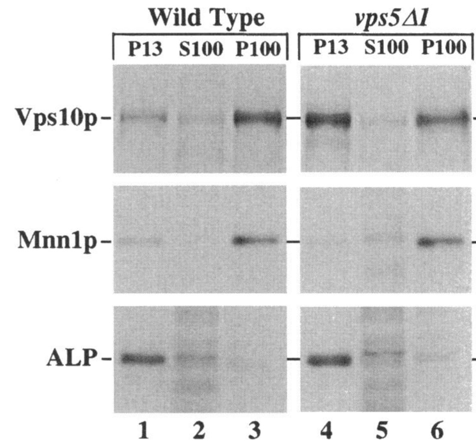

Figure 6. Subcellular fractionation of Vps10p. Spheroplasts generated from wild-type (SEY6210) or *vps5Δ1* cells (BHY152) were pulse labeled with [^{35}S]methionine and [^{35}S]cysteine for 15 min and chased for 45 min. The spheroplast were gently lysed by Dounce homogenization in a hypotonic buffer and the lysates were fractionated by sequential centrifugation as described in the text to generate a P13 pellet fraction, S100 supernatant fraction, and P100 pellet fraction. Vps10p, Mnn1p, and ALP were immunoprecipitated from each fraction, resolved on a 8% SDS-polyacrylamide gel, and visualized by fluorography.

structures, but these observations implicate Vps5p function, directly or indirectly, in the biogenesis or maintenance of vacuole structure. Four other class B *vps* mutants have been identified, *vps17*, *vps39*, *vps41*, and *vps43* (Vater *et al.*, 1992). Interestingly, only *vps17* mutant cells share morphological defects that are closely related to *vps5* mutant cells. *vps39*, *41*, and *43* mutant cells exhibit a diffuse FM4–64 staining pattern consistent with a more extreme fragmentation of vacuole-related compartments (Emr, unpublished observation). This suggests that Vps5p and Vps17p may function at the same point in the vacuolar protein-sorting pathway, distinct from the site of action of Vps39p, Vps41p and Vps43p.

The Subcellular Localization of the CPY-sorting Receptor Is Altered in vps5Δ1 Cells

The sorting of p2CPY from the late Golgi-sorting compartment to the prevacuolar endosome is mediated by Vps10p, a transmembrane receptor that cycles between these two compartments (see INTRODUCTION). Because SNX1 seems to play a role in the trafficking of EGF receptor from the cell surface to lysosomes in mammalian cells and *vps5* mutants show a strong defect in CPY sorting but a limited defect in PrA localization, Vps5p's role in Vps10p trafficking was examined by subcellular fractionation. Differential centrifugations can be used to easily separate yeast subcellular membranes. In the experiments shown in Figure 6, spheroplasts generated from wild-type and *vps5Δ1* cells were labeled for 15 min with [^{35}S]methionine/[^{35}S]cysteine, chased for 45 min with unlabeled methionine and cysteine, and then lysed. First, unbroken spheroplasts were removed by a 500 × *g* centrifugation to generate the supernatant S5 fraction. The cleared lysates were spun at 13,000 × *g* generating a supernatant (S13) and pellet fraction (P13). The S13 fractions were then spun at 100,000 × *g* to generate the final S100 supernatant and P100 pellet fractions. The presence of Vps10p, the Golgi resident protein, mannosyltransferase (Mnn1p), and the vacuolar membrane protein, ALP, in the various cell fractions was determined by immunoprecipitation using protein-specific antibody. In wild-type cells, Vps10p was found primarily (~80%) in the P100 cell fraction, a fraction enriched in Golgi membrane marker Mnn1p (Figure 6, lane 3). A small fraction of Vps10p was also found in the P13 which also contained the vacuolar membrane marker ALP (lane 1). The fractionation pattern of Vps10p changed in *vps5Δ1* cells (Figure 6, lanes 4–6). A significant portion of Vps10p (~30%) shifted from the P100 to the P13. Importantly, this effect did not extend to all Golgi localized proteins as the distribution of Mnn1p was unaltered in *vps5Δ1* cells (lane 6). Similarly, the fragmentation of the vacuole observed in *vps5Δ1* cells did not change the fractionation of the vacuolar membrane marker, ALP, which remained localized almost exclusively to P13 membranes (lane 4). When the P13 membranes from the *vps5Δ1* cells were separated on sucrose density gradients, Vps10p was found associated with membranes of high buoyant density, consistent with colocalization with vacuolar membranes (our unpublished results). The shift of Vps10p from a Golgi-enriched membrane fraction to a fraction enriched in vacuolar membranes indicates Vps5p function is required to maintain the normal localization pattern of Vps10p.

Vps5p Is Phosphorylated on a Serine Residue(s)

A trpE-Vps5p hybrid protein was used to generate polyclonal antiserum directed against Vps5p and this antiserum was used to identify the *VPS5* gene product. Wild-type and *vps5*Δ1 cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 10 min at 30°C. Unlabeled methionine and cysteine were added, and an aliquot of the cells was removed and immediately processed for immunoprecipitation while the remainder of the culture was incubated for an additional 60 min before processing. As shown in Figure 7A, this antiserum detected a protein of 90 kDa in wild-type cell extracts (lane 2) that was absent in cells carrying the *vps5* null allele (lane 1). Vps5p appeared to be stable; after a 60-min chase period, no significant decrease in the amount of newly synthesized Vps5p was detected (lane 3). The apparent molecular mass of 90 kDa is larger than that predicted by the primary amino acid sequence of Vps5p (76 kDa). This discrepancy may be due to the highly charged nature of Vps5p (pI = 5.04).

Due to its aberrant gel migration pattern, we examined the possibility that Vps5p was phosphorylated (Figure 7A, lanes 4–6). The Vps5p-specific antiserum detected a 90-kDa phosphoprotein in ^{32}P-labeled wild-type cell extracts (lane 5) that was not seen in ^{32}P-labeled extracts generated from cells carrying the *vps5* null allele (lane 4). Furthermore, when *VPS5* was placed in a multicopy vector (2 μ), an increase in phosphorylated Vps5p (~10 fold) was seen (lane 6). To determine the nature of the phosphorylation event, two-dimensional thin-layer electrophoresis was carried out on an acid hydrolyzed sample of ^{32}P-labeled Vps5p (Figure 7B). By comparing the migration pattern of ^{32}P-labeled phosphoamino acids generated from Vps5p with that of stained phosphoserine, phosphothreonine, and phosphotyrosine standards, it was found that Vps5p was phosphorylated on a serine residue(s). Like Vps5p, the class B *VPS* gene product, Vps17p, has also been shown to contain phosphoserine residues (Köhrer and Emr, 1993).

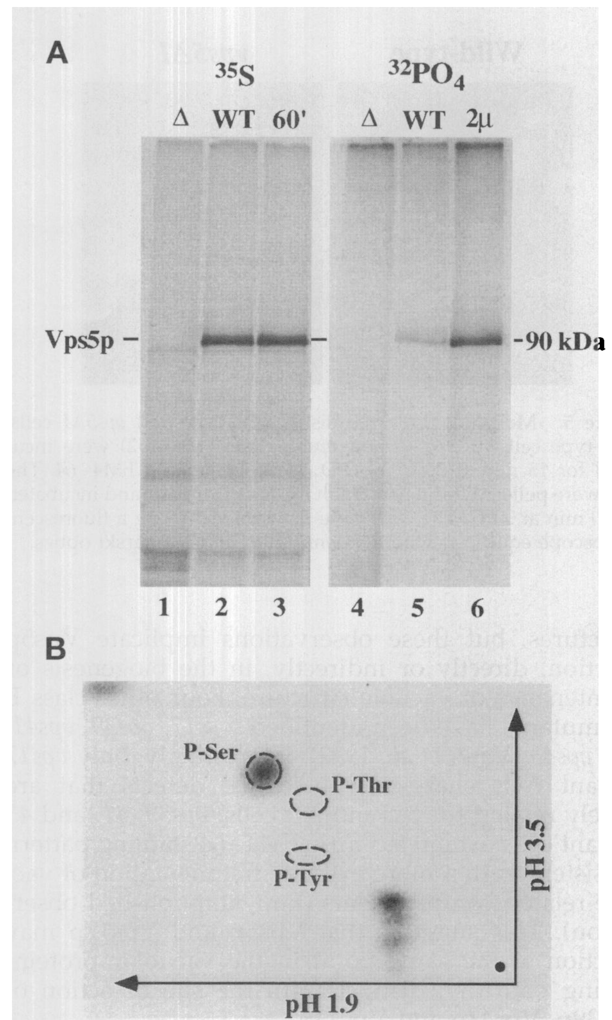

Figure 7. Identification and characterization of the *VPS5* gene product. (A) Wild-type (SEY6210) cells (lanes 2 and 5), wild-type cells carrying *VPS5* on a multicopy plasmid (pBHY5–43; lane 6), and *vps5Δ1* cells (BHY152; lanes 1 and 4) were labeled for 10 min with Tran^{35}S-label at 30°C (lanes 1–3) or 30 min with ortho^{32}PO$_4$ (lanes 4–6). The labeling was terminated by the addition of trichloroacetic acid immediately (lanes 1, 2, and 4–6) or after a 60-min chase period (lane 3). Cell extracts were generated and Vps5p was isolated by immunoprecipitation. The immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography or autoradiography. (C) Phosphoamino acid analysis of Vps5p. Vps5p was immunoprecipitated from ^{32}P-labeled cell extracts and resolved by SDS-PAGE. Vps5p was eluted and acid hydrolyzed. The hydrolysates were mixed with unlabeled phosphoamino standards and separated by two-dimensional thin-layer electrophoresis. The thin-layer plates were autoradiographed and the position of the labeled phosphoamino acid was compared with ninhydrin-stained standards.

Vps5p Fractionates with 100,000 × g Membranes

Vps5p is predicted to be a very hydrophilic protein, being comprised of 31% charged amino acids and yielding a net charge of −25. Vps5p lacks any obvious hydrophobic stretches capable of serving as a transmembrane domain or as a signal sequence. Despite its hydrophilic nature, Vps5p was found to be associated with cellular membranes. In the analysis shown in Figure 8, A and B, ^{35}S-labeled yeast spheroplasts from wild-type cells and cells overexpressing Vps5p were lysed and the lysate was subjected to a set of sequential centrifugations at 500 × *g*, 13,000 × *g*, and 100,000 × *g* as described earlier. The presence of Vps5p and organelle marker proteins in each of these fractions (S5, the 500 × *g* supernatant, the S13 and P13, the 13,000 × *g* supernatant and pellet fraction respectively, as well as the S100 and P100, the supernatant and pellet fractions of the 100,000 × *g* spin) was determined by immunoprecipitation. Vps5p fractionated away from the P13 cell pellet fraction (Figure 8A, lane

3) which contained plasma membranes, ER, and vacuolar membranes (our unpublished results). However, a significant portion of Vps5p was found in the high-speed cell pellet fraction (P100) in wild-type cells (lane 5), cofractionating with the Golgi markers Mnn1p (Figure 6) and Kex2p (our unpublished results). When Vps5p was overexpressed (Figure 8B), the vast majority of the overexpressed material was found in a soluble cell fraction (S100), indicating that the association of Vps5p with the P100 fraction could be saturated. Because *vps5* and *vps17* mutants show the same protein-sorting and vacuole morphology phenotypes, the fractionation pattern of Vps5p also was examined in *vps17Δ1* cells (Figure 8C). Interestingly, Vps5p's association with the P100 cell fraction was dependent on the presence of Vps17p. The vast majority of Vps5p shifted from the P100 to the S100 cell fraction in cells that lacked Vps17p (Figure 8C). In addition, Vps5p could be quantitatively extracted from the P100 fractions with 1 M NaCl, indicating that Vps5p was peripherally associated with this cell fraction (our unpublished results).

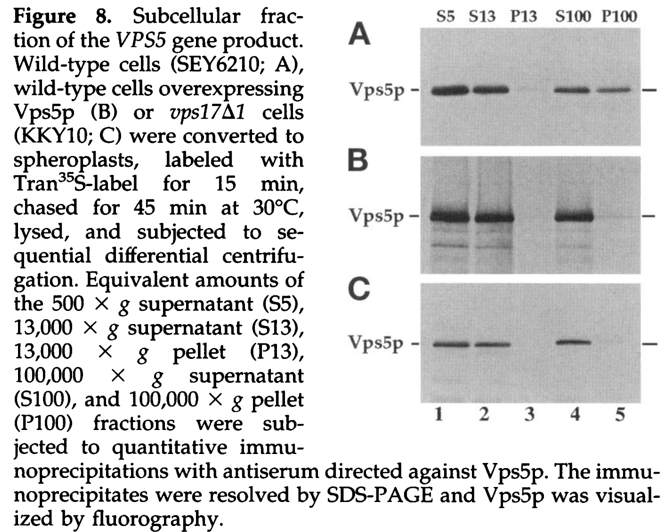

Figure 8. Subcellular fraction of the *VPS5* gene product. Wild-type cells (SEY6210; A), wild-type cells overexpressing Vps5p (B) or *vps17Δ1* cells (KKY10; C) were converted to spheroplasts, labeled with Tran^{35}S-label for 15 min, chased for 45 min at 30°C, lysed, and subjected to sequential differential centrifugation. Equivalent amounts of the 500 × *g* supernatant (S5), 13,000 × *g* supernatant (S13), 13,000 × *g* pellet (P13), 100,000 × *g* supernatant (S100), and 100,000 × *g* pellet (P100) fractions were subjected to quantitative immunoprecipitations with antiserum directed against Vps5p. The immunoprecipitates were resolved by SDS-PAGE and Vps5p was visualized by fluorography.

Vps5p/Vps17p Cofractionate with Dense Cellular Membranes

To further characterize the membrane compartment with which Vps5p associates, the distribution of Vps5p was examined using Accudenz density gradients. Spheroplasts generated from wild-type cells were labeled for 15 min with [^{35}S]methionine and [^{35}S]cysteine and chased for another 45 min. The pulse-labeled spheroplasts were lysed, the lysate was cleared by centrifugation at 13,000 × *g*, and the supernatant was subjected to a 100,000 × *g* centrifugation. The resultant pellet was resuspended and loaded onto a 9–37% Accudenz gradient and spun to equilibrium. Fractions were collected, and Vps5p, Vps17p, Vps10p, as well as the Golgi membrane protein Kex2p, were recovered by immunoprecipitation and then resolved by SDS-PAGE. As shown in Figure 9, Vps5p and Vps17p cofractionated with virtually identical patterns (A), concentrating in fractions 11 and 12. Furthermore, Vps5p and Vps17p associated with membranes with a density distinct from those that contain the late-Golgi marker protein Kex2p which were concentrated in fractions 8 and 9 (Figure 9A). Vps10p was found predominantly in the Kex2p-positive fractions and to a lesser degree in lighter membrane fractions that most likely represent a prevacuolar endosomal compartment (Becherer *et al.*, 1996). When the P100 membranes were loaded on a 20–50% Accudenz gradient, Vps5p migrated to a midway position in the gradient corresponding to a density of 1.18 g/cm^3 (our unpublished results), which was similar to the density observed in Figure 9. These data indicate that Vps5p and Vps17p are associated with the same dense membrane fraction, distinct from that of Golgi membranes.

Vps5p and Vps17p Are Part of a Protein Complex

The shared phenotypic characteristics of *vps5* and *vps17* mutants and the fact that both proteins appear to be associated with dense membranes indicates that Vps5p and Vps17p may function at a similar point in the vacuolar-sorting/biogenesis pathway. Although no genetic interactions have been uncovered between *vps5* and *vps17* mutants, the possibility that Vps5p and Vps17p physically interact was explored using chemical cross-linking agents. In the experiments shown in Figure 10, the homobifunctional cross-linking agent Dithiobis(succinimidylpropionate) (DSP) was used. DSP contains a disulfide bond in the linker between its two functional groups so that once isolated, the individual components of a cross-linked complex can be released by treating samples with a reducing agent. ^{35}S-labeled wild-type cells, *vps5Δ1* or *vps17Δ1* cells were gently lysed, the crude extracts were treated or not treated with DSP for 30 min and the cross-linking reaction was quenched with hydroxylamine. The extracts were then subjected to immunoprecipitation with Vps5p antiserum under denaturing conditions and resolved by SDS-PAGE (Figure 10A). A 70-kDa protein could be cross-linked to Vps5p in wild-type cell extracts (lane 2). This protein did not coimmunoprecipitate in the absence of cross-linking agent (lane 1) or when Vps5p was absent (lane 3). The 70-kDa protein was also absent if the cross-linking reaction was performed with *vps17Δ1* cell extracts, suggesting that this protein was Vps17p (which migrates at 70 kDa). To confirm that Vps5p and Vps17p formed a complex, a second set of cross-linking studies were performed (Figure 10B). ^{35}S-labeled wild-type cell ex-

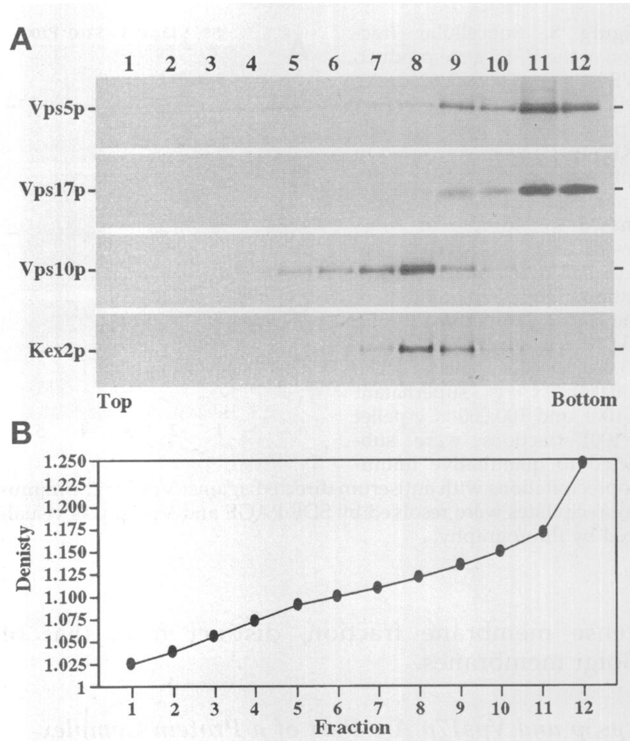

Figure 9. Localization of Vps5p and Vps17p by Accudenz density gradient analysis. Spheroplasts generated from TVY614 cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 15 min and then chased for 45 min. After lysis, large membranes were removed by centrifugation at 13,000 × *g* for 10 min, and the cleared lysate was subjected to centrifugation at 100,000 × *g* for 60 min. The resultant pellet (P100) was suspended in lysis buffer, loaded onto a 9–37% Accudenz gradient, and spun to equilibrium. Twelve fractions were collected, and Vps5p, Vps17p, Kex2p, and Vps10p were immunoprecipitated from each fraction; the immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. The density of each gradient fraction was derived from its refractive index.

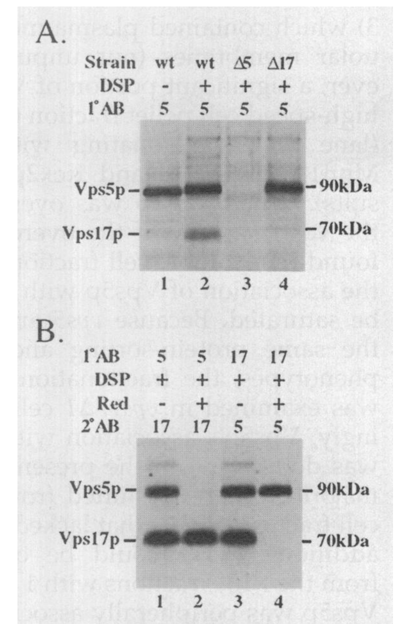

Figure 10. Cross-linking Vps5p and Vps17p. (A) Spheroplasts generated from wild-type cells (wt), *vps5Δ1*, and *vps17Δ1* cells (Δ5 and Δ17, respectively) were labeled with [^{35}S]methionine and [^{35}S]cysteine for 10 min and then chased for 30 min. Labeled spheroplasts were osmotically lysed and the lysates were untreated (−) or treated (+) with the cross-linking agent DSP. The extracts were immunoprecipitated with antiserum directed against Vps5p, the immunoprecipitates were reduced with SDS sample buffer, resolved by SDS-PAGE, and the cross-linked products were visualized by fluorography. (B) Spheroplasts were labeled and cross-linked as described above. Cross-linked extracts were subjected to sequential immunoprecipitations under denaturing but nonreducing conditions using Vps5p or Vps17p antiserum (1° AB). The immunoprecipitates there then treated with a reducing or nonreducing buffer (Red+, Red−, respectively) and then reimmunoprecipitated with a second antiserum as indicated (2° AB). The final immunoprecipitated material was treated with a reducing sample buffer, resolved by SDS-PAGE, and visualized by fluorography.

tracts were treated with DSP and then subjected to sequential immunoprecipitations with Vps5p- and Vps17p-specific antiserum or vice versa under reducing or nonreducing conditions. In the first set of experiments, cross-linked proteins were immunoprecipitated with Vps5p (lane 2) or Vps17p (lane 4) antiserum under denaturing conditions. The DSP was reduced and samples were reimmunoprecipitated with Vps17p (lane 2) or Vps5p (lane 4) antiserum. Vps5p and Vps17p coimmunoprecipitated in both cases demonstrating a physical interaction between these two proteins. In a second set of experiments, sequential immunoprecipitations were performed as described above, except the cross-linker was not reduced between the immunoprecipitations. Under these conditions only the Vps5p and Vps17p present in a complex would be isolated and an estimate of Vps5p and Vps17p stoichiometry was generated (lanes 1 and 3). In both cases, approximately the same amount of Vps17p and Vps5p was coimmunoprecipitated, indicating these proteins are complexed in equimolar amounts. The presence of the Vps5p/Vps17p protein complex was further confirmed by native immunoprecipitation (our unpublished results).

DISCUSSION

Vps5p is a highly charged 76-kDa phosphoprotein that peripherally associates with a dense membrane fraction in a manner that is dependent on the presence of Vps17p, another component of the vacuolar protein delivery system (Köhrer and Emr, 1993). Chemical cross-linking studies demonstrate that Vps5p and Vps17p physically interact. Cells that lack Vps5p function exhibit defects in vacuolar protein sorting. Although the vast majority of CPY is missorted in these mutants, the sorting of another soluble hydrolase, PrA, is only partially affected. The sorting defects in *vps5* mutants appear to result, in part, from the mislocalization of the vacuolar protein-sorting receptor, Vps10p. These data indicate that Vps5p together with Vps17p may participate in the intracellular trafficking of the Vps10-sorting receptor.

Further support for the role of Vps5p function in CPY-sorting receptor trafficking comes from the observation that Vps5p and the mammalian protein SNX1

share sequence homology. SNX1 was isolated in a yeast two-hybrid screen designed to uncover proteins that interact with a cytoplasmic domain of the EGF receptor. SNX1 was shown to interact with a domain of the EGF receptor that included the predicted lysosome targeting signal (Tyr-Leu-Val-Ile) (Kurten *et al.*, 1996). Subsequent overexpression of SNX1 resulted in a decrease in the amount of EGF receptor at the cell surface of CV-1 cells and an overall decrease in the total amount of receptor found in the cells, consistent with its degradation in the lysosome. These results indicate that SNX1 participates in EGF receptor trafficking to the lysosome (Kurten *et al.*, 1996). Vps5p may play a similar role in the vacuolar protein-sorting system by regulating the trafficking of the CPY-sorting receptor (see below). However, a direct physical interaction between Vps5p and the CPY-sorting receptor has yet to be demonstrated.

Vps5p also shares a limited homology with another yeast protein Mvp1p. Like most *vps* mutants, cells that lack Mvp1p function missort CPY yet exhibit wild-type vacuolar morphology (Ekena and Stevens, 1995). *MVP1* was originally identified as a multicopy suppressor of a *vps1* dominant mutant (Ekena and Stevens, 1995). *VPS1* codes for a protein that is 45% identical to the mammalian GTPase dynamin and is thought to function in the formation of transport vesicles that facilitate vacuolar protein targeting (Rothman *et al.*, 1990; Vater *et al.*, 1992; Wilsbach and Payne, 1993). The role Mvp1p plays in vacuolar protein sorting is unknown, but it has been suggested that Mvp1p may regulate GTP binding to Vps1p or GTP hydrolysis (Ekena and Stevens, 1995).

The Vps5/Vps17 Protein Complex

Since Vps5p and Vps17p form a heteromeric complex, they appear to function together in the vacuolar protein-sorting pathway. A large portion of this complex is peripherally associated with a dense membrane fraction. Interestingly, the association of Vps5p with this membrane fraction is dependent on Vps17p. It is unclear if Vps17p recruits Vps5p to the membrane or if only the Vps5p/Vps17p complex is capable of membrane association. Although these two proteins lack significant sequence homology, Vps5p and Vps17p share two interesting physical characteristics; both proteins are very hydrophilic and they are both phosphorylated on a serine residue(s). An analysis of the Vps5p phosphorylation state in other *vps* mutant backgrounds have failed to identify any potential modulators of phosphorylation (e.g., protein kinases or phosphatases; our unpublished results), and the role these phosphorylation events play has yet to be determined. However, phosphorylation may be involved in regulating Vps5p and Vps17p function by serving to modulate the membrane association/dissociation of the Vps5p/Vps17p complex or by participating in the recruitment of other potential members of this complex. The exact nature of the membrane fraction with which the Vps5p/Vps17p complex is associated is unknown, but it is clearly distinct from Golgi, endosomal, and vacuolar membranes. The density of the Vps5p/Vps17p containing membranes are strikingly similar to that observed for COPI- (1.18 g/cm^3) and COPII- (1.19 g/cm^3) coated vesicles (Serafini, *et al.*, 1991; Schekman, personal communication). We are currently examining the possibility that Vps5p and Vps17p may be associated with transport vesicles that function in the endosome to Golgi recycling pathway (see below).

Vps5p and Receptor Trafficking

The role of Vps10p in yeast vacuolar protein sorting is similar to that of the M6P receptor in lysosomal protein sorting in mammalian cells (Horazdovsky *et al.*, 1995; Stack *et al.*, 1995). Both receptors bind their protein ligands in a late Golgi compartment or TGN. The receptor/ligand complexes are packaged into transport vesicles which are then delivered to an intermediate endosomal compartment where the ligands dissociate from the receptor. The ligands/hydrolases are then delivered to the vacuole or lysosome, whereas the receptors recycle back to the late Golgi for another round of sorting. The process of receptor recycling appears to be highly regulated (Cereghino *et al.*, 1995; Cooper and Stevens, 1996). In yeast, a number of *VPS* gene products have been implicated specifically in Vps10p recycling including Vps29p, Vps30p, Vps35p, and Vps27p (Piper, *et al.*, 1995; Seaman *et al.*, 1997). As seen in *vps5* mutants, mutations in *VPS29, 30,* and *35* result in a shift of Vps10p from a Golgi-enriched fraction to the vacuole membrane (Seaman *et al.*, 1997). This has led to the proposal that these three proteins act in the retrieval of Vps10p from the prevacuolar endosome to the late Golgi. Thus, in *vps29, 30,* and *35* mutants, Vps10p accumulates in endosomes and is eventually missorted to the vacuole. Interestingly, unlike *vps5* mutants, *vps29, 30,* and *35* mutant cells contain normal-appearing vacuoles. This key difference indicates that Vps5p may be involved in a different aspect of Vps10p trafficking. One possibility is that Vps5p functions as a more generalized factor involved in the formation of recycling vesicles at the endosome that return Vps10p back to the Golgi or Vps5p may be a critical component of the recycling vesicles themselves. In cells that lack Vps5p, Vps10p would become trapped in the prevacuolar endosome and ultimately be missorted to the vacuole. However, the missorting of Vps10p alone cannot explain the fragmented appearance of the vacuole in *vps5* mutants since *vps29, 30,* and *35* mutants exhibit a more severe mislocalization of Vps10p yet contain morphologically normal

vacuoles. It is likely that other proteins mislocalized in *vps5* mutants (but not in *vps29*, *vps30*, and *vps35* mutants) are required for normal vacuolar integrity.

Although the exact function of Vps5p in vacuolar protein localization is still unknown, the evidence presented in this study is consistent with its participation in Vps10p trafficking events. It is becoming increasingly clear that many aspects of vesicle-mediated protein sorting are conserved in a wide variety of eukaryotic systems. The homology between Vps5p and mammalian SNX1 suggests that the basic mechanisms involved in intracellular receptor protein trafficking are also shared. Determining the precise role Vps5p plays in trafficking the Vps10-sorting receptor between the endosome and Golgi should lead to insights into the role this family of proteins plays in a wide variety of receptor trafficking events.

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REFERENCES

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

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

Becherer, K.A., Rieder, S.E., Emr, S.D., and Jones, E.W. (1996). Novel syntain homologue, Pep12p, required for the sorting of lumenal hydrolases to the lysosome-like vacuole in yeast. Mol. Biol. Cell *7*, 579–594.

Cereghino, J.L., Marcusson, E.G., and Emr, S.D. (1995). The cytoplasmic tail of the vacuolar protein sorting receptor Vps10p and a subset of *VPS* gene products regulate receptor stability, function, and localization. Mol. Biol. Cell *6*, 1089–1102.

Cooper, A.A., and Stevens, T.H. (1996). Vps10p cycles between the late-Golgi and prevacuolar compartments in its function as the sorting receptor for multiple vacuolar hydrolases. J. Cell Biol. *133*, 529–541.

Cowles, C.R., Emr, S.D., and Horazdovsky, B.F. (1994). Mutations in the *VPS45* gene, a *SEC1* homologue, are defective for vacuolar protein sorting and accumulate clusters of membrane vesicles. J. Cell Sci. *107*, 3449–3459.

Dieckmann, C.L., and Tzagoloff, A. (1985). Assembly of the mitochondrial membrane system. J. Biol. Chem. *260*, 1513–1520.

Ekena, K., and Stevens, T.H. (1995). The *Saccharomyces cerevisiae MVP1* gene interacts with *VPS1* and is required for vacuolar protein sorting. Mol. Cell. Biol. *15*, 1671–1678.

Graham, T.R., and Emr, S.D. (1991). The Sec18/NSF protein is required at multiple steps in the secretory pathway: mutants define compartmental organization of protein modification and sorting events in the yeast Golgi. J. Cell Biol. *114*, 207–218.

Guthrie, C., and Fink, G.R. (eds.) (1991). Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology, New York: Academic Press.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. *166*, 557–580.

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

Herman, P.K., Stack, J.H., DeModena, J.A., and Emr, S.D. (1991). A novel protein kinase homolog essential for protein sorting to the yeast lysosome-like vacuole. Cell *64*, 425–437.

Herman, P.K., Stack, J.H., and Emr, S.D. (1991). A genetic and structural analysis of the yeast Vps15 protein kinase: evidence for a direct role of Vps15p in vacuolar protein delivery. EMBO J. *10*, 4049–4060.

Horazdovsky, B.F., Busch, G.R., and Emr, S.D. (1994). *VPS21* encodes a rab5-like GTP binding protein that is required for the sorting of yeast vacuolar proteins. EMBO J. *13*, 1297–1309.

Horazdovsky, B.F., DeWald, D.B., and Emr, S.D. (1995). Protein transport to the yeast vacuole. Curr. Opin. Cell Biol. *7*, 544–551.

Ito, H., Fukada, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. *153*, 163–168.

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

Jones, E. (1984). The synthesis and function of proteases in *Saccharomyces*: genetic approaches. Annu. Rev. Genet. *18*, 233–270.

Jones, E.W. (1977). Proteinase mutants of *Saccharomyces cerevisiae*. Genetics *85*, 23–33.

Kleid, D.G., Yansura, D., Small, B., Dowbenko, D., Moore, D.M., Grubman, M.J., McKercher, P.D., Morgan, D.O., Robertson, B.H., and Bachrach, H.L. (1981). Cloned viral protein vaccine for foot-and-mouth disease. Science *214*, 1125–1129.

Klionsky, D.J., Banta, L.M., and Emr, S.D. (1988). Intracellular sorting and processing of a yeast vacuolar hydrolase: proteinase A propeptide contains vacuolar targeting information. Mol. Cell. Biol. *8*, 2105–2116.

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

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

Köhrer, K., and Emr, S.D. (1993). The yeast *VPS17* gene encodes a membrane-associated protein required for the sorting of soluble vacuolar hydrolases. J. Biol. Chem. *268*, 559–569.

Kornfeld, S. (1992). Structure and function of the mannose 6-phosphate/insulin like growth factor-II receptors. Annu. Rev. Biochem. *61*, 307–330.

Kornfeld, S., and Mellman, I. (1989). The biogenesis of lysosomes. Annu. Rev. Cell Biol. *5*, 483–525.

Kurten, R.C., Cadena, D.L., and Gill, G.N. (1996). Enhanced degradation of EGF receptors by a sorting nexin, SNX1. Science *272*, 1008–1010.

Marcusson, E.G., Horazdovsky, B.F., Lin Cereghino, J., Gharakhanian, E., and Emr, S.D. (1994). The sorting receptor for yeast vacuolar carboxypeptidase Y is encoded by the *VPS10* gene. Cell *77*, 579–586.

Meisenhelder, J., and Hunter, T. (1991). Phosphorylation of phospholipase C in vivo and in vitro. Methods Enzymol. *197*, 288–305.

Miller, J. (1972). Experiments in Molecular Genetics, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Paravicini, G., Horazdovsky, B.F., and Emr, S.D. (1992). Alternative pathways for the sorting of soluble vacuolar proteins in yeast: a *vps35* null mutant missorts and secretes only a subset of vacuolar hydrolases. Mol. Biol. Cell *3*, 415–427.

Piper, R.C., Cooper, A.A., Yang, H., and Stevens, T.H. (1995). VPS27 controls vacuolar and endocytic traffic through a prevacuolar compartment in *Saccharomyces cerevisiae*. J. Cell Biol. *131*, 603–617.

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

Roberts, C.J., Raymond, C.K., Yamashiro, C.T., and Stevens, T.H. (1991). Methods for studying the yeast vacuole. In: Guide to Yeast Genetics and Molecular Biology, vol. 194, ed. C. Guthrie and G.R. Fink, New York: Academic Press, 644–661.

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

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

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

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

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Seaman, M.N.J., Marcusson, E.G., Cereghino, J.L., and Emr, S.D. (1997). Endosome to Golgi retrieval of the vacuolar protein sorting receptor, Vps10p, requires the function of the *VPS29*, *VPS30*, and *VPS35* gene products. J. Cell Biol. *137*, 79–92.

Serafini, T., Stenbeck, G., Brecht, A., Lottspeich, F., Orci, L., Rothman, J.E., and Wieland, F.T. (1991). A coat subunit of Golgi-derived non-clathrin-coated vesicles with homology to the clathrin-coated vesicle coat protein B-adaptin. Nature *349*, 215–220.

Sherman, F., Fink, G.R., and Lawrence, L.W. (1979). Methods in Yeast Genetics: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics *122*, 19–27.

Stack, J., Horazdovsky, B., and Emr, S. (1995). Receptor-mediated protein sorting to the vacuole in yeast: roles for a protein kinase, a lipid kinase and GTP-binding proteins. Annu. Rev. Cell Dev. Biol. *11*, 1–33.

Stack, J.H., Herman, P.K., Schu, P.V., and Emr, S.D. (1993). A membrane-associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole. EMBO J. *12*, 2195–2204.

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

Valls, L.A., Hunter, C.P., Rothman, J.H., and Stevens, T.H. (1987). Protein sorting in yeast: the localization determinant of yeast vacuolar carboxypeptidase Y resides in the propeptide. Cell *48*, 887–897.

Vater, C.A., Raymond, C.K., Ekena, K., Howald, S.I., and Stevens, T.H. (1992). The VPS1 protein, a homolog of dynamin required for vacuolar protein sorting in *Saccharomyces cerevisiae*, is a GTPase with 2 functionally separable domains. J. Cell Biol. *119*, 773–786.

Vida, T.A., Graham, T.R., and Emr, S.D. (1990). In vitro reconstitution of intercompartmental protein transport to the yeast vacuole. J. Cell Biol. *111*, 2871–2884.

Vida, T.A., Huyer, G., and Emr, S.D. (1993). Yeast vacuolar proenzymes are sorted in the late Golgi complex and transported to the vacuole via a prevacuolar endosome-like compartment. J. Cell Biol. *121*, 1245–1256.

Vida, T.V., and Emr, S.D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. J. Cell Biol. *128*, 779–792.

Vogelstein, D., and Gillespie, D. (1979). Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA. *76*, 615–619.

Wilsbach, K., and Payne, G.S. (1993). Vps1p, a member of the dynamin GTPase family, is necessary for Golgi membrane protein retention in *Saccharomyces cerevisiae*. EMBO J. *12*, 3049–3059.