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Alternative Pathways for the Sorting of Soluble Vacuolar Proteins in Yeast: A *vps35* Null Mutant Missorts and Secretes Only a Subset of Vacuolar Hydrolases

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vps35 mutants of *Saccharomyces cerevisiae* exhibit severe defects in the localization of carboxypeptidase Y, a soluble vacuolar hydrolase. We have cloned the wild-type *VPS35* gene by complementation of the vacuolar protein sorting defect exhibited by the *vps35-17* mutant. Sequence analysis revealed an open reading frame predicted to encode a protein of 937 amino acids that lacks any obvious hydrophobic domains. Subcellular fractionation studies indicated that 80% of Vps35p peripherally associates with a membranous particulate cell fraction. The association of Vps35p with this fraction appears to be saturable; when overproduced, the vast majority of Vps35p remains in a soluble fraction. Disruption of the *VPS35* gene demonstrated that it is not essential for yeast cell growth. However, the null allele of *VPS35* results in a differential defect in the sorting of vacuolar carboxypeptidase Y (CPY), proteinase A (PrA), proteinase B (PrB), and alkaline phosphatase (ALP). proCPY was quantitatively missorted and secreted by Δ*vps35* cells, whereas almost all of proPrA, proPrB, and proALP were retained within the cell and converted to their mature forms, indicating delivery to the vacuole. Based on these observations, we propose that alternative pathways exist for the sorting and/or delivery of proteins to the vacuole.

INTRODUCTION

Eucaryotic cells contain a number of distinct compartments characterized largely by the unique set of proteins residing within them. This high degree of organization requires specific mechanisms to sort and deliver proteins from their site of synthesis in the cytoplasm to their final intracellular or extracellular destination. The pathway for proteins destined for the lysosomal/vacuolar compartment of eukaryotic cells represents a well-studied example of an intracellular protein sorting pathway (Kornfeld and Mellman, 1989; Klionsky *et al.*, 1990). Lysosomal proteins, like proteins destined for secretion, are first translocated across the membrane of the endoplasmic reticulum and then are transported to the Golgi complex. Within the Golgi apparatus, the pathways diverge. Secretory protein traffic proceeds by a bulk flow, or default mechanism, to the cell surface, whereas lysosomal proteins contain additional sorting information that directs their delivery to the lysosome (Kornfeld, 1986; Kornfeld and Mellman, 1989).

In a variety of mammalian cells, the N-linked carbohydrate side chains on soluble lysosomal proteins are modified with mannose-6-phosphate residues that are recognized by specific membrane receptors that mediate lysosomal delivery (Kaplan *et al.*, 1977; Kornfeld and Mellman, 1989). The targeting signals on soluble proteins destined for the lysosome-like vacuole in the yeast *Saccharomyces cerevisiae* are not comprised of specific carbohydrate modifications but instead appear to reside directly within the polypeptide backbone of these proteins (Clark *et al.*, 1982; Schwaiger *et al.*, 1982; Johnson *et al.*, 1987; Valls *et al.*, 1987; Klionsky *et al.*, 1988).

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Table 1. Strains used in this study

Strain	Genotype	Reference
S. cerevisiae		
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al.*, 1988
SEY6211	*MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9*	Robinson *et al.*, 1988
SEY6210.5	*MATα/MATa leu2-3,112/leu2-3,112 his3-Δ200/his3-Δ200 ura3-52/ura3-52 trp1-Δ901/rp1-Δ901 suc2-Δ9/suc2-Δ9 ADE2/ade2-101 lys2-801/ LYS2*	Herman *et al.*, 1990
SEY35-17	SEY6210 *vps35-17*	Robinson *et al.*, 1988
GPY1135	SEY6211 *vps35Δ1::HIS3*	This study
DKY6224	*MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 pep4Δ1::LEU2*	Klionsky *et al.*, 1988
BHY11	SEY6211 *leu2-3::pBHY11(CPY-Inv LEU2)*	Horazdovsky, unpublished data
BHY157	SEY35-17 *leu2-3::pBHY11(CPY-Inv LEU2)*	This study
BHY158	BHY11 *vps35-17::pVPS35-10 (VPS35 TRP1)*	This study
E. coli		
JM101	*Δ(lac-pro) supE thi-1 F' traΔ36 lacI^q ΔM15 proAB*	Casadaban and Cohen, 1980
MC1066	*F$^-$ ΔlacXZY hsr- hsm$^+$ spsL galW galK trpC9830 leuB600 pyrF::Tn5*	Casadaban *et al.*, 1983
JF1754	*hsdR metB leuB hisB lac gal*	McNeil and Friesen, 1981
DH5α	*F$^-$ Φ80dlacZΔM15 endA1 hsdR17(r_k^- m_k^+) supE44 thi-1 recA1 gyrA96 relA1 Δ(lacZYA-argF)U169 deoR λ$^-$*	Bethesda Research Laboratories, Gaithersburg, MD

In an attempt to identify cellular components required for vacuolar protein sorting in *S. cerevisiae*, several genetic selection procedures have been employed. Each has resulted in the isolation of mutants that exhibit defects in vacuolar protein processing and/or localization (Jones, 1977; Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989). These vacuolar protein sorting (*vps*) mutants missort vacuolar enzyme precursors to the cell surface. Together, these mutants define more than 45 complementation groups. In addition to the observed sorting defects, analysis of vacuolar morphology by light and electron microscopy has revealed that several of the *vps* mutants possess abnormal vacuolar structures (Banta *et al.*, 1988). Based on these morphological studies, the *vps* mutants were assigned to three distinct classes. The majority of the mutants (called class A *vps* mutants) contains normal or slightly enlarged vacuole structures. A second class of mutants, class B, is characterized by the presence of multiple small vacuole-like organelles. Finally, class C mutants lack any compartment that resembles a wild-type vacuole.

Thirty-two mutant alleles of the *VPS35* locus were originally isolated using a gene fusion-based selection scheme for mutants that missort and secrete a carboxypeptidase Y-invertase (CPY-Inv) hybrid protein. *vps35* mutant cells also exhibited defects in the sorting of the authentic soluble vacuolar hydrolase carboxypeptidase Y (CPY) (Robinson *et al.*, 1988). Light and electron microscopic analysis revealed that *vps35* mutant cells contain morphologically normal vacuoles (class A) (Banta *et al.*, 1988). This indicates that *vps35* cells are competent for vacuole assembly and suggests that the *VPS35* gene product may be involved in the sorting of only a subset of vacuolar enzymes.

In an effort to better understand the role of the *VPS35* gene product in vacuolar protein sorting and delivery, we report here on the cloning and sequencing of the *VPS35* gene, the phenotypic consequences of a *vps35* null allele, and the identification and localization of the *VPS35* gene product. We describe a simple plate assay technique that we devised for cloning the *VPS35* gene. This technique should prove generally useful for the cloning of many other genes that affect the sorting or retention of proteins in the secretory pathway. Based on our findings, we suggest that the Vps35 protein (Vps35p) is a component of a membrane associated multiprotein complex that is required for the sorting of only a subset of vacuolar proteins.

MATERIALS AND METHODS

Strains and Media

The *S. cerevisiae* and *Escherichia coli* strains used are listed in Table 1. Bacterial strains were grown on standard media (Miller, 1972). Yeast strains were grown on yeast extract, peptone, dextrose (YPD) medium, on synthetic medium (SM) supplemented as necessary (Sherman *et al.*, 1979), or on Wickerham's minimal proline medium (WiMP) (Wickerham, 1946) supplemented with 0.2% yeast extract (WiMPYE).

Reagents

DNA restriction and modifying enzymes were from either New England BioLabs (Beverly, MA) or Boehringer Mannheim Biochemicals (Indianapolis, IN). Zymolyase 100T was obtained from Seikagako Kogyo (Tokyo, Japan). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside and isopropyl-β-D-thiogalactopyranoside were from Boehringer Mannheim Biochemicals. The Sequenase sequencing kit was from

United States Biochemical (Cleveland, OH). Deoxynucleotides and the Miniprep Plus kit were products of Pharmacia (Piscataway, NJ). Tran^{35}S label was from ICN (Irvine, CA). All other radiochemicals and the Multiprime DNA labeling kit were from Amersham (Arlington Heights, IL). The Elutrap Electro-Separation Chamber was purchased from Schleicher & Schuell (Keene, NH) and used as instructed by the manufacturer. GeneScreen was obtained from New England Nuclear (Boston, MA). Freund's complete and incomplete adjuvants were obtained from GIBCO (Grand Island, NY). All other chemicals including antiserum to glucose-6-phosphate dehydrogenase were from Sigma Chemical (St. Louis, MO). Antibodies to Kex2p were a gift from Kyle Cunningham, and the antisera to CPY, proteinase A (PrA), and alkaline phosphatase (ALP) were described previously (Klionsky *et al.*, 1988; Klionsky and Emr, 1989). The antisera to protease B (PrB) was a generous gift from Elizabeth Jones, Carnegie-Mellon University, Pittsburgh, PA.

Yeast Genetics and Transformation

Genetic crosses, sporulation of diploids, and dissection of tetrads were performed as previously described (Sherman *et al.*, 1979). Yeast transformations were performed by the method of Ito *et al.* (1983). Typically, 1 μg of plasmid DNA was added to 100 ml of competent cells. For integrative transformations, 200 ng of linear DNA fragment were used together with 10 μg of circular pBluescript plasmid (Stratagene, La Jolla, CA) as carrier DNA. For gene disruption, the 2.7-kb *Nci* I/*Xba* I fragment from pGPY55 (see below) was transformed into the diploid strain SEY6210.5. His$^+$ transformants were sporulated and the resultant asci dissected. Integrative mapping strains were constructed as follows. pVPS35-10 (see below) was linearized with *Asp*718I and used to transform BHY11 to Trp$^+$, yielding BHY158. BHY158 was crossed with BHY157, diploids were selected, sporulated, and the resultant asci dissected.

Cloning of VPS35 With an Invertase Plate Assay

SEY35-17 cells (*vps35-17 leu2-3,112*) carrying a plasmid encoding the CPY-Inv fusion protein (pCYI-50) (Johnson *et al.*, 1987) were transformed with a yeast genomic library contained in a pBR322-based *LEU2/CEN4/ARS1* shuttle vector (a generous gift from Philip Hieter, Johns Hopkins University School of Medicine, Baltimore, MD), and Leu$^+$ transformants were selected. Leu$^+$ colonies were replica plated onto selective SM fructose medium and incubated overnight at 26°C. An invertase assay solution containing 125 mM sucrose, 100 mM sodium acetate (pH 5.5), 10 μg/ml horseradish peroxidase, 8 units/ml glucose oxidase, 2 mM O-dianisidine (Johnson *et al.*, 1987; Klionsky *et al.*, 1988) was mixed with an equal volume of a 2.4% agar solution (at 50°C) and immediately poured over the replica colonies. After 5 min at room temperature, colonies secreting the CPY-Inv fusion protein turned red (indicating Vps$^-$ phenotype), and colonies with internal invertase activity remained white (Vps$^+$). Plasmids conferring the Vps$^+$ phenotype were isolated from these yeast cells as described previously (Braus *et al.*, 1985).

Vectors and Plasmid Constructions

E. coli plasmids pBluescript KS(+) and (−) were obtained from Stratagene. The *E. coli*-yeast shuttle vector pPHYC16 has been described by Herman and Emr (1990) and the 2μ plasmid pBHY10 by Horazdovsky, and Emr, (unpublished data). The *HIS3* gene was isolated from a plasmid kindly provided by E. Phizicky, Dept. of Biochemistry, University of Rochester, Rochester, NY. The pATH1 vector used in constructing the *trpE*-Vps35 fusion protein has been described elsewhere (Dieckmann and Tzagoloff, 1985). A 5.8-kb *Sph* I-*Xba* I complementing fragment from the originally isolated *vps35* complementing plasmid was subcloned into pPHYC16 to generate pGPY35. Plasmid pGPY45 was constructed by excising the 3.8-kb *Nci* I/*Sca* I fragment from pGPY35, filling in the *Sca* I site with Klenow and ligating the complementing fragment into the *Sma* I site of pBHY10. The integrative mapping plasmid, pVPS35-10, was constructed by inserting the *Nci* I/*Sca* I *VPS35*-containing fragment into the integrative mapping vector pPHY110 (Herman and Emr, 1990). For the deletion construct pGPY55, the 2.45-kb *Sna*BI fragment of pGPY35 was replaced by a blunt-ended 1.35-kb *Bam*HI/*Xho* I *HIS3* fragment. Recombinant *HIS3* plasmids were selected directly in the *hisB E. coli* strain JF1754 on M63 minimal medium lacking histidine.

Northern Blot Analysis

For Northern blot analysis, yeast RNA was prepared as previously described (Zitomer and Hall, 1976) and enriched for poly(A)$^+$ RNA according to Aviv and Leder (Aviv and Leder, 1972). RNA was separated on a formaldehyde-agarose gel as in Rave *et al.* (1979), transferred to GeneScreen membranes, and hybridizations were as described in Thomas (1980). The 2.45-kb *Sna*BI *VPS35* fragment used as probe was labeled using the Amersham Multiprime kit.

Sequence Analysis

A 2.8-kb *Sph* I/*Bam*HI and a 3-kb *Bam*HI/*Xba* I fragment from pGPY35 were cloned into the *E. coli* plasmid pBluescript KS(−) to generate pGP30 and pGP40. Exonuclease III-mung bean nuclease deletions were performed on both plasmids from both ends of the inserts as described in the Stratagene Bluescript manual. Double-stranded DNA from a number of deletion plasmids was isolated from *E. coli* strain DH5α using the Miniprep Plus kit from Pharmacia. Plasmid DNA was denatured and sequenced using the Sequenase sequencing kit (United States Biochemicals). The predicted protein sequence of Vps35p was compared with the contents of the NBRF, Genbank, and EMBL databases, using the FASTA and TFASTA programs (Pearson and Lipman, 1988) of the University of Wisconsin Genetics Computer Group sequence analysis package (Devereux *et al.*, 1984).

Preparation of Vps35p Specific Antisera

A gene fusion between the *E. coli trpE* gene and *VPS35* was constructed by subcloning a 0.5-kb *Eco*RV/*Cla* I fragment encoding amino acids 630–808 of Vps35p into *Sma* I/*Cla* I digested pATH1 (Dieckmann and Tzagoloff, 1985), generating an in-frame fusion gene. The fusion point was sequenced to ensure correct in-frame fusion. This *trpE*-Vps35 fusion protein was induced and prepared as previously described by Kleid *et al.* (1981) with the following modifications: induction was overnight, cells were broken in a freeze-thaw step, and 2% Triton X-100 was used instead of Nonidet P-40. After sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, the predominant 55-kDa band representing the fusion protein was excised and the protein was electroluted from the gel slices using a Schleicher & Schuell Electro-Separation chamber. Approximately 300 μg of the purified fusion protein were emulsified with Freund's complete adjuvant and injected intramuscularly and subcutaneously into a young male New Zealand white rabbit. After 4, 6, 8, and 10 wk the rabbit was boosted with ~50 μg of the fusion protein in an emulsion with Freund's incomplete adjuvant. Antisera were collected and screened by immunoprecipitation.

Cell Labeling and Immunoprecipitations

For whole-cell radiolabeling, cells were grown in WiMPYE supplemented with the appropriate amino acids to an optical density (OD) at 600 nm of 1. To initiate labeling, Tran^{35}S label was added (at final concentration of 150 mCi/ml) to cells at 10 OD_{600nm}/ml in WiMP, 1 mg/ml bovine serum albumin and the cells were incubated 5 min at 30°C. When necessary, the labeling reaction was chased by adding cold methionine to 10 mM for the appropriate amount of time. The reaction was terminated by adding trichloroacetic acid to a final concentration of 5%. For labeling of spheroplasts (Vida *et al.*, 1990), cells were grown in WiMP as above. Zymolyase 100T 10 μg/2 × 10^7 cells

were added, and the culture was incubated 30 min at 30°C. Spheroplasts were washed in 1.2 M sorbitol and labeled as described above in WiMP, 1.2 M sorbitol. The immunoprecipitations were as described (Klionsky *et al.*, 1988), except that three washes were performed: once with buffer A (50 mM tris(hydroxymethyl)aminomethane [Tris]-HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA) containing 0.5% Triton X-100, once with buffer A plus 2 M urea, and then again with buffer A. Samples were separated in 10% SDS-polyacrylamide gels as previously described (Laemmli, 1970).

Subcellular Fractionation

SEY6210 (wild-type) cells were spheroplasted and labeled 5 min as described above. The cultures were chased with cold methionine (10 mM) for 5 min, and the chase was stopped by diluting the labeled culture in one volume of ice-cold WiMP and incubating it on ice for 5 min. The spheroplasts were sedimented 3 min at 500 × *g*, resuspended in 2 ml of lysis buffer (10 mM triethanolamine, pH 7.2, 0.8 M sorbitol, 1 mM EDTA) containing a protease inhibitor cocktail (1 mM phenylmethylsulfonylfluoride [PMSF], 50 μg/ml leupeptin, 50 μg/ml pepstatin A, 100 μg/ml α_2-macroglobulin, 50 μg/ml antipain), and dounced 20–25 times in a glass tissue homogenizer. The lysate was centrifuged 3 min at 500 × *g*, and the unlysed spheroplasts were reextracted in the same buffer. The combined supernatants were centrifuged 15 min at 13 000 × *g* to generate a pellet (P13) and a supernatant (S13) fraction. Typically, 0.5-ml aliquots of the S13 fraction were adjusted to the following concentrations of one of several reagents: lysis buffer, 1 M NaCl, 4 M urea, or 1% Triton X-100. Extracts were incubated 10 min on ice and centrifuged 45 min at 100 000 × *g*. The supernatant (S100) was removed and precipitated in 5% trichloroacetic acid, the pellet (P100) was resuspended in 100 μl boiling buffer (50 mM Tris-HCl, pH 7.5, 1% SDS, 6 M urea, 1 mM EDTA). The trichloroacetic acid precipitations were held on ice for 20 min and then centrifuged 2 min at 13 000 × *g*. The trichloroacetic acid pellets were washed twice with acetone, dried, and resuspended in 100 μl boiling buffer. The samples were then immunoprecipitated as described above.

Sucrose Gradients with Vps35p

SEY6210 wild-type spheroplasts were labeled and lysed as described above, except the chase period was for 30 min. After removing the unlysed spheroplasts (3 min at 500 × *g*), the lysate was centrifuged for 45 min at 100 000 × *g*. The pellet fraction was suspended in 2.5 ml 60% (wt/wt) sucrose, 50 mM Tris-HCl, pH 7.2, loaded on the bottom of a 5-ml ultra-clear Beckman centrifugation tube, and overlaid with 2.5 ml of 35% (wt/wt) sucrose, 50 mM Tris-HCl, pH 7.2. The gradient was centrifuged for 18 h at 170 000 × *g* and was harvested from top to bottom in 0.45-ml fractions. The fractions were precipitated in 5% trichloroacetic acid and immunoprecipitated with the appropriate antisera as described above.

RESULTS

Cloning of the VPS35 Gene

Previously, we have described a selection procedure for the isolation of *vps* mutants in yeast that exploited the sorting behavior of a CPY-Inv hybrid protein (Bankaitis *et al.*, 1986; Robinson *et al.*, 1988). In wild-type cells (deleted for all endogenous genes encoding invertase, Δ*suc*), the targeting information contained in the CPY portion of the fusion protein leads to efficient sorting of the fusion protein to the vacuole (Johnson *et al.*, 1987). However, in *vps* mutant cells, the CPY-Inv hybrid protein is mislocalized and secreted from the cell. The secreted invertase activity leads to a selectable phenotype, the ability of these mutant cells to utilize sucrose, an invertase substrate, as a sole carbon source (Bankaitis *et al.*, 1986; Robinson *et al.*, 1988).

To clone the wild-type *VPS35* gene, we devised a simple plate overlay assay that directly tests single colonies for secretion of the CPY-Inv hybrid protein. SEY35-17 (*vps35-17 leu2-3,112*) cells containing a CPY-Inv fusion (pCYI-50) (Johnson *et al.*, 1987) were transformed with a yeast genomic library contained in a pBR322-based *LEU2/CEN4/ARS1* shuttle vector. Leu^+ transformants were selected on SM glucose plates supplemented with the appropriate amino acids. After 72 h, the transformants were replicated onto selective SM fructose plates and incubated for 24–36 h. The replica-plates were then overlaid with an invertase assay solution containing 1.2% agar (see MATERIALS AND METHODS). Invertase catalyzes the hydrolysis of sucrose to glucose and fructose (Goldstein and Lampen, 1975). This activity can be easily detected by colorimetric assays designed to quantitate the release of glucose from sucrose hydrolysis (Bankaitis *et al.*, 1986; Robinson *et al.*, 1988). Because yeast cells do not transport sucrose across the plasma membrane, this enzyme assay detects only the invertase activity that has been secreted from the cells. Transformant colonies containing cells that secreted the CPY-Inv hybrid protein produced glucose and turned red after ~5 min, indicating a Vps^- phenotype, whereas transformants that restored normal localization of the hybrid protein to the vacuole (Vps^+) remained white for >30 min (Figure 1).

Among 35 000 Leu^+ transformants assayed for CPY-Inv hybrid protein secretion, 17 remained white after the overlay assay, indicating that they represented potential *VPS35* gene clones. Plasmid DNA was extracted from eight of these transformants and amplified in *E. coli*. On retransformation, the plasmids restored intracellular localization of the CPY-Inv hybrid protein in strains carrying either of two different *vps35* alleles. Restriction enzyme analysis of the complementing plasmids demonstrated that each contained largely overlapping genomic DNA inserts. A restriction map of the smallest of these inserts, a 7.5-kb complementing fragment, is shown in Figure 2A. Subcloning and complementation analysis localized the complementing activity to a 3.5-kb *Nci* I/*Sca* I fragment. The complementing *Nci* I/*Sca* I fragment (see Figure 2A) also was subcloned into the 2μ vector pBHY10 to generate the plasmid pGPY45. Yeast transformants harboring this plasmid overproduced the *VPS35* gene product ~30-fold (see below). Transformation of SEY35-17 or wild-type strains with pGPY45 resulted in a Vps^+ phenotype, and growth rates of the strains were unaffected, indicating that severalfold overproduction of the *VPS35* gene product was not detrimental to cell growth.

To confirm that the cloned genomic fragment contained the *VPS35* locus, a *Nci* I/*Sca* I fragment (Figure 2A) was subcloned into an integrative plasmid (pPHYI10) containing the *TRP1* selectable marker. The

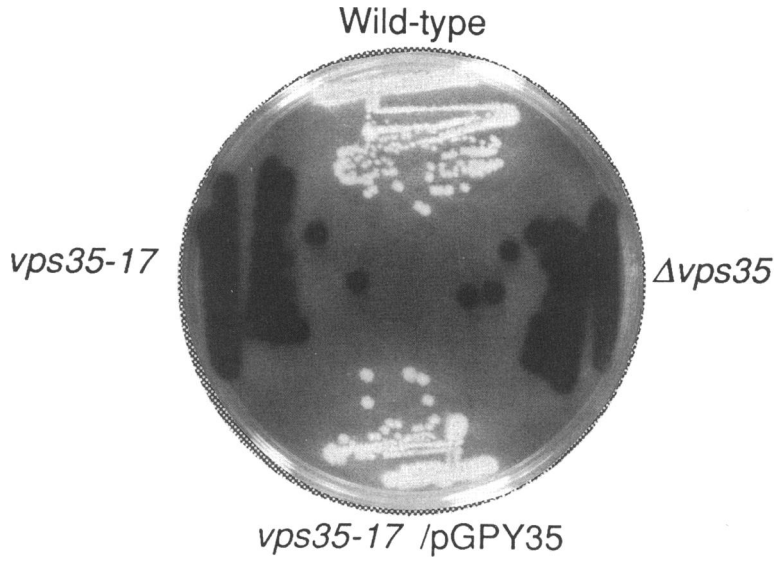


Figure 1. Invertase plate assay to detect complementation of *vps35-17* by the cloned *VPS35* gene. The parental strain SEY6210 (wild-type), the mutant strain SEY35-17 (*vps35-17*), the mutant strain SEY35-17 carrying plasmid pGPY35 (*vps35-17*/pGPY35), and the deletion strain GPY1135 (Δ*vps35*) were streaked on SM glucose plates. After incubation at 30°C for 72 h, the cells were replica plated onto SM fructose. The replica plates were then overlaid with an invertase assay solution containing 1.2% agar. All the strains carried a plasmid encoding a CPY-Inv fusion protein. Colonies secreting the fusion protein turned red (indicating Vps^- phenotype), whereas the others remained white (indicating a Vps^+ phenotype).

plasmid was linearized at an unique restriction enzyme site within the *VPS35* complementing fragment and was used to transform BHY11 (*VPS35 trp1*) to Trp^+. Trp^+ transformants (BHY158) were then crossed with BHY157 (*vps35 trp1*), and diploids were selected and sporulated. Twenty tetrads were dissected and gave 2: 2 segregation of Trp^+:Trp^- and of Vps^+:Vps^-. Trp^+ cosegregated with Vps^+ in every case, indicating that the cloned DNA fragment mapped to the *VPS35* locus.

Disruption of VPS35

To examine the phenotypic consequences of deleting the *VPS35* gene, we replaced a 2.45-kb *Sna*BI fragment of the minimum complementing clone with the *HIS3* gene (Figure 2B). An *Nci* I/*Xba* I fragment containing this deletion construct was used to transform the parental diploid strain SEY6210.5, which is homozygous for the *his3-Δ200* mutation. Two independent His^+ transformants were subjected to tetrad analysis. All four spores from one tetrad were analyzed by Southern blot to verify correct integration and subsequent segregation of the deletion construct. Sixteen spores of four more tetrads were transformed with the *PRC1-SUC2* gene fusion on a low copy-number plasmid (Johnson *et al.*, 1987) and assayed for secretion of the CPY-Inv hybrid protein. As expected for the disruption of the *VPS35* gene, a segregation pattern of two Vps^- (CPY-Inv secreting), His^+:2 Vps^+ (CPY-Inv non-secreting), His^- spores was observed. After sequencing the minimum complementing fragment (see below), we determined that the deleted fragment comprised >85% of the *VPS35* coding region (Figure 2B). As implied in the results above, all haploid progeny were viable, and therefore, the *VPS35* gene is not essential for yeast cell growth or spore germination. Also, Δ*vps35* cells were viable at 37°C, consistent with the observation that none of the 32 previously isolated *vps35* alleles results in a temperature-sensitive growth defect.

To examine vacuolar morphology in the deletion strain, Δ*vps35* cells were stained with 5(6)-carboxy-2′,7′-dichlorofluoresceon diacetate to label the vacuoles (Pringle *et al.*, 1989). Strains harboring a disrupted *vps35* gene invariably contained one to three large vacuolar compartments similar to those observed in wild-type cells or other *vps35* mutants (Banta *et al.*, 1988). Therefore, the *VPS35* gene product is not required for maintaining the integrity or structure of the vacuole. Moreover, a vacuole was also observed in all newly forming buds, indicating that vacuolar inheritance/segregation is not affected in Δ*vps35* mutants.

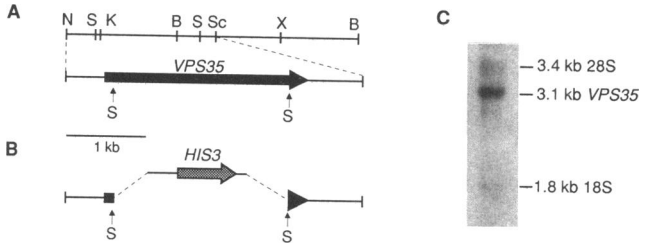


Figure 2. Characterization and disruption of the *VPS35* locus. (A) Restriction map of the entire 7.5-kb *VPS35* complementing fragment. The black arrow represents the *VPS35* open reading frame in an enlargement of the 3.5-kb *Nci* I/*Sca* I *VPS35* minimum complementing fragment. Restriction enzymes are as follows: *Bam*HI, B; *Kpn* I, K; *Nci* I, N; *Sca* I, Sc; *Sna*BI, S. (B) *VPS35* gene disruption/deletion. The *VPS35* open reading frame was largely deleted by replacing a 2.45-kb *Sna*BI internal fragment with the yeast *HIS3* gene. The stippled arrow represents the *HIS3* coding region. (C) Identification of the *VPS35* transcript. Poly(A)$^+$ RNA was separated on a formaldehyde-agarose gel, transferred to GeneScreen, and probed with a [α-^{32}P]labeled *VPS35* DNA fragment internal to the coding sequence. Yeast rRNAs 18S and 28S were used as size markers.

Sorting of native CPY in the wild-type strain, the Δ*vps35* mutant strain, and the Δ*vps35* strain transformed with the complementing plasmid pGPY35 also was examined. Spheroplasts of the appropriate strains were labeled with Tran^{35}S label for 5 min and chased for 60 min. The cultures were then fractionated into supernatant and pellet fractions, and CPY was immunoprecipitated. In wild-type yeast cells, >95% of the newly synthesized CPY was present as a 61-kDa mature species in an intracellular fraction (Figure 3), indicating correct localization to the vacuole. In contrast, Δ*vps35* spheroplasts contained <5% of the CPY as the mature species. The majority of the CPY was present as the Golgi-modified 69 kDa (p2) precursor molecule, and >90% of this p2CPY was secreted by the mutant cells (Figure 3). This severe sorting defect was corrected by introducing the complementing plasmid pGPY35 (*CEN, VPS35*) into the Δ*vps35* mutant strain (Figure 3).

To determine whether the protein sorting defect of Δ*vps35* cells extended to vacuolar proteins other than CPY, we also examined the sorting of proteinase A (PrA), proteinase B (PrB), and alkaline phosphatase (ALP). As is shown in Figure 4, ~80% of the vacuolar membrane protein ALP was converted to the mature enzyme (mALP) in the Δ*vps35* strain, consistent with proper delivery to the vacuole. Because of its retention as an integral membrane protein, no detectable levels of unprocessed precursor or mALP was found in the extracellular media fraction. A limited defect in the sorting and/or processing of ALP has also been observed with several other *vps* mutants, such as *vps15* (Robinson *et al.*, 1988; Klionsky and Emr, 1989; Herman *et al.*, 1991), indicating that membrane-associated proteins may be sorted to the vacuole by a pathway different from that utilized by soluble vacuolar proteins. However, unlike *vps*15 mutants, which are defective in the sorting and processing of all soluble hydrolases examined (CPY, PrA, and PrB), up to 80% of precursor PrA (proPrA) and ~80% of precursor PrB (proPrB) was converted to the mature vacuolar forms in Δ*vps35* spheroplasts after 30 min of chase (mPrA, mPrB) (Figure 4). The small portion of proPrA and proPrB found in the extracellular media was secreted from Δ*vps35* spheroplasts at essentially the same rate as that seen for p2CPY (proCPY). In addition, the maturation kinetics

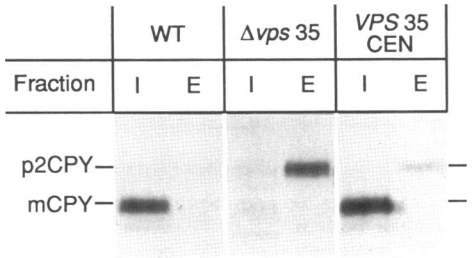


Figure 3. Intracellular sorting of CPY. Yeast spheroplasts were labeled with Tran^{35}S label for 5 min and then chased for 60 min with 10 mM cold methionine. The labeled cultures were centrifuged for 5 min at 13 000 × *g* and separated into pellet (I, intracellular) and supernatant (E, extracellular) fractions. The level of CPY in each fraction was assessed by quantitative immunoprecipitation. The strains used were SEY6210 (WT), GPY1135 (Δ*vps35*), and GPY1135 harboring pGPY35 (*VPS35 CEN*). The positions of p2CPY (69 kDa) and mature CPY (mCPY; 61 kDa) are indicated.

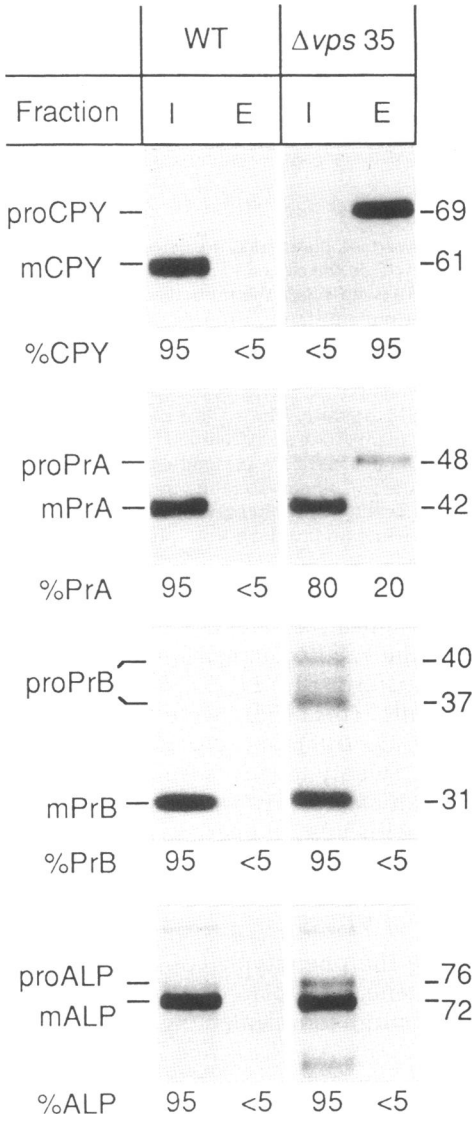


Figure 4. Sorting and processing of the vacuolar enzymes CPY, PrA, PrB, and ALP. Spheroplasts of the yeast strains SEY6210 (WT) and GPY1135 (Δ*vps35*) were labeled with Tran^{35}S label for 10 min and then chased for 30 min with 10 mM cold methionine and 0.2% yeast extract. Centrifugation at 13 000 × *g* for 5 min separated the cultures into pellet (I, intracellular) and supernatant (E, extracellular) fractions. Protein levels in each fraction were determined by quantitative immunoprecipitation with the appropriate antisera. The approximate molecular weights of the precursor (pro) and mature (m) forms of CPY, PrA, PrB, and ALP are indicated (Stevens *et al.*, 1982; Klionsky, *et al.*, 1988; Klionsky and Emr, 1989; Moehle, *et al.*, 1989).

for PrA and PrB in Δ*vps35* cells were also similar to those seen in wild-type cells. Thus, although CPY sorting was completely dependent on the *VPS35* gene product, the vast majority of proPrA and proPrB was apparently properly sorted and processed in the absence of the Vps35p.

These results differ somewhat from those seen in an earlier study (Robinson *et al.*, 1988). Far more proPrA was originally reported to be missorted in *vps35* mutant cells. To rectify this discrepancy, the vacuolar protein sorting defect of the *vps35* mutant cells used in the original study was compared with the sorting defect of the Δ*vps35* cells used in this study. In all cases the extent of maturation of CPY, PrA, PrB, and ALP in cells carrying the original *vps35* mutant allele was the same as in cells carrying the disrupted allele. Unlike the growth conditions used in the previous study, cells and spheroplasts used here were propagated in minimal media containing yeast extract before and after the labeling period. This helped to preserve the metabolic integrity and stability of the cells. The exclusion of yeast extract during growth and chase periods, therefore, is likely to have resulted in the exaggerated PrA maturation and sorting defect seen in the earlier study.

VPS35 Sequence Analysis

The nucleotide sequence of the *VPS35* minimum complementing fragment was determined by sequencing a series of nested exonuclease III generated deletion templates by the dideoxynucleotide chain termination method. The complete nucleotide sequence of both DNA strands was determined. The DNA sequence contained one long open reading frame (ORF) of 2810 bp that has the potential to encode a protein of 937 amino acids with a predicted molecular weight of 108 344 (Figure 5). Upstream of the translational initiation codon, at position −50, a TATATAA element was identified that closely resembles the yeast consensus TATAAA sequence for transcription initiation (Struhl, 1987). A sequence TAG . . . TAAG . . . TTT that closely approximates the proposed yeast transcriptional termination sequence according to Zaret and Sherman (1982) was found starting 89 nucleotides downstream of the ORF stop codon TAG (Figure 5). Northern analysis was performed using an internal fragment of the *VPS35* ORF as a probe to identify the *VPS35* transcript. Consistent with the ORF size predicted from the DNA sequence, a RNA species of 3100 bases was detected (Figure 2C).

Analysis of the deduced protein sequence indicated that Vps35p is relatively hydrophilic and contains nine potential N-linked glycosylation sites. No obvious N-terminal signal sequence or membrane-spanning domains were detected by hydropathy analysis. A comparison of the predicted protein sequence with sequences in the Genbank, EMBL, and NBRF databases failed to detect any sequence similarities of obvious significance (Lipman and Pearson, 1985; Pearson and Lipman, 1988). A search for sequence similarity using the 5′ flanking sequence of the *VPS35* gene revealed that this sequence is identical to the 3′ untranslated region of the *INO1* gene (Dean-Johnson and Henry, 1989). Using the TFASTA algorithm (Pearson and Lipman, 1988), it could be shown that the sequence overlap extended to the C-terminal amino acids of the Ino1 protein. Because the *INO1* locus has previously been mapped to the left arm of chromosome X (Mortimer *et al.*, 1989), we conclude that the yeast *VPS35* gene also maps to chromosome X.

Identification of the Vps35 Protein

To characterize the *VPS35* gene product, we prepared a polyclonal antiserum against a *trpE*-Vps35 fusion protein. A fragment encoding 178 amino acids (amino acids 630–808 in Figure 5) of the *VPS35* gene product was cloned into a *trpE* expression vector (Dieckmann and Tzagoloff, 1985) to generate an in-frame gene fusion. On induction, *E. coli* cells carrying this *trpE-VPS35* gene fusion produced a novel protein of 55 kDa. This hybrid protein was purified and used to immunize rabbits, and the resulting antiserum was utilized in immunoprecipitation experiments to detect Vps35p. The antibodies recognized a unique polypeptide of ~110 000 molecular weight from radiolabeled wild-type yeast extracts (Figure 6, lanes 3–6). This protein was ~30-fold more abundant when the *VPS35* gene was present on a multicopy plasmid (Figure 6, lane 7) and was not detected in a Δ*vps35* strain (GPY1135) or by the preimmune serum (Figure 6, lanes 1 and 2, respectively). These data indicated that the polyclonal antiserum specifically recognized the protein product of the *VPS35* gene. Pulse-chase analysis indicated the turnover rate for Vps35p is relatively slow; after a 60-min chase, there was only a slight decrease in the amount of labeled Vps35 protein present in wild-type cells (Figure 6, lane 5). Cells also were labeled after treatment with tunicamycin, an inhibitor of N-linked glycosylation. The size of Vps35p was unaffected by the drug, indicating that none of the nine potential sites for N-linked carbohydrate modification are utilized (Figure 6, lane 6) (The two minor protein species with a faster mobility than Vps35p seen after tunicamycin treatment were occasionally seen in cell extracts generated from Δ*vps35* strains and, therefore, do not represent a Vps35p species.) This result, together with the absence of any obvious N-terminal signal sequence or transmembrane domains, suggests that Vps35p does not enter the secretory pathway. Densitometric analysis of the levels of Vps35p relative to CPY suggested that Vps35p comprises ~0.01% of total cell protein in exponentially growing yeast cells. The overproduction of Vps35p did not interfere with processing and sorting

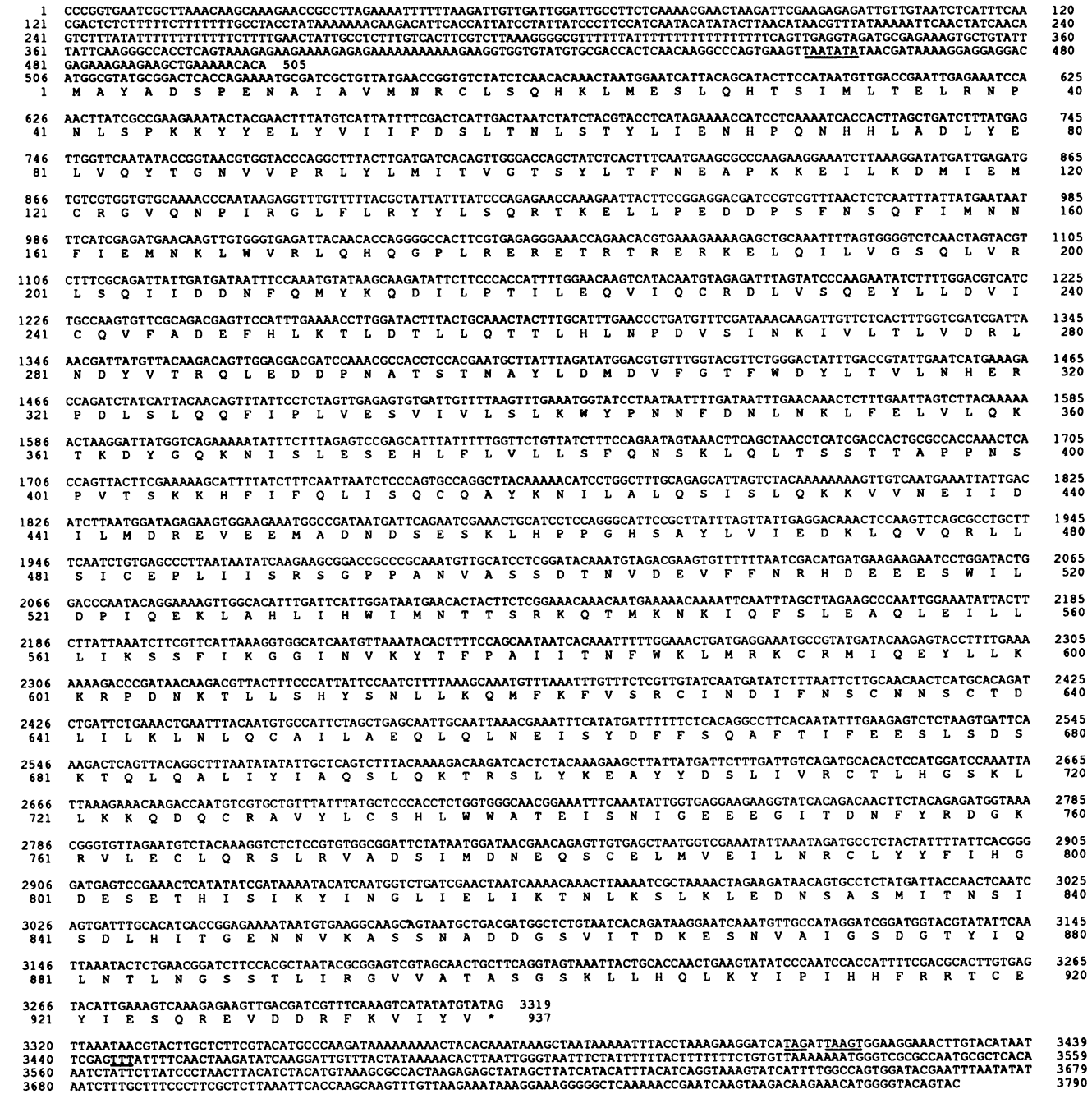


Figure 5. Sequence of the *VPS35* locus. The nucleotide sequence and the deduced amino acid sequence of the *VPS35* are shown. Putative transcription initiation and termination sequences are underlined.

of CPY or PrA. In addition, cells overproducing Vps35p did not exhibit any other obvious phenotype, such as temperature-sensitive growth or sensitivity to osmotic stress.

Protease protection experiments indicated that Vps35p is in contact with the cytosol. In these experiments, DKY6224 (Δ*pep4*) spheroplasts were labeled with Tran^{35}S label and gently lysed by the addition of DEAE-dextran under conditions that disrupt the plasma membrane but not internal organelles (Klionsky and Emr, 1990). After this lysis, Vps35p was found to be degraded by exogenously added protease K, whereas the precursor forms of CPY, p1 and p2, residing in ER and the Golgi compartments, respectively (Stevens *et al.*, 1982;

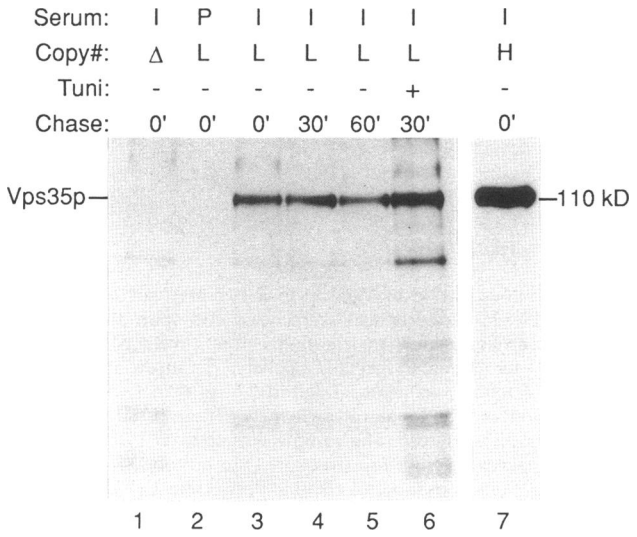


Figure 6. Identification and characterization of the Vps35 protein. Whole cells were labeled with Tran^{35}S label for 5 min and chased for the time indicated. Immunoprecipitations were performed with the clarified cell extracts using preimmune serum (P; lane 2) or the Vps35p immune serum (I; lanes 1 and 3–7). Copy number refers to the gene dosage of *VPS35* in the strains used: L is SEY6210 (single copy, lanes 2–6), Δ is GPY1135 (no copy, lane 1), and H is GPY1135 with plasmid pGPY45 (multicopy, lane 7). Exposure time of lane 7 containing overproduced Vps35p is only 1/10 of lanes 1–6. Where indicated (+), Tunicamycin was added to 20 μg/ml 15 min before radiolabeling (lane 6). The position and size of Vps35p is indicated.

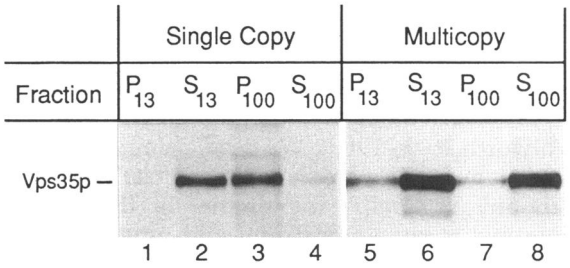
Franzusoff and Schekman, 1989), were resistant to proteolysis. These results indicate that Vps35p is not sequestered in the lumen of an intracellular organelle.

Subcellular Fractionation of the Vps35 Protein

Differential centrifugation techniques were used to more precisely determine the intracellular location of Vps35p. SEY6210 spheroplasts (single copy *VPS35*) were radiolabeled, lysed under conditions that maintain the structural integrity of internal organelles, and the crude lysates were spun at 500 × *g* to remove unlysed spheroplasts. This supernatant was centrifuged at 13 000 × *g* to generate a supernatant (S13) and a pellet (P13) fraction. The S13 supernatant was spun at 100 000 × *g* to obtain another set of supernatant (S100) and pellet (P100) fractions. The relative level of Vps35p in each fraction was assayed by immunoprecipitation (Figure 7, A and B). When wild-type cell lysates were examined, a small portion of Vps35p (15%) was found in the P13 pellet, but the majority of the protein (80%) was sedimented only at 100 000 × *g* (Figure 7, A, lanes 1–4, and B). In contrast, a vacuolar membrane marker protein, mALP, was found predominantly in the P13 fraction (Figure 7B). Under comparable conditions, the P13 pellet has been shown to contain other intracellular organelles, such as nuclei (Hurt *et al.*, 1988) and mitochondria (Goud *et al.*, 1988; Walworth *et al.*, 1989). Interestingly, p2 CPY and Kex2p, which most likely reside in a late Golgi compartment (Julius *et al.*, 1984; Graham and Emr, 1991), are mainly found in the P100 pellet (Figure 7B). In addition to Golgi compartments, the P100 fraction has also been found to contain vesicular intermediates that transit between secretory pathway organelles (Walworth *et al.*, 1989). Thus, if Vps35p is associated with an organelle, possible candidates include transport vesicles and the Golgi apparatus but, importantly, not the vacuole.

In a similar experiment, lysates of SEY6210 cells harboring the *VPS35* gene on the multicopy plasmid pGPY45 were separated into pellet and supernatant fractions at 13 000 and 100 000 × *g*, and the distribution of Vps35p was also determined (Figure 7, A, lanes 5–8, and B). Unlike in the wild-type strain, the vast majority of Vps35p was found in the supernatant S100 fraction in cells overproducing this protein. Apparently, Vps35p associates with only a limited number of sites present in the P100 fraction. These interaction sites ap-

A



B

Protein	P13	P100	S100
Vps35p (single copy)	15%	80%	5%
Vps35p (multicopy)	20%	10%	70%
ALP (vacuole memb.)	90%	10%	<2%
Kex2p (late Golgi)	15%	85%	<2%
p2CPY (late Golgi)	10%	65%	25%
G6PDH (cytosol)	5%	5%	90%

Figure 7. Subcellular fractionation of the Vps35 protein. (A) Spheroplasts of SEY6210 (single copy *VPS35*, lanes 1–4) and GPY1135 harboring plasmid pGPY45 (multicopy *VPS35*, lanes 5–8) were labeled, osmotically lysed, clarified (500 × *g*), and separated into pellet (P13) and supernatant (S13) fractions by centrifugation at 13 000 × *g*. A portion of the S13 fraction was then centrifuged at 100 000 × *g* to generate a second set of pellet (P100) and supernatant (S100) fractions. Vps35p was isolated by immunoprecipitation. The exposure time for lanes 1–4 was 120 h. The exposure time for lanes 5–8 was 15 h. (B) Quantitation of the amount of Vps35p and subcellular marker proteins present in the cell fractions described in A. The marker proteins include alkaline phosphatase (ALP), the Kex2 protease (Kex2p), the Golgi modified precursor of CPY (p2 CPY), and glucose-6-phosphatase dehydrogenase (G6PDH).

pear to be saturated in strains that overproduce Vps35p, and the excess Vps35p remains in the 100 000 × *g* supernatant.

The nature of the association of Vps35p with the particulate fraction P100 was investigated by treating the S13 fraction containing the Vps35p with various reagents for 10 min at 4°C: 1 M NaCl, 4 M urea, and 1% Triton X-100. The lysates were then centrifuged at 100 000 × *g* for 45 min, and the pellet and supernatant fractions were assayed for the presence of Vps35p by immunoprecipitation (Table 2). High salt concentrations and urea extracted 95 and 90% of Vps35p from the particulate fraction, respectively. These findings suggest that ionic and hydrophobic interactions may stabilize the association of Vps35p with a pelletable structure. In addition, treatment of the cell lysates with Triton X-100 also solubilized the vast majority of the particulate Vps35p, indicating that Vps35p may be associated with a cellular membrane fraction.

To further address the role of membranes in the association of Vps35p with the P100 fraction, the partitioning of Vps35p with cellular membranes was examined using sucrose density gradients. Sucrose gradients can separate organelles and membranous structures on the basis of their equilibrium density. This approach has been used successfully to localize proteins to particular compartments in the secretory pathway (Walworth *et al.*, 1989). We used a simple two-step sucrose gradient to establish whether Vps35p is associated with any organellar membrane. In this experiment spheroplasts generated from wild-type cells were radiolabeled and lysed. After removal of unbroken cells at 500 × *g*, the lysate was separated into pellet and supernatant fractions at 100 000 × *g* for 45 min. The pellet was resuspended in 60% sucrose and overlaid with a 35% sucrose solution. After centrifugation of the gradient at 170 000 × *g* for 18 h, fractions were collected and the amount of Vps35p and the vacuolar membrane marker ALP in each fraction was determined by immunoprecipitation. ALP migrated to the top of the 60% sucrose layer and into the 35% sucrose layer (Figure 8). The same result was found for a membrane protein of a late Golgi compartment, Kex2p. In similar experiments membrane proteins of other organelles, such as the plasma membrane ATPase, Sec4p (a protein located on the cytoplasmic surface of secretory vesicles), and mitochondrial cytochrome c-oxidase, were shown to migrate out of the 60% sucrose solution into fractions of lesser density (Goud *et al.*, 1988; Walworth *et al.*, 1989). A small portion of Vps35p remained in the 60% sucrose layer, unaffected by the centrifugation. However, most of Vps35p migrated out of the 60% sucrose solution and into the 35% sucrose layer similar to ALP. Thus, the results from the fractionation studies suggest that Vps35p is associated with an intracellular membrane, most likely as a component of a large multiprotein complex.

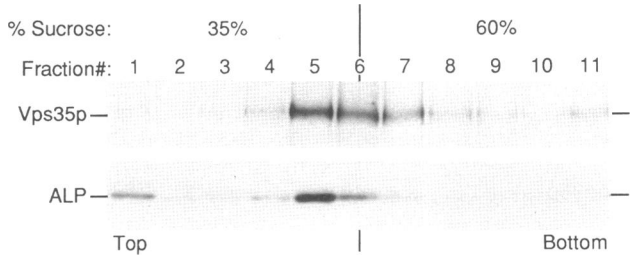


Figure 8. Localization of Vps35p in a two-step sucrose gradient. Labeled SEY6210 spheroplasts were lysed and spun at 500 × *g* to remove unbroken cells. The clarified extracts were centrifuged at 100 000 × *g* for 45 min and yielded a pellet and a supernatant fraction. The pellet fraction was resuspended in 2.5 ml 60% sucrose, loaded on the bottom of a Beckman ultra-clear centrifuge-tube, and overlaid with 2.5 ml 35% sucrose. The sample was spun at 170 000 × *g* for 18 h and harvested from top (fraction 1) to bottom (fraction 11) in 0.45-ml fractions. Vps35p and alkaline phosphatase (ALP) were isolated from each gradient fraction by quantitative immunoprecipitation with the appropriate antisera.

Table 2. Characterization of the particulate Vps35 protein[a]

Treatment	S100[b] (%)	P100[b] (%)
Control	<5	95
1 M NaCl	95	<5
4 M Urea	90	10
1% Triton X-100	95	<5

[a] The relative levels of the Vps35 protein in the P100 and S100 subcellular fractions were determined after the treatment of wild-type clarified cell lysate with the reagents listed above. See Figure 7 and text for complete experimental details.

[b] Percent recovery of Vps35p in the S100 vs. P100 fraction.

DISCUSSION

To gain a better understanding of the role the *VPS35* gene product plays in vacuolar protein sorting, the *VPS35* gene was cloned, its protein product analyzed, and the phenotypic consequences of a *vps35* null allele were assessed. Several *VPS* genes have been isolated by complementation of a temperature-sensitive growth defect associated with certain *vps* mutants (Banta *et al.*, 1990; Herman and Emr, 1990; Herman *et al.*, 1990; Rothman *et al.*, 1990). However, none of the 32 originally isolated *vps35* alleles result in a temperature-conditional growth defect, so an alternative assay was devised to isolate the *VPS35* gene from a yeast vacuole (Bankaitis *et al.*, 1986; Robinson *et al.*, 1988). However, *vps* mutants do not sort the CPY-Inv fusion protein to the vacuole but instead secrete the hybrid from the cell. This extracellular invertase activity can be easily detected by overlaying mutant yeast colonies with a soft agar solution containing reagents that detect invertase activity. Using this simple assay, we were able to identify

a yeast genomic clone capable of complementing the CPY-Inv mislocalization phenotype associated with *vps35* cells and have shown that the complementing DNA fragment contains the *VPS35* gene. Recently, several other *VPS* genes have been isolated with the help of this assay (Horazdovsky and Emr, unpublished data; Koehrer and Emr, unpublished data; Gharakanian and Emr, unpublished data), and we believe that this method will be useful for cloning many other genes that regulate the intracellular sorting or retention of proteins in the secretory pathway.

Some insights into the possible function of Vps35p were provided by subcellular fractionation studies. Several lines of evidence indicate that Vps35p resides in the cytoplasm of the yeast cell as part of a membrane associated, multiprotein complex. 1) Vps35p sediments with a particulate fraction isolated from yeast lysates. 2) The *VPS35* gene product does not contain an obvious signal sequence or transmembrane domains and N-linked carbohydrates are not added to Vps35p, even though it has nine potential sites for N-linked glycosylation. 3) Vps35p is degraded by protease added externally to intact organelles isolated from gently lysed spheroplasts and is, therefore, not sequestered in the lumen of an organelle. 4) Vps35p is released from the particulate fraction by several reagents, including urea and high salt, that disrupt protein–protein interactions, and is also released by treatment with Triton X-100. 5) Vps35p has been shown to partition with cellular membranes in sucrose gradients. Based on these observations, we conclude that Vps35p's association with the high-speed pellet results from interactions with a membrane associated protein or protein complex. Interestingly, the interaction of Vps35p with the particulate fraction is saturable. In wild-type yeast cells, Vps35p is largely pelleted at 100 000 $\times$ *g* (80%), whereas in cells overproducing Vps35p, the vast majority remains in the supernatant fraction (Figure 7). This observation indicates that the association site(s) for Vps35p is specific and limiting. The exact composition of the Vps35p association complex is unknown but probably includes other proteins, as overproduced Vps35p fractionates as a soluble protein. Attempts to localize Vps35p in wild-type cells using indirect immunofluorescence techniques have failed thus far. In a strain overproducing Vps35p, only cytoplasmic staining was observed, supporting the finding that in these cells most of the overexpressed Vps35p is in the soluble fraction.

Unexpectedly, our analysis of the vacuolar protein sorting defects in Δ*vps35* mutants indicates that Vps35p function may only be required for the sorting of a small subset of soluble vacuolar proteins. Even though Δ*vps35* cells contain a morphologically wild-type vacuole, CPY sorting is almost completely blocked in the *vps35* null mutant; >95% of CPY is secreted as its Golgi modified p2 precursor form (proCPY, Figure 4). However, the vast majority of two other soluble proteases, PrA and PrB, are retained and matured in Δ*vps35* mutant cells (Figure 4). This result indicates that CPY sorting is completely dependent on the presence of functional Vps35p. Yet, the sorting and processing of other soluble proteases (PrA and PrB) can occur in a Vps35p-independent manner. Apparently, only a subset of soluble vacuolar proteins, like CPY, may require Vps35p for their efficient sorting to the vacuole.

We provide three possible models to explain the Vps35p dependent/independent sorting of soluble vacuolar hydrolases. First, soluble vacuolar proteins could use two (or more) completely independent delivery systems. In *vps35* mutant cells, the specific sorting system responsible for vacuolar delivery of CPY and presumably other unknown proteins would be inactivated, yet an independent PrA and PrB delivery system would remain intact. As a result, CPY would be secreted from the cell, whereas PrA and PrB would be delivered to the vacuole and converted to their mature forms. The presence of completely independent systems for the localization of different subsets of vacuole proteins cannot be ruled out. However, because a number of *vps* mutants mislocalize CPY, PrA, and PrB to the same extent (Robinson *et al.*, 1988; Herman *et al.*, 1991; Horazdovsky, and Emr, unpublished data), truly independent delivery systems seem unlikely. In the second model, the active sorting of soluble vacuolar proteins may involve a single receptor complex that is required for the recognition of all soluble vacuolar proteins. This receptor complex could possess different affinities for soluble vacuolar proteins (high affinity for proPrA and proPrB but a lower affinity of proCPY), or, alternatively, different vacuolar proteins may occupy the receptor compartment for differing periods of time providing some proteins increased access to the receptor (such as proPrA and proPrB). If receptor complexes were to become limiting (in the absence of Vps35p) then proCPY, being unable to efficiently compete for receptor binding, would be secreted from the cell. This seems unlikely, however, as it has been shown previously that overexpression of PrA does not result in the missorting or secretion of CPY (Rothman *et al.*, 1986). If a single receptor complex with a higher affinity for PrA than for CPY is involved in the sorting pathway, the overexpression of PrA would be expected to result in the secretion of CPY. This is not the case. In addition, the near normal maturation kinetics of PrA and PrB in Δ*vps35* cells (as well as the similar secretion rates for p2CPY and the small amount of proPrA and proPrB that are secreted in this mutant) further indicate that the delivery of PrA and PrB is largely unaffected by the loss of *VPS35* gene product function and a longer residence time in the proposed sorting compartment cannot account for their vacuolar delivery. Finally, in the third model, CPY, PrA, and PrB could use the same transport system (vesicle carriers) to mediate their Golgi to vacuole delivery but may utilize different membrane receptors for their selective pack-

aging into a common carrier. One receptor complex may recognize CPY, whereas a second receptor complex may recognize PrA and PrB. Though Vps35p does not resemble a receptor molecule, it may play a role in the function, modification, or packaging of the CPY-specific receptor complex. Loss of Vps35p function would inactivate the CPY-specific receptor and result in default secretion of CPY. PrA, PrB, and ALP localization would be largely unaffected in this model, as their delivery would depend on a different receptor complex(es). Present data are most consistent with this final model. Direct proof for the role of multiple receptor complexes in this protein-sorting process will require the identification of these complexes. Unfortunately, no good candidate for a receptor molecule has yet been identified among the presently characterized *VPS* gene products. To learn more about the detailed biochemical function of Vps35p, we plan to assay its role in a recently developed in vitro reconstitution assay for vacuolar protein sorting (Vida *et al.*, 1990), as well as identify cellular components that interact with the *VPS35* gene product using both genetic and biochemical approaches.

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