The Yeast VPS17 Gene Encodes a Membrane-associated Protein Required for the Sorting of Soluble Vacuolar Hydrolases*

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vps17 mutants missort and secrete several vacuolar hydrolases. To analyze the role of the VPS17 gene in vacuolar protein delivery, we have cloned this gene by complementation of the vacuolar protein sorting defects of a vps17–5 mutant. Disruption of the VPS17 gene had no effect on the viability of haploid yeast cells, although they show an obvious defect in vacuolar morphology. vps17-disrupted cells contain numerous small vacuole-like compartments and also exhibit a severe defect in the sorting of carboxypeptidase Y (CPY), a soluble vacuolar hydrolase. 95% of CPY is missorted and secreted from the mutant cells. Vacular sorting of two other soluble hydrolases, proteinase A and proteinase B, is also affected, but to a lesser extent. Delivery and maturation of the vacuolar membrane protein alkaline phosphatase does not appear to be affected in a Δvps17 strain.

The DNA sequence of the VPS17 clone indicates that the gene encodes a 551-amino-acid protein with a calculated molecular mass of 63.1 kDa. The protein sequence is hydrophilic and contains no obvious N-terminal signal sequence or hydrophobic membrane-spanning domains, indicating that the Vps17p does not enter the secretory pathway. Using a Vps17p-specific polyclonal antiserum, we have demonstrated that the Vps17 protein is not modified with N-linked carbohydrates at any of its four potential N-linked glycosylation sites. The Vps17 protein, however, fractionates to a particulate fraction after centrifugation at 100,000 × g. Vps17p can be released from this particulate fraction by treatment with either Triton X-100 or urea, indicating that the Vps17p is peripherally associated with a crude membrane fraction.

Based on these results, we propose that the Vps17p functions on the trans-Golgi network and the endoplasmic reticulum to facilitate the sorting and delivery of soluble vacuolar hydrolases. Vacular membrane protein traffic, however, appears to occur by a mechanism that is independent of Vps17p function.

Eukaryotic cells are highly compartmentalized containing many different membrane-enclosed organelles that are specialized for different tasks. These specialized compartments are largely maintained by a constant flow of a unique set of proteins that must be accurately sorted and delivered to each compartment. The five major targets for noncytoplasmic proteins in most eukaryotic cells include the secretory system, mitochondria, peroxisomes, lysosomes, and the nucleus. Proteins destined for the cell surface and the lysosome transit together through the early stages of the secretory pathway. After translocation into the ER1 and addition of core oligosaccharides, proteins that are not destined to be retained in the ER are transported to and through the Golgi complex via transport vesicles (Novick et al., 1980, 1981; Kaiser and Schekman, 1990). In the Golgi, most proteins undergo further post-translational modifications (addition of outer chain carbohydrates, elongation of O-linked oligosaccharides (Tanner and Lehle, 1987) prior to transport out of the late Golgi. Protein transport to the cell surface appears to occur by a default or bulk flow mechanism, whereas the transport of lysosomal proteins requires specific sorting signals (Kornfeld, 1986; Kornfeld and Mellman, 1989). Soluble lysosomal proteins of mammalian cells are modified with mannose 6-phosphate residues on N-linked carbohydrate chains, which are recognized by receptors that mediate sorting and delivery to the lysosome (Kaplan et al., 1977; von Figura and Hasilik, 1986; Kornfeld, 1987). In contrast, the sorting information present in yeast lysosomal/vacuolar proteins is not associated with any specific carbohydrate modification and instead appears to reside within the polypeptide backbone of these proteins (Clark et al., 1982; Schaizer et al., 1982; Valls et al., 1987; Johnson et al., 1987; Klionsky et al., 1988, 1990; Klionsky and Emr, 1990; Winther et al., 1991).

Genetic selections in yeast have identified more than 40 vps (ups for vacuolar protein sorting defective) complementation groups that are required for the sorting and delivery of vacuolar hydrolases (Bankaitis et al., 1986; Rothman and Stevens, 1988; Robinson et al., 1988; Rothman et al., 1989a, 1989b). All vps mutants missort and secrete vacuolar enzyme precursors instead of delivering them to the vacuole. Since protein secretion and post-translational modification seems to be normal in most of the vps mutants, these mutants are presumed to be either defective in components of the sorting
machinery or in the assembly and maintenance of the acceptor compartment, the vacuole. The *ups* mutants have been grouped into three major classes, according to their vacuolar morphology (Banta et al., 1988). Class A *ups* mutants appear to have normal vacuoles, whereas class B mutants contain several small fragmented vacuole-like compartments, as shown by vacuole-specific fluorescent staining procedures (Banta et al., 1988). Class C mutants lack vacuolar structures and instead accumulate other membrane compartments including, vesicles, multilamellar membrane structures, and Golgi-like structures (Banta et al., 1988). Thus far, eight of the *VPS* genes have been cloned and characterized (Dulic and Riezman, 1989; Banta et al., 1990; Herman and Emr, 1990; Raymond et al., 1990; Rothman et al., 1990; Wada et al., 1990; Woolford et al., 1990; Herman et al., 1991a; Preston et al., 1991; Robinson et al., 1991; Paravicini et al., 1992).

Using a gene fusion-based selection scheme for *ups* mutants (Bankaitis et al., 1988; Robinson et al., 1988), we identified 11 alleles of the *ups17* locus, and each was found to missort precursor forms of the soluble vacuolar hydrolases carboxypeptidase Y (CPY), proteinase A (PrA), and proteinase B (PrB) (Robinson et al., 1988). Further complementation analysis revealed that *ups17* and *pep21* define the same complementation group (Rothman et al., 1989a). Light and electron microscopic analysis have demonstrated that representative alleles of the *ups17* complementation group exhibit class B vacuolar morphology. Many small vacuole-like compartments (10–30) are present in the mutant cells, rather than the one to three large vacuoles normally seen in wild-type cells (Banta et al., 1988).

In this study, we report on the cloning and sequencing of the *VPS17* gene, the identification and localization of the *VPS17* gene product, and the biochemical and morphological consequences of a *ups17* null allele. Our results support the model that there are alternative delivery mechanisms to the vacuole yeast and that the Vps17p appears to be required for the efficient sorting of only a subset of vacuolar proteins.

### EXPERIMENTAL PROCEDURES

**Materials**—Boehringer Mannheim and New England BioLabs (Beverly, MA) were the suppliers for all DNA restriction and modifying enzymes used in this study. Deoxynucleotides, 5-bromo-4-chloro-3-indolyl-β-D-galactoside and 5(6)-carboxy-iodoacetamido-4-methylumbelliferyl-β-D-galactoside were purchased from ICN Chemicals (Irvine, CA). α-[35S]dATP, ['32P]orthophosphate, [3H]thymidine, deoxyadenosine triphosphate, and the multiprime DNA labeling kit from EN Radiochemicals (Irvine, CA). [α-35S]dATP, ['32P]dATP, ['32P]dCTP, and the multiprime DNA labeling kit from Amersham Corp. Pall Biodyne transfer membranes were purchased from ICN Chemicals & Radioisotope Division (Irvine, CA). The Elutrap Electro-Separation Chamber was obtained from Schleicher & Schuell. Zymolyase 100T (Kirin Brewery Co.) is a product from Seikagaku Kogyo Co. (Tokyo, Japan). 5(6)-Carboxy-2'-7'-dichlorofluorescein diacetate (DCDFA) was from Molecular Probes, Inc. (Beaverton, OR). Freund's complete and incomplete adjuvants were obtained from GIBCO. The cellulose DC plates were from Merck (Darmstadt, Germany). All other chemicals including the antiserum to glucose-6-phosphate dehydrogenase were purchased from Sigma. The antiserum to PrB was a gift from Elizabeth Jones (University of California, Los Angeles, Los Angeles, CA). Antiserum to CPY, PrA, and ALP were described previously (Klionsky and Emr, 1989; Klionsky et al., 1988).

**Strains and Media**—Table 1 describes the S. cerevisiae and *Escherichia coli* strains used in this study. Yeast cells were grown at 30 °C in YPD-rich medium or standard minimal medium, supplemented as necessary (Sherman et al., 1986) or in Wickerham's minimal proline medium (WIMP) (Wickerham, 1949), supplemented with 0.2% yeast extract (WIMPYE) and the necessary amino acids. All bacterial cells were grown at 37 °C in standard LB medium (Miller, 1972), supplemented with ampicillin (100 μg/ml).

**Genetic Procedures**—Crosses, sporulation of diploids, and tetrad analyses were performed by standard genetic techniques (Sherman et al., 1986). One-step gene disruptions and integration experiments were done according to Rothstein (1983) and Orr-Weaver et al. (1983), respectively. Yeast cells were transformed using the lithium acetate method of Ito et al. (1983). *E. coli* transformation was carried out as described by Hanahan and coworkers (1983).

**Cloning of VPS17**—The *VPS17* gene was cloned by transforming the yeast strain S1075–5 containing the CPY-invertase fusion plasmid pCYI-50 (CEN4, ARS1, URA3) (Johnson et al., 1987) with a yeast genomic library, a pYC50 derivative (CEN4, ARS1, LEU2) containing large Saccharomyces yeast genomic inserts. The library was a generous gift from Phil Hieter (Johns Hopkins University School of Medicine, Baltimore, MD). Leu+ transformants were selected on SM glucose plates, replica plated onto SM fructose plates, incubated overnight at 26 °C, and subsequently analyzed by an invertebrate plate assay (see "Results") (Klionsky et al., 1988; Paravicini et al., 1992). Vpl+ transformants were restreaked; the complementing plasmids were isolated as described by Sherman et al. (1986), and amplified in E. coli.

**Nucleic Acid Techniques**—Genomic yeast DNA was isolated from *S. cerevisiae* essentially as described by Sherman et al. (1986) and used for Southern blot analysis (Southern, 1975). Yeast mRNA was isolated by a hot phenol extraction as described previously (Köhler and Domdey, 1991). Radioactively labeled DNA probes were generated according to the method of Feinberg and Vogelstein (1984). Double-stranded plasmid DNA templates used for sequencing were isolated by a small scale boiling method (Willimczik, 1985) and denatured prior to sequencing. DNA sequencing techniques were performed as described in Ausubel et al. (1990).

**Sequence Analysis**—The DNA sequence of *VPS17* was obtained by generating exonuclease III-mung bean nuclease deletion constructs from both ends of plasmid p26 (plasmid KS+) containing the 2.6-kb *VPS17* BglII-Smal fragment) and pCla21 (plasmid KS+, containing the 2.1-kb *VPS17* ClaI fragment) as described in the Stratagene manual. Overlapping clones were sequenced with T3- (5'-ATTTACGCTCACTAAG-3') and T7- (5'-AATACGACTCACTATAG-3') specific primers according to the double-stranded plasmid DNA sequencing method (Sanger et al., 1977) using a Sequenase sequencing kit (U.S. Biochemicals). The sequence of the *VPS17* gene, as well as its predicted protein sequence were compared to the entries of the NBRF, GenBank, and EMBL databases, using the FASTA and TFASTA programs of the University of Wisconsin Genetics Computer Group sequence analysis package (Devereux et al., 1984) and at the NCBI using the BLAST network service (Altschul et al., 1990, Karlin and Altschul, 1990).

**Vectors and Plasmids Construction**—The CPY-invertase fusion plasmid pCYI-50 was described earlier (Johnson et al., 1987). Plasmid pKKY17 represents the originally isolated 9-kb genomic clone that encodes the sorting of the PrB precursor proteins in the mutant cells. A partial restriction map of the genomic DNA insert is shown in Fig. 1A. Several subclones were generated from pKKY17 using the yeast centromeric shuttle vector pPHYC6 (Herman and Emr, 1990) (see Fig. 1B). To generate pKKY17–5, a 6-kb EcoRI fragment was inserted into the EcoRI site of the plasmid pPHYC6. To generate pKKY17–4, a 4.5-kb BamHI fragment and in the case of pKKY17–5, a 4-kb *BglII*-Sphi fragment was cloned into the BamHI-Sphi sites of the vector. The construct pKKY17–7 contains a 4-kb HindIII fragment, and pKKY17–6 was made by cloning a 2.5-kb EcoRI fragment into the EcoRI site of the vector. pKKY17–1 contains a 4-kb HindIII fragment, inserted into the SmaI-digested and phosphatase-treated vector pPHYC6. An overexpressing 2-μm construct of *VPS17* (pKKY17–2) was generated by cloning this 2.6-kb HindIII fragment, into the Sac1/HindIII site of the plasmid pCYI-50 (CEN4, ARS1, URA3, LEU2). The 5-kb BamHI fragment from pKKY17 was cloned into the corresponding sites of pRS2 (Promega, Madison, WI) and named pK7–1. The constructs pK7–9 and pK7–10 are BlueScript KS(+) derivatives containing either a 1.6- or 2.1-kb *Clal* fragment. Plasmid pK7–11 contains the 2.6-kb *BglII*-Smal fragment in the BamHI/Smal sites of pBlueScript KS(+). The sequence of the completed 2.1-kb BamHI-Sphi fragment is shown in Fig. 1A. Several subclones were generated from pKKY17–5 containing the 2.6-kb *BglII*-Sphi fragment and pKKY17–9 was amplified in E. coli, and subsequently analyzed by an invertebrate plate assay (see "Results") (Klionsky et al., 1988; Paravicini et al., 1992). Vpl+ transformants were restreaked; the complementing plasmids were isolated as described by Sherman et al. (1986), and amplified in E. coli.

**Plasmid pKK17–13 was constructed to disrupt the *VPS17* gene. It was constructed in the following way. The recessed ends of the 3.5-kb *NdeI*-SacI fragment of pKK17–9 were filled in with Klcan polymerase and were treated with calf intestine phosphatase. In a triple ligation reaction, this 3.5-kb fragment was ligated with a 1.6-
protein A-Sepharose beads which were washed three times with buffer.
munoprecipitations were performed as described above except for the
pKK17-14. After induction with indoleacrylic acid, the insoluble
in water for 30 min, fixed for
is used in a 1/200 dilution. Prior to electrophoresis, PrA immuno-
pair EcoRI-BamHI fragment, containing the C-terminal part of the
was excised and treated for phosphoamino acid analysis as described
by Meisenhelder and Hunter (1991) with the following modifications.
The first and second dimension of the thin layer electrophoresis of
hydroylated protein were run at 7 V/cm.
Subcellular Fractionation of Vps17p—Fractionation experiments were
done either with the wild-type strain BHY10 or with the same
strain transformed with the overexpressing Vps17p construct
(pKKY17-2). 50 units of cells at a OD_{600 nm} = 1 were harvested,
spheroplasted with Zymolyase 100T, labeled with Tran32P04 for 15 min,
and chased for 45 min with WIMPVE containing 10 mM methionine,
as described above. The spheroplasts were sedimented in a clinical
centrifuge at 13,000 x g for 15 min at 4 °C to generate a P13 pellet
and a S13 supernatant fraction. 0.5 ml of the S13 supernatant were
precipitated. 0.5 ml of the S13 supernatant fraction were directly
the pellets were directly dissolved in
of BHY10, harboring
Phosphoamino Acid Analysis—10 OD_{600} units of BHY10, harboring
the VPS17 gene on a 2-μm plasmid (pKYY17-2) were labeled with
as described above. The immunoprecipitated Vps17p was run
with 5% SDS-polyacrylamide gel, dried onto Whatman 3MM paper,
detected by autoradiography. The Vps17p containing gel piece was
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that the temperature-sensitive growth phenotype was tightly linked to the *ups17* locus, but actually caused by a mutation in a neighboring gene. Therefore, we chose the *ups17*-5 allele to clone the wild-type VPS17 gene. The CPY-invertase sorting defect exhibited by the *ups17*-5 mutant was used to identify prospective VPS17 clones. In wild-type cells (deleted for all endogenous genes encoding invertase, *dscu*), the targeting information contained in the CPY portion of a CPY-invertase fusion protein leads to efficient sorting of the fusion protein to the vacuole (Johnson et al., 1987). However, *ups* mutant cells missort and secrete the fusion protein. The secreted invertase activity leads to a selectable phenotype, the ability to grow on sucrose, an invertase substrate, as a sole carbon source (Bankaitis et al., 1986; Robinson et al., 1988).

In order to clone the VPS17 gene, the strain SEY17-5 (*ups17*-5, *leu2*-3, 112, harboring the CPY-invertase hybrid construct pCY850 (Johnson et al., 1987)) was transformed with a YCP50-based genomic yeast library (CEN4, ARS1, LEU2). 15,000 *Leu*+ transformants were replicated on SM fructose plates and were assayed for secreted invertase by a simple plate overlay assay (Paravicini et al., 1992). Using this assay, one can easily distinguish between colonies that secrete the CPY-invertase fusion protein (*Vps*- colonies turn red after a few minutes) and colonies that sort the CPY-invertase hybrid protein to the vacuole (*Vps* colonies remain white). Among the 15,000 screened colonies only one colony remained white in the plate overlay assay, indicating that this transformant contained a plasmid that complemented the *ups17* sorting defect. Curing this transformant of the library plasmid uncovered the *Vps*- phenotype. Subsequently, the complementing plasmid was isolated and amplified in *E. coli*. Reintroduction of the rescued plasmid into SEY17-5 (*ups17*-5) and other alleles of the *ups17* complementation group (*ups17*-2, *ups17*-4) proved that this genomic clone, designated pKKY17, was able to complement the protein sorting defect exhibited by all *ups* mutants tested. This confirmed that the plasmid complemented the *Vps*- phenotype.

DNA restriction analysis of pKKY17 identified a 9-kb genomic insert (Fig. 1A), and further subcloning and complementation experiments allowed us to localize the complementing activity to a 2.6-kb *HindIII* fragment (Fig. 2B).

Integrative mapping was used to determine if this genomic fragment corresponds to the authentic VPS17 locus. We subcloned the complementing 5-kb BamHI/Sph1 DNA fragment (see Fig. 1B) into the integrating plasmid pRS304 (Sikorski and Hieter, 1989), which carries the selectable *TRP1* marker. This integrating plasmid (pKKK17-12) was linearized at a single *SmaI* site within the complementing fragment to facilitate homologous recombination (Orr-Weaver et al., 1988). Following transformation of the yeast strain SEY17-5 (harboring the CPY-invertase fusion plasmid pCY150) with the linearized plasmid, *Trp*+ transformants were selected and subsequently crossed to the parental wild-type strain SEY6210. Following sporulation of the diploid strain, multiple tetrads were dissected and the spores analyzed. All analyzed ascii showed the expected 2:2 Trp*/Trp* segregation pattern, but none of the spores was *Vps*-. We also analyzed 90 random spores and found that 43 were *Trp*+ and 47 were *Trp*-. None of the 90 spores exhibited a *Vps* phenotype. These results demonstrated that the complementing clone was tightly linked to the VPS17 locus. The integrative mapping analysis also confirmed the earlier finding that the VPS17 gene is linked to the ADE2 locus (Robinson et al., 1988), which has been mapped to the right arm of chromosome XV (Mortimer et al., 1989).

**VPS17 Sequence Analysis**—Both strands of the 2.8-kb

![Fig. 1. Characterization and disruption of the VPS17 locus.](image-url)

A, restriction map of the isolated genomic 9-kb Sau3A fragment. Restriction enzymes are: *BamHI* (B), *BglII* (Bg), *ClaI* (C), *EcoRI* (E), *HindIII* (HI), *KpnI* (K), *PstI* (P), *SmaI* (S), and *SphI* (Sp). B, complementation analysis of different subclones. + indicates if this fragment complements (+) or does not complement (−) the *ups* phenotype of SEY17-5. C, VPS17 gene disruption/deletion. A 1.6-kb *NdeI*/SmaI fragment of the VPS17 ORF was replaced by a 2-kb blunt-ended *HIS3* gene. Restriction enzymes are: *BglII* (Bg), *ClaI* (C), *EcoRI* (E), *HindIII* (HI), *NdeI* (N), *SmaI* (S), and *SphI* (Sp).

ByII-HindII complementing fragment were sequenced (see "Experimental Procedures") and one long open reading frame (ORF) of 1853 base pairs was identified. This ORF has the potential to code for a 551-amino-acid polypeptide (Fig. 2A). Using a 1.6-kb *ClaI* fragment (see Fig. 1C), which is specific for this ORF, as a probe, we detected a single 1.9-kb polyadenylated RNA species on a Northern blot (Fig. 3B). The size of this mRNA is consistent with the predicted size of the VPS17 ORF.

Analysis of the deduced protein sequence indicated that the molecular mass of the Vps17 protein is 63.1 kDa, and according to the Kyte-Doolittle algorithm (Kyte and Doolittle, 1982), it appeared to be a very hydrophilic polypeptide (Fig. 2B). 30% of the amino acids in Vps17p are charged, and the protein has a predicted isoelectric point of 7.7. It contains four potential N-linked glycosylation sites (Asn-X-Ser/Thr) (Marshall, 1972), but no apparent N-terminal signal sequence or obvious hydrophobic membrane-spanning domains are predicted. Homology searches using the FASTA and TFASTA algorithms (Pearson and Lipman, 1988) of the UWGCG sequence analysis package (Devereux et al., 1984), or the BLAST program (Altschul et al., 1990; Karlin and Altschul, 1990), did not reveal any significant sequence similarity between Vps17p and any sequences in the GenBank, EMBL, or NBRF databases.

*ups17* Null Mutant Strains Exhibit a Severe Vacuolar Protein Sorting Defect and Contain Numerous Small Vacuoles—The one-step gene disruption technique (Rothstein, 1983) was used to construct a Δ*ups17* allele in the diploid strain SEY6210.5. Using the construct pKK17-13, we were able to replace almost all of VPS17 (except amino acids 1-10) by the *HIS3* gene (Fig. 1C). His*+* transformants were sporulated and subjected to tetrad analyses. A segregation pattern of 2 Vps*, His*/2 Vps*, His*+* spores among all tetrads dissected was
FIG. 2. Sequence of the VPS17 gene. A, the nucleotide sequence and the deduced amino acid sequence of the VPS17 gene are shown. Putative N-linked glycosylation sites are marked with an asterisk. B, hydropathy analysis of the Vps17p. The Vps17 protein sequence was subjected to hydropathy analysis according to the Kyte-Doolittle algorithm using a window of 19 amino acids. The amino acids are numbered along the horizontal axis. Hydrophobic values lie above the horizontal axis; hydrophilic values lie below.

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observed, indicating that the VPS17 null allele is not lethal, but results in a vacuolar protein sorting defect. There was also no temperature-sensitive growth defect associated with the Δups17 null mutant. All the spores were able to grow at 37°C. The gene disruption was confirmed by isolating genomic DNA from all four segregants of one tetrad and analyzing it by Southern blot hybridization (Fig. 3A). Backcrossing the Δups17 strain to SEY17-5 demonstrated that the disruption indeed belongs in the ups17 complementation group. VPS17 gene disruptions were also performed in the two haploid strains BHY10 and BHY11; yielding the vps17 null mutant strains KKY10 and KKY11.

To further analyze the vacuolar protein sorting defect of the vps17 null mutant and to compare it with the originally isolated vps17-5 allele, we analyzed the sorting of CPY in the wild-type strain BHY10 and the two ups17 mutant strains SEY17-5 (ups17-5) and KKY10 (Δups17). After radioactively labeling yeast spheroplasts with Tran35S for 15 min and chasing them for 60 min, the spheroplasts were fractionated into a pellet (I, intracellular) and a media (E, extracellular/secreted) fraction, CPY was immunoprecipitated from both fractions and analyzed on SDS-polyacrylamide gels (Fig. 4).

Compared to the wild-type strain, where most of the newly synthesized CPY (>95%) is matured to the 61 kDa form after the 60-min chase, the ups17 null mutant and the vps17-5 strain exhibited a strong sorting defect (Fig. 4). The majority of CPY (95%) was present as the Golgi-modified 69-kDa p2 form in the extracellular media fraction (Fig. 4). Only a hint of mature CPY (~5%) could be detected inside the cell (Fig. 4). This vacuolar sorting defect of the null mutant strain was completely complemented when the wild-type VPS17 gene

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2 B. Horazdovsky, unpublished results.
**Fig. 3.** Southern and Northern blot analysis of the VPS17 gene. A, Southern blot analysis of a diploid strain with one disrupted vps17 allele. Disruption of the VPS17 gene was performed in the diploid strain SEY6210.5, using the plasmid pKK17–13. Upon sporulation, tetrad dissection, and germination, genomic DNA was isolated from all four segregants and digested with ClaI to completion. The cleaved DNA was separated on a 1.2% agarose gel, blotted, and probed with a radioactively labeled 2.6-kb BglII–SmaI fragment obtained from pKKY17. In wild-type segregants (1 and 2), a 1.6- and a 2.1-kb fragment hybridized to the probe, whereas in the vps17-disrupted segregants (3 and 4), only a 3-kb ClaI fragment was detected. The numbers on the left side indicate the position of DNA markers in kb. B, identification of the VPS17 RNA transcript by Northern blot analysis. Poly(A)⁺ RNA was isolated from SEY6210, separated in a 1.2% agarose gel, blotted, and probed with a radioactively labeled 1.6-kb ClaI fragment obtained from pKKY17–8. Numbers on the right side indicate the position of RNA size markers in kb. The arrow indicates the position of the 1.9-kb VPS17-specific mRNA.

**Fig. 4.** Intracellular sorting of CPY. Yeast spheroplasts were labeled with Tran³²S label for 15 min and chased for 60 min. The cultures were separated into a pellet (I, intracellular) and a supernatant (E, extracellular) fraction by a 1-min centrifugation at 13,000 × g. The amount of CPY in each fraction was determined by immunoprecipitation. The strains used were BHY10 (WT), KKY10 (∆vps17), KKY10 harboring pKKY17–1 (VPS17 CEN), and KKY10 harboring pKKY17–2 (VPS17-2μ). The migration position of p2CPY (69 kDa) and mCPY (61 kDa) are indicated.

**Fig. 5.** Vacuole morphology of wild-type (WT) and ∆vps17 null mutant strains. Logarithmically growing yeast cells were labeled for 15 min with CDCFDA at 30 °C in YPD media buffered with 1 m citrate-KOH to pH 3.8. Stained cells were carefully sedimented by a 5-min centrifugation at 500 × g and resuspended in YPD. Panels on the left show Nomarski optics, and the corresponding fluorescence optics are shown on the right side.

**Fig. 6.** Intracellular sorting and processing of CPY, PrA, and ALP in wild-type (WT) and ∆vps17 null mutant strains. Spheroplasts of BHY10 (WT) and KKY10 (∆vps17) were labeled with Tran³²S label for 10 min and then chased for 0 or 60 min. The cultures were separated into a pellet (I, intracellular) and a supernatant (E, extracellular) fraction by a 1-min centrifugation at 13,000 × g. The amount of CPY in each fraction was determined by immunoprecipitation. The migration positions of the precursor and mature forms of CPY (p1CPY, p2CPY, mCPY), PrA (proPrA, mPrA), and ALP (proALP, mALP) are indicated.

vps17 Mutants Exhibit Differential Defects in the Sorting of Vacuolar Proteins—To assess the extent of the sorting defect of additional vacuolar proteins in the ∆vps17 null mutant, we examined the sorting and processing behavior of two other soluble vacuolar enzymes, PrA and PrB, and one vacuolar membrane protein, ALP. Spheroplasts of strains BHY10 (WT) and KKY10 (∆vps17) were labeled with Tran³²S label for 10 min and chased for 0 or 60 min. Each sample was fractionated into a pellet (I, intracellular) and a supernatant (E, extracellular) fraction, and then split into four equal aliquots. Each aliquot was immunoprecipitated with antisera specific either to CPY, PrA, PrB, or ALP. The three panels in Fig. 6 show the results of the immunoprecipitates for CPY, PrA, and ALP in the wild-type and ∆vps17 strains. The processing and sorting of CPY in the wild-type cells (WT) was normal; after the 10-min pulse period, all three forms of CPY, the ER-specific p1 form (p1CPY), the Golgi-specific p2 form (p2CPY), and the vacuole-specific mature form (mCPY)
were labeled, and after the 60-min chase, all CPY was matured and found inside the cell (Fig. 6). The \( \Delta \text{ups17} \) null mutant showed the expected severe sorting defect for CPY. After the 10-min pulse period, a significant portion of the Golgi-specific p2CPY was already secreted, and after the 60-min chase, about 90% of p2CPY was in the medium fraction (Fig. 6). Only a minor fraction of mCPY (<5%) could be detected inside the mutant cells (Fig. 6). Since the conversion of p1CPY to p2CPY is normal in the \( \text{ups17} \) mutant cells, there is no obvious defect in protein transport from the ER to the Golgi. However, Golgi to vacuole transport is almost completely defective.

The vacuolar protein sorting defect for another soluble vacuolar hydrolase, PrA, however, was different from that of CPY. In the wild-type strain, we observed normal processing and sorting of PrA; after the 60-min chase, proPrA was all matured and found inside the cell (Fig. 6). The \( \Delta \text{ups17} \) null mutant, however, showed an unexpected sorting defect for PrA. After the 60-min chase, there was a significant amount (60%) of mature proteinase A (mPrA) inside the cells and only a minor fraction (10%) of proPrA was secreted (Fig. 6). 30% of PrA accumulated inside the cells and remained as the pro-form. This demonstrated that the sorting and processing defect for PrA in the \( \Delta \text{ups17} \) null mutant is not as severe as that seen for CPY. Essentially the same sorting/processing defect was observed for another soluble hydrolase PrB. Approximately 60% mPrB and 30% proPrB accumulated inside the mutant cells, whereas less than 10% of proPrB were secreted (data not shown). In a more detailed kinetic analysis of PrA and PrB processing in wild-type and \( \Delta \text{ups17} \) mutant cells, we also could show that the maturation of precursor forms of PrA and PrB to mature PrA and PrB occurs about 2 times slower than in wild-type cells (data not shown), which indicates that either the movement of newly synthesized PrA and PrB through the delivery pathway to the vacuole, or its processing, is delayed in the \( \Delta \text{ups17} \) mutant strain. This kinetic delay in PrA processing also explains the results reported previously for PrA processing in the original \( \text{ups17} \) alleles (Robinson et al., 1988). Since only a 30-min chase point was analyzed, the previous study overestimated the actual sorting defect.

The processing of ALP (Fig. 6) was surprisingly unaffected in \( \text{ups17} \) null mutant cells. After the 60-min chase period, in both wild-type and \( \Delta \text{ups17} \) mutant cells, all the radioactively labeled ALP was completely matured. Therefore, disruption of the \( \text{ups17} \) gene seems to have a differential effect on the sorting and delivery of different vacuolar proteins.

**Identification and Characterization of the Vps17 Protein**

To characterize the \( \text{VPS17} \) gene product, we raised polyclonal antiserum against a \( \text{trpE-Vps17} \) fusion protein. This \( \text{trpE} \) fusion protein contained 183 C-terminal amino acids of the \( \text{Vps17} \) protein (amino acids 369–551). The fusion protein was expressed in \( \text{E. coli} \) cells, purified, and used to raise a polyclonal antiserum in a rabbit (see "Experimental Procedures"). Using this antiserum in immunoprecipitations, it was possible to detect a single 70-kDa protein in radiolabeled yeast cell extracts (Fig. 7A). This protein was about 5–8-fold more abundant when the \( \text{VPS17} \) gene was present on a multicopy plasmid, and the protein could not be detected in \( \Delta \text{ups17} \) cell extracts, or when preimmune serum was used (Fig. 7A). Based on these results, we conclude that this polyclonal antiserum specifically recognizes the \( \text{Vps17} \) protein. Densitometric analysis of the levels of \( \text{Vps17} \) relative to CPY (approximately 0.1% of total cell protein) suggested that \( \text{Vps17} \) comprises <0.01% of total cell protein in logarithmically growing yeast cultures. The \( \text{Vps17} \) appears to be a relatively stable protein, since in pulse-chase labeling experiments, we did not detect significant turnover of the protein even after a 90-min chase period (Fig. 7B).

The predicted molecular mass of \( \text{Vps17} \) is 63 kDa, however, the observed molecular mass on a 9% SDS-polyacrylamide gel was 70 kDa. Although it is possible that the hydrophilic structure of the protein itself could account for this mobility shift, we also tested if the \( \text{Vps17} \) protein undergoes any post-translational modifications. Thus, wild-type yeast cells were labeled with Tran\(^{35}S\) label after 15 min of pretreatment with tunicamycin, a drug that inhibits N-linked glycosylation (Elbein, 1987). The size of the \( \text{Vps17} \) protein was unaffected by this treatment, indicating that the four potential N-linked glycosylation sites are not used (Fig. 7B).

Because the \( \text{VPS15} \) gene has been shown to encode a novel Ser/Thr protein kinase (Herman et al., 1991a) which plays a crucial role in the regulation of vacuolar protein sorting, we also tested if the \( \text{Vps17} \) protein is phosphorylated \textit{in vivo}. Whole yeast cells were labeled with \(^{32}P\) orthophosphate. The cells were lysed with glass beads, and the \( \text{Vps17} \) was immunoprecipitated from the clarified extracts. The phosphorylation analysis was done with the wild-type strain \( \text{BHY10}, \text{BHY10} \) harboring \( \text{pKKY17}-2 \). The migration position of the \( \text{Vps17} \) is indicated. The numbers on the right side represent the migration positions of molecular mass markers. B, whole yeast cells were labeled with Tran\(^{35}S\) label for 10 min and chased for the indicated times. Where indicated (+), tunicamycin was added 15 min prior to the labeling reaction. The position of \( \text{Vps17} \) is indicated.

**FIG. 7. Identification and characterization of the Vps17 protein**. A, whole yeast cells were labeled with Tran\(^{35}S\) label for 15 min at 30 °C. The cells were lysed with glass beads, and immunoprecipitations were performed with the clarified lysates, either with the preimmune serum (P), or the \( \text{Vps17} \) specific antiserum (I). Copy number refers to the gene dosage of \( \text{Vps17} \) in the strains used: \( \Delta \) (no copy) KKY10, L (single copy) \( \text{BHY10} \), and \( \text{H} \) (multicopy) \( \text{BHY10} \) harboring \( \text{pKKY17}-2 \). The migration position of the \( \text{Vps17} \) is indicated. The numbers on the right side represent the migration positions of molecular mass markers. B, whole yeast cells were labeled with Tran\(^{35}S\) label for 15 min of pretreatment with tunicamycin, a drug that inhibits N-linked glycosylation (Elbein, 1987). The size of the \( \text{Vps17} \) protein was unaffected by this treatment, indicating that the four potential N-linked glycosylation sites are not used (Fig. 7B).
clarified lysates using the Vpsl7p-specific antiserum. Strains are: with glass beads, and immunoprecipitations were performed with the 
with [32P]orthophosphate for 30 min at 30°C. After removing unbroken cells by a brief low speed spin (500 × g), this S5
lysat was spun at 13,000 × g, yielding a P13 pellet and a S13 supernatant fraction. The latter was subsequently spun at 100,000 × g to give rise to a P100 pellet and a S100 supernatant. The relative levels of Vps17p in each fraction were then assayed by quantitative immunoprecipitations. As shown in Fig. 10A, about 95% of Vps17p was found in the S13 supernatant fraction, and about 75% of this material pellets after the 100,000 × g spin. The remaining 25% appeared to be soluble, present in the S100 fraction, together with other soluble cytosolic proteins, e.g. glucose-6-phosphate dehydrogenase (G6PDH) (Fig. 10C). A protein unrelated to Vps17p with slower mobility (which is normally only weakly detected by the Vps17 antibody) is also seen in the P100 fraction, presumably because it is highly enriched in this fraction. In previous experiments we have shown that 90% of the ER-specific marker p1CPY is found in the P13 pellet fraction (Herman et al., 1991a), and this fraction is also known to be enriched in nuclei, mitochondria, and vacuoles (Goud et al., 1988; Hurt et al., 1988; Walworth et al., 1989; Herman et al., 1991a). The lack of Vps17 protein in the P13 pellet fraction indicates that the Vps17 protein was not associated with these yeast organelles. Most of the Vps17p (75%) is in the P100 fraction where most of the Golgi marker enzyme Kex2p (95%) (Cunningham and Wickner, 1989; Redding et al., 1991) fractionated (Fig. 10C). This raises the interesting possibility that the particulate Vps17p might be associated with a Golgi compartment or possibly with small transport vesicles that sediment at 100,000 × g (Walworth et al., 1989). If the Vps17 protein interacts with a membranous compartment, then it should be possible to disrupt this interaction with Triton X-100 and the four possible N-linked glycosylation sites of Vps17p were not glycosylated. Based on these results, we conclude that the Vps17 protein does not enter the secretory pathway.

To determine the intracellular location of the Vps17p more precisely, we used differential centrifugation which is outlined in Fig. 9. Yeast spheroplasts (BHY10) were radioactively labeled with Tran35S label and lysed under conditions that do not destroy the integrity of internal organelles (Walworth et al., 1989) (see “Experimental Procedures”). After removing unbroken cells by a brief low speed spin (500 × g), this S5 lysate was spun at 13,000 × g, yielding a P13 pellet and a S13 supernatant fraction. The latter was subsequently spun at 100,000 × g to give rise to a P100 pellet and a S100 supernatant. The relative levels of Vps17p in each fraction were then assayed by quantitative immunoprecipitations. As shown in Fig. 10A, about 95% of Vps17p was found in the S13 supernatant fraction, and about 75% of this material pellets after the 100,000 × g spin. The remaining 25% appeared to be soluble, present in the S100 fraction, together with other soluble cytosolic proteins, e.g. glucose-6-phosphate dehydrogenase (G6PDH) (Fig. 10C). A protein unrelated to Vps17p with slower mobility (which is normally only weakly detected by the Vps17 antibody) is also seen in the P100 fraction, presumably because it is highly enriched in this fraction. In previous experiments we have shown that 90% of the ER-specific marker p1CPY is found in the P13 pellet fraction (Herman et al., 1991a), and this fraction is also known to be enriched in nuclei, mitochondria, and vacuoles (Goud et al., 1988; Hurt et al., 1988; Walworth et al., 1989; Herman et al., 1991a). The lack of Vps17 protein in the P13 pellet fraction indicates that the Vps17 protein was not associated with these yeast organelles. Most of the Vps17p (75%) is in the P100 fraction where most of the Golgi marker enzyme Kex2p (95%) (Cunningham and Wickner, 1989; Redding et al., 1991) fractionated (Fig. 10C). This raises the interesting possibility that the particulate Vps17p might be associated with a Golgi compartment or possibly with small transport vesicles that sediment at 100,000 × g (Walworth et al., 1989). If the Vps17 protein interacts with a membranous compartment, then it should be possible to disrupt this interaction with Triton X-100 and

substrate of the Vps15 kinase, a detailed biochemical and mutational analysis will be required to further test this.

To determine if the Vps17p is phosphorylated on serine, threonine, or tyrosine, we performed a phosphoamino acid analysis. In vivo [32P]orthophosphate-labeled Vps17p was immunoprecipitated and extracted from an SDS-polyacrylamide gel. After acid hydrolysis, the amino acid mixture was separated by two-dimensional thin layer electrophoresis, together with the non-radioactive phosphoamino acid standards phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY). The phosphoamino acid standards were stained with ninhydrin, and their migration positions are indicated by dashed circles. The open circle (O) indicates the loading position.

Subcellular Fractionation Studies Suggest That The Vps17p Is Associated with a Membrane Fraction—We performed protease protection experiments to test if the Vps17p protein enters a membranous compartment in the yeast cell. Wild-type yeast spheroplasts were radioactively labeled and gently lysed with DEAE-dextran under conditions which disrupt the plasma membrane, but not internal organelles (Klionsky and Emr, 1989). Adding proteinase K, we found that Vps17p was rapidly degraded, whereas p1CPY and p2CPY, which reside in the ER and Golgi compartments, respectively (Franzusoff and Schekman, 1989; Stevens et al., 1982), were resistant. p1CPY and p2CPY were degraded only in the presence of Triton X-100 and proteinase K (data not shown). This indicates that the Vps17 protein is exposed to the cytoplasm of the yeast cell which is consistent with the observation that
Yeast Vps1p Protein

associated with a membranous component of the P100 fraction, since the interaction is sensitive to the detergent Triton X-100 and because the Vps17p cofractionated with membranes in the sucrose step gradient analysis. It seems likely that this association is stabilized by hydrophobic interactions as urea treatment efficiently solubilized Vps17p, but high salt treatment released only a portion of Vps17p from the particulate P100 fraction.

**DISCUSSION**

We have shown earlier that vps17 mutants exhibit severe vacuolar protein sorting defects (Bankaitis et al., 1986; Robinon et al., 1988). To better understand the molecular function of the Vps17 protein in vacuolar protein delivery, we have cloned the wild-type VPS17 gene and analyzed its gene product. The gene codes for a hydrophilic protein of 551 amino acids that lacks an N-terminal signal sequence and hydrophobic membrane spanning domains. Protease protection experiments have shown that it is exposed to the cytoplasm, which is also supported by the observation that none of the four possible N-linked glycosylation sites are modified.

Some insight into the possible function of the Vps17 protein came from subcellular fractionation studies. When wild-type yeast cells were fractionated by differential centrifugation, we found two pools of Vps17p, one completely soluble pool (25% of Vps17p) and a particulate pool (75%) that sediments at 100,000 g. Five to eight-fold overexpression of Vps17p leads to an increase in the soluble pool which suggests a saturable interaction of Vps17p with the 100,000 g pellet fraction (data not shown). Several lines of evidence indicate that Vps17p associates with a membrane. First, 75% of the Vps17p is found in the particulate fraction after a 100,000 g centrifugation. Second, the interaction of Vps17p with the particulate fraction is sensitive to Triton X-100. Third, in sucrose density gradients, a significant portion of Vps17p fractionates together with cell membranes. Despite the fact that the Vps17p is very hydrophilic and has many charged amino acids, we believe that this association is mediated by a hydrophobic interaction, since Vps17p can efficiently be extracted from the particulate fraction with urea but not with high salt.

One important question is, what is the nature of this membranous material? Based on the differential centrifugation experiments, we can exclude the possibility that Vps17p is associated with mitochondria, nuclei, or vacuoles (Fig. 10), since it is found exclusively in the S13 supernatant fraction and not in the P13 pellet fraction, where most of the vacuolar, mitochondrial, and nuclear marker proteins accumulate. Our data indicate that consistent with a role in Golgi to vacuole protein sorting, Vps17p cofractionates with a Golgi marker. The majority of Vps17p (75%) pellets at 100,000 × g, together with the late Golgi compartment that contains Kex2p protein (Graham and Emr, 1991; Wilcox and Fuller, 1991). This late Golgi compartment is predicted to be the site where vacuolar protein sorting takes place (Graham and Emr, 1991). Because this P100 fraction also contains small transport vesicles (Walworth et al., 1989), we cannot exclude an interaction with an intermediate in the transport reaction. Based on these results, we assume that the Vps17p protein functions at an early stage of the sorting process. Whether it is involved in the actual sorting, packaging, or transport of vacuolar proteins is not yet clear.

**In vivo** phosphorylation and phosphoamino acid analyses have demonstrated that the Vps17p is phosphorylated on serine residue(s). This post-translational modification could account for the difference between the calculated molecular mass, based on the primary protein sequence (63 kDa) and the calculated mass, based on its primary sequence (63 kDa) and

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**FIG. 10. Subcellular fractionation of Vps17 protein.** The fractionation scheme we applied to the wild-type yeast strain BHY10 is outlined in Fig. 9. A, spheroplasts of BHY10 were labeled with Tran35S label for 15 min, chased for 45 min, and osmotically lysed. The crude extract was spun at 500 g for 45 min, and the supernatant and pellet were treated with various reagents (1 M NaCl, 2% Triton X-100, 2 M urea) for 30 min on ice. The lysates were then spun at 100,000 g for 45 min, and the supernatant and pellet fractions were immunoprecipitated with Vps17p-specific antisera. Triton X-100 and urea treatment extracted most of the Vps17 protein from the P100 pellet, whereas high salt treatment (1 M NaCl) only partially affected the association of Vps17p with the particulate P100 fraction (Fig. 10B). Additional evidence for a membrane association came from sucrose flotation experiments (Walworth et al., 1989), where we could show that a significant fraction of Vps17p fractionated together with other cell membrane markers (e.g. Kex2p) in a sucrose step gradient (60, 50, 30, 0% sucrose from bottom to top). Together, these findings suggest that the Vps17p is

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**Table:**

| Fraction | Vps17p |
|----------|--------|
| S13      | >95%   |
| P13      | <5%    |
| S13      | 75%    |
| P100     | 25%    |
| S100     | <2%    |

**Fig. 10A:**

**Fig. 10B:**

**Fig. 10C:**

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**A**

| Fraction | Vps17p |
|----------|--------|
| S6       | 100%   |
| P13      | <5%    |
| S13      | 95%    |
| P100     | 25%    |
| S100     | <2%    |

**B**

| Fraction | Vps17p |
|----------|--------|
| S6       | 100%   |
| P13      | <5%    |
| S13      | 95%    |
| P100     | 25%    |
| S100     | <2%    |

**C**

| Fraction | Vps17p |
|----------|--------|
| P13      | <5%    |
| P100     | 95%    |
| S100     | <2%    |
the observed molecular mass on denaturing SDS-polyacrylamide gels (70 kDa). The finding that the Vps17 protein is phosphorylated on serine residues is of particular interest because another Vps protein, Vps15p, was shown to encode a novel Ser/Thr protein kinase that acts very early in the delivery pathway of vacuolar proteins (Herman et al., 1991a, 1991b). Our finding that the Vps17p is phosphorylated in a Δups15p background, however, indicates that the Vps17p may not be a substrate of the Vps15 kinase. Further detailed biochemical and mutational studies of the phosphorylation site(s) of Vps17p will be required to rigorously rule out a role for Vps15p in the phosphorylation of the Vps17p. Mutational analyses of the Vps17p phosphorylation site(s) also should clarify the importance of this modification.

Gene disruption experiments have shown that a ups17 null mutant is viable, which is not too surprising, given that all ups genes analyzed to date have been found not to be essential for vegetative growth (Dulic and Riezman, 1988; Banta et al., 2000; Herman and Emr, 1990; Rothman et al., 1990; Wada et al., 1990; Woolford et al., 1990; Herman et al., 1991a; Preston et al., 1991; Robinson et al., 1991; Paravcini et al., 1992). Unlike several other ups mutants (ups11, 15, 16, 18, 33, and 34) (Robinson et al., 1988) that show a temperature-sensitive growth defect, Δups17 null mutants are able to grow at elevated temperatures. However, stress analyses of the VPS17 gene do show a defect in vacuolar morphology (Banta et al., 1988). Comparing wild-type and Δups17 mutant strains, we could demonstrate that the null mutant accumulates many small vacuole-like structures (Banta et al., 1988). Our detailed analysis of the vacuolar protein sorting defect in the Δups17 null mutant strain indicates that the Vps17p is only required for the sorting of soluble vacuolar proteins. The sorting and processing of ALP, a vacuolar membrane protein, was not blocked in the mutant cells, whereas sorting and processing of the soluble vacuolar protein CPY was almost completely blocked. More than 95% of the Golgi-modified precursor form of CPY was missorted and secreted by the ups17 mutant. The missing sorting and processing defects for two other soluble vacuolar hydrolases, PrA and PrB, were partially affected (80% mature, 40% precursor). The observation that ALP processing is completely independent of Vps17p is consistent with previous observations with other ups mutants. Since ups35 and ups15 mutant cells also do not affect ALP sorting and/or maturation (Herman et al., 1991b; Paravcini et al., 1992), it raises the question of whether there are different delivery pathways to the yeast vacuole. In this respect, it is interesting to note that there is also more than one delivery pathway to the mammalian lysosome, a monocyte 6-phosphate-dependent pathway, used by many soluble lysosomal enzymes, and a monocyte 6-phosphate-independent pathway, followed by several lysosomal membrane markers (von Figura and Hasilik, 1986; Kornfeld, 1987; Kornfeld and Mellman, 1989). In yeast, however, analyses of other vacuolar membrane proteins as they are identified, will need to be examined to obtain further evidence that vacuolar membrane proteins travel to the vacuole by a Vps17p-independent pathway. Independent pathways do not necessarily mean that different vacuolar proteins travel to the vacuole by different delivery pathways, although we cannot exclude this possibility. However, because a number of ups mutants appear to mislocalize CPY, PrA, PrB, and ALP to the same extent (Robinson et al., 1988; Klionsky and Emr, 1989), completely independent delivery pathways appear to be unlikely. Based on the fact that the sorting signals in CPY (Johnson et al., 1987; Valls et al., 1987), PrA (Klionsky et al., 1988), and ALP (Klionsky and Emr, 1989) appear to be different, as they do not show any primary sequence similarity, these proteins may travel to the vacuole via the same vesicle but bind to different membrane receptor sites in the sorting compartment. Because the Vps17p does not resemble a membrane receptor, we propose that the Vps17 protein may associate directly or indirectly with a receptor complex(es) that participates in CPY, PrA, and PrB sorting to the vacuole. Receptor function is compromised in the absence of Vps17p such that CPY sorting is completely defective while PrA and PrB sorting is only partially blocked. Biochemical purification of the Vps17 protein together with any associated proteins or protein complexes should enable us to address this model.

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