A Novel RING Finger Protein, Vps8p, Functionally Interacts with the Small GTPase, Vps21p, to Facilitate Soluble Vacuolar Protein Localization*

(Received for publication, June 27, 1996, and in revised form, October 10, 1996)

Bruce F. Horazdovsky‡‡, Christopher R. Cowles§§, Peg Mustol¶¶, Michael Holmes¶¶, and Scott D. Emr††††‡‡‡‡

From the ‡Department of Biochemistry, Texas Southwestern Medical Center, Dallas, Texas 75235-9038 and §Division of Cellular and Molecular Medicine and Howard Hughes Medical Institute, University of California, San Diego, School of Medicine, La Jolla, California 92037-0668

Genetic analyses of vacuolar protein sorting in Saccharomyces cerevisiae have uncovered a large number of mutants (eps) that missort and secrete soluble vacuolar hydrolases. Here we report the characterization of the gene product affected in one of these mutants, Vps8p. Polyclonal antiserum raised against a trpE-Vps8 fusion protein specifically detects a 134-kDa protein in labeled yeast cell extracts. Subcellular fractionation studies demonstrate that Vps8p is distributed between a low speed membrane pellet fraction and a high speed membrane pellet fraction. The lack of a hydrophobic domain in Vps8p suggests that Vps8p peripherally associates with a membrane(s). This association was found to depend on the function of Vps21p, a member of the Rab/Ypt/Sec family of small GTPases. In vps21 null mutant cells, Vps8p is found in the cytosol. In addition, overexpression of Vps21p partially suppresses a eps null mutant, indicating that Vps8p and Vps21p functionally interact. Vps8p contains a C-terminal cysteine-rich region that conforms to the H2 variant of the RING finger Zn$^{2+}$ binding motif. Truncation of this C-terminal region partially compromises Vps8p function. While vps8 null mutants strains missort and secrete soluble vacuolar hydrolases, the integral vacuolar membrane protein, alkaline phosphatase (ALP), is sorted to the vacuole and matured normally. In addition, when eps8 mutants are combined with endocytic or late secretory pathway mutants (end3 or sec1, respectively), ALP is still delivered to the vacuole. These observations indicate that ALP is sorted to the vacuole in a Vps8p-independent manner, possibly via an alternative vesicle carrier.

Intracellular protein localization is carried out by specific and often highly regulated delivery systems. One of the best characterized is the lysosomal hydrolase sorting pathway (1, 2). In mammalian cells, soluble lysosomal hydrolases enter the secretory pathway via translocation across the endoplasmic reticulum (ER)
1. In the lumen of the ER, these proteins are modified with core oligosaccharides and are then transported to the Golgi complex. In the Golgi, the core oligosaccharides are further processed, including the specific addition of the mannose 6-phosphate recognition marker. When lysosomal proteins reach the trans Golgi network (TGN), they are selectively sorted away from the secretory protein pool; this sorting event is largely mediated by two receptors that recognize the mannose 6-phosphate carbohydrate modification (2). The mannose 6-phosphate receptors, or MPRa, and their coupled ligands are then packaged into clathrin-coated vesicles and delivered to a prelysosomal endosome. Receptor-ligand complexes dissociate in the endosome (probably due to the acidic environment of this compartment), and lysosomal proteins proceed to the lysosome, whereas the receptors recycle to the TGN for another round of sorting or are diverted to the plasma membrane to scavenge extracellular lysosomal proteins.

Localization of soluble proteins to the lysosome-like vacuole of yeast follows a similar itinerary. This is exemplified by the delivery of the soluble vacuolar hydrolase carboxypeptidase Y (CPY) (3, 4). When CPY is translocated into the lumen of the yeast ER, it receives four core oligosaccharides, generating the p1 precursor form of the enzyme (5). p1CPY then transits the Golgi complex, where the core oligosaccharides are extended with additional mannose residues, generating the p2 precursor form of CPY. In a late Golgi compartment, probably analogous to the TGN, p2CPY binds the vacuolar protein sorting receptor Vps10p and is actively sorted away from secretory proteins and delivered to the vacuole via a prevacuolar endosome compartment (6, 7). Vps10p then recycles from the endosome to the Golgi for another round of sorting (8, 9), and p2CPY moves on to the vacuole. Upon arrival in the vacuole, the prosegment of p2CPY is removed, generating the mature active form of the enzyme (mCPY). However, unlike the MPRa, Vps10p does not recognize a carbohydrate modification but rather recognizes a peptide-sorting signal present at the N terminus of the CPY prosegment (10, 11).

Several selections and screens have been used to identify mutants that affect the vacuolar protein sorting (eps) pathway (12–15) or show deficiencies in vacuolar peptidase activity (pep) (16). Complementation analysis of the eps and pep mutants revealed an extensive genetic overlap. Together, the eps and pep alleles were thereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

* This work was supported in part by National Institutes of Health Grant GM-32703 (to S. D. E.) and a grant from the American Cancer Society IRG-142L (to B. F. H.). The costs of publication of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ The first three authors contributed equally to this work.
† Member of the Biomedical Sciences Graduate Program and a Lucille P. Markey Charitable Trust Predoctoral Fellow.
‡‡ Supported as an investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed. Tel.: 619-534-6462; Fax: 619-534-6414.

1 The abbreviations used are: ER, endoplasmic reticulum; TGN, trans Golgi network; MPR, mannose 6-phosphate receptor; CPY, carboxypeptidase Y; YPD, yeast extract-peptone-dextrose; YPF, yeast extract-peptone-fructose; SD, synthetic dextrose; PrA, proteinase A; ALP, alkaline phosphatase; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pair(s).
Vps21p function. suggesting that Vps8p maybe a positive modulator of thermore, overexpression of Vps21p can partially bypass Vps8p dependent on the presence of the small GTPase, Vps21p. Furthermore, enlarged vacuole structure that is not properly acidified. Vps8p ining vacuolar structure), cells that completely lack Vps8p function in the vesicle targeting/fusion event. Although originally assigned to the class D mutant group (normal appearance), several gene products (Vps21p, Vps45p, Pep12p) that have been implicated in vesicle targeting and/or fusion events in the Golgi to endosome step of this transport pathway. Vps21p is a small GTPase of the Rab family (21, 25), Vps45p is a Sec1 homologue (23, 26), and Pep12p (Vps6p) is a syntaxin homologue (27). Similar proteins have been found to be involved in vesicle targeting and/or fusion events in mammalian cells. A novel gene product affected in a class D mutant, Vps8p, has also been implicated in this Golgi-to-endosome vesicle targeting/fusion reaction as a potential modulator of Vps21p function (28).

Here we describe the VPS8 gene product that also appears to function in the vesicle targeting/fusion event. Although originally assigned to the class A vps mutant group (normal appearing vacuolar structure), cells that completely lack Vps8p function exhibit several of the class D mutant phenotypes, including a temperature-sensitive growth defect and a single, enlarged vacuole structure that is not properly acidified. Vps8p is a hydrophilic protein that is associated with a particulate cell fraction containing cellular membranes, and this association is dependent on the presence of the small GTPase, Vps21p. Furthermore, overexpression of Vps21p can partially bypass Vps8p function, suggesting that Vps8p may be a positive modulator of Vps21p function.

**EXPERIMENTAL PROCEDURES**

**Media and Reagents—**Escherichia coli cells were grown in LB and M9 media supplemented with appropriate antibiotics and amino acids (31). Saccharomyces cerevisiae was propagated in yeast extract-peptone-dextrose (YPD), yeast extract-peptone-fructose (YPF), or synthetic dextrose (SD) supplemented with amino acids as required (32). The strains used in this study are listed in Table I. Restriction and modification enzymes were purchased from Boehringer Mannheim, New England Biolabs, and Stratagene. Zymolyase 100-T (Krin Brewery Co.) was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Glusulase was from Dupont. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside, phenylmethylsulfonyl fluoride, a-mercaptoethanol, aprotinin, leupeptin, pepstatin, and isopropyl-β-D-thiogalactopyranoside were acquired from Boehringer Mannheim. Tran32S-labeled DNA was obtained from ICN Biochemicals. 32P-orthophosphate (γ)[32P]dATP was provided by Amersham Corp. Production of antiserum to vacuolar hydrolases (CPY, alkaline phosphatase (ALP), and protease A (PrA)) has been described previously (33, 34). All other reagents were purchased from Sigma.

**Plasmid Constructions—**Plasmid constructions were performed using previously described recombinant DNA manipulation methods (35). The class A yeast method of Volgelstein and Gillespie (36) was employed for DNA fragment isolation. The CEN-based VPS8 plasmid pPS81 was generated by subcloning the VpsI-Scal fragment of library plasmid pH8Y–1 (containing VPS8, referred to figure 1A) to the SmaI site of pRS416 (37). The 2 micron-based plasmid pPS82 was fashioned by ligating the VpsI-Scal fragment of pH8Y–1 into pRS426 (37). Integrate mapping plasmid pPS84 was made by inserting the VPS8-containing Accl-Aci fragment of pH8Y–1 in pRS304 (37). The VPS8 deletion/disruption plasmid pPS83 was made by subcloning the Accl-Aci fragment of pH8Y–1 into pBluescript KS (Stratagene) and then replacing the ClaI-EcoRI site of the resulting plasmid with the HIS3 gene. For preparation of a trp-EVps8 fusion protein, pPS81 was digested with XhoI and HindIII, yielding a 1.3-kb fragment, which was purified and ligated into XhoI-HindIII digested pTH2 vector (containing trpE coding sequences) (38) to generate pPS88. Construction of pH8Y21–28 (2 µg VPS21) was previously detailed (21). The vps8 C-terminal deletion mutant was constructed by digesting VPS8 containing DNA with NcoI, filling in the NcoI sites with Klenow and ligating the blunted NcoI fragment into the Smal site of pRS424 to generate pCCY8–AC31.

**Nucleic Acid and Genetic Manipulations—**Bacterial DNA transformations were accomplished using the method of Hanahan (39). Standard yeast genetic procedures were adhered to as described previously (32, 40). Yeast transformations employed a LiAc treatment protocol (41). pH8Y1 (CPY-invertase::LEU2) (21) was integrated at the leu2-3,122 locus of SEY8–30 to produce strain SEY8–30.11. Integrative mapping studies of cloned VPS8 were initiated by linearizing pPS84 with SmaI and transforming BHY10 cells. Trp+ transformants (SEY–4.304) were mated with SEY8–30.11. Diploid colonies were selected, sporulated, and 20 of the resulting ascospores were dissected. Trp+ regants were selected on the basis of secretion of p2CPY at 38°C, while spores containing a deletion were selected on the basis of secretion of p2CPY at 30°C (42).

**Isolation of the VPS8 Gene—**A plasmid-based yeast genomic DNA library (LEU2, CEN; a gift from Philip Hieter) was employed to transform SEY8–4 cells harboring a plasmid encoding a CPY-invertase fusion protein (21). Transformant cells were replica-plated to YPF medium, incubated at 30°C overnight, then subjected to an assay designed to detect extracellular invertase activity (21). Plasmids were isolated from Vps+ cells and used to transform SEY8–4 to confirm complementing activity. Isolated plasmids were digested and pieces containing genetic sequence were subcloned and tested for their ability to complement the SEY8–4 mutant phenotype for the purpose of identifying the minimum complementing DNA fragment shown in Fig. 1A. Initial sequencing of
the minimum complementing fragment employed T3 and T7 primers and defined an open reading frame (YAL002W) covering base pairs 143990–147520 of chromosome I in the S. cerevisiae genome data base. Several oligonucleotide primers spanning the VPS8 coding sequence were utilized to verify sequence of the Vps8 gene.

Antiserum Preparation—Bacterial JM101 cells were transformed with trpE-VPS8 gene fusion plasmid pPS82. Induced production and purification of fusion protein followed the method of Kleid et al. (43), as modified by Herman and Emr (22). Immunization of New Zealand White rabbits with fusion protein was executed as described previously (44). CNBr-activated Sepharose (Pharmacia Biotech Inc.) was coupled to purified trpE-Vps8 fusion protein and used to affinity-purify harvested antiserum according to manufacturer’s instructions. Eluted antiserum was screened and titrated by immunoprecipitation of labeled yeast cell extracts.

Cell Labeling and Immunoprecipitation—Yeast cells were grown in SD containing required amino acids to an A600 of 0.8. For experiments involving immunoprecipitation of vacuolar hydrolases, 5 A600 units of cells were harvested by centrifugation and suspended in 1 ml of SD medium containing 1 mg/ml bovine serum albumin. Cells were incubated for 10 min at 30 °C; and where temperature shifts were employed, an additional 15 min preincubation at permissive or nonpermissive temperature was performed. Labeling was initiated via addition of 100 μCi of Tran35S-label and allowed to proceed for 10 min. Chase periods were then started by adding methionine, cysteine, and yeast extract to final concentrations of 5 mM, 1 mM and 0.2%, respectively. For experiments involving separation of pellet and medium fractions, cells were converted to spheroplasts prior to cell labeling and chase periods (17, 18). Following the labeling and chase cultures were then centrifuged at 13,000 × g for 1 min to yield an intracellular (P) pellet fraction and an extracellular (E) medium fraction. The presence of CPY, PAI, and ALP proteins in each fraction was determined by immunoprecipitation (2,000 × g for 5 min) and spheroplasts were generated as described previously (45, 46). Cultures were incubated at 30 °C for 10 min prior to addition of 1.2 mM of Tran35S-label. Labeling proceeded for 30 min at 30 °C and was followed by a chase period of 30-min duration, as detailed above. Harvesting and lysis of spheroplasts, as well as differential centrifugation of resulting lysates, was performed as previously detailed (23). Levels of Vps8p, ALP, glucose-6-phosphate dehydrogenase, and Kex2p in each subcellular fraction were determined by immunoprecipitation as described previously (44).

Microscopy—Wild-type cells (SEY6210) or cells lacking the VPS8 gene (PSY83) were grown in rich medium (YPD) to mid-log phase. The cells were harvested and resuspended at 20 A600 units/ml in YPD medium. FM4-64 was added to 20 μM, and the cells were incubated with shaking for 30 min at 30 °C. The cells were harvested and resuspended in YPD medium, placed on standard slides and viewed with a Nikon Microphot-SA microscope equipped with a 100 × CF N Plan DIC achromatic objective and a 546-nm filter for visualizing FM4-64 fluorescence.

RESULTS

Characterization of the VPS8 Locus—Many of the vps mutants were isolated using a CPY-invertase hybrid protein-based selection scheme (10, 13). The hybrid proteins consisted of the N-terminal portion of CPY fused with the normally secreted enzyme, invertase. In wild-type cells, the fusion proteins were correctly delivered to the vacuole due to the vacuolar protein sorting information found in the CPY portion of the molecule. However, in mutant cells that were unable to correctly localize vacuolar proteins, the CPY-invertase was missorted and delivered to the cell surface by default via the secretory pathway. The mislocalization of CPY-invertase led to a selectable phenotype, the ability of these mutant cells to grow on medium containing sucrose, an invertase substrate, as a sole
carbon source (12). One mutant identified using this technique was \textit{ups8}. \textit{ups8} mutant cells secreted the vast majority of CPY-invertase fusion proteins and this mislocalization phenotype was exploited to clone the wild-type \textit{VPS8} locus. \textit{ups8} mutant cells (SEY8–3) carrying a CPY-invertase gene fusion were transformed with a yeast genomic library (\textit{CEN LEU2}). Leu+ transformants were selected and the transformants were replica plated onto rich medium containing fructose as a carbon source (YPF). After a 16-h incubation at 30 °C, the plates were overlaid with a soft agar solution containing reagents to detect extracellular invertase activity (21). Those transformants that contained complementing library plasmids properly sorted the CPY-invertase hybrid to the vacuole, resulting in white colonies. Transformants that contained noncomplementing library plasmids missorted the CPY-invertase hybrid to the cell surface, resulting in brown colonies. Using this screening method, four complementing library plasmids were isolated, all of which contained common genomic DNA inserts. Various portions of the genomic inserts were subcloned, and a minimal DNA fragment responsible for \textit{ups8} complementing activity was identified (Fig. 1A). DNA sequence generated from the 5' and 3' ends of this 5.6-kb \textit{PvuII-AccI} fragment was compared to sequences in the \textit{S. cerevisiae} genome database and were found to correspond to a segment of chromosome I. A single large open reading frame had been defined in this fragment (YAL002W). Using the sequence available in the database, oligonucleotides were designed to serve as primers for sequence analysis of the cloned \textit{ups8} complementing genomic DNA fragment. No differences were observed between the sequence of the cloned genomic fragment and the data base sequence for this region of chromosome I. Integrative mapping studies demonstrated that the cloned 5.6-kb fragment contained the \textit{VPS8} locus (see “Experimental Procedures”). \textit{VPS8} was predicted to encode a protein of 1176 amino acids with a molecular mass of 134,304 daltons (Fig. 1C). No hydrophobic stretches that could serve as a signal sequence or transmembrane domains were detected in Vps8p. Sequence comparisons with other known proteins and protein sequence motifs revealed that Vps8p contains a cysteine-rich region that conforms to a subclass of the RING finger zinc binding domain (Fig. 1D). RING finger domains are thought to mediate protein-protein interactions and have a consensus sequence of \textit{Cys-X<sub>2</sub>-Cys-X<sub>2</sub>-Cys-X<sub>2</sub>-Cys-X<sub>2</sub>-Cys-X<sub>4</sub>-Cys-X<sub>2</sub>-Cys} (47). As shown in Fig. 1D, Vps8p contains the RING H2 variant where Cys<sub>4</sub> is replaced by a His residue (Cys-X<sub>2</sub>-Cys-X<sub>2</sub>-Cys-X<sub>2</sub>-His-X<sub>2</sub>-Cys-X<sub>2</sub>-Cys-X<sub>2</sub>-Cys) (47). Several other yeast proteins contain H2 variant RING finger motifs including Vps15p, End1p (Vps11p), and Ste6p (Fig. 1D) (12, 48–51). Interestingly, Vps15p and Vps11p, like Vps8p, are involved in vacuolar protein localization. A \textit{VPS8} gene disruption/deletion (\textit{ups8ΔI}) was constructed, and the consequences of a \textit{ups8} null allele on cell viability, vacuolar protein sorting, and vacuole morphology were determined. The \textit{VPS8} deletion construct was made by replacing the vast majority of the \textit{VPS8} coding sequence with a \textit{HIS3} gene cassette (Fig. 1A). Cells that harbored this \textit{ups8} null allele were viable, but showed a slow growth phenotype when incubated at elevated temperature (Fig. 1B). This temperature dependence slow growth phenotype was completely complemented by the cloned \textit{VPS8} gene on a \textit{CEN}-based plasmid vector (Fig. 1B). The ability of \textit{ups8ΔI} cells to localize native vacuolar proteins was also examined. The delivery of vacuolar proteins can be monitored easily due to compartmental-specific modification and processing of these proteins as they travel through the sorting pathway (see Introduction) (5). In experiments shown in Fig. 2, spheroplasts were generated from wild-type cells, \textit{ups8ΔI} cells or \textit{ups8ΔI} cells carrying the cloned \textit{VPS8} gene on a \textit{CEN}- or 2μ-based expression vector. The spheroplasts were pulse-labeled with [\textit{35S}]{methionine}/[\textit{35S}]{cysteine} and chased with unlabeled methionine and cysteine for 30 min. The labeled cultures were separated into a spheroplast pellet (internal, I) and a medium (external, E) fraction. The soluble vacuolar proteins CPY and PrA were immunoprecipitated from these fractions, and the immunoprecipitates were resolved by SDS-PAGE. Wild-type cells properly localized both CPY and PrA to the vacuole, as evidenced by the presence of the mature vacuolar forms of these proteins inside the cell (Fig. 2; I, mCPY, and mPrA). In contrast, the vast majority of CPY and PrA was not delivered to the vacuole in \textit{ups8ΔI} cells, as evidenced by the lack of maturation of these two proteases (p2CPY and proPrA, respectively). Most of the p2CPY was secreted into the medium fraction by \textit{ups8ΔI} cells (E), whereas a smaller portion of proPrA was secreted by these mutants. The reason for this differential secretion phenotype is unclear, but it has been observed in a large number of \textit{ups} mutant strains. The missorting phenotype was complemented by the presence of the cloned \textit{VPS8} gene on a \textit{CEN}-based (low copy) plasmid vector (\textit{ups8ΔI/CEN}). However, when the cloned \textit{VPS8} gene was present in a multi-copy vector (\textit{ups8ΔI/2μ}), a small (<15%) portion of Golgi-modified p2CPY was secreted from these cells, demonstrating that overexpression of Vps8p resulted in a very modest vacuolar protein missorting phenotype. To determine if vacuolar membrane proteins were also mislocalized in \textit{ups8} mutant cells, sorting of the transmembrane vacuolar protein ALP was examined in wild-type and \textit{ups8ΔI} cells (Fig. 3A). In these experiments cells were labeled with [\textit{35S}]{methionine} and [\textit{35S}]{cysteine} for 10 min and chased for 45 min in the presence of unlabeled methionine and cysteine. Cells were harvested at 0 min of chase and 45 min of chase and cell lysates were generated. These lysates were subjected to immunoprecipitation using antisera directed against ALP, and the immunoprecipitates were resolved using SDS-PAGE. In wild-type cells both mature and Golgi-modified precursor ALP (mALP and proALP, respectively) were observed at the 0-min chase point (Fig. 3A, lane 1). Following the 45-min chase period, only mature vacuolar ALP was seen (lane 2). When ALP localization was examined in \textit{ups8ΔI} cells, the same sorting phenotype was observed; ALP was matured in \textit{ups8ΔI} cells after a 45-min chase period, indicating vacuolar delivery (lane 3).
Vps8p Is Required for Soluble Vacuolar Protein Localization

Figure 3. Intracellular sorting of vacuolar alkaline phosphatase. A, wild-type (SEY6210) and vps8Δ1 cells (PSY83) were labeled for 10 min with Tran35S-label and the incubation was continued for 45 min at 30 °C (45-min chase) prior to harvesting. Cell extracts were generated and ALP was isolated by immunoprecipitation. The immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. B, vps8Δ1/sec1 Δ (PSY85) or vps8Δ1/end3 Δ (PSY86) double mutant cells were preincubated at 30 or 37 °C for 15 min. Labeling was initiated by the addition of Tran35S-label and the incubation was continued for 10 min. Unlabeled methionine and cysteine were added and incubation was continued for 45 min. ALP was immunoprecipitated as described in A.

Unlike soluble vacuolar proteins that were missorted in vps8Δ1 cells, ALP localization did not appear to be dependent on Vps8p function.

In a recent report, it has been shown that ALP is delivered to the vacuole via the cell surface in vps1 mutant cells (52). To see if ALP was trafficking via the cell surface in vps8 mutant cells, the sorting of ALP was examined in vps8Δ1/sec1 Δ and vps8Δ1/end3 Δ double mutants. The SEC1 gene product is required for the delivery of proteins from the Golgi to the cell surface (53). END3 gene product function is required for the internalization of proteins from the cell surface via the endocytic pathway (54). The sec1 Δ and end3 Δ mutant alleles that were used in this study show a temperature-dependent mutant phenotype. At the permissive temperature of 30 °C, both Sec1p and End3p function properly in the secretory and endocytic pathway, respectively. However, at the nonpermissive temperature of 37 °C, these mutant proteins become nonfunctional, and movement through the late secretory (sec1 Δ) or endocytic pathway (end3 Δ) is blocked (at a nonpermissive temperature, ALP expression was induced approximately 3–4-fold relative to permissive temperature). By examining ALP trafficking in vps8Δ1/sec1 Δ or vps8Δ1/end3 Δ double mutants at the nonpermissive temperature, the contribution of protein movement to (sec1 Δ) or from (end3 Δ) the plasma membrane can be determined. As shown in Fig. 3B, only mature vacuolar ALP was detected following the 45-min chase period at 30 or 37 °C, despite the fact that trafficking via the plasma membrane was blocked in these double mutants at a nonpermissive temperature (lanes 2 and 4). These results indicate that the localization of vacuolar membrane proteins such as ALP does not involve movement to or from the plasma membrane and that membrane trafficking from the Golgi apparatus to the vacuole continues in vps8 mutant cells.

Despite the fact that membrane is delivered to the vacuole, the morphology of this organelle was slightly perturbed in vps8Δ1 cells. Vacuoles can be visualized in a number of ways. Using Nomarski optics, the vacuole often appears as a large indentation(s) in the cell. In addition, there are a number of fluorescent dyes that accumulate in or associate with the vacuole. One of these fluorescent labels is the lipophilic dye, FM4-64 (55). This dye can be introduced into growing yeast cells by simply adding the dye to a cell suspension. The dye intercalates into the plasma membrane and then is endocytosed and transported to the vacuole, where it stably accumulates in the vacuolar membrane with no obvious deleterious effects on the cell. In wild-type cells, this dye revealed the presence of one to five vacuolar structures that are often distorted and interconnected by tubular structures (Fig. 4). These fluorescent structures were often coincident with the vacuolar indentations seen by Nomarski optics. In contrast, the vast majority of vacuoles seen in vps8Δ1 cells were single, round, and slightly enlarged structures (Fig. 4). In addition, mother-to-daughter vacuole segregation structures were largely missing in vps8Δ1 cells, and vacuolar acidification as measured by quinacrine uptake was defective in the mutant (data not shown). These vacuolar phenotypes are representative of those seen in class D vps mutants (18).

RING finger Zn2+ binding domains are thought to mediate protein-protein interactions. In an initial effort to examine the role this domain plays in Vps8p function, a mutant was constructed in which the last 31 C-terminal amino acids of Vps8p were eliminated (vps8ΔC31). This truncation eliminated 4 Cys/His residues that comprise the RING finger motif and would be predicted to eliminate the protein’s ability to coordinate Zn2+. Plasmids coding for Vps8ΔC31p or wild-type Vps8p were used to transform vps8Δ1 Δ mutant cells and the ability of these proteins to facilitate vacuolar localization of CPY was determined (Fig. 5A). vps8Δ1 Δ cells and vps8Δ1 Δ cells carrying wild-type Vps8p or truncated Vps8p were labeled for 10 min with [35S]methionine and [35S]cysteine and chased with unlabeled methionine and cysteine for 30 min. Whole cell lysates were generated from these cultures and CPY was immunoprecipitated from the cell extracts and resolved by SDS-PAGE. As seen previously, vps8Δ1 Δ cells mislocalized the vast majority of CPY, which accumulated as its Golgi-modified precursor form (Fig. 5A, lane 1). When wild-type Vps8p was expressed in these cells, the sorting defect was completely complemented as indicated by the presence of mature vacuolar CPY (lane 2). Expression of the truncated version of Vps8pΔC91p only partially complemented the CPY sorting defect seen in vps8Δ1 Δ cells. Approximately 20% of the CPY remained as the Golgi-modified precursor (lane 3). These results indicate that the C-terminal portion of Vps8p which contains the RING finger Zn2+ binding motif participates in Vps8p function.
Vps8p Is Required for Soluble Vacuolar Protein Localization

**Fig. 5. Partial complementation of the vps8Δ1 CPY missorting phenotype by Vps8pΔ1C31 and suppression of vps8Δ1 CPY missorting by overexpression of Vps21p.** A, vps8Δ1 cells (PSY83) (lane 1) and vps8Δ1 cells carrying wild-type VPS8 (lane 2) or the vps8ΔC31 truncation mutant (lane 3) on a CEN plasmid vector were labeled with Tran35S-label for 10 min at 30 °C. Unlabeled methionine and cysteine were added, and the incubation was continued for 30 min. Cells were lysed, and CPY was isolated by immunoprecipitation, resolved by SDS-PAGE, and visualized by fluorography. B, CPY was immunoprecipitated from extracts prepared from wild-type (SEY6210) cells (lane 1), vps8Δ1 (PSY83) cells (lane 2) and vps8Δ1 cells carrying VPS21 on a high copy number plasmid (lane 3) as described in A. The migration positions of Golgi modified CPY precursor (p2CPY) and mature vacuolar form of CPY (mCPY) are shown.

 share a number of phenotypes that suggest they may function at a similar step in the vacuolar protein sorting pathway. Though vps8 mutants were originally classified as class A mutants (vacuole morphology indistinguishable from wild-type), examination of vps8 deletion mutant cells clearly demonstrates that the vacuole seen in these cells has a morphology similar to other class D mutants. We examined the possibility of a genetic link between vps8 mutants and other genes that when mutated result in a class D phenotype. One of these, VPS21, encodes a small GTPase of the Rab family whose function has been implicated in vesicle trafficking in the vacuolar protein sorting pathway. *vps8Δ1* cells were transformed with a multicopy plasmid vector (2µ) containing the VPS21 gene and the effect of elevated Vps21p levels on the sorting of native CPY was examined in these cells. Wild-type cells, *vps8Δ1* cells and *vps8Δ1* cells carrying the 2µ VPS21 plasmid were labeled with [35S]methionine and [35S]cysteine and chased as described above. CPY was immunoprecipitated from cell lysates generated from these cultures and resolved by SDS-PAGE. As seen in Fig. 5B, wild-type cells (WT) properly delivered CPY to the vacuole, as demonstrated by the presence of mature vacuolar CPY (lane 1). In *vps8Δ1* cells, the vast majority of CPY was not delivered to the vacuole and was found in its Golgi-modified p2 precursor form (lane 2). However, when Vps21p was overexpressed in *vps8Δ1* cells, a significant portion of CPY was converted to the mature vacuolar form (lane 3). This suppression was not seen when VPS21 was present on a low copy, CEN vector or when *vps8Δ1* cells were transformed with the multicopy vector lacking the VPS21 sequences (data not shown). These results demonstrated that overexpression of Vps21p can partially suppress the CPY missorting phenotype associated with *vps8Δ1* mutant cells. Overexpression of other class D Vps proteins, including Vps45p, Pep12p, and Vps9p, did not suppress the *vps8Δ1* sorting defect and overexpression of Vps8p did not suppress the sorting defect associated with vps21Δ (data not shown).

**Characterization of the VPS8 Gene Product—Polyclonal antiserum raised against a trpE·Vps8p fusion protein was used to probe labeled cell extracts in order to identify the VPS8 gene product.** Wild-type cells, *vps8Δ1* cells and wild-type cells transformed with a multicopy plasmid carrying the VPS8 coding sequence were labeled with [35S]methionine and [35S]cysteine for 0 or 60 min, and the extracts were subjected to immunoprecipitation using the Vps8p antiserum. Molecular mass of Vps8p and Vps10p was shown to the right and were determined by comparing their migration pattern to that of stained protein standards. Exposure time for the Vps8p panel including lanes 4 and 5 was 1/50 of that for the panel including lanes 1–3. The exposure time for the Vps10p panels were the same.

**Fig. 6. Identification of the VPS8 gene product.** Wild-type (SEY6210) cells (lanes 2 and 3), wild-type cells carrying VPS8 on a multicopy plasmid (pPSY82) (lanes 4 and 5) and *vps8Δ1* cells (PSY83) (lane 1) were labeled for 10 min with Tran35S-label at 30 °C. The labeling was terminated by the addition of trichloroacetic acid immediately (lanes 1, 2, and 4) or following a 60-min chase period (lanes 3 and 5). Cell extracts were generated and Vps8p was isolated by immunoprecipitation. The immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. Vps10p was isolated by immunoprecipitation from duplicate samples of labeled cell extract. Molecular mass of Vps8p and Vps10p are shown to the right and were determined by comparing their migration pattern to that of stained protein standards. Exposure time for the Vps8p panel including lanes 4 and 5 was 1/50 of that for the panel including lanes 1–3. The exposure time for the Vps10p panels were the same.
Vps8p Is Required for Soluble Vacuolar Protein Localization

33613

Fig. 7. Subcellular fraction of the VPS8 gene product. Wild-type cells (SEY6210) (A) or vps21Δ cells (GBY10) (B) were converted to spheroplasts, labeled with Tran35S-label for 30 min, chased for 30 min at 30 °C, lysed, and subjected to sequential differential centrifugation. Equivalent amounts of the 13,000 × g pellet (P13), 100,000 × g supernatant (S100), and 100,000 × g pellet (P100) fractions were subjected to gel electrophoresis. Vps8p was visualized by fluorography. Percentage of the total Vps8p present in each fraction is shown beneath the panels and was determined by densitometry.

Because a genetic interaction was uncovered between VPS8 and VPS21, we also tested the effect of a vps21Δ mutation on Vps8p fractionation. When the Vps8p fractionation pattern was examined in cells lacking VPS21 (vps21Δ), it was found that the majority of Vps8p was no longer associated with cellular membranes. Approximately 80% of Vps8p was found in the soluble S100 cell fraction in vps21Δ cells (Fig. 7), in contrast to the 10% of Vps8p found in the S100 fraction of wild-type cells (Fig. 7). When Vps8p subcellular fractionation was examined in other class D mutant cells (Δvps45 and Δpep12), the majority of Vps8p remained associated with the P100 (date not shown). These data suggest that Vps21p is directly or indirectly responsible for the membrane association of Vps8p.

DISCUSSION

The work presented in this study describes the initial characterization of Vps8p. Vps8p is a hydrophilic 134-kDa protein required for the sorting of soluble vacuolar proteins. Cells that lack this protein missort and secrete the vast majority of the soluble vacuolar hydrolase CPY, while sorting of the vacuolar membrane protein, ALP, is unaffected. vpsΔ cells also exhibit a slow growth phenotype at 38 °C and contain a slightly enlarged, single vacuolar structure. Interestingly, most of Vps8p is associated with a particulate cell fraction that contains cellular membranes, even though the protein lacks any obvious hydrophobic domains that could serve as a transmembrane domain. This association is dependent on the presence of the small GTPase, Vps21p. Furthermore, overexpression of this GTPase partially suppresses the need for Vps8p function. Together these data indicate: 1) Vps8p participates in the transport of soluble vacuolar proteins from the Golgi to the prevacuolar endosome, 2) Vps8p may modulate Vps21p function, and 3) Vps8p may directly interact with Vps21p.

Morphological analysis has allowed the classification of the $\text{ups}$ mutants into distinct groups. Though vps8 mutant alleles were originally classified as class A (containing a normal appearing vacuolar compartment), vps8 null mutant cells exhibit several class D mutant phenotypes, distinguishing them from other class A $\text{ups}$ mutants (18). These phenotypes include the missorting of multiple soluble vacuolar proteins, a slight accumulation of aberrantly processed soluble vacuolar hydrolases, temperature sensitive growth at 38 °C, as well as a single, enlarged vacuolar structure that shows a decreased number of mother-to-daughter vacuole segregation structures and an acidification defect. Many of the wild-type VPS gene products affected in the class D mutants have been implicated in the vesicle-mediated transport of vacuolar proteins from the Golgi to the prevacuolar endosome. The Vps15p-Vps34p protein kinase-lipid kinase complex appears to play a role in the packaging and/or formation of intermediate transport vesicles at the surface of the Golgi (4, 57). The small GTPase of the Rab family, Vps21p (21, 25), and the Sec1p homologue, Vps45p (23, 26), appear to participate in the targeting and/or fusion of these transport vesicles with the endosome. The newly characterized gene product of VPS9 also appears to function in this vesicle transport event and may be involved in modulating Vps21p activity (28). In addition, Pep12p (Vps6p), a syntaxin homologue, is part of the class D group and likely mediates the docking of Golgi-derived transport vesicles with the prevacuolar endosome (27). The genetic interaction between VPS21 and VPS8 described here clearly implicates Vps8p function at the Golgi-to-endosome step in the vacuolar protein delivery pathway.

Clues as to the specific function of Vps8p in vacuolar protein sorting come from the observation that overexpression of Vps21p can partially bypass Vps8p function. One interpretation of this result is that Vps8p is a positive modulator of Vps21p function. Simply by increasing the overall levels of Vps21p in the cell, the function of Vps8p would be abrogated. Since Vps21p is a member of the Rab family of small GTPases, a number of activities that regulate its function may be envisioned. Vps8p could serve as a nucleotide exchange factor, enhancing Vps21p intrinsic nucleotide exchange rate and facilitating the conversion of Vps21p in its GDP-bound form to its GTP-bound form. Since Vps21p possesses a relatively high intrinsic nucleotide exchange activity,2 overexpression of this GTPase may provide the cell with enough Vps21p in its GTP-bound form to facilitate vacuolar protein sorting. A number of nucleotide exchange factors for members of the Rab, Ras, Rho, and Arf families of small GTPases have been described. However, Vps8p shares no significant sequence homology with these GEFs, and Vps8p-dependent nucleotide exchange activity has yet to be demonstrated. Alternatively, Vps8p may modulate Vps21p function by facilitating the interaction of Vps21p with other cellular component(s). For example, Vps8p may not be an exchange factor but may mediate the interaction between

---

2. B. Horazdovsky, unpublished results.
Vps21p and an exchange factor.

The similar subcellular fractionation pattern between Vps21p and Vps8p, as well as the dependence of Vps8p's membrane association on the presence of Vps21p, indicates that these two proteins physically interact with one another (directly or indirectly) and associate with similar membrane pools. In this regard, Vps21p may recruit Vps8p to the membrane in a manner analogous to the recruitment of Rabaptin-5 by Rab5 (58) or Rabphilin-3A by Rab3A (59, 60). Though sequence comparisons fail to reveal any extensive homology between Vps8p and Rabaptin-5 or Rabphilin-3A, Rabphilin-3A does contain a Cys-rich amino-terminal domain (four pairs of Cys-X2-Cys) (59, 61) that is required for its interaction with Rab3A (62). Similarly, Vps8p's Cys-rich RING finger domain may facilitate its interaction with Vps21p and/or other proteins. Consistent with this hypothesis, a truncated Vps8p lacking the Cys-rich motif exhibited reduced function. Closer examination of the interaction between Vps8p and Vps21p, as well as the membrane fraction with which Vps8p is associated, will be required to determine the potential role Vps8p plays in modulating Vps21p function.

Interestingly, Vps8p function is required for only the sorting of soluble vacuolar proteins; the localization of the vacuolar membrane protein, ALP, is unaffected in vps8 mutants (52). VPS1 codes for a high molecular weight GTPase that shows extensive homology with dynamin (63). Members of the dynamin family have been implicated in the formation of transport vesicles in mammalian cells and in yeast (64, 65). Like vps8 mutants, cells that lack Vps1p missort and secrete a large portion of the soluble vacuolar protein CPY but deliver ALP to the vacuole (52). In the case of vps1 mutants, ALP is delivered to the vacuole via the plasma membrane. One explanation of this result is that the loss of Vps1p function results in the mislocalization of vacuolar membrane proteins to the cell surface where they are subsequently delivered to the vacuole through the endocytic pathway (52). However, the localization of ALP in vps8 mutants that also contained the sec1 or end3 temperature sensitive mutation demonstrated that delivery of ALP in these mutants does not involve transit via the plasma membrane. ALP is delivered to the vacuole despite the fact that movement to (sec1) and from (end3) the plasma membrane is blocked in the vps1/sec1 and vps8/end3 double mutants. One possibility is that vacuolar membrane proteins like ALP may utilize a different transport intermediate whose delivery is not dependent on Vps8p function. Our current studies are directed toward dissecting the transport system utilized for the delivery of vacuolar membrane proteins.

Work with the Vps proteins affected in the class D vps mutants already has provided many new insights into the formation of Golgi-derived transport vesicles as well as the targeting and fusion of these vesicles with the prevacuolar endosome. Many of these proteins have been found to share significant sequence homologies with other proteins known to participate at other vesicle-mediated transport events throughout the secretory and endocytic pathways. This conservation suggests that many of the basic functions involved in vesicle targeting events are carried out by distinct sets of related proteins (66, 67). Some of these components are shared (e.g. NSF), while others, though related, are specific to discrete vesicle targeting events (e.g. Rab, Sec7 homologues, and SNAREs). Among these groups of proteins, Vps8p appears to be unique (no homologues yet identified). Determining Vps8p's precise function in the vacuolar protein sorting pathway therefore should provide additional insights into the mechanisms and regulation of vesicular trafficking events.
Vps8p Is Required for Soluble Vacuolar Protein Localization

53. Novick, P., Ferro, S., and Schekman, R. (1981) Cell 25, 461–469
54. Benedetti, H., Rath, S., Crausz, F., and Riezman, H. (1994) Mol. Biol. Cell 5, 1023–1037
55. Vida, T. V., and Emr, S. D. (1995) J. Cell Biol. 128, 779–792
56. Marcussen, E. G., Horazdovsky, B. F., Cereghino, J. L., Gharakhanian, E., and Emr, S. D. (1994) Cell 77, 1023–1037
57. Stack, J. H., Herman, P. K., Schu, P. V., and Emr, S. D. (1993) EMBO J. 12, 2195–2204
58. Stenmark, H., Vitale, G., Ullrich, O., and Zerial, M. (1995) Cell 83, 423–432
59. Shirataki, H., Kaibuchi, K., Sukoda, T., Kishida, S., Yamaguchi, T., Wada, K., Miyazaki, M., and Takai, Y. (1993) Mol. Cell. Biol. 13, 2061–2068
60. Stahl, B., Chou, J. H., Li, C., Sudhof, T. C., and Jahn, R. (1996) EMBO J. 15, 1799–809
61. Li, C., Takei, K., Geppert, M., Daniell, L., Stenius, K., Chapman, E. R., Jahn, R., De Camilli, P., and Sudhof, T. C. (1994) Neuron 13, 885–898
62. Yamaguchi, T., Shirataki, H., Kishida, S., Miyazaki, M., Nishikawa, J., Wada, K., Numata, S., Kaibuchi, K., and Takai, Y. (1993) J. Biol. Chem. 268, 27164–27170
63. Vater, C. A., Raymond, C. K., Ekena, K., Howaldstevenson, I., and Stevens, T. H. (1992) J. Cell Biol. 119, 773–786
64. Takei, K., McPherson, P. S., Schmid, S. L., and De Camilli, P. (1995) Nature 374, 186–190
65. Hinshaw, J. E., and Schmid, S. L. (1995) Nature 374, 190–192
66. Bennett, M. K., and Scheller, R. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2559–2563
67. Rothman, J. E., and Orci, L. (1992) Nature 355, 409–415