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A Yeast Protein Related to a Mammalian Ras-Binding Protein, Vps9p, Is Required for Localization of Vacuolar Proteins

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In the yeast *Saccharomyces cerevisiae*, mutations in vacuolar protein sorting (*VPS*) genes result in secretion of proteins normally localized to the vacuole. Characterization of the VPS pathway has provided considerable insight into mechanisms of protein sorting and vesicle-mediated intracellular transport. We have cloned *VPS9* by complementation of the vacuolar protein sorting defect of *vps9* cells, characterized its gene product, and investigated its role in vacuolar protein sorting. Cells with a *vps9* disruption exhibit severe vacuolar protein sorting defects and a temperature-sensitive growth defect at 38°C. Electron microscopic examination of Δ*vps9* cells revealed the appearance of novel reticular membrane structures as well as an accumulation of 40- to 50-nm-diameter vesicles, suggesting that Vps9p may be required for the consumption of transport vesicles containing vacuolar protein precursors. A temperature-conditional allele of *vps9* was constructed and used to investigate the function of Vps9p. Immediately upon shifting of temperature-conditional *vps9* cells to the nonpermissive temperature, newly synthesized carboxypeptidase Y was secreted, indicating that Vps9p function is directly required in the VPS pathway. Antibodies raised against Vps9p immunoprecipitate a rare 52-kDa protein that fractionates with cytosolic proteins following cell lysis and centrifugation. Analysis of the *VPS9* DNA sequence predicts that Vps9p is related to human proteins that bind Ras and negatively regulate Ras-mediated signaling. We term the related regions of Vps9p and these Ras-binding proteins a GTPase binding homology domain and suggest that it defines a family of proteins that bind monomeric GTPases. Vps9p may bind and serve as an effector of a rab GTPase, like Vps21p, required for vacuolar protein sorting.

In eukaryotic cells, the identities and functions of organelles are determined largely by their unique repertoire of resident proteins. Proper intracellular organization, therefore, requires sorting and transport of proteins from their common site of synthesis to the appropriate resident organelle. Studies of vacuolar protein biosynthesis in *Saccharomyces cerevisiae* have provided an extremely useful experimental system for studying protein localization. Genetic selections for mutants that mislocalize vacuolar proteins (vacuolar protein sorting [*vps*] mutants) have led to the identification of more than 40 genes required in this process (2, 34–36). Ongoing analysis of *VPS* genes and *vps* mutants continues to provide considerable insight into mechanisms of protein sorting and intracellular trafficking of macromolecules.

The vacuole of *S. cerevisiae* is an acidic organelle containing large amounts of degradative enzymes and is therefore analogous to the lysosome of animal cells (21). Of all vacuolar proteins, the biosynthesis of carboxypeptidase Y (CPY) is best understood, and it serves as a prototype in models of vacuolar protein sorting. CPY is an abundant, soluble, vacuolar protease whose trafficking status can be easily assessed by identification of electrophoretically distinct forms generated during its biosynthesis (44). CPY enters the secretory pathway at the endoplasmic reticulum (ER), where core glycosyl chains are added (forming p1CPY [67 kDa]), and then transits through the Golgi apparatus, where it is further modified by glycosylation (forming p2CPY [69 kDa]). The vacuolar targeting signal of CPY, a short stretch of amino acids found in the pro segment (19, 46), is bound in a late Golgi compartment by its sorting receptor encoded by *VPS10* (10, 24, 48), effectively sorting p2CPY from the pool of secretory proteins (24). In poorly understood ensuing steps, receptor-bound CPY is packaged into transport vesicles and is delivered to a prevacuolar endosomal compartment (30, 48). Subsequent transport to the vacuole results in proteolytic processing of p2CPY to the mature active form, mCPY (61 kDa). Despite the wealth of information regarding the biosynthesis of CPY, important fundamental questions regarding the vesicular packaging mechanism and the underlying transport pathway to the vacuole remain unanswered.

A growing number of observations indicate that transport from the late Golgi to the vacuole is mechanistically similar to other secretory transport steps. First, ER-to-Golgi, Golgi-to-plasma membrane, and Golgi-to-vacuole transport is vesicle mediated, and second, sequence analysis of *VPS* genes indicates that several encode proteins which are homologous to proteins of the secretion machinery. For example, *VPS21*, *PEP12*/*VPS6*, and *VPS45* encode a member of the rab family of regulatory GTP hydrolases, a putative vesicle receptor (T-SNARE), and a Sec1p homolog, respectively, protein families implicated in secretory transport (3, 7, 16, 31). Mutations in each of these genes, as well as several other *VPS* genes comprising the class D *vps* group, result in similar phenotypes; the mutant cells are characterized by having a single, enlarged vacuole compared with wild-type cells, exhibit vacuolar inheritance defects, mislocalize soluble vacuolar hydrolases, and fail to properly assemble the vacuolar ATPase (32, 34). The common set of phenotypes that result from mutations in class D *VPS* genes suggests that they affect a common segment of the

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference or source
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	34
SEY6211	*MAT***a** *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ90 ade2-101 suc2-Δ9*	34
BHY10	SEY6210; *leu2-3,112*::pBHY11(CPY-Inv *LEU2*)	16
BHY11	SEY6211; *leu2-3,112*::pBHY11(CPY-Inv *LEU2*)	16
SEY9-2	SEY6210; *vps9-2*	34
SEY9-5	SEY6211; *vps9-5*	34
CBY1	SEY6210; *vps9Δ1*::*HIS3*	This study
CBY2	BHY10; *vps9Δ1*::*HIS3*	This study
CBY10	BHY10; *VPS9/VPS9*::*TRP1*	This study
CBY20	BHY10; *vps9Δ2*::*HIS3*	This study
CBY21	BHY11; *vps9Δ2*::*HIS3*	This study
CBY22	SEY6211; *vps9Δ2*::*HIS3*	This study
CBY23	SEY6210; *vps9Δ2*::*HIS3*	This study
CBY24	*MATα/MAT***a** *leu2-3,112*::pBHY11(CPY-Inv *LEU2*)/*leu2-3,112 his3-Δ200/his3-Δ200 ura3-52/ura3-52 trp1-Δ901/trp1-Δ901 suc2-Δ9/suc2-Δ9 ADE2/ade2-101 lys2-801/LYS2VPS9/VPS9*::*TRP1vps9-5*	This study

Golgi-to-vacuole pathway, and therefore they provide a useful genetic framework with which to investigate vesicle-mediated transport.

We describe here the identification, cloning, and characterization of *VPS9*. Mutations in *VPS9* result in missorting of soluble vacuolar protein precursors, and experiments using a temperature-conditional allele of *vps9* indicate that Vps9p is directly involved in vacuolar protein trafficking. The DNA sequence of *VPS9* predicts a protein product that is related to mammalian Ras-binding proteins.

MATERIALS AND METHODS

Strains and media. *S. cerevisiae* strains used for these studies are listed in Table 1. Yeast strains were grown in standard yeast extract-peptone-dextrose (YPD) (38), yeast extract-peptone-fructose (YPF), or synthetic medium supplemented with 2% Casamino Acids and essential amino acid supplements (38) as required for maintenance of plasmids. Standard bacterial medium (26) was used for *Escherichia coli* cultures.

Yeast and bacterial methods. Transformation of *S. cerevisiae* strains was done by the lithium acetate method of Ito et al. (18), with single-stranded DNA used as the carrier (37). *E. coli* transformations were done by the method of Hanahan (12).

Standard techniques were used for genetic manipulations of *S. cerevisiae* (38). Integrative mapping of cloned *VPS9* was accomplished as follows. A *Hin*dIII fragment carrying *VPS9* was cloned into the integrative vector pRS304 (39), which carries the *TRP1* locus, to generate pCB99. This plasmid was cut at the unique *Nru*I site immediately upstream of the *VPS9* open reading frame (ORF) and used to transform BHY10 to tryptophan prototrophy, generating strain CBY10. CBY10 was crossed to SEY9-5 (34), and the resulting diploid (CBY24) was sporulated. Forty-four asci were dissected, and the haploid progeny were scored for CPY sorting and *TRP1* prototrophy. We observed the expected segregation pattern, indicating that the cloned DNA and the *VPS9* locus are tightly linked, presumably representing the same site.

DNA methods. Standard DNA manipulations (23) were used with restriction endonucleases and modification enzymes from Boehringer Mannheim, New England Biolabs, or U.S. Biochemical Corporation. The DNA sequence of the *VPS9* ORF and several hundred base pairs on each side of it were obtained by using Sequenase II (U.S. Biochemical Corp.) as instructed by the manufacturer. Oligonucleotides complementary to adjacent plasmid sequences were used for sequencing of subcloned fragments of the complementing *Hin*dIII fragment. In some cases, oligonucleotide primers complementary to *VPS9* were used. MacVector (Kodak) sequence analysis software was used to compile sequence data, and BLAST was used to search the GenBank database with the predicted protein sequence of Vps9p (1). To search for protein sequence motifs within Vps9p, BLOCKS (13), COILS (22), and BEAUTY (50) computer programs were used. Amino acid sequence alignments were done with the assistance of CLUSTAL W (45).

Cloning of *VPS9*. The wild-type *VPS9* locus was cloned by complementation of the CPY sorting defect of SEY9-2. A centromeric yeast library (*LEU2*, *CEN*; kindly provided by Philip Hieter) containing 10- to 15-kb DNA fragments was used to transform SEY9-2. Using a previously described colorimetric plate assay to detect secreted CPY-invertase (29), approximately 25,000 transformants were screened for complementation of the Vps$^-$ sorting defect. Five Vps$^+$ colonies were obtained, and plasmids were recovered from each them. Restriction mapping revealed that each plasmid possessed overlapping DNA fragments, and testing of smaller common fragments narrowed the complementing region to an approximately 2.5-kb *Hin*dIII fragment. This fragment was cloned into pRS416 and pRS426 (5) to generate pPS91 and pPS92, respectively.

Preparation of antiserum against Vps9p. A *trpE-VPS9* hybrid gene, encoding the carboxy-terminal 138 amino acids of Vps9p, was used to produce a fusion protein in *E. coli* JM101. The fusion protein was purified from bacterial extracts by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and used to immunize New Zealand White rabbits as previously described (17).

Metabolic labeling, immunoprecipitations, and subcellular fractionation. Yeast cultures were radiolabeled by using previously published procedures (16, 17). Immunoprecipitations were done by the method of Klionsky and Emr (20). For subcellular fractionation studies, spheroplasts made from wild-type cells (SEY6210) were labeled and processed as previously described (16). After clearing of the lysed spheroplast extract (500 × *g* for 10 min), the resulting supernatant was immediately centrifuged at 100,000 × *g* for 1 h to generate pellet (P100) and supernatant (S100) fractions from which various proteins were immunoprecipitated.

Construction of mutant *vps9* alleles. Two different deletion/disruption alleles of *vps9* were constructed in the following manner. An *Nla*III fragment containing the *VPS9* ORF was cloned into the *Sph*I site of pUC18. The 678-bp *Sna*BI-*Cla*I fragment was then replaced with the *HIS3* gene (Fig. 1). PCR was used to amplify the Δ*vps9*::*HIS3* construct, using PCR primers complementary to sequences at the beginning and end of the *VPS9* ORF. Homologous recombination-mediated transformation of SEY6210, SEY6211, BHY10, and BHY11 yielded corresponding Δ*vps9*::*HIS3* strains CBY23, CBY22, CBY20, and CBY21. In a second deletion/disruption construct, a 1,640-bp *Sna*BI fragment (Fig. 1) was removed from the cloned 2.5-kb *Hin*dIII fragment and replaced with the *HIS3* gene. The deletion/disruption construct was gel purified after digestion with *Hin*dIII and used for homologous recombination-mediated transformation of SEY6210 and BHY10 to generate CBY1 and CBY2. Disruption of the *VPS9* locus was confirmed by a PCR-based test (14) using oligonucleotide primers complementary to *VPS9* DNA sequences.

A modified PCR-based procedure was used to construct a temperature-sensitive for function (*tsf*) *vps9* allele (27, 40). The pPS91 *Hin*dIII fragment containing *VPS9* was amplified with skewed deoxyribonucleotide triphosphate concentration conditions (0.02 mM dATP; 0.2 mM dGTP, dTTP, and dCTP) with primers complementary to plasmid sequences immediately adjacent to the cloned yeast DNA fragment. The resulting PCR product was cotransformed with pPS91 which had been digested with *Sna*BI and gel purified to remove the *VPS9*-containing *Sna*BI fragment. Approximately 6,000 CBY1 Ura$^+$ plasmid transformants, containing gapped plasmids which were repaired by homologous recombination between overlapping plasmid and PCR fragment sequences, were selected and replica plated to two sets of YPF plates. After overnight growth at 26°C, one set of replicas was transferred to 38°C for 5 h, and the other set was maintained at 26°C. A colorimetric invertase plate assay (29) was used to identify colonies that were Vps$^+$ at 26°C and Vps$^-$ at 38°C. Putative *vps9*^{tsf} colonies were picked and retested, and plasmid linkage of the *tsf* missorting phenotype was confirmed after retransformation into CBY1 and CBY22.

Electron microscopy analysis. SEY6210 and CBY23 were grown in YPD to an optical density at 600 nm (OD_{600}) of 0.5 and then harvested by centrifugation. Previously described procedures (16) were used for fixing, staining, and viewing cross sections of each strain, except that the time of cell wall digestion was reduced to 1 h. The frequency of occurrence of honeycomb lattice structures in CBY23 cells was quantitated in 102 cells. A lattice was scored if it had at least two clearly recognizable elements of the lattice.

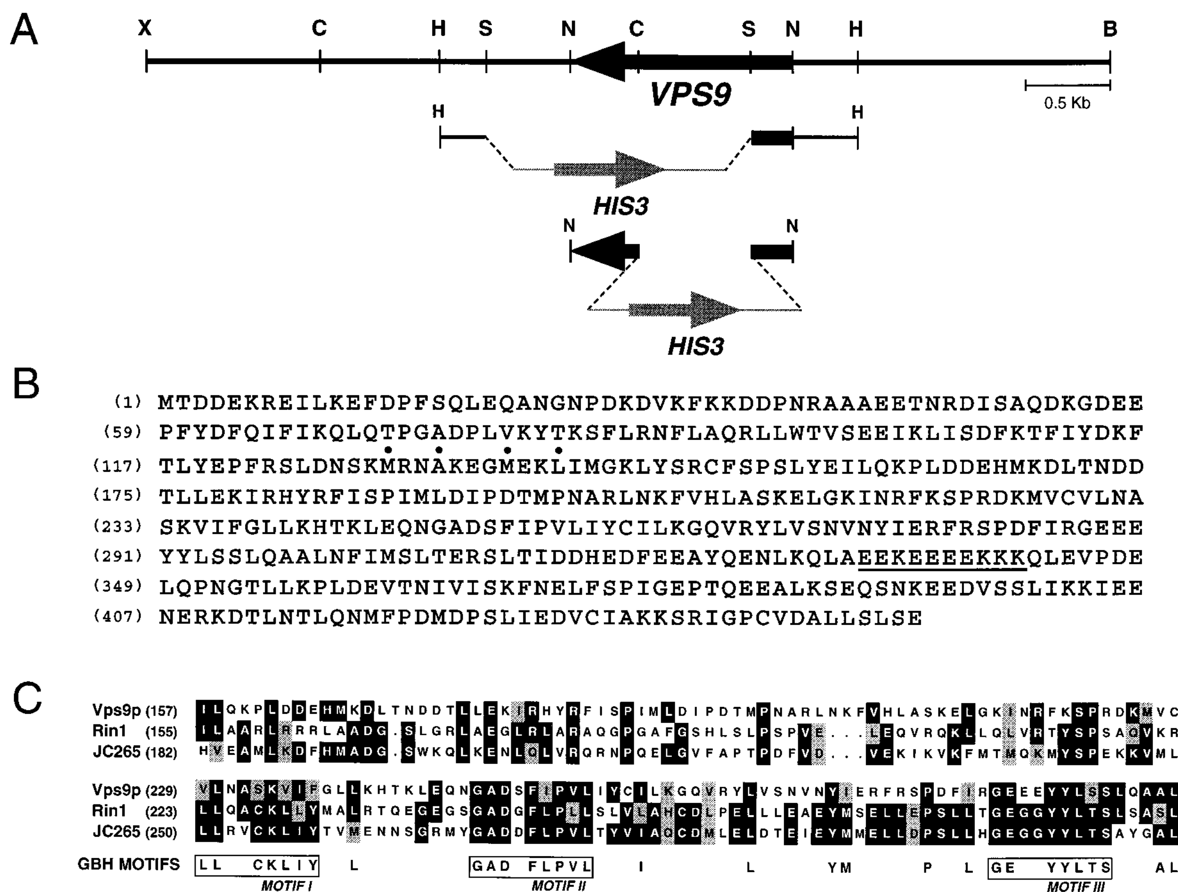

FIG. 1. Characterization of the *VPS9* locus. (A) Representation of a plasmid clone containing genomic DNA that complements mutations in *vps9*. Restriction endonuclease cleavage sites used in this study are indicated by vertical lines. Schematic representations of two deletion/replacement constructs used to make Δ*vps9*::*HIS3* strains CBY1 and CBY2 (middle) and CBY20, CBY21, CBY22, and CBY23 (bottom) are indicated. Abbreviations for restriction enzymes: C, *Cla*I; B, *Xba*I; H, *Hin*dIII; S, *Sna*BI; X, *Xho*I. (B) Predicted sequence of Vps9p in single-letter amino acid code. The region underlined constitutes a highly charged patch of glutamic acid and lysine residues. Amino acids indicated with dots above them conform to the heptad repeat motif. (C) Amino acid sequences of the related regions of Vps9p, Rin1, and JC265 (6). We have termed these regions GBH domains. Proteins are identified on the left, and the position of the first amino acid on each line follows in parentheses. Identities between sequences are indicated with black boxes, and conservative amino acid substitutions are indicated with grey boxes. A GBH domain consensus sequence is listed for the highly conserved carboxy-terminal region of the GBH domain. GBH motifs, the most highly conserved sequences of the consensus, are boxed.

Nucleotide sequence accession number. The GenBank accession number for the sequence presented in this paper is U50142.

RESULTS

Identification of *VPS9*. We have previously described a genetic selection that makes use of a hybrid gene encoding the vacuolar targeting signal of CPY fused in frame to the coding sequence of invertase to identify strains that mislocalize vacuolar proteins (2). Proper functioning of the VPS pathway results in the delivery of the CPY-invertase fusion protein to the vacuole, whereas in mutant *vps* cells, the fusion protein is secreted (2). In one screen, 20 alleles of *vps9* were obtained (34), and one of these strains (SEY9-2) was used to clone the wild-type *VPS9* locus. To do so, SEY9-2 was transformed with an *S. cerevisiae* centromeric DNA library to generate approximately 25,000 Leu^+ transformants, and they were subsequently screened for complementation of the Vps^- phenotype by using a colorimetric plate assay that detects secreted invertase (29). Five colonies that exhibited a plasmid-linked Vps^+ phenotype were obtained, and restriction analysis of the plasmid in each of these transformants indicated that they contained overlapping yeast DNA segments. A series of subclones was generated from the smallest complementing plasmid, and each was further tested for complementation. The minimal complementing DNA fragment tested was an approximately 2.5-kb *Hin*dIII fragment (Fig. 1), and integrative mapping techniques confirmed that the *VPS9* locus was indeed contained on this DNA fragment (see Materials and Methods).

We determined the sequence of part of the 2.5-kb *Hin*dIII DNA fragment described above and found a single ORF with the capacity to encode a protein of 451 amino acids (predicted molecular mass of 52,393 Da and predicted pI of 5). Hydropathy analysis (data not shown) of this ORF predicted a hydrophilic protein with no hydrophobic regions long enough to span a lipid bilayer; thus, Vps9p is predicted to be a soluble protein. The predicted sequence of Vps9p was used to search the DNA sequence databases with BLAST (1) for genes with related primary structure. This search revealed nearly complete identity (99%) with two yeast genome database entries (accession numbers U20373 and Z46660) that appear to correspond to the same gene. Several differences between the *VPS9* ORF that we sequenced and the putative *VPS9* sequences in the database were found; careful examination of

our DNA sequencing gels and resequencing of problematic regions confirm the sequence presented here.

The predicted sequence of Vps9p also displayed significant sequence similarity to proteins encoded by two highly related human cDNAs, termed JC265 and Rin1 (BLAST *P* values of 2.3×10^{-9} and 7.5×10^{-8}, respectively), which were identified as inhibitors of a mutant *ras2* allele in *S. cerevisiae* (6). A comparison of the regions of Rin1, JC265, and Vps9p that may be related is shown in Fig. 1C. Over this 144-amino-acid region (Vps9p numbering), these three sequences are 19% identical and 35% similar when conservative amino acid changes are considered. The carboxy-terminal 72 amino acids are more highly related than the amino-terminal 72 amino acids (25% identical and 46% similar versus 13% identical and 24% similar). Close examination of the aligned sequences revealed three nine-amino-acid sequences (in the carboxy-terminal half of the alignment) that are nearly identical all of the proteins. Figure 1C shows a consensus sequence that describes the more highly related carboxy-terminal regions; we have termed these nonamer sequences GTPase binding homology (GBH) motifs I, II, and III. Interestingly, GBH motif II includes a region of JC265 and Rin1 previously suggested to be distantly related to GTPase-activating proteins of human Ras (6).

We also examined the sequence of Vps9p for previously defined protein sequence motifs. This analysis revealed a small region of Vps9p, encompassing amino acids 130 to 143, containing a heptad repeat which is strongly predicted to form an amphipathic alpha helix capable of participating in coiled-coil interactions with other proteins (22). The amino acids comprising the conserved positions of the heptad repeat are indicated with dots in Fig. 1B. Amino acids 331 to 340 of Vps9p (underlined in Fig. 1B) constitute a highly charged patch of 10 contiguous aspartate and lysine residues that resemble in character the highly charged complexin family of proteins implicated in neuronal exocytosis (25).

Characterization of a *vps9* null mutant. Strains containing a null *vps9* allele were generated by integrative disruption of the *VPS9* locus with the *HIS3* gene (see Materials and Methods and Fig. 1). Transformation of a wild-type strain with a DNA fragment containing either of two deletion/replacement constructs resulted in histidine prototrophy and loss of Vps9p, as judged by immunoprecipitation (see Fig. 6). Disruption of the *VPS9* locus was also confirmed by a PCR-based test (data not shown).

The results described above indicate that *VPS9* is not an essential gene under optimal growth conditions. Null alleles in some other *vps* genes result in a growth defect at elevated temperatures; therefore, we tested the ability of the Δ*vps9*::*HIS3* strain (CBY23) to grow at different temperatures. At or below 37°C, CBY23 grew nearly as well as a wild-type strain (SEY6210); at 38°C, however, growth of CBY23 was severely impaired (Fig. 2). This phenotype is shared with a number of other characterized class D *vps* null mutants, including Δ*pep12*/*vps6*, Δ*vps21*, Δ*vps45*, and Δ*vac1*/*vps19* (3, 7, 16, 31, 49). A centromeric plasmid carrying *VPS9* (pPS91) complemented the growth defect of CBY23, indicating that the growth defect of the null strain is caused by the disruption of *VPS9* (Fig. 2). Temperature-sensitive growth of *vps9* strains, as well as other *vps* strains, is likely to result from the combined stress of growth at elevated temperature and defects in vacuolar protein sorting rather than from temperature-sensitive function of an essential component in these mutant strains (34).

Six distinct morphological classes of *vps* mutants have been identified. Genes in each class encode proteins that are thought to act within a common segment of the VPS pathway. Indeed, proteins encoded by two class D *VPS* genes, *VPS15* and *VPS34*, form a complex required for vacuolar protein sorting (40, 41). Previous studies of mutant *vps9* strains revealed that these cells contain a single large vacuole and lack vacuolar segregation structures (32), hallmarks of class D *vps* mutants. We examined cross sections of Δ*vps9* cells (CBY23) by electron microscopy and observed several striking morphological changes associated with the loss of *VPS9*. Compared with wild-type cells, the majority of cross sections contained elevated but variable numbers of approximately 40-nm-diameter vesicles (Fig. 3A and C). These vesicles are the same size as those that accumulate in the cells of some other class D *vps* strains such as Δ*vps21*, Δ*vps45*, and Δ*pep12*/*vps6* mutants (3, 7, 16, 31). In addition, approximately 10% of the sections contained reticulated membrane networks that took on the appearance of a honeycomb lattice, with an average distance between individual elements of the lattice of approximately 60 to 70 nm (Fig. 3A and C to F). In many sections, we also observed strings of evenly spaced (by approximately 60 to 70 nm) tubulovesicular structures that may be perpendicular cross sections of the networks shown in Fig. 3. Several other heterogeneous structures were also observed in Δ*vps9* cells, including tubular "fingers" emanating from a central body resembling a vacuole (Fig. 3H) and membrane rings similar to Berkeley bodies (28) (Fig. 3G). Finally, nearly all Δ*vps9* cells examined possessed a single large vacuole typical of class D *vps* mutants.

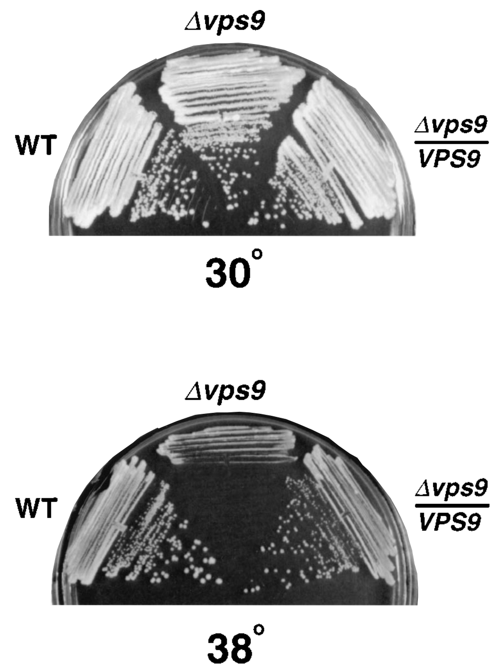

FIG. 2. Null mutations in *VPS9* result in temperature-sensitive growth. The indicated yeast strains (wild type [WT], SEY6210; Δ*vps9*, CBY23; Δ*vps9*/VPS9, CBY23 transformed with pPS91, encoding Vps9p) were streaked onto YPD plates and incubated for 3 days at the indicated temperature.

The original alleles of *vps9* were identified because of defects in the delivery of a CPY-invertase fusion protein to the vacuole. To determine the extent of the vacuolar protein sorting defect in cells completely lacking Vps9p, trafficking of four vacuolar proteins, CPY, proteinase A (PrA), alkaline phosphatase (ALP), and carboxypeptidase S (CPS), was analyzed in the Δ*vps9* strain (Fig. 4). For these experiments, cultures were pulse-labeled with Tran^{35}S-label for 10 min to label newly synthesized vacuolar precursors, then chase medium was

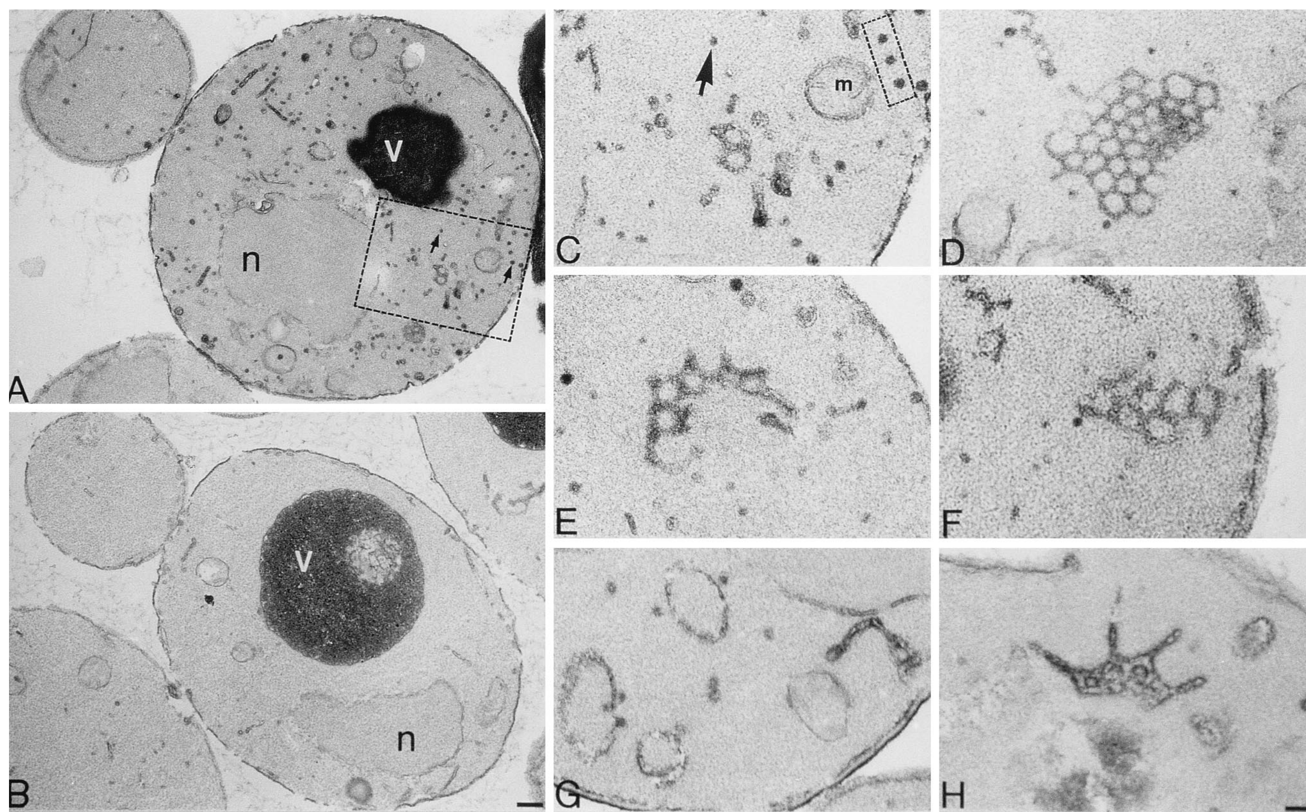

FIG. 3. Electron microscopic analysis of CBY23 (Δ*vps9*) cells. (A) Cross section of a typical mutant cell. Arrows point to examples of approximately 40-nm-diameter vesicles that accumulate in this mutant. (B) Cross section of a wild-type cell (SEY6210). A region of the cell shown in panel A (dashed box) is enlarged in panel C, and an evenly spaced string of three vesicles is enclosed within the dashed box. (D to F) Honeycomb lattice structures from three different CBY23 cell cross sections. (G) Example of the membrane rings found in some CBY23 cross sections. (H) Example of the vacuolar structures seen in some CBY23 cells. In panels A and B, the nucleus (n) and vacuole (v) are labeled; in panel C, a mitochondrion (m) is indicated. In panels A and B, the magnification is ×13,140 (the scale bar in panel B represents approximately 0.27 μm); in panels C to H, the magnification is ×32,850 (the scale bar in panel H represents approximately 0.11 μm).

added, and the cells were incubated for a further 40 min. Cells were subsequently converted to spheroplasts and fractionated to yield an intracellular and extracellular fraction, and antibodies specific for the individual vacuolar proteins were then used to immunoprecipitate the proteins from each fraction. The Δ*vps9* strain accumulated all newly synthesized CPY as the Golgi-modified p2CPY precursor and secreted approximately 80% of it during the chase period. A small portion of CPY was present as a smear which may represent intracellular degradation or aberrant glycosylation of the mistargeted protein. Another soluble vacuolar protease, PrA, also accumulated as its Golgi-modified precursor, and approximately 50% of it was secreted during the chase period. Small amounts of several other processed forms of PrA, including a form that comigrates with mature PrA, are also apparent; however, it is not known if this form represents bona fide mature PrA in the vacuole, as some was observed in the extracellular fraction. Maturation of the integral membrane proteins ALP and CPS was completely blocked in Δ*vps9* cells (Fig. 4).

Isolation of a *tsf* allele of *vps9*. The results described above suggest that Vps9p is required for sorting of vacuolar proteins. These experiments, however, do not distinguish if Vps9p is directly required for vacuolar protein sorting or if the sorting defects observed with mutant *vps9* cells is an indirect consequence of the loss of *VPS9* function. These possibilities can be distinguished with the use of a *tsf* allele of *vps9*; if Vps9p is directly required for vacuolar protein sorting, then inactivation of Vps9p function (by a temperature shift) should result in rapid missorting of vacuolar protein precursors. If not, we expect to observe a significant time lag before the onset of the *vps* phenotype. A PCR-based mutagenesis strategy (27, 40) was used to obtain several temperature-conditional alleles of *vps9*, and one was chosen for further analysis. At the permissive temperature (26°C), *vps9^{tsf}* cells delivered approximately 90% of newly synthesized CPY to the vacuole (Fig. 5A). In contrast, in *vps9^{tsf}* cells incubated at the nonpermissive temperature (38°C) for 30 min before labeling and then throughout the course of the experiment, more than 50% of newly synthesized CPY accumulates as the Golgi-modified p2CPY precursor, indicating that this fraction of p2CPY is not delivered to the vacuole. We isolated several other putative *vps9^{tsf}* alleles but did not obtain any that had a CPY sorting defect more severe than that of the *vps9^{tsf}* allele characterized here (see below).

The onset kinetics of the *vps9^{tsf}* missorting phenotype after a shift from the permissive to the nonpermissive temperature was determined. To do so, *vps9^{tsf}* cells were pulse-labeled with Tran^{35}S-label for 5 min at 26°C. The culture was then transferred to 38°C, and a prewarmed chase solution containing unlabeled methionine and cysteine was added. At various times after initiation of the chase, aliquots of the culture were removed, extracellular and intracellular fractions were generated, and CPY was immunoprecipitated from each fraction. As expected, at the end of the short labeling period, most CPY was found in the intracellular fraction as its ER-modified p1 precursor, with a small amount of Golgi-modified p2CPY also present (Fig. 5B, t_0). Ten minutes after the temperature shift,

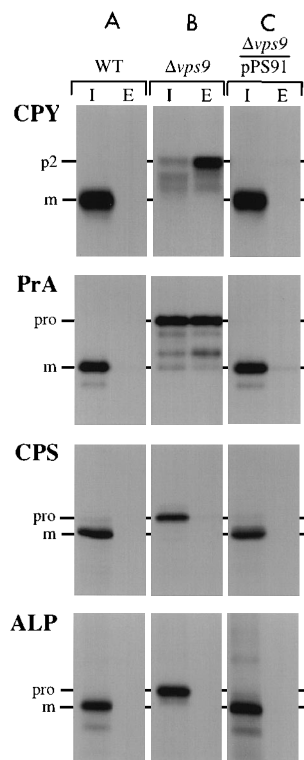

FIG. 4. Vacuolar protein sorting in Δ*vps9* mutant cells. Cultures of the indicated strains were pulse-labeled with Tran^{35}S-label for 10 min at 30°C, chase medium was added, and the cultures were incubated for a further 40 min. Cells were then spheroplasted, and intracellular (I) and extracellular (E) fractions were generated. Antibodies specific for the indicated vacuolar proteins were used to immunoprecipitate proteins from each fraction, and the recovered material was then subjected to SDS-PAGE and visualized by fluorography. Before electrophoresis, CPS samples were treated with endoglycosidase H. (A) Sorting analysis of wild-type (WT; SEY6210) cells; (B) analysis of Δ*vps9* (CBY2) cells; (C) analysis of Δ*vps9* cells transformed with pPS91, encoding Vps9p. The higher-molecular-weight precursor form (pro) and smaller mature forms (m) of each vacuolar protein are indicated to the left.

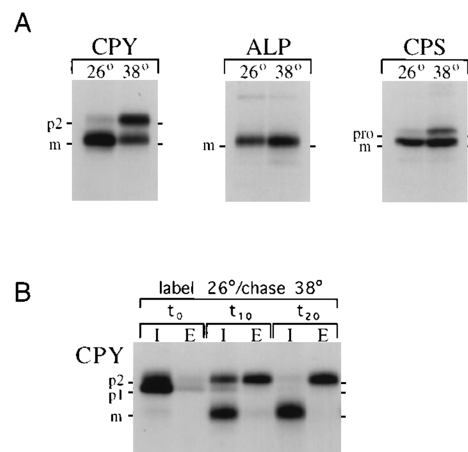

FIG. 5. Vacuolar protein sorting in *vps9^{tsf}* cells. (A) Mutant *vps9^{tsf}* cells were preincubated at 26 or 38°C for 30 min prior to labeling with Tran^{35}S-label for 10 min. Prewarmed chase solution was then added to each culture, and the cells were chased for 40 min at the indicated temperature and processed as described in Materials and Methods. (B) CBY20 (Δ*vps9*) cells transformed with a centromeric plasmid (pCB105) encoding a temperature-conditional *vps9* allele were pulse-labeled for 5 min at 26°C. The culture was then transferred to 38°C, and prewarmed chase solution was added. Equal aliquots of the culture were harvested at the indicated time points (t_0, time of chase addition) and processed as described in the legend to Fig. 4.

more than 50% of p2CPY was found in the extracellular fraction, indicating that it had been missorted and secreted (Fig. 5B, t_{10}). Twenty minutes after the addition of chase, nearly all p2CPY was found in the extracellular fraction. In wild-type cells subjected to the same experimental regimen, only mature CPY was present inside the cells (data not shown [40]).

The *vps9^{tsf}* allele also allowed us to test if Vps9p function is required for the delivery of vacuolar membrane proteins to the vacuole. The vacuoles of *vps9^{tsf}* cells grown at the permissive temperature contain soluble vacuolar hydrolases and are fully competent to mature vacuolar ALP and CPS (Fig. 5A). Therefore, after *vps9^{tsf}* cells are shifted to the nonpermissive temperature, newly synthesized ALP or CPS should be proteolytically processed to their mature forms unless the *vps9^{tsf}* defect blocks their delivery to the vacuole. We preshifted *vps9^{tsf}* cells to 38°C for 30 min prior to a 10-min label period followed by a 40-min chase period (at 38°C). Antibodies specific for ALP or CPS were then used to immunoprecipitate each protein from whole cell extracts, and processing of each enzyme was determined by SDS-PAGE. Under these conditions, processing of ALP was unaffected and CPS maturation was partially blocked in the *vps9^{tsf}* mutant (Fig. 5A).

Identification of the *VPS9* gene product. To identify the protein encoded by the cloned *VPS9* gene, we prepared a rabbit antiserum against a fusion protein containing the carboxy-terminal 138 amino acids of Vps9p fused to the bacterial TrpE protein. In wild-type cells, this antiserum detected a rare protein with a slightly heterogeneous electrophoretic mobility centered at about 52 kDa (Fig. 6A). This immunoreactive protein was not detected in Δ*vps9* cells (Fig. 6A), and the amount of it was substantially increased (at least 20-fold) in wild-type cells carrying *VPS9* on a multicopy vector (Fig. 6A), indicating that this plasmid does indeed direct the expression of *VPS9*.

We used the Vps9p antiserum to investigate the distribution

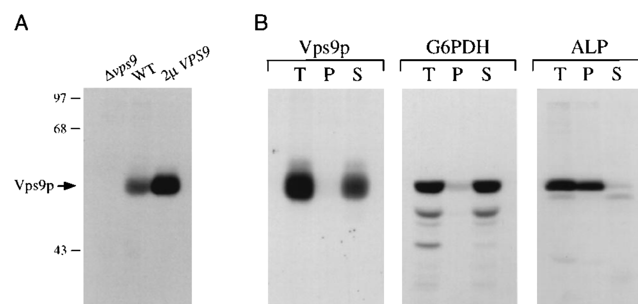

FIG. 6. Identification and fractionation of Vps9p. (A) Spheroplasted yeast cultures (10 OD_{600} equivalents) were pulse labeled at 30°C for 30 min and then chased for 90 min. Extracts of each culture were prepared, and a Vps9p-specific antiserum was used to immunoprecipitate Vps9p from each fraction. Immunoprecipitates were subjected to SDS-PAGE, and proteins were visualized by fluorography. Note that only 2 OD_{600} equivalents were run in the 2μ *VPS9* lane. Positions of migration of molecular size standards (in kilodaltons) are indicated to the left. (B) Wild-type cells (SEY6210) were pulse-labeled and chased as described above, and a spheroplast extract was prepared by osmotic lysis and gentle homogenization. The extract was then centrifuged at 100,000 × *g* for 60 min, and the supernatant (S) and pellet (P) fractions were precipitated with trichloroacetic acid. Antibodies against each of the proteins indicated at the top (G6PDH, glucose-6-phosphate dehydrogenase) were used to immunoprecipitate each protein from the fractions, and the samples were then subjected to SDS-PAGE and fluorography. Proteins immunoprecipitated from an equivalent amount of lysed cells used in the fractionation were run in the T lanes.

of Vps9p in extracts of wild-type cells. Spheroplasted cells were metabolically labeled with Tran^{35}S-label, chased for 90 min in the presence of unlabeled methionine and cysteine, and then lysed. After clearing, the cell extract was centrifuged (100,000 $\times$ g) to yield particulate (P100) and soluble (S100) fractions, and Vps9p or control marker proteins were immunoprecipitated from each fraction. All detectable Vps9p was found in the S100 fraction, as was the soluble enzyme glucose-6-phosphate dehydrogenase (Fig. 6B). In contrast, the vacuolar integral membrane protein ALP was found in the P100 fraction. With this fractionation protocol, Vps9p behaves as a soluble cytosolic protein.

DISCUSSION

A large number of *VPS* gene products are required for maintenance and function of the vacuole of *S. cerevisiae*. In an effort to understand the molecular mechanisms underlying vacuolar protein sorting and transport, we have cloned *VPS9* and characterized its gene product. Originally, the *vps9* strain was identified as a mutant which secretes a CPY-invertase fusion protein (34). The results of our experiments demonstrate that that *VPS9* is required for vacuolar localization of the soluble vacuolar hydrolases CPY and PrA. A temperature-conditional allele of *vps9* was generated and used to investigate whether Vps9p function is directly required for vacuolar protein sorting. We found that the protein encoded by *vps9^{tsf}* was inactivated rapidly after a temperature shift, and we observed a concomitant secretion of p2CPY. Temperature-conditional alleles of other *vps* genes that encode components of the vacuolar protein sorting machinery cause secretion of p2CPY within 1 to 15 min after a shift to the nonpermissive temperature (8, 15, 30, 31, 33, 40, 47). These experiments indicate that Vps9p is directly required for sorting of several vacuolar proteins and is therefore, likely to be a component of the vacuolar protein sorting machinery.

Conclusions regarding the sorting fate of the membrane proteins ALP and CPS in Δ*vps9* cells are complicated by the dependence of proteolytic processing (which is indicative of their delivery to the vacuole) on PrA activity. Since PrA is missorted in Δ*vps9* cells, it is possible that ALP and CPS are correctly localized but are not proteolytically matured. In fact, in *vps9^{tsf}* cells at the nonpermissive temperature, ALP was processed to its mature form and CPS maturation was partially blocked. Furthermore, a previous immunofluorescence study (32) suggested that ALP is localized to the vacuole in *vps9* mutant cells. These results argue that the vacuolar sorting of CPY, PrA, and CPS, but not ALP, may require Vps9p function. Since the *vps9^{tsf}* allele may be only partially inactivated by temperature shift (Fig. 5), we cannot rule out the possibility that Vps9p activity is required for the sorting of all vacuolar proteins, but that the residual activity of the *vps9^{tsf}* mutant (at the nonpermissive temperature) is sufficient for proper sorting of ALP. The role of *VPS9* in sorting of vacuolar membrane proteins will require further investigation.

The sequence of *VPS9* may provide insight into its function; a region of Vps9p is related to human proteins (Rin1 and JC265) that negatively regulate Ras-mediated signalling in *S. cerevisiae*. Rin1 has been shown to bind directly to Ras in a manner that competes with binding of Raf (a downstream effector of Ras), indicating that the effector domain of Ras is the principal binding site for Rin1 (11) and that Rin1 may act as a downstream effector of Ras. Conservation of amino acid sequence between Vps9p, Rin1, and JC265 implies that these amino acids may represent a common structural and functional domain. One intriguing possibility for the role of the conserved amino acids of this domain is that they define a surface for binding monomeric GTPases and that sequence divergence within this region indicates ligand-specific differences between the proteins. The homology between Vps9p, Rin1, and JC265 may be indicative of a larger protein family, whose members bind small GTPases. We term this region a GBH domain. The presence of a GBH domain in Vps9p suggests that Vps9p may bind one or more GTPases, such as a rab protein, involved in vacuolar protein sorting. Since available evidence indicates that Rin1 does not modulate the enzymatic activity of Ras (11), it is likely that Rin1 serves solely as an effector of Ras-GTP. In an analogous manner, Vps9p may serve as a downstream effector of a rab protein required for vacuolar protein sorting. The protein encoded by *VPS21*, a class D *VPS* gene, is a GTP-binding protein homologous to rab5 and is therefore a candidate target for interaction with Vps9p. In fact, deletion of *VPS21* and deletion of *VPS9* result in a common set of phenotypes (see below).

Electron microscopic examination of Δ*vps9* cells revealed that many cells contained a regular tubular lattice of unknown origin. Smaller structures with the same general features can be found in wild-type cells, albeit infrequently, suggesting that in Δ*vps9* cells, this structure is merely exaggerated rather than represents a novel structure that forms only in the absence of Vps9p. More importantly, Δ*vps9* cells accumulate approximately 40-nm-diameter vesicles and exhibit growth defects at 38°C, phenotypes shared with three other class D *vps* null mutants, the Δ*vps21*, Δ*vps45*, and Δ*pep12*/*vps6* mutants (3, 7, 16). Information regarding interactions between class D *VPS* gene products is currently lacking; however, from what we know about their amino acid sequences, we can construct a speculative model to explain their shared set of phenotypes. In the current view of the VPS pathway, class D *VPS* gene products are thought to be required for Golgi-to-endosome trafficking (42). *PEP12*/*VPS6* encodes an endosomal protein that may serve as a receptor (T-SNARE) for Golgi-derived vesicles carrying cargo destined for the vacuole (3). Like *pep12* mutants, *vps9* cells accumulate 40- to 50-nm vesicles, indicating that Vps9p function is required after vesicles have budded from the late Golgi. Since both Pep12p and Vps9p contain heptad repeats, they could physically interact via a coiled-coil interaction to promote the docking and/or fusion of transport vesicles. As members of the rab family of GTPases are found on transport vesicles (9), Vps9p may also associate with a rab protein, such as Vps21p, via its GBH domain. If Vps9p bound both proteins simultaneously, it would bridge the rab protein on the docked vesicle and the vesicle receptor, Pep12p. Such an interaction might represent an intermediate to vesicle fusion and could also provide a proofreading mechanism to ensure fusion of only appropriately docked vesicles.

The function of rab GTPases in vesicle-mediated transport has been enigmatic, but it was recently reported that a mutation in the effector domain of Sec4p, a rab protein required for secretion, can be efficiently suppressed by elevated expression of Sec9p, a plasma membrane protein that is a component of a multimeric vesicle receptor complex (4). This observation suggests that *SEC9* encodes an effector of *SEC4* (4). Interestingly, many other genetic suppressors of the *sec4* mutant were obtained (4), and so there exist other activities that require effector domain function of *SEC4*. One such type of activity may be fulfilled by rabaptin, a recently identified effector of rab5, a GTPase required for fusion of early endosomes (43). Although we have not found significant amino acid sequence similarity between Vps9p and rabaptin, they have common properties; each is predicted to participate in coiled-coil interactions (22, 43), and each is predominantly soluble at steady

state (reference 43 and Fig. 6). Thus, it is possible that rabaptin and Vps9p perform similar functions. With the *sec4* multicopy suppression studies (4) in mind, if our model of Vps9p function is correct, most vesicle-mediated transport events may require a similar activity. Further study of *VPS9* will aim to identify components of the vacuolar protein sorting machinery that interact with Vps9p and could provide considerable insight into the general mechanisms of vesicle-mediated protein transport.

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