Molecular Analysis of the Yeast VPS3 Gene and the Role of Its Product in Vacuolar Protein Sorting and Vacuolar Segregation during the Cell Cycle

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**Abstract.** vps3 mutants of the yeast Saccharomyces cerevisiae are impaired in the sorting of newly synthesized soluble vacuolar proteins and in the acidification of the vacuole (Rothman, J. H., and T. H. Stevens. Cell. 47:1041-1051; Rothman, J. H., C. T. Yamashiro, C. K. Raymond, P. M. Kane, and T. H. Stevens. 1989. J. Cell Biol. 109:93-100). The VPS3 gene, which was cloned using a novel selection procedure, encodes a low abundance, hydrophilic protein of 117 kD that most likely resides in the cytoplasm. Yeast strains bearing a deletion of the VPS3 gene (vps3-Δ1) are viable, yet their growth rate is significantly reduced relative to wild-type cells. Temperature shift experiments with strains carrying a temperature conditional vps3 allele demonstrate that cells rapidly lose the capacity to sort the vacuolar protein carboxypeptidase Y upon loss of VPS3 function. Vacuolar morphology was examined in wild-type and vps3-Δ1 yeast strains by fluorescence microscopy. The vacuoles in wild-type yeast cells are morphologically complex, and they appear to be actively partitioned between mother cells and buds during an early phase of bud growth. Vacuolar morphology in vps3-Δ1 mutants is significantly altered from the wild-type pattern, and the vacuolar segregation process seen in wild-type strains is defective in these mutants. With the exception of a vacuolar acidification defect, the phenotypes of vps3-Δ1 strains are significantly different from those of mutants lacking the vacular proton-translocating ATPase. These data demonstrate that the acidification defect in vps3-Δ1 cells is not the primary cause of the pleiotropic defects in vacuolar function observed in these mutants.

Eukaryotic cells possess several distinct membrane-bounded intracellular organelles, each of which is composed of unique proteins and other cellular components. It is of interest to understand how these structures are assembled, maintained, and partitioned during cell growth and cell division. The vacuole of the budding yeast Saccharomyces cerevisiae is one such organelle that has become the focus of genetic and biochemical investigation. Yeast vacuoles are similar in many respects to lysosomes in mammalian cells and vacuoles in plant cells (Rothman and Stevens, 1988). The vacuole occupies a significant fraction of the overall volume in a yeast cell and is thought to mediate many diverse processes such as intracellular protein degradation, amino acid and ion storage, pH and osmotic regulation, and degradation of internalized ligands (Rothman and Stevens, 1988; Jones, 1984). The interior of the vacuole is maintained at an acidic pH by a membrane-associated proton-translocating ATPase (H+-ATPase) (Preston, et al., 1989; Kane, et al., 1989). The vacuole contains many hydrolytic enzymes some of which are soluble in the lumen of the organelle while others are integral membrane proteins (Rothman and Stevens, 1988; Roberts, et al., 1989; Klionsky and Emr, 1989). All resident vacuolar proteins studied to date enter the secretory pathway, receive carbohydrate modifications characteristic of passage through the ER and Golgi apparatus, and are subsequently sorted away from nonvacuolar proteins within or shortly after transport through the Golgi apparatus (Stevens, et al., 1982; Moehle, et al., 1988; Roberts, et al., 1989; Klionsky and Emr, 1989). In certain well-characterized vacuolar proteins, separate cis-acting signals have been identified that direct entry into the ER and sorting to the vacuole (Valls, et al., 1987; Johnson, et al., 1987; Klionsky, et al., 1988). In one case, the vacuolar targeting signal has been localized to a few contiguous amino acid residues (Valls, et al., 1987, 1990; Johnson, et al., 1987; Rothman, et al., 1989b). Several vacuolar hydrolases traverse the secretory pathway as inactive precursors and are proteolytically processed to the active species upon delivery to the vacuole by a process that requires the activity of proteinase A (PrA), the product of the PEP4 gene (Jones, et al., 1982; 1. Abbreviations used in this paper: CDCFDA, 2',7'dichloro carboxyfluorescein diacetate; CPY, carboxypeptidase Y; ORF, open reading frame; PrA, proteinase A.)
Ammerer et al., 1986; Woolford et al., 1986; Mechler et al., 1987).

While the biosynthesis of many vacuolar proteins has been relatively well characterized, other aspects of vacuolar biology are poorly understood. Little is known about the components that recognize vacuolar targeting information, the manner in which sorting occurs, the processes whereby vacuoles are assembled and maintained, or the mechanism of organelle segregation during cell division. Several laboratories have isolated mutants that secrete newly synthesized soluble vacuolar proteins instead of efficiently localizing them to the vacuole. Vacuolar protein sorting (vps) mutants were isolated by selection for secretion of the vacuolar hydrolase carboxypeptidase Y (CPY) (Rothman and Stevens, 1986; Rothman et al., 1989a; Bankaitis et al., 1986; Robinson et al., 1988). Most of the pep mutants, isolated in a screen for mutants defective in CPY activity (Jones, 1977), also missort soluble vacuolar proteins (Rothman et al., 1989a). Complementation analysis between these mutants has shown that at least 49 genes are required for the proper sorting of vacuolar proteins (Rothman et al., 1989a; Robinson et al., 1988). Further phenotypic characterization has revealed that certain of these mutants are also defective in vacuolar acidification, organelle assembly, and/or protease maturation (Rothman et al., 1989a,c; Rothman and Stevens, 1986; Banta et al., 1988). Analysis of these mutants should ultimately identify the molecular components and mechanisms necessary for the biogenesis of vacuoles.

The mechanism by which vacuoles are segregated to daughter cells during cell division is also not well understood. Separate genetic and morphological studies have provided evidence that daughter cells receive vacuolar material from mother cells before cell division (Zubenko et al., 1982; Weisman et al., 1987; Weisman and Wickner, 1988). While it has been proposed that small transport vesicles might mediate this transfer process (Weisman and Wickner, 1988), the mechanism underlying vacuolar segregation remains unclear. In fact, even the issue of what constitutes normal vacuolar morphology has been the subject of recent controversy (Weisman et al., 1987; Pringle et al., 1990).

Previous reports have shown that vps3 cells secrete roughly 90% of newly synthesized CPY as well as substantial amounts of PrA, and the remaining intracellular CPY and PrA molecules accumulate primarily as unprocessed zymogens (Rothman and Stevens, 1986; Rothman et al., 1989a; unpublished results). The vps3 mutant was referred to as the vps3 mutant in these earlier reports, and the name was changed in a consolidation of vpl (Rothman and Stevens, 1986) and vpt (Bankaitis et al., 1986) mutant nomenclature (Robinson et al., 1988; Rothman et al., 1989a). vps3 mutant cells also have defects in vacuolar acidification. Vacuolar membranes purified from vps3 mutants retain only 5-10% of the wild-type vacuolar H+-ATPase activity, and they exhibit a corresponding deficiency in at least two subunits of this multi-subunit enzyme complex (Rothman et al., 1989c; Kane et al., 1989). These subunits are present at wild-type levels in total cell extracts, suggesting that the vacuolar H+-ATPase complex is incorrectly assembled onto the vacuolar membrane in vps3 mutants (Rothman et al., 1989c). Here we report on the characterization of the VPS3 gene, the VPS3 gene product (Vps3p), and the phenotypes of vps3 mutants in greater detail. Our studies indicate that Vps3p is a cytoplasmic protein whose function is likely to be intimately involved in vacuolar protein sorting. We also show that the vacuole in wild-type cells appears to be actively segregated between mother and daughter cells during cell division, and vps3 mutants are defective in this process.

Materials and Methods

Strains, Growth Conditions, and Materials

The genotypes of the S. cerevisiae strains used in this study are shown in Table I. The vps3-1 mutation was referred to as the vps3-1 mutation in a previous report (Rothman et al., 1989c). The SF838-1D pho8-Δ1 strain carries a substitution of the LEU2 gene within the PHO8 gene. Recombinant strains were constructed by single step gene replacement or integration (Rothstein, 1983), and the lithium acetate transformation procedure was used (Ito et al., 1983) to introduce DNA fragments or plasmids into yeast cells. Spheroplast transformation (Sherman et al., 1982) was used in the cloning of the VPS3 gene. The Escherichia coli strain used in this study was MC1061 (F' ΔhdaΔhdm + ηΔ139 (araA159-301 leu2-3,112)) lacYΔ7 xgalU galkI rpsL: Casadaban and Cohen, 1980). The growth media for yeast were rich medium (YPD) containing 1% Bacto yeast extract, 2% Bacto peptone (Difco Laboratories Inc., Detroit, MI), and 2% glucose or minimal media (SD) containing 0.67% yeast nitrogen base without amino acids (Difco Laboratories Inc.) and 2% glucose (Sherman et al., 1982) as specified, and the latter medium was supplemented with the appropriate nutrients (Stevens et al., 1986). Strains were grown at 30°C unless otherwise indicated. The negative selection, which must be conducted on Leu" yeast strains, was performed on supplemented SD plates lacking leucine and containing 1mM CBZ-phenylalanine and 0.03 mM F6eue. CBZ-phenylalanine was synthesized from CBZ-phenylalanine and F6eue by a standard peptide coupling procedure (Paul and Anderson, 1960).

F6eue was from Fairiel Chemical Co. (Blythewood, SC). Restriction endonucleases and DNA modifying enzymes were from Bethesda Research Laboratories (Gaithiersburg, MD), Boehringer Mannheim Biochemicals (Indianapolis, IN), or New England Biolabs (Beverly, MA). Glusulase was from DuPont Co. (Boston, MA), Carrier free H7350 and zymolyase 100T were from ICN Biomedicals Inc. (Irvine, CA). [35S]ETP and [35S]ATP were from New England Nuclear (Boston, MA). [125I]protein A was from Amersham Corp. (Arlington Heights, IL), and nitrocellulose was from Schleicher & Schuell, Inc. (Keene, NH). 2',7'dichlorofluorescein diacetate (CFDA) was from Molecular Probes (Eugene, OR). Multitest test slides were from Flow Laboratories, Inc. (McLean, VA). CPY antibody has been described previously (Rothman and Stevens, 1986), affinity-purified rabbit antiyeast alkaline phosphatase (Pho8p) antibody will be described elsewhere (Dr. G. Pohlig and Dr. T. Stevens, unpublished observations), alkaline phosphatase conjugated goat anti-rabbit IgG was from Promega Biotech (Madison, WI), alkaline phosphatase color development reagents, used as recommended, were from Bio-Rad Laboratories (Richmond, CA), and IgG Sorb was from the Enzyme Center (Boston, MA). 0.1% formaldehyde was from J. T. Baker Chemical Co. (Phillipsburg, NJ), urea was from International Biotechnologies, Inc. (New Haven, CT), SDS was from BDH Biochemicals Ltd. (Poole, UK), and all other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cloning, Sequencing, and DNA Manipulations

DNA manipulations were performed by routine procedures (Maniatis et al., 1982). The VPS3 gene was cloned by transforming mutants vps3-2 or vps3-10 (Table I) to Ura" with yeast genomic clone banks carried in the centromere vector Vyc50 (Rose et al., 1987) or the multicopy vector Y'Ep24 (Carlson and Botstein, 1982). Roughly 1 x 103 Ura" transformants derived from each library were pooled and replated on CBZ-phenylalanine and F6eue plates at a density of 1 x 104 cells/plate. Survivors arose at a frequency of 1 x 105 colonies/plate, 50 were tested for secretion of CPY by the immunoblot procedure (Rothman et al., 1986). Vps" colonies were observed at a frequency of 10-100%. Certain plasmids from independent Vps" isolates shared common restriction fragments, and these plasmids complemented the vps3 defect. One of these complementing plasmids isolated from the Y'Ep24 clone bank, WP53, was characterized further. An integrating plasmid was built from pvps3 by EcoRI digested fragments from this plasmid together to form a separate, smaller plasmid. The subcloning removed the 2 μm-based yeast origin of replication but retained
| Strains | Genotype | Source or reference |
|---------|-----------|---------------------|
| SF838-1D | MATa, ade6, his4-519, leu-2-3, leu-2-112, ura3-52, pep4-3, gal | Rothman and Stevens, 1986 |
| JHRY20-2C | MATa, his3Δ200, leu-2-3, leu-2-112, ura3-52 | Rothman et al., 1986 |
| SEY6211 | MATa, ade2-101, his3Δ200, leu-2-3, leu-2-112, trpl-1-A901, ura3-52, suc2-A9, lys2, pep4-3, gal | Robinson et al., 1988 |
| SF838-9D | MATa, ade6, his4-519, leu-2-3, leu-2-112, lys2, pep4-3, gal | Rothman et al., 1989a |
| SF838-9DRL1m1006 | MATa, his4-519, ura3-52, lys2, pep4-3, gal, vps3-2 | Rothman and Stevens, 1986 |
| SF838-9DRL1m1062 | same as above except vps3-A10 | Rothman and Stevens, 1986 |
| SF838-9Dm300 | same as SF838-9D except vps3-28 | Rothman et al., 1989a |
| SF838-9Dm301 | same as SF838-9D except vps3-29 | Rothman et al., 1989a |
| CRY1 | diploid from cross of JHRY20-2C and SF838-9D | This study |
| SF838-1D vps3-A1 | same as SF838-1D except vps3-A1::LEU2 | This study |
| SF838-1D vps3-A2 | same as SF838-1D except vps3-A2::LEU2 | This study |
| SF838-1D ptoh8-A1 | same as SF838-1D except ptoh8-A1::LEU2 | This study |
| JHRY20-2C vps3-A1 | same as JHRY20-2C except vps3-A1::LEU2 | This study |
| JHRY20-2C vps3-A2 | same as JHRY20-2C except vps3-A2::LEU2 | This study |
| JHRY20-2C vps3-A2p | same as JHRY20-2C vps3-A2 except vps3-A2::vps3-A1::URA3 (see Materials and Methods) | This study |
| SEY6211 vps3-A1 | same as SEY6211 except vps3-A1::LEU2 | This study |

Within the T4 lysozyme coding sequence (pCKR101). pEXP2 was created from pCKR101 by cloning an 8-bp Eco RI linker into the Sna BI site positioned 24 nucleotides downstream of the T4 lysozyme ATG start codon, digestion of the resulting plasmid with Eco RI, and religation. This placed the polylinker eight codons downstream of the ATG start site. pEXP3 was made by digesting pCKR101 with Sna BI and Eco RI, blunt ending, and religation. pEXP3 was built from pEXP2 by digesting with Sac I, blunt ending, and religation. Two plasmids that lead to the expression of a portion of the Vps3p protein were made. A 900-bp Pst I-Bgl II fragment from the middle of the Vps3p open reading frame (ORF) (see Fig. 2) and a 750-bp Pst I-Hind III from the same region were cloned into the Sma I site in pEXP2 (pCKR114 and pCKR115, respectively). A temperature-sensitive allele of the VPS3 gene was generated by in vitro hydroxylamine mutagenesis of plasmid pCKR68 (Schauer et al., 1985). The mutagenized plasmid DNA was used to transform a vps3-A1 strain to Ura-. 2,000 transformant colonies were screened for a Vps3p phenotype at 30°C and a Vps3p phenotype at 37°C. Eight putative conditional transformants were isolated, and one was characterized further. Subcloning of the putative vps3-A2 allele revealed that the conditional phenotype was linked to the VPS3 gene. The conditional allele carried on a 4.2-kb Bam HI fragment was subcloned into the Bam HI site of the integrating vector Ylp5 (Botstein et al., 1979) which was lacking the Sal I site (pCKR75). pCKR75 was digested at the Sal site within the VPS3 gene and used to transform JHRY20-2C vps3-A2 to Ura" (JHRY20-2C vps3-A2::vps3-A1::URA3 Table 1). The genomic integrants analyzed displayed the same conditional phenotype as the original isolate. Further analysis has suggested that the insertion maps to the 3' end of the VPS3 gene.

**Fluorescence Microscopy**

Vascular vital staining was performed on log-phase yeast cultures at a density of 1 - 10⁶ cells/ml in supplemented SD broth buffered to pH 50 with 50 mM citrate and containing 10 μM CDCFDA. Cultures were shaken at room temperature for ~30 min before observation. Double labeling with calcifluor was achieved by addition of 1 μg/ml calcifluor to the labeling media. Vectors were labeled with endogenous ade2 fluorophore, produced in ade2 yeast strains under conditions of limiting adenine, by the methods described by Weissman et al. (1987). ade2 pigment synthesis was depressed by 10-fold dilution of these cells into YEPD broth supplemented with 200 μg/ml adenine. All labeled cells were applied directly to Con A-coated slides (Pringle et al., 1990) and viewed or photographed using an Axioskop photomicroscope (Carl Zeiss Inc., Thornwood, NY) and TMAX-400 film (Eastman Kodak Co., Rochester, NY). Calcifluor was viewed through a DAPI filter set (Zeiss 487702; Carl Zeiss Inc.), CDCFDA through an FITC filter set (Zeiss 487709; Carl Zeiss Inc.) and ade2 pigment through a rhodamine filter set (Zeiss 487715; Carl Zeiss Inc.).
Antibodies, Immunoblotting, and Immunoprecipitation

The Vps3p antigen was expressed in E. coli from plasmid pCKRI14, purified, and used to immunize rabbits as described by Roberts et al. (1989). Rabbit anti-Vps3p antibodies were affinity purified against protein expressed from plasmid pCKRI15 as described by Hicke and Schekman (1989) except that antibody bound to the column was washed with 1 M NaCl, 0.1 M NaHCO3, pH 8.5, and 1 M NaCl, 0.1 M Na/H-OAc, pH 4.0 before elution, antibody binding and elution were performed twice in succession, and the final eluted antibody was resuspended in PBS containing 5 mg/ml BSA. Immunoblotting of total yeast protein extracts (prepared as described by Rothman et al., 1986c), subcellular fractions (prepared as described by Gould et al., 1988), or yeast vacuoles (prepared as described by Kane et al., 1989) were performed by the method of Rothman et al. (1989c). The blots were probed with a 1:1,000 dilution of rabbit anti-Vps3p antibody followed by either alkaline phosphatase-conjugated goat anti-rabbit IgG or [125I]protein A. Immunocomplexes were visualized according to the manufacturers’ suggestions.

For CPY immunoprecipitations, vps3-Δ1 cultures were grown in supplemented SD containing 50 μM sulfate at 30°C. At various times before labeling, aliquots were shifted to 37°C. The strains were then harvested, resuspended in 0.5 ml of sulfate-free SD containing 50 mM KH2PO4, pH 5.7, and 0.5 mg/ml BSA at a density of 1 × 107 cells/ml, and 200 pCi of H235SO4 was added. After 30 min of labeling, the cells were chased with 10 mM Na2SO4, 0.4 mM cysteine, and 0.3 mM methionine for 30 min. The cells were maintained at 37°C or 30°C throughout the pulse and chase as specified. The chase was terminated by the addition of 10 mM sodium azide, the cells were converted to spheroplasts (Stevens et al., 1986), the periplasmic and medium fractions were pooled to give the extracellular fraction (Vals et al., 1987), the spheroplasts were boiled for 2 min in 1% SDS, and both intracellular and extracellular fractions were adjusted to 1 ml in PBS, 0.1% SDS, 0.1% Triton X-100, 1 mM EDTA, 1 mM PMSF, and 10% IgG Sorb (prepared as suggested by the manufacturer) final concentrations. CPY was immunoprecipitated as described by Stevens et al. (1986). The IgG Sorb-immune complexes were washed twice in 10 mM Tris, pH 8.0, 0.1% Triton X-100, 0.1% SDS, and 0.1 mM EDTA, and the samples were analyzed by SDS-PAGE and fluorography as described previously (Stevens et al., 1986). The amount of radiolabeled CPY in each lane was quantified directly using an Ambis Radiographic Imaging System (Ambis Systems, Inc., San Diego, CA). CPY and Vps3p were immunoprecipitated from the intracellular fraction of H235SO4-labeled wild-type strain SF838-1D and quantified by the same procedures as above. Percent of total cell protein was calculated from the counts incorporated into CPY divided by the total incorporated counts ×100.

Results

Isolation and Sequence of the VPS3 Gene

We have described previously a selection procedure for ob-
taining vps mutants in yeast (Rothman and Stevens, 1986). The selection permitted leucine-independent growth of a vps leucine auxotroph by utilizing extracellular CPY activity to release leucine from the CPY specific substrate N-carbobenzoxy-l-phenylalanyl-l-leucine (CBZ-pheleu) (Rothman and Stevens, 1986). A negative selection against vps mutants was devised by exploiting the observation that the leucine analog p-t,5,5,5-trifluoroleucine (F3leu) is toxic to Leu+ yeast cells in the absence of exogenous leucine (Bussey and Umberger, 1970). The compound N-carbobenzoxy-l-phenylalaninyl-l,1,1,5,5,5-trifluoroleucine (CBZ-pheF3leu) inhibited the growth of a vps3 mutant relative to the isogenic wild-type strain (Fig. 1). The observed growth inhibition was typical of most vps mutants (data not shown). Growth was restored in a vps3 mutant which harbored the corresponding VPS3 gene on a plasmid (Fig. 1 and see below). However, the selection had certain limitations. vps mutants insensitive to CBZ-pheF3leu but still Vps+ arose at a frequency of 1 × 10−4−1 × 10−2. Furthermore, F3leu liberated by Vps− cells poisoned adjacent Vps+ cells at high plating densities (>10⁶ cells/plate). Finally, growth of some vps mutants was only partially inhibited by the selection (data not shown). Thus, the selection was ideal for high frequency events that required a low plating density, such as the cloning of a gene from a genomic library.

The VPS3 gene was cloned from wild-type yeast genomic clone banks contained in the centromere vector YCp50 (Rose et al., 1987) or in the multicopy, 2 µm-based vector YEp24 (Carlson and Botstein, 1982) by complementation of the CBZ-pheF3leu growth inhibition of vps3 mutants. Complementing plasmids were isolated from Vps+ survivors of the negative selection. All complementing plasmids shared characteristic restriction fragments, and the 10.5-kb complementing region from a YEp24 isolate, pVPS3, is shown in Fig. 2 A. To test whether the complementing sequences encompassed the VPS3 genetic locus, a URA3-marked integrating plasmid was constructed from pVPS3. This plasmid was digested at a unique restriction site within the complementing region and used to transform a VPS3 ura3 strain to Ura+. A representative transformant was crossed to two different vps3 ura3 mutants. Diploids from these crosses were sporulated and tetrads were dissected and analyzed. The Ura+ marker segregated 2:2 away from the vps3 defect in all 29 four spore tetrads analyzed, indicating that the cloned sequences map to the VPS3 locus. The position of the VPS3 gene within the pVPS3 genomic insert was determined by subcloning and transposon mutagenesis (Huisman et al., 1987). Transposon insertions which destroyed VPS3 complementation were clustered in a 3.6-kb region. This transposition system also permitted the direction of transcription and translation of the VPS3 gene to be determined (Fig. 2 A). A 3.9-kb Bam HI–Sna BI fragment encompassing the 3.6-kb cluster of insertions complemented the vps3 defect when subcloned into either single copy or multicopy shuttle vectors, suggesting that the entire VPS3 gene was localized to this region (Fig. 2 B).

The DNA sequence of 3.6 kb within this region was determined (Figs. 2 C and 3). It was found to contain a single, long ORF of 3,033 nucleotides which would encode a hydrophilic protein of 1,011 residues with a predicted molecular mass of ~117 kDa. Neither the VPS3 sequence nor the translated ORF had significant homology to other nucleic acid (GENBANK and EMBL) or protein sequences (SWISS PROT and PIR) available. Furthermore, there were no significant hydrophobic regions suggestive of membrane spanning domains, and the amino terminus of the protein did not appear to encode an ER signal sequence. This analysis suggested that the putative VPS3 gene product is a soluble cytoplasmic protein.

Construction and Characterization of vps3 Deletion Mutants

A null mutation of the VPS3 gene was created by replacing the entire VPS3 coding sequence with the LEU2 gene. This removed 224 nucleotides 5′ of the Vps3p start codon through

Figure 2. Physical map of the VPS3 gene, and DNA manipulations used in this study. (A) The 10.5-kb genomic DNA insert in pVPS3. The restriction map of this region is shown above the line, and the results of transposon mutagenesis are shown below the line. Filled symbols designate transposon insertions that disrupt VPS3 complementing activity, and open symbols represent insertions that do not affect complementation. Arrows indicate the orientation of insertions that express LacZ activity in yeast and thereby define the location and direction of translation of expressed ORFs. Round symbols denote insertions that do not express LacZ activity. B, Bam HI; C, Cla I; E, Eco RI; G, Bgl II; H, Hind III; N, Sna BI; P, Pst I; Sal I. (B) The smallest subclone that complements the vps3Δ mutation is the 3.9-Kb Bam HI–Sna BI fragment. (C) The 3.6-Kb Hind III–Bam HI segment was sequenced (III). The location and direction of the Vps3p ORF are represented by the large arrow (>). The region used to generate rabbit anti-Vps3p antibody is designated by diagonal bars within the arrow (ZZZ). (D and E) the LEU2 gene substitution deletion (vps3Δ1) and insertion (vps3Δ2) constructs, respectively.
Strains of the same genotypes in a JHRY20-2C genetic background ble spores. The 48 complete tetrads analyzed invariably grew into two large and two small colonies which were JHRY20-2C; Table I). Southern blots of Bam HI-digested dia, the difference in doubling rates increased to greater than data are available from EMBL/GenBank/DDBI under accession number M33314. we conclude that while the Table I). Three such diploids were used for tetrad analysis. required for wild-type growth rates, it is not an essential gene. The gene substitution construct was subsequently used to de-

Figure 3. Nucleotide and amino acid sequence of the VPS3 gene. A 3.6-kb region defined by Hind III and Bam HI sites was sequenced as described in Materials and Methods. The predicted amino acid sequence of the Vps3p protein is depicted in the one letter code. These sequence data are available from EMBL/GenBank/DDBI under accession number M33314.

Figure 4. Southern analysis of VPS3, vps3-Δ1, and vps3Δ0 strains. Genomic DNA from isogenic strains SF838-ID (lane 1), SF838-1D vps3-Δ1 (lane 2), and SF838-1D vps3Δ0 (lane 3) was digested with Bam HI, separated by electrophoresis, blotted, and probed with a radiolabeled 1.1-kb Bam HI-Sal I fragment from the 5' end of the VPS3 gene. The vps3Δ0 strain carries a tandem duplication of the VPS3 gene separated by the Ylp5 integrating plasmid. One copy is the vps3-Δ2 insertion allele (the upper, 6.3-kb band) and one copy is the vps3Δ0 allele (the lower, 4.2-kb band), which migrates with the wild-type VPS3 gene.

Strains of the same genotypes in a JHRY20-2C genetic background gave identical patterns of hybridizing fragments as those shown.

52 nucleotides 3' of the stop codon (designated vps3-Δ1). A fragment containing the gene substitution was used to con-

Figure 5. Identification of the Vps3p protein. 50 μg of total protein extracts from isogenic vps3-Δ1 (lane 1), wild-type (lane 2), and Vps3p overpro-

duction (lane 3) strains were electrophoresed on an 8% SDS-PAGE gel and blotted onto nitrocellulose. The blots were probed with affinity-puri-

fied rabbit anti-Vps3p antibody followed by alkaline phosphatase-conjugated goat anti-rabbit IgG antibody. All strains were in an SF838-1D genetic background.
The VPS3 Gene Encodes a Nonabundant Protein

The protein product of the VPS3 gene, Vps3p, was detected using antibodies directed against a portion of the protein. Roughly 1 kb of the VPS3 ORF (Fig. 2 C) was expressed in Escherichia coli as a hybrid protein which was then purified and used to raise antisera in rabbits; Vps3p-specific antibodies were subsequently affinity purified. A protein of ~140 kD was recognized in immunoblots of wild-type extracts (Fig. 5). This protein was absent in vps3ΔA extracts and increased in abundance in extracts of wild-type cells that carried the VPS3 gene on a multicopy plasmid (pVPS3). These data established that the 140-kD protein was Vps3p. This and other blots suggested that Vps3p was expressed at low levels in wild-type cells. To examine this further, Vps3p and CPY were immunoprecipitated from H2O2-labeled wild-type cell extracts, and the counts in each were quantified. Given that CPY constitutes roughly 0.1% of total cell protein (T. H. Stevens, unpublished observations), that the counts associated with Vps3p were slightly <5% of the counts in CPY, and adjusting for the fact that there are about three times more sulfur atoms in Vps3p than in CPY, we estimate that Vps3p constitutes 0.001% of total cell protein. In subcellular fractionation experiments, roughly 50% of the Vps3p was sedimentable when osmotically lysed spheroplasts were centrifuged at 10,000 g; under these same conditions, a soluble cytoplasmic protein (phosphoglycerate kinase) was found exclusively in the supernatant and a membrane protein (DPAP B) sedimented quantitatively (data not shown). Vps3p was not detected in samples of purified vacuolar membranes. Indirect immunofluorescence using Vps3p antibody in wild-type cells failed to produce a specific signal; however, the Vps3p product was readily detected in Vps3p-overproducing cells as a cytoplasmically diffuse, nuclear and vacuole-excluded signal (data not shown). While the possibility that Vps3p resides in a distinct subcellular compartment cannot be rigorously excluded, the hydrophilic nature of the putative Vps3p amino acid sequence taken together with the fractionation and immunofluorescence data are most consistent with an interpretation that Vps3p is a cytoplasmic protein which forms stable associations with intracellular membranes, cytoskeleton, or some other sedimentable subcellular structure.

A vps3 Temperature Conditional Mutant Rapidly Loses the Capacity to Sort CPY at the Nonpermissive Temperature

Mutations were generated in the VPS3 gene by in vitro mutagenesis and a temperature conditional allele of the gene was isolated. The vps3Δ mutant allele was integrated into a vps3ΔA disruption strain by tandem integration (JHRY20-2C vps3ΔA; Table I). Southern blots demonstrated that a single copy of the vps3Δ construct had integrated at the VPS3 locus (Fig. 4 and data not shown). Yeast strains carrying this mutant allele were viable at both the permissive and nonpermissive temperatures, but they were conditionally defective for the sorting of CPY. Upon shift from the permissive to the nonpermissive temperature, it was possible to monitor the time required for the CPY missorting defect to occur. We reasoned that if the appearance of the sorting defect was rapid, occurring in only a fraction of a cell generation, it would suggest that the VPS3 gene encodes a function that is in some way intimately required for vacuolar protein sorting. If the sorting defect took more than one cell generation to become apparent, this would imply that the function of the gene might be more distantly removed from the actual process of protein sorting.

Sorting of newly synthesized CPY was examined in the vps3Δ mutant and the isogenic wild-type strain. They were initially grown, pulsed with H2O2, and chased entirely at the permissive (30°C) or the nonpermissive temperature (37°C). The wild-type strain secreted <10% of the protein at either growth temperature (data not shown). By contrast, the temperature-sensitive mutant secreted 82% of the total labeled CPY at 37°C, while only 17% was extracellular at

Figure 6. A temperature conditional vps3Δ mutant rapidly loses the capacity to sort CPY upon shift to the nonpermissive temperature. (A) Strain JHRY20-2C vps3Δ was grown at 30°C and aliquots were shifted to 37°C at various times. Shifted cells were labeled at 37°C for 30 min with H2O2, and chased at 37°C for 30 min as described in Materials and Methods. CPY was immunoprecipitated from intracellular (I) and extracellular (E) fractions, and the immunoprecipitates were analyzed on 8% SDS-PAGE gels followed by fluorography. The 30°C sample was from cultures maintained at 30°C throughout the growth, pulse, and chase procedure. The 37°C sample was from a culture grown for several generations at 37°C before a pulse and chase at 37°C. The time of the shift was computed as the sum of the preshift time plus the 30-min pulse. (B) Quantitation of CPY in intracellular (o) and extracellular (●) fractions as a function of time (min). The amount of CPY was determined by direct counting of CPY-associated 35S in each fraction (see Materials and Methods). The 0-min point was from cells treated exclusively at 30°C, and the ∞ symbol represents cells treated exclusively at 37°C.
30°C (Fig. 6). The intracellular CPY was in the mature form while the extracellular CPY was in the unprocessed proCPY form (Fig. 6 a), demonstrating that the extracellular CPY had been secreted rather than released by cell lysis (Rothman and Stevens, 1986). Thus, there was a pronounced difference in the CPY sorting capacity of the vps3A mutant at the two different growth temperatures.

The vps3A mutant rapidly lost the ability to sort CPY upon shift to the nonpermissive temperature. We defined time zero in this experiment as the time when the chase period was initiated after labeling. Accordingly, a culture that was shifted to 37°C for 30 min before the 30-min pulse, 30-min chase regimen was defined as having been at the nonpermissive temperature for 1 h and so on. This was a reasonable definition since newly synthesized CPY reaches its ultimate destination with a half time of ~5 min (Hasilik and Tanner, 1978). Therefore, by the time the chase was begun most of the labeled CPY had already been routed to its final destination. By these criteria the defect in sorting upon shift to the nonpermissive temperature was evident at the earliest time points and was essentially complete after a 90-min shift (Fig. 6 b). The doubling time of the vps3A mutant under these conditions was roughly 4 h. Thus, the onset of the CPY missorting defect occurred in much less than the vps3A cell generation time.

The Vacular Morphology in vps3-A1 Cells Is Aberrant

Given that vps3-A1 mutants missort soluble vacuolar proteins and fail to acidify their vacuoles, we were interested to know whether the assembly and/or morphology of vacuoles were also perturbed in the mutant strains. To perform this study, vacuolar morphology was characterized in asynchronous cultures of wild-type cells (SF838-1D, JHY20-2C) and isogenic vps3-A1 mutants and then compared. Two techniques were used to label vacuolar structures with fluorescent probes for subsequent examination by fluorescence microscopy. Vacuoles were stained in live cells using the vacuolar vital stain CDCF/DA (Fig. 7); this nonfluorescent compound is hydrolyzed in yeast cells to the fluorescent molecule 2',7'-carboxyfluorescein, which accumulates specifically in the lumen of the vacuole (Pringle et al., 1990). This vital stain was particularly useful because it allowed cells to be examined in the same growth medium in which they were labeled. Indirect immunofluorescence was also used to view vacuolar membranes in fixed cells (Fig. 8). Existing immunofluorescence procedures (Kilmartin and Adams, 1984; Pringle et al., 1990) were modified in order to achieve an optimal combination of morphological preservation and satisfactory antibody penetration. Cells were fixed for longer periods of time, and treatment with an ionic detergent was used (Roberts et al., 1990). Vacuolar membranes were identified with antibodies that recognize alkaline phosphatase (Pho8p), a vacuolar membrane protein encoded by the PHO8 gene (Kaneko et al., 1987; Klionsky and Emr, 1989).

The vacuoles in wild-type cells generally appeared as irregularly shaped, morphologically complex organelles composed of several small, membrane-enclosed compartments (Figs. 7 a and b; 8, a and c). These structures were usually clustered in one region of the cell, and it was not possible to discern whether these compartments were physically separated by cytoplasm or joined by interconnected membrane channels. Photographs fail to record the entire extent of vacuolar material in cells because they are limited to a single focal plane. The morphology of the yeast vacuole was significantly influenced by environmental factors. When yeast cells were exposed to an energy poison, deprived of a carbon source, or merely centrifuged, it was observed that vacuoles tended to appear as single, spherical structures (Pringle et al., 1990; Makarow and Nevalainen, 1987; our unpublished results). Throughout this study, cultures were minimally perturbed before observation or fixation, and the combined approaches of vital staining and immunofluorescence provided essentially the same view of vacuolar structures. In addition, both wild-type strains gave similar results. Our results are also consistent with other published observations on vacuolar morphology (Wienken et al., 1970; Pringle et al., 1990; Preston et al., 1989).

Microscopy allowed examination of individual cells at various stages in the cell cycle, and thus it was possible to reconstruct probable changes in vacuolar morphology as wild-type yeast cells bud and divide. The most surprising discovery, revealed clearly by both microscopic techniques, concerns the segregation of vacuolar material into growing buds in a certain phase of the cell cycle. Shortly after bud emergence, through a later stage when the nucleus migrates to the neck of the bud and nuclear division begins, there was a high probability of observing continuous vacuolar structures extending from the mother cell through the bud neck and into the bud (Figs. 7 b and c, Table II). These mother cell to bud vacuolar networks were designated as "segregation structures." The morphology of these segregation structures varied, and some fairly prominent individual examples are shown. In other cases, the structure appeared as a thin line or a string of smaller round compartments which extended from vacuoles in the mother cell into the bud. Vacuolar segregation structures were seen in both wild-type strains used in this study as well as in all wild-type strains we have examined thus far. Quantitation of vacuolar morphology as a function of bud enlargement using indirect immunofluorescence is shown in Table II. While apparent segregation structures were observed during all phases of bud growth, they were observed most frequently in cells where the ratio of bud diameter to mother cell diameter ranged from 0.3 to 0.6 (77% of these cells had segregation structures). Mitochondrial segregation, monitored by the appearance of mitochondrial DNA in the bud, appears to occur in the same portion of the cell cycle, perhaps lagging slightly behind vacuolar segregation (Table II). However, since the mitochondrial organelle extends beyond the mitochondrial DNA (Pringle et al., 1990), the number of buds containing mitochondria must be regarded as a minimum estimate. The vacuoles in mother cells and buds rarely appeared connected in cells where mitotic events had commenced, suggesting that the initiation of nuclear division marks the end of the vacuolar segregation process (Fig. 8, b and c; Table II). Mitotic cells were defined as those large budded cells in which the nucleus had migrated into the neck of the bud, the nuclei were dividing, or there were separate nuclei in the mother and the bud. Of 72 mitotic cells quantified, only 13 (18%) had apparent segregation structures; only 4 of 45 cells undergoing or beyond the stage of mitosis and 0 of 26 cells with separate nuclei in the mother and bud had apparent segregation structures.

When the vacuoles in vps3-A1 cells were examined by the
same procedures, three significant differences from the vacuoles in the isogenic wild-type cells were observed. First, there was a difference in vacuolar morphology. Whereas wild-type vacuoles assumed a variety of convoluted shapes, the vacuoles in \textit{vps3-\Delta 1} cells, if present, were almost always nearly perfect spheres (Fig. 7, c and d, Fig. 8, d and f). While the relative volume occupied by vacuoles in wild-type cells appeared more or less uniform, the size of the vacuoles in \textit{vps3-\Delta 1} cells varied. In some \textit{vps3-\Delta 1} cells the vacuole seemed to occupy almost the entire cell, while in other cells the vacuoles were a small fraction of the total cell volume. Live mutant cells were double labeled with CDCFDA and calcofluor. Calcofluor stained bud scars and thus allowed determination of cell "age", that is, the number of budding cycles a mother cell has been through (Pringle et al., 1990). The ratio of vacuole diameter to cell diameter was measured in 40 cells with only one bud scar and in 40 cells that had at least three visible bud scars. In going from the former class of cells to the latter class of cells, the average vacuole diameter to cell diameter ratio increased from 0.42 to 0.66 and the average cell size increased slightly from 1.3 to 1.5 arbitrary units. These data may have interesting implications (see Discussion). Unfortunately, the nonuniform vacuolar morphology in wild-type cells precluded a similar comparative study.

The second unique characteristic of vacuoles in \textit{vps3-\Delta 1} mutants was the paucity of vacuolar material in developing buds and the virtual absence of vacuolar segregation structures in these cells (Figs. 7 d and 8 f, Table III). In wild-type cultures, vacuoles were observed in 99% of the buds of cells where the ratio of the bud to mother cell diameter exceeds 0.6 (Figs. 7 b and 8 c, Table II). Only 26% of the mutant buds in this stage of bud enlargement had detectable vacuoles (Table III). Where discernable vacuoles were present in \textit{vps3-\Delta 1} buds, they were very small. Examples of these minute bud vacuoles are visible in Figs. 7 d and 8 f. With regard to vacuolar segregation structures in \textit{vps3-\Delta 1} mutants, quantitation of immunofluorescence experiments revealed these structures in only 7% of the small-budded cells where the ratio of bud to mother cell diameter was 0.3–0.6 (Table III); by contrast, 77% of wild-type cells in this stage of bud growth had segregation structures. Even when present, the segregation structures in the mutant were less prominent than those observed in wild-type cells (Fig. 7 d). Mitochondrial segregation and nuclear division into the buds of \textit{vps3-\Delta 1} cells appears to proceed normally (Table III), although the latter may be partially delayed with respect to bud growth. This suggests that the defect in these mutants is specific to vacuolar segregation.

The third significant difference between the vacuoles in wild-type cells and \textit{vps3-\Delta 1} cells was that not all \textit{vps3-\Delta 1} cells had detectable vacuoles. Vital staining revealed that in \textasciitilde 15–30% of the \textit{vps3-\Delta 1} cells, CDCFDA failed to label a specific subcellular structure (Fig. 7 d, Table IV). Double labeling with CDCFDA and calcofluor revealed that the majority of mutant cells lacking apparent vacuoles were daughter cells (which lack bud scars; Table IV). The percentage of CDCFDA-labeled daughter cells in SF838-1D \textit{vps3-\Delta 1} cultures (41%) and JHRY20-2C \textit{vps3-\Delta 1} cultures (67%) was reproducibly different, suggesting that genetic background can influence the extent of the vacuole deficient phenotype in \textit{vps3-\Delta 1} mutants. By contrast, almost all mother cells in the mutant populations, and comparable percentages of all cells in the wild-type populations of cells possessed detectable vacuoles. Thus, vacuolar deficiencies were specific to \textit{vps3-\Delta 1} daughter cells. These vacuole deficient cells were likely to be live cells; when fresh cultures of wild-type cells or \textit{vps3-\Delta 1} mutants from either strain were plated and examined several hours later, \textasciitilde 90% of the plated cells had formed micro-colonies (data not shown). Significant differences in the percentage of colony forming units between wild-type and mutant cells were not detected. Furthermore, the percentage of mother cells versus daughter cells in the wild-type and mutant strains was roughly the same (Table IV). If a large number of daughter cells were inviable, they would be expected to constitute a greater proportion of the overall cell population. Immunofluorescence experiments yielded similar results. Those \textit{vps3-\Delta 1} cells that had visible vacuoles also had prominent labeling of the organelle by alkaline phosphatase antibody (Fig. 8 f). Once again, however, there was a subclass of cells that was deficient in vacuolar structures when viewed by Nomarski optics (Fig. 8 d). These same cells had a variety of labeling patterns. Some did not label, others had punctate labeling throughout the cell, some had specific labeling of a very small organelle, and some had a combination of specific and punctate labeling (Fig. 8 f, data not shown). A plausible explanation is that cells which had diffuse alkaline phosphatase staining were daughter cells in which nascent vacuolar material had failed to assemble into a coherent structure. However, it is not possible to differentiate between mother and daughter cells in immunofluorescence experiments since bud scars are lost when cells are spheroplasted (Pringle et al., 1990).

The vacuolar segregation defect in \textit{vps3-\Delta 1} cells is consistent with the observation that many daughter cells do not appear to possess coherent vacuoles. The fact that nearly all \textit{vps3-\Delta 1} mother cells have vacuoles suggests that vacuole assembly does occur in daughter cells that go on to divide, and this assembly event must occur during an early portion of the cell cycle. Incidentally, vacuolar assembly does not appear to be a prerequisite to budding since 5% of the fixed budding cells quantified in Table III did not appear to have a coherent vacuole but rather had punctate labeling throughout the cell characteristic of many unbudded mutant cells. Given that vacuolar assembly does proceed, the question arises as to whether these vacuoles are assembled from material contributed by the mother cell or whether the vacuoles are assembled by de novo synthesis. Previous reports have demonstrated that the endogenous, fluorescent pigment produced in \textit{ade2} yeast strains accumulates in vacuoles and is segregated.

\textbf{Figure 7.} Vacuolar morphology in live cells of wild-type and \textit{vps3-\Delta 1} strains. Vacuoles in living cells were stained with the vacuolar vital stain CDCFDA, and the stained cells were photographed by Nomarski optics (a and c) and by epifluorescence through an FITC filter set (b and d). The wild-type strain was JHR20-2C (a and b). The \textit{vps3-\Delta 1} strain was JHRY20-2C \textit{vps3-\Delta 1} (c and d). Bar, 5 \textmu m.
Table II. Segregation of Organelles into Buds in Wild-type Cells

| Bud/mother cell* | Vacuoles† | Nuclei§ | Mitochondria ||
|-----------------|-----------|----------|------------|
|                 | NV        | S        | VB         | PM | M | NB | B |
| 0.0-0.19        | 27        | 1        | 0          | 28 | 0 | 27 | 1 |
| 0.20-0.29       | 19        | 8        | 0          | 27 | 0 | 24 | 3 |
| 0.30-0.39       | 10        | 18       | 1          | 29 | 0 | 22 | 7 |
| 0.40-0.49       | 4         | 36       | 3          | 42 | 1 | 20 | 23|
| 0.50-0.59       | 1         | 38       | 9          | 40 | 8 | 10 | 38|
| 0.60-0.69       | 0         | 16       | 30         | 14 | 32| 1  | 45|
| 0.70-1.00       | 1         | 6        | 25         | 1  | 31| 0  | 32|

Wild-type strains were fixed and labeled by indirect immunofluorescence as described in Materials and Methods. 125 cells of SF838-1D and 125 cells of JHY20-2C were quantified, and the sum of these data is shown. The numbers represent the number of cells scored in each category (see below).

* The ratio of bud diameter to mother cell diameter.
† Vacuoles were scored by alkaline phosphatase indirect immunofluorescence. NV, No vacuole detected in the bud; VB, independent vacuoles in the mother cell and the bud.
§ Nuclei were scored by DAPI staining. PM, Premitotic nuclei, defined by apparent position in the mid-body of the mother cell; M, mitotic nuclei, defined by apparent migration to the neck of the bud, mitosis, or completion of nuclear division.
¶ Mitochondria were scored by DAPI staining of mitochondrial DNA. NB, No mitochondrial DNA detected in the bud; B, mitochondrial DNA detected in bud.

Table III. Segregation of Organelles into Buds in vps3-Δ1 Cells

| Bud/mother cell* | Vacuoles† | Nuclei§ | Mitochondria ||
|-----------------|-----------|----------|------------|
|                 | NV        | S        | VB         | PM | M | NB | B |
| 0.0-0.19        | 23        | 1        | 0          | 24 | 0 | 24 | 0 |
| 0.20-0.29       | 29        | 0        | 0          | 29 | 0 | 24 | 5 |
| 0.30-0.39       | 25        | 3        | 1          | 29 | 0 | 15 | 4 |
| 0.40-0.49       | 35        | 3        | 5          | 41 | 1 | 10 | 42|
| 0.50-0.59       | 32        | 3        | 4          | 31 | 8 | 5  | 34|
| 0.60-0.69       | 38        | 0        | 13         | 25 | 26| 2  | 49|
| 0.70-1.00       | 26        | 0        | 10         | 6  | 30| 2  | 34|

*¶¶ See footnotes for Table II. 125 cells of SF838-1D vps3-Δ1 and 125 cells of JHY20-2C vps3-Δ1 were quantified, and the sum of these data is shown. The numbers represent the number of cells scored in each category.

Discussion

Vacuolar protein sorting and vacuolar biogenesis in yeast is a complex problem. Characterization of genes that effect these processes will help unravel some of the complexity of these phenomena. A negative selection against vps mutants was devised which facilitates the cloning of VPS genes from a yeast genomic library. This selection was used to clone the VPS3 gene as well as several other VPS genes (Raymond, C. K., unpublished observations). Sequencing of VPS3 revealed an ORF with the potential to encode a hydrophilic protein with a predicted molecular mass of 117 kD. Antibodies were used to demonstrate that the VPS3 gene product, Vps3p, is expressed as a low abundance protein with an apparent molecular mass of ~140 kD by gel electrophoresis. The discrepancy between the predicted and apparent molecular weights is not uncommon (Sorger and Pelham, 1987; Novick et al., 1989), and the possibility of posttranslational modifications has not been pursued. Immunofluorescence and subcellular fractionation experiments suggest that Vps3p is neither a nuclear nor a vacuole-associated protein, and the data support the interpretation that Vps3p is a cytoplasmic protein that stably binds to, and may function in association with, intracellular membranes or cytoskeletal elements.

Mutations in the VPS3 gene were constructed in vitro and introduced into the yeast genome. Haploid cells that suffered a complete deletion of the VPS3 gene were viable, somewhat slow-growing cells with many characteristics in common with spontaneous mutants of vps3. Strains bearing an insertion mutation partway through the VPS3 gene had some phenotypic characteristics intermediate between wild-type cells and the complete deletion mutant. One hypothesis is that the truncated gene retains partial function.

vps3 mutants are defective in vacuolar protein sorting, vacuolar acidification, and processing of the zymogen form of PrA to its mature form (Rothman and Stevens, 1986; Roth-
The indicated strains were double labeled with CDCFDA and calcofluor as described in Materials and Methods. The percentages of mother cells and daughter cells were determined by counting the number of cells with bud scars in a total population of at least 110 cells chosen at random. The percentages of cells with CDCFDA-labeled vacuoles were determined from a population of at least 120 mother cells or 375 daughter cells for each of the strains listed above.

Table IV. Percentage of Mother Cells and Daughter Cells with Vacuoles in Wild-type and vps3-ΔI Strains

| Strain     | % Mother cells | % Daughter cells | % CDCFDA-labeled mother cells | % CDCFDA-labeled daughter cells |
|------------|----------------|------------------|-------------------------------|--------------------------------|
| SFR38-1D   | 55             | 45               | 94                           | 97                             |
| SFR38-1D   | 54             | 46               | 93                           | 41                             |
| JHRY20-2C  | 52             | 48               | 100                          | 95                             |
| vps3-ΔI    | 52             | 48               | 95                           | 67                             |

The two independent microscopic techniques used to examine vacuoles in wild-type and vps3-ΔI cells yielded consistent results. The organelle in wild-type cells generally appeared to be composed of several, irregularly shaped vacuolar compartments. Other studies on vacuolar morphology in wild-type yeast cells are consistent with the observations presented here (Wiemken et al., 1970; Pringle et al., 1989), and these data appear different than the previously reported finding that yeast cells possess single, major vacuoles throughout the cell cycle (Weisman et al., 1987). The differences may be due to the observation that the morphology of the vacuole is easily perturbed, especially by energy deprivation, and under these conditions it readily changes into a single, spherical organelle (Pringle et al., 1990; Raymond, C. K., unpublished observations). It is tempting to speculate that the vacuole maintains its unusual, convoluted morphology in growing cells through attachment to cytoskeletal elements, most notably actin. The same conditions that cause collapse of the actin network in yeast cells (Pringle et al., 1990; Novick et al., 1989) also lead to rearrangement of the vacuole into a spherical organelle (Raymond, C. K., unpublished observations). Microtubule structures, on the other hand, are not visibly altered by energy deprivation. The observed rearrangement of a complex vacuole into a single sphere may involve coalescence of several independent vacuolar compartments mediated by vacuolar membrane fusion. Alternatively, if a complex vacuole is an interconnected vacuolar network, then rearrangement may simply involve a change in shape.

Previous evidence had suggested that daughter cells receive vacuolar material from mother cells (Zubenko et al., 1982; Weisman et al., 1987; Weisman and Wickner, 1988). Our observations suggest that the transfer of vacuolar contents may occur through a continuous network of vacuolar structures which extends from the mother cell into the newly forming bud. The appearance of these segregation structures is generally constrained to a part of the cell cycle shortly after bud emergence but before migration of the nucleus into the neck of the bud. This proposed mechanism of vacuolar segregation is an alternative to previous suggestions that vacuolar transfer is mediated by small transport vesicles (Weisman et al., 1987; Weisman and Wickner, 1988; Weisman et al., 1990). Interrvacuolar exchange of vacuolar material may therefore be accomplished by a combination of continuous vacuolar structures and vacuolar transport vesicles. At least two plausible models can be envisioned to account for segregation structures. Vacuoles may extend into nascent daughter cells by active translocation of the organelle along a cytoskeletal track or other intracellular structure that leads into the bud. Another possibility is that fusion of new vacuolar material with the vacuolar structures in mother cells may be spatially constrained to a region of the organelle facing the bud such that all new synthesis leads to the polarized growth of vacuoles into the bud.

The vacuoles in vps3-ΔI cells differed from the wild-type organelle in several important respects. The vacuoles in vps3-ΔI cells almost always assumed a simple, spherical shape. Furthermore, the relative cellular volume occupied by the vacuole varied greatly. Some cells had little or no detectable vacuoles while other cells had vacuoles that occupied the majority of the cytoplasmic compartment. Finally, segregation structures were rarely observed in the mutant cell population. The aberrant vacuolar morphology in vps3-ΔI cells may be a general consequence of the vacuolar protein sorting defect in this mutant. If this were the case, we would expect all vps mutants to exhibit abnormal vacuoles similar to those in vps3-ΔI cells. This is not the case. Mutants representing 26 different vps complementation groups have been examined by immunofluorescence proce-
plementation groups also had an overall vacuole morphology similar to that seen in vps3-ΔI cells. Another mutant with vacuolar segregation defects was recently described in the literature (Weisman et al., 1990). The mutation was named vacI-1, and the mutant strain had many characteristics in common with the segregation defective vps mutants. Whether the vacI-1 mutation is allelic to any of the vps mutations is not yet known.

All of the aberrant morphological characteristics of vacuoles in vps3-ΔI cells are potentially interrelated. The characteristic spherical shape of the vacuole and the defect in segregation reflect the possibility that the organelle is detached from the structural elements that normally determine shape and segregation of vacuoles in wild-type cells. The lack of normal segregation might give rise to two effects. Mother cells, unable to segregate vacuoles to the bud, would continue to accumulate vacuolar material to the point where the organelle assumed a disproportionate amount of cell volume. Measurement of cell diameter and vacuolar diameter in populations of vps3-ΔI mother cells with either one bud scar or greater than three bud scars supports this possibility. Assuming that the cells and vacuoles are simple spheres, the data demonstrated that as vps3-ΔI cells “aged,” the relative vacuolar volume increased from 7 to 29% of total cell volume. On the other hand, an apparent consequence of the vacuolar segregation defect in vps3-ΔI cells is that many daughter cells are born with little or no vacuolar compartment. In a wild-type population, the maternal cell contributes a substantial fraction of its lumenal vacuolar material to daughter cells (Weisman et al., 1987; this study). By contrast, the maternal cell contribution to daughter cell vacuoles in vps3-ΔI populations was marginal, at best. Similar observations have been made with the segregation defective vacI-1 mutant (Weisman et al., 1990). These data raise the possibility that the vacuoles which eventually appear in vps3-ΔI daughter cells arise by de novo synthesis from accumulated but unassembled vacuolar constituents synthesized in independent daughter cells. The presence of accumulated but unassembled vacuolar membrane constituents is suggested by the punctate labeling pattern seen within certain vps3-ΔI cells by immunofluorescence. An alternate possibility is that the mother cell contributes a small, and in many cases undetectable, vacuolar compartment to the growing bud. This tiny vacuole would then serve as an assembly point for the daughter vacuole, yet vacuole assembly is delayed in the mutant. Vacuolar assembly must eventually proceed as almost all cells that go through a budding cycle have a coherent vacuole.

Our data have demonstrated that the product of the VPS3 gene is required for sorting and processing of soluble vacuolar proteins, integrity of vacuolar morphology, efficient segregation of vacuolar material into the bud during the cell cycle, acidification of the vacuolar lumen, and assembly of the vacuolar H^+ -ATPase. How might Vps3p function be required for these many diverse aspects of vacuolar assembly and function? One model is that Vps3p may be involved in the assembly of (some) peripheral membrane proteins onto Golgi apparatus-derived transport vesicles that are destined to fuse with the vacuole. This is based on the assumption that vacuolar protein sorting, acidification, and morphology, including segregation structures, are dependent on the activities of peripheral membrane proteins. For example, peripheral membrane proteins may assist in the sorting, packaging, and delivery of newly synthesized soluble vacuolar proteins from the main secretory pathway to the vacuole, and these same activities may effect processing in some manner. Peripheral membrane proteins would likely be involved in the presumed attachment of the vacuolar membrane to the cytoskeletal network in the cell. Vacuolar acidification is already known to be dependent on the peripheral membrane proteins which constitute a major portion of the vacuolar H^+ -ATPase (Kane et al., 1989; C. T. Yamashiro and T. H. Stevens; unpublished observations). Given this model, the assembly of these peripheral membrane proteins would not occur in the absence of Vps3p function. This would account for the observed defects in sorting, acidification, and vacuolar morphology observed in vps3-ΔI mutants. Future experiments will be aimed at clarifying the role that the VPS3 gene product plays in the assembly and maintenance of this dynamic organelle.

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References

Ammerger, G., C. P. Hunter, J. H. Rothman, G. C. Saari, L. A. Vallis, and T. H. Stevens. 1986. PEP4 gene of Saccharomyces cerevisiae encodes proteinase A, a vacuolar enzyme required for processing of vacuolar precursors. Mol. Cell. Biol. 6:2490–2498.

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

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

Botstein, D., S. C. Falco, S. Stewart, M. Brennan, S. Scherer, D. T. Stinchcomb, K. Struhl, and R. Davis. 1979. Sterile host yeast (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene. 8:17–24.

Bussey, H., and H. E. Umbarger. 1970. Biosynthesis of the branch-chain amino acids in yeast: a trifluorocleucine-resistant mutant with altered regulation of leucine uptake. J. Bacteriol. 103:286–294.

Carlson, M., and D. Botstein. 1982. Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast inverterase. Cell. 27:145–154.

Casadaban, M. F., and S. N. Cohen. 1980. Analysis of gene control signals by DNA fusion and cloning in Escherichia coli. J. Mol. Biol. 138:179–207.

Goud, B., A. Salminen, N. C. Walworth, and P. J. Novick. 1988. A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. Cell. 53:753–768.

Hasilik, A., and W. Tanner. 1978. Biosynthesis of the vacuolar yeast glycoprotein carboxypeptidase Y: conversion of precursor into the enzyme. Eur. J. Biochem. 85:599–608.

Hickey, H., and R. Schekman. 1989. Yeast Sec23p acts in the cytoplasm to promote protein transport from the endoplasmic reticulum to the Golgi complex.
