Yeast vacuole protein targeting (vpt) mutants exhibit defects in the sorting and processing of multiple vacuolar hydrolases. To evaluate the impact these vpt mutations have on the biogenesis and functioning of the lysosome-like vacuole, we have used light and electron microscopic techniques to analyze the vacuolar morphology in the mutants. These observations have permitted us to assign the vpt mutants to three distinct classes. The class A vpt mutants (26 complementation groups) contain 1-3 large vacuoles that are morphologically indistinguishable from those in the parental strain, suggesting that only a subset of the proteins destined for delivery to this compartment is mislocalized. One class A mutant (vpl3) is very sensitive to low pH and exhibits a defect in vacuole acidification. Consistent with a potential role for vacuolar pH in protein sorting, we found that bafilomycin A1, a specific inhibitor of the vacuolar ATPase, as well as the weak base ammonium acetate and the proton ionophore carbonyl cyanide m-chlorophenylhydrazone, collapse the pH gradient across the vacuolar membrane and cause the missorting and secretion of two vacuolar hydrolases in wild-type cells. Mutants in the three class B vpt complementation groups exhibit a fragmented vacuole morphology. In these mutants, no large normal vacuoles are observed. Instead, many (20-40) smaller vacuole-like organelles accumulate. The class C vpt mutants, which constitute four complementation groups, exhibit extreme defects in vacuole biogenesis. The mutants lack any organelle resembling a normal vacuole but accumulate other organelles including vesicles, multilamellar membrane structures, and Golgi-related structures. Heterozygous class C zygotes reassemble normal vacuoles rapidly, indicating that some of the accumulated aberrant structures may be intermediates in vacuole formation. These class C mutants also exhibit sensitivity to osmotic stress, suggesting an osmoregulatory role for the vacuole. The vpt mutants should provide insights into the normal physiological role of the vacuole, as well as allowing identification of components required for vacuole protein sorting and/or vacuole assembly.

Eukaryotic cells are distinguished by their several discrete membrane-enclosed organelles. Each of these subcellular compartments has unique structural and functional characteristics which are conferred in large part by the distinct set of proteins that constitute that organelle. Thus, accurate sorting and trafficking of proteins from their site of synthesis in the cytoplasm to their correct noncytoplasmic destinations are essential for maintaining the functional and structural identity of each organelle.

In mammalian cells, the secretory pathway has been shown to mediate the modification, processing, and delivery of proteins destined for a variety of intracellular and extracellular compartments. Proteins destined for secretion, assembly into the plasma membrane, delivery to lysosomes, or retention within endoplasmic reticulum (ER) and Golgi compartments transit through all or a portion of the secretory pathway (11). Presently, available data are consistent with a model in which much of protein secretion occurs via a default mechanism (5). Proteins competent for entry into the ER but lacking any additional sorting information passively transit from the ER to the Golgi complex and then are secreted via a nonspecific bulk flow mechanism (58). However, proteins that depart from this pathway, such as lysosomal enzymes, contain additional sorting signals that permit specific recognition, modification, and subsequent delivery of these proteins from late Golgi compartments to the lysosome (53).

In the yeast Saccharomyces cerevisiae most protein secretion also appears to occur via a constitutive default pathway (55). However, like mammalian lysosomal enzymes, proteins destined for the yeast vacuole depend on the presence of additional protein sorting information (21, 25, 56). The yeast vacuole is a prominent intracellular organelle which shares functional characteristics with both mammalian lysosomes and plant central vacuoles. This organelle is believed...
to play an important role in the storage of amino acids and other small molecules (30). Like mammalian lysosomes, the yeast vacuole is an acidic compartment and contains a number of hydrolytic enzymes (33, 59). Certain of these hydrolases, including the glycoproteins proteinase A (PrA), proteinase B (PrB), and carboxypeptidase Y (CPY) have been shown to be synthesized at the level of the ER as inactive precursors. These proenzymes transit through the Golgi complex and are sorted to the vacuole, where they are processed to the mature active enzymes (16, 19, 32, 54). Sequence determinants have been defined within proCPY and proPrA that are necessary and sufficient to target these proteins to the vacuole (21, 25, 56). Mutational alterations in the proCPY sorting signal lead to missorting and secretion of the precursor form of this enzyme. Although the sorting signals in proCPY and proPrA lack any obvious primary sequence similarity, it is presumed that a common cellular protein-sorting apparatus mediates specific recognition and subsequent vacuolar localization of these enzymes. Each undergoes a similar set of compartment-specific modifications, and the kinetics for vacuolar delivery of both proteins are essentially the same (25). It seems likely that many soluble vacuolar proteins are segregated to this compartment via the same targeting mechanism.

In an effort to identify components of the vacuolar protein sorting apparatus, we recently isolated a number of mutants that exhibit defects in the proper localization and processing of several vacuolar proteins. These vacuolar protein targeting (vpt) mutants were identified using a gene fusion-based selection scheme. In wild-type cells, proCPY sequences fused to the gene for the normally secreted enzyme invertase (Inv) contain sufficient sorting information to divert delivery of enzymatically active Inv to the yeast vacuole (2, 21). Mutants have been selected that missort and secrete such CPY-Inv hybrid proteins. The ~600 mutants isolated thus far have been assigned to more than 33 complementation groups. The mutants exhibit hybrid protein-independent defects in the sorting of normal vacuolar enzymes including CPY, PrA, and PrB (46). Upon missorting, the precursor forms of these proteins are secreted, presumably because the selective vacuole protein delivery pathway is defective and the proteins then follow the default secretion pathway (2).

Given this large number of potential gene products that can influence the vacuolar protein-sorting process, it seemed likely that mutations in at least some of these genes might also affect biogenesis of a normal vacuole. Specifically, some of the proteins that are mislocalized in the vpt mutants may be essential in defining important structural and functional characteristics of this organelle. In addition, one might expect that lipid and protein constituents of the vacuole membrane would transit together with soluble vacuolar enzymes via a common vesicle carrier. Defects in vacuole membrane assembly therefore might also be expected in certain of the vpt mutants. To address these questions, we have assessed the vacuolar as well as other organellar morphologies in each of the vpt mutants. Using both light and electron microscopic techniques, we found that mutants in most of the vpt complementation groups still assembled morphologically normal vacuoles. However, mutants in three complementation groups accumulated what appeared to be multiple small vacuoles. Cells in four other vpt complementation groups exhibited extreme defects in vacuole biogenesis; these mutants accumulated vesicles and membrane-enclosed compartments that bore no resemblance to a normal vacuole. In addition, certain of the vpt mutants exhibited other phenotypes such as sensitivity to low pH or to osmotic stress. These observations provide insights into the mechanism(s) of vacuole biogenesis as well as the normal physiological role of this organelle.

**Materials and Methods**

**Strains and Media**

Mutants were isolated as described (46) from parental strains SEY6210 MATa ura3-52 leu2-3,112 his3-Δ200 trpl-Δ901 lys2-801 sec12-Δ9 and SEY6211 MATa ura3-52 leu2-3,112 his3-Δ200 trpl-Δ901 ade2-101 sec12-Δ9. Other strains used were HMSN1 MATa sec17-Δ90 (37), SEY5078 MATa sec17-Δ90 ade2-101 leu2-3,112 ura3-52 (this study), and SEY886 MATa sec17-Δ90 ade2-101 leu2-3,112 ura3-52 (this study). Cells were grown on standard yeast extract peptone dextrose (YPD) or synthetic dextrose (SD) (synthetic minimal, supplemented as necessary) media (49). ade2Δ strains were scored for the presence of red pigment after growth for 3-5 d on both standard YPD (containing 2% glucose) and YPD containing 8% glucose. Sensitivity to low pH was assessed on YPD adjusted to pH 3.5, 3.0, or 2.5 with 6 N HCl. Sensitivity to high osmotic pressure was determined on YPD containing 1.0 or 1.5 M NaCl, 1 M KCl, or 2.5 M glycerol (osmolarity ~1.7%). As noted previously by Singh (51), certain batches of hypertonic media stored more than a few days inhibited growth of the wild-type strains; therefore it was important to test the media with a strain known to be resistant to the osmotic stress conditions present.

Genetic crosses, sporation of diploids, and dissection of tetrads were performed as described by Sherman et al. (49).

**Labeling of Cells with Fluorescent Dyes**

All manipulations were performed at room temperature unless otherwise noted. Cells were labeled in the presence of FITC-dextran (FD) as described by Makarow (27), with the following modifications. Cells (5 ml) were grown in YPD to early log phase (1-2 × 10⁷ cells/ml), centrifuged for 2 min at 30°C, washed once, and resuspended in J.1M K-phosphate, pH 7.6. After 30°C, cells were centrifuged and mounted as above. Alternatively, cells were labeled with FITC as described previously (41). (2 × 10⁷) were resuspended in 1 ml YPD containing 50 mM Na-citrate, pH 5.5, and 10 μg/ml FITC in DMSO. After a 10-min incubation at 25°C with shaking, cells were centrifuged, washed once, and resuspended in 0.1 ml 100 mM K-phosphate, pH 7.5, containing 2% glucose. Cells were mounted as above.

For quinacrine labeling, cells were grown as described above and quinacrine was added to a final concentration of 175 μM in YPD, pH 7.6. After a 5-min incubation at 30°C, cells were centrifuged and mounted as above without washing. Ammonium acetate (200 μM final concentration) was added to the incubation mix where indicated (57).

For observation of the ade2Δ endogenous fluorophore, cells were grown as described by Weisman et al. (57) and mounted as for FD.

**Microscopy**

Cells were observed using a Carl Zeiss Inc. microscope (Thornwood, NY), with a 100x oil-immersion objective, equipped for Nomarski optics and epifluorescence. Fluorescence filters used were Carl Zeiss Inc. B3450-490 (excitation), FT510 (beam splitter), and BP520-560 (emission barrier). All fluorescent images were photographed for 40–60 s using Eastman Kodak Co. (Rochester, NY) Tri-X Pan ASA400 film, increased to ASA1600 by using Diafine developer.

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**Electron Microscopy**

Cells were prepared using a modification of the procedure of Byers and Goetz (6). Cells (100 ml) were grown in synthetic minimal medium to an O.D. of ~0.3, shifted to YPD medium, and allowed to grow for one generation. The cells were harvested by centrifugation (5 min in an International Equipment Co. clinical centrifuge), washed in dH₂O, and fixed for 2 h at room temperature in 2 ml of 0.1 M Na-cacodylate, pH 6.8, 5 mM CaCl₂ (Buffer A), containing 3% glutaraldehyde. The fixed cells were washed once in 100 mM Tris-HCl, pH 8.0, 25 mM dithiothreitol, 5 mM Na₂-EDTA, 1.2 M sorbitol, and then incubated in the same buffer for 10 min at 30°C. To remove cell walls, the cells were centrifuged, washed once with 1 ml 0.1 M K-phosphate (adjusted to pH 5.8 with citric acid) containing 1.2 M sorbitol, and resuspended in 0.5 ml of the same buffer containing 0.05 ml β-glucuronidase type H-2 and 2.5 mg zymolyase. This cell suspension was incubated for 2 h at 30°C. The spheroplasts were centrifuged and washed in 1 ml buffer A. ts strains were shifted to 37°C for 3 h before harvesting and were fixed at 37°C for analysis at the nonpermissive temperature; the rest of the procedure was identical to that for non-ts strains. The reduced osmium-thiocarbohydrazide-reduced osmium membrane-enhancement staining technique was adapted from Willingham and Rutherford (42), and observed on a transmission electron microscope (model 440; Philips Electronic Instruments, Inc., Mahwah, NJ).

**Immunoprecipitations**

Immunoprecipitations on labeled spheroplasts (46) were performed as described previously (25).

**Materials**

Bafilomycin A₁ was the generous gift of K. Altendorf, Universität Os- nabrück, Osnabrück, FRG. Tran 35S-label was purchased from ICN Radiochemicals, Irvine, CA. Gluteraldehyde, OsO₄, thiocarbohydrazide, and K-ferrocyanide were purchased from Polysciences, Inc., Warrington, PA. Zymolase was purchased from Seikagaku Kogyo Co., Ltd., Japan, and low melting point agarose was purchased from Bethesda Research Laboratories, Gaithersburg, MD. Diffract developer was the product of Acufine, Chicago, IL. London Ross White Embedding resin was purchased from Ted Pella Inc., Irvine, CA. FD (70 S), FITC, quinacrine, β-glucuronidase (type H-2), and all other chemicals not listed above were purchased from Sigma Chemical Co., St. Louis, MO.

**Results**

**Vacuole Morphology in vpt Mutants**

We have used light and fluorescence microscopy to determine the state of the vacuole in multiple vpt alleles from each of 33 complementation groups. In a wild-type strain grown in rich medium, yeast vacuoles occupy ~20% of the yeast cell volume and can often be visualized in the light microscope using Nomarski optics. However, small vacuoles can not be visualized by this technique and some cells do not appear to have any vacuole when observed by Nomarski optics (57). Therefore, in order to visualize even small vacuoles, we have taken advantage of a number of fluorescent dyes that specifically accumulate in this organelle.

FD as well as FITC² by itself have been used to label yeast vacuoles (27, 41). In wild-type cells, the vacuole appeared as a single large fluorescent spot or 2–3 spots of approximately equal size under the labeling conditions used (Fig. 1 A). A large vacuole was also visible in these cells using Nomarski optics. Unexpectedly, when the vpt mutants were examined using this dye, most of the mutants (26 complementation groups, see Table I) exhibited a vacuolar morphology indistinguishable from that of the parental strains (Fig. 1 B). We have grouped these mutants together and refer to them as class A vpt mutants. The remaining seven complementation groups exhibited an altered vacuolar morphology. In three of the groups (vpt3, 5, and 26), cells had multiple smaller organelles which were visible using Nomarski optics and which stained with FITC (Fig. 1 C). This group of mutants has been designated class B. The remaining four vpt complementation groups (vpt11, 16, 18, and 33) had no intracellular structures which stained in the presence of FD. When observed by Nomarski optics, cells in these complementation groups appeared to have rough surfaces, and no vacuoles were visible (Fig. 1 D). We have defined this group of mutants as class C vpt mutants (Table I). These observations have been confirmed using two other fluorescent dyes that also accumulate in yeast vacuoles (see below).

**Class A vpt Mutants Exhibit Wild-type Vacuole Morphology**

We have studied the vpt mutants exhibiting each of the three vacuolar morphologies in more detail. In ade2 strains of S. cerevisiae, an endogenous fluorophore accumulates in the vacuole and can be visualized using fluorescence microscopy (57). By this method, the parental strains and the class A vpt mutants had vacuolar morphologies identical to those observed using FD (Fig. 1 E and F).

Like mammalian lysosomes (38), yeast vacuoles have an acidic pH (33) and can be labeled by dyes, such as quinacrine (1, 57), chloroquine (26), and neutral red (20, 34), which accumulate in a pH-dependent manner. These weak bases are presumed to diffuse through membranes and accumulate in acidic compartments (9). The vacuole morphology of wild-type cells as observed using quinacrine was identical to that seen with FD or the ade2 endogenous dye (Figs. 2 A and 4 A). Most of the class A vpt mutants also contained 1–3 large vacuoles which accumulated quinacrine, although in many cases the fluorescence was less intense than that in the parental strain (not shown). Multiple alleles of three complementation groups (vpt10, 13, and 24), exhibited very little or no vacuolar staining with quinacrine, although vacuoles were clearly visible by Nomarski optics in these cells (Fig. 2 E). These vpt mutants, which had morphologically normal vacuoles but exhibited no pH-dependent accumulation of dye, might carry mutations which affect vacuole acidification. In plant cells, the vacuole plays an important role in pH homeostasis; a decrease in external pH results in a lowered vacuolar pH, while the cytoplasmic pH remains constant (3). We reasoned that mutants defective in vacuole acidification might also exhibit defects in the regulation of intracellular pH. To address this issue, we tested whether any of the vpt mutants were sensitive to low pH. Growth was assayed on of FITC and other contaminating impurities in the FD. We have repeated the vacuole labeling experiments in a few of the vpt mutants using FITC and have observed vacuolar morphologies similar to those reported here for FD.

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2. While these studies were in progress, Preston et al. (41) reported that the vacuolar staining associated with FD was in fact due to nonendocytic uptake
Figure 1. Vacuole morphology in vpt mutants labeled with FITC or the ade2 endogenous fluorophore. (A, B, C, and D) Nomarski (left) and fluorescence (right) photomicrographs of cells labeled in the presence of FD (see Materials and Methods). (E, F, G, and H) Nomarski (left) and fluorescence (right) photomicrographs of cells grown in SD containing limiting adenine (12 μg/ml) to allow production of the ade2 fluorophore. In each cell, the fluorescent spot corresponds to the vacuole, which appears as a large circular indentation using Nomarski optics (arrows). (A and E) Wild-type vacuole morphology as seen in the parental strains SEY6210 (A) or SEY6211 (E). (B and F) Representative class A vpt mutants, vpt10 (B) and vpt29 (F), in which the vacuole morphology is indistinguishable from that of the parent. (C and G) The class B vpt mutants, vpt5 (C) and vpt3 (G), contain multiple small vacuoles. (D and H) Representative class C vpt mutants, vpt18 (D) and vpt11 (H), which contain no structures that stain like vacuoles. Bar, 10 μm.

YPD media adjusted to pH 3.5, 3.0, or 2.5. The parental strains grew, although more slowly than on standard YPD, under these conditions. Strains in one complementation group, vpt13, were found to be extremely sensitive to low pH. Fourteen of 21 vpt13 alleles were unable to grow at pH 3.5, and 19 alleles of vpt13 were inhibited for growth at pH 3.0 (Fig. 3 A). Some alleles of other vpt mutants were also weakly sensitive to low pH (data not shown).

The existence of vpt mutants that exhibited possible defects in vacuole acidification led us to investigate the role of vacu-
lar pH in the localization of proteins to the vacuole in wild-type cells. The vacuolar membrane contains a proton-translocating Mg$^{2+}$-dependent ATPase which produces a proton gradient across the vacuolar membrane and acidifies the interior of the vacuole (24). The drug bafilomycin $A_1$ has been shown to be a specific inhibitor of the vacuolar membrane proton-translocating ATPase of *Neurospora crassa* (4). When wild-type yeast cells were treated with 10 $\mu$M bafilomycin $A_1$ for 10 min before staining with quinacrine, no vacuolar fluorescence was observed (Fig. 4 B). The inhibition of the pH-dependent quinacrine staining in these cells indicates that bafilomycin eliminates the pH gradient across the yeast vacuolar membrane, presumably by inhibiting the vacuolar membrane ATPase. To assess the role of the pH of the yeast vacuole in vacuolar protein localization, we next examined the effect of bafilomycin on the sorting and processing of vacuolar hydrolases. Spheroplasts were pretreated with bafilomycin (10 $\mu$M final concentration) for 10 min, radioactively labeled, and separated into intracellular and extracellular fractions before immunoprecipitation with CPY- and PrA-specific antisera. As shown in Fig. 4 C, in the absence of bafilomycin all of the CPY and PrA was processed to the mature enzyme form and remained associated with the yeast spheroplast fraction (0 $\mu$M, lanes I and E), indicating that these enzymes had been delivered to the vacuole (46, 59). In contrast, in the presence of bafilomycin, $\approx$50% of the CPY was present in the proenzyme form, and most of this proCPY was secreted into the extracellular space (Fig. 4 C, 10 $\mu$M, lanes I and E). Bafilomycin caused a similar defect in the processing and targeting of PrA. Other agents known to raise vacuolar pH, including the weak base ammonium acetate (200–400 mM; reference 57) and the proton ionophore carbonyl cyanide $m$-chlorophenylhydrazone (CCCP; 40 $\mu$M) also caused the mislocalization of CPY and PrA (data not shown). Significantly, protein traffic to the cell surface was not disrupted under these conditions, and the concentration of bafilomycin used in these experiments did not inhibit yeast cell growth. Together these data indicate that vacuole pH plays a role in the efficient delivery and maturation of at least some vacuolar hydrolases.

We next examined the class A *vpt* mutants by transmission EM, using a technique that results in enhanced staining of biomembranes and structures containing glycomolecules (see Materials and Methods). As seen in Fig. 5 A, the vacuole stained as a large electron-dense compartment using this procedure. Thin sections of wild-type cells typically contained one large or two to three smaller vacuoles per cell. Other intracellular structures such as the nucleus, mitochondria, and ER were also readily visible. The majority of the class A *vpt* strains, such as the ones shown in Fig. 5, B and C, exhibited typical wild-type morphology. In thin sections of both wild-type and class A strains, we often observed an apparent substructure within the vacuole that did not stain like the rest of the vacuole and remained electron transparent (e.g., see Fig. 5 B). The significance of this structure is unclear, although it may represent the polyphosphate precipitate often observed in freeze-etched yeast cells (30).

Electron microscopic analysis also revealed that certain class A *vpt* mutants accumulated aberrant structures in addition to the normal vacuole. Mutants in two complementation groups, *vpt15* and *vpt29*, contained organelles similar to those seen in yeast protein secretion (sec) mutants (37), including vesicles and Berkeley bodies (Bbs, structures presumably related to the Golgi complex; see reference 36). The electron micrographs in Fig. 6 show typical *vpt15* and *vpt29* cells and, for comparison, sec1 cells (accumulates vesicles), sec18 (accumulates ER), and sec17 (accumulates Bbs), prepared using the membrane-enhancement technique. The vacuoles in these *vpt15* and *vpt29* cells were abnormally large and occasionally contained inclusions (Fig. 6 C). This aberrant morphology was seen in every *vpt15* and *vpt29* allele examined. *vpt13* and *vpt26* cells also occasionally contained vesicles and Bbs (not shown).

In two complementation groups, *vpt7* and *vpt28*, stacks of lamellae and reticular membrane arrays were observed at a high frequency (Fig. 7). These organelles were more prevalent in *vpt7* and *vpt28* than in the parental strain (i.e., 0.8–1.0 structure per cell section vs. 0.4 for the parent in 30–50 sections examined for each strain). These structures, which were usually not associated with any other organelle, are likely to correspond to exaggerated Golgi complexes. Finally cells in one class A mutant, *vpt12*, accumulated vesicles similar to those seen in the class C mutants (see below).

### Table I. Summary of *vpt* Mutant Phenotypes

| Class A (*vpr1, 2, 4, 6, 7, 8, 9, 10, 12, 13, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 27, 28, 29, 30, 31, 32) | Wild-type vacuoles | *vpr15, 29* | *vpr13* | *vpr15, 29-Bbs, vesicles* | *vpr7, 28-"Golgi"* | *vpr12-vesicles* |
| Class B (*vpr3, 5, 26) | Fragmented vacuoles | *vpr26* | | | |
| Class C (*vpr11, 16, 18, 33) | No vacuoles | *vpr11, 16, 18, 33* | | | Multilamellar membrane structures, vesicles, Bbs |

**Class B *vpt* Mutants Exhibit an Altered Vacuole Morphology**

Unlike the class A *vpt* mutants, cells in the three class B complementation groups (*vpr3, 5, and 26*) contained multiple compartments that stained in the presence of FD and were visible using Nomarski optics (Fig. 1 C). These small "vacuoles" also accumulated the *ade2* endogenous fluorophore (Fig. 1 G). To determine whether these organelles had the lowered pH characteristic of wild-type vacuoles, we tested their ability to accumulate quinacrine. As shown in Fig. 2 C, the structures in the class B *vpt* mutants stained with quina-
Figure 2. Quinacrine accumulation in the vacuoles of wild-type cells and vpt mutants. Cells were incubated in YPD, pH 7.6, containing 175 μM quinacrine for 5 min in the absence (A, C, and E) or presence (B and D) of 200 mM ammonium acetate. (A and B) Nomarski (left) and fluorescence (right) images of the parental strain SEY6210. (C and D) Nomarski (left) and fluorescence (right) images of vpt5, which exhibits a typical class B vacuole morphology. (E) vpt13 exhibits no quinacrine staining (right), although vacuoles are clearly visible using Nomarski optics (left). Bar, 10 μm.

Figure 3. Growth defects associated with vprl3 and the class C vpt mutants. (A) Three alleles of vprl3 and the parental strain SEY6210 were streaked on YPD medium (left) or YPD medium adjusted to pH 3.0 with 6 N HCl (right). The plates were incubated at 30°C for 2 and 4 d, respectively. (B) A representative allele from each of the four class C vpt complementation groups was streaked on YPD medium (left) or YPD medium containing 1.5 M NaCl (right). The plates were incubated at 30°C for 2 and 5 d, respectively. The parental strain SEY6211 and a class A vpt mutant, vpr1, are shown for comparison.

Crine, suggesting that these compartments had a pH similar to that of the vacuole in the parental strain. This hypothesis was further tested by labeling with quinacrine in the presence of ammonium acetate (57). Under these conditions, no fluorescent staining was observed in the parental strain or the class B vpt mutant (Fig. 2, B and D). This supports the hypothesis that the fluorescent staining observed in class B cells is due to the acidic pH of the compartments stained rather than to some nonspecific accumulation of dye.

The class B vpt mutants were also examined at the ultrastructural level to confirm the multiple-vacuole morphology observed by light and fluorescence microscopy. As seen in Fig. 5 D, a representative class B vpr5 mutant allele contained multiple small organelles that stained like wild-type vacuoles. The number of vacuoles per cell section was quantitated for vpr5 and for the parental strain (using 30–40 cell sections per strain). The average number of vacuole-like structures per cell section for the class B vpr was 5.7, while the number for the wild-type strain was 1.8. 71% of the class B cells, as compared to 16% of the parental cells, had 3 or more vacuoles. On the basis of the size of the vacuoles and the thickness of the sections, we have calculated that an average class B cell contains ~35 of these small vacuoles, while wild-type cells contain one to four vacuoles per cell. Representatives of the other two class B complementation groups (vpr3 and vpr26) exhibited similar vacuolar morphologies when observed by EM.

Yeast which carry a mutation in the β-tubulin gene (tub2)
Bafilomycin was added to cells containing quinacrine. Cells (2.5 × 10^7/ml) were incubated for 10 min at 25°C in YPD, pH 7.6, in the absence (A) or presence (B) of 10 μM bafilomycin (in DMSO). An aliquot of cells (50 μl = 5 × 10^7 cells) from each sample was added to 400 μl of YPD, pH 7.6, containing 200 μM quinacrine without (A) or with (B) 10 μM bafilomycin. Cells were incubated for 5 min at 30°C, resuspended, and mounted as described in Materials and Methods. Bar, 10 μm.

Figure 4. Effect of bafilomycin on vacuole staining with quinacrine and on vacuolar protein sorting. (A and B) Nomarski (left) and fluorescence (right) images of parental strain SEY6210 stained with quinacrine. Cells (2.5 × 10^7/ml in 0.25 ml) were preincubated for 10 min at 25°C in YPD, pH 7.6, in the absence (A) or presence (B) of 10 μM bafilomycin (in DMSO). An aliquot of cells (50 μl = 5 × 10^7 cells) from each sample was added to 400 μl of YPD, pH 7.6, containing 200 μM quinacrine without (A) or with (B) 10 μM bafilomycin. Cells were incubated for 5 min at 30°C, resuspended, and mounted as described in Materials and Methods. Bar, 10 μm.

(C) Strain SEY6210 was enzymatically converted to spheroplasts as described (46). Bafilomycin was added to 10 μM final concentration as indicated 10 min before the addition of radioactive label. Cells were labeled with Tran-35S-label for 20 min at 30°C and chased for 30 min by the addition of 5 mM methionine. Cultures were then separated into (I) intracellular (spheroplast) and (E) extracellular (periplasm and media) fractions (46). Immunoprecipitations with antisera to CPY and PrA were performed as described (25). The predicted locations and approximate molecular sizes of the different forms of CPY and PrA are indicated. Increased concentrations (100 μM) of bafilomycin did not increase the amount of missorted precursor CPY or PrA. Importantly, bafilomycin had no effect on mitochondrial protein import (data not shown), which requires an electrochemical potential across the mitochondrial inner membrane (17).
of these conditional lethal strains at both permissive (25°C) and nonpermissive (37°C) temperatures. Surprisingly, the vacuolar morphology of each of the ts strains examined was identical at 25°C and 37°C; aberrant organelles accumulated to the same extent at either temperature.

The existence of mutants in several complementation groups that lacked any apparent vacuole allowed us to begin to analyze the requirements for vacuole biogenesis in vivo. If a vacuole can be formed via a de novo synthetic pathway, diploids made by mating two class C mutants from different complementation groups should contain a normal vacuole. If, however, a preexisting normal template vacuole is necessary to direct the synthesis of the new organelle, diploids heterozygous for two class C mutations may be incapable of generating a vacuole despite the presence of both wild-type gene products. When we examined diploids made by crossing a ts vpt11 allele with a ts vpt18 allele, we observed that the diploid was temperature resistant, competent to sort vacuolar proteins, and contained a normal-appearing vacuole which stained with FITC. Homozygous diploids made by crossing two ts alleles of the same class C complementation group exhibited typical class C morphology and were ts, as expected. Furthermore, when the mating pairs were examined within 4–6 h of mixing, heterozygous class C zygotes were observed which clearly contained a vacuole in each of the conjugating cells, as well as in the diploid bud emerging from the zygote (Fig. 9). Ultrastructural analysis confirmed the presence of vacuoles in these heterozygous mating pairs.
Figure 6. Electron micrographs of vpt15 and vpt29, class A mutants which exhibit aberrant organelles similar to those seen in certain of the sec mutants. Cells were prepared as described in the legend to Fig. 5. vpt15 (A) and vpt29 (B) cells contain Bbs (large arrows) and vesicles (small arrows) throughout the cytoplasm. (C) A typical vpt15 cell in which inclusions are seen in the vacuole. (D, E, and F) sec mutants (37) prepared using the membrane-enhancement technique, are shown for comparison. Each of the mutant strains was incubated at 37°C for 3 h before fixation. (D) Exaggerated tubular networks of membranes, presumably corresponding to ER, are clearly visible in this typical sec18 cell. (E) Accumulated Bbs are seen in this high magnification view of a portion of a representative sec7 cell. (F) A high magnification view of part of a sec1 cell shows an accumulation of secretory vesicles. These vesicles have a different appearance than those seen in vpt15 and vpt29 (compare with A and B). Bars: (A, B, C, and D) 0.5 μm; (E and F) 0.1 μm.

(not shown). These observations suggest that vacuole biogenesis can occur de novo in the absence of a normal template organelle. This assembly is rapid, occurring within one generation after conjugation. We cannot at present, however, exclude the possibility that the class C mutants contain tiny degenerate vacuole forms that are capable of functioning as templates or targets for new vacuolar protein and membrane delivery.

**Class C Mutants are Defective in Several Vacuolar Functions**

The vacuole has been implicated in a number of diverse cellular functions in wild-type cells, including osmoregulation (29), storage of amino acid reserves (30), endocytosis (43), and adaptation to adverse growth conditions (48). Like mammalian lysosomes and plant vacuoles (10, 28), the yeast vacuole may also mediate the normal intracellular turnover of macromolecules. Protein degradation increases dramatically during sporulation, and mutants lacking PrA or PrB activity are partially or completely defective in sporulation (22). We postulated that the class C vpt mutants, which exhibited extreme aberrations in vacuole assembly and morphology, might be defective in other cellular functions that may be vacuole related.

If the vacuole is required for adaptation to a change in external osmosity, one might expect that cells that lack a vacuole would be unable to survive in the presence of even a small increase in ionic or osmotic pressure. We assessed the growth of representative vpt alleles on YPD medium supplemented with 1.0 or 1.5 M NaCl, 1.0 M KCl, or 2.5 M glycerol. None of the class C mutants were able to survive, although the parental strains grew, under these conditions (Fig. 3 B). In addition, vpt 15, 26, and 29 were somewhat sensitive to the presence of 1.0 M NaCl or 2.5 M glycerol and were completely unable to grow on YPD medium containing 1.5 M NaCl. None of the other vpt mutants were sensitive to any of the osmotic stress conditions tested.

The class C mutants also exhibited other physiological
Figure 7. A gallery of electron micrographs of vpt28 and vpr7, which accumulate Golgi-like structures (arrows). Cells were prepared as described in the legend to Fig. 5. A shows vpt7 cells; B shows a vpt28 cell. C and D are high magnification views of two Golgi-like structures representative of those seen in these mutants. Bars: (A and B) 1 μm; (C and D) 0.2 μm.

defects, including poor growth on nonfermentable carbon sources such as glycerol or lactate, poor growth in minimal media containing proline as the sole nitrogen source, poor sporulation of homozygous diploids, and low frequencies of DNA transformation. Furthermore, the class C vpt mutants contained smaller pools of basic amino acids than the class A or B vpt mutants, as judged by a filter assay for basic amino acids (reference 8; data not shown). Other cellular functions in the class C mutants, however, appeared to be normal. Based on the ultrastructural analysis, these mutants exhibited wild-type morphology of organelles such as the nuclei and mitochondria (see Fig. 8 B). The microtubules in these mutants appeared normal, as judged by immunofluorescence. Protein secretion also appeared to be unaffected in the class C as well as the other vpt mutants (46).

All vpt Phenotypes Cosegregate in Genetic Crosses

If the pleiotropic phenotypes discussed above all result from the vpt mutation in question, each of the phenotypes should cosegregate in genetic crosses of the mutants with wild-type cells. To confirm this, representative vpt mutants were back-crossed to the parental strain of the opposite mating type, the diploids were sporulated and tetrads were dissected. In this way, the vacuole morphology associated with each of the class B and class C vpt mutants was shown to cosegregate with the vpt defect (data not shown). The segregation of each of the other phenotypes associated with the class C mutants was also examined. In crosses between ts class C mutants and parental strains, all phenotypes showed the expected 2:2 segregation pattern. In each cross, the temperature sensitivity, osmotic sensitivity, and block in ade2 red pigment formation all cosegregated with the vacuole protein sorting defect. The results of a typical tetrad analysis for a ts class C mutant, vpt16, are shown in Fig. 10. Similar tetrad analyses also demonstrated that the low pH sensitivity exhibited by vpt13 cosegregated with the vpt sorting defect (data not shown).

Discussion

We have analyzed in detail the morphology and growth properties of a number of mutants defective in vacuole protein targeting. Three distinct vacuolar morphologies associated with the vpt mutants have been observed. The class A mutants, constituting 26 complementation groups, resembled the wild-type parent strains in that they had one or a few large vacuoles which were easily observed using light and fluorescence microscopy (Fig. 1, A and B). A second class of mutants,
class B, consisted of three complementation groups and was characterized by an altered morphology in which the vacuole was highly fragmented (Fig. 1 C). Mutants in the four class C complementation groups had no discernible vacuoles (Fig. 1 D), but accumulated small vesicles and other novel membrane-enclosed structures throughout the cytoplasm (Fig. 8).

The majority of the class A vpt mutants showed no apparent abnormalities in the vacuole itself or in other cellular features, as determined by electron microscopic analysis. In these mutants, at least some proteins presumably must continue to be properly targeted to the vacuole. Consistent with this idea, many of the class A vpt mutants mislocalize only a small fraction of CPY, PrA, PrB, or a CPY-Inv hybrid protein that contains vacuolar sorting information (46). It is possible that these mutants define functions which are only peripherally involved in vacuole protein targeting. However, other class A vpt mutants (vpt 1, 4, 6, 7, 15, 17, 29, and 30) exhibit gross defects in the localization and processing of

Figure 8. Electron micrographs of class C vpt mutants, which exhibit extreme defects in vacuole biogenesis. Cells were prepared as described in the legend to Fig. 5. (A, B, and C) Typical class C cells lack a discernible vacuole but accumulate aberrant organelles, including vesicles (small arrow), Bbs (large arrow), and large membranous structures (asterisk). A and B show vpt16 cells; C shows vpt11 cells. D shows a high magnification view of the vesicles that accumulate in class C cells (compare with vesicles in sec1 mutant, Fig. 6 F). E shows a high magnification view of the complex multilamellar arrays that accumulate in the cytoplasm in the class C vpt mutants. N, nucleus; L, lipid droplet; M, mitochondrion. Bars: (A, B, and C) 0.5 μm; (D and E) 0.1 μm.
vacuolar proteins, secreting as much as 70–100% of the CPY (46). These class A mutants still contain intact vacuoles and secrete <5% of the activity of a vacuolar membrane marker enzyme, a-mannosidase, suggesting that different pathways may exist for the sorting of soluble and membrane vacuolar proteins (see below).

Mutants in one of the class A complementation groups, vpt 13, exhibited extreme sensitivity to low pH (Fig. 3 A), suggesting that these cells may be unable to regulate their intracellular pH. The vpt 13 mutants were also defective in the pH-dependent accumulation of quinacrine in the vacuole (Fig. 2 E). In mammalian cells, endosomal acidity has been implicated in the proper localization of proteins to this organelle. Compounds such as amines that raise intralysosomal and endosomal pH (40) cause lysosomal enzymes to be secreted. The increase in pH appears to inhibit the uncoupling of lysosomal enzymes from their receptor carrier(s), resulting in a saturation of the available receptor sites (13). Furthermore, mammalian cell mutants have been described which appear to be defective in acidification of the endosome; these mutants secrete increased amounts of lysosomal hydrolases (45). In yeast, vacuolar pH may also play an important role in vacuole protein targeting. Treatment of wild-type yeast cells with ammonium acetate, the proton ionophore CCCP, or bafilomycin A1, a drug which specifically inhibits the vacuolar ATPase, resulted in a block in the pH-dependent accumulation of quinacrine in the vacuole as well as the missorting of the vacuolar ATPase, resulting in a block in the pH-dependent accumulation of quinacrine in the vacuole as well as the missorting of the vacuolar ATPase, resulting in a block in the pH-dependent accumulation of quinacrine in the vacuole as well as the missorting of the vacuolar ATPase, resulting in a block in the pH-dependent accumulation of quinacrine in the vacuole as well as the missorting of the vacuolar ATPase, resulting in a block in the pH-dependent accumulation of quinacrine in the vacuole as well as the missorting of the vacuolar ATPase, resulting in a block in the pH-dependent accumulation of quinacrine in the vacuole as well as the missorting of the vacuolar ATPase, resulting in a block in the pH-dependent 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partment of the secretory pathway (36). The accumulation of Golgi-like structures or Bbs observed in these and certain other vpr mutants is consistent with a backup of vacuole proteins at the Golgi, the site of segregation for these proteins (54). In addition, the cytoplasm in these cells was filled with vesicles and complex lamellar arrays that might represent remnant vacuolar material or intermediates in vacuole biogenesis (Fig. 8). Several scenarios could account for the accumulation of organelles in these mutants. The gene products defined by the class C vpr mutants might correspond to essential components of a vacuolar protein sorting apparatus or to structural proteins of the vacuole itself. Alternatively, the class C VPT gene products might be involved in the regulation of organelle biogenesis, a process about which very little is known. Like its mammalian counterpart, the yeast vacuole may play an important role in the intracellular turnover of macromolecules. Perhaps in the class C mutants, organelles and membrane fragments accumulate because the cells lack a vacuole to perform this digestive function. The vesicles observed in these vpr mutants might represent intermediates in endocytic traffic that, in the absence of a vacuole, have no suitable target destination. On the other hand, the structures which accumulate in the class C mutants might represent actual intermediates in vacuole biogenesis. However, because these organelles are present at both permissive and nonpermissive growth temperatures, we are at present unable to test whether they correspond to actual reversible intermediates in the vacuole assembly pathway or are dead-end, nonreversible compartments. Additional experiments, such as immunoelectron microscopy or purification of the vesicles, will be required to address the nature of the content of these structures and their likely origin.

In plant cells, the large central vacuole plays an important role in regulating cell turgor pressure (3, 18, 61). Although not as extensively studied, it is possible that the vacuole may have a similar osmoregulatory function in yeast. High concentrations of solutes such as polyphosphates are stored in the yeast vacuole as osmotically inactive aggregates or polymers that could be converted into osmotically active forms by enzymatic digestion of the polymers. Large pools of sugars and basic amino acids (especially arginine) may also be present (29). Mutants in the four class C complementation groups were sensitive to osmotic stress (Fig. 3 B). Perhaps these cells are unable to accumulate the compounds normally used to generate high internal osmotic pressures. Alternatively, the osmotic-sensitive phenotype may not be directly related to vacuolar function. A number of seemingly diverse mutants, including nonsense suppressors, plasma membrane ATPase mutants, and actin mutants are sensitive to osmotic stress (31, 35, 51). Singh and Sherman (52) have suggested that, like ts mutations, alterations in a variety of essential proteins may make cells unusually sensitive to stressful growth conditions, in this case hypertonicity. Two class A vpr mutants (vpr 15 and 29) were partially inhibited for growth in the presence of hypertonic stress. The vacuoles in these strains were abnormally large and occasionally appeared to contain inclusions. Like class C vpr mutants, cells in these groups accumulated vesicles and Bbs (Fig. 6), and many of these strains are ts for growth. Furthermore, these mutants are severely defective in the processing and sorting of CPY and PrA (46). On the basis of the class C-like phenotypes exhibited by vpr 15 and 29, we propose that these mutants may represent an intermediate between the class A and C morphologies.

The class C vpr mutants are among the most defective in targeting CPY, PrA, and PrB to the vacuole (46). Unlike other vpr mutants, however, the class C mutants secrete 30-50% of the vacuolar membrane marker enzyme, α-mannosidase (46), indicating that the sorting defect in these mutants extends to membrane, as well as luminal, proteins. Taken together, the data suggest that different recognition systems may participate in the sorting of soluble vacuolar proteins, such as CPY and PrA, and vacuole membrane proteins (e.g., α-mannosidase). However, both sets of proteins may transit via common carrier vesicles or other intermediate compartments en route to the vacuole. According to such a model, the class C vpr mutants might affect protein sorting at the level of the common compartment. In contrast, the defects observed in many of the class A mutants, which mislocalize only a subset of the vacuole proteins, are likely to affect more specific components in the pathway, such as protein receptors.

A set of vacuolar protein localization (vpl) mutants similar to those described here has been isolated by selecting for the presence of active CPY in the periplasm (47). Some of these mutants also exhibit aberrant organelles such as Bbs and multivesicular bodies in addition to a normal vacuole. Complementation analysis has revealed that several of the vpl mutations fail to complement various vpr mutations. However, none of the vpl mutants exhibits the extreme defects in vacuole morphology seen in the class C vpr mutants (46, 47; Rothman, J., and T. Stevens, personal communication).

Two mutants which are defective in the accumulation of an endocytic marker, lucifer yellow carboxyhydrazide, and in pheromone response have been described by Chvatchko et al. (7). One of these mutants, end 1, has a morphology similar to that of the class C vpr mutants in that it lacks a vacuole and accumulates many small vesicles in the cytoplasm. This mutant is also defective in CPY processing (44) and sorting (46). Like the class C vpr mutants, end 1 grows poorly on glycerol (Dulic, V., and H. Riezman, personal communication) and is unable to grow under conditions of osmotic stress or high temperature. Crosses between end 1 and vpr 11 mutants have demonstrated that these two mutations define a single complementation group (46; Dulic, V., and H. Riezman, personal communication). This finding suggests that the vacuolar protein sorting and endocytic pathways may converge and that some gene functions may be common to both pathways. Geuze et al. have suggested that lysosomal enzymes are directed to the lysosome via a prelysosomal compartment which is also the site of uncoupling of endocytosed ligands and receptors (12). More recently, Griffiths et al. have identified a compartment in rat kidney cells which, by immunolocalization studies, appears to be shared by the lysosomal targeting and endocytic pathways (14). The extreme defects in vacuole biogenesis, aberrations in vacuolar and cellular morphology, and increased sensitivity to suboptimal growth conditions observed in certain of the vpr mutants suggest strongly that vacuole structure and/or function are impaired in these strains. However, functions such as secretion and microtubule assembly appear to be normal in all of the vpr mutants (46). Although the class C vpr mutants, as well as vpr 15 and 29, accumulate organelles resembling those seen in certain sec mutants, complementa-
tion analysis has indicated that these vpt mutants and the sec mutants are not allelic (46). Other yeast mutations have been described which result in vpt-like morphological defects. A ts mutation in the single yeast actin gene (act1) leads to an accumulation of Bbs and vesicles similar to that seen in vpt15 and 29 (35); however, complementation analysis indicates that none of the ts vpt mutants is allelic to act1 (unpublished results). Likewise, although a deletion of the clathrin heavy chain gene in yeast causes severe morphological and growth defects, these cells continue to sort vacuolar proteins properly (39). A recently isolated mutant, spl1, exhibits a vpt-like morphology in that it lacks a central vacuole but contains many small vesicles throughout the cytoplasm (24a). We have not yet been able to test the allelism of this mutation with any of the vpt mutations.

The class B and C vpt mutants, which exhibit altered vacuolar morphologies, may define functions required for specific stages of vacuole biogenesis. These mutants may be especially useful for in vitro studies directed at reconstituting different steps in vacuole assembly. Molecular cloning of the VPT genes and characterization of the encoded gene products, coupled with the development of an in vitro system in which these gene products can be assayed, should help elucidate the roles these proteins play in vacuolar function, protein targeting, and organelle biogenesis.

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