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Morphological Classification of the Yeast Vacuolar Protein Sorting Mutants: Evidence for a Prevacuolar Compartment in Class E *vps* Mutants

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The collection of vacuolar protein sorting mutants (*vps* mutants) in *Saccharomyces cerevisiae* comprises of 41 complementation groups. The vacuoles in these mutant strains were examined using immunofluorescence microscopy. Most of the *vps* mutants were found to possess vacuolar morphologies that differed significantly from wild-type vacuoles. Furthermore, mutants representing independent *vps* complementation groups were found to share aberrant morphological features. Six distinct classes of vacuolar morphology were observed. Mutants from eight *vps* complementation groups were defective both for vacuolar segregation from mother cells into developing buds and for acidification of the vacuole. Another group of mutants, represented by 13 complementation groups, accumulated a novel organelle distinct from the vacuole that contained a late-Golgi protein, active vacuolar H^+-ATPase complex, and soluble vacuolar hydrolases. We suggest that this organelle may represent an exaggerated endosome-like compartment. None of the *vps* mutants appeared to mislocalize significant amounts of the vacuolar membrane protein alkaline phosphatase. Quantitative immunoprecipitations of the soluble vacuolar hydrolase carboxypeptidase Y (CPY) were performed to determine the extent of the sorting defect in each *vps* mutant. A good correlation between morphological phenotype and the extent of the CPY sorting defect was observed.

INTRODUCTION

Organelle biogenesis in eukaryotic cells requires the selective assembly of specific proteins, lipids, and other constituents from a common pool of biosynthetic intermediates. The biogenesis of the yeast vacuole was chosen as a model system for organelle assembly because most vacuolar functions are dispensible for the growth of vegetative cells, and hence it was possible to generate a large collection of mutants with defects in vacuole assembly (reviewed in Klionsky *et al.*, 1990; Raymond *et al.*, 1992a). Most soluble and integral membrane proteins of the yeast vacuole studied to date enter the endoplasmic reticulum and traverse the secretory pathway through the Golgi complex (Stevens *et al.*, 1982; Klionsky *et al.*, 1988; Klionsky and Emr, 1989; Moehle *et al.*, 1989; Roberts *et al.*, 1989; Hirsh *et al.*, 1991). Vacuolar proteins appear to be sorted from cell surface and resident Golgi proteins in a late Golgi compartment. This was elegantly documented in the case of the soluble vacuolar glycoprotein carboxypeptidase Y (CPY), which was shown to be sorted to the vacuole after passage through the most distal Golgi compartment characterized in *Saccharomyces cerevisiae* (Graham and Emr, 1991).

The precursor form of CPY carries positive sorting information that directs the protein to the vacuole (Johnson *et al.*, 1987; Valls *et al.*, 1987, 1990). In the absence of this sorting signal, the protein is secreted (Stevens *et al.*, 1986). To identify genes whose products participate in the sorting process, positive selections for mutants that secrete CPY were devised (Bankaitis *et al.*, 1986; Rothman and Stevens, 1986). Complementation testing among a large collection of such vacuolar protein sorting (*vps*) mutants has indicated that as many as 40

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VPS genes influence CPY sorting (Robinson *et al.*, 1988; Rothman *et al.*, 1989a). Additional analyses have shown that all of the *vps* mutants exhibit defects in the sorting of several other soluble vacuolar proteins (Robinson *et al.*, 1988; Rothman and Stevens, 1986). The large number of *vps* complementation groups demonstrates that vacuole assembly is a complex process.

Phenotypic characterization of the *vps* mutants suggests that a wide range of vacuolar biogenesis defects may influence the fidelity of soluble protein sorting. Certain mutants were found to have gross alterations in vacuolar morphology or to lack an acidic vacuolar lumen (Rothman and Stevens, 1986; Banta *et al.*, 1988; Rothman *et al.*, 1989a,b). Initial characterization of vacuolar morphology in the *vps* mutant collection was conducted at the level of the light microscope using the vacuolar lumenal marker fluorescein isothiocyanate (FITC) and Nomarski optics, as well as by thin-section electron microscopy (Banta *et al.*, 1988). These techniques were sufficient to divide the *vps* mutant collection into three classes based on vacuolar morphology (Banta *et al.*, 1988). Most of the *vps* mutants, class A, contained vacuoles that appeared similar to those in wild-type cells. Certain mutants, class B, contained numerous small vacuole-like compartments. Four *vps* mutants, class C, exhibited the most extreme defect in vacuolar morphology and appeared to lack vacuoles altogether.

Although initial studies indicated that the vast majority of *vps* strains possessed relatively normal vacuoles (Banta *et al.*, 1988; Rothman *et al.*, 1989a), recent advances in fluorescence microscopy and the availability of antibodies that recognize vacuolar membrane proteins have provided powerful new tools for phenotypic analysis (Pringle *et al.*, 1989; Roberts *et al.*, 1991). These techniques provided a clear demonstration that vacuole inheritance in wild-type cells occurs via vacuolar segregation structures; the term "vacuole inheritance" refers to the process by which daughter cells receive vacuolar material from mother cells (Zubenko *et al.*, 1982; Weisman *et al.*, 1987), and the term vacuolar "segregation structure" refers to the morphologically distinct projection of mother cell vacuoles into developing buds during a specific period in bud enlargement (Weisman and Wickner, 1988; Raymond *et al.*, 1990; Weisman *et al.*, 1990; Gomes de Mesquita *et al.*, 1991; Shaw and Wickner, 1991). Microscopic analysis demonstrated that *vps*3 and *vps*34 mutants were defective for vacuolar segregation (Herman and Emr, 1990; Raymond *et al.*, 1990). The fact that both mutants were originally thought to have wild-type vacuoles prompted us to reexamine the *vps* mutant collection using indirect immunofluorescence microscopy. We report that the vacuoles in most of the *vps* mutants differ significantly from the wild-type organelle and that the entire collection of *vps* mutants can be subdivided into specific groups based on the morphological traits of their vacuoles.

MATERIALS AND METHODS

Strains, Media, Materials, and Antibodies

The yeast strains used in this study are listed in Table 1. New vacuolar protein localization (*vpl*) complementation groups were identified by selection for mutants (Vps^-) that secrete the vacuolar protein CPY followed by complementation testing as described previously (Rothman and Stevens, 1986; Rothman *et al.*, 1989a), except that CPY secretion was monitored using a plate overlay immunoblotting technique (Rothman *et al.*, 1986; Roberts *et al.*, 1991). The *vps*27-Δ1::LEU2 mutation was constructed by substituting the *LEU2* gene for a portion of the *VPS27* open reading frame and by transplacement of this construct into the indicated yeast strains. DNA was transformed into yeast strains by electroporation using a Bio-Rad (Richmond, CA) gene pulser as recommended by the manufacturer. Rich growth medium was yeast peptone dextrose (YPD), minimal medium was synthetic dextrose (SD) supplemented with appropriate nutrients, and sporulation medium was a standard recipe, all as described in Sherman *et al.* (1982). The *Escherichia coli* strain used in this study was MC1061 (Casadaban and Cohen, 1980).

Antibodies that recognize alkaline phosphatase (ALP), the product of the *PHO8* gene, were generated by subcloning the ~1000 base pair (bp)*Xba* I – *Sal* I fragment of *PHO8* (Kaneko *et al.*, 1987) into the *E. coli* protein expression vector pEXP2 (Raymond *et al.*, 1990). The 38-kDa ALP protein fragment produced from this plasmid, pGP100, was purified from *E. coli* and injected into rabbits as described in Roberts *et al.* (1989). A second overlapping ~875 bp *Bgl* II – *Sal* I fragment from the *PHO8* gene was also subcloned into pEXP2 to give pGP102, and the resulting 35-kDa protein fragment was used to construct an affinity column by linking it to cyanogen bromide-activated Sepharose (Sigma Chemical, St Louis, MO). ALP-specific antibodies were affinity purified from rabbit serum as described by Raymond *et al.* (1990), and microscopy-grade adsorbed antibodies that recognize ALP were prepared as described by Roberts *et al.* (1991). Monoclonal antibodies that recognize the 60-kDa subunit of the vacuolar H^+-adenosine triphosphatase (ATPase) (V-ATPase) were prepared by Kane *et al.* (1989, 1992). For immunofluorescence, rabbit antibodies directed against yeast CPY were affinity-purified using a CPY-Sepharose column, as described above. Polyclonal rabbit anti-CPY serum used for immunoprecipitations was prepared by subcloning the 1150-bp *Bam*HI fragment from the CPY structural gene, *PRC1* (Stevens *et al.*, 1986), into the *E. coli* protein expression vector pEXP2 (Raymond *et al.*, 1990). The 42-kDa CPY fragment was purified from *E. coli* extracts and injected into rabbits as described by Roberts *et al.* (1989). Secondary antibodies for indirect immunofluorescence were purchased from Jackson ImmunoResearch (West Grove, PA) and prepared according to the recommendations of the manufacturer. Other reagents were from Sigma Chemical, except as specified below.

Fluorescence Microscopy

Vacuolar morphology was examined in fixed yeast strains by indirect immunofluorescence techniques essentially as described by Roberts *et al.* (1991). Ten-milliliter log phase cultures at a density of 2.5–5 × 10^6 cells/ml in YPD at 30°C were fixed by the addition of 1.2 ml 37% formaldehyde (J. T. Baker Chemical, Phillipsburg, NJ) with gentle shaking. After 30 min, the cells were transferred to 2 ml of standard fixative (Roberts *et al.*, 1991) and shaken overnight (12–16 h) at room temperature. The cells were then harvested in a clinical centrifuge, resuspended in 1 ml of 200 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.0, 20 mM EDTA, and 1% β-mercaptoethanol, transferred to a microfuge tube, and incubated for 10 min. Pelleted cells were resuspended in spheroplast buffer containing 1.2 M sorbitol, 50 mM KPO_4, pH 7.3, 1 mM $MgCl_2$, 15 μg/ml zymolyase 100T (ICN Biomedicals, Irvine, CA), and 25 μl/ml glusulase (DuPont, Boston, MA) and incubated with occasional shaking for 30 min at 30°C. Spheroplasted cells were harvested by centrifugation at ~5000 × *g* for 30 s, washed once in 1 ml of 1.2 M sorbitol, and split into two 500 μl aliquots. One aliquot was treated with 5% sodium dodecyl sulfate (SDS) for 5 min for ALP staining (by adding 500 μl 10% SDS in 1.2 M sorbitol), and

the other was treated with 1% SDS for 30 s for 60-kDa V-ATPase subunit staining. Both treated aliquots were subsequently washed twice with 1 ml of 1.2 M sorbitol and resuspended in 1 ml 1.2 M sorbitol.

Forty-microliter aliquots of fixed treated cells were placed on polylysine-coated multiwell test slides (ICN Biomedicals) that were prepared by placing a 1 mg/ml solution of polylysine bromide (Sigma Chemical) into each well for 30 s followed by six washes with water and air drying. After 10 min, the wells were washed three times with phosphate-buffered saline containing 5 mg/ml bovine serum albumin (PBS-BSA) (Roberts *et al.*, 1991), and the slides were incubated in a humid chamber for 15 min. Antibodies were then added. For ALP staining, the primary antibody was used at a 1:10 dilution, and the secondary antibodies were used at a 1:100 dilution of biotinylated goat anti-rabbit followed by a 1:100 dilution of FITC-conjugated streptavidin. For 60-kDa V-ATPase subunit staining, the primary antibody was used at a 1:20 dilution, and the secondary antibodies were used at a 1:200 dilution of biotinylated goat anti-mouse followed by a 1:200 dilution of FITC-streptavidin. For the ALP/60-kDa double-labeling experiment (Figure 7), 1:10 ALP and 1:10 60-kDa V-ATPase subunit antibodies were added in the first incubation, followed by 1:100 biotinylated goat anti-rabbit and 1:100 rhodamine-conjugated goat anti-mouse in the second incubation and 1:100 FITC-streptavidin in the final incubation. For the CPY/60-kDa subunit double-labeling experiment (Figure 7), 1:1000 CPY and 1:10 60-kDa V-ATPase subunit antibodies were added in the first incubation, followed by 1:100 FITC-goat anti-rabbit and 1:200 biotinylated goat anti-mouse in the second incubation, and 1:200 rhodamine-streptavidin in the final incubation. The concentration of primary antibody solutions was not determined, and the dilutions used for optimal staining were determined empirically. Secondary antibodies were supplied or prepared as 1-mg/ml solutions. All antibody incubations were for 1 h, and several PBS-BSA washes were conducted between antibodies. After antibody labeling was complete and the wells were washed several times, mounting medium (Pringle *et al.*, 1989) was added to each well, and the slides were covered with a coverslip. The cells were viewed and photographed as described by Raymond *et al.* (1990).

Quinacrine staining of live cells was conducted as described previously (Roberts *et al.*, 1991).

CPY Immunoprecipitation

Yeast cultures were grown overnight in SD-Met to an optical density at 600 nm of ~1 (OD_{600} = 1). Cells were centrifuged and resuspended at OD_{600} = 1 in SD-Met, 50 mM KPO_4, pH 5.7, and 2 mg/mL BSA. The cultures were pulse-labeled for 10 min at 30°C with 200 μCi/1 OD_{600} of cells with [^{35}S]MET[^{35}S]CYS (EXPRE^{35}S^{35}S label, New England Nuclear, Boston, MA). Labeled cultures were chased with 80 μg/ml of Met and Cys at 30°C for 30 min. The pulse-chase was terminated by the addition of NaN_3 to 10 mM. The cells were spheroplasted, separated into intracellular and extracellular fractions, and CPY was immunoprecipitated from each fraction as described by Stevens *et al.* (1986) with the following modifications. Intracellular and extracellular fractions were heated for 5 min at 100°C after the addition of 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.13% SDS, and 0.13% Triton X-100 (final concentrations). Samples were preadsorbed with 50 μl of 10% IgGSORB (The Enzyme Center, Malden, MA) for 15 min on ice. The supernatants resulting after centrifugation were incubated with anti-CPY polyclonal antibody for 1 h on ice. Another aliquot of IgGSorb was added, and after 1 h on ice the precipitated immune complexes were washed twice with 10 mM Tris HCl, pH 8.0, 0.1% SDS, 0.1% Triton X-100, and 2 mM EDTA. Immunoprecipitated samples were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The radioactivity of immunoprecipitated CPY in the intracellular and extracellular fractions was quantified using an AMBIS Radioanalytic Imaging System (Ambis Systems, San Diego, CA).

RESULTS

Analysis of Vacuolar Morphology and Protein Sorting in the vps Mutant Collection

The *vps* mutants, all of which were selected for secretion of CPY or a CPY fusion protein, were generated in two different laboratories and originally designated as vacuolar protein localization (*vpl*) mutants (Rothman and Stevens, 1986; Rothman *et al.*, 1989a) or vacuolar protein targeting (*vpt*) mutants (Bankaitis *et al.*, 1986; Robinson *et al.*, 1988). The common *vps* nomenclature was introduced because many of the *vpl* and *vpt* mutants defined the same complementation groups, i.e., they carried mutations in many of the same genes (Robinson *et al.*, 1988; Rothman *et al.*, 1989a). There have been several revisions to the *vps* mutant list since the last available publication (Klionsky *et al.*, 1990), and the current compilation of *vps* complementation groups is shown in Table 2.

Vacuoles were visualized using indirect immunofluorescence techniques (Pringle *et al.*, 1989; Roberts *et al.*, 1991). Two antibody preparations that recognize either ALP or the 60-kDa subunit of the vacuolar H^+-ATPase (V-ATPase) were used. ALP is an integral membrane protein of the vacuole (Kaneko *et al.*, 1987; Klionsky and Emr, 1989), and antibodies directed against ALP clearly delineate the vacuolar membrane (Raymond *et al.*, 1990; Roberts *et al.*, 1991). The 60-kDa V-ATPase subunit is a peripheral membrane protein that associates as part of a multi-subunit complex with the cytoplasmic face of the vacuole (Kane *et al.*, 1989, 1992; Yamashiro *et al.*, 1990). Antibodies directed against the 60-kDa V-ATPase subunit stain the vacuolar membrane in wild-type cells, and they yield a diffuse cytoplasmic staining pattern in mutants that fail to assemble an active V-ATPase complex (Kane *et al.*, 1992). Vital staining with quinacrine was also used as a qualitative assay of vacuolar acidification in live cells. Quinacrine is a basic fluorescent compound that accumulates in acidified intracellular compartments such as the yeast vacuole; loss of vacuolar acidification due to dissipation of the pH gradient with 200 mM ammonium acetate, inhibition of the V-ATPase with the drug bafilomycin A_1, or mutations that perturb V-ATPase function largely abolish quinacrine staining (Weisman *et al.*, 1987; Banta *et al.*, 1988; Rothman *et al.*, 1989b; Yamashiro *et al.*, 1990). Vacuolar protein sorting was monitored by pulse-chase labeling of cells, immunoprecipitation of CPY from intracellular and extracellular fractions, and quantitation of CPY in the immunoprecipitates.

At least two mutant strains from each complementation group were examined by the techniques described above. Additional alleles were examined for groups where the initial two mutants gave inconsistent results. The pairs of *MATα* and *MAT*a *vps* mutant alleles reported in this study are shown in Table 1. Within the 41 *vps* mutant groups studied, six different classes of mutant vacuolar morphology were observed that could be readily differentiated from one another. To be consistent with previous publications, the nomenclature of Banta *et al.* (1988) was used and extended to include additional classes, as described in detail in later sections.

Table 1. Yeast strains used in this study

Strains	Genotype	Source
SF838-9D	*MATα, ade6, his4, leu2, ura3, pep4*	Rothman *et al.*, 1989a
SF838-9DR2L1	*MATa, his4, leu2, lys2, ura3, pep4*	Rothman
JHRY20-2C	*MTAa, his3, leu2, ura3*	Rothman *et al.*, 1986
SEY6210	*MATα, his3, leu2, lys2, suc2, trp1, ura3*	Robinson *et al.*, 1988
SEY6211	*MATa, ade2, his3, leu2, suc2, trp1, ura3*	Robinson *et al.*, 1988
SF838-9D *vps1-Δ2*	same as SF838-9D except *vps1-Δ2::LEU2*	Rothman *et al.*, 1990
JHRY20-2C *vps1-Δ2*	same as JHRY20-2C except *vps1-Δ2::LEU2*	Rothman *et al.*, 1990
vpl2-7, vpl2-2[a]	same as SF838-1D, same as SF838-9DR2L1	Rothman and Stevens, 1986
vpl3-1, vpl3-2	same as SF838-1D, same as SF838-9DR2L1	Rothman and Stevens, 1986
vpl4-1, vpl4-7	same as SF838-1D, same as SF838-9DR2L1	Rothman and Stevens, 1986
vpl5-21, vpl5-8	same as SF838-1D, same as SF838-9DR2L1	Rothman and Stevens, 1986
vpl6-27, vpl6-18	same as SF838-1D, same as SF838-9DR2L1	Rothman and Stevens, 1986
vpl7-1, vpl7-2	same as SF838-1D, same as SF838-9DR2L1	Rothman and Stevens, 1986
vpl8-24, vpl8-19	same as SF838-1D, same as SF838-9DR2L1	Rothman and Stevens, 1986
vpl9-13, vpl9-2	same as SF383-9D, same as SF383-9DR2L1	Rothman *et al.*, 1989a
vpl10-7, vpl10-27	same as SF383-9D, same as SF383-9DR2L1	Rothman *et al.*, 1989a
vpl11-58, vpl11-2	same as SF383-9D, same as SF383-9DR2L1	Rothman *et al.*, 1989a
vpl12-1, vpl12-3	same as SF383-9D, same as SF383-9DR2L1	Rothman *et al.*, 1989a
vpl13-2, vpl13-11	same as SF383-9D, same as SF383-9DR2L1	Rothman *et al.*, 1989a
vpl14-6, vpl14-12	same as SF383-9D, same as SF383-9DR2L1	Rothman *et al.*, 1989a
vpl15-9, vpl15-18	same as SF383-9D, same as SF383-9DR2L1	Rothman *et al.*, 1989a
vpl16-2, vpl16-9	same as SF383-9D, same as SF383-9DR2L1	Rothman *et al.*, 1989a
vpl17-7, vpl17-2	same as SF383-9D, same as SF383-9DR2L1	Rothman *et al.*, 1989a
vpl18-9, vpl18-3[b]	same as SF383-9D, same as SF383-9DR2L1	Rothman *et al.*, 1989a
vpl19-10, vpl19-3	same as SF383-9D, same as SF383-9DR2L1	Rothman *et al.*, 1989a
vpl20-6, vpl20-1	same as SF383-9D, same as SF383-9DR2L1	This study
vpl21-13, vpl21-1	same as SF383-9D, same as SF383-9DR2L1	This study
vpl22-7, vpl22-1[b]	same as SF383-9D, same as SF383-9DR2L1	This study
vpl23-5, vpl23-2	same as SF383-9D, same as SF383-9DR2L1	This study
vpl24-4, vpl24-1	same as SF383-9D, same as SF383-9DR2L1	This study
vpl25-6, vpl25-1	same as SF383-9D, same as SF383-9DR2L1	This study
vpl26-2, vpl26-1	same as SF383-9D, same as SF383-9DR2L1	This study
vpl27-3, vpl27-1	same as SF383-9D, same as SF383-9DR2L1	This study
vpl28-4, vpl28-1	same as SF383-9D, same as SF383-9DR2L1	This study
vpl30-2, vps30-1	same as SF383-9D, same as SF383-9DR2L1	This study
vpl31-2, vpl31-1	same as SF383-9D, same as SF383-9DR2L1	This study
CKRY2-8A[c]	*MTAa, ade6, (his3 and/or his4), leu2, ura3, pep4, pho8-Δ1::LEU2, vps27-Δ1::LEU2*	This study
vpt1, vpt1[d]	same as SEY6210, same as SEY6211	Robinson *et al.*, 1988
vpt2, vpt2	same as SEY6210, same as SEY6211	Robinson *et al.*, 1988
vpt3, vpt3	same as SEY6210, same as SEY6211	Robinson *et al.*, 1988
vpt4, vpt4	same as SEY6210, same as SEY6211	Robinson *et al.*, 1988
vpt5, vpt5	same as SEY6210, same as SEY6211	Robinson *et al.*, 1988
vpt6, vpt6	same as SEY6210, same as SEY6211	Robinson *et al.*, 1988
vpt7, vpt7	same as SEY6210, same as SEY6211	Robinson *et al.*, 1988
vpt16	same as SEY6210, same as SEY6211	Robinson *et al.*, 1988
vpt18	same as SEY6210, same as SEY6211	Robinson *et al.*, 1988
vpt21, vpt21	same as SEY6210, same as SEY6211	Robinson *et al.*, 1988
vpt30, vpt30	same as SEY6210, same as SEY6211	Robinson *et al.*, 1988
vpt31, vpt31	same as SEY6210, same as SEY6211	Robinson *et al.*, 1988

[a] Pairs of *vpl* mutants used in this study are listed by allele number, with the *MATα* mutant followed by the *MAT***a** mutant.

[b] *vpl18* and *vpl22* mutants were found to define a single complementation group that was designated *vpl18;* all four alleles were examined in this study.

[c] CRY2-8A is the outcrossed haploid progeny of a diploid made by crossing JHRY20-2C *pho8-Δ1* (Raymond et al., 1990) X SF838-9D *vps27-Δ1*. The diploid was sporulated and haploid progeny that were both *pho8Δ* and *vps27Δ* were used for further study. Because the parents of the diploid strain were both His^-, but at two different *HIS* loci (*his3* or *his4*), it is known that CRY2-8A is His^-, but the genotypes of the *HIS* loci have not been established.

[d] Pairs of *vpt* mutants used in this study are listed by allele number, with the *MATα* mutant followed by the *MAT***a** mutant. Although all of *vpt* mutants were examined, only those *vpt* strains that do not overlap genetically with *vpl* mutants are shown.

Table 2. The *vpt* and *vpl* mutants define 41 *vps* complementation groups[a]

vps mutant	*vpt* mutant	*vpl mutant*
vps1	*vpt26*	*vpl1*
vps2	*vpt14*	*vpl2*
vps3	*vpt17*	*vpl3*
vps4	*vpt10*	*vpl4*
vps5	*vpt5*	—
vps6	*vpt13*	*vpl6*
vps8	*vpt8*	*vpl8*
vps9	*vpt9*	*vpl31*
vps10	*vpt1*	—
vps11	*vpt11*	*vpl9*
vps13	*vpt2*	—
vps15	*vpt15*	*vpl19*
vps16	*vpt16*	—
vps17	*vpt3*	—
vps18	*vpt18*	—
vps19	*vpt19*	*vpl21*
vps20	*vpt20*	*vpl10*
vps21	*vpt21*	—
vps22	*vpt22*	*vpl14*
vps23	*vpt23*	*vpl15*
vps24	*vpt24*	*vpl26*
vps25	*vpt25*	*vpl12*
vps26	*vpt4*	—
vps27	*vpt27*	*vpl23*
vps28	*vpt28*	*vpl13*
vps29	*vpt6*	—
vps30	*vpt30*	—
vps31	*vpt31*	—
vps32	*vpt32*	*vpl5*
vps33	*vpt33*	*vpl25*
vps34	*vpt29*	*vpl7*
vps35	*vpt7*	—
vps36	—	*vpl11*
vps37	—	*vpl16*
vps38	—	*vpl17*
vps39	—	*vpl18*
vps41	—	*vpl20*
vps43	—	*vpl24*
vps44	—	*vpl27*
vps45	—	*vpl28*
vps46	—	*vpl30*

[a] There have been several revisions to the list of *vps* mutants since the last compilation was published (Klionsky et al., 1990). They are as follows. 1) Ten new *vpl* complementation groups were identified that have not been previously published (*vpl20* through *vpl31*, except *vpl22* and *vpl29*, which were eliminated). Five of these *vpl* groups overlap with established *vps* groups and five define five new *vps* complementation groups. 2) Four previously undetected overlaps between *vpl* and *vpt* mutants were discovered. Based on comparison of restriction maps of cloned genes, *VPS7* is the same as *VPS34* and *VPS40* is the same *VPS15* (Herman and Emr, 1990; Herman *et al.*, 1991; Raymond and Stevens, unpublished data). In addition, *vps2* mutants failed to complement *vps14* mutants and *vps12* mutants failed to complement *vps21* strains. When diploids from these crosses were sporulated and haploid progeny analyzed (from at least 15 four spore tetrads), the Vps$^-$ phenotype segregated 4:0. This demonstrates that both sets of overlaps are the consequence of allelic mutations in the same gene. 3) Diploids formed between several alleles of *vpl5* and *vpt5* complemented one another, indicating that the two groups define separate genes. However, the *vpl5* mutants failed to complement *vpt32* mutants, and thus these two sets of mutants form one complementation group. To incorporate these changes *vps5* = *vpt5* and *vps32* = *vpl5* = *vpt32*.

Class A vps Mutants Have Vacuoles Similar to Those of Wild-type Cells

The morphology of vacuoles in wild-type yeast strains and the changes the organelle undergoes during the growth of vegetative cells or on entry into stationary phase have been described extensively elsewhere (Weimken *et al.*, 1970; Weisman *et al.*, 1987; Weisman and Wickner, 1988; Pringle *et al.*, 1989; Raymond *et al.*, 1990; Gomes de Mesquita *et al.*, 1991). The wild-type parent strains of the *vps* mutants (Table 1) all possess similar vacuolar morphologies (Figure 1, A–D; Raymond, 1990). The organelle generally appears to have 1–10 lobes that cluster together in one region of the cell. During the cell cycle, portions of the mother vacuole appear to actively partition into developing buds (Figure 1,A–D). The characteristic mother to bud vacuolar extensions have been termed vacuolar "segregation structures" (Raymond *et al.*, 1990; Gomes de Mesquita *et al.*, 1991), and the process whereby maternal vacuolar material is passed to daughter cells has been termed "vacuole inheritance" (Weisman *et al.*, 1987, 1990; Weisman and Wickner, 1988; Shaw and Wickner, 1991). In wild-type cells, the ALP staining pattern and the 60-kDa subunit staining pattern are virtually indistinguishable (Figure 1, A–D), indicative of the fact that both ALP and the V-ATPase reside predominantly on the vacuolar membrane.

Mutants from nine *vps* complementation groups were found to have vacuoles similar to wild-type, and in keeping with previous work these were designated as having a "class A" morphological phenotype (Table 3) (Banta *et al.*, 1988). In some mutants (*vps10, vps13, vps44, vps46*), there was no detectable difference between the vacuoles in the mutants and those in the wild-type parents. In the remaining mutant strains (*vps8, vps29, vps30, vps35, vps38*), the morphology of the vacuole was slightly perturbed, as is shown for the *vps35* mutant in Figure 1, E–H. Most of these mutant cells appeared to have one to two vacuoles per cell with a single round vacuole per cell being the predominant morphology. Although vacuolar segregation structures were far less prominent in this set of mutants than in wild-type strains, all large buds possessed distinct vacuoles indicative of normal vacuole inheritance. Furthermore, antibodies directed against the 60-kDa subunit of the V-ATPase decorated the vacuolar membrane, and quinacrine accumulation was normal in these class A *vps* mutants, suggesting that the V-ATPase complex was properly assembled. The amount of newly synthesized CPY secreted from the class A *vps* mutants was somewhat variable (Table 3), particularly among the strains with vacuoles that were most similar to wild-type.

Class B vps Mutants Possess Fragmented Vacuoles

Certain *vps* mutants displayed a fragmented vacuole phenotype. These strains were designated previously

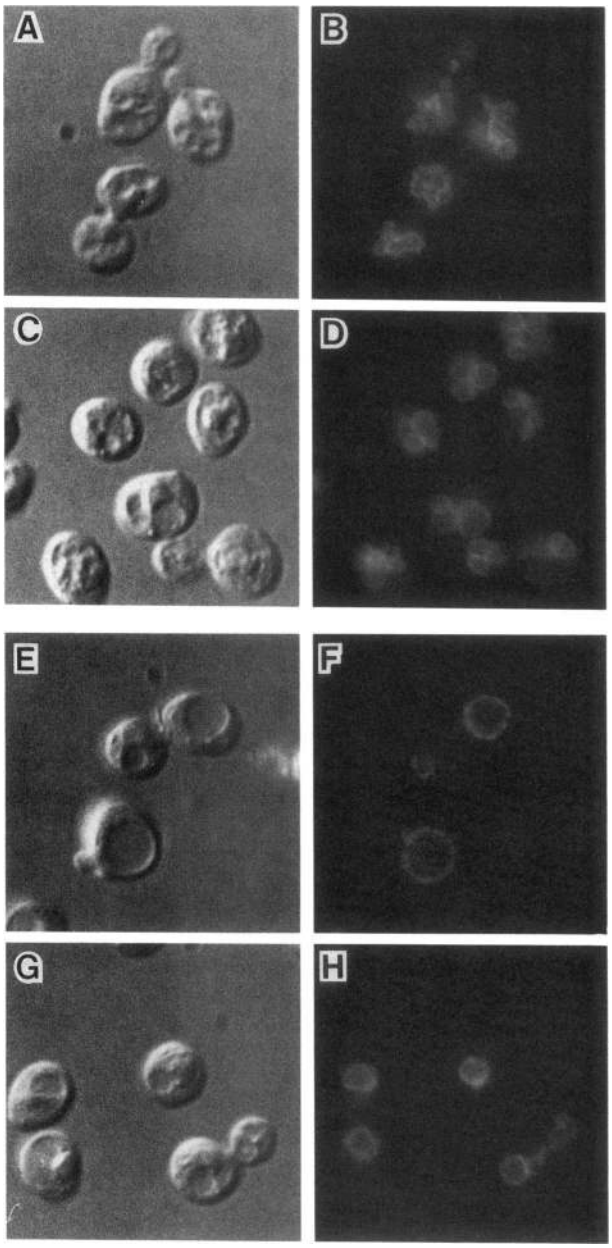

Figure 1. Vacuole morphology in the wild-type strain SF838-9D (A–D) and the class A mutant *vps35* (*vpt7*; E–H). ALP staining is shown in B and F, 60-kDa V-ATPase subunit staining in D and H, and the corresponding Nomarski images of whole cells in A, C, E, and G. One centimeter equals ~5 μm.

as class B *vps* mutants (Banta *et al.*, 1988). Class B mutant cells had a large number of small vacuolar compartments that were visible by Nomarski optics and by staining with either ALP or 60-kDa V-ATPase subunit antibodies (Figure 2). Quinacrine accumulated in multiple small compartments in all of the class B mutants, suggesting that functional V-ATPase was assembled onto the membranes of the fragmented vacuoles. However, in certain class B mutants (*vps41*; Figure 2H), the diffuse appearance of the V-ATPase staining pattern may indicate a partial defect in the assembly of the V-ATPase. Mutants representing five complementation groups were designated class B (Table 3). One mutant complementation group originally designated as class B, *vps1* (Banta *et al.*, 1988), was similar to class B mutants but appeared to have a distinct phenotype as described in a later section.

Within the class B group of mutants, there appeared to be two subgroups. The many small vacuoles in *vps5* and *vps17* mutants retained the wild-type characteristic of clustering within one region of the cell (Figure 2, A–D). Inspection of a large number of labeled cells revealed that vacuole fragments in these mutants were often aligned along the mother cell to bud axis in a configuration reminiscent of wild-type vacuolar segregation structures. In contrast, the fragmented vacuoles in *vps39*, *vps41*, and *vps43* class B mutants appeared to be randomly dispersed throughout the cell (Figure 2, E–H). The class B *vps* mutants secreted a consistently high level of newly synthesized CPY (Table 3).

Class C vps Mutants Lack Coherent Vacuoles

Mutants from four *vps* complementation groups did not possess an organized vacuole (Table 3). These class C *vps* mutants (Banta *et al.*, 1988) have been the subject of several investigations (Chvatchko *et al.*, 1986; Banta *et al.*, 1988, 1990; Dulic and Reizman, 1989; Wada *et al.*, 1990; Woolford *et al.*, 1990; Robinson *et al.*, 1991; Preston *et al.*, 1991). ALP staining of the class C mutants yielded a scattered punctate pattern (Figure 3B), suggesting that this integral membrane protein may be distributed among the multiple vesicle-like structures seen in electron micrographs of these mutants (Chvatchko *et al.*, 1986; Banta *et al.*, 1988; Rothman *et al.*, 1989a; Klionsky *et al.*, 1990; Wada *et al.*, 1990; Woolford *et al.*, 1990). No cell surface staining with the ALP antibody was apparent, indicating that the majority of this vacuolar membrane protein remains sequestered intracellularly. The steady-state level of ALP in class C mutants was comparable with that seen in wild-type cells, as determined by quantitative Western blot analysis. Thus, the reduced fluorescence intensity of the ALP-specific signal in class C mutants relative to other cells was probably a consequence of its highly dispersed intracellular distribution. The staining pattern of the 60-kDa V-ATPase subunit was diffuse (Figure 3D). The subtle differences between certain class B *vps* mutants (Figure 2, E–H; *vps39*, *vps41*, and *vps43*) and the class C mutants (Figure 3) observed by indirect immunofluorescence using ALP and 60-kDa V-ATPase subunit antibodies are more readily apparent using electron microscopic techniques (Banta *et al.*, 1988). As reported previously, all four of the class C *vps* mutants are strongly defective for CPY sorting (Table 3).

Table 3. Morphological classification and CPY secretion among the *vps* mutants[a]

Class A	% CPY	Class B	% CPY	Class C	% CPY	Class D	% CPY	Class E	% CPY	Class F	% CPY
vps8	66, 67	*vps5*	97, 69	*vps11*	84, 80	*vps3*	69, 65	*vps2*	30, 30	*vps1*	78, 82
vps10	62, 84	*vps17*	74, 79	*vps16*	69	*vps6*	67, 70	*vps4*	43, 48	*vps26*	72, 76
vps13	21, 30	*vps39*	57, 44	*vps18*	79	*vps9*	58, 45	*vps20*	48, 45		
vps29	73, 68	*vps41*	58, 31	*vps33*	72, 86	*vps15*	67, 72	*vps22*	21, 29		
vps30	66, 72	*vps43*	52, 34			*vps19*	77, 68	*vps23*	31, 31		
vps35	67, 81					*vps21*	35, 23	*vps24*	29, 40		
vps38	62, 59					*vps34*	54, 53	*vps25*	34, 30		
vps44	40, 34					*vps45*	71, 69	*vps27*	33, 43		
vps46	27, 23							*vps28*	37, 38		
								vps31	9, 16		
								vps32	33, 54		
								vps36	39, 37		
								vps37	28, 20		

[a] % CPY represents the percentage of newly synthesized CPY that is secreted from each ^{35}S labeled *vps* mutant, as determined by quantitative immunoprecipitations and determination of the amount of radioactive CPY in the intracellular and extracellular fractions. The pairs of numbers are the percentage of newly synthesized material secreted from the *MATα* and *MATa* alleles of each *vps* mutant used in this study, respectively.

Class D vps Mutants Display Defects in Vacuole Inheritance and Acidification of the Vacuole

The class D *vps* mutants have been found to exhibit defects in several aspects of vacuole assembly. Two members of this group (*vps3* and *vps6*) initially attracted interest because they were unable to generate a pH gradient across the vacuolar membrane, and they failed to assemble peripheral membrane subunits of the V-ATPase onto the cytoplasmic surface of the vacuole (Rothman *et al.*, 1989b). The latter phenotype is illustrated for *vps45* in Figure 4D, where antibodies for the 60-kDa V-ATPase subunit, which is present at wild-type levels, produced a diffuse cytoplasmic staining pattern. Further characterization of *vps3* and *vps34* mutants revealed that these mutants are also defective for vacuole inheritance (Herman and Emr, 1990; Raymond *et al.*, 1990). The vacuoles in the mother cells of class D *vps* mutants appeared as single large spheres that failed to form segregation structures (Figure 4, B and F). Buds appeared to receive little or no vacuolar material from mother cells, and antibodies against ALP sometimes produced dispersed punctate staining patterns in daughter cells, similar to class C cells (Raymond *et al.*, 1990). This suggests that nascent vacuolar material produced in these cells transiently accumulates as unfused vesicles. The fact that nearly all mother cells possess vacuoles raises the possibility that vacuoles can arise de novo from unassembled vacuole precursors within daughter cells.

Mutants from eight *vps* complementation groups displayed class D phenotypes. Independent screens produced four additional vacuole segregation (*vac*) mutants with defects in vacuole inheritance (Weisman *et al.*, 1990; Shaw and Wickner, 1991; Weisman and Wickner, 1992). However, complementation testing between *vac* and *vps* mutants identified *vac1* as *vps19*, *vac3* as *vps36*, and *vac4* as *vps15*.

Within the class D *vps* strains there appeared to be subgroups of mutants with somewhat distinct characteristics. Yeast strains carrying *vps3*, *vps6*, *vps9*, *vps21*, or *vps45* mutations had phenotypes indistinguishable from those of *vps3Δ* strains described previously (Figure 4, A–D) (Raymond *et al.*, 1990). The morphological phenotypes of *vps15* and *vps34* mutants (Figure 4, E–H), although indistinguishable from one another, were slightly different from other class D mutants. In addition to vacuolar membrane staining, antibodies against ALP yielded punctate cytoplasmic labeling in almost all *vps15* and *vps34* cells, and the percentage of (daughter) cells that lacked apparent vacuoles and had a class C-like staining pattern was several-fold higher than in other class D mutants (Figure 4F). These data are consistent with the interpretation that ALP is present in the membranes of the multiple vesicular bodies seen in electron micrographs of these mutants (Banta *et al.*, 1988). Furthermore, antibodies that recognize the 60-kDa V-ATPase subunit labeled the vacuolar membrane and the cytoplasm in *vps15* and *vps34* strains, indicating that the V-ATPase assembly defect in these mutants was less pronounced than in other class D strains (compare Figure 4, D and H). In support of this, measurements of V-ATPase specific activities in isolated vacuolar membranes prepared from *vps34* strains were 20–40% of the wild-type levels, significantly higher than the levels measured in *vps3* and *vps6* mutants (Rothman *et al.*, 1989b; Yamashiro, 1990). The similarity between *vps15* and *vps34* mutants is consistent with the finding that Vps15p and Vps34p interact physically and that genetic interaction between these genes has been observed (Herman *et al.*, 1991). All of the class D *vps* mutants secreted high percentages of newly synthesized

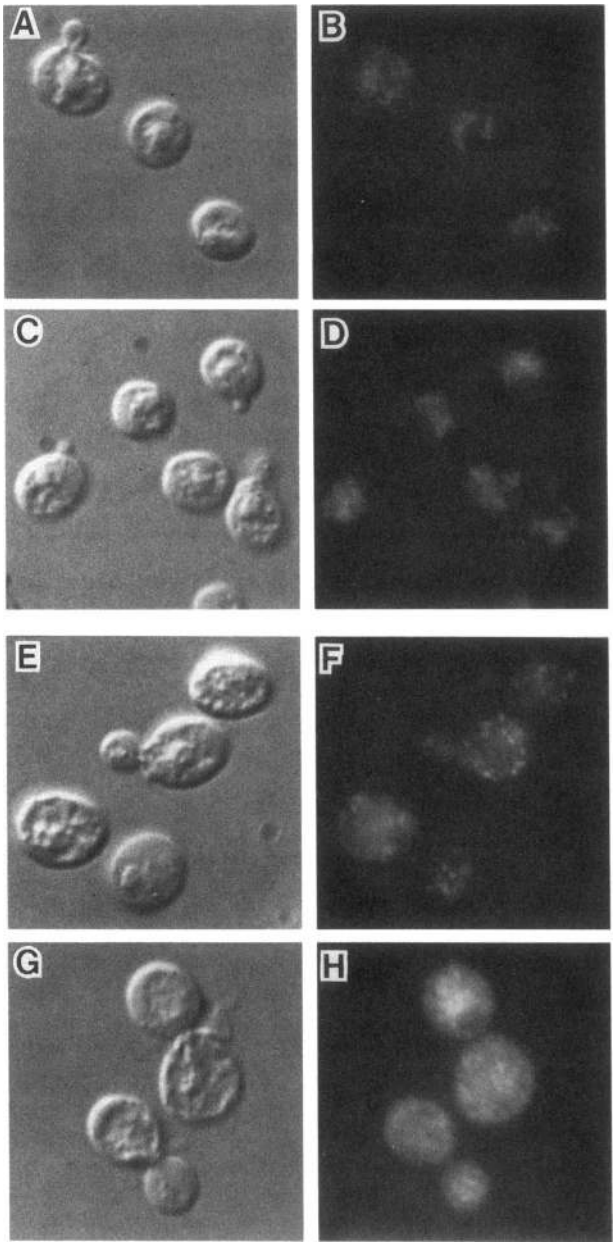

Figure 2. Class B mutants *vps17* (*vpt3;* A–D) and *vps41* (*vpl20-6;* E–H) exhibit similar but distinct classes of fragmented vacuoles. ALP staining is shown in B and F, 60-kDa vacuolar H^+-ATPase subunit staining in D and H, and the corresponding Nomarski images of whole cells in A, C, E, and G. One centimeter equals ~5 μm.

CPY except for *vps21* mutants (Table 3). The morphological phenotypes of *vps21* strains are otherwise identical with the subgroup of mutants that includes *vps3*.

A Novel Prevacuolar-like Organelle is Prominent in Class E vps Mutants

Antibodies directed against ALP, as well as visualization of vacuoles by Nomarski optics, revealed that the vacuoles of class E *vps* mutants were relatively normal in appearance (Figure 5, A and B). Antibodies specific for the 60-kDa V-ATPase subunit revealed some vacuolar and diffuse staining, but the majority of protein appeared to be sequestered in a separate compartment (termed the "class E compartment") that was distinct from the vacuole (Figure 5, C and D). Two lines of evidence suggested that the extravacuolar 60-kDa V-ATPase subunit was part of active V-ATPase complexes that were clustered around the class E compartment. First, quinacrine accumulated in the vacuole at low levels, but very high levels accumulated in the smaller class E compartment(s) adjacent to the vacuole (Figure 6, A and B). Quinacrine labeling of the extravacuolar compartment in class E *vps* mutants was sensitive to the addition of 200 mM ammonium acetate (Raymond, 1990), suggesting that quinacrine accumulated in the extravacuolar compartment because it was acidified (Weisman *et al.*, 1987; Rothman *et al.*, 1989b). Second, the V-ATPase specific activities measured in membranes prepared from several class E mutants were very similar to the specific activity measured in wild-type membranes (Yamashiro, 1990; Yamashiro, Rothman, and Stevens, unpublished observation).

The distinct distributions of ALP and the 60-kDa V-ATPase subunit in class E cells suggested that the novel class E compartment defined by the V-ATPase may represent exaggerated prevacuolar or endosome-like organelles. This hypothesis was investigated further by examining the distribution of a membrane protein of

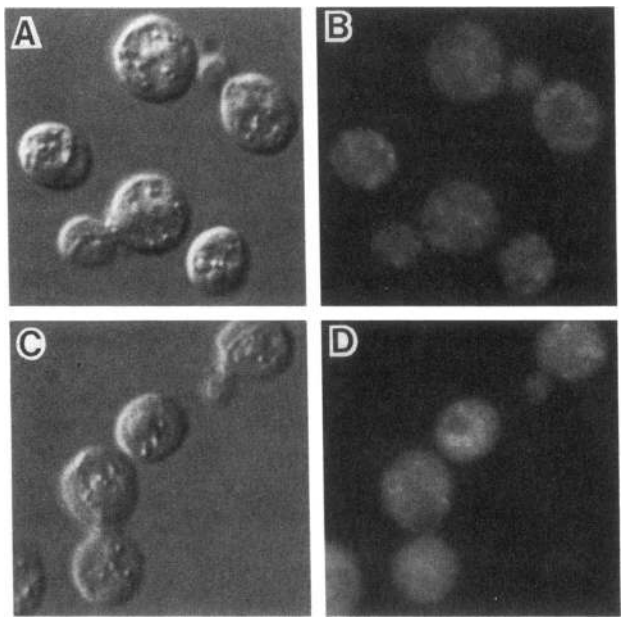

Figure 3. The class C mutant *vps11* (*vpl19-13*) lacks organized vacuolar structures. ALP staining is shown in B, 60-kDa V-ATPase subunit staining in D, and the corresponding Nomarski images of whole cells in A and C. One centimeter equals ~5 μm.

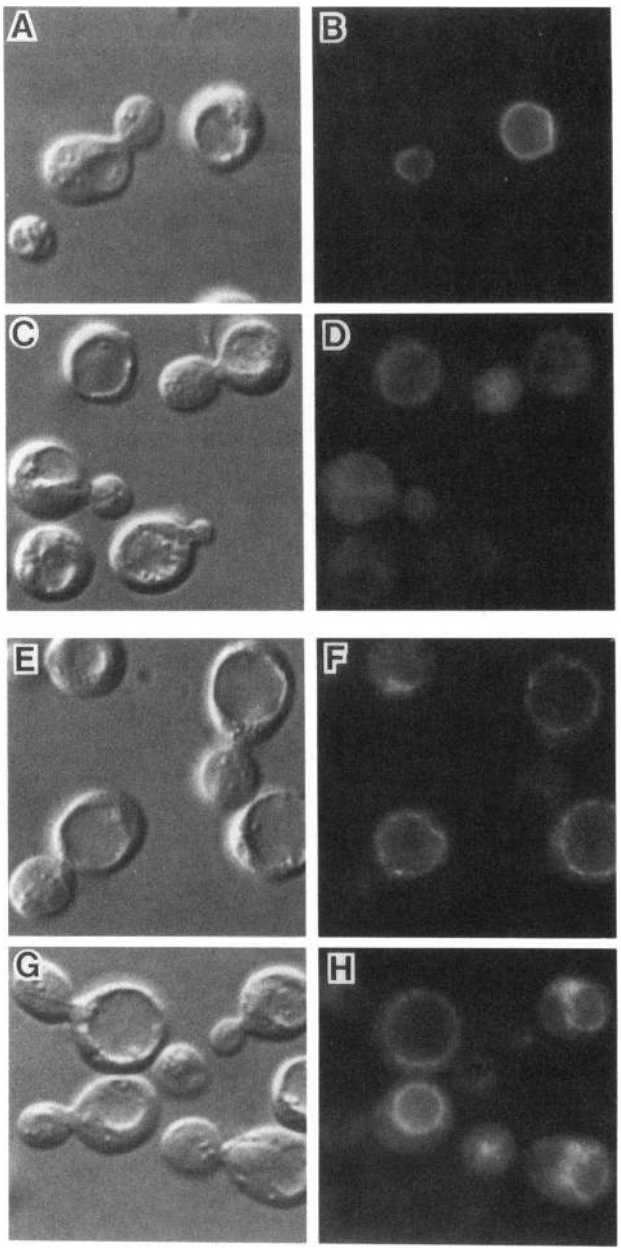

Figure 4. Class D mutants *vps45* (*vpl28-4*; A–D) and *vps15* (*vpl19-10*; E–H) exhibit defects in vacuole inheritance and assembly of the 60-kDa subunit of the V-ATPase onto the vacuolar membrane. ALP staining is shown in B and F, 60-kDa V-ATPase subunit staining in D and H, and the corresponding Nomarski images of whole cells in A, C, E, and G. One centimeter equals ~5 μm.

the late-Golgi compartment and soluble vacuolar proteins in class E mutant cells using indirect immunofluorescence (Figure 7). The late-Golgi marker was an integral membrane fusion protein that carries the cytoplasmic tail of dipeptidyl aminopeptidase A (DPAP A) fused to the transmembrane and lumenal domains of ALP (A-ALP). This protein was shown to reside in

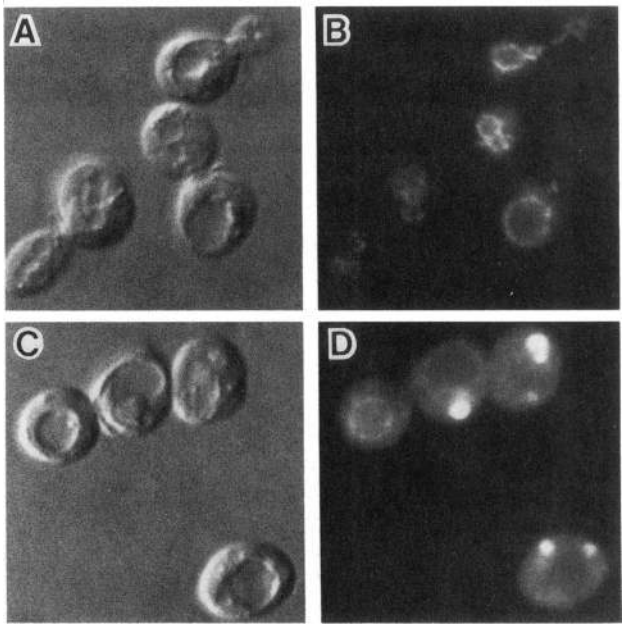

Figure 5. The class E mutant *vps27* (*vpl23-5*) possesses a novel 60-kDa V-ATPase subunit stained organelle distinct from the ALP staining vacuole. ALP staining is shown in B, 60-kDa V-ATPase subunit staining in D, and the corresponding Nomarski images of whole cells in A and C. One centimeter equals ~5 μm.

the late-Golgi (as defined by Kex2p co-localization) (Redding *et al.*, 1991) compartment of wild-type cells (Roberts, 1991; Roberts *et al.*, 1992; Nothwehr and Stevens, unpublished observations). In class E mutants, a significant portion of the A-ALP fusion protein was localized to the novel class E compartment, as demonstrated in the double-label immunofluorescence images shown in Figure 7, A–C. The remaining signal from the A-ALP fusion protein appears localized to punctate regions typical of Golgi staining in wild-type yeast cells (Redding *et al.*, 1991). Similar double-labeling procedures with antibodies that recognize CPY and antibodies

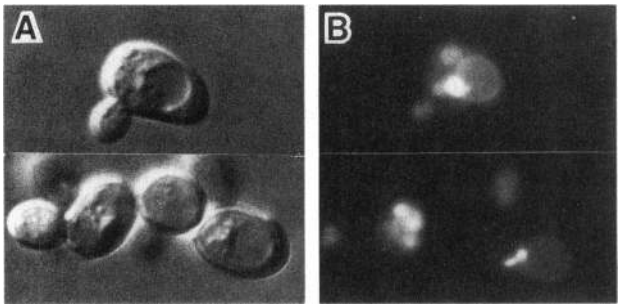

Figure 6. Quinacrine accumulates to high levels in a novel compartment of the class E mutant *vps27* (*vpl23-5*). Quinacrine staining is shown in B and the corresponding Nomarski image of whole cells in A. One centimeter equals ~5 μm.

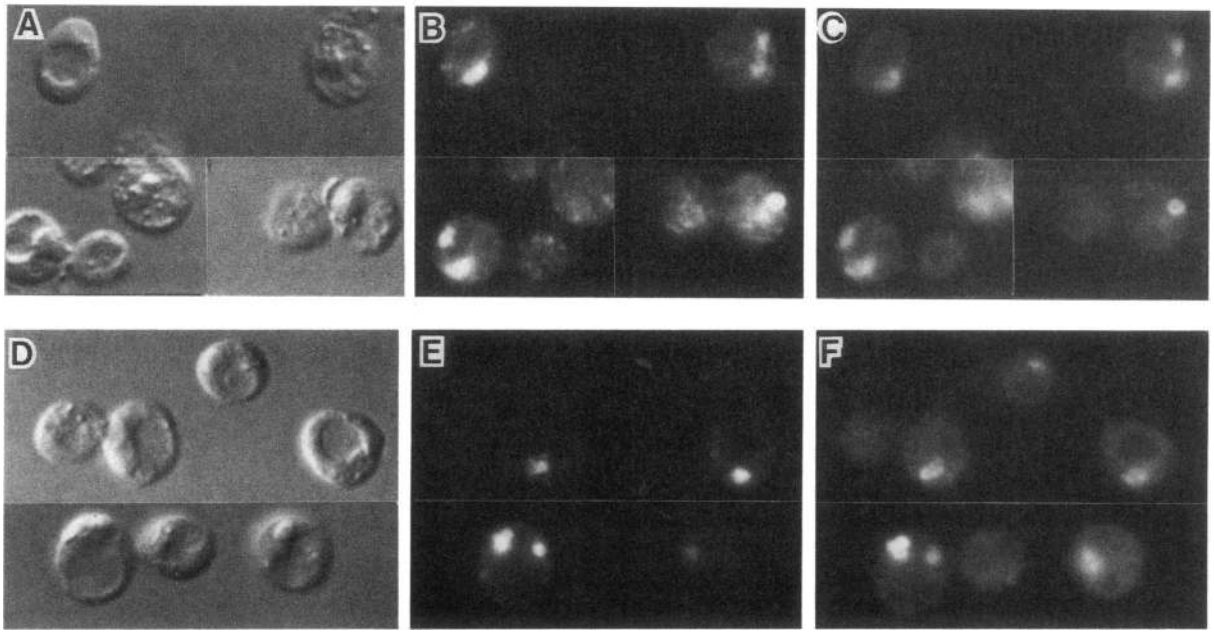

Figure 7. Double-labeling experiments demonstrate that significant pools of A-ALP and CPY, proteins normally found in the late Golgi and the vacuoles of wild-type cells, respectively, accumulate in the novel prevacuolar organelle of the class E mutant *vps27* (CRY2-8A, A–C; *vpl23-5*, D–F). The distribution of the A-ALP fusion construct is revealed by ALP antibodies in B. Note that the yeast strain shown is a *vps27Δpho8Δ* double mutant (Table 1), and thus the endogenous vacuolar signal from ALP is absent. CPY staining is shown in E. Both proteins colocalize with the 60-kDa V-ATPase subunit, which defines the novel class E compartment, as shown in C and F. Nomarski images are shown in A and D. One centimeter equals ~5 μm.

for the 60-kDa V-ATPase subunit showed that most of the intracellular CPY also is localized within the class E compartment (Figure 7, D–F). The soluble vacuolar proteins proteinase A (PrA) and proteinase B were also found in the novel class E organelle. The observations presented in Figure 7 indicate that the novel class E compartment possesses marker proteins of both the late-Golgi and the vacuole, consistent with the interpretation that it is an exaggerated prevacuolar organelle.

Mutants from thirteen *vps* complementation groups exhibited a class E phenotype (Table 3). All of the class E *vps* mutants secreted modest amounts of newly synthesized CPY (Table 3).

Vacuoles in Class F vps Mutants are Encircled by Smaller Vacuolar Compartments

Mutants from two *vps* complementation groups, *vps1* and *vps26*, have vacuolar morphologies intermediate between wild-type cells and the vacuolar morphology of class B mutants (Figure 8); *vps1* mutants were, in fact, originally described as class B mutant strains (Banta *et al.*, 1988). Unlike class B strains, however, cells in both class F mutants typically contain a large central vacuole (Figure 8, A and C). The labeling pattern of ALP and 60-kDa V-ATPase subunit reveal that this central compartment is often surrounded by smaller vacuole-like compartments (Figure 8, B and D). Although segregation structures are seldom observed in *vps1* and *vps26* cells, large buds contain vacuoles indicating that vacuole inheritance occurs in these strains. The observations that the 60-kDa subunit is assembled onto membranes and that vacuoles from both mutants label with quinacrine (Banta *et al.*, 1988; Rothman *et al.*, 1989b) suggest that V-ATPase assembly is not influenced by the *vps1* or the *vps26* mutations. Both class F mutants exhibited severe CPY sorting defects (Table 3).

DISCUSSION

Indirect immunofluorescence microscopy was used to characterize the vacuolar morphology in *vps* mutants representing 41 complementation groups. As with previous studies (Banta *et al.*, 1988; Rothman *et al.*, 1989a), we encountered *vps* mutants that exhibited profound perturbations in vacuole assembly, and we found that groups of mutants from different complementation groups shared similar aberrant morphological phenotypes. However, the major conclusion drawn in the previous reports was that majority of *vps* mutants had wild-type class A vacuoles. The greater resolution of the analytical tools utilized in this study indicates that many of the mutants originally placed in the class A group have vacuoles that are significantly different from the wild-type organelle. Once again, groups of mutants from different complementation groups were found to

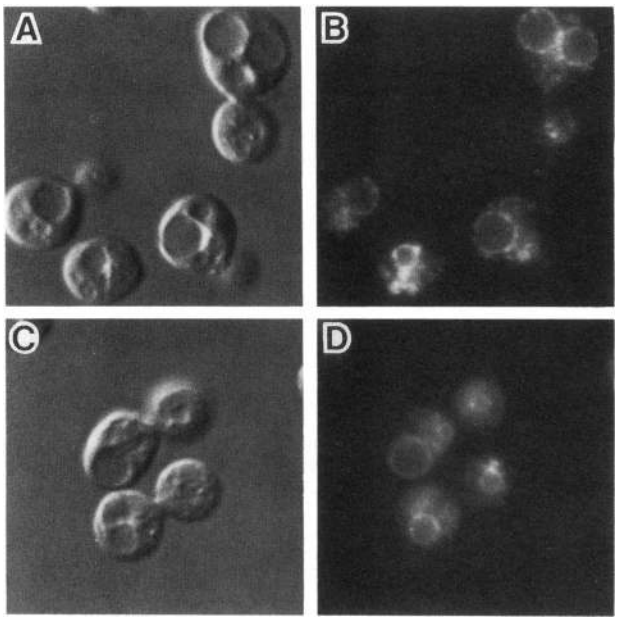

Figure 8. Vacuole-like compartments cluster around a central vacuoles in the class F mutant *vps1* (SF838-9D *vps1*-Δ2). ALP staining is shown in B, 60-kDa V-ATPase subunit staining in D, and the corresponding Nomarski images of whole cells in A and C. One centimeter equals ~5 μm.

share similar traits, and so the class A, class B, and so on, nomenclature of Banta *et al.* (1988) was extended to include these new groups. Overall, six classes of distinct morphological phenotype could be differentiated among mutants of the 41 *vps* complementation groups (Table 4). Quantitative analysis of the CPY missorting defects in the same collection of mutants suggests there was, in most cases, a good correlation between morphological phenotype and the severity of the sorting defect within a given class of *vps* mutants.

Our findings represent a significant contribution toward understanding vacuolar biogenesis in *S. cerevisiae* and thus to the more general problem of organelle assembly in eukaryotic cells. The analysis presented here demonstrates that most *vps* mutants can be differentiated from their isogenic wild-type parents based on morphological phenotypes and that groups of mutants appear to share similar aberrant characteristics. Many of the phenotypic features of individual class A, B, C, D, and F *vps* mutants have been described previously (reviewed in Klionsky *et al.*, 1990; Raymond *et al.*, 1992a), but an overview of the entire *vps* mutant collection that accounted for newly discovered morphological phenotypes (e.g., class D, E, and F phenotypes) had not been conducted. An important conclusion drawn from the current analysis is that this large collection of *vps* mutants can be subdivided into a relatively small number of morphological groups. The fact that there are at least six distinct classes of *vps* mutants suggests there also may be at least six "pathways" or distinct biochemical "processes" required for vacuolar protein sorting and vacuole assembly. As a corollary, the wild-type gene products affected in mutants that share similar phenotypes may act in the same pathway. This is exemplified by the *vps15* and *vps34* mutants. These mutants share identical vacuolar morphologies, and the wild-type Vps15p and Vps34p products appear to act as a complex (Herman *et al.*, 1991).

The other significant aspect of this work is that the morphological characteristics of a given mutant suggest possible functions for corresponding wild-type gene products. For example, mutations that specifically affect the soluble protein sorting apparatus would not be anticipated to perturb other events in vacuole assembly, and, thus, such mutants might be expected to have a class A phenotype. Mutations in a putative soluble vacuolar protein sorting receptor complex eventually may be identified among the class A *vps* mutants, particularly those with a pronounced sorting defect.

In class B mutants there are many small vacuolar compartments, and in class C mutants there are no apparent vacuoles, yet no cell surface ALP staining was observed in these classes (or any other class) of *vps* mutants. These data underscore the fundamental differences between the sorting of soluble and integral membrane vacuolar proteins. Recent evidence suggests that membrane proteins lacking specific sorting information are delivered to the vacuole by default (Roberts, 1991; Cooper and Bussey, 1992; Raymond *et al.*, 1992a,b; Roberts *et al.*, 1992; Wilcox *et al.*, 1992). The fact that none of the *vps* mutations results in delivery of ALP to the cell surface, even in class C mutants, which have an extreme vacuole assembly phenotype, supports this conclusion.

The phenotype of class E *vps* mutants, which accumulate a novel compartment with protein constituents from both the late Golgi and the vacuole, has not been described previously. These mutants secrete modest amounts of newly synthesized CPY in its precursor form, and the CPY that is retained intracellularly is processed to its mature form (see Rothman and Stevens, 1986; Robinson *et al.*, 1988; Vater, Raymond, and Stevens, unpublished observations). Because a large pool of CPY was found within the novel class E compartment, by inference this material is in its processed enzymatically active form. This supposition is supported by the fact that the A-ALP fusion protein, which contains a C-terminal propeptide segment that is proteolytically cleaved by vacuolar hydrolases (Klionsky and Emr, 1989), partially localizes to the class E compartment and is partially processed in the class E mutant *vps27* (Raymond, Nothwehr, and Stevens, unpublished data). These data argue that the class E compartment is a post-Golgi organelle and not an exaggerated form of the late Golgi because only p2 precursor form of CPY

Table 4. Phenotypic characteristics of the morphological groups that define the *vps* mutants

Class	Characteristics
Class A	Wild-type vacuolar morphology. Vacuoles frequently appear as 3–10 subcompartments that cluster to one region of the cytoplasm. In premitotic budding cells, the vacuole is likely to extend between mother cell and developing bud along the cell axis. ALP and V-ATPase staining patterns of the vacuole are superimposable.
Class B	Mutants have highly "fragmented" vacuoles exhibiting >20 vacuole-like compartments per cell. In some mutants, these subcompartments are clustered, whereas in others they are distributed randomly throughout the cell. ALP and V-ATPase staining does not appear particularly discrete, owing in part to the dispersed nature of the vacuole-like compartments. Quinacrine staining in the class B *vps* mutants sugests that some active V-ATPase complexes are assembled.
Class C	Mutant cells do not have an organelle readily identifiable as a vacuole. Multiple impressions visible by Nomarski optics are presumably a mixture of vesicle-like organelles and lipid droplets seen previously in electron microscopy studies (Banta *et al.*, 1988; Wada *et al.*, 1990). ALP staining is distinctly punctate, suggesting that this membrane protein is distributed among vesicle-like membrane compartments. V-ATPase staining is more diffuse, suggesting that class C mutants are at least partially defective for the assembly of the V-ATPase.
Class D	These mutants are defective for vacuolar segregation, meaning mother cells have a large, single, spherical vacuolar compartment that fails to extend into developing buds, and newly formed daughter cells often lack coherent vacuoles and assume the appearance of class C mutants (Raymond *et al.*, 1990; Weisman *et al.*, 1990; Shaw and Wickner, 1991). The V-ATPase fails to assemble properly onto the vacuolar membrane, resulting in diffuse cytoplasmic localization of peripheral membrane V-ATPase subunits.
Class E	ALP antibodies label vacuoles that are readily visible by Nomarski optics, whereas V-ATPase antibodies label a prominent organelle distinct for the vacuole and from ALP staining structures; V-ATPase labeling is also weakly evident around the ALP-staining vacuole and as diffuse staining throughout the cytoplasm. Significant portions of soluble vacuolar hydrolases (CPY and PrA) and Golgi membrane proteins (DPAP A and A-ALP) also localize to the novel organelle in class E mutants. These data indicate that class E mutants may possess an exaggerated form of a prevacuolar endosome-like compartment.
Class F	These mutants often have a large central vacuole surrounded by a number of class B-like fragmented vacuolar structures. As such, these mutants exhibit vacuolar morphology distinct from class A and class B cells. ALP and V-ATPase antibodies yield superimposable staining patterns.

is secreted, yet mature CPY is present in this novel compartment.

Class E *vps* mutants possess two distinct organelles with vacuolar markers, and the question arises as to which organelle is the vacuole and how these separate compartments are generated. The large organelle that is apparent by Nomarski optics and that also stains with ALP antibodies has the appearance normally associated with a yeast vacuole. On the other hand, the new compartment has active V-ATPase complexes and large pools of active vacuolar hydrolases associated with it. We envision at least two models for how these compartments are generated (Figure 9). In the first model, proteins sorted to a common vacuolar compartment in wild-type cells enter one of (at least) two separate sorting pathways (Figure 9A). These pathways converge in the vacuole in wild-type cells. In class E mutants, these pathways fail to merge and lead to the generation of separate compartments. To accomodate experimental observations, it is also necessary to postulate that late Golgi proteins (e.g., A-ALP) enter one of these divergent pathways as a consequence of the class E *vps* mutation.

In the second model, proteins sorted in the late Golgi and bound for the vacuole enter a prevacuolar or endosome-like compartment. Furthermore, proteins that function in the late Golgi are hypothesized to enter the prevacuolar compartment, and in wild-type cells, these proteins are recycled back to the Golgi. Vacuolar proteins, on the other hand, are transported from the class E compartment to the vacuole. In class E *vps* mutants, protein traffic from the prevacuolar endosome-like organelle is restricted for most of these proteins. This leads to the accumulation of proteins in the prevacuolar compartment and the morphological exaggeration of the organelle itself. We cannot, at present, distinguish between these models. However, evidence has been presented that wild-type cells possess a distinct compartment along the endocytic pathway (Singer and Riezman, 1990).

Two additional observations lead us to favor the interpretation that the class E compartment is a prevacuolar endosome-like organelle. Antibodies directed against Vps1p, which is thought to associate with the yeast Golgi but does not appear to associate with vacuolar membranes (Raymond, 1990; Rothman *et al.*, 1990), label the class E organelle (Raymond and Stevens, unpublished observations). In addition, the a-factor receptor, Ste3p, which is endocytosed from the cell surface and delivered to the vacuole in wild-type strains, accumulates in the prevacuolar endosome-like organelle of the class E mutant *vps2* (Davis and Sprague, personal communication). The fact that there are 13 class E *vps* complementation groups indicates that a large number of components are required for conversion of the class E organelle into normal wild-type vacuoles.

In summary, our work has shown that the large collection of *vps* mutants is composed of subclasses of mutant strains that share similar morphological phenotypes and CPY sorting defects. These data support a model

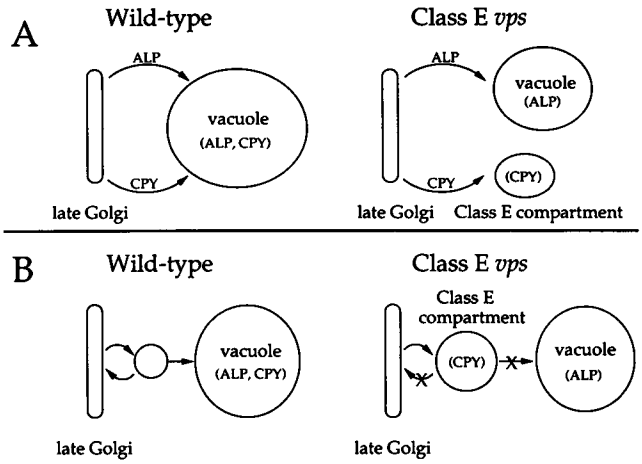

Figure 9. Two models for the formation of the new compartment in class E *vps* mutants. (A) Proteins bound for the vacuole in wild-type cells are normally packaged into at least two separate classes of transport vesicles that subsequently fuse with the vacuole. In class E mutants, these pathways fail to merge. In addition, late Golgi proteins such as A-ALP enter the pathway defined by CPY as a consequence of the class E *vps* mutation. (B) Proteins bound for the vacuole in wild-type cells normally pass through a prevacuolar endosome-like compartment. Late Golgi proteins are hypothesized to cycle between the late Golgi compartment and the prevacuole. In class E mutants, most protein traffic (an exception being ALP) from the putative endosome-like organelle either to the vacuole or back to the late Golgi is restricted, leading to morphological exaggeration of the organelle. The steady-state resident proteins for each compartment are shown in parenthesis.

in which the different classes of mutants affect specific and discrete processes, pathways, or steps required for vacuole biogenesis. Similarly, mutants with indistinguishable morphological phenotypes are likely to act along the same or similar vacuole assembly pathway. These observations should facilitate the choices of *VPS* genes that are subjected to further molecular characterization. Finally, we have discovered that a large number of class E *vps* mutants possess a novel organelle that contains proteins from the late Golgi, the vacuole and a protein endocytosed from the cell surface. These are the characteristics expected of a prevacuolar endosomal compartment, and they support previous evidence that wild-type yeast cells possess endosomes (Singer and Riezman, 1990).

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