Multiple Classes of Yeast Mutants Are Defective in Vacuole Partitioning yet Target Vacuole Proteins Correctly

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In *Saccharomyces cerevisiae* the vacuoles are partitioned from mother cells to daughter cells in a cell-cycle-coordinated process. The molecular basis of this event remains obscure. To date, few yeast mutants had been identified that are defective in vacuole partitioning (*vac*), and most such mutants are also defective in vacuole protein sorting (*vps*) from the Golgi to the vacuole. Both the *vps* mutants and previously identified non-*vps vac* mutants display an altered vacuolar morphology. Here, we report a new method to monitor vacuole inheritance and the isolation of six new non-*vps vac* mutants. They define five complementation groups (*VAC8–VAC12*). Unlike mutants identified previously, three of the complementation groups exhibit normal vacuolar morphology. Zygote studies revealed that these *vac* mutants are also defective in intervacuole communication. Although at least four pathways of protein delivery to the vacuole are known, only the Vps pathway seems to significantly overlap with vacuole partitioning. Mutants defective in both vacuole partitioning and endocytosis or vacuole partitioning and autophagy were not observed. However, one of the new *vac* mutants was additionally defective in direct protein transport from the cytoplasm to the vacuole.

INTRODUCTION

Cell division requires the partitioning of each cytoplasmic organelle into the daughter cells (Palade, 1983; Warren and Wickner, 1996). For example, during mitosis in mammalian cells, the nuclear envelope, endoplasmic reticulum (ER), and Golgi vesiculate and disperse throughout the cytoplasm, later to reassemble in the newly formed daughter cells (Rothman and Warren, 1994).

In the budding yeast *Saccharomyces cerevisiae*, vacuole segregation is a highly regulated process. Unlike the mammalian Golgi apparatus and ER, the yeast vacuole does not undergo a cell-cycle-specific frag-

mentation (Weisman *et al.*, 1987). During early S phase, the maternal vacuole normally forms membranous tubules or a series of connected vesicles, termed the “segregation structure,” that projects into the emerging bud. Vacuole inheritance in wild-type cells occurs via this segregation structure; a substantial portion of the mother vacuole is transferred into the bud (Weisman and Wickner, 1988; Weisman *et al.*, 1990; Raymond *et al.*, 1990; Gomes de Mesquita *et al.*, 1991). In both vegetative cells and zygotes, vacuole segregation begins shortly after bud emergence and is always directed into the daughter cells, indicating that this process is spatially regulated and coordinated with the cell cycle.

In addition to inheritance, the yeast vacuole receives its constituents through four other pathways. 1) Many

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vacuole proteins are directed to the vacuole through the secretory pathway. They are translocated into the ER and then transit to the Golgi apparatus, where they are sorted from secretory proteins and delivered to the vacuole (for review, Conibear and Stevens, 1995; Horazdovsky *et al.*, 1995). 2) Proteins can also be delivered to the vacuole by endocytosis (Riezman, 1993). 3) Some proteins target directly to the vacuole after they are synthesized in the cytoplasm (Yoshihisa and Anraku, 1990; Klionsky *et al.*, 1992; Harding *et al.*, 1995). 4) Under nutrient-deficient conditions, proteins can be sequestered in the vacuole through autophagy (Takeshige *et al.*, 1992; Thumm *et al.*, 1994).¹

Vacuole segregation and protein transport pathways are interrelated processes. For example, vacuolar proteins required for vacuole partitioning must be correctly localized to the vacuole. In addition, some components may be involved both in protein transport and vacuole segregation. The Class D vacuolar protein-sorting (*vps*)¹ mutants, which mis-sort vacuole proteins from the Golgi to the cell surface, are also defective in vacuole segregation (Raymond *et al.*, 1992). Because of the protein-sorting defect, it is unclear whether a given mutation in a Class D *vps* mutant has a direct effect on vacuole segregation. For this reason, non-*vps vac* mutants have been isolated and characterized. These include *vac2*, *vac5*, *vac6*, and *vac7*. *vac2* was obtained by screening a collection of temperature-sensitive yeast mutants by microscopic examination (Shaw and Wickner, 1991). *vac5* was isolated through a screen that enriched for dense yeast mutants (Nicolson *et al.*, 1995). *vac5* is allelic with *PHO80*. Pho80p is a cyclin that forms a complex with a cdc-like kinase, Pho85p. *vac6* and *vac7* were isolated by screening for mutants that do not sustain vacuolar proteolytic activities upon proteinase A depletion (Gomes de Mesquita *et al.*, 1996). The *VAC7* gene has no significant homology with other known genes (Bonangelino, Catlett, and Weisman, unpublished data).

The existence of so few non-*vps vac* mutants has hampered studies of vacuole segregation. Moreover, all four of the previous non-*vps vac* mutants have altered vacuole morphology, namely a single, round vacuole such as those found in Class D *vps* mutants. To isolate a large population of *vac* mutants, it was necessary to devise a new mutant screen.

The fluorescent styryl dye, (N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl)-hexatrienyl) pyri-

dinium dibromide (FM4-64), has been used to study endocytosis and vacuole dynamics in yeast (Vida and Emr, 1995). Previous studies showed that, when cells were incubated with FM4-64 and then transferred to fresh medium, the fluorophore first labeled the plasma membrane, then small vesicles, and finally the vacuole. Once the fluorophore reached the vacuole, it persisted for several generations (Vida and Emr, 1995). Here we report that inheritance of vacuoles labeled with FM4-64 can be monitored by fluorescence-activated cell sorting (FACS). Moreover, with the use of FACS, *vac* mutants can be isolated efficiently. Characterization of the new *vac* mutants reveals that they may be assigned to three classes on the basis of vacuole morphology. These mutants show no overlap with *vps*, endocytosis, or autophagy mutants and will be useful in elucidating the molecular basis of vacuole inheritance.

MATERIALS AND METHODS

Media, Yeast Strains, and Genetic Methods

All the yeast strains described in this report are listed in Table 1. Rich medium (1% yeast extract, 2% peptone, and 2% dextrose [YPD]), minimal yeast medium, synthetic complete (SC), and sporulation medium were made, and genetic manipulations were performed as described (Kaiser *et al.*, 1994). YCM medium is YPD medium buffered with citric acid/K₂HPO₄, pH 4.5 (Rogers and Bussey, 1978). Strain LIS14 was a meiotic segregant from diploid JSY2B-3.1 × W3094 and will be described elsewhere (Gomes de Mesquita, Shaw, and Woldringh, unpublished data). The plasmid (pBVH17) containing *PEP4* under its own promoter was described previously (van den Hazel *et al.*, 1993).

Vacuole Staining with FM4-64

Vacuoles of growing yeast were labeled with FM4-64 by a method similar to that described previously (Vida and Emr, 1995). Strains were grown at 23°C to an optical density (OD) of 0.5–1.2 OD₆₀₀ in YPD medium. Aliquots of 250 μl of cells were incubated with aeration in medium containing 80 μM FM4-64 for 1 h or the time indicated at 23°C. The cells were then harvested at 13,000 × g for 1 min, washed by resuspending in 1 ml YPD to remove free FM4-64, collected by centrifugation at 13,000 × g for 1 min, and resuspended in 5 ml of YPD. The cells were then allowed to grow for 3–3.5 h at 23°C. After this chase period, the cells were harvested at 13,000 × g for 1 min and viewed with a Zeiss Axioskop microscope equipped with a Pan-Neofluor ×100, 1.3/NA oil immersion objective. The filter set used excites the cells at 546 nm and allows detection of emission from 580–630 nm for FM4-64 fluorescence. Photographs were taken with an attached 35 mm camera with Kodak T-Max 400 film.

Yeast cells to be labeled with carboxy-2',7'-dichlorofluorescein diacetate (CDCFDA; Preston *et al.*, 1989) were harvested as above and resuspended in YCM medium containing 6 mM CDCFDA and incubated with aeration for 15 min. Cells were then harvested and viewed with a fluorescence microscope. The filter set for this fluorophore excites the cells at 450–490 nm with emission of wavelength above 520 nm. Note that the concentration of CDCFDA used is 200-fold higher than what we used previously (Weisman *et al.*, 1989). This higher concentration was chosen to ensure that all vacuoles could be detected.

¹ Abbreviations used: ALP, alkaline phosphatase; API, aminopeptidase I; CDCFDA, carboxy-2', 7'-dichlorofluorescein diacetate; CPY, carboxypeptidase Y; *cvt*, cytoplasm-to-vacuole-targeting mutant; FM4-64, (N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl)-hexatrienyl) pyridinium dibromide; PrA, proteinase A; PGK, phosphoglycerate kinase; TTBS, tris-buffered saline with 0.1% Tween 20; *vac*, vacuole-partitioning mutant; *vps*, vacuolar protein-sorting mutant; YPD, 1% yeast extract, 2% peptone, and 2% dextrose media.

Table 1. Yeast strains used in this study

Strains	Genotype	Source
RHY6210	MAT α leu2,3-112 ura3-52 his3- Δ 200 trp- Δ 901 lys2-801 suc2- Δ 9 pep4- Δ 1137	D.S. Gomes de Mesquita <i>et al.</i> , 1996
LWY7210	MAT α leu2,3-112 ura3-52 trp Δ 901 lys2-801 suc2- Δ 9 Δ ade8::HIS3 pep4- Δ 1137	This study
LWY7213	MAT α leu2,3-112 ura3-52 trp-901 lys2-801 suc2- Δ 9 Δ ade8::HIS3	This study
LWY152	MAT α vac1- Δ 2::TRP1 trp1-2891 leu2,3-112 ura3-52 his4-519	Weisman <i>et al.</i> , 1992
JSY2B	MAT α vac2-1 ura3-52 ade2-101 lys2-801	Shaw and Wickner, 1991
LIS14	MAT α vac2-1 leu2,3-112 ura3-52 his3- Δ 200 pep4- Δ 1137	D.S. Gomes de Mesquita <i>et al.</i> , unpublished data
W3094	MAT α pep4- Δ 1137 ura3-52 his3- Δ 200 leu2,3-112	van den Hazel <i>et al.</i> , 1992
TN.3B	MAT α vac5-1::URA3 ura3-52 his3- Δ 200	Nicolson <i>et al.</i> , 1995
JBY007	RHY6210 vac7-1	D.S. Gomes de Mesquita <i>et al.</i> , 1996
JBY009	RHY6210 vac6-1	D.S. Gomes de Mesquita <i>et al.</i> , 1996
LWY1116	RHY6210 vac8-1	This study
LWY2077	LWY7213 vac8-1	This study
LWY1103	RHY6210 vac9-1	This study
LWY2130	LWY7213 vac9-1	This study
LWY1701	RHY6210 vac10-1	This study
LWY2135	LWY7213 vac10-1	This study
LWY1045	RHY6210 vac11-1	This study
LWY1050	RHY6210 vac12-1	This study

Cell-sorting Analysis of Vacuole Inheritance

Cells (2×10^6) were labeled as above and allowed to grow for the time point indicated in the absence of the dye. At each time point, 1 ml of cells was removed and analyzed with a fluorescence-activated cell sorter (Coulter EPICS 753; Coulter Electronics, Hialeah, FL). The cells were excited at 488 nm, and the emitted light was passed through a 670/14 bandpass filter. Cells ($\sim 1 \times 10^4$) were counted in each analysis.

vac Mutant Enrichment and Screening

To facilitate complementation analysis, two parent strains were used to screen for *vac* mutants. Construction of the first, RHY6210, which is virtually isogenic with SEY6210 but lacks a functional *PEP4* gene, will be described elsewhere (Gomes de Mesquita *et al.*, 1996). For construction of the second, LWY7210, the mating type of RHY6210 was switched to MAT α by transformation and subsequent loss of pGAL-HO (Herskowitz and Jensen, 1991). A MAT α strain was selected, and its *ADE8* gene was disrupted with *HIS3* via homologous integration. Thus, the two parental strains are virtually isogenic, differing only at two nutritional loci.

Cultures were mutagenized with ethyl methane sulfonate (EMS; Sigma Chemical, St. Louis, MO) to 60% viability. Viable, mutagenized cells (4×10^6) were resuspended in 10 ml of YPD and incubated for 12 h at 23°C. During this incubation the cells doubled approximately three times. The cells were then incubated with medium containing 80 μ M FM4-64 for 1 h. FM4-64 was removed from the medium, and the cells were allowed to continue to grow for 5 h in fresh medium, followed by FACS analysis. Cells were collected from the most-labeled population and the least-labeled population of the sorting profile. Each of these two populations represented 2% of the total cells. Collected cells (~ 2000 -4000) were plated on YPD plates and incubated at 23°C. Individual colonies were selected, grown in liquid culture overnight, and screened with a fluorescence microscope. Mutants whose buds contained little or no fluorescent material were selected for further study.

Yeast Cell Blots of CPY Secretion

Immunoblot detection of carboxypeptidase Y (CPY) secretion was performed as described previously (Roberts *et al.*, 1991). Patches of yeast were incubated on YPD plates for 1 d at 23°C. Nitrocellulose filters were applied to the top of the patches and incubated overnight at 23°C. The filters were removed, and adhering cells were washed off with water. The filters were then immunoblotted with polyclonal antibody against CPY, followed by secondary antibody coupled to horseradish peroxidase (Sigma). Immunoreactive protein bound to the filter was detected by epichemiluminescence (ECL) (Amersham, Buckinghamshire, UK).

Cell Labeling and Immunoprecipitations

To examine localization of vacuolar proteases from whole cells, we labeled yeast as described previously (Klionsky *et al.*, 1992) for 5 min in SMD (0.067% yeast nitrogen base, 2% glucose, vitamins, and required amino acids) in the presence of bovine serum albumin (BSA; 2.5 mg/ml). Nonradioactive chase was initiated by the addition of 1 mM methionine, 2 mM cysteine, and 0.1% yeast extract. Samples were removed at the times indicated; the cells were killed by the addition of 10 mM NaN₃ and separated into cell and medium fractions by a 10 sec centrifugation at $12,500 \times g$. Each fraction was then precipitated by the addition of 8 vol of cold acetone. CPY, proteinase A (PrA), and alkaline phosphatase (ALP) were immunoprecipitated (Harding *et al.*, 1995) from the resultant extracts and analyzed by SDS-PAGE.

Primary antisera to API and PrA were used at 1:15,000, antiserum to CPY at 1:30,000, and antiserum to PGK at 1:75,000 in TTBS with 0.5% non-fat milk and incubated with the blot for a period from 30 min to 2 h. After incubation in primary antiserum, all blots were washed in TTBS three times and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antiserum (Cappel, Durham, NC) at a dilution of 1:30,000 for 30–45 min. The filters were then washed three times in TTBS, and immunoreactive bands were visualized by using a chemiluminescent substrate (Gallagher, 1994). Primary antiserum incuba-

tions of 2 h were performed at 4°C. All other incubations were performed at room temperature.

Cell Fractionation and Protease Treatment for Determination of Aminopeptidase I (API) Localization

Cell fractionation was performed essentially as described (Scott and Klionsky, 1995). Yeast cells (10 OD₆₀₀ U) were harvested at OD₆₀₀ of 0.8–1.2, washed once in 10 mM Tris-SO₄, pH 9.4, and 10 mM dithiothreitol, and resuspended in this buffer to an OD₆₀₀ of ~2.0. The cells were incubated for 15 min at 30°C with constant shaking (200–250 rpm) and then harvested by centrifugation and resuspended in 1 M sorbitol, 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), pH 6.8 (spheroplasting buffer) to an OD₆₀₀ of ~2.0. Zymolyase 20T (0.2 mg) was added, and the sample was mixed gently by inversion. Spheroplasting was performed at 30°C for no more than 20 min with gentle mixing every 4–5 min. This procedure routinely yielded 85–95% spheroplasts in 15 min.

Spheroplasts were harvested by centrifugation at 3000 × *g* for 3 min, resuspended in spheroplasting buffer, and transferred to microcentrifuge tubes. The spheroplasts were harvested again at 3000 × *g* for 2 min, gently resuspended in 0.5 ml of lysis buffer (200 mM sorbitol and 20 mM PIPES, pH 6.8), and incubated at room temperature for 10 min, inverting once at 5 min. A 100 µl aliquot was then removed (total sample) and precipitated with 10% vol/vol trichloroacetic acid (TCA). So that the protease sensitivity of the accumulated proAPI could be examined, two 100 µl aliquots were removed to tubes and subjected to treatment with 50 µg/ml proteinase K for 30 min at room temperature in the presence or absence of 0.2% Triton X-100. The protease reaction was stopped by precipitation with 10% TCA. The remaining 200 µl aliquot of spheroplasts was centrifuged at 6000 × *g* for 5 min; 100 µl of the supernatant was removed and precipitated with 10% TCA (cytosol-enriched). The pellet was sonicated in 200 µl of water; then a 100 µl sample (vacuole-enriched) was precipitated as above. All precipitated samples were washed twice with acetone, dried, and resuspended in sample buffer (125 mM Tris-HCl, pH 6.8, 0.4% SDS, 1% glycerol, and 5% β-mercaptoethanol) (Laemmli, 1970) at 50 µl per initial OD₆₀₀ unit of cells. Equivalents of each fraction (0.2 OD₆₀₀ and 0.02 OD₆₀₀) were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) filter; API/PrA and phosphoglycerate kinase (PGK) bands were detected by Western analysis (Harding *et al.*, 1995). Antiserum to peptides within the mature region of API was described previously (Klionsky *et al.*, 1992). Antiserum to PGK was generously contributed by Dr. Jeremy Thorner (University of California, Berkeley, CA) (Baum *et al.*, 1978).

Indirect Immunofluorescence

Cells were processed for indirect immunofluorescence microscopy as described (Raymond *et al.*, 1992). A monoclonal antibody against the 60-kDa vacuolar ATPase (V-ATPase) subunit was used at a 1:10 dilution, followed by a 1:200 dilution of FITC-conjugated secondary antibody. The V-ATPase monoclonal antibodies were provided by Dr. T. Stevens (University of Oregon, Eugene, OR) and purchased from Molecular Probes (Eugene, OR).

Labeling of Nuclei and Mitochondria and Accumulation of Lucifer Yellow in Vacuole

DAPI (4',6-diamidino-2-phenylindole) staining of nuclei was performed on fixed cells as described (Kaiser *et al.*, 1994). Mitochondria were stained in living cells with DiOC₆(3) (3,3'-dihexyloxycarbocyanine iodide) as described (Pringle *et al.*, 1989). Accumulation of Lucifer yellow in the vacuoles was performed as described (Dulic *et al.*, 1991).

Autophagy Assay

Autophagy was examined as described (Takeshige *et al.*, 1992). Cells of proteinase A-deficient strains were transferred from rich medium (YPD) to defined medium with no nitrogen source SD-(N) (Takeshige *et al.*, 1992) and incubated for 3 h. The accumulation of autophagic bodies in the vacuole was examined with Nomarski optics.

Monitoring Vacuole Inheritance in Yeast Zygotes

For each mating, one mating-type strain was labeled with FM4-64. After washing, the labeled cells were mixed with an equal number of cells of the opposite mating type. The cells were incubated with shaking at 23°C for 4–4.5 h (Weisman *et al.*, 1990). Evidence of FM4-64 labeling in the originally unlabeled parent cell vacuole and bud vacuole were scored with a fluorescence microscope.

RESULTS

Vacuole Inheritance Can Be Monitored by FACS

During budding in wild-type yeast, the vacuole contents of the mother cell are divided ~50-50 between mother and bud (Weisman *et al.*, 1987). Therefore, a population of wild-type yeast labeled with FM4-64 should start with a specific level of fluorescence, and after one doubling, there should be a diminution of the fluorescence intensity per cell. Each mother cell should lose a significant amount of FM4-64, whereas each daughter cell should inherit FM4-64. In contrast, mutants defective in vacuole inheritance should retain fluorescence primarily in the mother cell vacuole. To test this hypothesis, wild-type, *vac6*, and *vac7* cells were labeled with FM4-64 for 1 h; the FM4-64 was removed from the medium, and the cells were allowed to continue to grow. Cells were collected at the indicated time points and subjected to cell-sorting analysis. The histograms for each strain and time point are shown (Figure 1).

Immediately after the fluorophore is removed from the medium (zero time), fluorescence profiles of wild-type cells and *vac* mutants are very similar. During the first cell doubling, the FM4-64 migrates from the plasma membrane to vesicular intermediates and ultimately to the vacuole membrane (Vida and Emr, 1995). Thus, there is not much difference in fluorescence intensity in the zero time point and the 2 h time point (Figure 1). After 4 h in medium, the wild-type cells continue to behave as a single peak. However, the population has significantly less fluorescence than the starting cells. This is because the mother cells have donated much of their vacuolar membranes to the daughter cells. This pattern is consistent with the observation that wild-type cells transfer ~50% of their vacuolar material to the daughter cell vacuoles in each generation. In contrast, the profiles of *vac6* and *vac7* show a bimodal fluorescence distribution. The highly labeled cells are the mother cells that are unable to donate vacuolar material to the buds; the peak with less fluorescence contains the daughter cells that have

Figure 1. Vacuole inheritance can be followed by FACS. Cells were incubated at 23°C for 1 h with 80 μ M FM4-64 in YPD medium. The cells were harvested, and the free dye was removed; then cells were resuspended in fresh YPD medium and allowed to grow for the times indicated. At each time point, $\sim 1 \times 10^4$ cells were subjected to FACS analysis. Each panel indicates a separate strain and time point. Numbers for hours after removal of FM4-64 are shown at the top. The top panels are the wild-type parent (RH6210). The middle panels are *vac7*, and the bottom panels are *vac6*.

inherited little or no vacuole. Cell-sorting analysis clearly distinguishes between a wild-type strain and *vac* mutant strains.

The presence of a number of cells with intermediate fluorescence intensity (between the two peaks) in *vac6* and *vac7* mutants occurs because there is some dilution of the labeled parental vacuole. In each generation some mother cells still donate a little material to daughter cells.

vac Mutant Enrichment and Screening

The cell-sorting analysis demonstrated that flow cytometry may be effective for the enrichment of *vac* mutants. When a population of mutagenized cells labeled with FM4-64 is allowed to undergo approximately two doublings in fresh medium, the original (mother) *vac* mutant will be present with the most highly labeled cells, and daughters of the *vac* mutants will be among the least-labeled cells. Three independent populations of cells were mutagenized, labeled with FM4-64, and allowed to continue to grow for 5 h after FM4-64 was removed from the medium. The cells were sorted in the flow cytometer. The most highly labeled cells and the least-labeled cells were collected and allowed to form colonies on plates. A total of 1070 colonies were subsequently screened by fluorescence microscopy. Seventeen isolates were found to be defective in vacuole segregation. To identify any Class D *vps* mutants, we examined cells for the presence of

secreted CPY (see MATERIALS AND METHODS). Nine isolates were identified that secrete a large amount of CPY (Figure 2). These nine Class D *vps* mutants include four *vac1* alleles, two *vps34* alleles, and one *vps15* allele (Eric Whitters and Tom Stevens, personal communication). Complementation analysis of two of the *vps* mutants remains to be determined. The remaining eight isolates (L1, L5, L19, R3, R45, R46, R14, and R17) are non-*vps* *vac* mutants. Six isolates were backcrossed to the original parental strain at least three times. In each case the defect was found to be caused by a single recessive mutation. For two isolates, R3 and L5, the heterozygous diploids would not sporulate.

Complementation Analysis of *vac* Mutants

Diploids were generated by pair-wise crossing of the eight isolates and examined for complementation of the vacuole inheritance defect. This complementation analysis suggested that R45 and R46 are in one complementation group. None of the new *vac* mutants is allelic with any of the previously identified *vac2*, *vac5*, *vac6*, or *vac7* mutants. Thus, we define L1, L19, R45/46, R14, and R17 as *vac8*, *vac9*, *vac10*, *vac11*, and *vac12*, respectively. That *vac6*, *vac11*, and *vac12* each define new complementation groups was confirmed by their unlinked segregation in tetrad dissection. Most of the new *vac* mutants were isolated from the highly labeled population;

Figure 2. Secretion of vacuolar CPY in yeast mutants that are defective in vacuole inheritance. Patches of 17 isolates as well as wild-type (RHY6210), *vac1*- Δ 2, *vac2*, *vac6*, and *vac7* strains were grown on YPD plates for 1 d and then overlaid with a nitrocellulose filter for 12 h at 23°C. The filter was probed with CPY antiserum to visualize the secreted CPY.

vac9 was isolated from the least-labeled population. FACS enrichment from the highly labeled population was effective (4 *vac* mutants from 630 colonies). This can be compared with *vac2* as the only *vac* mutant identified among 800 temperature-sensitive strains (Shaw and Wickner, 1991).

vac Mutants Can Be Grouped into Three Classes on the Basis of Vacuole Morphology

The Class D *vps* mutants were found to possess vacuolar morphologies that differed significantly from wild-type vacuoles (Raymond *et al.*, 1992). The previously identified *vac* mutants also have a similar alteration in their vacuolar morphology (Shaw and Wickner, 1991; Weisman and Wickner, 1992; Nicolson *et al.*, 1995; Gomes de Mesquita *et al.*, 1996). *vac2*, *vac5*, and *vac6* have a major, round vacuole and fail to form segregation structures. (*vac1*, *vac3*, and *vac4* are allelic with known *vps* Class D mutants and have the expected vacuole morphology.) *vac7* also has a large round vacuole, yet its morphology is distinct from the mutants listed above.

Vacuole morphology in the new *vac* mutants (*vac8*–*vac12*) as well as in all the previously identified *vac* mutants was examined by fluorescence microscopy after cells were labeled with FM4-64. Interestingly, unlike the mutants listed above, *vac8*, *vac9*, *vac10*, R3,

and L5 have vacuolar morphologies close to wild type; the vacuoles frequently appear as several lobes that cluster in one region of the cytoplasm. However, unlike wild type, the buds inherit little or no vacuole. Mutants with this vacuole morphology are now called Class I mutants (Figure 3b). In contrast, *vac11* and *vac12* show identical vacuole morphologies to that of *vac6* and the Class D *vps* mutants. In all of these mutants, the vacuole is rounder. When labeled with FM4-64, *vac* mutants that we designate as Class II exhibit a heavily labeled region or nodule on the vacuole membrane (Figure 3c). Often this region is oriented toward the bud and may be an “arrested” segregation structure. Note that the size of this nodule varies considerably among individual cells. Our first guess was that, as the mother cell ages and continues to produce buds, this nodule would grow. Therefore we examined the size of the nodule relative to the number of bud scars on the mother cell. However, we did not observe any correlation between the age of the mother cell and the size of the nodule. Among the Class II mutants, most of the buds seem to inherit a vacuole, but the vacuole is much smaller than normal (see below). *vac5*, *vac6*, *vac11*, and *vac12* are Class II *vac* mutants.

vac7 also has an enlarged, round vacuole, but it looks very different from the Class II mutants. The vacuole in *vac7* cells is more enlarged, and the nodule found in the Class II mutants is not observed. Over 50% of the buds seem to lack a vacuole (Bonangelino, Catlett, and Weisman, unpublished data). Most important, *vac7* seems to be defective in vacuole membrane scission. First, no vacuolar lobes form. Second, vacuole membranes in the neck between the mother and bud often do not fuse, creating a shape like an open figure eight. *vac7* defines the Class III mutants (Figure 3d). Interestingly, *fab1*, a mutant isolated in a nonrelated mutant hunt (Yamamoto *et al.*, 1995), is phenotypically very similar to *vac7* and may also be considered a Class III *vac* mutant. *fab1* and *vac7* are not allelic with each other. Each new *vac* mutant was examined and assigned to one of the three classes (Table 2).

Vacuole inheritance in the new *vac* mutants was assessed by counting random fields of cells labeled with FM4-64. After removing FM4-64 from the medium, cells were allowed to grow for one doubling time. The presence of FM4-64-labeled vacuoles in the buds was scored (Table 3). *vac8* was the most severe mutant, with 94% of the population displaying no detectable fluorescence in the bud. In the Class II mutants *vac11* and *vac12*, 70% of buds have less than normal inheritance. In contrast, 96% of buds in the wild-type strain have normal vacuole inheritance.

All of the above mutants are defective in vacuole inheritance to varying extents, yet they all have the ability to grow a new vacuole. FM4-64 labeling of the

Figure 3. *vac* mutants can be grouped into three classes on the basis of vacuole morphology. Cells were labeled with FM4-64 for 1 h. Free dye was removed, and cells were resuspended in fresh YPD medium and allowed to grow for 3 h at 23°C. The vacuole morphology and inheritance were then viewed by fluorescence microscopy. A representative *vac* mutant from each class is shown. (a) Wild type (RHY6210); (b) *vac8* (Class I); (c) *vac11* (Class II); (d) *vac7* (Class III). Bar, 5 μ m.

vacuoles, followed by a chase period, allows the assessment of vacuole inheritance. In contrast, the fluorescent dye CDCFDA labels all existing vacuoles (Preston *et al.*, 1989). In the strongest mutant, *vac8*, two-thirds of the buds have CDCFDA-labeled vacuoles, whereas only 6% of those buds inherit at least some detectable vacuolar material from the mother cells (Table 3). This suggests that synthesis

of a new vacuole in the bud occurs rapidly, even before its separation from the mother cell. CDCFDA labeling of the other mutants revealed similar results. Rapid generation of a vacuole had also been observed previously for *vac1-1* (Weisman *et al.*, 1990). These observations suggest that de novo vacuolar synthesis occurs; however, it is possible that even in *vac8* there is still some minimal inheritance of FM4-64 that is not detected by fluorescence microscopy.

In addition to examination by microscopy, the vacuole inheritance in the new *vac* mutants was assessed by FACS analysis. As expected, the bimodal fluorescence distribution was also observed in the new mutants. The histograms of two examples, *vac8* (Class I) and *vac12* (Class II), are shown (Figure 4).

Table 2. Three classes of non-*vps* *vac* mutants

Class	<i>vac</i> mutants	Description of vacuole
I	<i>vac8</i> , <i>vac9</i> , <i>vac10</i>	multilobed
II	<i>vac2</i> , <i>vac5</i> , <i>vac6</i> , <i>vac11</i> , <i>vac12</i>	rounded, nodule on membrane
III	<i>vac7</i> , <i>fab1</i>	enlarged, scission defect

Table 3. Quantitation of vacuole inheritance in the *vac* mutants

Strain
WT	96%	2%	2%
<i>vac8</i>	5%	1%	94%
<i>vac9</i>	12%	2%	86%
<i>vac10</i>	30%	8%	62%
<i>vac11</i>	10%	74%	16%
<i>vac12</i>	12%	73%	14%
<i>vac8</i> (CDCFDA label)	12%	54%	34%

The first six strains were labeled with FM4-64 to measure vacuole inheritance. The last strain, *vac8* was labeled with CDCFDA (as a control) to indicate all vacuoles present. For FM4-64, cells were resuspended in fresh YPD medium after labeling and allowed to grow for one doubling time. For CDCFDA, the presence of the vacuoles in the buds was examined immediately after labeling. Random fields of cells were examined, and the percentage of cells with the indicated vacuole phenotype is shown. More than 170 cells were counted for each strain.

Examination of Vacuole Partitioning in Zygotes

To determine whether *vac* mutations affect intervacuole transfer, we examined the ability of parental vacuoles to exchange material by monitoring movement of FM4-64. Cells of one mating type were labeled with FM4-64, mixed with equal amounts of cells of the opposite mating type, and incubated at 23°C for 4–4.5 h. Vacuole inheritance in the zygotes was scored via

fluorescence microscopy (Table 4). As expected, in homozygous matings most of the parental vacuoles did not transfer the labeled vacuole material to the bud or to the originally unlabeled parent vacuole. In *vac9*, *vac10*, and *vac11* heterozygotes, most wild-type parental vacuoles (which were not labeled initially) received *vac* mutant parental vacuole material via the bud vacuole, suggesting that the vacuole segregation defect in the *vac* mutants can be rescued by soluble components from the wild-type parent. Although *vac8-1* and *vac12-1* are recessive alleles, vacuole segregation did not occur from the *vac8* parent or the *vac12* parent in heterozygous matings, suggesting that *VAC8* and *VAC12* each encode proteins that are not freely diffusible in the zygotes. Interestingly, vacuole inheritance in *vac1* heterozygous zygotes showed a similar pattern to *vac8* and *vac12* (Weisman *et al.*, 1990).

Protein Sorting from the Golgi to the Vacuole in the New *vac* Mutants Is Normal

Results from the CPY colony blot suggest that the new *vac* mutants do not mis-sort CPY to the cell surface. So that the fidelity of protein sorting from the Golgi to the vacuole could be tested further, cells were labeled for 5 min and then subjected to a nonradioactive chase. Samples were removed at the times indicated and separated into cell and medium fractions, as stated in

Figure 4. FACS analysis of vacuole inheritance in the new *vac* mutants. Cells were incubated at 23°C for 1 h with 80 μ M FM4-64 in YPD medium. The cells were harvested, and free dye was removed. At each doubling time point, cells were subjected to FACS analysis. Approximately 1×10^4 cells were counted in each analysis. Each panel indicates a separate strain and time point. Numbers for doubling times after removal of FM4-64 are shown at the top.

Table 4. Vacuole inheritance in zygotes

Cross
	Normal transfer	Transfer from labeled parent to bud only	No transfer from labeled parent
wt × wt	98%	2%	0%
<i>vac8</i> × <i>vac8</i>	0%	0%	100%
<i>vac9</i> × <i>vac9</i>	22%	17%	61%
<i>vac11</i> × <i>vac11</i>	16%	16%	68%
<i>vac12</i> × <i>vac12</i>	10%	6%	84%
<i>vac9</i> × wt	93%	1%	6%
<i>vac10</i> × wt	97%	0%	3%
<i>vac11</i> × wt	73%	8%	19%
<i>vac8</i> × wt	14%	9%	77%
<i>vac12</i> × wt	10%	2%	88%

The strains listed on the left in **bold** were labeled with FM4-64. After removing FM4-64 from the medium, the labeled cells were mixed with an equal number of cells of the opposite mating type. Cultures were incubated with shaking at 23°C for 4–4.5 h. Cells were examined by fluorescence microscopy and scored for the presence of labeled vacuoles. Vacuole segregation in the *vac10* homozygous cross was not scored because of the low frequency of zygote formation.

MATERIALS AND METHODS, and the extracts were immunoprecipitated sequentially with antiserum to CPY, PrA, and ALP. Transit of these proteins through the secretory pathway can be monitored by the appearance of intermediate forms. *vps16*, a Class C mutant (Banta *et al.*, 1988), was used as a control for *vps*-type secretion. WT and *vac* mutants exhibit similar kinetics of maturation for CPY, PrA, and ALP (Figure 5). Also, none of the *vac* mutants secretes precursor CPY or PrA to the medium fraction when compared with *vps16*. Therefore, we conclude that *vac8*, 9, 10, 11, and 12 have no significant defect in protein targeting to the vacuole via the secretory pathway.

V-ATPase Is Assembled in the vac Mutants

In Class D *vps* mutants, the vacuolar ATPase (V-ATPase) fails to assemble on the vacuolar membrane (Raymond *et al.*, 1992). Failure to assemble the V-ATPase results in a cytoplasmic localization of VMA2 gene product, the 60-kDa V-ATPase subunit. Indirect immunofluorescence microscopy was used to analyze the localization of the 60-kDa subunit in the new *vac* mutants. The 60-kDa subunit localizes to the vacuole membrane both in wild-type cells and in the mutants (Figure 6). As a control, in *vac1* cells the V-ATPase was nonvacuolar and diffuse (Figure 6e). Note, however, that the altered vacuolar morphology of the Class II mutant *vac6* (Figure 6c) and the Class III mutant *vac7* (Figure 6d) can be clearly observed. All of the new *vac* mutants were tested for the localization of the 60-kDa V-ATPase subunit. In each case the subunit was local-

ized on the vacuole membrane. Correct assembly of the V-ATPase is an indication that the vacuole inheritance defect in these mutants is not due to a general defect in vacuole biogenesis.

vac8 Is Also Defective in API Transport to the Vacuole

Aside from the secretory pathway, some proteins enter the vacuole directly from the cytoplasm (Yoshihisa and Anraku, 1990; Klionsky *et al.*, 1992). Recently, several cytoplasm-to-vacuole-targeting (*cvt*) mutants have been isolated that are defective in aminopeptidase I (API) transport from the cytoplasm to the vacuole (Harding *et al.*, 1995). Considering the large overlap of *vps* mutants and *vac* mutants, we tested for an overlap between cytoplasm-to-vacuole targeting and vacuole segregation. Defects in the Cvt pathway were followed by examining the processing of API. Cell extracts were prepared, resolved by SDS-PAGE, and probed with anti-mAPI antiserum. Although *vac6*, *vac7*, and most of the new *vac* mutants showed normal API processing, *vac8* was found to accumulate a significant amount of proAPI (Figure 7, lane 6). Complementation analysis suggested that *vac8* is not allelic with any of the previously identified *cvt* mutants. In addition, none of the *cvt* mutants shows a defect in vacuole segregation (our unpublished results). Analysis of 16 tetrads from two backcrosses of *vac8* to the parental strain revealed that the vacuole segregation defect cosegregates with the API processing defect.

To determine whether *vac8* is defective in API transport from the cytoplasm to the vacuole, a differential lysis fractionation experiment was performed (Scott and Klionsky, 1995). Yeast cells were converted to spheroplasts and subjected to gentle osmotic lysis to break the plasma membrane while leaving the vacuole membrane intact. Lysates were fractionated by centrifugation and then subjected to Western blot analysis. All the accumulated proAPI in *vac8* was found in the supernatant fraction, as was the majority of the cytosolic marker phosphoglycerate kinase (PGK; Figure 7, lane 8). In contrast, in wild-type cells, the accumulated mature API is found in the pellet fraction, as was PrA (Figure 7, lane 2). Furthermore, the proAPI in *vac8* was accessible to proteinase K in the absence of detergent (Figure 7, lane 9). As had been observed with the original *cvt* mutants (Harding *et al.*, 1995), the proAPI is digested to a lower molecular weight, corresponding to the mature protease-resistant form. These data suggest that, like the *cvt* mutants, *vac8* is blocked in API transport into the vacuole.

The Endocytosis and Autophagy Pathways Are Normal in the New vac Mutants

Endocytosis is another pathway of membrane movement to the vacuole. As a first assay for endocytosis,

the new *vac* mutants were labeled with Lucifer yellow, a marker for fluid phase endocytosis (Dulic *et al.*, 1991). All of the new *vac* mutants, except *vac9*, accumulated levels of Lucifer yellow similar to that seen in our wild-type strain (our unpublished results). However, analysis of α -factor uptake in *vac9* indicated that it, too, undergoes nearly wild-type rates of endocytosis (our unpublished results). We also examined vacuole segregation in the known endocytosis mutants (*end3–end13*) (Raths *et al.*, 1993; Munn and Riezman, 1994; Munn *et al.*, 1995). Most of them did not show a vacuole segregation defect. The exception was *end12*, which is allelic with *vps34*, a Class D *vps* mutant.

The new *vac* mutants were then analyzed for their ability to perform autophagy as assessed by their ability to accumulate autophagic bodies. The *vac* mutants do accumulate autophagic vesicles when transferred from rich medium to nitrogen starvation medium (our unpublished results). Thus endocytosis and autophagy in these new *vac* mutants seem to be normal. Together with the normal protein sorting from the Golgi to the vacuole, these results suggest that the new *vac* mutants are specifically defective in vacuole seg-

regation but not in other membrane trafficking processes. It is of interest that, although there are at least four pathways of protein movement to the vacuole, to date only the *vps* mutants commonly have a vacuole-partitioning defect.

Nuclei and Mitochondria Are Inherited Normally in the *vac* Mutants

We examined whether there are any effects of *vac* mutations on mitochondrial or nuclear partitioning. Staining with the mitochondria-specific dye DiOC₆(3) showed that mitochondria in the *vac* mutants have normal morphology and can be transferred into the growing buds (our unpublished results). DAPI staining revealed that nuclear segregation is also normal (our unpublished results).

DISCUSSION

Vacuole partitioning during the cell cycle is a highly regulated process. Shortly after bud emergence, a structure forms on the mother vacuole membrane that

Figure 5. Intracellular sorting of vacuolar proteinases in the new *vac* mutants. WT (LWY7213), *pep4* (LWY7210), *vps16*, *vac9* (LWY2130), *vac10* (LWY1701 bearing a single-copy *PEP4* plasmid), and *vac11* (LWY1045 bearing a single-copy *PEP4* plasmid) were labeled in the presence of 2.5 mg/ml BSA for 5 min by using [³⁵S]methionine, followed by a nonradioactive chase for the times indicated. At each time point, the cells were pelleted in the presence of 1 mM sodium azide and separated into cell and medium fractions. Radiolabeled cell extracts were immunoprecipitated as described in MATERIALS AND METHODS. Cell and medium fractions were analyzed by SDS-PAGE. (A) Cell fractions and the 30-min medium fraction were immunoprecipitated with CPY and PrA antisera. (B) Cell fractions from the 30-min chase point were immunoprecipitated with ALP antiserum. Positions of each precursor and mature protein species are shown; M, medium fraction from the 30-min chase point. Samples from *vac8* and *vac12* look essentially like *vac11*.

Figure 6. The localization of the 60-kDa V-ATPase subunit is normal in the three classes of *vac* mutants. Cells were fixed, converted to spheroplasts, and stained with monoclonal antibody to the 60-kDa V-ATPase subunit. A representative mutant from each class as well as wild type and *vac1-Δ2* are shown; (a) wild type (RHY6210); (b) *vac8* (Class I); (c) *vac6* (Class II); (d) *vac7* (Class III); (e) *vac1-Δ2*. The wild-type cells without primary antibody incubation are also shown (f). Bar, 5 μ m.

rapidly extends into the bud. The mother cell vacuole and recently transferred bud vacuole remain connected for ~20% of the cell cycle (Gomes de Mesquita *et al.*, 1991) and in this time continue to exchange their contents (Weisman and Wickner, 1988). In some respects, vacuole partitioning is very similar to other forms of membrane traffic. In all cases, membranes need to deform, there are fission events and fusion events, and there is a specific direction in which movement occurs. Other aspects of vacuole partitioning are more unique. For instance, rather than a vesicle moving from a donor membrane to an acceptor membrane, the segregation structure travels to a location where no previous organelle has been detected. Moreover, movement is restricted to a specific portion of the cell cycle.

We have recently begun to identify molecules involved in vacuole inheritance and have shown that actin, profilin, and a Class V myosin are all required for this event (Hill, Catlett, and Weisman, unpublished data). However, most of the molecules that are essential to vacuole partitioning remain to be determined. One effective approach toward the identifica-

tion of required genes and toward the isolation of protein complexes is the isolation of mutants defective in a specific process.

Initially, there were no effective ways to isolate *vac* mutants. First, there were no obvious plate assays that would allow the selection of *vac* mutants. Identification of *vac* mutants required a method of labeling the vacuole and screening candidate strains by microscopy. Recently, a plate assay for vacuole inheritance has been developed and was successfully used to isolate two mutants, *vac6* and *vac7* (Gomes de Mequita *et al.*, 1996). A second problem is the significant overlap between mutants that are defective in vacuole protein sorting and vacuole partitioning. *vac1*, the first mutant identified as having a defect in vacuole partitioning, is now known to be allelic with *pep7/vps19* (Zhang *et al.*, 1992). In the case of the phenotypic lag plate assay, 14 candidates were identified, but again, most had *vps* mutant phenotypes, and only two were non-*vps* *vac* mutants (Gomes de Mequita *et al.*, 1996). *vac2*, the first *vac* mutant identified with no apparent defect in vacuole protein sorting, was isolated by screening 800 temperature-sensitive strains. However three of the

Figure 7. The *vac8* mutant accumulates proAPI outside of the vacuole. Wild-type (LWY7213) and *vac8* (LWY2077) strains were grown to an OD₆₀₀ of ~1, and 10 OD₆₀₀ units were harvested. The cells were converted to spheroplasts and subjected to differential osmotic lysis; samples were prepared, and protease treated as stated in MATERIALS AND METHODS. Approximately 0.2 OD₆₀₀ (anti-API and anti-PrA blots) or 0.02 OD₆₀₀ equivalents (anti-PGK blot) of protein from each fraction were separated on three identical 8% SDS-PAGE gels, transferred to PVDF membrane, and probed as described in MATERIALS AND METHODS. Positions of each protein species are shown. Numbers at the right indicate the molecular weight in kDa; T, total before osmotic lysis; P, membrane pellet from 6000 × g centrifugation; S, supernatant from 6000 × g centrifugation; Pr, total fraction treated with proteinase K; Tx, total fraction treated with proteinase K in the presence of 0.2% Triton X-100.

four *vac* mutants identified in that screen were also defective in vacuole protein sorting (Shaw and Wickner, 1991). A third problem is that yeast are able to grow a vacuole in a bud that has received little or possibly no vacuolar material (Weisman *et al.*, 1990). For example, although *vac1* mother cells with large buds contained an essentially normal-sized vacuole, the buds and unbudded cells often seemed to contain no vacuole or contained a smaller-than-normal vacuole. Our observation in *vac8* that approximately two-thirds of buds have a detectable, but not inherited, vacuole also supports this finding (Table 3). It was proposed that, in the absence of significant inheritance, the new vacuole arises from Golgi-derived vesicles or from endocytosis (Weisman and Wickner, 1990). Moreover, formation of a normal-sized vacuole in a *vac2* unbudded cell with no apparent vacuole is indeed rapid, occurring in <50 min, which is significantly less than the doubling time of a wild-type strain (Gomes de Mesquita, Shaw, and Woldringh, unpublished data). This capacity to form a new vacuole mitigates the severity of any vacuole inheritance defect.

The fluorophore FM4-64 has greatly aided our ability to score a defect in vacuole inheritance. CDCFDA, FITC, and quinacrine had all been used previously to visualize yeast vacuoles (Preston *et al.*, 1987, 1989;

Weisman *et al.*, 1987; Pringle *et al.*, 1989). However, because these compounds reversibly cross the vacuolar membrane, none could be used to measure vacuole inheritance. The endogenous *ade2* fluorophore had been used for monitoring vacuole inheritance (Weisman *et al.*, 1987). Whereas the *ade2* fluorophore persists in the vacuole for several generations, conditions under which the fluorophore forms greatly perturb vacuole morphology and size. Moreover, the fluorophore only accumulates when *ade2* yeast are incubated in the absence of adenine, a condition in which the strain cannot grow. FM4-64 can be added exogenously to exponentially growing cells. Once the fluorophore reaches the vacuole membrane, it is stable. Furthermore, vacuole morphology and size are not perturbed (Vida and Emr, 1995).

We have developed a procedure to use FACS as a means to monitor vacuole inheritance and enrich for *vac* mutants in yeast. FACS analysis offers a means of analyzing a large population of cells. In a typical analysis, ~1 × 10⁴ cells are examined. As expected, when *vac* mutants are labeled with FM4-64 and then reintroduced into fresh media, during the chase period a bimodal distribution is observed. The distance between the two peaks and the depth of the valley between them correlates with the severity of the inheritance defect. For example, as assessed by microscopy, *vac8* was the most severe mutant isolated in this screen (Table 3). As measured by FACS, *vac8* was also the most severe mutant (Figure 4; our unpublished results).

One of the interesting findings from our new screen is that *vac* mutants may be divided into at least three classes based on vacuole morphology. The first group, Class I mutants, had not been described previously. Class I mutants have relatively normal vacuole morphology; the vacuole seems to be multilobed. Three of the five new complementation groups we identified have been placed into this new group. Because vacuole morphology is normal and other measurable aspects of vacuole biogenesis are normal, it is likely that study of this class of mutants will be particularly revealing.

The first *vac* mutants studied we now designate as Class II mutants. *vac2*, *vac5*, *vac6*, *vac11*, *vac12*, and all the Class D *vps* mutants look very similar when examined by fluorescence microscopy. Rather than a vacuole composed of many lobes, there is generally a single major vacuole. Earlier examinations were performed by CDCFDA and FITC. Re-examination of these strains with FM4-64 revealed more detail, and these strains still seem morphologically very similar. With FM4-64, one can additionally see a spot or a nodule on the vacuole membrane (Figure 3c). This region is often oriented toward the bud. Perhaps this region represents an arrested segregation structure.

Once we identify proteins specific for the segregation structure, we will be able to test this hypothesis.

vac7, a Class III mutant, has a rounded vacuole but is very different in appearance when compared with the Class II mutants. *vac7* vacuoles are much rounder and larger, but most important, when a vacuole is present in the bud, it is often connected to the mother vacuole (Figure 3d). The vacuole membrane is constricted in the neck of the yeast, but the vacuoles are delayed in pinching off and separating from each other. It seems that the *vac7* vacuole is defective in membrane scission/periplasmic fusion. This type of defect would explain both the lack of vacuole lobes and also the persistent connection between mother and bud vacuoles. We are pursuing studies of the Class III mutants. Preliminary analysis suggests that membrane scission is the primary defect and that normal vacuole partitioning does not occur because of the inability of these mutants to form a normal segregation structure (Bonangelino, Catlett, and Weisman, unpublished results).

One of the ways that we examined the new *vac* mutants was to test vacuole partitioning in zygotes formed between a given mutant and the corresponding wild-type parent. When wild-type haploids mate and form a zygote, the original parental vacuoles never fuse. However, after formation of the first zygotic bud, each parental vacuole forms a segregation structure. The segregation structures each migrate to the bud and there fuse with each other (Weisman and Wickner, 1988). When the vacuole of one parent is originally labeled with a fluorophore, fusion of each parental segregation structure results in transfer of label from the mother to the bud and subsequently to the other parental vacuole. Failure to form the segregation structure in either parent blocks the exchange of the vacuolar material between the two parents. All of the new *vac* alleles are recessive; therefore, the heterozygous diploids display wild-type vacuole partitioning.

Examination of vacuole partitioning in zygotes resulting from a heterozygous cross tests whether the defective gene product is freely soluble or attached to a complex (Weisman *et al.*, 1990). Because bud emergence occurs shortly after nuclear fusion, it is unlikely that a defect would be corrected because of the synthesis of a new gene product. Rather, complementation of a defect in a heterozygous zygote would most likely be due to diffusion of the wild-type protein to the formerly defective vacuole. In fact, most of the new *vac* mutants are complemented in this manner. However, two of the new mutants, *vac8* and *vac12*, were not corrected in a heterozygous mating. As described below, additional information suggests that the *vac8* gene product may in fact reside on the vacuole membrane.

Cytoplasm-to-vacuole protein targeting does not seem to be the primary mode of transport to the vacuole for proteins involved in vacuole partitioning. We screened seven *cvt* mutants that are defective in transporting API to the vacuole (Harding *et al.*, 1995) and found that all were wild type for vacuole partitioning. However, one of our new mutants, *vac8*, does have a defect in the localization of API. We propose that *vac8* is essential for both API targeting and vacuole partitioning. As such, it is possible that Vac8p resides on the vacuolar membrane. This localization would be consistent with the heterozygous zygote studies. We are currently working to clone the *VAC8* gene.

Earlier studies indicated that there would be significant overlap between the *vps* mutants and *vac* mutants. Indeed, of our original 17 isolates, 9 were Class D *vps* mutants. Fortuitously, the proportion of *vps* mutants isolated in our screen was lower than that reported previously. Although there are at least four pathways in addition to vacuole inheritance that transport material to the vacuole, only mutants defective in the Vps pathway have turned up in significant numbers in *vac* mutant screens. We would like to understand the significance of this overlap between *vac* mutants and *vps* mutants. The simplest explanation is that molecules essential for vacuole partitioning transit to the vacuole via the Vps pathway. Although this hypothesis may be correct, there is at least one puzzling observation. The *vps* Class E mutants accumulate an exaggerated late endosome and, like the Class D mutants, are defective in the targeting of many vacuolar proteins to the vacuole (Raymond *et al.*, 1992). However, unlike the Class D mutants, *vps* Class E mutants have no apparent defect in vacuole partitioning. It is still more than a formal possibility that some of the gene products that are required for accurate vacuole protein sorting also function directly in vacuole partitioning.

Because of the large overlap between the *vps* Class D mutants and *vac* mutants, we expected to find an overlap between endocytosis mutants and *vac* mutants and between autophagy mutants and *vac* mutants. So far the only significant overlap between *end* mutants and *vac* mutants is that *vps* Class D mutants display defects in vacuole partitioning and endocytosis. An overlap between non-*vps* *end* mutants and non-*vps* *vac* mutants has not been observed. In addition to screening our new mutants for defects in endocytosis, we examined the available *end* mutants for defects in vacuole segregation. *end3–end11* and *end13* all displayed wild-type vacuole partitioning. *end12* is defective in vacuole partitioning, but it is allelic with *vps34*, a Class D *vps* mutant. Interestingly, *end7-1*, which does not have a defect in vacuole partitioning, is allelic with *ACT1*. From previous studies we have identified actin alleles that are defective in vacuole partitioning (Hill,

Catlett, and Weisman, unpublished data). This actin allele specificity further supports our observations that, aside from selected *vps* mutants, there is little overlap between mutants defective in endocytosis and vacuole partitioning. None of the new *vac* mutants reported here is allelic with *ACT1*.

Our development of a rapid and simple screen for mutants defective in vacuole partitioning will greatly enhance our ability to elucidate the molecular basis of this process. Moreover, we have a relatively rapid and objective way to measure the degree of a vacuole inheritance defect in a large population of cells. Our method of isolating *vac* mutants may also be useful for the isolation of mutants defective in the partitioning of other organelles.

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