Protein Sorting in *Saccharomyces cerevisiae*: Isolation of Mutants Defective in the Delivery and Processing of Multiple Vacuolar Hydrolases

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Using a selection for spontaneous mutants that mislocalize a vacuolar carboxypeptidase Y (CPY)-invertase fusion protein to the cell surface, we identified vacuolar protein targeting (*vpt*) mutants in 25 new *vpt* complementation groups. Additional alleles in each of the eight previously identified *vpt* complementation groups (*vpt1* through *vpt8*) were also obtained. Representative alleles from each of the 33 *vpt* complementation groups (*vpt1* through *vpt33*) were shown to exhibit defects in the sorting and processing of several native vacuolar proteins, including the soluble hydrolases CPY, proteinase A, and proteinase B. Of the 33 complementation groups, 19 were found to contain mutant alleles that led to extreme defects. In these mutants, CPY accumulated in its Golgi complex-modified precursor form which was secreted by the mutant cells. Normal protein secretion appeared to be unaffected in the *vpt* mutants. The lack of significant leakage of cytosolic markers from the *vpt* mutant cells indicated that the vacuolar protein-sorting defects associated with these mutants do not result from cell lysis. In addition, the observation that the precursor rather than the mature forms of CPY, proteinase A, proteinase B were secreted from the *vpt* mutants was consistent with the fact that mislocalization occurred at a stage after Golgi complex-specific modification, but before final vacuolar sorting of these enzymes. Vacuolar membrane protein sorting appeared to be unaffected in the majority of the *vpt* mutants. However, a subset of the *vpt* mutants (*vpt11*, *vpt16*, *vpt18*, and *vpt33*) was found to exhibit defects in the sorting of a vacuolar membrane marker enzyme, α-mannosidase. Up to 50% of the α-mannosidase enzyme activity was found to be mislocalized to the cell surface in these *vpt* mutants. Seven of the *vpt* complementation groups (*vpt3*, *vpt11*, *vpt15*, *vpt16*, *vpt18*, *vpt29*, and *vpt33*) contained alleles that led to a conditional lethal phenotype; the mutants were temperature sensitive for vegetative cell growth. This temperature-sensitive phenotype has been shown to be recessive and to cosegregate with the vacuolar protein-sorting defect in each case. Tetrad analysis showed that *vpt3* mapped to the right arm of chromosome XV and that *vpt15* mapped to the right arm of chromosome II. Intercrosses with other mutants that exhibited defects in vacuolar protein sorting or function (*vpl*, *sec*, *pep*, and *end* mutants) revealed several overlaps among these different sets of genes. Together, these data indicate that more than 50 gene products are involved, directly or indirectly, in the process of vacuolar protein sorting.

A basic and fundamental question in biology is that of how a cell is constructed spatially from the information encoded by its genes and from maternally inherited cues and structures. This spatially segregated cellular organization is critical for normal cell functioning. To understand the process, it is important to know not only how the cytoskeletal elements fit together and define the overall shape and structure of the cell but also how proteins, structural and otherwise, find their way to appropriate locations within the cell. An analysis of the assembly and maintenance of each organelle individually could provide a view of the mechanism underlying the cellular construction process.

Protein sorting to mammalian lysosomes represents one of the best-characterized protein delivery pathways (19, 37). Much is known about the route that is followed by lysosomal enzymes, and a few components of the sorting machinery have been characterized at the biochemical and molecular levels. However, this is a complex process and we still lack a detailed understanding of the molecular mechanisms underlying this sorting pathway. We chose to work on protein sorting to the yeast equivalent of the mammalian lysosome, the vacuole. The yeast *Saccharomyces cerevisiae* is the

organism of choice because we wished to take a genetic approach to solving the vacuolar protein targeting problem. The vacuole is an acidic compartment that is involved in amino acid and inorganic ion storage, as well as various degradative and nutrient recycling functions, especially under starvation conditions (20, 43). It contains a variety of hydrolytic enzymes, including the soluble glycoproteins carboxypeptidase Y (CPY), proteinase A (PrA), and proteinase B (PrB), and several membrane-bound enzymes, including an α-mannosidase, a proton-translocating ATPase, dipeptidylaminopeptidase B, and several permeases (43, 44).

Vacuolar proteins transit along with proteins destined for secretion through the early stages of the secretory pathway (38). The transport of the luminal enzymes CPY and PrA has been studied in the greatest detail (18, 18a, 35a). Each of these enzymes is synthesized initially as an inactive preproenzyme that is translocated into the lumen of the endoplasmic reticulum, where the transient N-terminal signal peptide is removed and the proteins are modified with an N-linked core oligosaccharide (12). These intermediate precursor forms are referred to as p1CPY and p1PrA. The p1 forms are then delivered to the Golgi complex, where further glycosyl modifications convert them to larger p2 forms. At this stage, it is presumed that proteins on their way to the vacuole are sorted from other secretory proteins destined for

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secretion or assembly into the plasma membrane (9, 11, 38). Vesicular carriers are presumed to mediate Golgi complex-vacuole transfer of these enzymes. At present there is no evidence concerning the existence of an intermediate compartment between the Golgi complex and the vacuole in *S. cerevisiae* analogous to the endosomal compartment detected in mammalian cells. Just prior to, or more likely upon, arrival in the vacuole, the p2 forms of CPY and PrA are proteolytically cleaved to their mature forms (22, 23). This proteolytic maturation is dependent on the presence of active vacuolar PrA, and involves the removal of N-terminal prosegments from each protein (13). Similarly, other vacuolar enzymes such as PrB also appear to be made and transported in their precursor forms and to undergo PrA-dependent proteolytic maturation in the vacuole (21, 22; C. Moehle, C. K. Dickxon, and E. Jones, J. Cell Biol., in press).

The observations that mutations in the CPY vacuole sorting signal or high gene dosage-induced overproduction of CPY and PrA lead to missorting and secretion of these proteins indicate that a failure to sort vacuolar proteins results in their delivery to the cell surface (15, 39, 41). This rationale was used by Rothman and Stevens (35) to identify mutants that were defective in vacuolar protein sorting by isolating yeast strains that secreted CPY. Through this approach, they identified mutants that were assigned to eight complementation groups (referred to as *vpl*, for vacuolar protein localization-defective mutants). Independently, we used a gene fusion-based selection scheme to isolate mutants defective in vacuolar protein targeting (*vpt*) (2). By this approach, mutants defective in the vacuolar sorting of a CPY-invertase (Inv) hybrid protein (CPY-Inv433) (15) were selected. Eight *vpt* complementation groups were identified and shown to missort and secrete the CPY-Inv hybrid protein as well as the wild-type CPY protein (2). The advantages of the Inv fusion approach include the following. (i) The identification of mutants is not dependent on their secretion of active CPY; (ii) the gene fusion approach, in theory, could be applied to any vacuolar protein whether its biochemical activity was known or not; and (iii) the absence or presence of external Inv activity can be easily assayed, and powerful genetic selection procedures have been developed that demand the presence of external Inv activity.

In this study we greatly extended the gene fusion approach to isolate and biochemically characterize some 500 new *vpt* mutants. Here we describe the genetic and biochemical analysis of these vacuolar protein-targeting mutants. Our results indicate that many different gene functions participate in the events associated with normal vacuolar protein sorting. This process is likely to involve several distinct steps, including the selective recognition of vacuolar proteins, their packaging into transport vesicles, delivery of these vesicles to the correct target organelle, recognition and fusion with the target, release of the vacuolar proteins, and recycling of transport components for additional rounds of protein sorting.

MATERIALS AND METHODS

Yeast strains. *S. cerevisiae* SEY6210 (*MATα leu2-3,112 ura3-52 his3-Δ200 trpl-Δ901 lys2-801 suc2-Δ9 GAL*) and SEY6211 (*MATa leu2-3,112 ura3-52 his3-Δ200 trpl-Δ901 ade2-101 suc2-Δ9 GAL*) were constructed by standard genetic methods (36). Both strains contained a complete deletion (*suc2-Δ9*) of the chromosomal copy of *SUC2* and contained no other unlinked Inv structural genes (7).

SEY6210 and SEY6211 were transformed to uracil independence with either of two CPY-Inv fusion-encoding plasmids, pCYI-50 and pCYI-433 (15). *vpt* mutant alleles *vpt1-1* through *vpt8-1* (2) in the SEY2101 (*MATa ura3-52 leu2-3,112 suc2-Δ9 ade2-101*) (7) strain background were used for complementation tests with new *vpt* mutants isolated in strain SEY6210 (this study). They were also crossed with strain SEY6210 to give *MATα lys2-801 vpt1-1* through *vpt8-1* strains for crossing with new *vpt* mutants isolated from SEY6211.

Media and genetic methods. YPD medium, minimal yeast medium, sporulation medium, and bromocresol purple indicator plates were prepared as described previously (36). The bromocresol purple plates contained 2% sucrose as the sole carbon source and were spread with 0.2 mg of antimycin A in 95% ethanol just before use (2). The minimal medium of Wickerham (42), which was modified as described previously (15), was used for cell growth before and during labeling with $Na^{35}SO_4$ (ICN Radiochemicals). Standard genetic methods (36) were used throughout this study. Replica plating to test phenotypes of segregants was done with a 48-pronged replicator by using dilution in sterile 96-well microtiter dishes (24). Rapid complementation analysis was carried out by using the pronged replicator to transfer arrays of 48 new *vpt* mutants to microtiter dishes containing *vpt* tester strains of the opposite mating type suspended in liquid YPD medium. Mating and diploid growth were allowed to occur in the wells for 2 days at 26°C. Replicas were then made onto minimal medium, which was supplemented to select for diploid strains. The resulting patches of diploid strains were transferred without dilution to YP fructose and assayed for external Inv activity by using a rapid filter replica assay method (15, 18) to score complementation.

Enzyme assays. The procedures and unit definitions used for assays of invertase and α-glucosidase (15), CPY (39), PrA (1), α-mannosidase (28), and glyceraldehyde-3-phosphate dehydrogenase (Worthington Enzyme Manual; Worthington Diagnostic, Freehold, N.J.) (3) have been described previously.

Labeling, fractionation, and immunoprecipitation. Spheroplasts were prepared and labeled by a modification of a previously described method (32). Cells were grown to the mid-log phase in the minimal medium of Wickerham (42) that was supplemented with 0.2% yeast extract. Four units of cells at an optical density at 600 nm were then centrifuged and suspended in 0.1 M Tris sulfate (pH 9.4)–10 mM dithiothreitol and incubated at 30°C for 5 min, centrifuged again, and suspended in the minimal medium of Wickerham (42) that was adjusted to pH 7.4 and that contained 1.3 M sorbitol. Lyticase was added to a final activity of 30 U per optical density unit at 600 nm, and the cultures were incubated at 30°C for 20 min to remove the cell wall. Spheroplasts were pelleted, suspended in the minimal medium of Wickerham (42) containing 1.3 M sorbitol, and labeled with Tran^{35}S label (0.2 mCi/ml) for 20 min at 30°C. Chase was initiated by the addition of methionine (final concentration, 50 μg/ml). The labeled culture was separated into spheroplast (intracellular) and medium (extracellular) fractions. To examine proteins in the medium fraction, cells were grown and labeled as described previously (15). The whole cells were then separated from the medium fraction by centrifugation, precipitated with 5% trichloroacetic acid, suspended in loading buffer, and run on sodium dodecylsulfate–10% polyacrylamide gels. Antisera to PrA, CPY, and Inv were prepared as described previously (18a).

Materials. Lyticase was obtained from Enzogenetics,

Tran^{35}S label was from ICN Radiochemicals and all other chemicals were from Sigma Chemical Co. (St. Louis, Mo.). Antiserum to PrB was a gift from C. Moehle and E. Jones.

RESULTS

Isolation of 25 new *vpt* complementation groups. Previously, we observed that short amino-terminal domains of the CPY sequence, when fused to mature enzymatically active Inv, can quantitatively divert delivery of this normally secreted enzyme to the yeast vacuole (15). Yeast cells require external Inv activity to grow on sucrose as a sole fermentable carbon source, so we could exploit the sorting behavior of the CPY-Inv hybrid protein to select mutants that missort and secrete the fusion protein. Yeast strains deleted for all endogenous genes encoding invertase (Δ*suc*) but containing a low-copy-number (*CENIV, ARSI*) plasmid encoding an appropriate CPY-Inv hybrid protein as their only source of Inv activity could not grow on sucrose, because all the Inv activity was sequestered in the vacuole (Fig. 1). Suc$^+$ derivatives of such cells can be isolated simply by selecting for growth on sucrose-containing medium. Based on earlier results, many of these mutants were expected to mislocalize the native vacuolar enzyme CPY as well, because targeting of the fusion proteins to the vacuole is dependent on the presence of the CPY vacuolar sorting signal at the amino-terminal end of the CPY-Inv hybrid proteins (15). In the present study, we decided to select such mutants using two different CPY-Inv hybrid proteins: CPY-Inv50 (encoded by plasmid pCYI-50) and CPY-Inv433 (encoded by plasmid pCYI-433) (Fig. 1). These gene fusions were composed of the coding sequence for the N-terminal 50 and 433 amino acids of proCPY, respectively, which were fused in-frame to a truncated form of the *SUC2* gene carried on the *Escherichia coli-S. cerevisiae* shuttle vector pSEYC306 (15). This modified *SUC2* gene lacked its 5'-regulatory sequences and the coding sequence for its amino-terminal signal peptide (15) (Fig. 1).

Suc$^+$ mutant strains were isolated from the Δ*suc2* strains SEY6210 and SEY6211 harboring either pCYI-50 or pCYI-433. To select Suc$^+$ mutants, single colonies of the four parental strains were plated onto YP medium with sucrose as the sole carbon source, antimycin A as an inhibitor of respiration, and bromocresol purple as a pH indicator; bromocresol purple turns yellow when sucrose is being fermented. Only one spontaneous mutant derived from each parental colony was picked, to ensure that each mutation event was independent. A total of 505 Suc$^+$ mutants were obtained and characterized: 241 *MATa* Suc$^+$ *vpt* mutants (isolated from SEY6211) and 264 *MATα* Suc$^+$ *vpt* mutants (isolated from SEY6210). We confirmed that these *vpt* mutants secreted the CPY-Inv hybrid protein by carrying out a rapid filter assay for external Inv activity. This assay involved patching the mutants onto YP plates containing fructose, incubating the plates at 26°C for 24 to 36 h, and then making replicas onto Whatman no. 1 filter papers (Whatman, Inc., Clifton; N.J.) that were presoaked in an Inv assay solution (15, 18). External Inv reacted with the assay mixture to produce a red spot at the position of the appropriate cell patch. The presence of internal Inv activity was confirmed by exposing the cell patches to chloroform vapor, which lyses the yeast cells, prior to transfer onto the assay filter. This simple method permitted us to get an initial indication of the extent of the mislocalization defect for each new *vpt* mutant. All of the *vpt* mutants were found to express external Inv activity by this assay. In the parental control
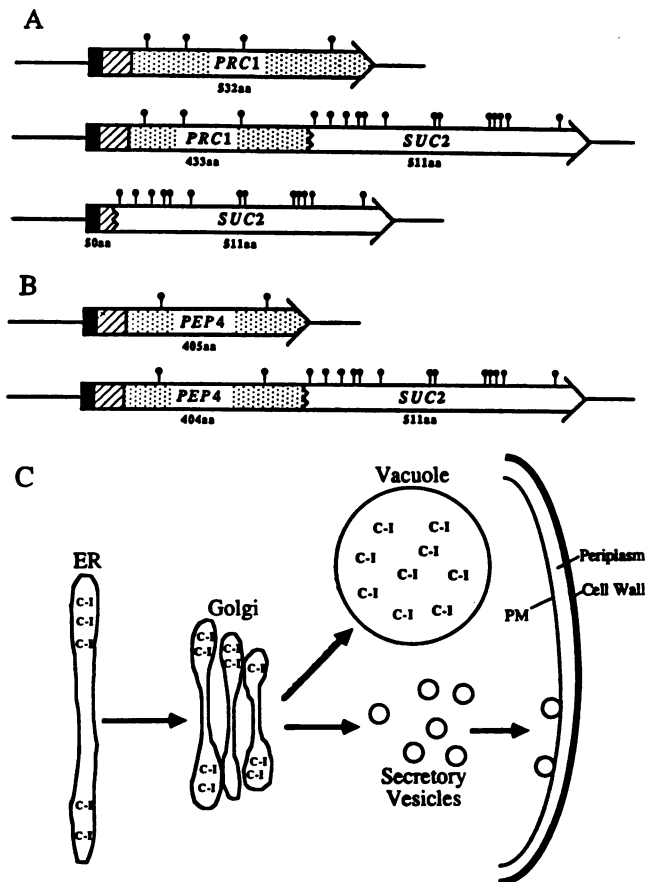

FIG. 1. Selection scheme for *vpt* mutants. (A) *PRC1-SUC2* gene fusions encoding CPY-Inv hybrid proteins. At the top is a schematic representation of the gene encoding CPY (*PRC1*). The signal sequence is indicated by a solid black box, the prosegment is indicated by hatched lines, and the mature sequences are indicated by dots (15). The positions of the oligosaccharide addition sites are indicated by tadpole-shaped forms. The two fusions of *PRC1* sequences to *SUC2* used to select *vpt* mutants are shown below. The 50-amino-acid fusion received extensive glycosyl modification, whereas the 433-amino-acid fusion did not become hyperglycosylated. (B) The gene encoding PrA (*PEP4*) and a representation of the *PEP4-SUC2* gene fusion tested for its localization in the *vpt* mutants (Table 1). (C) A simple diagram showing the vacuolar protein delivery route followed by the CPY-Inv hybrid protein (C-I). The *vpt* mutants were selected for their mislocalization of some or all of this CPY-Inv hybrid protein to the cell surface. For some of the *vpt* mutants, secretion of the mislocalized CPY-Inv was shown to be dependent on *SEC1* gene function, indicating that delivery to the surface is mediated by secretory vesicles (2). ER, Endoplasmic reticulum; aa, amino acid; PM, plasma membrane.

strains, all of the Inv activity was internal, as determined by the assay.

The Inv filter assay was also used to determine whether the *vpt* mutations were dominant or recessive in diploid strains derived by backcrossing each of the *vpt* mutants to the appropriate parental strain (SEY6210 or SEY6211). Recessive *vpt* mutants were then intercrossed, diploid strains were isolated, and the filter assay was used to assign the *MATa vpt* × *MATα vpt* strains to complementation groups (Fig. 2). Complementing pairs of recessive *vpt* mutants exhibited no external Inv activity (white patch on Inv filter assays), whereas noncomplementing pairs of *vpt* mutants gave rise to red patches on the filters corresponding to
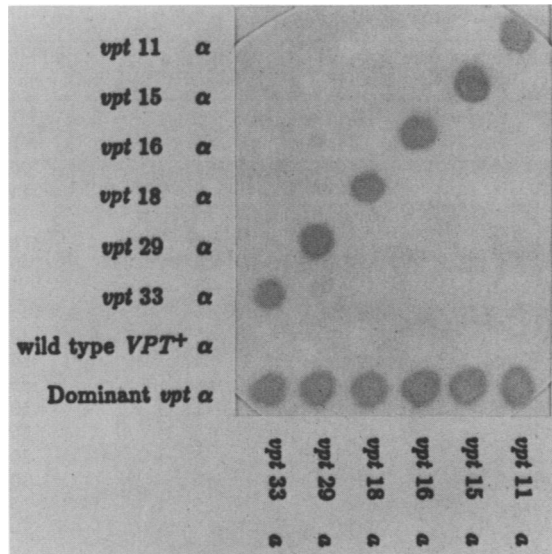
FIG. 2. Complementation analysis of vpt mutants. Secreted Inv activity of intact cells. Diploids were constructed by crossing vpt MATα lys2 with vpt MATa ade2 mutants and selecting Ade⁺ Lys⁺ diploid strains on minimal medium. These diploid strains were patched in an array onto YP medium containing fructose and screened by the Inv filter assay. Noncomplementation was seen as a red spot, indicating secretion of the CPY-Inv hybrid protein, while complementation was observed as no reaction (white).

the secretion of Inv activity. In general, the assay gave unambiguous results, as shown in Fig. 2. Of the 505 new vpt mutants, 17 were found to contain mutations that led to a dominant Suc⁺ phenotype. Of six of these dominant mutants tested, only one was found to harbor a mutation linked to the fusion plasmid. The other mutants apparently contained dominant chromosomal mutations. These have not yet been characterized further. The remaining 488 mutants contained recessive mutations. Of these, 184 (84 MATa and 100 MATα) could be assigned to the previously identified vpt complementation groups (vpt1 through vpt8) (2). The remaining MATa vpt mutants were systematically crossed with new MATα vpt mutants until they could be assigned to new complementation groups (arbitrarily defined as containing one MATa and one MATα allele). Twenty-five new vpt complementation groups were identified (vpt9 through vpt33) (Table 1). Mutant alleles in each of the 33 complementation groups identified were obtained with both the pCYI-50 and the pCYI-433 fusion plasmids. A total of 21 recessive MATa vpt mutants and 36 recessive MATα vpt mutants were crossed with at least one allele from each of the 33 vpt complementation groups and, in addition, were crossed with all the remaining unassigned vpt mutants of the opposite mating type. All 21 unassigned MATa mutants complemented each of the remaining 36 unassigned MATα vpt mutants. We did not construct MATa and MATα alleles of each of these 57 unassigned vpt mutants and continue the complementation analysis further.

Because the vpt mutants were selected as spontaneous events, we expected that they would correspond to single-site mutations. To obtain further support of this, many of the vpt mutants (vpt3, vpt4, vpt7, vpt9, vpt11, vpt14, vpt15, vpt16, vpt18, vpt29, and vpt33) were crossed with parental VPT strains and subjected to tetrad analysis. For each mutant tested, the vpt defect and all the other markers scored in the cross segregated with the expected 2:2 Men-

delian inheritance pattern of simple single-locus mutations. This confirmed that these vpt mutants represent single-site changes and further indicated that the genetic defects associated with these mutants do not result in any gross mating or chromosomal abnormalities. In the course of these analyses, we noticed that two of the vpt mutants appeared to be linked to auxotrophic markers that were heterozygous in the crosses. The segregation data for vpt3 and ade2 (17 parental ditype:1 tetratype:0 nonparental ditype) indicated that vpt3 was linked to ade2. The approximate map distance (calculated as described previously [36]) between ade2 and vpt3 was ~2.8 centimorgans. This locates the VPT3 locus on the right arm of chromosome XV, where ADE2 has been mapped (25). Another of the vpt mutants, vpt15, appeared to be linked to lys2 (17 parental ditype:5 tetratype:0 nonparental ditype), in this case less tightly (~11.4 centimorgans). The LYS2 gene has been mapped to the right arm of chromosome II (25).

Mislocalization of vacuolar enzymes in the vpt mutants. At least one mutant from each of the 33 vpt complementation groups was subjected to a series of enzyme assays to quantitate the extent of the mislocalization defect and to test whether mislocalization of the CPY-Inv fusion protein was due to cell lysis.

The extent of mislocalization of the CPY-Inv hybrid protein was determined for several alleles of each complementation group by assaying external Inv (in whole cells) as well as total Inv (in lysed cells) and then calculating the percentage of Inv activity that was secreted (15). The results obtained for a representative vpt mutant allele from each complementation group are presented in Table 1. In most cases other alleles from the same complementation group secreted the CPY-Inv hybrid protein to a similar extent. Two to five alleles from each vpt complementation group were assayed. The alleles for which assay data are shown in Table 1 were used in all subsequent analyses.

The most obvious unrelated defect that might lead to the apparent secretion of vacuolar proteins is cell lysis. For this reason, assays of a cytoplasmic marker enzyme, glyceraldehyde-3-phosphate dehydrogenase, were carried out to determine whether any of the vpt mutants exhibited significant levels of external glyceraldehyde-3-phosphate dehydrogenase activity. In no case was there evidence of significant leakage of this cytoplasmic marker enzyme into an extracellular fraction. Furthermore, the level of cell-associated glyceraldehyde-3-phosphate dehydrogenase activity detected after detergent lysis of vpt mutant cells was similar to that detected in the parental strains (SEY6210 and SEY6211) (Table 1). In addition, the mutants were tested for the presence of external α-glucosidase, another cytoplasmic enzyme. Again, no evidence for lysis was found (data not shown). As discussed below, the vpt mutants secreted precursor forms of the vacuolar proteins rather than the mature forms, as would have been expected if cell lysis were responsible for the observed secretion.

As an initial crude test to determine whether the new vpt mutants also secrete CPY, we assayed CPY enzyme activity in each mutant. Since CPY is normally processed from the inactive p2 zymogen to the mature, active form inside the vacuole, it is expected that if CPY is missorted into the media, it will not become activated or will be activated poorly (38, 39). Thus, the more extreme the vpt mislocalization defect, the lower the cell-associated CPY activity is expected to be relative to the activities of the parental strains SEY6210 and SEY6211. CPY activity levels ranged from 2% of parental activity in vpt5 up to 82% in vpt23 (Table 1). CPY

TABLE 1. *vpt* complementation groups and enzyme assay data[a]

Complementation groups	Total no. of alleles	No. of *ts* alleles	% CPY-Inv secreted	% of parent CPY activity	% PrA-Inv secreted	% External G3PDH	% External α-mannosidase
vpt1	46		85	10	45	5	<5
vpt2	29		25	65	ND	ND	ND
vpt3	11	1	80	10	75	<5	<5
vpt4	15		75	5	65	<5	<5
vpt5	9		100	<5	65	<5	5
vpt6	6		70	5	45	<5	<5
vpt7	32		90	30	55	<5	10
vpt8	33		65	25	65	<5	<5
vpt9	20		65	35	55	<5	<5
vpt10	29		50	30	35	<5	20
vpt11	8	2	90	10	60	<5	25
vpt12	3		65	70	55	<5	<5
vpt13	26		100	25	110	<5	10
vpt14	8		50	45	35	<5	15
vpt15	14	8	80	25	65	5	20
vpt16	5	2	75	10	60	<5	30
vpt17	40		80	20	65	<5	<5
vpt18	8	3	100	30	90	<5	30
vpt19	16		80	20	100	<5	<5
vpt20	5		35	75	30	5	5
vpt21	2		85	45	105	<5	5
vpt22	4		35	50	25	<5	<5
vpt23	.		25	80	30	<5	<5
vpt24	8		40	50	50	<5	10
vpt25	4		35	50	30	5	<5
vpt26	4		95	15	70	<5	15
vpt27	9		40	40	40	<5	20
vpt28	7		35	65	40	<5	5
vpt29	8	2	70	15	90	<5	10
vpt30	4		60	40	70	<5	5
vpt31	2		15	65	30	<5	5
vpt32	5		35	55	35	<5	5
vpt33	7	5	90	10	85	<5	20
VPT[+]			5	100	5	<5	<5

[a] The *vpt* mutants were assigned to 33 complementation groups on the basis of standard complementation analysis. Also shown is the number of temperature-sensitive *vpt* mutants in each of the seven complementation groups that had Ts alleles. Inv assays were performed on several alleles of each complementation group. Whole cells were assayed for external Inv activity, and lysed cells were assayed for total Inv activity. The percentage of external Inv activity was calculated for each mutant allele. A strongly defective allele (high extracellular percentage of Inv) was chosen for further analysis. Levels of other enzyme activities secreted by, or associated with, these representative alleles are presented. Abbreviations: G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ND, not determined.

activities were inversely proportional to the level of the CPY-Inv hybrid protein secreted from most of the *vpt* mutants (Table 2). For example, some mutants such as *vpt5* and *vpt33* were very defective by both criteria, whereas others, including *vpt2* and *vpt20*, did not show major defects by either assay. This result indicates that the behavior of the CPY-Inv hybrid protein in the *vpt* mutants accurately reflects the effect that these mutations have on CPY activity and, presumably, on its localization.

The availability of PrA-Inv hybrid proteins that were efficiently targeted to the vacuole (Fig. 1) (18, 18a) offered a

simple way for us to test the effect of *vpt* mutations on the localization of another protein that is found in the vacuole in wild-type yeast. To this end, the representative *vpt* alleles were cured of the plasmids encoding CPY-Inv fusions and retransformed with a vector (pP4I-404) encoding a fusion protein, PrA-Inv404, consisting of the N-terminal 404 amino acids of PrA fused to mature active Inv. The hybrid protein expressed by pP4I-404 is delivered efficiently to the vacuole in *S. cerevisiae* VPT strains (18a). Inv assays showed that in most of the *vpt* mutants significant amounts of PrA-Inv hybrid protein were secreted instead of being targeted to the

TABLE 2. Grouping of *vpt* mutants based on CPY and CPY-Inv localization[a]

% of CPY-Inv activity secreted	*vpt* complementation group with the following CPY activities (% of parent):			
	<15%	15–30%	30–50%	>50%
>90%	**5, 26, 33**	**7, 13, 18**		
65–90%	**1, 3, 4, 6, 11, 16**	**15, 17, 19, 29**	21	
40–65%		**8**, 10	**9, 30**, 14, 24	12
<40%			27	2, 20, 22, 23, 25, 28, 31, 32

[a] Grouping of *vpt* mutants based on severity of CPY and CPY-Inv fusion protein localization defects. The percentage of CPY enzyme activity was broken into four levels, as was the percentage of CPY-Inv hybrid protein secreted. The 33 *vpt* complementation group numbers are shown in the appropriate spaces. *vpt* mutants that showed severe mislocalization defects, processing defects, or both types of defect for both CPY and PrA, as shown by cell fractionation and immunoprecipitation of these proteins (Fig. 3), are given in boldface.
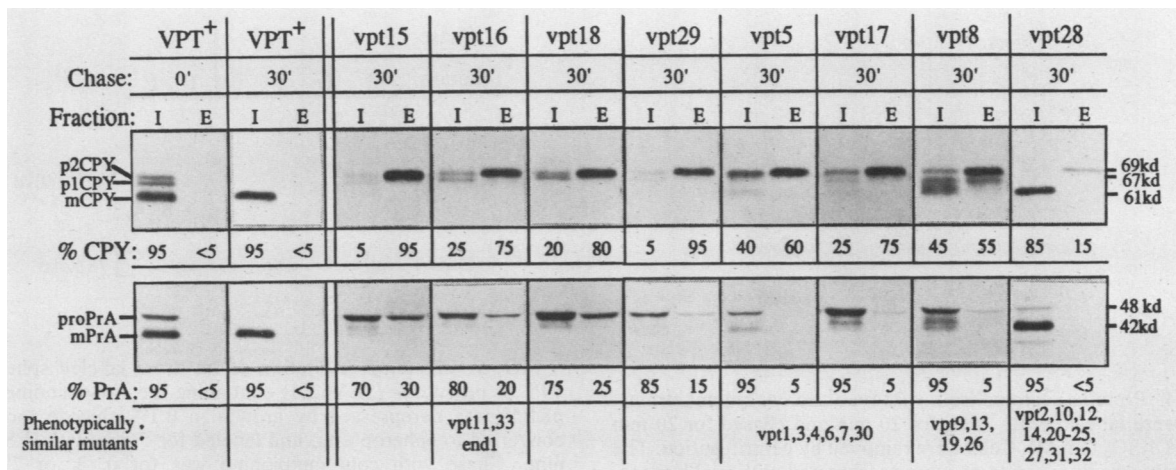
	VPT+		VPT+		vpt15		vpt16		vpt18		vpt29		vpt5		vpt17		vpt8		vpt28	
Chase:	0'		30'		30'		30'		30'		30'		30'		30'		30'		30'	
Fraction:	I	E	I	E	I	E	I	E	I	E	I	E	I	E	I	E	I	E	I	E
p2CPY, p1CPY, mCPY																				
% CPY:	95	<5	95	<5	5	95	25	75	20	80	5	95	40	60	25	75	45	55	85	15
proPrA, mPrA																				
% PrA:	95	<5	95	<5	70	30	80	20	75	25	85	15	95	5	95	5	95	5	95	<5
Phenotypically similar mutants*							vpt11,33 end1						vpt1,3,4,6,7,30				vpt9,13, 19,26		vpt2,10,12, 14,20-25, 27,31,32	

(CPY bands labeled: 69kd, 67kd, 61kd. PrA bands labeled: 48kd, 42kd.)

FIG. 3. Immunoprecipitation of CPY and PrA in wild-type (SEY6210 and SEY6211) and *vpt* mutant yeast strains. Spheroplasts were labeled (as described in the text) and chased for the indicated times (in minutes). The spheroplast pellet made up the internal (I) fraction and the supernatant constituted the external (E) fraction in each case. Immunoprecipitations were carried out with CPY or PrA antisera. Densitometer tracings were used to calculate the approximate percentages of CPY and PrA in the internal and external fractions for each mutant shown. The *vpt* mutants with similar phenotypes to those shown are listed. Distribution patterns of CPY and PrA for *vpt1*, *vpt3*, *vpt4*, *vpt6*, *vpt7*, and *vpt30* ranged between those shown for *vpt5* and *vpt17*. The migration positions of precursor and mature forms of CPY (mCPY) and PrA (mPrA) and their approximate molecular sizes (in kilodaltons [kd]) are indicated. For many of the mutants a protein form intermediate in size between p2CPY and mature CPY was immunoprecipitated by anti-CPY antisera. This band migrated at approximately the same position as p1CPY on our gels. The p1 and p2 forms of PrA could not be resolved under the gel conditions used.

vacuole (Table 1). The most defective *vpt* mutants, as estimated by the reduction in CPY activity levels and secretion of the CPY-Inv hybrid proteins, were also the mutants that exhibited the largest defects in PrA-Inv sorting (Table 1). It is possible that both PrA and CPY use a common delivery pathway.

We were also interested in determining whether, in addition to the sorting of soluble vacuolar hydrolases, sorting of vacuolar membrane proteins was affected in any of the *vpt* mutants. One vacuolar membrane enzyme that has been well characterized is α-mannosidase. This enzyme is not dependent on PrA processing for its activation (17), and thus would be expected to remain active even when it was mislocalized (to the cell surface, for example). The *vpt* mutants were tested for the presence of cell surface α-mannosidase activity by assaying whole, as well as detergent-lysed, cells. In all of the *vpt* mutants, total α-mannosidase activity levels were comparable to that of the wild type (*VPT*). The only *vpt* mutants that showed significant levels of external cell-associated α-mannosidase activity were *vpt10*, *vpt11*, *vpt15*, *vpt16*, *vpt18*, *vpt27*, and *vpt33* (Table 1). It is interesting that these mutants, with the exception of *vpt10* and *vpt27*, were severely defective by several other criteria, including CPY and PrA mislocalization (Fig. 3), and the possession of additional phenotypes such as temperature sensitivity (Table 1) and abnormal vacuolar morphology (L. M. Banta, J. S. Robinson, D. J. Klionsky, and S. D. Emr, J. Cell. Biol., in press). All of the representative *vpt* alleles shown in Table 1 were non-temperature-sensitive alleles. To further address the α-mannosidase localization defect, we also assayed the potentially more severe temperature-sensitive (Ts) alleles of each complementation group for which they were available (*vpt11*, *vpt15*, *vpt16*, *vpt18*, and *vpt33*). Interestingly, we observed as much as 40 to 50% of the α-mannosidase activity at the cell surface in some Ts alleles of *vpt11*, *vpt16*, *vpt18*, and *vpt33*, even when the cells were grown at the permissive growth temperature (25°C). No evidence for cell lysis of the Ts alleles was obtained.

We suspected that some of the *vpt* mutants might secrete an altered spectrum of proteins compared with that of wild-type *S. cerevisiae* for two reasons. First, since secretory and vacuolar proteins transit together through portions of the secretory pathway (38), some gene products affecting the delivery of molecules to the vacuole might also affect the transport of other molecules destined for secretion. Thus, if the *vpt* mutations affected the secretion pathway, some extracellular proteins might be missing or reduced in the medium isolated from certain of the *vpt* mutant strains. Second, and conversely, additional protein species may accumulate in the growth medium, since the *vpt* mutants were selected for their ability to secrete vacuolar contents. To address these questions, we examined the secretion of radioactively labeled proteins by some *vpt* mutants into the growth medium. Cells were labeled with $^{35}SO_4$ for 20 min and then chased for 20 min in the presence of excess unlabeled SO_4. Media proteins were precipitated in trichloroacetic acid, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and examined by autoradiography (Fig. 4). No striking differences between the media fractions isolated from wild-type or *vpt* mutant cells were observed. Comparable amounts of a similar set of proteins were observed in extracts from both wild-type and *vpt* mutant cells. Two of the mutants (*vpt7* and *vpt11*) exhibited an extra protein band, in addition to the set observed in *VPT S. cerevisiae* (Fig. 4). We did not determine whether either of these new bands corresponded to a known vacuolar protein. Based on their apparent molecular weights, these proteins were not CPY or PrA. Because we analyzed the medium from pulse-labeled cells, we also inferred that the rate of normal protein secretion was not grossly altered in the *vpt* mutants. The lack of new proteins corresponding to CPY or PrA in the medium was probably due to the instability of these mislocalized proteins (2, 38), as well as to their relatively low abundance compared with that of the proteins in the medium observed under these experimental conditions.

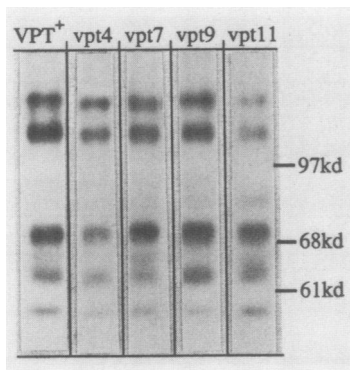In addition, many of the mutants were transformed with

FIG. 4. Proteins secreted from wild-type and *vpt* mutant strains. Strains were labeled with $^{35}SO_4$ for 20 min and chased for 20 min with $(NH_4)_2SO_4$ (1 mM). Cells were removed by centrifugation. The supernatant (medium) proteins were precipitated with trichloroacetic acid and run on a 10% polyacrylamide gel. Size standards (in kilodaltons [kd]) are as indicated.
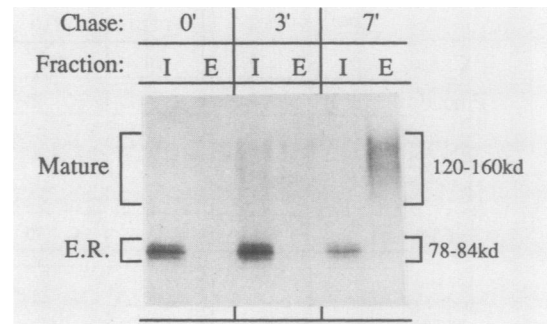

FIG. 5. Immunoprecipitation of Inv from labeled spheroplasts. VPT *S. cerevisiae* (SEY6211) containing the Inv-encoding plasmid pRB58 was derepressed by growth in 0.1% glucose for 30 min, converted to spheroplasts, and labeled for 5 min with [^{35}S]methionine. Chase with cold methionine was for 0, 3, or 7 min, as indicated. Spheroplasts were separated into pellet (I) and supernatant (E) fractions by centrifugation and immunoprecipitated with Inv antisera. The core glycosylated Inv found in the endoplasmic reticulum (E.R.) migrated as a set of bands that were converted to a heterogeneous mixture of highly glycosylated molecules in the Golgi complex prior to secretion from the cell. kd, Kilodaltons.

an Inv-encoding plasmid (pRB58) and tested for normal Inv secretion (assay and calculations were as described previously [27]). The *vpt* mutants were found to secrete Inv at the same levels as wide-type *S. cerevisiae*. This provides further evidence that the *vpt* mutants carry out secretion normally.

vpt mutants exhibit defects in the sorting and processing of several vacuolar proteins. To assess directly the extent of the sorting defect in the various *vpt* mutants, we labeled and fractionated *vpt* mutant cells. Antisera directed against CPY, PrA, and PrB were used to detect the amount and form of these different vacuolar proteins in each cell fraction. Initially, cells were labeled and fractionated into medium, periplasm, and spheroplast fractions, with subsequent immunoprecipitation by anti-CPY and anti-PrA antisera. These data (not shown) allowed us to categorize the *vpt* mutants based on the severity of their CPY and PrA localization and processing defects. However, we noticed that during the fractionation, presumably because of the incubation time required to remove the cell wall enzymatically, some fraction of these proteins was being degraded or modified. To avoid this, we converted the mutant cells to spheroplasts before they were labeled and fractionated. In this way we were able to look at mislocalization by isolating spheroplast (pellet) and medium fractions by centrifugation. The medium fraction corresponded to all material secreted from the cells, including those proteins that are normally retained in the periplasm. A further advantage of labeling spheroplasts is that because lysed spheroplasts do not incorporate the label, it reduces the background of labeled vacuolar proteins in the medium resulting from cell lysis during spheroplast preparation. To confirm that the spheroplast-labeling technique did not alter normal protein secretion or protein sorting to the vacuole, we initially analyzed the modification and processing of the secreted enzyme (Inv) and the vacuolar proteins (CPY and PrA) in spheroplasts prepared from a wild-type strain. Both protein secretion and vacuolar protein sorting and processing appeared to be normal (Fig. 3 and 5). Indeed, we observed that the level of protein expression and the kinetics of protein modification and delivery appeared to be normal by the spheroplast-labeling technique (8, 15, 18, 35b). This argues that secretory protein traffic occurs normally in yeast spheroplasts.

Wild-type and *vpt* mutant spheroplasts were labeled with [^{35}S]methionine for 20 min and chased with cold methionine

for 30 min. Supernatant (external) and pellet (internal) fractions were immunoprecipitated with anti-CPY antisera. We observed two significant effects of the *vpt* mutants on the biogenesis of the vacuolar protein CPY (Fig. 3). First, processing was altered; 14 of the mutants exhibited no mature CPY at all (*vpt1, vpt3, vpt4, vpt5, vpt6, vpt7, vpt11, vpt15, vpt16, vpt17, vpt18, vpt29, vpt30, vpt33*), and an additional 5 *vpt* mutants processed less than 30% of CPY to the mature forms (*vpt8, vpt9, vpt13, vpt19, vpt26*). Second, missorting and secretion of CPY were observed. Two of the *vpt* mutants (*vpt15* and *vpt29*) secreted more than 95% of CPY into the periplasmic and medium fraction. An additional 12 *vpt* mutants (*vpt1, vpt3, vpt4, vpt5, vpt6, vpt7, vpt11, vpt16, vpt17, vpt18, vpt30, vpt33*) were almost as defective; they secreted between 60 and 80% of CPY (Fig. 3). These 14 most defective mutants exhibited a complete defect in maturation of the small amount of CPY that remained associated with the spheroplast fraction, as discussed above, indicating that the accumulated proCPY was not sorted to the vacuole or, possibly, that the vacuole of these mutants was so defective that it was not competent for the processing of proCPY. Many of the remaining *vpt* mutants exhibited less severe defects in the sorting of the CPY protein. In these mutants, a substantial portion of CPY remained associated with the spheroplast and was processed to the mature active enzyme, indicating that some of the proCPY was sorted correctly to the vacuole (Fig. 3).

To determine whether this protein-sorting defect is specific for CPY or extends to other vacuolar proteins, we examined sorting and processing of two additional vacuolar proteins, PrA and PrB, in the *vpt* mutants. Relative to CPY, lower levels of both PrA and PrB were secreted by the *vpt* mutants. However, we noted that the extent of the defect in maturation of these proteins paralleled that observed for CPY in each of the *vpt* mutants (Fig. 3 and 6). The absence of CPY in the vacuole cannot explain the accumulation of precursor forms of PrA and PrB, as maturation of these proteases is not dependent on CPY function. This suggests that the defect in vacuolar sorting of PrA and PrB may be similar to that observed for CPY, but that secretion of the missorted PrA and PrB is blocked. We reasoned that the reduced level of proPrA and proPrB in the media fraction
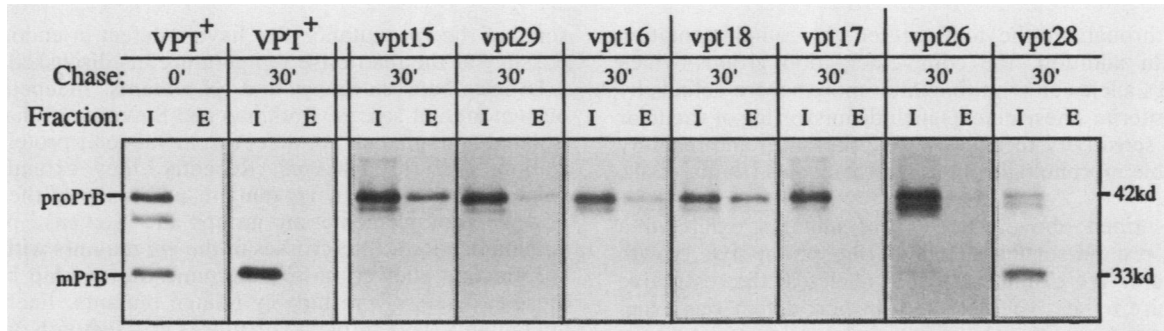
FIG. 6. Immunoprecipitation of PrB in wild-type (SEY6210 and SEY6211) and *vpt* mutant yeast strains. Spheroplasts were labeled as described in the text and chased for the indicated times (in minutes). I, Internal (spheroplast pellet) fraction; E, external (supernatant) fraction. PrB antiserum was used for the immunoprecipitations. The migration positions of precursor (proPrB) and mature (mPrB) forms of PrB and their approximate molecular sizes (in kilodaltons [kd]) are indicated. Note the extra band at 0 min; presumably, it was an intermediate in PrB maturation.

might be due to a reduced rate in secretion of these missorted proteins. To address this possibility, we extended the length of the chase period from 30 to 90 min and otherwise performed the fractionation and immunoprecipitation procedures as described above. When this experiment was carried out on *vpt5*, *vpt11*, and *vpt15*, no increases in the levels of proPrA or proPrB were observed in the medium after this extended chase period (data not shown). In this experiment we also observed that no additional processing of the proPrA and proPrB occurred during the chase.

An alternative explanation is that the lack of these proteins in the medium fraction might not be due to a block in secretion of the mislocalized zymogens, but rather to the specific or nonspecific association of these proteins with the spheroplast membrane surface. To test this possibility, we treated the labeled spheroplasts with 500 μg of proteinase K per ml for 30 min at 0°C to determine whether any of the cell-associated proPrA and proPrB was accessible to the exogenously added protease. Several *vpt* mutants (*vpt5*, *vpt17*, and *vpt26*) were analyzed in this way, and in each case, PrA and PrB were unaffected by the addition of proteinase K. As expected, secreted CPY was degraded under these conditions. Both PrA and PrB were, however, subject to degradation if the detergent Triton X-100 (1%) was added prior to protease addition. Taken together, these experiments indicate that proPrA and proPrB accumulate in some intracellular compartment. The present data do not rule out the vacuole as the site in which these proteins reside; however, the lack of processing of the accumulated proteins suggests that this may not be case. This lack of processing does not eliminate the vacuole as the site of accumulation of PrA and PrB, because of the observation that in missense PrA mutants, CPY activity is normal but PrA activity is decreased (23).

Several *vpt* mutants are temperature sensitive for growth. It is not clear whether vacuolar protein sorting is essential for yeast cell growth. The *pep4* mutants (45) are defective in enzyme activities of many soluble vacuolar hydrolases, yet they grow normally at all temperatures. We selected our mutants at 26°C to allow us to score for potential temperature-conditional phenotypes. Thus, after the mutants were selected for their ability to grow on sucrose-containing medium at 26°C, they were tested for their ability to grow at 37°C on YPD medium. Of the 505 mutants isolated, 23 (approximately 5%) were Ts for growth at 37°C (i.e., they did not form colonies at 37°C). These Ts mutants belonged to six *vpt* complementation groups (*vpt11*, *vpt15*, *vpt16*, *vpt18*,

vpt29, and *vpt33*). In addition, one allele of *vpt3* had a partial Ts phenotype (very small colonies at 37°C). Genetic crosses and tetrad analysis were used to confirm that in each case the Ts phenotype cosegregated with the *vpt* protein sorting defect. One Ts allele from each complementation group was crossed to the parent strain (SEY6210 or SEY6211), sporulated, and subjected to tetrad dissection. Ten or more tetrads were examined for each cross (an example of the cosegregating phenotypes is shown in Fig. 7). The remaining Ts alleles in a given complementation group were inferred to be linked to the appropriate *vpt* locus by complementation testing at 37°C. It is interesting that of the *vpt* complementation groups that contained Ts alleles (not including *vpt3*), 22 of 50 total alleles (i.e., 44%) in these complementation groups were Ts. Thus, the Ts alleles are not scattered at
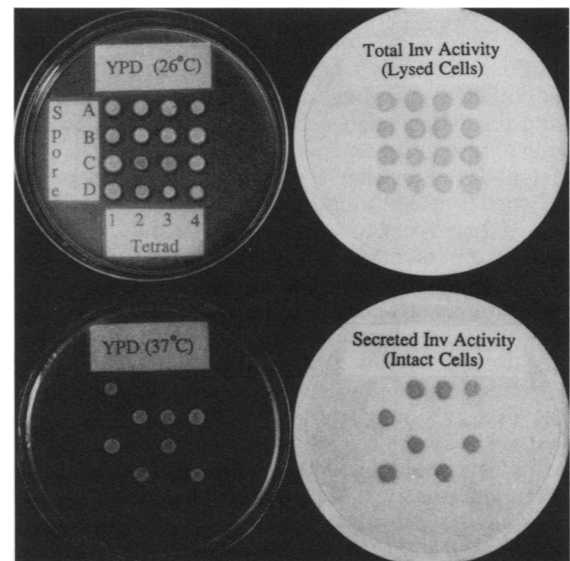

FIG. 7. Demonstration that the Ts growth phenotype and the *vpt* mislocalization phenotype cosegregate in four representative tetrads from a cross of a Ts allele of *vpt16* with strain SEY6210. The filter assay of the lysed segregant patches showed that each spore had Inv activity. Below, the Inv filter assay of intact segregant patches showed that the ability to secrete Inv activity (i.e., the CPY-Inv hybrid protein) segregated 2:2. Similar cosegregation of the Ts and *vpt* defects was seen for *vpt3*, *vpt11*, *vpt15*, *vpt18*, *vpt29*, and *vpt33*. At least 10 tetrads were analyzed in each case.

random throughout the total set of *vpt* complementation groups. In addition, the complementation groups which possess Ts alleles all tend to exhibit more severe defects by several criteria. These defects include missorting of vacuolar proteins, sensitivity to high osmotic pressure, and inability to assemble morphologically normal vacuoles (Banta et al., in press).

As described above, the Ts *vpt* mutants exhibited a vacuolar protein-sorting defect at the permissive growth temperature. We examined the Ts alleles at the restrictive temperature to see whether the transport defect was exaggerated further and thereby might explain their inviability at 37°C. The mutants were preincubated for 60 min at 37°C and then labeled for 20 min with $^{35}SO_4$ and chased for 20 min, both at 37°C. Immunoprecipitation with CPY antibody showed that there was no apparent difference in CPY processing or secretion at the restrictive temperature (data not shown), but it should be noted that all of these Ts *vpt* mutants exhibited extreme defects in vacuolar protein sorting, even at the permissive temperature. Since the Ts *vpt* mutants secreted Inv at 37°C, secretion apparently is not blocked at this temperature (see below). Because the defect in CPY sorting and processing was nearly complete even at the permissive growth temperature, the absence of growth at the nonpermissive temperature may be explained by the appearance of a more complete sorting defect at the high temperature, which alters the sorting of additional vacuolar proteins and possibly also affects secreted proteins that are essential for growth. Alternately, it may be that the combined stress of growth at a high temperature together with a defect in vacuolar protein sorting may, in an additive fashion, prevent cell growth at 37°C. For example, it is possible that a loss of vacuole protein localization at a high temperature may result in a block in secretion or endocytosis, or both, since these defects have been shown to be lethal (27, 34). To address these questions, the Ts *vpt* mutants were tested in several ways for possible secretory or endocytic defects. First, the Ts *vpt* mutants were transformed with a plasmid (pRB58) carrying the entire wild-type structural gene and regulatory sequences of *SUC2* and tested for Inv secretion at restrictive and permissive temperatures. For the experiment at the restrictive temperature, cells were shifted to 37°C for 60 or 120 min prior to the assay. Activities in whole and lysed cells were measured and compared to determine the percentage of Inv secreted (27). The mutants that were tested were not found to be significantly different from the parental strain SEY6210 harboring the same *SUC2* plasmid (data not shown). This implies that the Ts *vpt* mutants do not exhibit an Inv secretion defect at 37°C. Second, the Ts *vpt* mutants were tested for genetic overlap with the Ts secretion-defective mutants *sec1-sec23* (27) and *sec53* (10). The *sec* mutants are defective for the secretion of Inv at 37°C and are blocked for transport at various stages of the secretory pathway in *S. cerevisiae* (26). *sec* × *vpt* diploid strains were selected and tested for complementation of the temperature sensitivity at 37°C. In each case complementation was observed, implying that there is no genetic overlap between these two sets of mutants. The Ts *vpt* mutants were also crossed with the Ts endocytosis-defective mutant *end1* (5). Diploid strains were again tested for Ts growth defects. It was found that *vpt11* did not complement *end1*, implying that these two mutants are allelic to one another. In addition, the *END1* gene (a gift from H. Riezman) was found to rescue the Ts defect of the *vpt11* allele tested (P. Herman, unpublished data). This genetic overlap raises the possibility that

some of the *vpt* mutants may have a defect in endocytosis, possibly at the restrictive temperature, as discussed above.

Overlap between the *vpt* and *vpl* mutants. Independent of our *vpt* mutant screen, Rothman and Stevens (35) have also isolated mutants that are defective in vacuolar protein localization, *vpl1* through *vpl8*. Recently, they extended this work and selected new *vpl* mutants and assigned them to 11 new *vpl* complementation groups (T. Stevens, personal communication). Intercrosses of the *vpt* mutants with all the *vpl* mutants allowed us to determine the overlap between these two sets of presumably related mutants. Each of the *vpl* mutants (*vpl1* through *vpl19*) was crossed with our Suc⁻ parental strain, diploid strains were selected and sporulated, and Suc⁻ *vpl* segregants were identified. These Δsuc *vpl* mutants were then crossed to representative *vpt* mutant alleles from each complementation group, which harbored the CPY-Inv433 hybrid protein-encoding plasmid. Mislocalization of the hybrid protein from the diploid strains was scored by Inv filter assays. The results of these complementation studies are shown in Table 3. We observed that 12 of the 33 *vpt* mutants overlapped with *vpl* complementation groups. This overlap was confined in most cases to *vpt* complementation groups with the less severe vacuolar protein localization defects (Table 1 and 3). At present, we do not have an explanation for this observation. Differences in the selection schemes used or in the starting strains may have affected in part the final set of mutants obtained. J. H. Rothman and T. H. Stevens (unpublished data) have used another complementation assay technique and have independently confirmed each of these results. To avoid future confusion regarding these two classes of mutants, we have decided to use the general term *vps* for vacuolar protein sorting to describe both classes of mutants (Table 3).

Some differences were seen between *vpl* and *vpt* mutants in terms of the levels of missorting of vacuolar proteins. For example, *vpt5* and *vpl5* were in the same complementation group (*vps5*). The *vpl5* allele analyzed by Rothman and Stevens (35) showed a weaker protein sorting defect for CPY and PrA than did *vpt5* (2) (Fig. 3). This result may indicate allele differences. However, since the two mutations were in unrelated genetic backgrounds, the discrepancy also could be due to strain background differences.

Another class of pleiotropic mutants, the *pep* mutants, also has been shown to affect vacuolar protein processing (16, 17). Complementation analysis has shown that there is genetic overlap between subsets of the *pep*, *vpt*, and *vpl* mutants (T. Stevens and E. Jones, personal communication). The overlap between the *vpt* and *pep* mutants is as follows: *vpt3* = *pep21*, *vpt4* = *pep8*, *vpt5* = *pep10*, *vpt11* = *pep5*, *vpt13* = *pep12*, and *vpt17* = *pep6*.

DISCUSSION

In an effort to identify additional mutants that exhibit novel defects in vacuolar protein sorting and to develop an appreciation for the number of gene functions that may contribute to this process, we exploited a CPY-Inv fusion-based selection scheme (2) to isolate more than 500 new *vpt* mutants. The mutants were assigned to 33 different complementation groups, 7 of which contained Ts alleles. Even though this was an unexpectedly large number of complementation groups, our data suggest that many additional gene functions are likely to influence the vacuolar protein delivery pathway. Several of the newly isolated *vpt* mutants could not be assigned to any of these 33 complementation groups. In addition, genetic comparison of the *vpt* mutants

TABLE 3. Complementation analysis of vpt and vpl mutants

vpt mutant	vpl mutant	Common complementation group name
vpt26	vpl1	vps1
	vpl2	vps2
vpt17	vpl3	vps3
vpt10	vpl4	vps4
vpt5	vpl5	vps5
vpt13	vpl6	vps6
	vpl7	vps7
vpt8	vpl8	vps8
vpt9		vps9
vpt1		vps10
vpt11	vpl9	vps11
vpt12		vps12
vpt2		vps13
vpt14		vps14
vpt15		vps15
vpt16		vps16
vpt3		vps17
vpt18		vps18
vpt19		vps19
vpt20	vpl10	vps20
vpt21		vps21
vpt22	vpl4	vps22
vpt23	vpl15	vps23
vpt24		vps24
vpt25	vpl12	vps25
vpt4		vps26
vpt27		vps27
vpt28	vpl13	vps28
vpt6		vps29
vpt30		vps30
vpt31		vps31
vpt32		vps32
vpt33		vps33
vpt29		vps34
vpt7		vps35
	vpl11	vps36
	vpl16	vps37
	vpl17	vps38
	vpl18	vps39
	vpl19	vps40

a The vpt mutants were crossed with the vpl mutants (that had been made Δsuc2 by crossing them with strain SEY6210) and tested for their ability to complement the CPY-Inv433 sorting defect. Extensive overlapping was seen, and it is proposed that to avoid confusion in the future, these strains should be referred to as vps mutants (for vacuolar protein sorting) and the numbers should be as listed here (see also J. H. Rothman and T. H. Stevens, manuscript in preparation).

with similar mutants from other laboratories (sec, pep, vpl, end [5, 10, 16, 27, 35]), affecting secretory protein delivery or processing, indicated that although there was overlap among some of these different sets of mutants, more than 50 gene functions are implicated in the vacuolar protein sorting process.

This large number of genes can be interpreted to indicate that vacuolar protein sorting is a very complex process involving a series of distinct and precisely controlled sorting events. These include translocation into the endoplasmic reticulum, sorting to and through the Golgi complex, separation from proteins destined for secretion or Golgi complex retention, and selective delivery to the vacuole, possibly via an intermediate endosomelike compartment. Because each of these steps is likely to be catalyzed by proteins that are reused through several rounds of protein transfer, any mutations that affect their level of expression, stability, activity, or ability to recycle back to the appropriate compartment of

action may lead to sorting defects. Alternatively, the large number of mutants identified may suggest that many unrelated physiological and cellular defects can interfere with vacuolar protein sorting by indirectly influencing the process. That the former argument (that the vpt mutations cause specific changes in the targeting machinery) may indeed be the case for the more defective vpt mutants is supported by the following observations. (i) All of the late-acting sec mutants, which exhibited major defects in protein secretion, did not alter the delivery or maturation of CPY (38). (ii) Yeast strains deleted for the gene encoding the clathrin heavy chain did not exhibit defects in vacuolar protein biogenesis, even though the growth rate and intracellular morphology of these mutants were dramatically different from those of wild-type cells (29, 30). (iii) Cell fractionation and enzyme assay data ruled out the release of vacuolar proteins as a consequence of yeast cell lysis. (iv) Though many of the vpt mutants exhibited major defects in the targeting and maturation of vacuolar proteins, protein secretion as well as many other cell functions, including cell division, mating, and respiratory growth, remained largely unaffected. Direct evidence concerning the specificity of the events affected by the vpt mutants depends on the identification of the various VPT gene products, their site of action in the cell, and their individual biochemical activities.

Several observations led us to suspect that if vacuolar enzymes were not efficiently targeted to the vacuole, they would appear in the medium. Both CPY and PrA travel through the initial part of the secretory pathway (38, Klionsky et al., in press), so it can be postulated that if CPY or PrA fails to be diverted to the vacuole at the appropriate step, they might continue to be carried through the secretory pathway and end up at the cell surface. Indeed, much evidence has accumulated suggesting that many proteins are secreted by a default mechanism, while vacuolar and lysosomal enzymes must be actively sorted away from these proteins (4, 31). In addition, gene dosage-induced overproduction of CPY or PrA or mutational changes in the vacuolar sorting signals of these proteins lead to their missorting and secretion (15, 18a, 39, 41), suggesting that if the vacuolar targeting apparatus is overloaded or unable to recognize vacuolar proteins, the default reaction for these proteins is secretion. A similar overproduction-induced mislocalization phenotype has been observed for the vacuolar CPY-Inv and PrA-Inv hybrid proteins (2, 18a). Finally, an initial set of vacuolar protein mislocalization mutants which missort and secrete vacuolar enzymes has already been obtained by this scheme (2, 35).

The vpt mutants were selected on the basis of their ability to missort and secrete a hybrid vacuolar protein, CPY-Inv. The sorting of the artificial hybrid protein CPY-Inv fairly accurately reflects the sorting of the native CPY protein. Several lines of evidence support this conclusion. CPY-Inv hybrid proteins, carrying the CPY sorting signal, are very efficiently localized to the vacuole, as is true for native CPY (15, 18a). Mutants selected as defective in the vacuolar sorting of CPY-Inv hybrid proteins (vpt) also exhibit similar defects in the sorting of wild-type CPY (2). Enzyme assay data from this and previous studies (2) demonstrate that the extent of mislocalization of both the CPY-Inv hybrid proteins and native CPY protein correlate closely in each of the vpt mutants (Table 2). The vpt mutants genetically overlapped with the vpl mutants, which were selected by an independent scheme for defects in CPY sorting (35) (Table 3). The gene fusion approach also offers the advantage that it can be generally applied to the study of other gene products

independent of any knowledge of their biochemical activity (6, 7, 14).

It is of interest that although the extents of the sorting defects seen in different vpt mutants may vary, each mutant individually appears to exhibit qualitatively similar defects in the sorting and processing of the three proteins that were analyzed: CPY, PrA, and PrB. Although the vpt mutants did not secrete the same level of proCPY, proPrA, or proPrB, they all appeared to exhibit similar proteolytic processing defects for each of these proteins (Fig. 3 and 6). The vpt mutants caused significantly higher levels of proCPY to be mislocalized to the medium than the levels observed for either proPrA or proPrB. Most of the vpt mutants secreted p2CPY forms, indicating that this protein passes correctly through the Golgi complex. Although some level of secretion of proPrA and proPrB was observed in many of the vpt mutants, the bulk of each of these precursor proteins appeared to accumulate in an intracellular compartment distinct from the vacuole. They remained associated with the spheroplast cell fraction and were not degraded when extracellular proteases were added to the labeled spheroplasts. The proteins accumulated in their precursor forms, even though in many vpt mutants mature forms were also present, indicating that the vacuoles of these mutants were still competent for precursor maturation (Fig. 3 and 6). We do not know where in the cells the precursors accumulated or why they were not secreted like proCPY. The PrA-Inv hybrid protein was efficiently secreted by many of the vpt mutants (Table 1), suggesting that PrA itself is missorted in the mutants but cannot transit through later stages of the secretory pathway as efficiently as the PrA-Inv hybrid protein can. (Inv is normally secreted by S. cerevisiae and may facilitate transit of the PrA-Inv hybrid.) proPrA and proPrB may associate specifically or nonspecifically with some component of an intracellular membrane, or they may be diverted into a compartment that does not communicate with the cell surface. The vacuolar protein-sorting signals in both proCPY and proPrA have been identified, but they appear to share no significant primary sequence similarities (15, 18, 41) and therefore may be recognized by different carrier systems.

An important unresolved question regarding protein sorting to mammalian lysosomes or to the yeast vacuole is whether luminal proteins transit together with integral membrane proteins, and if so, does a common sorting apparatus participate in directing the delivery of these two distinct sets of proteins? In the case of I-cell disease, many lysosomal luminal enzymes are missorted; however, certain membrane proteins continue to be faithfully sorted to the vacuole (33, 37, 40). When we assayed for the location of the vacuolar membrane marker enzyme α-mannosidase, we found that it was not mislocalized to the cell surface in most of the vpt mutants. Vacuoles isolated from two of these mutants (vpt3 and vpt4) retained normal levels of this enzyme activity (2). However, a small subset of the vpt mutants did exhibit significant defects in the sorting of α-mannosidase activity. Alleles of vpt11, vpt16, vpt18, and vpt33 secreted up to 50% of their α-mannosidase activity to the cell surface. Interestingly, this same set of vpt mutants also exhibited other phenotypes that were consistent with the fact that these mutants have defects in both luminal and membrane vacuolar protein sorting. These phenotypes include the absence of a detectable vacuole in the mutant cells, as determined by light or electron microscopy, and accumulation of novel membrane-enclosed structures that may correspond to intermediates in vacuole membrane biogenesis (Banta et al., in

press). These results indicate that at least some vacuolar membrane proteins may share a common sorting step or compartment with soluble vacuolar proteins en route to the vacuole.

A useful observation is that seven of the vpt complementation groups had alleles exhibiting a Ts growth defect (on YPD medium at 37°C). In each case, this Ts growth defect was shown to be genetically linked to the vpt phenotype. It is probably not coincidental that these mutants all exhibited interesting additional vacuole-related defects. These Ts vpt mutants could be divided into three groups on the basis of their additional phenotypes and characteristics. (i) vpt15 and vpt29 were the two complementation groups with the most severe localization defect for CPY and PrA (Fig. 3). In addition, they both exhibited abnormal morphologies, including the accumulation of vesicular and Golgi complex-related structures, and apparently enlarged vacuoles relative to wild-type S. cerevisiae (Banta et al., in press). (ii) vpt11, vpt16, vpt18, and vpt33 also exhibited severe defects in the sorting of CPY and showed the accumulation of most of their PrA in the precursor form, indicating that it probably also did not reach the vacuole. These mutants exhibited the most striking structural defects of any of the vpt mutants, since they did not contain a morphologically identifiable vacuole. (iii) vpt3 had one Ts allele and was moderately defective in CPY and PrA localization, as assessed by immunoprecipitation (Fig. 3). The vpt3 mutant is interesting morphologically because it appears to contain highly fragmented vacuoles (Banta et al., in press).

One of the vpt mutants (vpt11) was found to be allelic to the Ts endocytosis-defective mutant end1 (5). This observation is interesting because it provides the first genetic evidence for a link between the endocytotic and biosynthetic delivery routes to the yeast vacuole. This result was not unexpected, because some mutants defective in the secretory pathway (sec) are also defective in endocytosis (34), and as discussed above, early stages of vacuolar protein targeting are sec dependent (38). It will be of interest to address the endocytic competence of the vpt mutants.

We identified many new genes whose products are involved in the process of vacuolar protein targeting. We expect that many of the gene products act directly in the targeting and transport of molecules to the yeast vacuole. More detailed information about how the vacuolar targeting pathway operates is anticipated on cloning of the VPT genes and detailed biochemical characterization of the gene products.

ACKNOWLEDGMENTS

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