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Genetics

Isolation of yeast mutants defective in protein targeting to the vacuole

(carboxypeptidase Y/invertase/gene fusion/protein sorting)

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ABSTRACT **We have constructed a *PRC1–SUC2* gene fusion that directs the synthesis in *Saccharomyces cerevisiae* of a hybrid polypeptide consisting of a 433-residue amino-terminal domain derived from the yeast vacuolar protease carboxypeptidase Y (CPY; EC 3.4.16.1) and a 511-residue carboxyl-terminal domain derived from the secreted yeast enzyme invertase (EC 3.2.1.26). Fractionation data indicated that this amount of CPY primary sequence is sufficient to quantitatively divert invertase to the yeast vacuole. The phenotypic consequence of localizing active invertase to the vacuole has enabled us to select for mutants that "mislocalize" the hybrid protein to the cell surface. The corresponding mutations that lead to this effect are all trans-acting and recessive, and they define at least eight complementation groups. These vacuolar protein targeting (*vpt*) mutants also exhibit hybrid protein independent defects in wild-type CPY delivery to the yeast vacuole. Precursor forms of CPY accumulate in the mutants and are secreted into the yeast periplasm and extracellular medium. The *vpt* mutants should provide useful information pertaining to the mechanisms by which yeast cells regulate vacuolar protein traffic.**

A property of all living cells is their ability to maintain a proper level of biochemical compartmentalization. This ordered state is in large part achieved by cellular processes that faithfully direct the efficient localization of defined subsets of proteins from their cytoplasmic site of synthesis to their various noncytoplasmic destinations. A major portion of intracellular protein traffic in eukaryotes is mediated by the secretory pathway. This pathway consists of a series of membranous organelles that mediate the events associated with the sorting, packaging, transport, and exocytosis of glycoproteins. The basic structure of the yeast secretory pathway has been elucidated and shown to be conserved with respect to the secretory pathways of higher eukaryotes (1). Furthermore, it has been demonstrated that yeast secretory, plasma membrane, and vacuolar proteins transit at least certain portions of the secretory pathway en route to their ultimate subcellular compartments (2–4).

To better understand how the various classes of proteins that transit the secretory pathway are sorted from each other, we have undertaken an analysis of protein targeting to the yeast vacuole. The yeast vacuole seems analogous to the mammalian lysosome from the standpoint that a number of glycoprotein hydrolases are sequestered within the organelle (5). The intracellular passage of one such vacuolar enzyme, carboxypeptidase Y (CPY; EC 3.4.16.1), has been analyzed in yeast cells that exhibit temperature-conditional defects at various points in the secretory pathway (4). From these and other studies it has been established that CPY is synthesized as an inactive zymogen that is translocated into the lumen of the endoplasmic reticulum, where it receives four N-glycosidically linked core oligosaccharides (4, 6). Delivery to the Golgi complex follows, and further glycosyl modifications of the pro-CPY take place in this organelle. Finally, the pro-CPY is targeted to the vacuole, where an 8000-kDa propeptide is removed to yield the active mature enzyme (7, 8). This maturation of CPY is dependent upon the presence of a functional *PEP4* gene product (8). The nature of the information elaborated by pro-CPY that directs its localization to the vacuole is not known. However, glycosylation does not appear to play a role in the targeting process, as unglycosylated CPY is still faithfully delivered to the yeast vacuole (9).

To date, the available data suggest a common itinerary for yeast secretory and vacuolar glycoproteins through the early stages of the secretory pathway. These proteins are presumably sorted and directed to their respective targets at the level of the Golgi complex (1, 4). We have devised a gene fusion-based selection for mutants that are defective in CPY delivery to the yeast vacuole. These mutants mislocalize this vacuolar hydrolase to the cell surface. In this report, we describe the isolation and initial characterization of such vacuolar protein targeting (*vpt*) mutants.

MATERIALS AND METHODS

Yeast Strains. *Saccharomyces cerevisiae* strains SEY2101 (*MATa ura3-52 leu2-3,-112 suc2-Δ9 ade2-1*) and SEY2102 (*MATα ura3-52 leu2-3,-112 suc2-Δ9 his4-519*) have been described (10). Standard methods (11, 12) were employed to construct yeast strains SEY2108 (*MATα ura3-52 leu2-3,-112 suc2-Δ9 Δprc1::LEU2*$^+$), SEY2109 (*MATa ura3-52 leu2-3,-112 suc2-Δ9 Δprc1::LEU2*$^+$), and SEY2101.1 (*MATa ura3-52 leu2-3,-112 suc2-Δ9 ade2-1 Δpep4::LEU2*$^+$).

Media and Reagents. YPD and minimal yeast media have been described (11). Minimal media were supplemented with glucose (2%) and proline (1%) as sole carbon and nitrogen sources, respectively. For sulfate-free media, chloride salts were substituted for all sulfate salts. Bromcresol purple indicator medium (11) was supplemented with sucrose (2%) and antimycin A (10 μg/ml; Sigma). $H_2{}^{35}SO_4$ was obtained from Amersham and lyticase was from Enzogenetics (Corvallis, OR). Tunicamycin and Ficoll 400-DL were purchased from Sigma.

Transformation. Yeast were transformed to uracil-independence with the appropriate plasmids by the lithium acetate method (13).

Radiolabeling and Immunoprecipitation. Procedures for radiolabeling yeast with $^{35}SO_4^{2-}$ in the presence or absence of tunicamycin, cell fractionation, immunoprecipitation with anti-CPY serum (gift of T. Stevens), and NaDodSO$_4$/PAGE have been described (14).

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Abbreviations: CPY, carboxypeptidase Y; mCPY, mature CPY; Inv, invertase.

Fractionation. Yeast vacuoles were purified from cells grown in glucose minimal proline medium. Yeast were converted to spheroplasts in 0.2 M imidazole/0.6 M sorbitol buffer (pH 6.5) with zymolyase as described by Daum *et al.* (15). Spheroplasts were resuspended in 0.2 M sorbitol/0.2 M imidazole/15% Ficoll/10 mg of bovine serum albumin per ml. Lysis of spheroplasts was induced by addition of DEAE-dextran to 30 μg/100 OD_{600} units of cells (16). Vacuoles were resolved on a discontinuous Ficoll step gradient (4) with the one modification that an extra 4% Ficoll step was added. Purified vacuoles were collected at the 4%/0% Ficoll interface.

Conversion of yeast cells to spheroplasts with lyticase and the collection of spheroplast and extracellular fractions has been described (14).

Enzyme Assays. The assay procedures and unit definitions for yeast invertase (17), α-mannosidase (18), α-glucosidase (19), and NADPH–cytochrome *c* reductase (20) have been described. Protein estimations were performed by the method of Lowry *et al.* (21).

RESULTS

Construction of the *PRC1–SUC2* Gene Fusion. The *PRC1* gene of yeast encodes the vacuolar serine protease CPY (8). *PRC1* has been cloned and its nucleotide sequence has been determined (ref. 22; T. Stevens, personal communication). *SUC2* encodes the periplasmic glycoprotein invertase (EC 3.2.1.26; abbreviated Inv in fusion proteins). This gene also has been cloned and its nucleotide sequence determined (23, 24). The coding sequence for a substantial amino-terminal moiety (433 amino acids) of pro-CPY (532 amino acids) was fused in-frame to a truncated form of the *SUC2* gene, lacking its 5′ regulatory sequences and the coding sequence for its amino-terminal signal peptide, to yield the pCYI433 shuttle plasmid (Fig. 1*A*). When radiolabeling of yeast strain SEY2101 (pCYI433) was performed in the presence of tunicamycin, an inhibitor of core glycosylation, a CPY-related polypeptide of some 105–110 kDa was precipitated (Fig. 1*B*). The size of this protein was in very good agreement with that expected (105–106 kDa) for the unglycosylated CPY-Inv hybrid protein, as deduced from the nucleotide sequence of the gene fusion. The apparent molecular mass of this polypeptide was shifted to approximately 135–140 kDa when the experiment was performed in the absence of tunicamycin. Essentially identical results were observed when invertase antiserum, rather than CPY antiserum, was used for the immunoprecipitation (data not shown). We interpreted these data to indicate that pCYI433 directed the synthesis of a CPY-Inv hybrid protein of 105–110 kDa apparent molecular mass that was core glycosylated at most, if not all, of the 16 possible asparagine-linked glycosylation sites. Each core glycosyl chain would be expected to increase the apparent molecular mass of the fusion protein by 2–2.5 kDa. No difference in apparent hybrid protein molecular mass was detected in *PEP4*$^+$ relative to Δ*pep4* yeast strains (Fig. 1*B*), suggesting that the pro portion of the hybrid protein was not processed.

CPY-Inv433 Is Targeted to the Yeast Vacuole. The CPY-Inv433 hybrid protein exhibited invertase activity, which we used as a marker to detect the cellular location of the hybrid protein. When whole yeast cells are assayed for wild-type invertase, essentially all of the activity is found to be secreted to the yeast periplasm (ref. 25; data not shown). However, quite different results were obtained with the invertase activity elaborated by CPY-Inv433. When intact yeast were assayed for external CPY-Inv activity, only some 2–5% of the total invertase activity (260 units/OD_{600} unit of cells) was measured at the cell surface. These values were comparable to the background enzyme activities measured at the cell

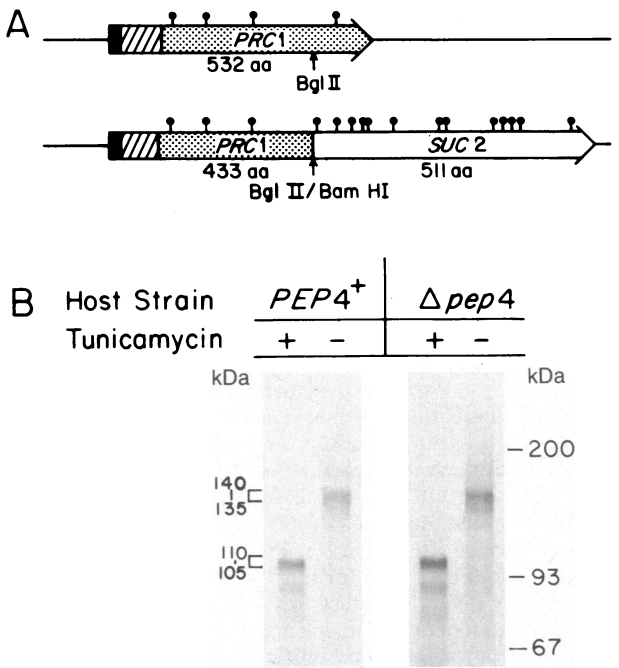

FIG. 1. (*A*) *PRC1–SUC2* gene fusion. A 3.2-kilobase-pair *Eco*RI–*Bgl*II DNA restriction fragment carrying the promoter and coding sequence for the amino-terminal 433 amino acid residues of the *PRC1* gene product was ligated into the *SUC2* gene fusion vectors pSEYC306 (*CEN4–ARS1 URA3*) and pSEY304 (2-μm circle *URA3*) that had been digested with the restriction enzymes *Eco*RI and *Bam*HI. This generates an in-frame fusion between the *PRC1* and *SUC2* genes. The *SUC2* fusion vectors pSEY304 and pSEYC306 contain the coding sequence for 511 amino acids of mature invertase. These vectors lack the coding sequence for the 19-amino acid invertase signal sequence and the first two amino acids of the mature enzyme. (Construction of these vectors will be described elsewhere.) The *PRC1* gene is divided into the coding regions for the presumed signal peptide (filled-in box), the propeptide (hatched box), and the mature CPY enzyme (stippled box). The approximate positions of the asparagine-linked core oligosaccharides as determined from the predicted amino acid sequences of the CPY and invertase proteins are indicated above the *PRC1* and *SUC2* genes. (*B*) Yeast strains SEY2101 and SEY2101.1 (Δ*pep4*) harboring the *PRC1–SUC2* gene fusion were labeled for 20 min with $^{35}SO_4^{2-}$ in the presence and absence of tunicamycin (10 μg/ml). The cells were lysed with glass beads prior to immunoprecipitation of the CPY-Inv hybrid protein with anti-CPY serum. Precipitated proteins were subjected to $NaDodSO_4$/PAGE and autoradiography. The hybrid protein appears to be susceptible to proteolytic cleavage or degradation that is not dependent on *PEP4* gene function.

surface for α-mannosidase, a vacuolar membrane marker (18), and α-glucosidase, a cytoplasmic enzyme (19). Thus, the amino-terminal 433 amino acids of pro-CPY were sufficient to redirect invertase to an intracellular compartment. The fidelity of intracellular localization of CPY-Inv433 was a function of gene dosage. Efficient intracellular compartmentalization of CPY-Inv433 required that the hybrid gene copy number be low. In the case of yeast strains harboring pCYI433, the gene fusion was present at 1–2 copies per cell. When an 8- to 12-fold overproduction of CPY-Inv433 was achieved by introducing the gene fusion into yeast on a multicopy 2-μm circle plasmid vector (pSEY304), some 20–25% of the total invertase activity was secreted (data not shown). In this respect, CPY-Inv433 mimicked the behavior of wild-type CPY (22).

To determine whether the proCPY sequences present in CPY-Inv433 were directing invertase to the yeast vacuole, we isolated vacuoles from fusion-bearing yeast strains. Enzyme markers for the vacuole membrane (α-mannosidase), the

endoplasmic reticulum (NADPH–cytochrome *c* reductase), and the cytoplasm (α-glucosidase) were assayed in crude cell extracts and vacuole-enriched fractions. Total protein was determined as well (Table 1). α-Mannosidase activity was monitored to identify the vacuoles and to gauge vacuole recovery during the fractionation (4, 18). Almost half (43%) of the total α-mannosidase activity loaded onto the discontinuous Ficoll gradient was recovered at the 4%/0% Ficoll interface after centrifugation. An essentially identical recovery (41%) of the total invertase activity was also measured in this vacuole fraction. Thus, α-mannosidase and CPY-Inv433 cofractionated. The purified vacuoles were substantially free of contamination by endoplasmic reticulum and cytoplasmic markers, and they represented an approximately 45- to 50-fold enrichment of vacuolar proteins with respect to total cell protein applied to the gradient (Table 1). These data indicated that CPY-Inv433 was targeted to the yeast vacuole. Further evidence in support of the vacuolar localization of CPY-Inv433 was obtained from indirect immunofluorescence experiments (26) with invertase antibody that revealed vacuole fluorescence for strain SEY2108 (pCYI433) but not for strain SEY2108 (data not shown).

Isolation of Mutants that Secrete CPY-Inv433. The efficient delivery of CPY-Inv433 to the yeast vacuole by strains having no other source of invertase activity resulted in the inability of such strains to grow on media with sucrose as their sole fermentable carbon source. Presumably, the sucrose-negative phenotype reflected the inability of the cells to transport sucrose into the yeast vacuole efficiently, thereby providing a ready selection for mutants that mislocalize CPY-Inv433 to the cell surface.

A total of 100 spontaneous sucrose-fermenting mutants were isolated with strains SEY2101, SEY2102, SEY2108, and SEY2109 bearing the pCYI433 plasmid. The selection was performed in the presence of antimycin A, an inhibitor of yeast respiratory metabolism (27). All but 2 of these independently obtained mutants exhibited sucrose-fermenting phenotypes that were recessive in heterozygous diploids. All of the corresponding mutations were found to be unlinked to the gene fusion. Complementation analyses identified at least eight complementation groups which we have designated *vpt1–8* for vacuolar protein targeting (see below). Of the 98 recessive mutations analyzed, 57 were assigned to a complementation group, with *vpt1* being most commonly represented, with 26 members.

To quantitate the level of CPY-Inv433 mislocalization to the cell surface in the *vpt* mutants, we measured the relative amounts of invertase activity that were secreted in a number of the *vpt* mutants with respect to the low levels of externalized invertase measured in the isogenic VPT^+ parents.

Table 1. Subcellular localization of CPY-Inv433

Enzyme	Total units in crude extract	Units recovered in vacuole fraction	% total activity in vacuole fraction
α-Mannosidase	880	376	43
CPY-Inv433	17,000	7000	41
α-Glucosidase	2,230	10	0.4
NADPH-cyt *c* reductase	763	21	2.7
Protein	3.6 mg	0.03 mg	0.9

Vacuoles were purified from SEY2108 pCPY-Inv433. Greater than 90% of the total enzyme activities loaded onto the gradient were recovered. α-Mannosidase, α-glucosidase, and NADPH–cytochrome *c* reductase represented markers for yeast vacuole membrane, cytoplasm, and endoplasmic reticulum, respectively. Enzyme units were defined as nmol of substrate hydrolyzed per min.

Yeast strains were cultured in glucose minimal proline medium and converted to whole cell, spheroplast, and periplasmic fractions. The fractions were subsequently assayed for the appropriate enzyme activities. Total activities measured in the whole cell fractions were normalized to 100%, and the total enzyme activities measured for the spheroplast and periplasmic fractions were expressed as portions thereof. All of the *vpt* mutants tested exhibited a dramatic mislocalization of invertase activity to the cell surface. The parental SEY2108(pCYI433) strain exhibited very little invertase, α-mannosidase, or α-glucosidase activity at the cell surface (see above). In contrast, the isogenic *vpt3-2*, *vpt4-2*, and *vpt6-2* strains secreted some 60%, 70%, and 65% of the total cellular invertase activity, respectively. In all cases, the recovery of the periplasmic and spheroplast activities accounted for greater than 90% of the total enzyme activities. It is important to note that α-mannosidase and α-glucosidase activities (*i*) cofractionated with the *vpt* spheroplasts, (*ii*) were not present in the periplasmic fractions at significantly higher quantities than those measured for the VPT^+ strain, and (*iii*) the specific activities for α-glucosidase and α-mannosidase in the *vpt* mutants were not significantly different from those of the parental VPT^+ strain. Furthermore, we obtained recoveries of 25–35% of the α-mannosidase activity with vacuoles purified from VPT^+, *vpt3-2*, and *vpt4-2* yeast, indicating that α-mannosidase continues to cofractionate with the *vpt* mutant vacuoles. These data indicated that the *vpt* mutants are selective in their mechanisms of CPY-Inv433 secretion and are not unusually prone to cell lysis or some other form of general leakiness. The specific activity of invertase was the same in the isogenic VPT^+ and *vpt* strains, suggesting that the *vpt* mutants do not overproduce CPY-Inv433 and secrete some fraction of the hybrid molecule population by this mechanism.

***vpt* Mutants Accumulate Precursor Forms of CPY.** If the *vpt* mutants are defective in host functions that interact with information elaborated by CPY sequences present in CPY-Inv433, one might expect that these mutants would also suffer defects in the proper localization of wild-type CPY. To test this possibility, SEY2108 derivative strains were cured of pCYI433 and made $PRC1^+$ by transformation with the *CEN4–ARS1*-based vector pCEN-PRC1. Normally, three forms of CPY can be detected in wild-type yeast: the transit intermediates p1 and p2, and the mature vacuolar form (mCPY). The p1 species represents core glycosylated pro-CPY (67 kDa) and is found at both the endoplasmic reticulum and the Golgi complex (4). In the Golgi complex p1 undergoes further glycosyl modification to generate the 69-kDa p2 form. Since PEP4-dependent conversion of p2 to mCPY is considered to occur in the vacuole (7, 8), we investigated the kinetics of CPY maturation in VPT^+ and *vpt* yeast as a first approximation of the fidelity of CPY localization in the *vpt* mutants (Fig. 2).

Previous studies of CPY delivery to the vacuole have indicated a half-time for this process of some 6 min (7). Kinetic analyses of CPY maturation in our VPT^+ strain are consistent with that estimate (Fig. 2). The mature species was stable throughout the entire 60-min chase period. Isogenic *vpt* strains exhibited markedly different CPY profiles. All of the *vpt* mutants represented in Fig. 2 were defective in CPY maturation, and they accumulated predominantly the 69-kDa (p2) precursor form. Reduced amounts of a CPY species that was electrophoretically indistinguishable from mCPY were also observed in these strains. The *vpt1-3* strain seemed to generate the greatest fraction of mCPY. In kinetics experiments where chase times of short duration (<20 min) were analyzed, some 30% of the radiolabeled CPY antigen was estimated to have been matured in this mutant. Other mutants, such as the *vpt3-2* and *vpt7-2* strains, were estimated to have converted as little as 10% of the recovered CPY to the

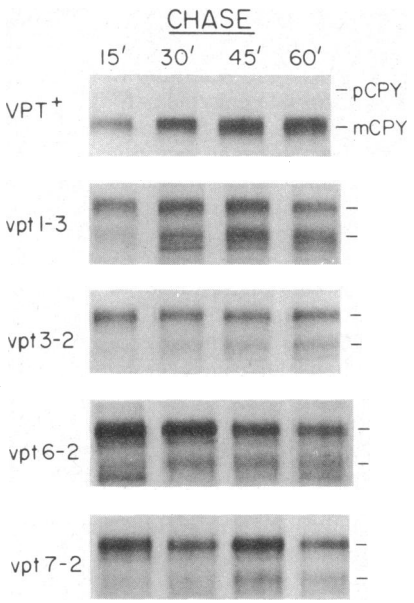

FIG. 2. Kinetics of CPY maturation in wild-type and *vpt* mutant yeast. Cells grown in glucose minimal proline medium were labeled with $^{35}SO_4^{2-}$ for 15 min. Chase was initiated by adding unlabeled SO_4^{2-} to 10 mM and terminated at the indicated times (min) by adding trichloroacetic acid to a final concentration of 5%. CPY was immunoprecipitated and analyzed by NaDodSO$_4$/PAGE and autoradiography (4). The mature (mCPY) and p2 precursor (pCPY) forms are indicated.

mature form. Mutants representative of *vpt* complementation groups not included in the experiment shown in Fig. 2 exhibited defects in CPY maturation similar to those presented here (data not shown). The p2 CPY that accumulated in the *vpt* mutants was unstable. This susceptibility to degradation was a result of p2 CPY mislocalization to the cell surface (see below). Since equivalent amounts of protein were loaded on the NaDodSO$_4$ gels in Fig. 2, the fractions of mCPY observed at late chase points represents an overestimation of the true amount of p2 CPY that was matured.

***vpt* Mutants Mislocalize CPY to the Periplasm and the Culture Medium.** Since the *vpt* mutants secreted a large percentage of CPY-Inv433 to the yeast periplasm and appeared to exhibit fusion-independent defects in CPY localization, isogenic *VPT*$^+$ and *vpt* yeast were fractionated to determine the ultimate fate of the mislocalized CPY (Fig. 3). In these experiments, the whole culture (W) fractions served as indicators of total quantities of CPY antigen. Note that in the *VPT*$^+$ strain the great majority of labeled CPY recovered from the W fraction was mCPY that fractionated with the intact spheroplasts, a property consistent with the vacuolar residence of this CPY form in wild-type yeast. Very little CPY antigen was recovered from the periplasmic and culture medium fractions. The mCPY observed in the periplasmic fraction was probably due to a small amount (<5%) of spheroplast lysis during the fractionation procedure. The CPY detected in the culture medium, however, was exclusively in the p2 form and its presence was not due to cell lysis. Our evidence indicates that the *VPT*$^+$ strain utilized in these studies normally secretes trace quantities (2–5%) of p2 CPY into the extracellular medium.

Fractionation of isogenic *vpt3-2* and *vpt4-2* strains demonstrated a striking mislocalization of CPY to the cell surface in both of these mutants. In each case, the predominant form of CPY recovered from the W fraction was p2. Reduced amounts of mCPY and the p1 form were observed also. Note that the spheroplast fractions derived from the *vpt3-2* and *vpt4-2* mutants were substantially devoid of radiolabeled CPY antigen. Rather, both the accumulated p2 species and the normally vacuolar mCPY were recovered from the periplasmic and medium fractions in an essentially quantitative manner. Secretion of CPY to the culture supernatant exhibited rapid kinetics (not shown). These mislocalized CPY species exhibited roughly equivalent distributions between the two extracellular fractions obtained from either mutant. The secreted CPY retained its native glycosylation pattern and did not appear to take on the heterogeneous character observed for normally secreted glycoproteins as a result of outer chain oligosaccharide addition (1). Similar patterns were observed for representative mutants in the other *vpt* complementation groups (data not shown). We believe the secreted mCPY is the authentic vacuolar form of the enzyme since the *vpt* strains exhibited low levels of CPY activity at the cell surface whereas the *VPT*$^+$ strains did not. To test whether cellular functions normally required for protein traffic from the Golgi complex to the cell surface were similarly required for the transit of CPY to the cell surface in *vpt* mutants, the *sec1-1* allele (2) was crossed into a *vpt3-1* strain. The *vpt3-1 sec1-1*^{ts} double mutant did not secrete CPY at the restrictive growth temperature (37°C) but efficiently secreted CPY at the permissive temperature (not shown).

DISCUSSION

We have employed the *SUC2* gene fusion approach as an initial strategy to genetically analyze the mechanism employed by yeast to properly deliver CPY to the vacuole. Gene fusion technology has been successfully applied in both prokaryotic and eukaryotic systems for purposes of mapping specific localization determinants within proteins and for devising genetic schemes for the identification of cellular components involved in particular localization processes (12, 28–30). The data reported here indicate that the vacuolar targeting problem should also be amenable to analysis using gene fusions.

We have demonstrated that the amino-terminal 433 residues of CPY are sufficient to redirect invertase, a normally secreted enzyme, to the yeast vacuole. It would seem, therefore, that the vacuolar targeting determinant that is elaborated by CPY must reside within this rather large

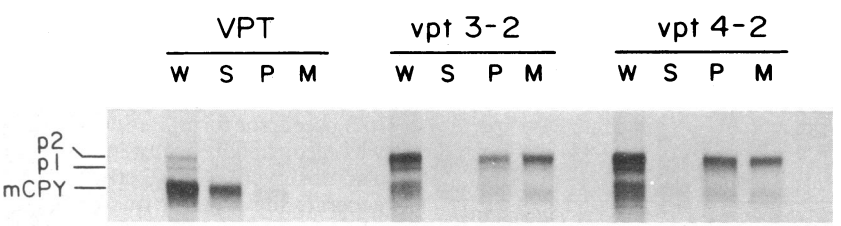

FIG. 3. Fractionation of CPY in *VPT*$^+$ and mutant *vpt* yeast. Cells were radiolabeled for 30 min with $^{35}SO_4^{2-}$ in medium that contained bovine serum albumin at 1 mg/ml. Incorporation of label was terminated by the addition of NaN_3 (10 mM). Cells were converted to whole culture (W), spheroplast (S), periplasmic (P), and culture medium (M) fractions. Radiolabeled CPY was precipitated from each and analyzed by NaDodSO$_4$/PAGE and autoradiography. The amount of each cell fraction analyzed is representative of equal numbers of labeled cells.

amino-terminal segment of the protein. The CPY targeting determinant(s) must therefore exhibit a functional dominance over any secretory information that may be present in mature invertase. We have recently obtained evidence that as few as 50 amino-terminal CPY residues are sufficient to divert invertase to the vacuole (unpublished data). We believe that CPY-Inv433 delivery to the vacuole provides an accurate representation of the route that is taken by wild-type CPY to this organelle. Other than the observation that both CPY and CPY-Inv433 are targeted to the vacuole, there are two reasons for concluding that this is so. First, CPY-Inv433 exhibited the same overproduction-related mislocalization phenotype that has been observed for wild-type CPY (24). Second, unlinked mutations that resulted in mislocalization of vacuolar CPY-Inv433 to the cell surface resulted in a similar, fusion-independent, mislocalization of wild-type CPY.

The *vpt* mutants exhibit major defects in the proper localization of CPY. As such, these mutants appear to be altered in cellular components that play an important role in mediating protein traffic to the yeast vacuole. Our initial results indicate that vacuolar protein traffic is a complex process that requires the participation of at least eight gene products. Rothman and Stevens (31) have employed a different approach to isolate vacuolar protein localization mutants that behave similarly to those we have described here. Preliminary comparisons of the two sets of mutants suggest that at least 14 complementation groups have been defined (unpublished results). The large number of complementation groups uncovered in these mutant analyses suggests that there may be several points in the vacuole delivery pathway where targeting can be blocked, with ensuing mislocalization to the cell surface, and that many gene products are involved. Indeed, it remains to be determined whether the *vpt* mutants are defective in protein sorting events (possibly at the level of the Golgi complex) or if they are affected in postsorting delivery events (e.g., vesicular traffic to the vacuole). Significantly, secreted CPY did not take on the hyperglycosylated form of normally secreted glycoproteins (Fig. 3). Our results do not rule out the possibility that the mislocalized CPY is secreted via a route alternative to that taken by other secretory glycoproteins such as invertase. However, the dependence of CPY secretion in a *vpt3-1* strain on *SEC1* function suggests that normal secretion and *vpt*-mediated secretion of CPY share at least one component of the late portion of the yeast secretory pathway. Secretion of wild-type invertase as well as bulk media and periplasmic polypeptides appear to be unaltered in *vpt* strains. It also seems that the *vpt* mutants may be pleiotropically defective in vacuolar protein biogenesis, as a *PEP4–SUC2* fusion protein was secreted in these mutants, and we have observed that proteinase B activity is depressed in these mutants, an expected consequence of proteinase B mislocalization (D. Klionsky and S.D.E., unpublished results). Furthermore, we have observed the appearance of several polypeptide species in bulk periplasmic and media fractions of *vpt* mutants that were not observed in fractions derived from the isogenic *VPT* parent.

An interesting feature of the *vpt* mutants reported here is that many of these strains show no significant growth defects or conditional phenotypes. Indeed, some *vpt* strains exhibit quite normal vacuoles on the basis of morphology and fractionation properties. Thus, even though CPY, and perhaps several other vacuolar components, is secreted in *vpt* strains, these mutants are still capable of assembling a vacuole. There may exist several pathways for vacuolar protein traffic in yeast and we have perturbed only one of these. Nevertheless, it appears that the *vpt* mutants have provided us with an initial genetic handle on how the cell delivers proteins to the yeast vacuole. We hope that through continued genetic analyses of vacuole assembly, coupled with a more detailed biochemical understanding of the many *VPT* gene products, we will ultimately be able to develop a molecular appreciation of the mechanisms by which intracellular protein traffic to the yeast vacuole is regulated.

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