The Cytoplasmic Tail Domain of the Vacuolar Protein Sorting Receptor Vps10p and a Subset of *VPS* Gene Products Regulate Receptor Stability, Function, and Localization

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VPS10 of *Saccharomyces cerevisiae* encodes a type I transmembrane receptor protein required for the sorting of the soluble vacuolar hydrolase carboxypeptidase Y (CPY). To characterize the essential structural features and intercompartmental transport itinerary of the CPY receptor, we have constructed mutant forms of Vps10p that alter the carboxy-terminal cytoplasmic tail of the protein. In addition, we have analyzed the effect these mutations as well as mutations in several *VPS* genes have on the function, stability, and localization of Vps10p. Although wild-type Vps10p is very stable over a 3-h chase period, overproduction of Vps10p results in *PEP4*-dependent degradation of the receptor. Immunofluorescence studies indicate that overexpressed receptor is delivered to the vacuole. A mutant form of Vps10p, in which 157 residues of the 164-residue cytoplasmic tail domain have been deleted, missorts CPY and is degraded rapidly. Additional mutations in the carboxy-terminus of Vps10p, including a deletion of a putative retention/recycling signal (FYVF), also result in CPY missorting and *PEP4*-dependent receptor instability. Because the cytoplasmic tail domain may interact with other factors, possibly *VPS* gene products, Vps10p stability was examined in a number of *vps* mutants. As was observed with the late Golgi protein Kex2p, Vps10p is unstable in a *vps1* mutant. However, instability of Vps10p is even more severe in the class E *vps* mutants. Double mutant analyses demonstrate that this rapid degradation is dependent upon vacuolar proteases and a functional vacuolar ATPase. Fractionation studies of Vps10p in class E *vps* mutant strains indicate that the turnover of Vps10p occurs in a compartment other than the vacuole. These data are consistent with a model in which the cytoplasmic tail of Vps10p directs cycling of the receptor between a late Golgi sorting compartment and a prevacuolar endosome-like compartment, an exaggerated form of which is present in the *vps* class E mutants.

INTRODUCTION

The compartmentalized nature of eukaryotic cells is necessary to sequester often competing biochemical reactions such as proteolysis and protein synthesis.

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The secretory pathway mediates the proper delivery and sorting of proteins to a variety of subcellular compartments. One of the best understood pathways involves the sorting of proteins to the lysosome. Soluble lysosomal proteins are synthesized in the cytosol and cotranslationally inserted into the endoplasmic reticulum (ER) along with proteins destined for secretion. Lysosomal proteins then transit through the Golgi apparatus where they receive a mannose 6-phosphate moiety that acts as a recognition signal

for the mannose 6-phosphate transmembrane receptor (MPR) in the trans-Golgi network (TGN). In the TGN, the MPR-ligand complexes are sorted into transport vesicles that fuse with the endosome. The low pH of the endosome then triggers the release of ligand from the receptor, the ligands move on to the lysosome, and the unoccupied receptors are recycled back to the TGN for further rounds of sorting (Kornfeld, 1992).

In the yeast *Saccharomyces cerevisiae*, several selection schemes (Jones, 1977; Bankaitis et al., 1986; Rothman and Stevens, 1986) have been undertaken to identify mutants defective for the delivery of proteins to the vacuole (the fungal equivalent of the lysosome). More than 45 complementation groups of vacuolar protein sorting (*vps*) mutants have now been identified (Jones, 1977; Robinson et al., 1988; Rothman et al., 1989). The hallmark of these mutants is that they mislocalize and secrete soluble vacuolar proteins, such as carboxypeptidase Y (CPY). The *vps* mutants can be divided into six groups based on their vacuolar morphology (classes A–F) (Banta et al., 1988; Raymond et al., 1992). We have previously shown that one of the *VPS* genes, *VPS10*, encodes a type I transmembrane glycoprotein that is localized to a Golgi-enriched fraction and serves as the sorting receptor for CPY (Marcusson et al., 1994). Thus, Vps10p and the MPRs serve analogous functions in yeast and mammalian cells.

The mechanism by which soluble proteins, such as CPY, reach the vacuole in yeast appears to be very similar to the mechanism of lysosomal protein sorting in mammalian cells. CPY is cotranslationally translocated into the ER where it is core glycosylated to generate the p1 precursor form of CPY (p1CPY). It next traverses the Golgi complex where its oligosaccharides are elongated to create p2CPY (Stevens et al., 1982). In the Golgi apparatus, a sorting signal in the propeptide portion of CPY is recognized by Vps10p, and a receptor-ligand complex is formed (Marcusson et al., 1994). This interaction is likely to occur in a distal Golgi compartment. Studies with a *sec* mutant that blocks intra-Golgi traffic have shown that a late Golgi compartment (TGN like) is the site at which p2CPY is sorted away from proteins destined to be secreted from the cell (Graham and Emr, 1991). Cell fractionation studies have also demonstrated the existence of a compartment containing p2CPY that lies between the Golgi apparatus and the vacuole. This compartment also appears to contain endocytosed α-factor and presumably represents an endosome-like intermediate (Vida et al., 1993). We have proposed that Vps10p delivers CPY to this endosomal compartment where it releases p2CPY before recycling back to the trans-Golgi for further rounds of sorting (Marcusson et al., 1994).

Vps10p presumably has a cellular itinerary similar to that of the MPRs, which have been shown to reside mainly in the TGN but also cycle through the endo-some and plasma membrane (Kornfeld, 1992). Transit of the MPRs through the cell is controlled by sequences in the carboxy-terminal tail of the MPRs that are likely to interact with cytosolic proteins (e.g. clathrin adaptor proteins). Signals in the cytoplasmic tail of the MPRs that control the cellular itinerary of these receptors have begun to be elucidated: a tyrosine-based signal has been shown to physically interact with clathrin adaptor protein complexes (Glickman et al., 1989), and a dileucine motif near the carboxy-terminus plays a role in the exit of the receptors from the trans-Golgi network (Johnson and Kornfeld, 1992).

Like Vps10p, two other late Golgi proteins, Kex2p and DPAP A, are also believed to cycle between the Golgi and the endosome in yeast. The stability of these proteins has been shown to be dependent on signals in their cytoplasmic tail domains (Wilcox et al., 1992; Nothwehr et al., 1993). The signals in these proteins are similar to the tyrosine-containing signals in the MPRs in that they require aromatic residues. A common set of cytosolic proteins may interact with the cytoplasmic tails of Kex2p, DPAP A, and Vps10p to control the cellular itinerary of these proteins. It is likely that such proteins could be among the more than 45 *VPS* gene products, as any mutation that causes Vps10p to be displaced from its normal cellular pathway should result in the secretion of CPY.

In this paper, we show that the cytoplasmic tail domain of Vps10p is essential for the proper function and stability of this receptor molecule. A mutant form of Vps10p missing the cytoplasmic tail is nonfunctional and mislocalized to the vacuole, where it is rapidly degraded in a *PEP4*-dependent fashion. We further show that a signal containing aromatic amino acids in the tail domain plays a role in Vps10p function and stability. A survey of the six different classes of *vps* mutants reveals that two *vps* classes (E and F) cause instability of Vps10p. The proteolytically processed form of Vps10p in the class E mutants is shown to reside in a compartment other than the vacuole. Therefore, this compartment may represent the E compartment that has been shown to contain active vacuolar hydrolases (Raymond et al., 1992).

MATERIALS AND METHODS

Yeast and Bacterial Methods

Standard yeast genetic techniques for diploid formation, sporulation, tetrad dissection, and gene disruption were used throughout (Sherman et al., 1979). Yeast cells were transformed using the alkali cation treatment method (Ito et al., 1983) or the procedure of Elble (1992). *Escherichia coli* transformations were performed using the method of Hanahan (1983). All yeast strains were grown in yeast extract, peptone, dextrose (YPD), or yeast nitrogen base (YNB) medium supplemented as necessary with the appropriate nutrients (Sherman et al., 1979). Strains lacking vacuolar ATPase (Δvma4) were grown in YNB medium supplemented with 0.2% yeast extract and buffered to pH 5.5 with 50 mM 2-[N-morpholino] ethanesulfonic acid (MES) (Morano and Klionsky, 1994).

Strain and Plasmid Construction

S. cerevisiae strains used in this study are listed in Table 1. Deletion-disruption strains were constructed as follows. To disrupt the *PEP4* gene in SEY28-2 (SEY6210 *vps28*), the plasmid, pP1::LEU2 (Ammerer *et al.*, 1986), was digested with *Bam*HI and transformed into SEY28-2 on YNB agar plates without leucine. Individual transformants were then picked, restreaked at least three times, and then assayed for CPY maturation to confirm the absence of proteinase A activity, yielding the strain JCY2801. *VMA4* knockout strains, such as JCY2802 (*vps28 Δvma4::URA3*), were created by transforming yeast with an *Eco*RI–*Bam*HI digestion of a disruption construct (pUC19/*Δvma4::URA3*) kindly pro-

vided by K. Morano and D. Klionsky (University of California, Davis, CA). Proper transformants were selected by presence of the *URA3* marker, inability to grow in medium buffered to pH 7.5, and absence of quinacrine staining of the vacuole, using 175 mM quinacrine (Banta *et al.*, 1988). *VPS10* deletion strain EMY20 (*Δvps10::TRP1*) was generated by transforming the wild-type strain SEY6210 with an *Xho*I–*Bam*HI restriction fragment of the pEMY10–105. The construct pEMY10–105 was made by deleting the 4.4-kb *Sal*I–*Stu*I portion of *VPS10* from the pBLUESCRIPT (Stratagene, La Jolla, CA) derivative pEMY10–102 (Marcusson *et al.*, 1994) and replacing it with the *TRP1* gene. To generate a *Δvps35* strain, the *Nci*I–*Xba*I fragment from plasmid pGPY35

Table 1. Strains used in this study

Name	Description	Source or Reference
SEY6210	*MATα leu2-3, 112 ura3-52 his3Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al.*, 1988
SEY6211	*MATα leu2-3, 112 ura3-52 his3Δ200 trp1-Δ901 ade2-101 suc2-Δ9*	Robinson *et al.*, 1988
SEY6210.5	SEY6210/SEY6211 diploid	Herman *et al.*, 1990
SEY1-1	SEY6210 *vps1-1*	Robinson *et al.*, 1988
SEY3-23	SEY6210 *vps3-23*	Robinson *et al.*, 1988
SEY4-15	SEY6210 *vps4-15*	Robinson *et al.*, 1988
SEY6-4	SEY6210 *vps6-4*	Robinson *et al.*, 1988
SEY8-3	SEY6210 *vps8-3*	Robinson *et al.*, 1988
SEY9-2	SEY6210 *vps9-2*	Robinson *et al.*, 1988
SEY11-6	SEY6211 *vps11-6*	Robinson *et al.*, 1988
SEY13-24	SEY6210 *vps13-24*	Robinson *et al.*, 1988
SEY15-8	SEY6210 *vps15-8*	Robinson *et al.*, 1988
SEY18-1	SEY6210 *vps18-1*	Robinson *et al.*, 1988
SEY19-1	SEY6210 *vps19-1*	Robinson *et al.*, 1988
SEY20-1	SEY6210 *vps20-1*	Robinson *et al.*, 1988
SEY22-2	SEY6210 *vps22-2*	Robinson *et al.*, 1988
SEY23-2	SEY6210 *vps23-2*	Robinson *et al.*, 1988
SEY24-2	SEY6210 *vps24-2*	Robinson *et al.*, 1988
SEY25-2	SEY6210 *vps25-2*	Robinson *et al.*, 1988
SEY26-1	SEY6210 *vps26-1*	Robinson *et al.*, 1988
SEY27-1	SEY6210 *vps27-1*	Robinson *et al.*, 1988
SEY28-2	SEY6210 *vps28-2*	Robinson *et al.*, 1988
SEY28-5	SEY6211 *vps28-5*	Robinson *et al.*, 1988
SEY32-1	SEY6210 *vps32-1*	Robinson *et al.*, 1988
SEY33-5	SEY6210 *vps33-5*	Robinson *et al.*, 1988
SEY34-1	SEY6210 *vps34-1*	Robinson *et al.*, 1988
BHY10	SEY6210 *leu2-3, 112*::pBHY11 (CPY-Inv *LEU2*)	Horazdovsky *et al.*, 1994
BHY110	SEY6210 *vps16Δ1::HIS3*	Horazdovsky and Emr, 1993
BHY152	SEY6210 *Δvps5::HIS3*	B. Horazdovsky, unpublished data
BWY100	SET6211 *Δvps24::HIS3*	B. Wendland, unpublished data
GBY10	SEY6210 *Δvps21::HIS3*	Horazdovsky *et al.*, 1994
KKY10	BHY10 *Δvps17::HIS3*	Koehrer and Emr, 1993
KKY20	BHY10 *Δvps28::URA3*	K. Koehrer, unpublished data
NY774	*MATα ura3-52 leu2-3, 112 sec4-8*	P. Novick, unpublished data
PSY129	SEY6210 *Δvps29::HIS3*	P. Scott, unpublished data
TVY1	SEY6210 *Δpep4::LEU2*	T. Vida, unpublished data
EMY3	SEY6210 *Δvps10::HIS3*	Marcusson *et al.*, 1994
EMY14	SEY6210 *Δpep4::LEU2 Δvps10::TRP1*	This study
EMY18	SEY6210 *Δvps35::HIS3*	This study
EMY20	SEY6210 *Δvps10::TRP1*	This study
EMY21	SEY6211 *Δvps10::TRP1 sec4-8*	This study
JCY104	SEY6211 *sec4-8*	This study
JCY2402	BWY100 *Δvma4::URA3*	This study
JCY2404	JCY104 *Δvps24::HIS3*	This study
JCY2502	SEY25-3 *Δvma4::URA3*	This study
JCY2801	SEY28-2 *Δpep4::LEU2*	This study
JCY2802	SEY28-5 *Δvma4::URA3*	This study
JCY2804	JCY104 *Δvps28::URA3*	This study
JCY3000	SEY6210 *Δvps30::HIS3*	J. Cereghino, unpublished data

(Paravicini *et al.*, 1992) was used to transform SEY6210 cells, yielding the strain EMY18.

To create a temperature-sensitive *sec4* strain that would carry more auxotrophic markers, NY774 (*MATα ura3–52 leu 2–3, 112 sec4–8*), generously provided by Peter Novick (Yale University, New Haven, CT), was mated to SEY6211. The resulting diploids were sporulated. Spore clones were chosen for the proper markers and temperature-sensitive growth at 37°C, yielding JCY104. Other strains containing the *sec4–8* mutation were then created by crossing with JCY104 and choosing segregants with the proper markers and temperature sensitivity.

pEMY10–14 was created by subcloning the *VPS10*-containing 8-kb *XhoI–BamHI* fragment from pEMY10–1 into pRS414 (Sikorski and Hieter, 1989). p6 MYC (a generous gift from Randolph Hampton, University of California, Berkeley) was cut with *ClaI*, filled-in with Klenow fragment, digested with *SacI*, and then blunted with T4 DNA polymerase. The 325-bp blunt end fragment thus generated was then subcloned into the *StuI* site at the 3′ end of *VPS10* in pEMY10–14 to create pEMY10–21. This construct encodes a Vps10p that has six *myc* epitopes (EQKLISEEDL) in tandem near the C-terminus. A 2μ version of this construct was then generated by cloning the 6-kb *BglII* fragment from pEMY10–21 into pRS426 (Sikorski and Hieter, 1989).

Oligonucleotide Site-directed Mutagenesis

Site-directed mutagenesis was performed using the method of Deng and Nickoloff (1992) with reagents from Clontech (Palo Alto, CA). pEMY10–1 (Marcusson *et al.*, 1994) was used as the template for all mutagenesis reactions. In the plasmid pEMYm1–10 (ΔC157), the codons for G1421 and G1422 were mutated to stop codons (GGG→TAG and GGA→TGA, respectively), resulting in the deletion of the last 157 amino acids of Vps10p. The codon for N1420 was also changed from AAT to AAC to create a *SpeI* site, which was used to help screen for mutants. The plasmids pEMYm7–10 (ΔTail1) and pEMYm8–10 (ΔTail2) were created by looping out the DNA encoding amino acids 1420–1497 and 1497–1576, respectively. pEMYm11–10 (ΔFYVY) was made by looping out the codons for amino acids 1454–1457. In this mutant, the codon S1458 was changed from TCA to TCC to create a *BamHI* site for screening.

Cell Labeling and Protein Immunoprecipitation

Protein stability assays, cell labeling, and immunoprecipitation were performed essentially as described in Gaynor *et al.* (1994). Yeast cells were grown to mid-logarithmic phase (A_{600} = 0.7–1.0) in YNB medium supplemented with the appropriate nutrients. Growth medium of Δ*vma4* strains was additionally supplemented with yeast extract to 0.2% and buffered with 50 mM MES, pH 5.5. Cells (2–3 OD_{600} equivalents per ml) were labeled in YNB medium, without yeast extract, with 20–30 μCi of Tran[35]S-label (ICN Biochemicals, Costa Mesa, CA) per OD equivalent of cells. Chase was initiated by adding a 10 × chase solution (50 mM methionine, 10 mM cysteine, 4% yeast extract, 20% glucose) to a 1 × final concentration. A 2 × chase solution diluted in medium was also used in some cases, without any variation in result. Chase was terminated at indicated times by adding trichloroacetic acid to a final concentration of 5–10%.

Immunoprecipitation of the samples was carried out as previously described (Klionsky *et al.*, 1988) with the following modifications. Before glass bead lysis, dried cell pellets were resuspended in boiling buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% sodium dodecyl sulfate [SDS], 6 M urea). Immunoprecipitates were washed in a succession of four buffers: Tween-20 IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.5% Tween-20), Tween-20 IP buffer plus 2 M urea, 0.1% SDS, and finally, Tris-buffered saline (TBS; 50 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.1 mM EDTA). Immunoprecipitates were resuspended in urea protein sample buffer (100 mM Tris-HCl, pH 6.8, 4.5% SDS, 6 M urea, 8% β-mercaptoethanol, 0.005% bromophenol blue) and heated to 65°C for 5 min. Samples were then electrophoresed as previously described by Laemmli (1970) in 8 or 9% SDS-polyacrylamide gels. Antibodies to alkaline phosphatase (ALP), α1,3-mannosyltransferase, and Vps10 protein have been previously described (Klionsky and Emr, 1989; Graham *et al.*, 1994; Marcusson *et al.*, 1994). Kex2 antibody was a generous gift from R. Fuller. Quantitation of stability and CPY sorting assays was performed with a Molecular Dynamics Phosphorimager (Sunnyvale, CA) and ImageQuant version 3.3 software.

Subcellular Fractionation

Yeast strains were grown in YNB medium and converted to spheroplasts as previously described (Vida *et al.*, 1990) except 5 μg/OD zymolyase 100T (Seikagaku, Tokyo, Japan) and 0.2% glusulase (Dupont, Wilmington, DE) were used. Five OD equivalents/milliliter of spheroplasts resuspended in YNB containing 1 M sorbitol were labeled for 30 min at 30°C and chased for 45 min with the chase solution described above. Labeled spheroplasts were harvested and resuspended at 10 OD equivalents/milliliter in cold lysis buffer (0.2 M sorbitol, 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, and protease inhibitors [phenylmethylsulfonyl fluoride (1 mM), $α_2$-macroglobulin (5 μg/ml), aprotinin (1 μg/ml), leupeptin (1 μg/ml), and pepstatin (1 μg/ml)]). The spheroplast suspension was dounced 5–10 times with a glass tissue homogenizer, and then centrifuged (500 × g, 5 min) to remove unbroken spheroplasts. The resulting supernatant (S5) was subsequently centrifuged at 13,000 × g for 15 min to generate pellet (P13) and supernatant (S13) fractions. The S13 was centrifuged at 100,000 × g for 45 min to create a high speed pellet (P100) and supernatant (S100) fraction. Five OD-equivalents of each pellet or supernatant fraction was precipitated with a final concentration of 10% trichloroacetic acid. The presence of Vps10 protein, ALP, or Kex2 protease was determined by immunoprecipitation and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography.

Immunofluorescence

For immunofluorescence using diploid wild-type yeast cells (SEY6210.5), the method of Redding *et al.* (1991) was used with the following modifications. Cells were fixed for 14–18 h, spheroplasted with 45 μg/ml Zymolyase 100T (Seikagaku), then treated with 1% SDS instead of 0.5%. Fixed spheroplasts were incubated in either 2 ng/ml anti-myc monoclonal (Cambridge Research Biochemicals, Wilmington, DE) or 10 ng/ml anti-60 kDa vacuolar ATPase subunit (Molecular Probes, Eugene, OR) as the primary antibody, followed by serial incubations of 0.5 μg/ml goat anti-mouse IgG, 1.0 μg/ml rabbit anti-goat IgG, and a 1/100 dilution of FITC-conjugated goat anti-rabbit IgG, all from Jackson ImmunoResearch, West Grove, PA.

RESULTS

Turnover of Vps10 Protein

A major assumption in models of receptor-mediated protein sorting is that receptors must be used multiple times to efficiently transport their cargo. Recycling is likely to be involved in the mechanism of Vps10-mediated CPY sorting, because the synthesis rate of Vps10p is at least 10-fold lower than that of its ligand, carboxypeptidase Y (Marcusson *et al.*, 1994; Marcusson and Emr, unpublished data). Consequently, the stability and recycling of Vps10p would contribute significantly to efficient sorting of CPY. To assess the stability of Vps10p, we carried out an extended pulse-chase analysis of metabolically labeled receptor pro-

tein. Whole cells from the wild-type strain SEY6210 were labeled for 15 min with Tran^{35}S-label, then chased with the addition of cold methionine and cysteine for 2 h. Equivalent samples were removed at 0-, 30-, 60-, and 120-min intervals. Cell extracts generated from these timepoints were immunoprecipitated with polyclonal antiserum specific to Vps10p (Marcusson *et al.*, 1994). Labeled Vps10p was then visualized by SDS-PAGE and subsequent autoradiography. Wild-type chromosomal Vps10p was found to be stable over a 2-h chase period (Figure 1, lanes 1–4). Additionally, longer chases have indicated that the half-life of wild-type Vps10 protein is far greater than 3 h (see Figure 7).

To determine whether overexpression of Vps10p influenced protein stability, wild-type cells containing *vps10* on a 2μ-based multicopy plasmid pEMY10-2 (Marcusson *et al.*, 1994), were pulse labeled for 15 min with Tran^{35}S and chased for 2 h. Increasing the intracellular pool of Vps10p 10- to 20-fold resulted in the appearance of a proteolytically clipped intermediate concomitant with the disappearance of full-length Vps10p. This proteolytic product of Vps10p has a molecular mass of approximately 170 kDa, 20 kDa less than intact Vps10p. Although other peptides are sometimes observed, this 170-kDa Vps10p product appeared to be a fairly stable intermediate that was only slowly proteolyzed. After a 2-h chase, approximately 30% of the overexpressed Vps10p was degraded to the lower molecular weight form (Figure 1, lanes 5–8).

Overexpression of a number of *S. cerevisiae* membrane proteins including Ste6p (the **a**-factor transporter) (Berkower *et al.*, 1994), the α1,3 mannosyltransferase (Mnn1p) (Graham *et al.*, 1994), and three proteases involved in the final proteolytic maturation of α-factor mating pheromone precursor, Kex1p (Cooper and Bussey, 1992), Kex2p (Wilcox *et al.*, 1992), and DPAP A (Roberts *et al.*, 1992), leads to an increased degradation rate for these various proteins. In proteinase A-minus strains ($\Delta pep4$), Kex1p, Kex2p, Ste6p, and DPAP A are stabilized and accumulate in the vacuole, the major site of protein degradation in yeast (Jones, 1984; Klionsky *et al.*, 1990; Jones and Murdock, 1994). Without proteinase A, yeast cells are pleiotropically deficient in the activities of the major vacuolar pro-
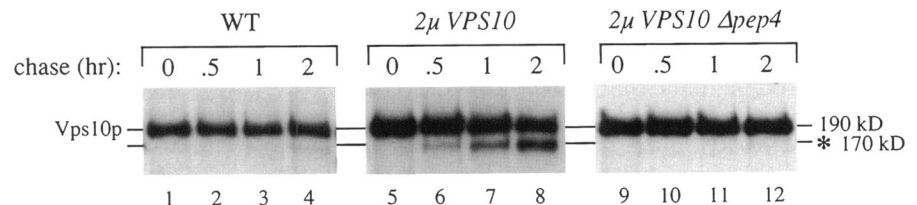
teases (Jones *et al.*, 1982). Therefore, because we wanted to determine the role of vacuolar proteases in degradation of overexpressed Vps10p, we performed a pulse-chase stability analysis of overexpressed Vps10p in a $\Delta pep4$ strain. We found that overexpressed Vps10p was stabilized ($t_{1/2}$>>2 h) in an isogenic $\Delta pep4$ strain (Figure 1, lanes 9–12), demonstrating that *PEP4*-dependent vacuolar proteases were involved in the degradation of Vps10p.

Overexpressed Vps10 Protein Accumulates in the Vacuole

The *PEP4*-dependent degradation of Vps10p indirectly indicated that the overexpressed receptor was delivered to the vacuole. To test directly for the presence of Vps10p in the vacuole, the subcellular distribution of overexpressed Vps10p in a $\Delta pep4$ strain was examined by differential centrifugation. Spheroplasts were labeled for 20 min at 30°C and chased for 45 min. Cells were osmotically lysed in the presence of protease inhibitors with five strokes of a Dounce homogenizer. After homogenization, the lysate was cleared of unbroken cells and centrifuged sequentially at 13,000 $\times$ *g* and 100,000 $\times$ *g*. The resulting pellets and supernatants were then immunoprecipitated for Vps10p, the vacuolar membrane marker protein ALP, or the late Golgi marker protein Kex2 protease. Previously, we reported that the majority (> 90%) of Vps10p in wild-type cells fractionated in the high speed pellet, which contains Golgi membranes in addition to small vesicular structures (Marcusson *et al.*, 1994). Although a large proportion of overexpressed Vps10p did fractionate with the P100 pellet (70%), a significant portion of Vps10p (30%) cofractionated with the vacuolar marker ALP (Figure 2). The distribution of Kex2p in the high speed pellet (P100) in the presence of overexpressed Vps10p was not altered in comparison with the wild-type strain (Figure 2).

As an independent means of directly examining the steady-state distribution of overexpressed Vps10p, indirect immunofluorescence of an epitope-tagged version of Vps10p was performed. The *myc* epitope, iterated six times, was inserted into the *Stu*I restriction endonuclease site at the carboxy terminus of the Vps10 protein. The insertion did not affect the reading

Figure 1. Stability of wild-type Vps10 protein. Yeast cells were pulse-labeled with Tran^{35}S for 15 min and chased for 2 h. Vps10p was immunoprecipitated from these fractions and resolved by SDS-PAGE and fluorography. The strains used were as follows: SEY6210 (WT), SEY6210 + 2 μVPS10 (2μ VPS10), and TVY1 + 2μ VPS10 (2μ VPS10 $\Delta pep4$). The "*" indicates the major proteolytic fragment of Vps10p.

WT   2μ VPS10 Δpep4

P13 S100 P100   P13 S100 P100
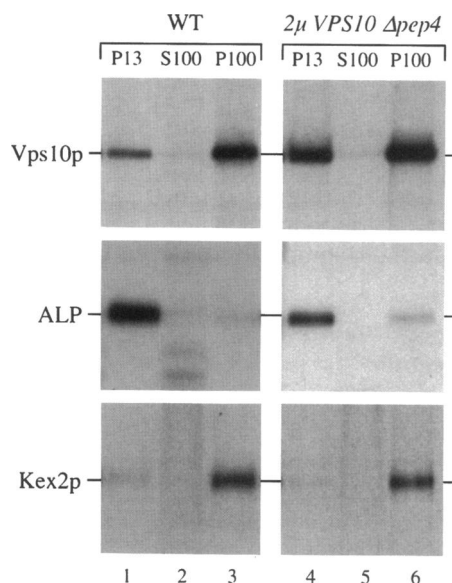
Vps10p

ALP

Kex2p

1   2   3   4   5   6

Figure 2. Subcellular fractionation of Vps10p. Spheroplasts from wild-type cells (SEY6210) or 2μ *VPS10 Δpep4* (TVY1 + 2μ *VPS10*) were labeled with Tran³⁵S for 20 min, chased for 45 min, and used to generate subcellular fractions as described in the text. The presence of Vps10p and marker proteins for the vacuole (ALP) and the late Golgi compartment (Kex2p) in the P13, S100, and P100 fractions was determined by quantitative immunoprecipitation.

Figure 3. Indirect immunofluorescence of MYC-tagged Vps10p. Wild-type diploid cells (SEY6210.5) containing no plasmid (A), or *CEN* (B), or 2μ (C) *VPS-MYC* containing plasmids were prepared for immunofluorescence as described in MATERIALS AND METHODS. Cells were stained with antibodies against MYC (A, B, and C) or the 60-kDa subunit of vacuolar ATPase (D).

frame or the stability of Vps10p and the epitope-tagged receptor efficiently complemented the CPY sorting defect of a Δvps10 disruption strain. This construct was subcloned into *CEN* and 2μ vectors and transformed into wild-type diploid cells. Indirect immunofluorescence of the *CEN* version of Vps10p-MYC with monoclonal anti-MYC antibody revealed a punctate pattern, possibly Golgi or endosomal, not observed in diploid cells without Vps10p-MYC (Figure 3, A and B). When Vps10p-MYC was overexpressed, immunofluorescence revealed localization to the vacuole in addition to nonvacuolar, punctate structures (Figure 3C). In the control, using a monoclonal antibody to the 60-kDa peripheral subunit of vacuolar ATPase, the expected pattern of vacuole membrane was seen. Punctate staining was not observed (Figure 3D).

The Cytoplasmic Tail Domain of Vps10p Is Required for Retention and Recycling of the Receptor

The carboxy-terminal tail domains of both the 300- and 46-kDa mannose 6-phosphate lysosomal protein sorting receptors (MPRs) contain leucine- and tyrosine-based signals that direct transport and recycling of the receptors between the Golgi, plasma membrane, and endosomes. These domains are thought to interact with components of the lysosomal delivery system (i.e., clathrin-associated adaptor molecules) to
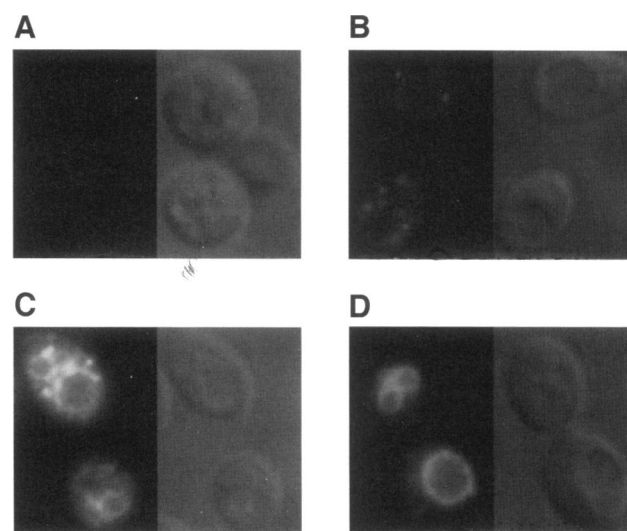
direct loaded receptors into transport vesicles destined for the endosome (Pearse and Robinson, 1990; Kornfeld, 1992). Similar in structure to MPRs, Vps10p appears to be a type I integral membrane protein with 164 carboxy-terminal amino acids exposed to the cytoplasm (Marcusson *et al.*, 1994). To examine the functional requirement of Vps10p's cytoplasmic domain, a mutant was constructed in which two stop codons were inserted into the sequence of *VPS10* after codon 1420. This resulted in the production of a truncated Vps10 protein lacking 157 C-terminal cytoplasmic residues but leaving the transmembrane domain intact, including a few charged amino acids on the cytoplasmic side of the membrane to properly anchor the protein. A *CEN* plasmid containing the *VPS10* carboxy-terminal deletion construct (pΔC157) was introduced into the Δvps10 strain EMY3, and the ability of the transformants to sort CPY properly was determined. Wild-type and ΔC157 strains were labeled with Tran³⁵S for 10 min and chased for 30 min by the addition of cold methionine and cysteine. Cells were converted to spheroplasts, and cultures were split into spheroplast pellet (intracellular, I) and medium (extracellular, E) fractions, and CPY was then immunoprecipitated from each fraction (Figure 4A). EMY3 cells carrying wild-type *VPS10* on a *CEN* vector correctly localized a majority of CPY (≈90%) to the vacuole, as indicated by the presence of CPY inside the cell in its mature vacuolar form (mCPY, Figure 4A, lanes 1 and 2). Similar to the phenotype of a Δvps10 strain (Marcusson *et al.*, 1994), Vps10p ΔC157 mislocalized and

Figure 4. Deletion of the cytoplasmic tail of Vps10p results in missorting of CPY and instability of the Vps10 protein. (A) Yeast spheroplasts were pulse-labeled for 10 min with Tran³⁵S, chased for 30 min, and separated into intracellular (I) and extracellular (E) fractions. Carboxypeptidase Y (CPY) was immunoprecipitated from the resulting fractions and visualized by SDS-PAGE and subsequent fluorography. Strains derived from EMY3 (Δvps10) harboring plasmids containing either wild-type VPS10 (CEN VPS10) or the deletion mutant (pΔC157) were used. (B) Yeast cells were labeled with Tran³⁵S for 10 min and chased for 2 h. Equal aliquots of cells were removed at the indicated intervals. The presence of Vps10p or the ΔC157 deletion protein was determined by quantitative immunoprecipitation. The strains used were as follows: SEY6210 (WT), SEY6210 + pΔC157 (WT + ΔC157), or TVY1 + pΔC157 (Δpep4 + ΔC157). The "*" indicates the major proteolytic fragments of Vps10p ΔC157.
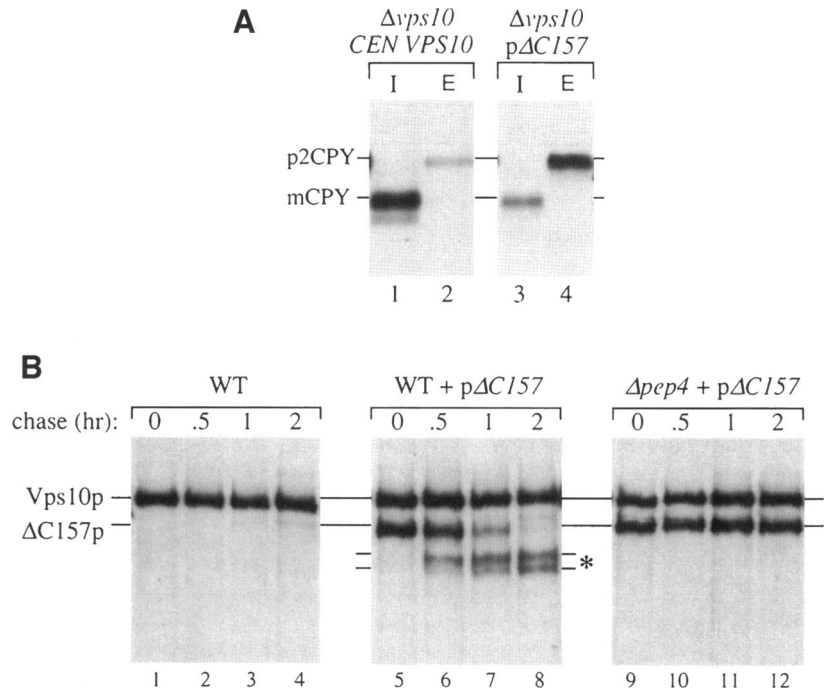
secreted to the medium approximately 70% of Golgi-modified p2CPY (Figure 4A, lanes 3 and 4).

We further analyzed Vps10p ΔC157 by testing whether expression or stability of the mutant receptor was altered. The metabolic stability of Vps10p ΔC157 was determined by pulse-chase experiments. Wild-type cells transformed with a plasmid carrying the ΔC157 mutation were labeled with Tran³⁵S for 10 min, then chased with cold methionine and cysteine for 0, 30, 60, or 120 min. Wild-type as well as mutant Vps10 proteins were immunoprecipitated. Wild-type and mutant Vps10p ΔC157 were produced at comparable levels (0 min chase, Figure 4B, lane 5). However, at later time points, Vps10p ΔC157 exhibited marked instability ($t_{1/2} \approx 1$ h), as demonstrated by the disappearance of the full-length protein and the appearance of smaller proteolytic products (Figure 4B, lanes 6–8). In the absence of active vacuolar proteases, degradation of Vps10p ΔC157 was blocked (Figure 4B, lanes 9–12), suggesting that the mutant receptor was delivered to the vacuole. Similar results were obtained for ΔC157 Vps10p in a Δvps10 strain; therefore, the presence or absence of wild-type Vps10p has no influence on the stability of the mutant receptor. Additionally, although it is possible that Vps10p is first delivered to the plasma membrane, then transported to the vacuole by endocytosis, we have found that the amount of degradation of the cytoplasmic tail deletion mutant protein ΔC157 Vps10p is not altered by blocking traffic to the plasma membrane at the restrictive temperature in a sec4ᵗˢ mutant. Using a sec1 mutant, it has been

shown that vacuolar delivery of mutant or wild-type Kex2 or DPAP A proteins is not disrupted in the absence of vesicular transport from the Golgi to the plasma membrane (Roberts et al., 1992; Wilcox et al., 1992; Nothwehr et al., 1993).

Mutations in the Vps10p Cytoplasmic Domain Affect Receptor Stability and Function

To define specific domains in the cytoplasmic tail of Vps10p that may direct transport and/or recycling of the receptor, oligonucleotide site-directed mutagenesis was used to generate three deletions in addition to ΔC157 (pictured in Figure 5): ΔTail1 (Δ1420–1497), ΔTail2 (Δ1497–1576), and ΔFYVF (Δ1454–1457). ΔTail1 and ΔTail2 were created to subdivide the cytoplasmic domain into two halves. The sequence FYVF starting at residue 1454 was deleted because it resembles the Golgi retention motif F/Y-X-F/Y found in other late Golgi proteins such as DPAP A (Roberts et al., 1992; Nothwehr et al., 1993) and Kex2p (Wilcox et al., 1992). CEN plasmids expressing these mutant forms of Vps10p were transformed into the Δvps10 strain EMY3 and tested for CPY sorting and Vps10 protein stability. Results of these assays are summarized in Figure 5. All mutations resulted in degradation of the mutant receptor protein and missorting of CPY. The relative defect in protein stability correlated with severity of the CPY missorting phenotype. The removal of 80 amino acids from the carboxy-terminus (ΔTail2) of Vps10p had a slight effect on CPY sorting. Deletion of
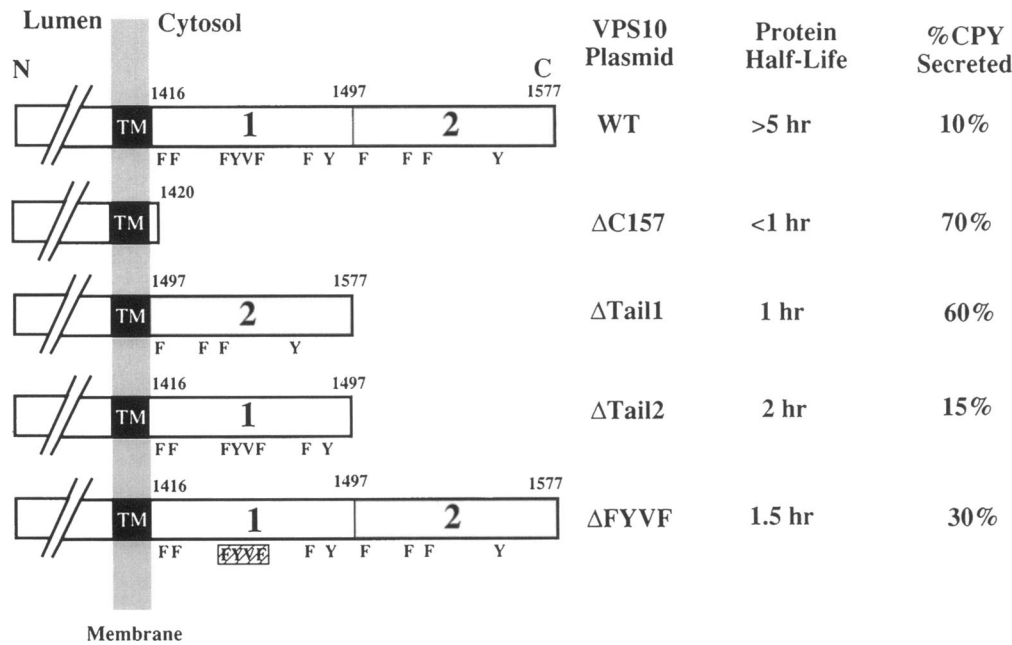
VPS10 Plasmid	Protein Half-Life	%CPY Secreted
WT	>5 hr	10%
ΔC157	<1 hr	70%
ΔTail1	1 hr	60%
ΔTail2	2 hr	15%
ΔFYVF	1.5 hr	30%

Figure 5. Determination of the stability and function of the Vps10p cytoplasmic tail mutants. Wild-type and mutant Vps10 proteins are shown schematically inserted into the membrane (shaded gray area). The amino terminus of Vps10p faces the lumen whereas the carboxy-terminal domain is cytosolic. The tail domain has been divided into two halves, 1 and 2. Numbers corresponding to residues defining these halves are shown above the cytosolic domains. Tyrosine (Y) and phenylalanine (F) residues are noted below. The ΔFYVF deletion is marked by the hatched box. Transmembrane (TM) segments are shown in black and the membrane itself is shaded in gray. Stability data were generated by labeling EMY3 (Δ*vps10*) cells containing each of the various constructs for 15 min. Samples were chased for a total of 3 h. Half-lives were determined through PhosphorImager quantitation of immunoprecipitated Vps10p. The same strains were pulse-labeled for 10 min and chased for 30 min to perform CPY sorting assays. % CPY secreted was determined by averaging values obtained from several experiments.

the first half of the cytoplasmic tail closest to the transmembrane domain (ΔTail1) or just the FYVF residues in this domain resulted in more instability of the mutant receptor and missorting of CPY. Just as was the case for a complete deletion of the cytoplasmic tail domain (Vps10p ΔC157), degradation of these tail mutant proteins is also *PEP4*-dependent, indicating each is delivered to the vacuole.

Subcellular Fractionation Demonstrates That Vps10p ΔC157 Cofractionates with Vacuolar Membranes

To determine the subcellular localization of Vps10p cytoplasmic tail domain mutants, we carried out differential centrifugation of Vps10p ΔC157 in Δ*vps10* Δ*pep4* cells as described above. Osmotically lysed yeast were cleared of unbroken cells (S5) then subjected to sequential fractionation steps to yield a 13,000 × *g* pellet (P13), a 100,000 × *g* pellet (P100), and the corresponding supernatant (S100). Vps10p, the late Golgi protein Kex2 protease, and the vacuolar membrane protein ALP were then quantitatively immunoprecipitated from the different fractions. As previously demonstrated in wild-type cells (Figure 2), ALP associated with the P13 pellet, and Kex2p was found in the P100 fraction in the Δ*vps10* Δ*pep4* strain (Figure 6). Even at a 45 min chase point (Figure 6), a substantial portion (50–60%) of truncated Vps10p is already associated with the P13 fraction. This supports the hy-

pothesis that the decreased half-life (≈1 h) of Vps10p ΔC157 is indicative of delivery to the vacuole. Although other organelles such as the endoplasmic reticulum and the plasma membrane have been found to fractionate in a 13,000 × *g* pellet (Marcusson *et al.*, 1994), the *PEP4*-dependent instability of Vps10p ΔC157 along with the fractionation results, strongly suggest that the mutant receptor is transported to the vacuole. A fractionation pattern similar to Vps10p ΔC157 was also observed for the ΔFYVF mutant.

Vps10p Is Unstable in Class E and Class F vps Mutant Strains

Previously, it has been proposed that Vps10p may interact with other components of the vacuolar transport machinery through its cytoplasmic domain (Marcusson *et al.*, 1994). To examine whether *VPS* gene products are necessary for Vps10p retention or recycling, we initiated a study of Vps10p stability in a number of *vps* mutant strains. Mutants from each of the six morphological classes of *vps* complementation groups were tested. Distinctions among these classes are based on differences in vacuolar features such as morphology, inheritance, protein composition, and function (Banta *et al.*, 1988; Raymond *et al.*, 1992). Class A *vps* mutants have one to three large vacuoles per cell, similar to wild-type cells. Class B mutants have many small, apparently fragmented, vacuole-like structures, whereas class C mutants appear to lack
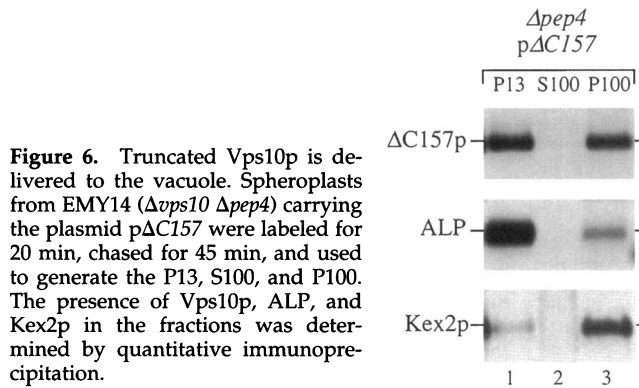
Figure 6. Truncated Vps10p is delivered to the vacuole. Spheroplasts from EMY14 (Δvps10 Δpep4) carrying the plasmid pΔC157 were labeled for 20 min, chased for 45 min, and used to generate the P13, S100, and P100. The presence of Vps10p, ALP, and Kex2p in the fractions was determined by quantitative immunoprecipitation.

Figure 7. Survey of Vps10p stability in *vps* mutants. Yeast cells were pulse-labeled with Tran35S for 15 min and chased for 3 h. Vps10p levels were determined by quantitative immunoprecipitation. Strains used were as follows: SEY6210 (WT), EMY18 (Δvps35), GBY10 (Δvps21), BWY100 (Δvps24), and SEY1–1 (vps1). The "*" denotes the major proteolytic fragment of Vps10p.

vacuoles altogether. In addition to exhibiting defects in vacuolar segregation, class D mutants have a single large, spherical, vacuole. Class E mutants have been proposed to contain an exaggerated prevacuolar compartment. Class F mutants are distinguished from class B mutants by a large central vacuole surrounded by fragmented vacuolar structures.

For Vps10p stability assays, whole cells were labeled for 15 min with Tran35S. Equal aliquots of labeled cells were removed 0, 1, 2, and 3 h after a chase was initiated with unlabeled methionine and cysteine. Vps10 protein was then immunoprecipitated and visualized by SDS-PAGE and subsequent autoradiography. Over a 3-h chase period, Vps10p remained quite stable in wild-type, Δvps35 (class A), or Δvps21 (class D) strains (Figure 7). This was also true for other *vps* mutants of classes A (*vps 8, vps 13, vps 29,* and *vps 30*) and D (*vps 3, vps 6, vps 9, vps 15, vps 19, vps 21,* and *vps 34*) as well as for classes B (Δvps5) and C (*vps 11, vps 16, vps 18,* and *vps 33*). In contrast, moderate degradation of Vps10p was observed in a *vps1* (class F) mutant; after 3 h of chase, about 40% of the labeled Vps10p had been degraded (Figure 7). All other alleles of *vps1* that were examined (SEY1–2 and SEY1–3) as well as the only other member of Class F, *vps26*, also displayed this instability phenotype.

Interestingly, Vps10p was most unstable in class E *vps* mutants. In Δvps24, as well as in all other class E mutants tested (*vps2, vps4, vps20, vps22, vps23, vps25, vps27, vps28,* and *vps32*), Vps10p was very unstable, exhibiting a half-life of less than 30 min (Figure 7). The dramatic instability of Vps10p in the class E but not other classes of *vps* mutants suggests that the class E mutants may alter either the trafficking of Vps10p or the composition and function of compartments through which the receptor normally cycles. As the intracellular itinerary of Vps10p is likely to involve transport to a prevacuolar endosome-like organelle followed by recycling back to the Golgi, the class E mutants may alter the endosomal compartment and therefore the stability of Vps10p.
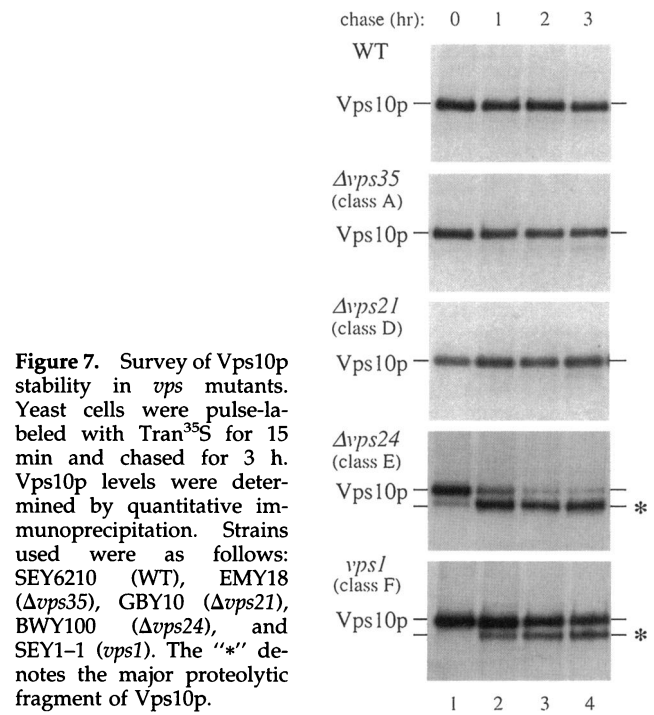
Degradation of Vps10 Protein Occurs in the Class E Compartment

The class E *vps* mutants have been demonstrated to contain an organelle distinct from the vacuole. Immunofluorescence studies have shown that although the vacuolar membrane protein ALP stained the vacuole in the class E mutants, the novel "E" compartment accumulated active vacuolar H+-ATPase complex, soluble vacuolar hydrolases, as well as a DPAP A (late Golgi protein)-ALP fusion (A-ALP) (Raymond *et al.*, 1992). To determine whether the degradation of the Vps10 receptor protein in the class E mutants might be a result of trafficking to the E compartment, we constructed double mutants to address the role of vacuolar proteases and the vacuolar ATPase activity on Vps10p instability. Double mutants were constructed between the class E *vps28* mutant and either Δpep4 or Δvma4. VMA4 encodes the 27-kDa peripheral subunit of the vacuolar H+-ATPase. Vps10p remained stable in the *vps28* Δpep4 double mutant (Figure 8A, lanes 5–8) indicating that active hydrolases are required for Vps10p degradation in this class E mutant. Additionally, disruption of VMA4 dramatically reduced the instability of Vps10p in the strains *vps28* Δvma4, Δvps24 Δvma4, and *vps25* Δvma4 (Figure 8A, lanes 9–12). The function of the vacuolar H+-ATPase is to maintain an acidic environment in the vacuole (Anraku *et al.*, 1992; Kane and Stevens, 1992; Nelson, 1992). Acidification of the vacuole has been implicated in the maturation of precursor proteins by stimulating
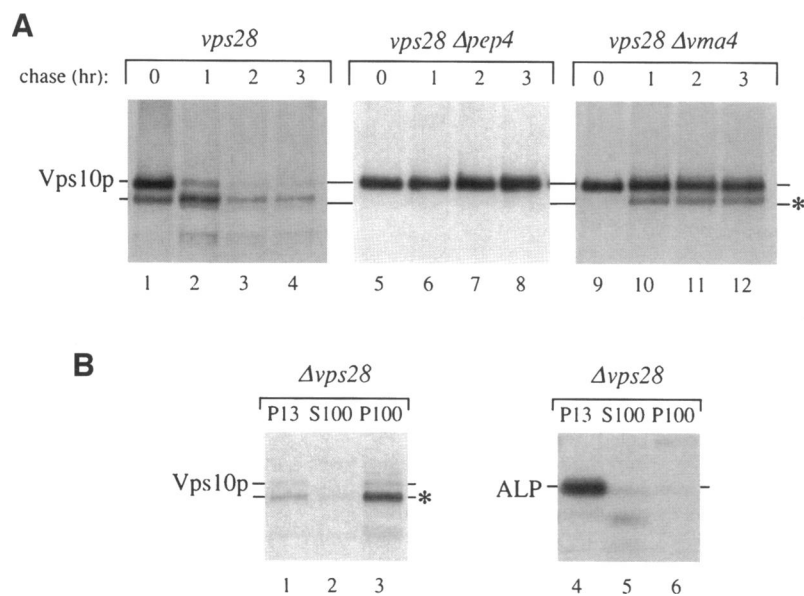
Figure 8. Vps10p is delivered to the E compartment in Δvps28. (A) Stability of Vps10p was examined by pulse-labeling cells for 15 min with Tran[35]S then adding a chase of unlabeled methionine and cysteine for 3 h. At 0, 1, 2, and 3 h equal aliquots were removed and Vps10p was immunoprecipitated from each sample. Strains used were: SEY28-2 (*vps28*), JCY2801 (*vps28 Δpep4*), and JCY2802 (*vps28 Δvma4*). (B) Yeast spheroplasts from KKY20 (Δ*vps28*) cells were labeled for 20 min, chased for 45 min, and used to generate the P13, S100, and P100 fractions. ALP and Vps10p levels were determined by immunoprecipitation. In both sets of gels, the "*" indicates the major proteolytic fragment of Vps10p.

the autocatalytic maturation of precursor proteinase A (Jones 1984; Klionsky *et al.*, 1990). Because inactivation of *VMA4* substantially reduced Vps10p instability, acidification of the class E compartment appears to play an important role in receptor degradation.

Class E mutants display a modest *vps* phenotype in that they secrete about 30–50% of newly synthesized CPY in its precursor form (Robinson *et al.*, 1988; Raymond *et al.*, 1992). The presence of active proteases in the E compartment presumably contributes to the rapid turnover of Vps10p, thus rendering the receptor unable to execute multiple rounds of protein sorting. We thought we might be able to rescue the class E mutant CPY sorting defect by taking advantage of the Δ*pep4 vps28* strain and protecting the receptor from degradation. However, the same quantity of CPY is secreted in Pep4+ as compared with Pep4− isogenic strains. Therefore, not only is Vps10p degraded, its intracellular itinerary is altered. Apparently, in the Class E *vps* mutants, protein traffic to the vacuole as well as traffic returning from the endosome is disrupted.

To determine the subcellular location of Vps10p in Δ*vps28* mutant cells, differential centrifugation of osmotically lysed spheroplasts was performed. Spheroplasts were labeled for 20 min and chased for 45 min to examine the fractionation of the processed form of Vps10p. As expected from previously reported immunofluorescence data (Raymond *et al.*, 1992), ALP was found in the vacuole-enriched P13 fraction (Figure 8B). The majority of both the clipped and remaining intact forms of Vps10p were fractionated in the high-speed 100,000 × *g* pellet (P100) (Figure 8B), indicating that the Vps10 protein was degraded in a compart-

ment other than the vacuole, possibly the E compartment.

Kex2p Also Exhibits Instability in the vps28 Class E Mutant

As previously noted, other Golgi proteins such as the fusion A-ALP (DPAP A fused to ALP) have been localized by immunofluorescence to the E compartment (Raymond *et al.*, 1992). To determine whether the instability observed for Vps10p was perhaps common to other Golgi proteins, we also examined the stabilities of the Kex2 protein, a late Golgi enzyme, and Mnn1p, a medial Golgi α1,3-mannosyltransferase. Like Vps10p, Kex2p is a type I integral membrane protein whose Golgi localization is dependent upon signals in its carboxy-terminal cytoplasmic tail domain (Wilcox *et al.*, 1992). Mnn1p, on the other hand, is a type II membrane protein of 89 kDa; its short cytoplasmic tail (18 amino acids) is not necessary for Golgi retention (Graham *et al.*, 1994). In wild-type cells, Vps10p, Kex2p, and Mnn1p all appeared to be quite stable, even at the 90-min chase time (Figure 9, lane 4). Both Kex2p and Mnn1p exhibit an increase in molecular weight during the 90-min chase period due to the slow acquisition of additional carbohydrate modifications (Wilcox and Fuller, 1991; Graham *et al.*, 1994). However, in Δ*vps28* cells, Vps10p and Kex2p were rapidly degraded. In fact, after a 30-min chase, most of the Vps10p (≈ 80%) and Kex2p (> 90%) had been proteolytically digested (Figure 9, lane 6). Mnn1p was stable in the Δ*vps28* strain and appeared to gradually increase in molecular weight as observed in wild-type cells.
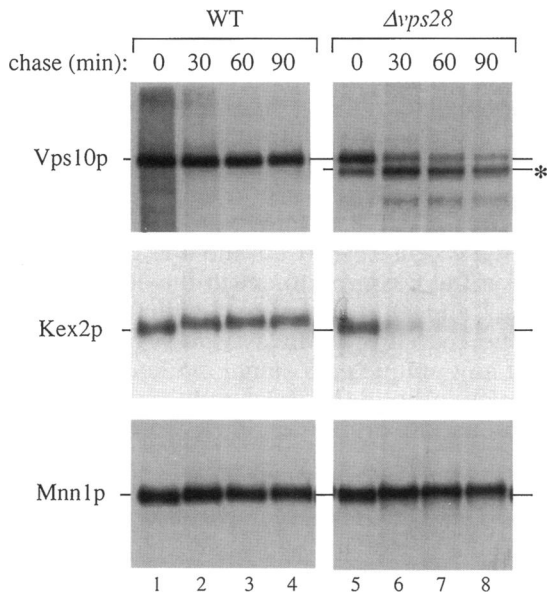
Figure 9. Stability of Kex2 and MnnI proteins in Δvps28. Yeast cells from WT (SEY6210) or Δvps28 (KKY20) strains were labeled for 15 min and chased for 0, 30, 60, or 90 min. Antibodies against Vps10p, Kex2p, and MnnIp were used to immunoprecipitate samples. The "*" denotes the position of the major proteolytic fragment of Vps10p.

DISCUSSION

We have previously described the cloning and initial characterization of the Vps10 protein as the first membrane receptor involved in the sorting of soluble vacuolar hydrolases in *Saccharomyces cerevisiae* (Marcusson *et al.*, 1994). Here, we focus on the essential structural features in the cytoplasmic tail of Vps10p and the intercompartmental transport itinerary of wild-type and mutant receptors. Overexpression of wild-type Vps10 protein results in *PEP4*-dependent turnover of the CPY receptor (Figure 1). Excess Vps10p appears to accumulate in the vacuole as indicated by differential centrifugation and immunofluorescence (Figures 2 and 3). Similarly, large and small deletions of the cytoplasmic tail domain of Vps10p lead to rapid, *PEP4*-dependent degradation of mutant receptors (Figures 4 and 5). In addition, stability and subcellular fractionation experiments (Figures 7 and 8) in the class E *vps* mutants indicate that, during its intracellular transport, Vps10p travels to the protease-containing E compartment, which appears to correspond to an exaggerated prevacuolar endosome (Raymond *et al.*, 1992). Together, these data show that the cytoplasmic tail of Vps10p is essential for recycling of Vps10p and that this recycling appears to include a prevacuolar compartment that is altered in the class E *vps* mutants.

By analogy to the mechanism of lysosomal protein sorting in mammalian cells by mannose-6-phosphate/

insulin-like growth factor II receptors (Kornfeld, 1992), Vps10p is likely to execute multiple rounds of CPY sorting by recycling back to the late Golgi sorting compartment after transporting the CPY to a prevacuolar endosome-like compartment (Figure 10A). *vps* mutants from morphological classes E and F appear to disrupt the Vps10p recycling pathway. These two classes of mutants exhibit only partial defects in the sorting of vacuolar hydrolases. In the mutants tested in this study, 30–60% of CPY is sorted and matured. Most other classes of *vps* mutants exhibit stronger sorting defects. It may be that other *vps* mutants, such as the class C *vps* mutants, are so defective in vacuolar protein sorting and vacuolar function that measurements of Vps10p stability cannot be used to demonstrate alterations in receptor trafficking. Therefore, alternative approaches will be necessary to fully elucidate the roles of these *VPS* gene products in Vps10p retention and recycling.

VPS1 (class F) function is required for the retention/recycling of membrane proteins Kex2p and DPAP A in the Golgi (Wilsbach and Payne, 1993; Nothwehr *et al.*, 1995). Because Vps10p may reside in the same late Golgi compartment as these membrane proteins (Vida *et al.*, 1993; Marcusson *et al.*, 1994), it is not surprising that Vps10p is also degraded and presumably delivered to the vacuole in a *vps1* mutant. A common mechanism for the retention of late Golgi proteins in wild-type cells may involve recycling of membrane proteins from a prevacuolar compartment. Speculation that *VPS1* acts in the formation of vesicular structures (Pryer *et al.*, 1992; Kelly, 1995; Nothwehr *et al.*, 1995) fits in well with its apparent role in Vps10p and Golgi membrane protein recycling. It is not certain whether *VPS1* participates in vesicle formation during anterograde or retrograde traffic; however, it has recently been reported that the *VPS1* and *MVP1* (multicopy suppressor of a dominant-negative *vps1* mutant) gene products both localize to the E compartment in Δvps27 cells. Therefore, Vps1p may associate with a late Golgi compartment or an endosomal compartment or cycle between the two (Ekena and Stevens, 1995).

Indirect immunofluorescent detection of late Golgi (DPAP A-ALP fusion) and vacuolar (vacuolar ATPase, CPY, and proteinases A and B) marker proteins in the class E compartment are consistent with the view that this compartment represents an exaggerated prevacuolar organelle (Raymond *et al.*, 1992). Rapid degradation of Vps10p in the class E *vps* mutants demonstrates that the receptor does seem to follow an intercompartmental itinerary that includes cycling through a prevacuolar compartment (Figure 10C). How Vps10p arrives at this compartment is poorly understood. The pathway from the Golgi to the endosome may be direct, or it may involve mislocalization to the plasma membrane followed by transport to the endosome by
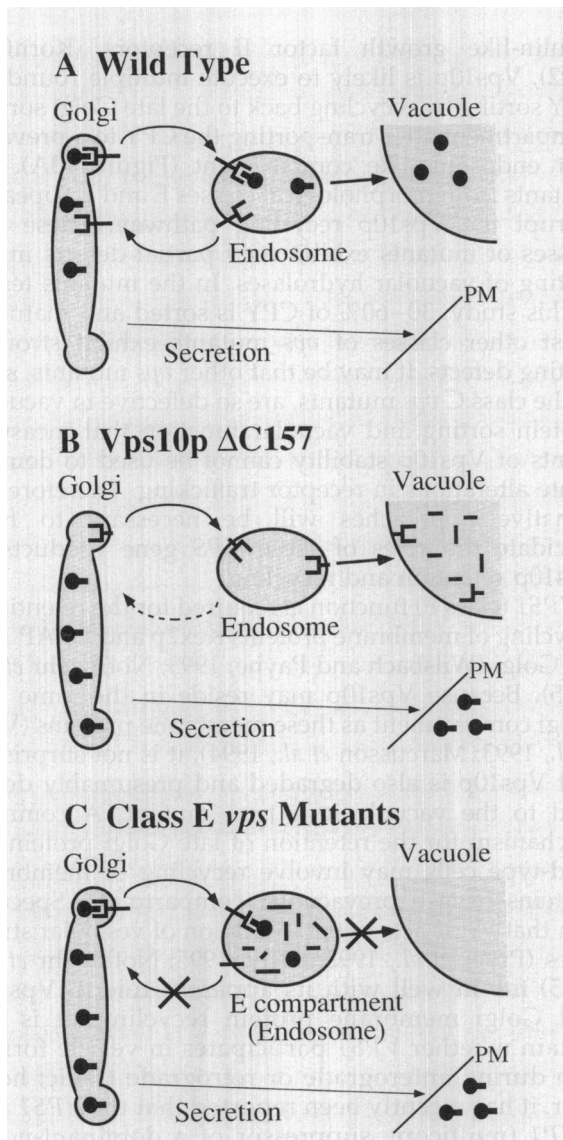
A Wild Type

Golgi   Vacuole

Endosome

PM

Secretion

B Vps10p ΔC157

Golgi   Vacuole

Endosome

PM

Secretion

C Class E *vps* Mutants

Golgi   Vacuole

E compartment
(Endosome)

PM

Secretion

Figure 10. Model of Vps10p-mediated CPY sorting. (A) In wild-type cells, Vps10 protein binds p2CPY through interactions involving the prosegment sorting signal of CPY. The receptor-ligand complexes are packaged into vesicles and transported to a prevacuolar endosomal compartment. At the endosome, Vps10p releases p2CPY, then the unoccupied receptor recycles back to the Golgi for additional rounds of sorting. Precursor CPY continues on to the vacuole where it is matured to its active form. (B) Although recycling of other proteins from the endosome to the Golgi may continue (as indicated by the dotted arrow), mutant receptors lacking cytoplasmic tail domains are delivered to the vacuole and degraded. Precursor CPY is secreted to the plasma membrane (PM). (C) An exaggerated form of a prevacuolar endosome-like compartment may exist in the *vps* class E mutants. Vps10p is rapidly degraded in this compartment due to the presence of active vacuolar hydrolases. Vesicular traffic back to the Golgi from the endosome, as well as traffic to the vacuole appears to be blocked in these mutants.

an endocytic mechanism. Vps10p stability experiments in *sec4^{ts}* and *sec4^{ts}/class E* (Δ*vps24* and Δ*vps28*) double mutants suggest that at the nonpermissive temperature, when vesicular traffic to the plasma membrane is blocked (Salminen and Novick, 1987), the rate of degradation of Vps10p is unchanged. Therefore, delivery of Vps10p to this exaggerated prevacuolar compartment is likely to occur directly from the late Golgi and not via endocytosis from the plasma membrane. Results demonstrating the localization of Vps10p to punctate structures by immunofluorescence in wild-type cells (Figure 3) and the degradation of Vps10p in the E compartment in the class E mutants (Figures 7 and 8) are consistent with a late Golgi to endosome recycling pathway. However, in the absence of any unique endosomal markers, direct localization of Vps10p to the yeast endosome by subcellular fractionation or immunofluorescence is not yet possible.

Mutation of the cytoplasmic tail or overproduction of Vps10p leads to delivery and subsequent degradation of the receptor in the vacuole (Figures 1–5 and 10B). These data seem to corroborate previously reported results involving Kex2p (Wilcox *et al.*, 1992), DPAP A (Roberts *et al.*, 1992; Nothwehr *et al.*, 1993), and mutant Wbp1-invertase fusion proteins (Gaynor *et al.*, 1994) suggesting that the vacuole, not the plasma membrane, is the default destination for membrane proteins of the yeast secretory pathway. Additional results from mutational analysis of the 164 amino-acid carboxy-terminus of *VPS10* indicate that the cytoplasmic tail domain plays a critical role in Golgi retention/recycling and thus in receptor function. Deletion of amino acid residues in the first half of the cytoplasmic tail (Δ1420–1497; ΔTail1) has a greater impact on the stability of the mutant receptor and CPY sorting than removal of the outer half (Δ1497–1576; ΔTail2) of the cytoplasmic tail (see Figure 5). Mutational analysis of the cytoplasmic tail domains of Kex1p, Kex2p, and DPAP A (Cooper and Bussey, 1992; Roberts *et al.*, 1992; Wilcox *et al.*, 1992) have shown that retention in or recycling back to the Golgi relies upon a signal containing aromatic residues. The FYVF sequence found in Vps10p matches the consensus Golgi retention/recycling signal (Y/F-X-Y/F) found in Kex2p and DPAP A as defined by Nothwehr (Nothwehr *et al.*, 1993; Nothwehr and Stevens, 1994). Aside from the tyrosine residue at position 1455 in the FYVF motif, there are only two other tyrosines in the entire tail domain. These other tyrosines appear to be less influential; one is contained within the ΔTail2 (Δ1497–1576) deletion and the other at position 1480 has no effect when mutated to an alanine (Marcusson and Emr, unpublished data). The deletion of the FYVF residues appears to result in a significant degree of instability, indicating that the FYVF motif may be one of the more important sequence elements in the tail domain. However, because deletion of the entire cytoplasmic tail domain has a greater effect on stability and sorting than any of the smaller deletions, multiple signals for

recycling may exist within the cytoplasmic domain of Vps10p.

The cytoplasmic tail of M6P/IGF-II, which also contains aromatic signals thought to be necessary for recruitment of the receptor into clathrin-coated pits at the plasma membrane (Canfield et al., 1991), has been shown to interact biochemically with Golgi and plasma membrane adaptor complexes (Glickman et al., 1989). Such interactions have not been demonstrated with Vps10p; however, it is possible that when these interactions are disrupted or saturated, Vps10p may be mislocalized to the vacuole. Speculation on a list of possible interacting partners of Vps10p includes clathrin and clathrin adaptor proteins, as well as other Vps proteins such as Vps29p and Vps35p. *vps29* and *vps35* mutants result in the same differential sorting defect as *vps10* mutants: precursor CPY is missorted whereas the majority of proteinase A, proteinase B, and ALP are converted to their mature forms (Paravicini et al., 1992; Marcusson and Emr, unpublished data). Subcellular fractionation experiments (Paravicini et al., 1992) have demonstrated the saturable association of Vps35p with the P100 membranous particulate cell fraction, which also contains Vps10p, but further data are not yet available. The tail domain, or more likely, proteins interacting with the cytoplasmic tail domain, may trigger activation of the Vps15 protein kinase/Vps34p phosphatidylinositol kinase complex (Stack et al., 1993, 1995; Chapman, 1994). This signal transduction complex is essential for protein sorting to the vacuole. Further experiments are underway to elucidate roles for Vps29p and Vps35p in regulating receptor traffic and to define interactions between Vps10p and other components of the machinery (such as Vps15p) required for the segregation, packaging, and delivery of receptor-ligand complexes in the vacuolar protein delivery system.

ACKNOWLEDGMENTS

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