Molecular Biology of the Cell
Vol. 11, 3737–3749, November 2000

Polar Transmembrane Domains Target Proteins to the Interior of the Yeast Vacuole

Fulvio Reggiori, Michael W. Black, and Hugh R. B. Pelham*

Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, United Kingdom

Submitted June 22, 2000; Revised August 10, 2000; Accepted August 10, 2000
Monitoring Editor: Juan Bonifacino

Membrane proteins transported to the yeast vacuole can have two fates. Some reach the outer vacuolar membrane, whereas others enter internal vesicles, which form in late endosomes, and are ultimately degraded. The vacuolar SNAREs Nyv1p and Vam3p avoid this fate by using the AP-3–dependent pathway, which bypasses late endosomes, but the endosomal SNARE Pep12p must avoid it more directly. Deletion analysis revealed no cytoplasmic sequences necessary to prevent the internalization of Pep12p in endosomes. However, introduction of acidic residues into the cytoplasmic half of the transmembrane domain created a dominant internalization signal. In other contexts, this same feature diverted proteins from the Golgi to endosomes and slowed their exit from the endoplasmic reticulum. The more modestly polar transmembrane domains of Sec12p and Ufe1p, which normally serve to hold these proteins in the endoplasmic reticulum, also cause Pep12p to be internalized, as does that of the vacuolar protein Cps1p. It seems that quality control mechanisms recognize polar transmembrane domains at multiple points in the secretory and endocytic pathways and in endosomes sort proteins for subsequent destruction in the vacuole. These mechanisms may minimize the damaging effects of abnormally exposed polar residues while being exploited for the localization of some normal proteins.

INTRODUCTION

The organization of the secretory pathway requires that membrane proteins be sorted: individual proteins have to be selectively incorporated into, or excluded from, the transport vesicles that move between organelles. This sorting is determined ultimately by the amino acid sequence, and the fate of many proteins is determined by motifs present in their cytoplasmic tails, which can interact directly with the various coat proteins that mold transport vesicles (Cosson and Letourneur, 1994; Ohno *et al.*, 1995; Honing *et al.*, 1998; Rapoport *et al.*, 1998; Rodionov and Bakke, 1998).

A second form of sorting is dependent on transmembrane domains (TMDs). In the case of Golgi enzymes, the length of the TMD has a powerful influence on their retention in the Golgi system, and this has been suggested to be due to their selective partitioning into subdomains of the lipid bilayer that differ in composition and hence thickness (Bretscher and Munro, 1993; Munro, 1995). TMD-dependent protein sorting has been demonstrated in both the late Golgi and the early endosomes of yeast cells (Rayner and Pelham, 1997; Lewis *et al.*, 2000). Some endoplasmic reticulum (ER) proteins, such as yeast Sec12p, are also retained by mechanisms that involve, at least in part, hydrophilic residues in their TMDs. A putative receptor, Rer1p, itself an integral membrane protein, is thought to be involved in recognizing the TMDs in the Golgi and mediating their retrograde transport to the ER (Sato *et al.*, 1996, 1997; Boehm *et al.*, 1997).

Recognition and sorting of TMDs may occur in part as a protective measure. Their sequences are usually hydrophobic, allowing them to mingle freely with lipids, but polar residues are sometimes present, and in the hydrophobic environment these have a strong tendency to form hydrogen bonds with each other (for discussion, see Bowie, 2000). This can lead to the stabilization of TMD interactions within multispanning proteins and between subunits of oligomers, but unpaired polar residues are potentially hazardous to the cell; like hydrophobic residues exposed to the aqueous environment, they may form promiscuous interactions that cause aggregation or interfere with the folding of multispanning proteins. It seems likely that cells have evolved mechanisms to "chaperone" individual polar TMDs, such as those in unassembled protein subunits.

A potential example is provided again by Rer1p, which in addition to sorting Sec12p has also been implicated in the retention in the ER of proteins with potentially charged residues in their TMDs (Letourneur and Cosson, 1998). ER retention of such proteins is well documented in animal cells, particularly among the individual subunits of the T cell receptor, which contain acidic and basic residues and are held in the ER until assembly masks them (Bonifacino *et al.*, 1990, 1991).

In this paper, we address the role of TMDs in the sorting of proteins in late endosomes. In yeast, as in animal cells,

* Corresponding author. E-mail address: hp@mrc-lmb.cam.ac.uk.

Table 1. Yeast strains

Strain	Genotype	Source
SEY6210	*MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	S. Emr
SEY4-1	*MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vps4-1*	M. Seaman
FRY024	*MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 apm3::HIS5spL*	This work
RH1597	*MATa ura3 leu2 his4 bar1 end4-1*	H. Riezman
Δ45	*MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vps45::HIS5spL*	Pelham laboratory
JHY005	*MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pep12::HIS3L*	Pelham laboratory
JHY024	*MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pep4::HIS3*	Pelham laboratory
FRY031	*MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Δpho13 Δpho8*	This work
SKY7	*MATα rer1::LEU2 mfα1::ADE2 mfα2::TRP1 bar1::HIS3 ura3 leu2 trp1 his3 lys2 ade2*	A. Nakano
BNY100	*MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vam3::HIS5spL*	Pelham laboratory

multivesicular bodies (MVBs) form in the endocytic pathway by a process in which vesicles invaginate from the outer endosomal membrane and pinch off to form discrete structures within the lumen. Fusion of the outer endosomal membrane with the vacuole then delivers these internal vesicles to the vacuolar interior, where they are eventually degraded (Burd *et al.*, 1998; Odorizzi *et al.*, 1998). Proteins destined for degradation enter these internal vesicles, whereas proteins that function on the vacuolar surface avoid this fate. One functional membrane protein, the vacuolar protease Cps1p, is also known to enter the internal vesicles (Odorizzi *et al.*, 1998).

The situation is complicated by the existence of at least two quite distinct transport routes to the vacuole for membrane proteins. Most proteins, whether they come from the Golgi complex or the plasma membrane, pass through late endosomes. Accordingly, they become trapped in an aberrant endosomal structure in so-called class E vacuolar protein sorting mutants such as *vps4*, which are defective in the late stages of this pathway (Raymond *et al.*, 1992; Rieder *et al.*, 1996; Babst *et al.*, 1997; Hicke *et al.*, 1997; Bryant *et al.*, 1998; Conibear and Stevens, 1998; Odorizzi *et al.*, 1998). However, a small number of proteins, including the vacuolar alkaline phosphatase (ALP) and the syntaxin Vam3p, have been shown to be transported directly from the Golgi to the vacuole, even in class E mutants, by a mechanism that depends on the AP-3 adaptor coat protein complex (Cowles *et al.*, 1997a,b; Piper *et al.*, 1997; Stepp *et al.*, 1997; Darsow *et al.*, 1998).

We have used SNARE proteins as models to test the features that determine whether proteins reach the vacuole surface or its interior. The latter process we refer to as internalization; to avoid confusion with endocytosis, we use the term only in this sense. In particular, we have studied the vacuolar proteins Nyv1p and Vam3p (Darsow *et al.*, 1997; Nichols *et al.*, 1997; Wada *et al.*, 1997; Srivastava and Jones, 1998) and the endosomal syntaxin Pep12p (Becherer *et al.*, 1996; Burd *et al.*, 1997; Lewis *et al.*, 2000). We show that Nyv1p, like Vam3p, uses the AP-3 pathway to reach the vacuole and that this is sufficient to prevent internalization, which evidently can occur only in late endosomes. Pep12p is present in late endosomes and can reach the vacuole from them, but it is not normally internalized. Strikingly, however, we find that the insertion of acidic residues in the cytoplasmic half of the TMD is sufficient to force internalization of Pep12p, and of Vam3p if this protein is routed through endosomes. Mutant TMDs that cause this internalization can also affect sorting at other locations. Depending on the protein to which they are appended, they can induce partial ER retention or transport of a protein from Golgi to endosomes, rather than to the cell surface. Testing of a variety of naturally occurring TMDs shows that internalization in late endosomes does not exclusively require acidic residues but is promoted by other polar residues, and that the features required are strikingly similar to those that, in other contexts, promote ER retention.

We suggest that cells have multiple quality control mechanisms that recognize proteins with abnormally hydrophilic TMDs. These mechanisms ensure either the retention of such proteins in the ER or their delivery to the interior of the vacuole for degradation.

MATERIALS AND METHODS

Yeast Strains

Yeast strains are described in Table 1. For gene disruption, the entire coding region was replaced with selectable markers with the use of PCR primers containing ~50 bases of identity to the regions flanking the ORF. *APM3* was disrupted with the *HIS5* gene of *Schizosaccharomyces pombe* flanked by coliphage *loxP* sites. A Δ*pho13* Δ*pho8* mutant was obtained by disrupting *PHO13* with *HIS5* flanked by *loxP* sites and subsequently disrupting *PHO8* with *TRP1* also flanked by coliphage *loxP* sites. The selectable markers were removed by excisive recombination at the *loxP* sites with the transient expression of *Cre* recombinase (Sauer, 1987).

Plasmids

Plasmids expressing SNARE chimeric constructs were all based on pRS406 or pRS416 vectors (Sikorski and Hieter, 1989). Unless indicated, PCR-generated derivatives were cloned as an *Eco*RI–*Bam*HI fragment behind sequences expressing the mut2 green fluorescent protein (GFP) variant (Cormack *et al.*, 1996) and the *TPI1* promoter as described by Wooding and Pelham (1998). Constructs shown in Figures 1 and 4 were cloned in pRS406, whereas constructs shown in all other figures were cloned into pRS416.

Plasmids expressing Snc1p and Snc1p–Nyv1p (here called GSN) have been described previously (Lewis *et al.*, 2000). GSV (*SNC1–VTI1*), GNS (*NYV1–SNC1*), and PSEC (*PEP12–SEC12*) and PCPS (*PEP12–CPS1*) constructs were obtained by PCR of *SNC1* (Lewis *et al.*, 2000), *NYV1* (Nichols *et al.*, 1997) and *PEP12* (Rayner and Pelham, 1997), respectively, with the use of primers encoding the appropriate transmembrane domain. *PEP12* deletions and point mutations were obtained by PCR with the use of appropriate primers. Point mutations in *PSSO* (*PEP12–SSO1*), *VAM3*, *SSO1*, and *SSSO* (*SNC1–SSO1*) were introduced also by PCR of the corre-

sponding constructs described elsewhere (Nichols *et al.*, 1997; Rayner and Pelham, 1997; Lewis *et al.*, 2000). For *PEP12–UFE1* chimeras, the cytosolic N terminus of Pep12p was cloned by PCR as an *Eco*RI–*Hin*dIII fragment. Previously described mutants of the *UFE1* TMD (Rayner and Pelham, 1997) were inserted as *Hin*dIII–*Bam*HI fragments. The *Hin*dIII site introduces the amino acids KL at the junction between the cytoplasmic domain and TMD. In all cases, changes were confirmed by sequencing.

A truncated version of the *PHO8* gene (lacking the first 60 N-terminal amino acids) was cloned as a *Stu*I–*Eco*RI fragment into the expression plasmid described above cut with *Hin*dIII, blunted with Klenow, and finally digested with *Eco*RI to eliminate GFP.

To check the ability of *PEP12* and *PSSO* constructs to complement the Δ*pep12* phenotype, the *TPI1* promoter and GFP were replaced with genomic sequence from the 820 base pairs (bp) immediately upstream of the *PEP12* ORF. Genomic sequence from the 300 bp immediately downstream of the *PEP12* ORF was also inserted as an *Bam*HI–*Xba*I fragment.

Imaging of Live Cells

Cells were grown to early log phase, harvested by centrifugation, and washed with water to promote homotypic fusion of fragmented vacuoles. They were placed onto slides coated with concanavalin A, sealed under coverslips, and examined with the use of either a Zeiss (Thornwood, NY) Axioskop microscope equipped with a Micromax charge-coupled device camera (Princeton Instruments, Princeton, NJ) (Figure 1 only) or an MRC-600 confocal microscope (Bio-Rad, Richmond, CA). Fluorescence intensity profiles were generated with the use of NIH Image software.

To stain the endocytic pathway, a 1-ml culture of early log phase cells was centrifuged and cells were resuspended in 100 μl of medium containing 20 μM FM4-64 (Molecular Probes, Eugene, OR). They were incubated for 15 min, harvested by centrifugation, resuspended in 1 ml of fresh medium, and incubated for another 30 min. They were collected by centrifugation, washed with 1 ml of water, and resuspended in water for microscopy.

Random PCR Mutagenesis

Random PCR mutagenesis of PSSO was performed as described (Cadwell and Joyce, 1992) with the use of primers flanking the *PEP12* ORF by 200 bp. PCR products were cotransformed with the expression plasmid described above that was linearized with *Eco*RI–*Bam*HI. Yeast colonies were scored microscopically for their ability to internalize the GFP–PSSO construct. To map the region responsible for internalization of the mutant protein, sequences coding for the first 101 amino acids (*Eco*RI–*Nhe*I), the central 152 amino acids (*Nhe*I–*Kpn*I), and the last 35 amino acids (*Kpn*I–*Bam*HI) were swapped with the unmutated version.

Protein Extraction and Immunoblotting

Early log phase cultures were lysed, and proteins were extracted as described previously (Reggiori *et al.*, 1997). Western blot analysis was performed with the use of rabbit polyclonal antibody to Pep12p (Holthuis *et al.*, 1998), to Vti1p (a gift from T. Stevens, University of Oregon, Eugene), or to GFP (Molecular Probes). All antibody incubations were carried out in PBS containing 3% dried milk. After incubation with anti-rabbit peroxidase-conjugated antibodies (Bio-Rad), detection was performed with the use of ECL reagents (Amersham, Arlington Heights, IL). Coimmunoprecipitation of Vam3p and Vti1p was carried out with anti-Vam3p antibody (Nichols *et al.*, 1997) as described (Holthuis *et al.*, 1998).

ALP Liquid Assay

This was derived from the procedure described by Toh-e *et al.* (1976). Early log phase cells (2.5 OD_{600}) were harvested by centrifugation, washed with 1 ml of ice-cold water, and resuspended in 100 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 5% DMSO, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 5 μg/ml pepstatin, 1 μg/ml antipain, 1 mM benzamidine, 200 μg/ml PMSF). Glass beads were added, and cells were disrupted by agitation at 4°C for 1 min. After pelleting of cell debris, 20 μl of lysate was mixed with 480 μl of a *p*-nitrophenyl phosphate solution (Sigma Fast *p*-nitrophenyl phosphate tablet sets, Sigma Chemical, St. Louis, MO) prewarmed at 37°C. Incubation was carried out at 37°C for 20 min and stopped by adding 500 μl of 10% trichloroacetic acid. Samples were neutralized by adding 1 ml of sodium sulfite–saturated water solution and centrifuged at 13,000 rpm for 10 min. One milliliter of supernatant was taken to measure the absorbance at either 420 or 550 nm. ALP activity was expressed in Miller units (units of activity per minute per OD_{600}): $1000 \times (A_{420} - 1.06A_{550})/(0.993 \times \text{min} \times \text{vol} \times OD_{600})$, where $OD_{600} = 25$ and vol = 0.02 ml.

RESULTS

The Cytoplasmic Domains of SNAREs Determine Their Route to the Vacuole

We set out to investigate the features of SNAREs, as model membrane proteins, that determine whether they remain on the outer membrane or are incorporated into internal vesicles when delivered to the vacuole. Specifically, we sought to determine whether sorting signals could be identified in their cytoplasmic domains, transmembrane domains, or both. The main focus of this study is on the membrane-spanning segments, but it was first necessary to determine the contributions of the cytoplasmic domains.

Initially, we compared two related SNAREs: Nyv1p, which is normally present on the outer vacuolar membrane, and Snc1p, an exocytic SNARE that can be redirected to the vacuole interior. The locations of these and other SNARE proteins in living cells can be monitored readily with the use of GFP chimeras. Figure 1A shows typical distributions of GFP–Snc1p, which is found on the plasma membrane and also on early endosome and Golgi compartments through which it recycles (Lewis *et al.*, 2000), and of GFP–Nyv1p. Previous studies have shown that when the transmembrane domain of Snc1p is altered, the protein fails to recycle from early endosomes to the Golgi and instead is mislocalized to the vacuole. The altered Snc1p is internalized in transit and, unlike Nyv1p, ends up in the vacuolar lumen (Lewis *et al.*, 2000). To determine whether the difference between the fates of Nyv1p and Snc1p was due to their cytoplasmic or transmembrane domains, we exchanged their TMDs. As shown in Figure 1B, the addition of the Nyv1p TMD to Snc1p (construct GSN) did not prevent its internalization. A similar result was obtained when the Snc1p TMD was replaced with that of Vti1p (GSV), another SNARE that is found on the vacuolar membrane (Ungermann *et al.*, 1999). Conversely, replacement of the Nyv1p TMD with that of Snc1p did not affect its localization (GNS). Hence, the fates of these SNAREs seem to be determined largely by their cytoplasmic domains.

A possible explanation is that the cytoplasmic domain of Nyv1p (like that of Vam3p) specifies transport via the AP-3 pathway, bypassing the site of internalization in late endosomes. In agreement with this, delivery of Nyv1p to the vacuole was unaffected in *vps4* cells (Figure 1A). It also reached the vacuole in a *vps45* mutant (our unpublished observations) in which delivery to late endosomes is blocked (reviewed by Conibear and Stevens, 1998). In *apm3* cells, it

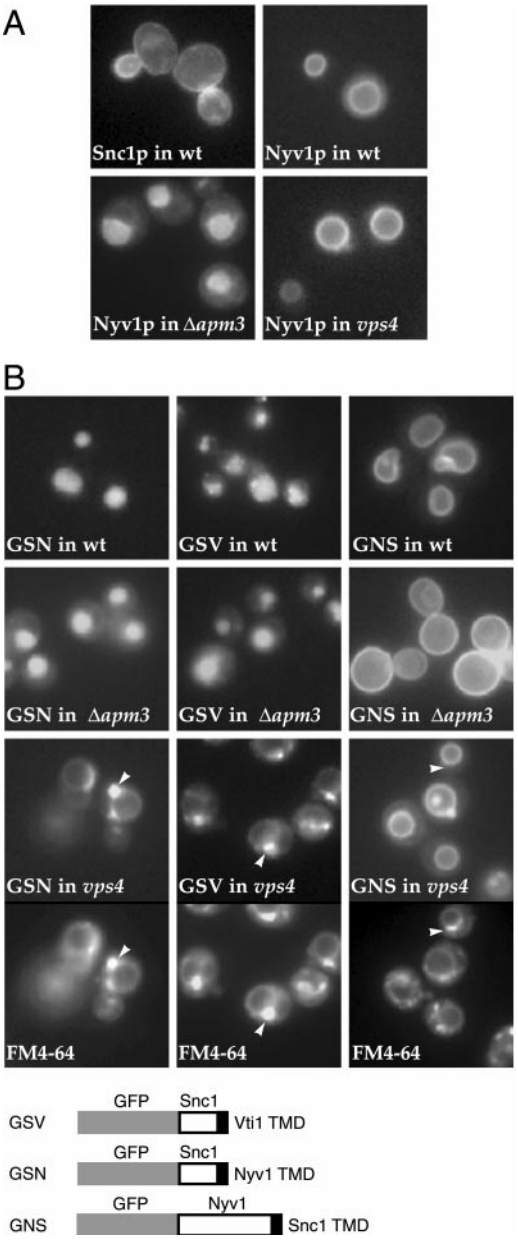

Figure 1. Nyv1p travels to the vacuole via the AP-3 pathway. (A) GFP-tagged versions of Snc1p and Nyv1p were expressed in wild-type cells (wt), and Nyv1p was expressed in *apm3* and *vps4* mutants. The structures visible with GFP–Nyv1p in wild-type and *vps4* cells are vacuoles. (B) GFP-tagged versions of Snc1p with the TMD from Nyv1p (GSN) or Vti1p (GSV), or GFP-tagged Nyv1p with the TMD from Snc1p (GNS), were expressed in the indicated strains. The *vps4* strains were also stained with FM4-64 to reveal the PVC and vacuolar membranes; arrowheads indicate equivalent positions in the FM4-64 and GFP images.

still reached the vacuole, but rather than being restricted to the outer vacuolar membrane, GFP fluorescence was visible within the vacuoles. This finding suggests that Nyv1p is rerouted to late endosomes under these conditions, as are other substrates of the AP-3 pathway (Cowles *et al.*, 1997a; Stepp *et al.*, 1997), and becomes partially internalized there.

As expected, sorting to the AP-3 pathway was determined by the cytoplasmic domain of Nyv1p rather than its TMD. Thus, Nyv1p with the Snc1p TMD (construct GNS) reached the vacuole in *vps4* cells but accumulated on the plasma membrane in *apm3* cells (Figure 1B), reflecting the fact that GNS lacks both an endocytic signal and the Golgi–endosome targeting information provided by the Nyv1p TMD (Lewis *et al.*, 2000). Conversely, Snc1p derivatives bearing the Nyv1p (GSN) or Vti1p (GSV) TMDs were delivered to the vacuolar lumen in *apm3* cells, but in a *vps4* mutant they accumulated in the abnormal prevacuolar compartments (PVCs) that accumulate in this mutant and can be visualized with the use of the endocytic tracer dye FM4-64 (Vida and Emr, 1995).

In the case of Vam3p and ALP, an acidic dileucine motif has been identified as an AP-3 pathway transport signal (Darsow *et al.*, 1998; Vowels and Payne, 1998). Inspection of the Nyv1p sequence revealed at least three potential motifs of this form (residues 97–102, 117–122, and 192–197). Preliminary deletion analysis suggested that at least two of these sequences contribute to its sorting (our unpublished observations), but we did not investigate these in detail.

We conclude that Nyv1p normally travels via the AP-3 pathway and that this ability to bypass the site at which MVBs form may account in part for the presence of Nyv1p on the vacuolar surface. When forced to travel instead via endosomes, GFP–Nyv1p appeared to be at least partially internalized.

Pep12p Avoids Internalization in Late Endosomes

To allow further investigation of the sorting signals active during the formation of MVBs, we sought an example of a SNARE that clearly avoids internalization in these structures. A good candidate is Pep12p, which is present in late endosomes and sometimes can be observed on vacuoles. Indeed, overexpression (by approximately eightfold) of a GFP-tagged version of Pep12p resulted in substantial vacuolar fluorescence, the protein clearly being present on the outer vacuolar membrane (Figure 2A). The different fates of Pep12p and Snc1p upon delivery to the vacuole are determined by their cytoplasmic domains: replacing the Pep12p TMD with that of the plasma membrane syntaxin Sso1p (Figure 2A, PSSO) (Aalto *et al.*, 1993) or of other SNAREs (Vam3p, Nyv1p, Vti1p, Tlg1p, or Snc1p) did not affect its location on the vacuolar membrane (Table 2), whereas when Snc1p was joined to the same set of TMDs it was entirely internalized in each case (Figure 1B) (Lewis *et al.*, 2000; our unpublished observations).

The different behavior of the Pep12p and Snc1p cytoplasmic domains implied either an internalization signal in Snc1p or an anti-internalization signal in Pep12p. To search for possible signals in Pep12p while excluding any possible influence of its normal TMD, we mutated the GFP–Pep12p construct bearing the Sso1p TMD (PSSO). Controls confirmed that this construct traveled to the vacuole via endosomes, because its passage was blocked in *vps4* cells but unaffected in *apm3* cells (Figure 2A). We found that removal of the first 129, 186, or 243 residues of the Pep12p sequence, or the last 69 residues (193–261, leaving the Sso1p TMD), did not prevent transport to the vacuole, and in each case trans-

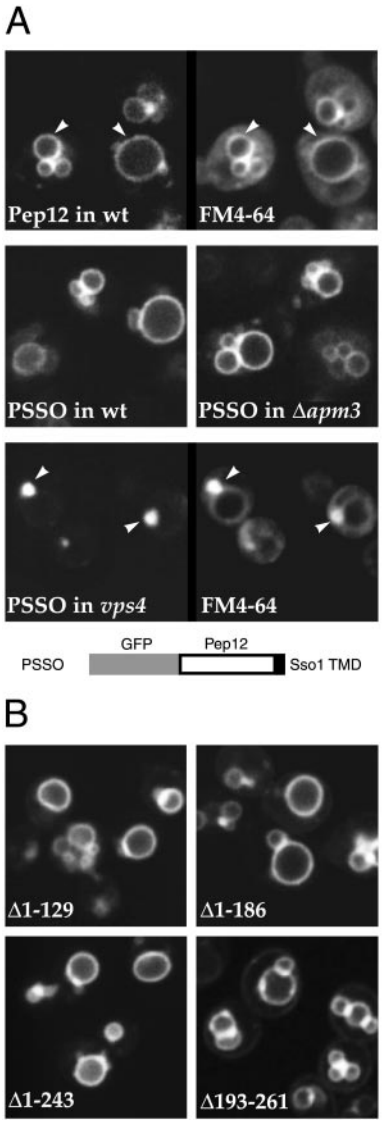

Figure 2. Pep12p with the Sso1p TMD (PSSO) reaches the outer vacuolar membrane via late endosomes. (A) Wild-type cells (wt) or indicated mutants expressing GFP–Pep12p or PSSO. GFP–Pep12p labels vacuoles, as shown by the FM4-64 staining; arrowheads indicate equivalent positions in these double-label images. PSSO labels vacuoles in wild-type and Δ*apm3* cells but labels perivacuolar PVC in *vps4* cells, which also labels with FM4-64 (arrowheads). (B) Deletion mutants of PSSO, lacking the indicated residues of Pep12p sequence, are also found on the outer vacuolar membrane. The junction with Sso1p occurs at residue 261.

port was still sensitive to *vps4* (our unpublished observations). However, none of these truncated proteins was internalized (Figure 2B). Next, we introduced random mutations into the Pep12p sequence by error-prone PCR and screened for mutants that were efficiently internalized into the vacuole. Although we obtained multiple simultaneous amino acid changes by this procedure (see below), no cytoplasmic mutations that led to internalization were identified.

Thus, Pep12p appears not to contain a cytoplasmic signal that prevents internalization. Instead, the small SNAREs Snc1p and Nyv1p may be specifically internalized; it is possible that these proteins, which in contrast to the syntaxins are likely to be largely unstructured (Hazzard *et al.*, 1999), are recognized by the cell as "unfolded" and hence suitable for degradation. In any case, it appears that Pep12p provides a robust model protein in which to test the potential effects of TMDs on internalization.

Acidic Residues in the TMD Cause Internalization

An initial clue that TMD sequences are indeed important came from the random mutagenesis of the Pep12–Sso1 construct. Three mutants were obtained that, when cells picked directly from yeast colonies were examined, showed internal vacuolar fluorescence. The mutations responsible were mapped to the TMD and adjacent sequences. DNA sequencing revealed two or three changes in this region, and in each case a negative charge had been introduced into the TMD (Figure 3A). Not all of the mutants had an equally strong phenotype: when grown exponentially in liquid culture, two of them had a much reduced phenotype but one, with an acidic residue at position 5 of the TMD, continued to show internal vacuolar fluorescence (Figure 3B). Thus, an internalization signal can be generated by specific changes to the TMD.

In the light of these results, we systematically introduced aspartic acid residues at alternative positions throughout the Sso1p TMD, which was appended to the Pep12p cytoplasmic domain. Figure 4 shows that single Asp residues were indeed sufficient to cause internalization of the chimera, but their position was crucial. At positions 3 and 5 of the TMD (counting from the cytoplasmic side), the effect was very obvious, but more C-terminal positions had a much weaker effect. This was confirmed by profiles of fluorescence intensity across single vacuoles (lower panel in Figure 4). Whereas the normal TMD resulted in clear peaks of fluorescence at the edges of the vacuole, with a dark interior, the 3D and 5D mutations resulted in maximum intensity at the center of the vacuole, with no detectable peaks at the edges. Other mutants typically gave a mixture of these two patterns, with membrane staining clearly detectable. Changing the Arg residue at position −1 to an Asp had a modest effect, but little change in distribution resulted from changing the Lys at −3 or from insertion of an Arg residue at position 3 or 5. All of the mutant proteins still passed through late endosomes, as demonstrated by their accumulation in PVCs in *vps4* cells; this is illustrated for one of the strongest alleles (PSSO3D) in Figure 4. The small proportion of the protein that reached the vacuole in these cells was not internalized, suggesting that functional endosomes are required for this effect.

The effects of the Asp insertions were not limited to the Pep12–Sso1p chimera, because introduction of an Asp at position 3 or 5 of the normal Pep12p TMD was also sufficient to cause internalization (Figure 5A). Remarkably, however, this did not prevent Pep12p from providing its function, as demonstrated by the restoration of growth at 37°C in a *pep12* null strain. The mutants showed activity comparable to that of the wild-type protein (Figure 5B), presumably functioning transiently as they passed through endosomes. The reten-

Table 2. Effects of TMD sequences on Pep12p localization

TMD	Sequence	Vacuolar location[a]	ER retention signal?[b]
PEP12	R VYLLIVLLVMLLFIFLIM K	Out	No
Sso1	R CWLIVFAIIVVVVVVVVVPAVV K	Out	No
Nyv1	K NITLLTFTIILFVSAAFMFFYLW	Out	No
Vti1	K FISYAIIAVLILLILLVLFS K	Out	No
Tlg1	D CCIGLLIVVLIVLLVLAFIA	Out	No
Snc1	K MCLALVIIILLVVIIVPIAV H	Out	No
Cps1	R AFISGIVALIIIGTFFLTSGL H	In	(No)
Sec12	K FFTNFILIVLLSYILQFSY K	In	Yes
Ufe1	K LTTYGAIIMGVFILFL D	In	Yes
Ufe1 mutC	K -LL---------- D	In	Yes
Ufe1 mutD	K ----LL---------- D	In	Yes
Ufe1 mutE	K --------L------- D	In	Yes
Ufe1 mutF	K -LL-----L------- D	In	Yes
Ufe1 mutG	K ----LLL-L------- D	In	Yes
Ufe1 mutH	K ---------L--VVV- D	In	Yes
Ufe1 mutI	K -LLLLL---L------ D	In/out	No
Ufe1/18	K ---------------- VL D	In/out	Yes
Ufe1/20	K ---------------- VLVL D	In/out	No
Ufe1/22	K ---------------- VLVLVL D	Out	No
Ufe1/24	K ---------------- VLVLVLVL D	Out	No

[a] Vacuolar staining was classified when the indicated TMD sequences were fused to GFP–Pep12p. For a vacuolar membrane designation (out), at least 80% of the large vacuoles showed brighter fluorescence at the rim than in the center. Internal (in) means at least 95% of large vacuoles showed brighter fluorescence in the center, with no visible rims. Where neither criterion was met, the designation was in/out; see Figure 10 for examples of these.

[b] ER retention depends on the cytoplasmic domain to which the TMD is attached; none of the sequences retains Pep12p in the ER. The first six sequences were tested on Snc1p (Lewis *et al.*, 2000; this work); the Sec12p TMD is well characterized (Sato *et al.*, 1996); the Ufe1 mutants were tested on the Golgi SNARE Sft1p, and the elongated Ufe1 sequences were tested on Ufe1p itself (Rayner and Pelham, 1997). The Cps1p sequence has not been tested on a cytoplasmic domain known to be capable of ER retention, but Cps1p itself is not an ER protein.

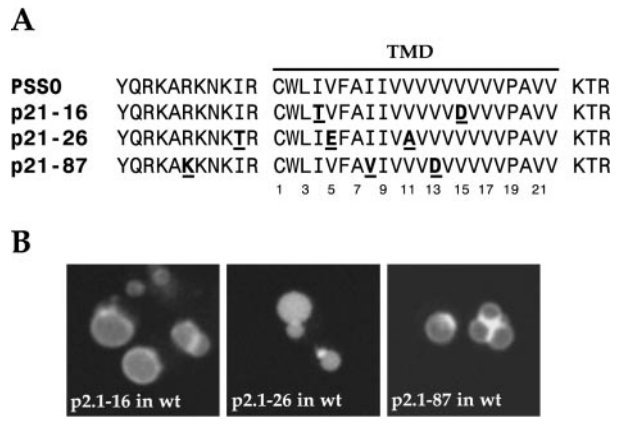

Figure 3. Mutants of PSSO that reach the interior of vacuoles. PCR-generated mutants were transformed into yeast, and cells from individual colonies were screened by fluorescence microscopy. (A) Sequence changes in the mutants. (B) Confocal images of cells grown to exponential phase in liquid culture (only vacuoles are visible). Note that under these conditions, only mutant p2.1-26 retains a strong internalization phenotype.

tion of activity suggests that the Asp insertions did not cause major misfolding or aggregation of Pep12p.

As a further test for charge-dependent internalization, we sought an alternative approach that does not depend on the presence of GFP. First, we established that the cytoplasmic domains of proteins targeted to the interior of the vacuole do eventually become exposed to vacuolar proteases, even though they should initially be protected by the membranes of the internal vesicles. Immunoblot analysis of a GFP–Snc1p chimera bearing the Nyv1p TMD, which is targeted to the vacuole interior (GSN, Figure 1B), revealed that 60% of it was proteolytically cleaved in wild-type cells (Figure 6A). This cleavage was abolished in cells lacking the vacuolar protease Pep4p, other minor cleavages then being detected. The total amount of GFP was similar in wild-type and *pep4* cells, indicating that GFP is substantially resistant to vacuolar proteases and thus is a reasonable marker for the vacuole delivery pathway. As a control, Figure 6A shows that 90% of a GFP–Nyv1p chimera, present on the outer membrane of the vacuoles (Figure 1A), remained intact even in wild-type cells. Thus, Pep4p cleavage can be used to measure the delivery of membrane proteins to the vacuolar lumen.

To avoid GFP altogether, we made use of ALP. ALP is synthesized as a precursor that is proteolytically activated

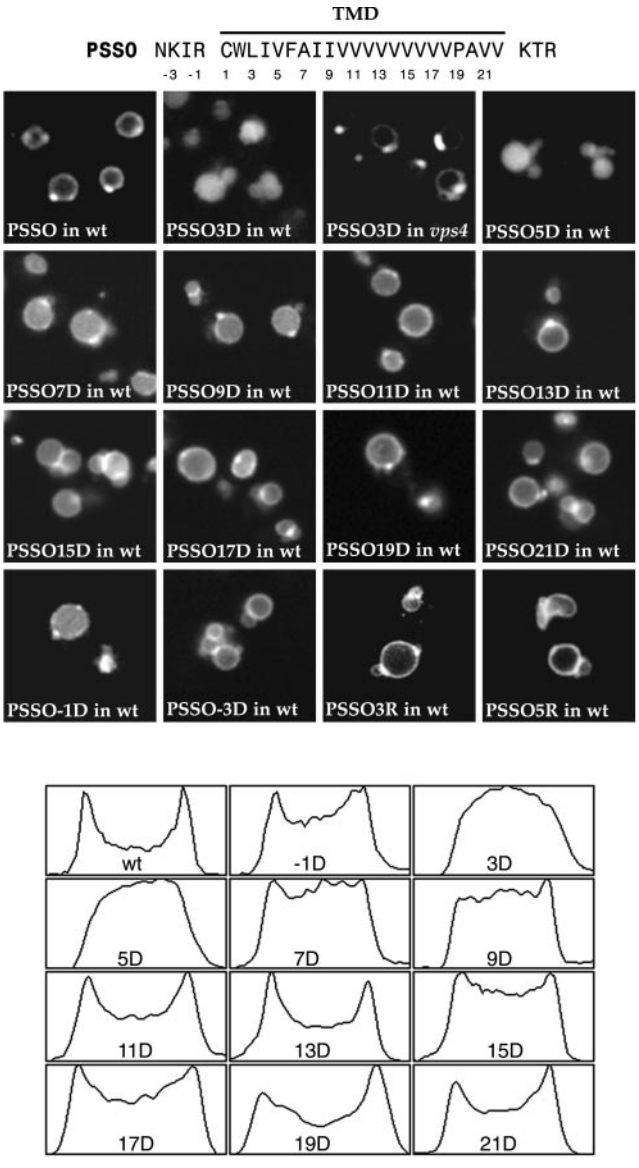

Figure 4. Single point mutations cause internalization of PSSO. Mutants are designated by the position of the change (numbering as indicated on the sequence) and the residue at that position (D or R). Confocal images are shown for constructs expressed in wild-type cells (only vacuoles are visible), and in the case of PSSO3D, also in *vps4* cells. The bottom panel shows profiles of fluorescence intensity taken across individual representative vacuoles for the Asp mutant series. The peaks visible in the wild-type (wt) profile correspond to the membrane at each side of the vacuole; intensity between these peaks indicates internal material.

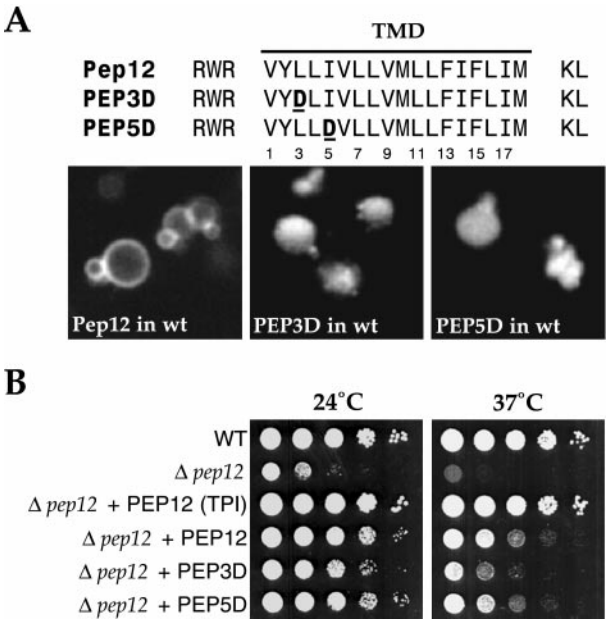

Figure 5. Mutations in the Pep12p TMD affect sorting but do not abolish function. (A) Aspartic acid mutations in Pep12p (as indicated) cause internalization. (B) Mutations do not block function. Drops containing serial 10-fold dilutions of cells were grown on agar at 24 or 37°C. The *pep12* mutation was complemented by a CEN plasmid expressing wild-type Pep12p from the TPI promoter. Other plasmids contained the *PEP12* promoter, driving PEP12 itself, or the mutant forms with Asp residues at the 3 or 5 position (PEP3D, PEP5D).

by Pep4p upon arrival at the vacuole (Klionsky and Emr, 1989). The precursor is a membrane protein that travels via the AP-3 pathway, but the activity resides in the lumenal domain, which is devoid of vacuolar targeting signals (Cowles *et al.*, 1997b; Vowels and Payne, 1998). We fused this domain to the Pep12p–Sso1p chimera and found that only low levels of phosphatase activity were produced (Figure 6B). This fits with the predicted location of the ALP sequences on the cytoplasmic side of the vacuolar membrane. Insertion of an Asp residue at position 3 of the TMD caused a substantial increase in phosphatase activity (Figure 6B). This finding confirms that the mutation induced internalization of the construct and its eventual exposure to vacuolar proteases.

We conclude that there is a specific mechanism that responds to the presence of acidic residues in the TMD, particularly the part of the TMD that spans the cytoplasmic leaflet of the membrane bilayer, and that directs the protein into the internal vesicles of MVBs.

Acidic TMDs Are Internalized Only in Endosomes

Having identified a strong sorting signal that functioned in endosomes, we investigated more specifically whether it could be recognized in vacuoles themselves. To do this, we introduced an Asp residue at position 3 of the TMD of a GFP–Vam3p chimera. Vam3p itself normally reaches the vacuole via the AP-3 pathway, as shown by its efficient delivery in a *vps4* mutant (Figure 7A). However, when this pathway is disrupted by the *apm3* mutation, Vam3p still reaches the vacuole and is found on its surface. Thus, like Pep12p, Vam3p is evidently able to pass through endosomes without being internalized. In *apm3* cells, it presumably uses a default pathway to endosomes, as it does when the AP-3 targeting signal is removed from it (Darsow *et al.*, 1998).

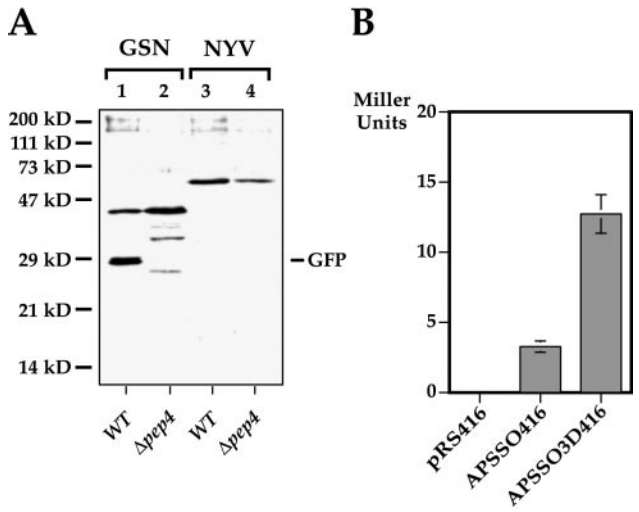

Figure 6. Proteolytic processing of chimeras in the vacuole. (A) GFP-tagged Snc1p with the Nyv1p TMD (GSN; apparent size 45 kDa) and GFP–Nyv1p (NYV; apparent size 60 kDa) were expressed in wild-type (WT) or *pep4* cells and detected by immunoblotting with anti-GFP. The position of free GFP generated by proteolysis of GSN is indicated. (B) *pho8 pho13* cells were transformed with vector alone (pRS416) or with plasmids expressing ALP–Pep12p–Sso1p chimeras without (APSSO416) or with (APSSO3D416) an aspartic acid at position 3 of the TMD. ALP activity was assayed in cell lysates.

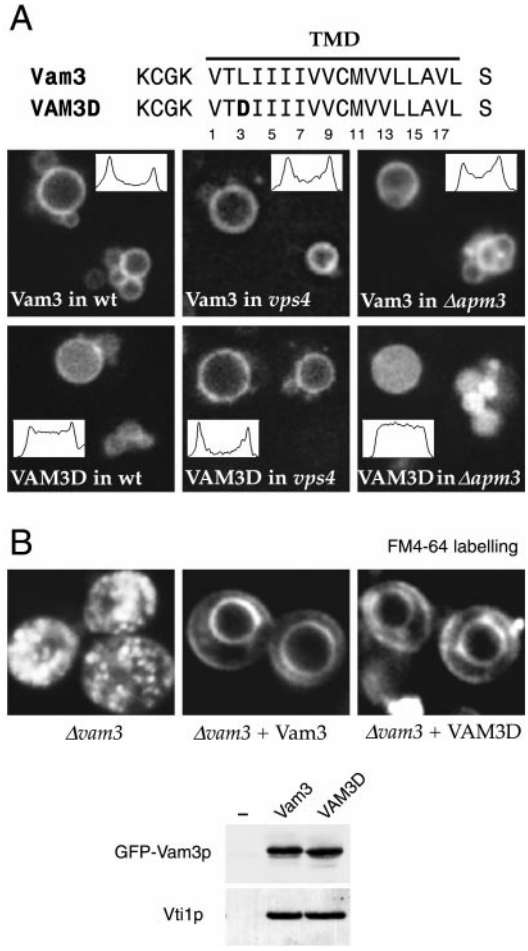

Figure 7. Aspartic acid residues cause Vam3p to be internalized only on passage through endosomes and do not affect its function. (A) GFP-tagged Vam3p with (VAM3D) or without (Vam3) an aspartic acid at position 3 of the TMD was expressed in wild-type (wt), *vps4*, or *apm3* cells. Confocal images of the vacuoles are shown. Insets show fluorescence profiles along a horizontal axis through the largest vacuole in each image. (B) Δ*vam3* cells, and cells of the same strain expressing either GFP–Vam3p or GFP–VAM3D from a plasmid, were labeled with FM4-64. Note the restoration of vacuole morphology by both proteins. The bottom panel shows immunoblots of extracts of the same cells probed for Vam3p and anti-Vam3p immunoprecipitates of these extracts probed for Vti1p.

When the acidic residue was present in its TMD, the fate of Vam3p was strongly influenced by the route it took. In *vps4* cells, it was distributed evenly around the vacuolar membrane, as normal. In *apm3* cells, on the other hand, it was almost entirely internal to the vacuole (see the intensity profiles of fluorescence in the optical sections shown in Figure 7A). This indicates that the acidic residue is recognized as a sorting signal only when the protein passes through endosomes. In wild-type cells, there was a mixture of surface and internal fluorescence, suggesting that the chimera traveled partially by the AP-3 pathway and partially via endosomes.

It has been reported that some proteins, notably the SNARE Vti1p, can be transported out of the vacuole to late endosomes; as a consequence, they become trapped in PVCs in class E *vps* mutants (Bryant *et al.*, 1998; Gary *et al.*, 1998). In the case of Vam3p, the bulk of the protein remained on the vacuolar membrane in *vps4* cells, and the same was true for the VAM3D mutant (Figure 7A). We infer that Vam3p does not normally cycle out of the vacuole, and the presence of a charge in its TMD does not induce its transport to PVCs in *vps4* cells. It appears that an acidic TMD has little consequence once the protein has reached the vacuolar membrane.

Although polar residues in TMDs could potentially cause inappropriate interactions resulting in aggregation or misfolding, we observed no effect on the functional properties of Vam3p. Thus, the VAM3D mutant was as effective as the wild-type protein at restoring normal vacuolar morphology to *vam3*Δ cells (Figure 7B). Vam3p is normally found in a SNARE complex that includes Vti1p (Ungermann *et al.*, 1999), and we found that identical amounts of Vti1p coprecipitated with the mutant and wild-type Vam3p (Figure 7B). The effects of the acidic residue on sorting, therefore, are not due to gross alterations to the physical or functional state of Vam3p.

Acidic TMD Residues Affect Sorting at Multiple Steps

It has been reported that acidic charges within a TMD can cause retention of a membrane protein in the ER due to *RER1*-dependent retrieval from the Golgi (Letourneur and Cosson, 1998), although we did not observe this effect with

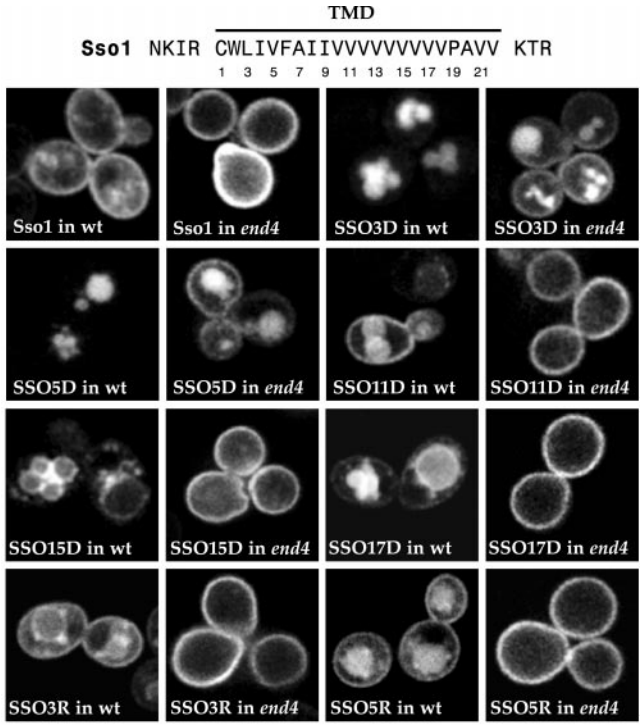

Figure 8. Effects of TMD mutations on Sso1p location. Confocal images are shown of cells expressing GFP-tagged Sso1p with the indicated representative mutations (D or R, at positions indicated by numbers). The distribution of each construct is shown in wild-type (wt) and *end4* cells. Note that 3D and 5D are the only mutant proteins that are not fully delivered to the plasma membrane in *end4* cells.

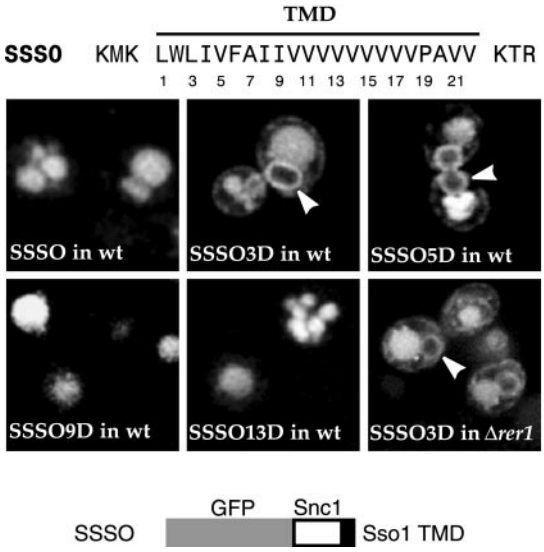

Figure 9. Effects of mutant Sso1p TMD when appended to Snc1p. GFP-tagged constructs consisting of Snc1p with the Sso1p TMD (SSSO), with Asp residues at position 3, 5, 9, or 13 as indicated, were expressed in wild-type (wt) or Δ*rer1* mutant cells, and confocal images were obtained. Much of the fluorescence is in vacuoles, but arrowheads indicate nuclear envelope staining with the 3D and 5D mutants (nuclei being identified by DAPI staining of the cells). Fainter fluorescence corresponding to peripheral ER was also visible in these cells.

the Pep12p chimeras. Furthermore, in studies of Snc1p sorting in the late Golgi, we observed that mutations that introduced acidic residues into the TMD caused missorting directly to the endosomal system (M. Lewis and M. Black, unpublished observations). To examine more thoroughly such additional effects on sorting, we tested the set of Asp insertions in the Sso1p TMD in two further contexts: in Sso1p itself, and appended to Snc1p.

With Sso1p, several distinct effects could be observed, which are illustrated by the examples shown in Figure 8. When the constructs were expressed in an *end4* mutant to eliminate the effects of endocytosis, it was clear that the 3D and 5D mutants were partially missorted directly from the Golgi to the endosomal pathway, ending up within the vacuole (Figure 8). Some missorting of the 7D mutant was also observed, but substitution of Asp residues at TMD position −1, 9, 11, 13, 15, 17, or 19, or of Arg residues at position 3 or 5, did not affect transport of Sso1p to the plasma membrane (see Figure 8 for examples).

In wild-type cells, an additional effect of the mutations was apparent: the GFP–Sso1p was cleared from the plasma membrane and delivered to the vacuole. It is likely that Sso1p is normally endocytosed at a modest rate (see the internal fluorescence of GFP–Sso1p in Figure 8) and, like Snc1p, retrieved from early endosomes. In the case of Snc1p, retrieval is dependent on the TMD sequence (Lewis *et al.*, 2000). Thus, alterations to the Sso1p TMD might be expected to prevent its retrieval; it is also possible that they can stimulate its endocytosis. Whatever the precise mechanism, removal of Sso1p from the cell surface was clearly a distinct phenomenon from the missorting in the late Golgi and the internalization in late endosomes. Unlike these two phenomena, transfer of Sso1p from the plasma membrane to the vacuole could be induced by Asp residues throughout the TMD and also by Arg residues. The difference is well illustrated by the 15D and 17D mutants (Figure 8), which are endocytosed to the vacuole yet inefficiently internalized. Vacuolar delivery was also observed with the 7D, 9D, 11D, 13D, 19D, 3R, and 5R mutants.

An Snc1p chimera bearing the Sso1p TMD normally passes to the plasma membrane, is endocytosed, and then, because it fails to be recycled to the Golgi, is delivered to the vacuole (Lewis *et al.*, 2000) (SSSO, Figure 9). However, insertion of an Asp residue into the TMD of this chimera at position 3, 5, or 7 had an additional effect: a substantial proportion of the protein was found in the ER (Figure 9). Some did reach the vacuole and did so without passing via the plasma membrane (as judged by expression in *end4* cells; our unpublished observations). Asp residues at other positions (9, 11, 13, 15, 17, 19, or 21) did not have this effect on ER retention (see Figure 9 for examples). Those mutants that did accumulate in the ER did so even in a *rer1* deletion mutant, indicating that Rer1p was not solely responsible for their retention (Figure 9).

These results indicate that introduction of an Asp residue into the Sso1p TMD can affect protein sorting at several different sites, depending on the protein to which the TMD

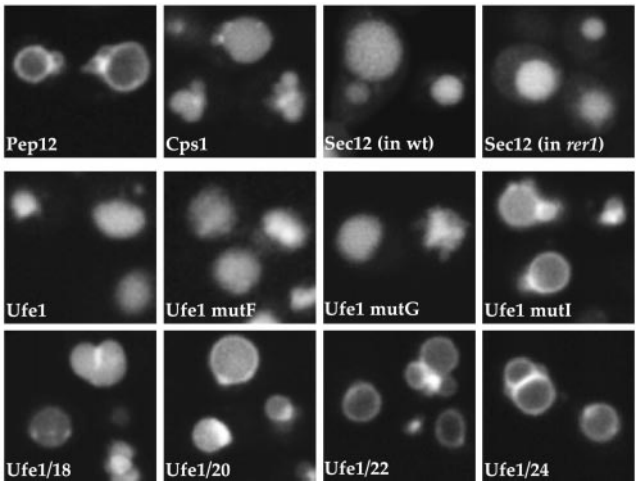

Figure 10. TMDs that cause internalization. Images are shown of vacuoles in cells expressing GFP–Pep12p with the indicated TMDs. See Table 2 for details of the sequences and Ufe1 mutations.

is appended. Remarkably, the position of the Asp residue has a similar effect on sorting at the ER/Golgi interface, the late Golgi, and late endosomes, maximal effects being achieved at positions 3 and 5. This suggests that similar physical properties of the TMD are recognized at each of these sites. In contrast, the sorting of Sso1p at the early endosome and/or plasma membrane, which is also affected by charges in the TMD, has a clearly distinct specificity.

Naturally Occurring Polar TMDs Are Sorted to the Vacuolar Interior

Acidic residues are quite rare in TMDs, but other hydrophilic residues are more common. To determine whether natural TMD sequences could also affect the fate of Pep12p, we prepared additional chimeras. First, we tested the TMD of the only protein known naturally to follow the internalization route into vacuoles, carboxypeptidase S (Cps1p). As shown in Figure 10, the Cps1p TMD targeted Pep12p to the vacuole interior. Given the similarities between ER retention and vacuole sorting, we also tested the TMD of Sec12p, which has an Asn residue at position 4 of the TMD. Although this is a substrate for the Rer1p-dependent retrieval system, when appended to Pep12p it did not cause ER retention but rather internalization into the vacuole, regardless of whether Rer1p was present. Similarly, the TMD of the ER syntaxin Ufe1p, which contains an Rer1p-independent ER retention signal, failed to keep Pep12p in the ER but targeted it to the vacuole interior (Figure 10).

An earlier study examined the effects of various mutations on the ability of the Ufe1p TMD to serve as an ER retention signal (Rayner and Pelham, 1997), and we tested these mutations also in the context of Pep12p. With regard to ER retention, replacement of pairs of polar residues in the first half of the TMD with leucines had little effect, but when all of them were altered some of the Pep12p could be found on the vacuole surface (Table 2; Figure 10). Lengthening the TMD from 16 to 20 residues proved a more effective means of saving Pep12p from internalization (Figure 10). In general, the features required for ER retention of Ufe1p and internalization of Pep12p were remarkably similar, although not identical (Table 2).

These experiments show that targeting to the vacuole lumen can readily be achieved by naturally occurring TMDs. The common features of these TMDs are quite difficult to discern, but most have a relatively short length and some hydrophilic residues, especially in their cytoplasmic half. Similar features are evidently recognized by the sorting machinery, which retains proteins in the ER and directs them to the vacuole lumen.

DISCUSSION

Internalization of Membrane Proteins in Endosomes

The yeast vacuole has multiple functions, one of which is to degrade proteins. For membrane proteins, this can be achieved by their internalization into late endosomes in small vesicles that are delivered to the vacuole lumen and ultimately destroyed (Burd *et al.*, 1998; Odorizzi *et al.*, 1998). Clearly, proteins that function on the outer vacuolar membrane have to avoid this fate; therefore, internalization must be specific. Selective sequestration into the internal membranes of MVBs is also important in animal cells for inactivation of endocytosed cell surface receptors (reviewed by Sorkin, 1998).

One way for a vacuolar protein to avoid internalization is to bypass the site at which it occurs by following the AP-3–dependent pathway directly from the Golgi to the vacuole. This strategy is followed by Vam3p (Cowles *et al.*, 1997a; Darsow *et al.*, 1998), and we now show that it is also used by Nyv1p. Our data support the view that internalization is restricted to endosomes: if there is direct invagination of vacuolar membrane, it is either a minor pathway under normal growth conditions or uses different sorting criteria from the endosomal event. Specifically, a Vam3p mutant that gets internalized does so only if it is forced to pass through the endosomal system. Furthermore, disruption of late endosomal function in *vps4* cells resulted in no detectable internalization of any construct tested.

Some proteins, e.g., Pep12p and Vam3p, can clearly pass through late endosomes and remain on the outer membrane. Our analysis of Pep12p revealed no specific cytoplasmic signal that might be required for this, but clearly the cytoplasmic domains of other SNAREs, such as Snc1p and Nyv1p, have features that cause them to be internalized (although neither would normally pass through late endosomes). We have been unable to identify any short internalization signal in these proteins, and we suspect that a global property, such as lack of tight structure, may be responsible (see below).

Polar Transmembrane Domains as Sorting Signals

Our most important conclusion is that sequences within the TMD can control the sorting of proteins in late endosomes, as they can at other points in the secretory and endocytic pathways. Specifically, a single aspartic acid residue in an otherwise hydrophobic TMD is sufficient to cause internalization, as are several natural TMDs that contain polar residues. These include that of Cps1p, which normally enters vacuoles in this way. This implies that the segregation mech-

anism is intimately connected to events within the lipid bilayer.

A rational explanation is that there is a quality control system that actively seeks membrane-spanning regions that contain inappropriately exposed residues and targets them for destruction. Polar residues exposed to the lipid bilayer could arise by mutation, misfolding, or incomplete assembly of membrane proteins. Such a system would be analogous to the proposed role of Vps10p as a receptor that can bind misfolded proteins in the Golgi lumen and carry them toward the vacuole (Hong *et al.*, 1996; Li *et al.*, 1999).

This idea is appealing, because our data indicate that there are at least three different sites in the endomembrane system at which similar features of a TMD are used to minimize the possibility of surface expression. The first example involves the well-known tendency for abnormal or unassembled proteins to be retained in the ER, a location where refolding is possible and from which degradation can be initiated. Polar residues within a TMD clearly accentuate retention, albeit in a manner that depends also on the nature of the rest of the protein. The second decision point occurs at the exit from the late Golgi: acidic residues within the TMD are sufficient to inhibit transport to the cell surface and instead direct proteins toward the endocytic pathway. Finally, in late endosomes, these same features dictate internalization and eventual destruction of the membrane protein. Although these mechanisms may have a primary role in protecting cells from damage, some normal cellular proteins evidently exploit them for their own localization, either to the ER (Sec12p, Ufe1p) or to the vacuole (Cps1p).

The similar specificity at each of the sorting points is striking. The strong effect of an acidic rather than a basic residue, within the cytoplasmic rather than the lumenal half of the bilayer, is one example. The common features of the Ufe1p TMD required for ER retention and internalization in endosomes is another. The specificity is not universal to all TMD-dependent sorting events, however; the recycling of Sso1p to the plasma membrane is also disrupted by charged residues within the TMD, but this is restricted neither to charges at the cytoplasmic end nor to negative residues. This sorting event is also different in nature: it retrieves proteins for reuse rather than targeting them for destruction.

The specificity suggests that polar TMDs are recognized by a protein receptor. Some other simple possibilities can be ruled out. For example, the TMD is not simply shortened by the insertion of a charge; if this were the only requirement, one would expect that both basic and acidic residues would suffice and that they could be placed near either end. Indeed, the Sso1p TMD is significantly longer than the Pep12p TMD, and introduction of an Asp at position 3 leaves a hydrophobic stretch of 19 residues, longer than the 18-residue Pep12p TMD. It seems more likely that the key feature is an acidic residue buried in the membrane, one to two helical turns from a positively charged amino acid that, by interacting with the acidic cytoplasmic face of the lipid bilayer, defines the start of the TMD. A single acidic residue, of course, is only one form of signal. The Sec12p TMD has an Asn at position 4, and both the Ufe1p and Cps1p TMDs have more dispersed polar features.

A likely reason for the cell to destroy proteins with abnormal TMDs is to prevent inappropriate aggregation or misfolding, and this raises the question of whether it is such gross aberrations that are recognized rather than specific TMD features. This seems unlikely in our experiments for several reasons. First, the TMDs of proteins such as Cps1p, Sec12p, and Ufe1p occur naturally in the cell, and these proteins have not been reported to form aggregates even when overexpressed. Second, there is no reason why such effects would have the observed specificity with regard to the location of acidic residues. Third, Vam3p containing an Asp in its TMD shows no sign of aggregation on the vacuole membrane when delivered there by the AP-3 pathway, is functional, and forms a complex containing Vti1p with an efficiency indistinguishable from that of the wild-type protein. Similarly, Pep12p mutants with acidic TMDs retain function, and the Sso1p mutants, which partially reach the plasma membrane, also show no sign of large-scale aggregation. It is difficult to see how more subtle effects, such as partial or transient dimerization, could lead to such efficient recognition by the internalization machinery. It seems more likely, and more of an advantage, that the cell recognizes a potentially disruptive TMD directly rather than the damage induced by it.

The effects of polar TMDs, however, are not independent of the proteins to which they are attached. Evidently, other properties of these proteins contribute to their overall fate, particularly in determining exit from the ER. Interestingly, the v-SNARE Snc1p seemed particularly prone to ER retention, and we observed a similar phenomenon when charges were introduced into the TMD of its relative Nyv1p (our unpublished observations). Previous work has also shown that the Golgi v-SNARE Sft1p is easily relocated to the ER by the Ufe1p TMD (Rayner and Pelham, 1997). In contrast, the three syntaxins we tested, Sso1p, Pep12p, and Vam3p, showed no sign of ER retention, even when acidic residues were introduced into their TMDs. The v-SNAREs were also subject to destruction in the vacuole regardless of the TMD, whereas the syntaxins were not. This may reflect in part the fact that Snc1p and its relatives have very little structure when not part of a SNARE complex and thus are effectively unfolded proteins, whereas syntaxins can assume a stable four-helix structure (Hazzard *et al.*, 1999; Misura *et al.*, 2000). Unfolded proteins are likely to be subject to additional quality control mechanisms at various points in the cell. Thus, the fate of any individual protein will be determined by a complex interplay of chaperones and sorting receptors, sometimes acting in concert and sometimes having opposite effects.

How might the mutant TMDs be sorted? By analogy with the ubiquitin system, the proteins might be marked for destruction by some modification, although none has been detected to date. Alternatively, they may be sorted directly by a protein receptor. If so, our data would suggest that related receptors patrol the bilayer at diverse locations. Alternatively, a single protein could act at more than one point, e.g., a "suicide" receptor might recognize a charged TMD in the Golgi and travel with it to endosomes and then into the vacuole. Such a receptor might itself segregate according to bilayer composition, not only in the Golgi but perhaps also in late endosomes. The internal membranes of MVBs in animal cells are reported to have a unique composition, being enriched in the unusual lipid *lyso*-bis-phosphatidic acid (Kobayashi *et al.*, 1998). In yeast, the formation of such structures requires Fab1p, a kinase that converts phosphati-

dylinositol 3-phosphate into phosphatidylinositol 3,5-bisphosphate, although whether the product is sorted selectively to internal membranes is not yet known (Odorizzi *et al.*, 1998).

The only receptor that has been implicated in the sorting of polar TMDs is Rer1p (Sato *et al.* 1996, 1997; Letourneur and Cosson, 1998). Although it is possible that Rer1p contributes to the sorting of the constructs we have used, it cannot be the sole receptor because it is not essential for internalization in endosomes, or for the retention of Snc1p TMD mutants in the ER, or for the TMD-dependent retention of Ufe1p (Lewis and Pelham, 1996). Although there are no close relatives of Rer1p in yeast, many membrane proteins of unknown function share its tetraspanning topology. It is possible that one or more of them shares its properties.

ACKNOWLEDGMENTS

We thank Mike Lewis for numerous plasmids and reagents and Sean Munro and Tim Levine for comments on the manuscript. F.R. was supported by a postdoctoral fellowship from the Swiss National Science Foundation and a European Molecular Biology Organization long-term fellowship. M.W.B. is a recipient of a Burroughs Wellcome Fund Hitchings-Elion Fellowship.

REFERENCES

Aalto, M.K., Ronne, H., and Keranen, S. (1993). Yeast syntaxins Sso1p and Sso2p belong to a family of related membrane proteins that function in vesicular transport. EMBO J. *12*, 4095–4104.

Babst, M., Sato, T.K., Banta, L.M., and Emr, S.D. (1997). Endosomal transport function in yeast requires a novel AAA-type ATPase, Vps4p. EMBO J. *16*, 1820–1831.

Becherer, K.A., Rieder, S.E., Emr, S.D., and Jones, E.W. (1996). Novel syntaxin homologue, Pep12p, required for the sorting of lumenal hydrolases to the lysosome-like vacuole in yeast. Mol. Biol. Cell *7*, 579–594.

Boehm, J., Letourneur, F., Ballensiefen, W., Ossipov, D., Demolliere, C., and Schmitt, H.D. (1997). Sec12p requires Rer1p for sorting to coatomer (COPI)-coated vesicles and retrieval to the ER. J. Cell Sci. *110*, 991–1003.

Bonifacino, J.S., Cosson, P., and Klausner, R.D. (1990). Colocalized transmembrane determinants for ER degradation and subunit assembly explain the intracellular fate of TCR chains. Cell *63*, 503–513.

Bonifacino, J.S., Cosson, P., Shah, N., and Klausner, R.D. (1991). Role of potentially charged transmembrane residues in targeting proteins for retention and degradation within the endoplasmic reticulum. EMBO J. *10*, 2783–2793.

Bowie, J.U. (2000). Understanding membrane protein structure by design. Nat. Struct. Biol. *7*, 91–94.

Bretscher, M.S., and Munro, S. (1993). Cholesterol and the Golgi apparatus. Science *261*, 1280–1281.

Bryant, N.J., Piper, R.C., Gerrard, S.R., and Stevens, T.H. (1998). Traffic into the prevacuolar/endosomal compartment of *Saccharomyces cerevisiae*: a VPS45-dependent intracellular route and a VPS45-independent endocytic route. Eur. J. Cell Biol. *76*, 43–52.

Burd, C.G., Babst, M., and Emr, S.D. (1998). Novel pathways, membrane coats and PI kinase regulation in yeast lysosomal trafficking. Semin. Cell Dev. Biol. *9*, 527–533.

Burd, C.G., Peterson, M., Cowles, C.R., and Emr, S.D. (1997). A novel Sec18p/NSF-dependent complex required for Golgi-to-endosome transport in yeast. Mol. Biol. Cell *8*, 1089–1104.

Cadwell, R.C., and Joyce, G.F. (1992). Randomization of genes by PCR mutagenesis. PCR Methods Appl. *2*, 28–33.

Conibear, E., and Stevens, T.H. (1998). Multiple sorting pathways between the late Golgi and the vacuole in yeast. Biochim. Biophys. Acta *1404*, 211–230.

Cormack, B.P., Valdivia, R.H., and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). Gene *173*, 33–38.

Cosson, P., and Letourneur, F. (1994). Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. Science *263*, 1629–1631.

Cowles, C.R., Odorizzi, G., Payne, G.S., and Emr, S.D. (1997a). The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. Cell *91*, 109–118.

Cowles, C.R., Snyder, W.B., Burd, C.G., and Emr, S.D. (1997b). Novel Golgi to vacuole delivery pathway in yeast: identification of a sorting determinant and required transport component. EMBO J. *16*, 2769–2782.

Darsow, T., Burd, C.G., and Emr, S.D. (1998). Acidic di-leucine motif essential for AP-3-dependent sorting and restriction of the functional specificity of the Vam3p vacuolar t-SNARE. J. Cell Biol. *142*, 913–922.

Darsow, T., Rieder, S.E., and Emr, S.D. (1997). A multispecificity syntaxin homologue, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole. J. Cell Biol. *138*, 517–529.

Gary, J.D., Wurmser, A.E., Bonangelino, C.J., Weisman, L.S., and Emr, S.D. (1998). Fab1p is essential for PtdIns(3)P 5-kinase activity and the maintenance of vacuolar size and membrane homeostasis. J. Cell Biol. *143*, 65–79.

Hazzard, J., Sudhof, T.C., and Rizo, J. (1999). NMR analysis of the structure of synaptobrevin and of its interaction with syntaxin. J. Biomol. NMR *14*, 203–207.

Hicke, L., Zanolari, B., Pypaert, M., Rohrer, J., and Riezman, H. (1997). Transport through the yeast endocytic pathway occurs through morphologically distinct compartments and requires an active secretory pathway and Sec18p/N-ethylmaleimide-sensitive fusion protein. Mol. Biol. Cell *8*, 13–31.

Holthuis, J.C., Nichols, B.J., Dhruvakumar, S., and Pelham, H.R. (1998). Two syntaxin homologues in the TGN/endosomal system of yeast. EMBO J. *17*, 113–126.

Hong, E., Davidson, A.R., and Kaiser, C.A. (1996). A pathway for targeting soluble misfolded proteins to the yeast vacuole. J. Cell Biol. *135*, 623–633.

Honing, S., Sandoval, I.V., and von Figura, K. (1998). A di-leucine-based motif in the cytoplasmic tail of LIMP-II and tyrosinase mediates selective binding of AP-3. EMBO J. *17*, 1304–1314.

Klionsky, D.J., and Emr, S.D. (1989). Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. EMBO J. *8*, 2241–2250.

Kobayashi, T., Stang, E., Fang, K.S., de Moerloose, P., Parton, R.G., and Gruenberg, J. (1998). A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. Nature *392*, 193–197.

Letourneur, F., and Cosson, P. (1998). Targeting to the endoplasmic reticulum in yeast cells by determinants present in transmembrane domains. J. Biol. Chem. *273*, 33273–33278.

Lewis, M.J., Nichols, B.J., Prescianotto-Baschong, C., Riezman, H., and Pelham, H.R. (2000). Specific retrieval of the exocytic SNARE snc1p from early yeast endosomes. Mol. Biol. Cell *11*, 23–38.

Lewis, M.J., and Pelham, H.R. (1996). SNARE-mediated retrograde traffic from the Golgi complex to the endoplasmic reticulum. Cell *85*, 205–215.

Li, Y., Kane, T., Tipper, C., Spatrick, P., and Jenness, D.D. (1999). Yeast mutants affecting possible quality control of plasma membrane proteins. Mol. Cell. Biol. *19*, 3588–3599.

Misura, K.M., Scheller, R.H., and Weis, W.I. (2000). Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. Nature *404*, 355–362.

Munro, S. (1995). An investigation of the role of transmembrane domains in Golgi protein retention. EMBO J. *14*, 4695–4704.

Nichols, B.J., Ungermann, C., Pelham, H.R., Wickner, W.T., and Haas, A. (1997). Homotypic vacuolar fusion mediated by t- and v-SNAREs. Nature *387*, 199–202.

Odorizzi, G., Babst, M., and Emr, S.D. (1998). Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. Cell *95*, 847–858.

Ohno, H., Stewart, J., Fournier, M.C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T., and Bonifacino, J.S. (1995). Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science *269*, 1872–1875.

Piper, R.C., Bryant, N.J., and Stevens, T.H. (1997). The membrane protein alkaline phosphatase is delivered to the vacuole by a route that is distinct from the VPS-dependent pathway. J. Cell Biol. *138*, 531–545.

Rapoport, I., Chen, Y.C., Cupers, P., Shoelson, S.E., and Kirchhausen, T. (1998). Dileucine-based sorting signals bind to the beta chain of AP-1 at a site distinct and regulated differently from the tyrosine-based motif-binding site. EMBO J. *17*, 2148–2155.

Raymond, C.K., Howald-Stevenson, I., Vater, C.A., and Stevens, T.H. (1992). Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E vps mutants. Mol. Biol. Cell *3*, 1389–1402.

Rayner, J.C., and Pelham, H.R. (1997). Transmembrane domain-dependent sorting of proteins to the ER and plasma membrane in yeast. EMBO J. *16*, 1832–1841.

Reggiori, F., Canivenc-Gansel, E., and Conzelmann, A. (1997). Lipid remodeling leads to the introduction and exchange of defined ceramides on GPI proteins in the ER and Golgi of *Saccharomyces cerevisiae*. EMBO J. *16*, 3506–3518.

Rieder, S.E., Banta, L.M., Kohrer, K., McCaffery, J.M., and Emr, S.D. (1996). Multilamellar endosome-like compartment accumulates in the yeast vps28 vacuolar protein sorting mutant. Mol. Biol. Cell *7*, 985–999.

Rodionov, D.G., and Bakke, O. (1998). Medium chains of adaptor complexes AP-1 and AP-2 recognize leucine-based sorting signals from the invariant chain. J. Biol. Chem. *273*, 6005–6008.

Sato, K., Sato, M., and Nakano, A. (1997). Rer1p as common machinery for the endoplasmic reticulum localization of membrane proteins. Proc. Natl. Acad. Sci. USA *94*, 9693–9698.

Sato, M., Sato, K., and Nakano, A. (1996). Endoplasmic reticulum localization of Sec12p is achieved by two mechanisms: Rer1p-dependent retrieval that requires the transmembrane domain and Rer1p-independent retention that involves the cytoplasmic domain. J. Cell Biol. *134*, 279–293.

Sauer, B. (1987). Functional expression of the cre-lox site-specific recombination system in the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. *7*, 2087–2096.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics *122*, 19–27.

Sorkin, A. (1998). Endocytosis and intracellular sorting of receptor tyrosine kinases. Front. Biosci. *3*, 729–738.

Srivastava, A., and Jones, E.W. (1998). Pth1/Vam3p is the syntaxin homolog at the vacuolar membrane of *Saccharomyces cerevisiae* required for the delivery of vacuolar hydrolases. Genetics *148*, 85–98.

Stepp, J.D., Huang, K., and Lemmon, S.K. (1997). The yeast adaptor protein complex, AP-3, is essential for the efficient delivery of alkaline phosphatase by the alternate pathway to the vacuole. J. Cell Biol. *139*, 1761–1774.

Toh-e, A., Nakamura, H., and Oshima, Y. (1976). A gene controlling the synthesis of non specific alkaline phosphatase in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta *428*, 182–192.

Ungermann, C., von Mollard, G.F., Jensen, O.N., Margolis, N., Stevens, T.H., and Wickner, W. (1999). Three v-SNAREs and two t-SNAREs, present in a pentameric cis-SNARE complex on isolated vacuoles, are essential for homotypic fusion. J. Cell Biol. *145*, 1435–1442.

Vida, T.A., and Emr, S.D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. J. Cell Biol. *128*, 779–792.

Vowels, J.J., and Payne, G.S. (1998). A dileucine-like sorting signal directs transport into an AP-3-dependent, clathrin-independent pathway to the yeast vacuole. EMBO J. *17*, 2482–2493.

Wada, Y., Nakamura, N., Ohsumi, Y., and Hirata, A. (1997). Vam3p, a new member of syntaxin related protein, is required for vacuolar assembly in the yeast *Saccharomyces cerevisiae*. J. Cell Sci. *110*, 1299–1306.

Wooding, S., and Pelham, H.R. (1998). The dynamics of Golgi protein traffic visualized in living yeast cells. Mol. Biol. Cell *9*, 2667–2680.