A Selective Transport Route from Golgi to Late Endosomes that Requires the Yeast GGA Proteins

Michael W. Black and Hugh R.B. Pelham

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

Abstract. Pep12p is a yeast syntaxin located primarily in late endosomes. Using mutagenesis of a green fluorescent protein chimera we have identified a sorting signal FSDSPEF, which is required for transport of Pep12p from the exocytic pathway to late endosomes, from which it can, when overexpressed, reach the vacuole. When this signal is mutated, Pep12p instead passes to early endosomes, a step that is determined by its transmembrane domain. Surprisingly, Pep12p is then specifically retained in early endosomes and does not go on to late endosomes.

By testing appropriate chimeras in mutant strains, we found that FSDSPEF-dependent sorting was abolished in strains lacking Gga1p and Gga2p, Golgi-associated coat proteins with homology to gamma adaptin. In the *gga1 gga2* double mutant endogenous Pep12p cofractionated with the early endosome marker Tlg1p, and recycling of Snc1p through early endosomes was defective. Pep12p sorting was also defective in cells lacking the clathrin heavy or light chain. We suggest that specific and direct delivery of proteins to early and late endosomes is required to maintain the functional heterogeneity of the endocytic pathway and that the GGA proteins, probably in association with clathrin, help create vesicles destined for late endosomes.

Key words: clathrin • endosomes • GGA proteins • Golgi apparatus • Pep12p

Introduction

The organization of the secretory pathway requires that membrane proteins are targeted to particular organelles. This process involves the selective incorporation of proteins into transport vesicles, and the specific fusion of these vesicles with their target organelle. A growing number of transport pathways within the cell are being defined, and many proteins carry multiple signals that determine their passage along these. The steady state distribution of any given protein is thus dictated by the particular combination of sorting signals that it bears.

Of particular importance is the sorting of those proteins that are involved in the transport process itself. Fusion of vesicles with their targets is mediated by soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs),[1] which are cytoplasmically oriented membrane proteins with the potential to form coiled-coil structures (Söllner et al., 1993; Rothman, 1994). SNAREs anchored in vesicles and target organelles form specific four-helix bundles, causing close apposition and eventual fusion of the membranes (Sutton et al., 1998; Weber et al., 1998). Each fusion event involves a member of a particular family of SNAREs termed the syntaxins. Yeast contains eight syntaxins with six distinct distributions: in the ER (Ufe1p), early Golgi (Sed5p), late Golgi/early endosomes (Tlg1p, Tlg2p), plasma membrane (Sso1p, Sso2p), late endosomes (Pep12p), and vacuole (Vam3p) (for reviews, see Nichols and Pelham, 1998; Pelham, 1999). Since syntaxins, like other membrane proteins, are initially inserted into the ER, this implies distinct sorting signals capable of directing them to each of these locations.

In this study, we have considered the delivery of the syntaxin Pep12p to endosomes. Two functionally distinct endosomal compartments can be distinguished, which we refer to as early and late endosomes. Early endosomes are the site from which some endocytosed proteins (such as the SNARE Snc1p) are retrieved to the Golgi (Lewis et al., 2000). Late Golgi proteins such as Kex2p and the SNAREs (Tlg1p and Tlg2p) are also thought to recycle through this compartment, which makes it difficult to distinguish from late Golgi cisternae. Late endosomes are easier to identify—they contain Pep12p and also show a characteristic coalescence into a large perivacuolar structure termed the prevacuolar compartment (PVC) in class E *vps* mutants (reviewed by Conibear and Stevens, 1998). In wild-type cells, late endosomes appear to mature into

Address correspondence to Hugh R.B. Pelham, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK. Tel.: 44-1223-402290. Fax: 44-1223-412142. E-mail: hp@mrc-lmb.cam.ac.uk

[1]*Abbreviations used in this paper:* CPY, carboxypeptidase Y; GFP, green fluorescent protein; PVC, pre-vacuolar compartment; SNARE, soluble *N*-ethylmaleimide–sensitive fusion protein attachment protein receptor; TMD, transmembrane domain.

© The Rockefeller University Press, 0021-9525/2000/10/587/14 $5.00

multivesicular bodies that then fuse with the vacuole (Odorizzi et al., 1998a), and this constitutes the main mechanism by which proteins reach the vacuole.

A quite different route is followed by a subset of vacuolar proteins, including alkaline phosphatase and the syntaxin Vam3p. These are transported directly from the Golgi to the vacuole, bypassing the PVC/late endosome completely. Access to this pathway requires a specific cytoplasmic signal and the AP-3 coat protein (reviewed by Odorizzi et al., 1998b). Unlike the related adaptors AP-1 and AP-2, AP-3 does not appear to require clathrin for its function.

In contrast, the coat proteins responsible for transport from Golgi to endosomes have proven hard to identify. AP-1 and AP-2 are not required, at least for those proteins whose transport has been studied (Huang et al., 1999). Furthermore, the role of clathrin itself is unclear. Shifting a temperature-sensitive clathrin heavy chain mutant to the nonpermissive temperature does cause a transient missorting of the vacuolar protease carboxypeptidase Y (CPY) to the cell surface, but after a brief period this defect disappears, implying that clathrin is not essential for Golgi-endosome traffic (Seeger and Payne, 1992a). Cells that lack clathrin altogether grow poorly but are viable and do not missort CPY.

We show here that transport of Pep12p to late endosomes requires a sorting signal in its NH_2-terminal domain, and that this signal is capable of diverting proteins directly from the exocytic pathway. Additional signals are also present: the transmembrane domain (TMD) serves to impede exocytosis, and the COOH-terminal region contains an endocytic signal. However, without the NH_2-terminal signal, Pep12p accumulates in early endosome/late Golgi structures, rather than late endosomes. Thus, there are distinct mechanisms for transport from the Golgi to early endosomes and to late endosomes.

The pathway defined by Pep12p is saturable and does not require the AP-1, AP-2, or AP-3 proteins. It is, however, defective in clathrin null mutants. It is also completely dependent on the presence of Gga1p or Gga2p, homologues of the GGA proteins originally identified in mammalian cells (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000; Poussu et al., 2000; Takatsu et al., 2000). These proteins show homology to the ear domain of gamma adaptin and are recruited to late Golgi membranes by the action of the GTPase ARF-1, but they are not associated with the majority of clathrin-coated vesicles. Our data indicates that they mediate transport from the Golgi to late endosomes. The phenotypes of *gga* null mutants suggest that this specific pathway is necessary to segregate the functions of early and late endosomes.

Materials and Methods

Yeast Strains

Yeast strains used in this study are listed in Table I. Gene disruptions were performed by replacing the entire open reading frame with the specified selectable markers using PCR primers containing 50–60 bases of identity to the flanking sequences.

Plasmids

Constructs used in this study were based on the pRS series of vectors (Sikorski and Hieter, 1989), and were centromeric except where indicated. All proteins were expressed using the *TPI1* promoter, except for the Pep12p derivatives in Figs. 3 and 4 *C* (below), which used the *PEP12* promoter. The Pep12-Sso1 chimera (replacing Pep12 K262-L288 with Sso1 R257-R290) was cloned downstream of the *TPI1* promoter (Wooding and Pelham, 1998) and mut2 green fluorescent protein (GFP) variant (Cormack et al., 1996) in the pRS416 vector. To identify mutations that sort this reporter to the cell surface, the *PEP12* sequence was randomly mutagenized using error-prone PCR (Cadwell and Joyce, 1992) using primers flanking this region by 200 bp. The product was cotransformed with the linearized *TPI*-GFP expression vector into the RH1597 strain (*end4-1*) and colonies screened for mutants missorting GFP to the cell surface. Residues R19–Q27 of Pep12 were specifically mutated by amplifying this sequence using oligonucleotides synthesized to introduce semirandom mutations in the 27-nucleotide region. At each position, 5% of the synthetic base precursor was replaced with one or more of the other three bases. Truncation of the Pep12 carboxy terminus (immediately upstream of the Pep12-Sso1 fusion) was performed using primers linking residues S2–I44 (2–44 construct) and S2–A192 (PNTS) of Pep12p to the Sso1p carboxy-terminal R257-R290. PNTP was made by replacing the Sso1 sequences in PNTS with the Pep12p car-

Table I. Yeast Strains

Strain	Genotype
SEY6210*	MATa *ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*
RH1597‡	MATa *ura3 leu2 his4 bar1 enΔ4-1*
JHY005§	MATa *ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pep12::HIS3L*
SEY4-1‖	MATa *ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vps4-1*
MBY001¶	MATa *aΔe2-1 trp1-1 can1-100 leu2-3,-112 his3-11,15 ura3-52 aΔe3-Δ853 ste18::HIS5spL*
BHY152‖	MATa *ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vps5::HIS3*
Δ45§	MATa *ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vps45::HIS5spL*
FRY027§	MATa *ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 apl2::HIS5spL*
FRY026§	MATa *ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 apl1::HIS5spL*
FRY024§	MATa *ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 apm3::HIS5spL*
JHY016§	MATa *ura3-52 his3-Δ200 leu2-3, -112 trp1-Δ901 suc2-Δ9 tlg1::TRP1*
MBY004¶	MATa *ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 gga1::HIS5spL gga2::TRP1*
vps1‖	MATa *ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vps1::HIS3*
Y04572**	MATa *his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 chc1::kanMX4*
Y04797**	MATa *his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 clc1::kanMX4*

*Provided by S. Emr, University of California, San Diego, CA.
‡Provided by H. Riezman, Biozentrum of the University of Basel.
§Provided by Pelham lab.
‖Provided by M. Seaman, University of Cambridge, Cambridge, UK.
¶This study.
**Provided by Euroscarf (the European *S. cerevisiae* archive for functional analysis).

boxy-terminal K262-L288, thus generating an internal deletion of residues 193–261 of Pep12p. To test for complementation of the Δpep12 phenotype, the *TPI1* promoter and GFP cassette from wild type and the NM09-NM40 Pep12-Sso1 fusions were replaced with the *PEP12* promoter (820 bp immediately upstream of the Pep12p open reading frame).

The S2-A192 region of Pep12p was also fused to the amino terminus of a *TPI1*-driven Ste18-Sso1 chimera containing the C106S and C107S mutations to disrupt the CaaX-directed isoprenylation (Finegold et al., 1990) and the R257–R290 transmembrane domain of Sso1p. Versions of these proteins were also made with GFP at the amino terminus. As a reporter for Ste18p activity at the plasma membrane, the *FUS1* promoter (450 bp upstream of the open reading frame) was cloned upstream of a β-galactosidase reporter in the 2-μm vector pRS424.

Plasmids expressing the GFP-tagged forms of Snc1p (Snc1, Snc1 end$^-$ and Snc1-Ufe18 end$^-$) have been described previously (Lewis et al., 2000). The GNS construct (see Fig. 7) was obtained by replacing the Nyv1 transmembrane domain with that of Snc1 under the control of the *TPI1* promoter with an amino-terminal GFP fusion (Reggiori et al., 2000).

Fluorescence Analysis

Yeast were grown to early-log phase and examined using an MRC-600 confocal microscope (Bio-Rad Laboratories). Images were smoothed and the contrast adjusted using Photoshop (Adobe Systems Inc.). To stain the endocytic pathway, cells were pelleted and resuspended in medium containing 20 μM FM4-64 (Molecular Probes). After a 15-min incubation at room temperature, cells were pelleted, washed once with water, and resuspended in either water (immediate visualization) or media for examining later time points.

β-Galactosidase Assay of FUS1 Expression

Transformants of the MBY001 strain carrying the *ste18* deletion were grown to early log phase, pelleted, and resuspended in fresh medium containing 2 μM α-factor (Sigma-Aldrich). A 5 ml culture was grown for 4 h at 30°C and the level of β-galactosidase from the *FUS1*-driven reporter was determined using an adaptation of a previously described procedure (Ausubel et al., 1995). Cells were pelleted, washed in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0), and resuspended in 5 ml of the same. A volume of 0.8 ml of cells (or a 10-fold dilution) was lysed using 50 μl of chloroform and 25 μl of 0.1% SDS and brought to 30°C before adding 0.16 ml of a 4 mg/ml *O*-nitrophenyl β-D-galactopyranoside (Sigma-Aldrich) in Z-buffer. The β-galactosidase units were determined using the following formula: $U = 1{,}000 \times [(OD\ 420) - (1.75 \times OD\ 550)]/[(t, \text{min}) \times (\text{vol, ml}) \times OD\ 600]$.

Subcellular Fractionation

Fractionation studies were carried out as described by Becherer et al. (1996). Cells were grown in 500 ml SD medium to midlogarithmic phase (OD 600 of 0.8–1.0), harvested, and resuspended in 50 ml 200-mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1% β-mercaptoethanol. After incubation at 30°C for 10 min, cells were harvested, resuspended in 50 ml synthetic medium containing 1.2 M sorbitol supplemented with 0.15 mg/ml zymolyase-20T (ICN Biomedicals), incubated at 30°C for 30 min and washed in 1.2 M sorbitol. Spheroplasts were lysed in 4–6 ml ice-cold hypo-osmotic buffer (50 mM KPO_4, pH 7.5, 200 mM sorbitol, 1 mM EDTA) containing freshly added Complete protease inhibitors (Boehringer) and 1 mM PMSF using 10–15 strokes in a Dounce homogenizer. The cell lysate was centrifuged sequentially at 500 *g* (5 min) and 13,000 *g* (15 min) and 2 ml of the supernatant was loaded on top of a 22–60% sucrose step gradient as described by Holthuis et al. (1998a,b). After centrifugation in an SW40Ti rotor (Beckman Coulter) at 170,000 *g* for 17–18 h, 0.78-ml fractions were collected from the top and used for SDS-PAGE and Western blot analysis. The gradient shown in Fig. 8 C (below) was prepared with 1 ml 60%, 1.5 ml each of 50, 45, 40, 37, and 34%, 1 ml 27%, and 1 ml 22% sucrose.

Immunoblotting

The carboxypeptidase Y secretion assay was performed as previously described (Roberts et al., 1991) by replica-plating freshly grown spots onto nitrocellulose for overnight growth. For Western blot analysis, all antibody incubations were carried out as in Holthuis et al. (1998a) using rabbit polyclonal antibodies against Pep12p, Vam3p, Tlg1p, Sso1p, and CPY (Nichols et al., 1997; Holthuis et al., 1998a,b), and GFP (a gift from Derek McCusker, MRC Cambridge). After incubation with peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories), detection was performed using enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech) and bands acquired from the fractionation experiments were quantified using National Institutes of Health Image software.

Results

Multiple Sorting Signals on Pep12p

To allow easy monitoring of the distribution of Pep12p, we tagged it at the amino terminus with GFP and expressed it from the strong *TPI1* promoter; the protein remained functional and was expressed at levels approximately five- to eightfold higher than normal (data not shown). Because lower levels of GFP were hard to image satisfactorily, all the GFP-tagged proteins described in this paper were expressed from this promoter.

By immunofluorescence, endogenous Pep12p typically appears as scattered or perivacuolar dots (Lewis et al., 2000). However, a substantial fraction of the GFP-tagged protein reached the outer vacuolar membrane, identified by staining with the tracer dye FM4-64 (Vida and Emr, 1995), presumably because it failed to be retrieved from endosomes before they fused with the vacuole (Fig. 1 A). This appeared to be due to saturation of the retrieval mechanism rather than the GFP tag itself, since endogenous Pep12p was also found in the vacuole under these conditions (data not shown). Though a consequence of overexpression, the exaggerated vacuolar staining is convenient: it provides a readily identifiable pattern that is clearly distinct from early endosomes and Golgi cisternae.

As expected, GFP-Pep12p travels to the vacuole via late endosomes, rather than by the AP-3 pathway. In *apm3* cells, which lack an AP-3 subunit, this pathway is blocked, but GFP-Pep12p still reached vacuoles (data not shown; Reggiori et al., 2000). Conversely GFP-Pep12p was found in the abnormal late endosome structures that accumulate in the class E mutant *vps4* (see Fig. 4 B).

The fate of membrane proteins leaving the late Golgi is strongly influenced by the nature of their transmembrane domains (Rayner and Pelham, 1997; Lewis et al., 2000). However, replacing the TMD of Pep12p with that of the plasma membrane syntaxin Sso1p did not result in its accumulation on the surface (Rayner and Pelham, 1997). We found that this was true even in an *end4* mutant, in which endocytosis is blocked (Fig. 1 B; Wesp et al., 1997), implying that Pep12p contains a cytoplasmic signal for direct transport from Golgi to endosomes.

To identify this signal, we performed random PCR mutagenesis of the Pep12-Sso1 chimera, transformed the mutagenized plasmids into an *end4* strain, and screened individual transformants by fluorescence microscopy. This protocol should have eliminated any TMD-dependent diversion from the exocytic pathway, and also prevented endocytosis. Hence, mutants lacking a direct Golgi-endosome transport signal should be found on the cell surface. A number of such mutants were obtained (see Fig. 1 B for examples). The corresponding plasmids were recovered and their phenotypes checked by retransformation.

When expressed in wild type rather than *end4* cells, almost all the mutant proteins were internalized, implying that Pep12p contains a signal for endocytosis. However, in one case (M48), some plasma membrane fluorescence was

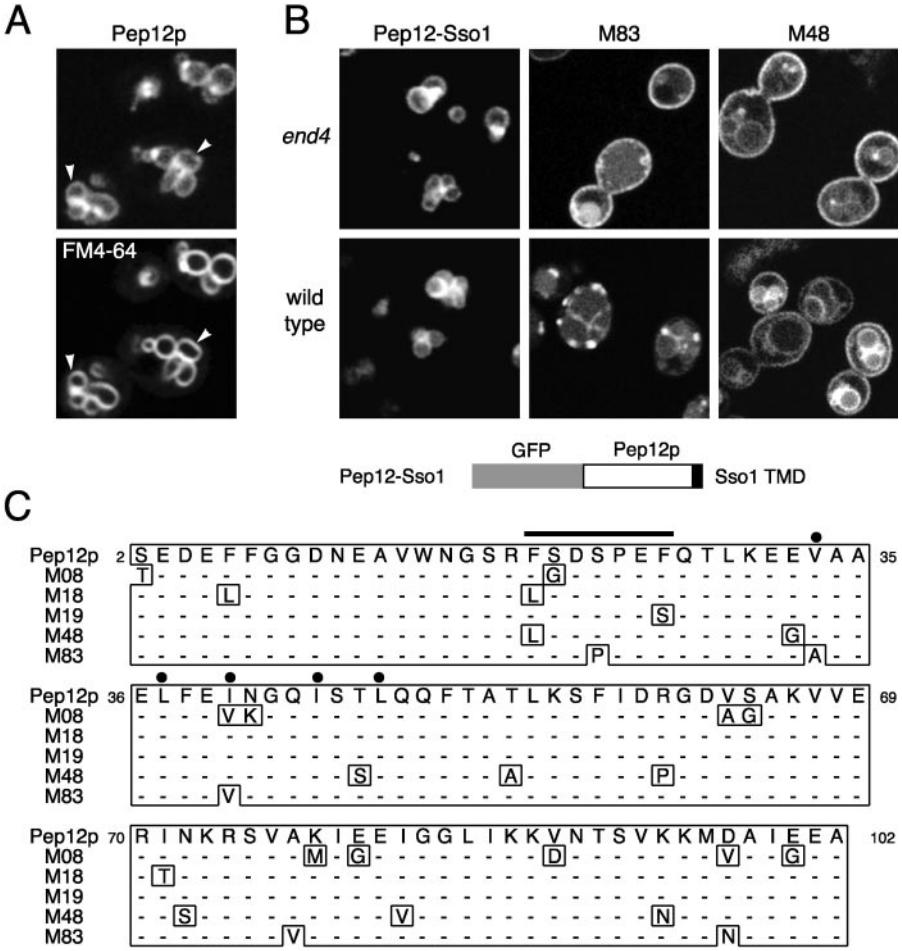


Figure 1. Mutations that affect targeting of Pep12p. (A) GFP-tagged Pep12p is on vacuolar membranes, identified by labeling with FM4-64. (B) GFP-tagged Pep12p bearing the Sso1p TMD (Pep12-Sso1), and examples of PCR-generated mutants, expressed in wild-type and *end4* cells. (C) Sequence changes in mutants. Changes within the region shown (residues 2–102 of Pep12p) were sufficient for mistargeting to the cell surface in *end4* cells. Black dots indicate the heptad repeat of the first predicted helix; the black bar indicates the smallest region that contains a change in each of the mutants sequenced.

retained (Fig. 1 B). Analysis of mutant/wild-type chimeras revealed that the inefficient endocytosis of this protein was due to changes in the COOH-terminal coiled coil domain, but we did not characterize the endocytic signal in detail.

A Signal for Transport from Golgi to Endosome

In contrast to the endocytic signal, the mutations responsible for initial transport to the cell surface could be traced in each case to the NH_2 terminus (residues 2–102). Fig. 1 C lists the amino acid changes in typical mutants that demonstrated a strong missorting phenotype. One (M19) contained only a single change, F26S, and we noticed that all of them had a mutation that lay between residues 20 and 26 (Fig. 1 C, bar).

The crystal structure of syntaxin 1A, to which Pep12p is related, shows that it has an NH_2-terminal domain consisting of a three-helix bundle. This can interact reversibly with the COOH-terminal helix that, when not bound in this way, interacts with other SNAREs (Misura et al., 2000; Sutton et al., 1998). Residues 20–26 of Pep12p are predicted to be adjacent to the first helix of the NH_2-terminal domain, revealed by a set of heptad repeats of hydrophobic residues (Fig. 1 C, dots); much of the corresponding region of syntaxin 1A is unstructured. Using synthetic oligonucleotides of mixed sequence, we introduced random mutations into this region of the Pep12-Sso1 chimera, screened them for their sorting phenotype in *end4* cells (Fig. 2 A), and sequenced 11 mutants that were strongly missorted together with four that were sorted normally (Fig. 2 B). Taken together, the results show that the sequence FSDSPEF is important for sorting. Mutations affecting each of these residues appeared to cause missorting, though the D could be changed to E without effect and some substitutions of the serine residues were tolerated. For brevity, we refer to this as the FSD motif.

It proved difficult to determine whether this sequence was sufficient for sorting, because deletion mutants that did not have the NH_2-terminal three-helix structure intact showed poor sorting, either alone or when fused to a heterologous protein. We also found that some of the original PCR mutants that showed a weak missorting phenotype had mutations in the helical region of Pep12p but not in the FSDSPEF sequence, which again suggests that the structural context of this motif is important for sorting. However, insertion of residues 2–44 of Pep12p between GFP and the Sso1p TMD did result in efficient transport to the vacuole, even in *end4* cells (Fig. 2 C). That this was a specific effect was shown by substituting the equivalent portions of two of the original mutants, M18 and M83. In each case, substantially more of the protein reached the plasma membrane (Fig. 2 C), implying disruption of the sorting signal. The residual vacuolar fluorescence even with the mutant peptides suggests that the construct is sorted by a second mechanism as well, perhaps being recognized by the cell as "unfolded."

Pep12p must interact with other proteins to function, and it might be that the FSD motif is required for one of these functional interactions and only indirectly for sort-

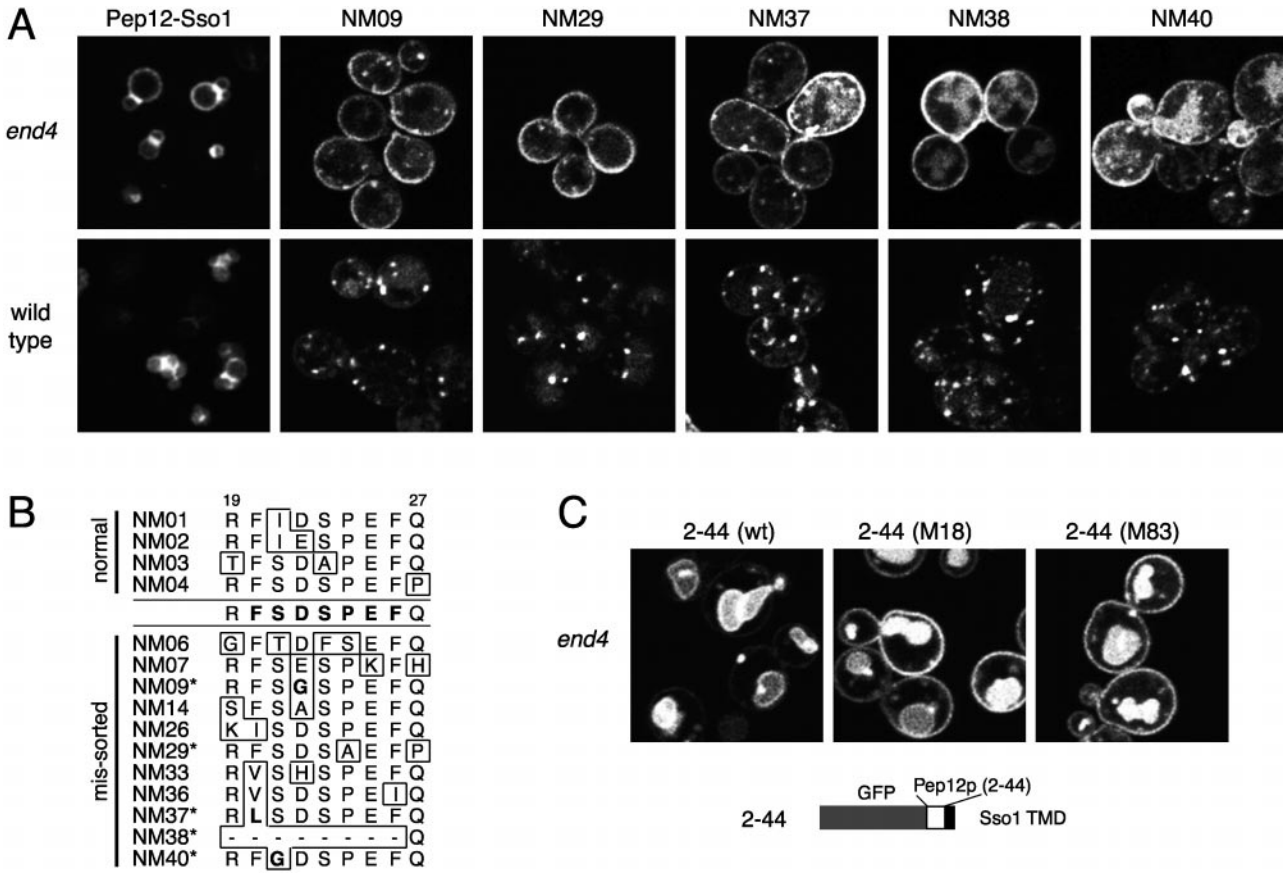
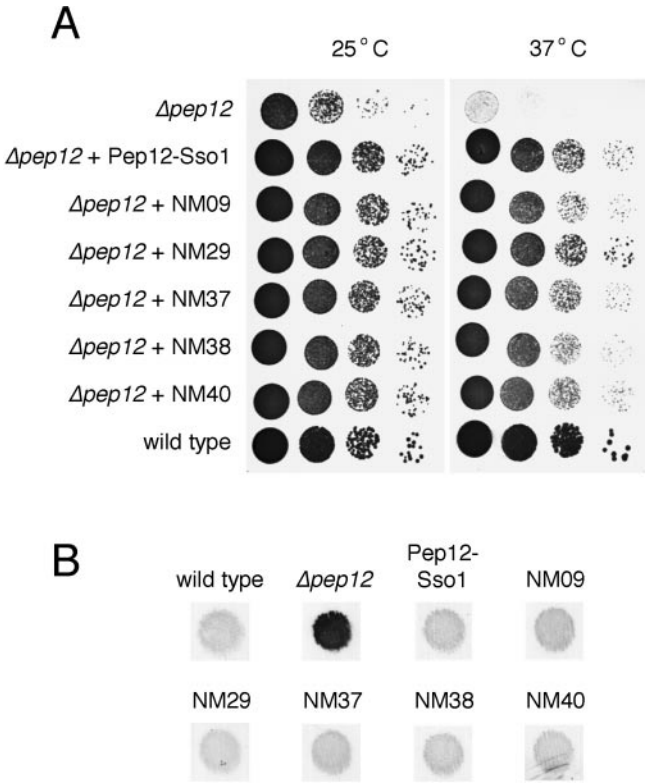


Figure 2. Targeted mutagenesis of the sorting signal. (A) Images of the parental construct (GFP-tagged Pep12-Sso1) and missorted mutants in wild type and *end4* strains. (B) Sequence changes in constructs that were missorted or showed a normal location. Dashes in the NM38 sequence indicate a deletion. *Mutants shown in A. (C) Distribution in *end4* cells of a construct containing only residues 2–44 of Pep12p (structure as diagrammed), or the M18 and M83 mutant versions (see Fig. 1).



ing. We therefore tested the point mutants for Pep12p function. For these experiments, we used the normal *PEP12* promoter and omitted the GFP tag. Null alleles of *pep12* show temperature-sensitive growth, a phenotype that could be rescued by the Pep12-Sso1 chimera, and roughly equally well by the various point mutants (Fig. 3 A). Pep12p is also required for sorting of CPY to the vacuole (Becherer et al., 1996), and secretion of pro-CPY by the null mutant can be detected by growing cells in contact with nitrocellulose, and then probing this with anti–CPY antibodies. As shown in Fig. 3 B, the point mutants all restored CPY sorting. This suggests that the mutant proteins not only retain their biochemical functions, but also can still reach endosomes, albeit largely by endocytosis rather than by direct transport from the Golgi.

Since most of the mutants had similar phenotypes, we selected NM37 as an example for further analysis. This carries a single change in the first residue of the FSD motif, F20L.

Figure 3. Missorted mutants retain function. (A) Serial 10-fold dilutions of wild-type, Δpep12, and the Δpep12 strain expressing untagged versions of Pep12-Sso1 or mutant derivatives (see Fig. 2) from the *PEP12* promoter were grown at 25° or 37°C. (B) CPY secreted by patches of the same cells was detected by antibody staining.

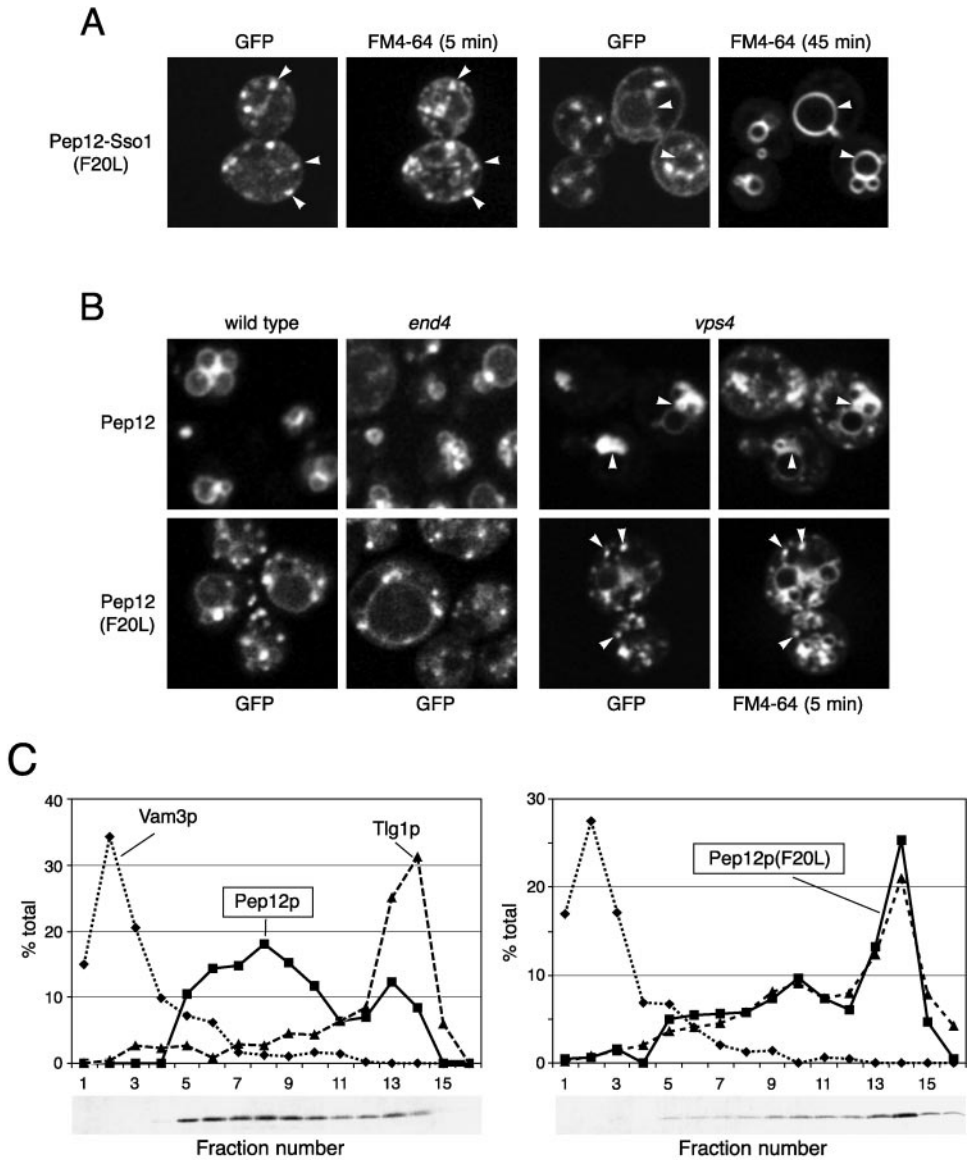


Figure 4. The F20L mutation allows Pep12p to accumulate in early endosomes. (A) Wild-type cells expressing GFP-tagged Pep12-Sso1 (F20L) were incubated with FM4-64 for 15 min, chased for 5 or 45 min as indicated, and imaged. FM4-64 labels the dots containing the GFP-labeled protein at early times, and then passes to the vacuole. (B) GFP-labeled Pep12p (F20L) with its own TMD is punctate in wild-type, *end4,* and *vps4* cells, whereas unmutated Pep12p is in vacuoles or, in *vps4*, the PVC. The *vps4* cells were incubated with FM4-64 and chased for 5 min to reveal labeling of punctate early endosomes containing the mutant protein. (C) Extracts of Δpep12 cells expressing untagged wild-type, Pep12p, or the F20L mutant at normal levels (from the *PEP12* promoter) were fractionated on sucrose density gradients. Fractions were assayed for Pep12p, Vam3p (vacuolar marker), and Tlg1p (early endosomal marker) by immunoblotting. The Pep12p blots are shown beneath the graphs.

Pep12p Lacking the NH_2-Terminal Targeting Signal Accumulates in Early Endosomal/Late Golgi Structures

Examination of the GFP-tagged mutants in wild-type cells, where endocytosis could occur, revealed that they accumulated in small punctate structures rather than the vacuole (Fig. 2 A). Analysis of the F20L mutant showed that these could be labeled rapidly by the endocytic tracer dye FM4-64, indicating that they were functional endosomes (Fig. 4 A). Evidently, the absence of the FSD signal caused the Pep12-Sso1 chimera to accumulate in an earlier endosomal compartment.

Restoration of the normal Pep12p TMD to the mutants prevented their passage to the cell surface, as judged by expression in *end4* cells (Fig. 4 B). This is consistent with previous work indicating that short TMDs prevent exocytosis (Lewis et al., 2000; Rayner and Pelham, 1997). Instead, the mutant Pep12p was again found in punctate structures (Fig. 4 B). Its failure to reach the vacuole suggested that these were not late endosomes, and to verify this we expressed the F20L version of GFP-Pep12p in a *vps4* mutant. In these cells, late endosomes form into a large perivacuolar structure termed the prevacuolar compartment, or PVC (Babst et al., 1997). Wild-type GFP-Pep12p accumulates in the PVC, which can be labeled with FM4-64 (Fig. 4 B). However, at early times FM4-64 can also be seen in dispersed dots corresponding to early endosomes (Fig. 4 B). The F20L mutant was found in these dispersed, FM4-64–labeled structures. Thus, whether delivered from the Golgi or from the plasma membrane, the mutant Pep12p accumulates in a compartment with the properties of an early endosome, rather than the late endosomal compartment (and thence the vacuole) to which Pep12p is normally delivered. In practice, early endosomes are very difficult to distinguish from the yeast equivalent of the TGN, and membrane proteins appear to cycle between the two (Lewis et al., 2000). Pep12p evidently possesses features that keep it in this late Golgi/early endosomal cycle when it fails to take its normal route to late endosomes.

To confirm this result, we examined the effect of the F20L mutation on the subcellular distribution of Pep12p itself, expressed at normal levels without a GFP tag. Mutant and wild-type proteins were expressed from the *PEP12* promoter in the absence of the endogenous gene and intracellular membranes separated on a sucrose den-

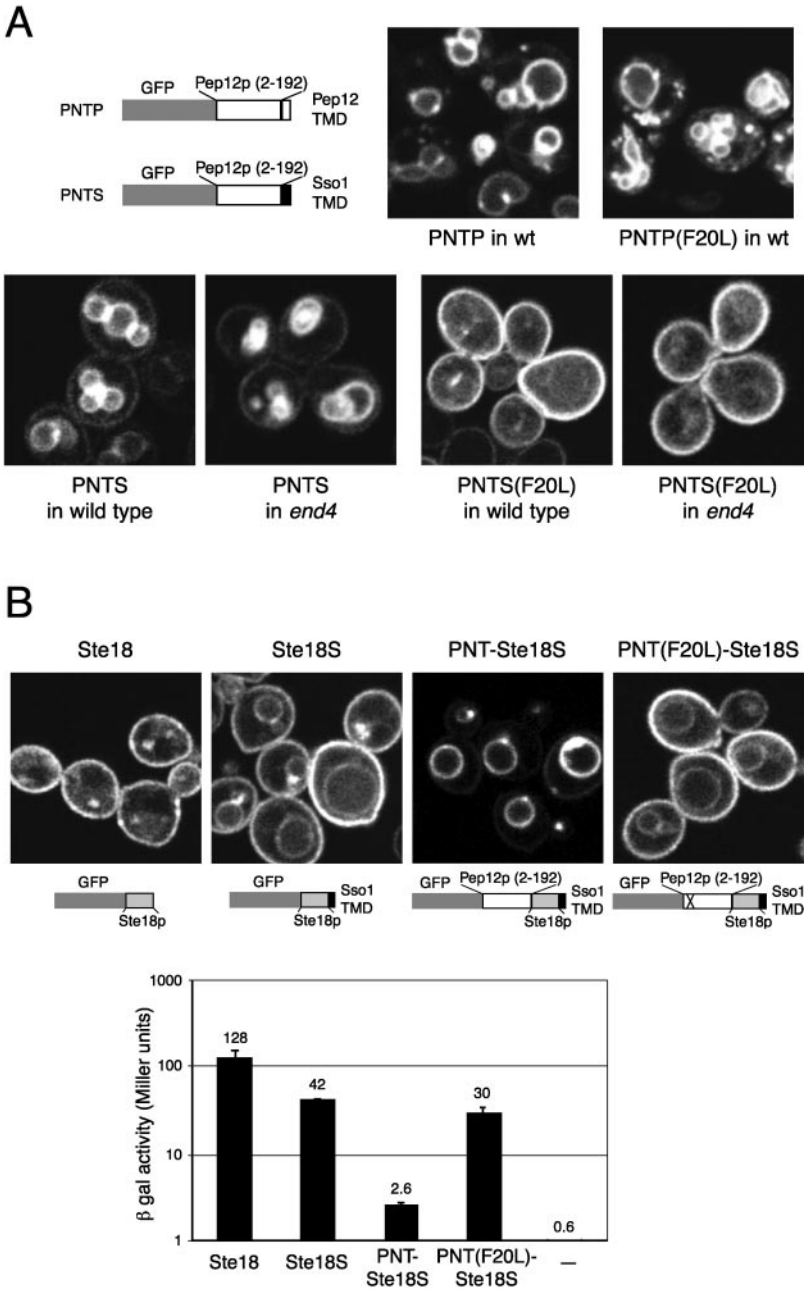


Figure 5. Properties of the NH_2-terminal domain of Pep12p. (A) GFP-tagged PNTP and the F20L mutant version both reach vacuoles in wild-type cells. PNTS, the equivalent construct with the Sso1 TMD, goes to vacuoles in wild-type and *end4* cells, whereas the F20L mutant goes to the plasma membrane. (B) GFP-labeled derivatives of Ste18p (as diagrammed) were expressed in a Δ*ste18* strain and imaged. Equivalent constructs without GFP, or no construct (−), were expressed in Δ*ste18* cells carrying a *FUS1*-lacZ reporter and the cells assayed for β-galactosidase activity. Note the log scale of the graph.

sity gradient. As shown previously (Becherer et al., 1996; Holthuis et al., 1998a) and in Fig. 4 C, the bulk of the wild-type Pep12p was found in fractions of intermediate density, separated from a vacuolar marker (Vam3p). However, a smaller peak was also observed in more dense sucrose, coincident with the presumptive early endosome marker Tlg1p (Becherer et al., 1996; Holthuis et al., 1998a,b; Lewis et al., 2000). Strikingly, the F20L mutant form of Pep12p was largely restricted to this region of the gradient, cofractionating very closely with Tlg1p (Fig. 4 C). Thus, the FSD signal is required under normal conditions to direct Pep12p to late endosomes.

Transport of GFP-Pep12p out of early endosomes became possible even without the FSD signal when the COOH-terminal coiled-coil domain (residues 193–261) was deleted. This construct, named PNTP (Pep12p NH_2 terminus with Pep12p TMD), comprised the GFP-tagged NH_2-terminal domain joined directly to the TMD. With the FSD signal, it was transported efficiently to vacuoles. When the FSD motif was mutated (F20L), it again reached vacuoles, though fluorescent dots were also visible (Fig. 5 A). This protein can therefore be transported to the vacuole by a pathway that is independent of the FSD signal. Evidently, removal of the COOH-terminal region disrupts an early endosome/late Golgi retention signal and allows the protein to travel by a default pathway through early and late endosomes.

Specific Assays for FSD-dependent Sorting

To make analysis of the FSD-dependent sorting pathway simpler, we designed a similar chimera bearing the Sso1p TMD instead of the Pep12p TMD (PNTS, Fig. 5 A). Since this construct lacks the coiled-coil domain that interacts with other SNAREs (Sutton et al., 1998), it is a nonfunctional reporter rather than an active SNARE. With an intact FSD motif, it was transported directly to the vacuole,

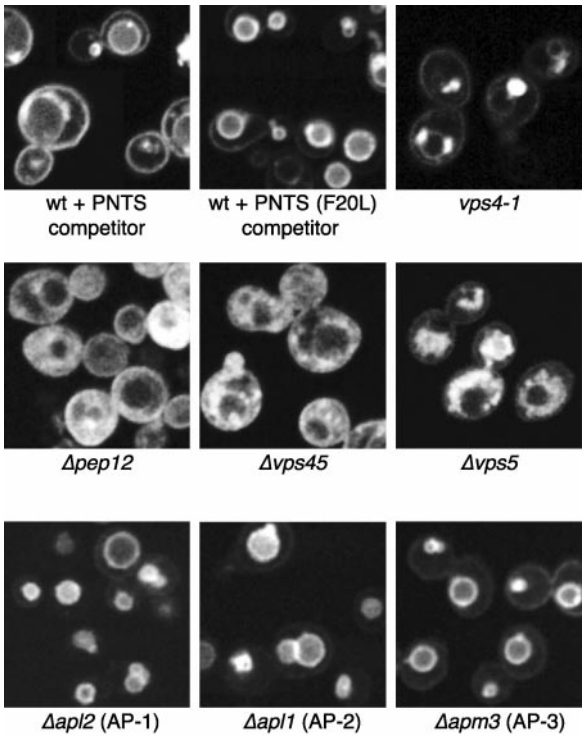


Figure 6. Sorting of the NH_2-terminal domain of Pep12p in various strains. GFP-tagged PNTS (see Fig. 5) was expressed in the indicated mutants, and in wild-type cells (wt) that coexpressed from a multicopy vector non–GFP-tagged versions (competitor) of either PNTS itself or the F20L mutant version.

as expected. However, when the FSD motif was mutated, PNTS accumulated on the cell surface (Fig. 5 A). This occurred in both *end4* and wild-type cells, indicating that removal of the COOH-terminal coiled-coil domain had abolished endocytosis, in agreement with our earlier finding that point mutations in this region also block endocytosis. Hence, fusion of residues 2–192 of Pep12p to the Sso1p TMD yields a protein whose destination is determined almost entirely by the FSD motif: without FSD, it travels to the plasma membrane and stays there; with FSD, it is diverted from the Golgi to late endosomes and the vacuole, where it accumulates. These destinations are easy to distinguish.

For a more quantitative estimate of the effects of the FSD signal, we made use of the fact that the transcriptional response of cells to alpha factor depends on the presence of Ste18p on the plasma membrane. Ste18p, the gamma subunit of a trimeric G protein, normally has a lipid membrane anchor but retains function when this is replaced by the Sso1p TMD. Its presence on the plasma membrane can then be assayed by the ability of cells to induce a *FUS1*-lacZ reporter gene when exposed to alpha factor (Pryciak and Huntress, 1998). Thus, if it can be diverted to the vacuole, this transcriptional response should be significantly reduced.

We expressed four different constructs in a *ste18* null strain carrying the *FUS1*-LacZ reporter; these lacked GFP, but we also made GFP-tagged versions to allow their locations to be assessed (Fig. 5 B). Ste18p itself was localized mostly to the plasma membrane and supported a strong alpha factor response. Addition of the Sso1p TMD reduced activity threefold, but the protein remained largely on the plasma membrane. Further addition of the NH_2-terminal domain of Pep12p reduced β-galactosidase activity by 94%, and rerouted the protein to the vacuole. Finally, introduction of the F20L mutation into this construct increased activity some 11-fold, to 70% of that obtained without the Pep12p domain at all, and also restored plasma membrane fluorescence (Fig. 5 B). We conclude that the NH_2-terminal domain of Pep12p can efficiently redirect another protein from the exocytic pathway to the endocytic one, and that the F20L mutation virtually abolishes this sorting function.

The FSD Pathway Requires Clathrin and the GGA Proteins

We used the PNTS construct to further characterize FSD-dependent sorting. First, we established that it is saturable, consistent with recognition by a specific protein. Thus, coexpression from a multicopy vector of a version of PNTS that lacked GFP caused significant mistargeting of the GFP version to the plasma membrane (Fig. 6). This effect was abolished by introducing the F20L mutation into the competitor construct.

We then tested a variety of mutants for their effects on this Golgi-endosome transport pathway. Delivery of PNTS to the vacuole was blocked by *vps4*, confirming that like Pep12p itself it travels via late endosomes (Fig. 6). Removal of endogenous Pep12p resulted in the accumulation of PNTS in a hazy pattern presumably corresponding to transport vesicles that are unable to fuse with their endosomal target. Removal of Vps45p, which is required for the function of both Pep12p and the early endosome/late Golgi SNARE Tlg2p (Nichols et al., 1998; Abeliovich et al., 1999), had a similar effect. In each case, very little PNTS reached the plasma membrane, indicating that the initial diversion from the exocytic pathway could still occur.

We tested known coat proteins that might be responsible for this sorting. Vps5p, a component of the retromer coat that retrieves proteins from late endosomes (Seaman et al., 1998), was not required (note that vacuoles are fragmented in *vps5* cells; Fig. 6). Similarly, transport was unaffected by mutations in the adaptors AP-1, AP-2, or AP-3. However, in cells lacking either clathrin heavy chain or the light chain, there was substantial missorting of PNTS to the plasma membrane (Fig. 7). A proportion of the protein was found inside the cells in membranes of uncertain identity, some of which appeared vacuolar, but, significantly, we could detect no difference between cell populations expressing PNTS or the F20L mutant version. This suggests that clathrin is required for the FSD motif to function as a sorting signal.

An effect was also seen in cells lacking Vps1p, a dynamin homologue previously implicated in Golgi-endosome transport (Nothwehr et al., 1995); dynamin in animal cells is thought to catalyze the pinching off of clathrin-coated vesicles (McNiven et al., 2000). Significant amounts of PNTS reached the cell surface in a *vps1* mutant, but comparison of PNTS with the F20L version showed that some recognition of the sorting signal was occurring in these cells: there was substantially more vacuolar and punctate fluorescence with the wild-type version than with the mutant (Fig. 7).

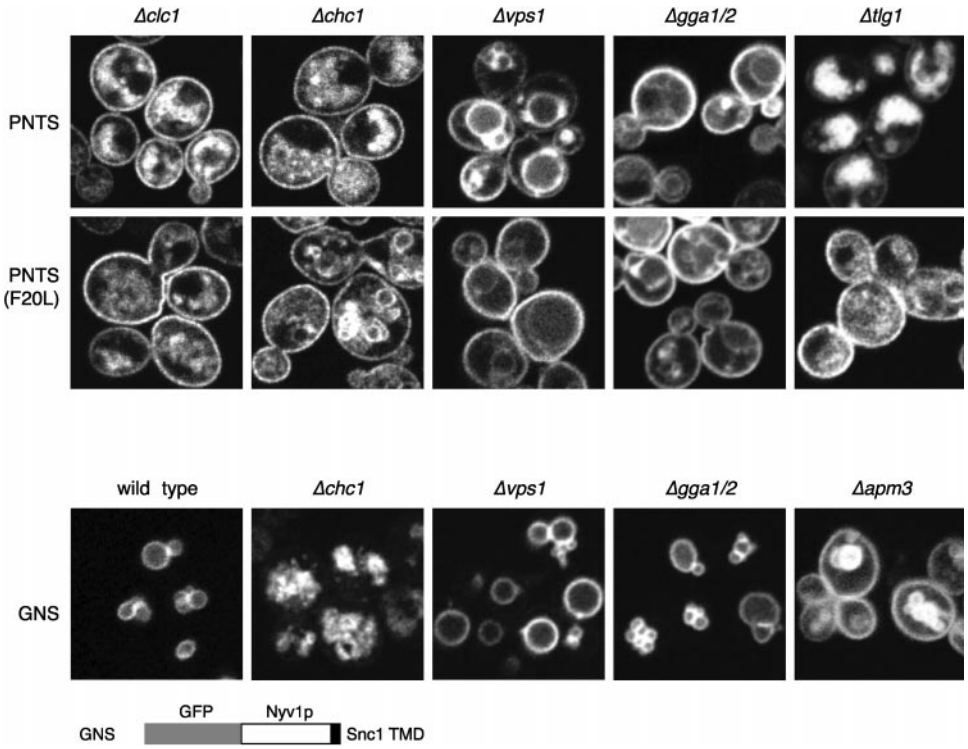


Figure 7. Missorting of PNTS by clathrin and *gga* mutants. GFP-tagged PNTS or the F20L mutant version were expressed in the indicated mutants. A marker for the AP-3 pathway to the vacuole, GNS (structure as diagrammed) was also expressed in the indicated mutants.

Recently, the GGA proteins, which have homology to the ear domain of the clathrin-associated protein gamma adaptin, have also been implicated in protein traffic from the Golgi (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000). In yeast, removal of the two GGA homologues affects sorting of CPY, but has only modest effects on growth (Dell'Angelica et al., 2000; Hirst et al., 2000). Single *gga* mutations have little effect, suggesting that they perform similar functions, though codon bias suggests that Gga2p is expressed at higher levels than Gga1p. Strikingly, a *gga1 gga2* double deletion mutant completely abolished FSD-dependent sorting of PNTS, although, as in the clathrin mutants, some of the protein did reach the vacuole in an FSD-independent manner (Fig. 7). The single *gga1* mutant had no effect, and *gga2* only a partial one (data not shown). These results suggest that the Gga proteins, perhaps in collaboration with clathrin, help to sort PNTS into vesicles that bud from the Golgi. Since loss of the GGAs is much less deleterious to growth than loss of clathrin, it may be that their function is limited to this one step.

An alternative explanation could be that the effects of proteins such as clathrin, Vps1p, and the GGAs are indirect, and result from a severe disruption of Golgi or endosome function. As one test of this, we expressed PNTS and its FSD-disrupted counterpart in cells lacking Tlg1p, which is required for normal early endosome function (Holthuis et al., 1998a,b). Though the vacuoles in these cells were fragmented, FSD-dependent sorting could still occur (Fig. 7). We also expressed a marker of the AP-3 pathway, which does not require clathrin (Seeger and Payne, 1992a). This marker, GNS, is a GFP-tagged version of the vacuolar SNARE Nyv1p bearing a TMD derived from the plasma membrane SNARE Snc1p (to disrupt a sorting signal found in the Nyv1p TMD; Lewis et al., 2000). It normally travels directly from Golgi to vacuole, but when the AP-3 pathway is disrupted by mutation of *apm3,* it can reach the plasma membrane (Fig. 7; Reggiori et al., 2000). We found delivery of GNS to the vacuole to be unaffected by deletion of *vps1*, or of *gga1* and *gga2* (Fig. 7). It also remained internal in *chc1* cells, though vacuolar membranes appeared highly fragmented in this strain (Fig. 7). These mutations do not therefore compromise all sorting events in the Golgi.

To confirm the lack of FSD-dependent sorting in *gga1/2* cells, we performed sucrose gradient fractionation of cells expressing GFP-tagged PNTS, with or without the FSD signal (Fig. 8 A). With wild-type cells, a substantial proportion of PNTS cofractionated at low density with the vacuolar marker Vam3p, while the FSD mutant form was mostly in high density fractions corresponding to plasma membrane (which is poorly separated from early endosomes in these gradients; Holthuis et al., 1998b). In contrast, the *gga1/2* mutant cells gave very similar profiles with PNTS and its mutated variant, with most of the protein being in the plasma membrane region. We did not obtain a clear vacuolar peak from Δ*chc1* cells, and thus were unable to compare the fractions of PNTS and PNTS(F20L) which reached "vacuoles" in this strain (data not shown).

We also used sucrose gradient fractionation to examine the location of endogenous Pep12p in these mutants. In wild-type cells, the bulk of Pep12p can readily be separated from the early endosome marker Tlg1p. However, in both *gga1/2* and *chc1* mutants, Pep12p and Tlg1p cofractionated at high density, consistent with the rerouting of Pep12p to early endosomes (Fig. 8 B). This effect was strikingly similar to the mistargeting of Pep12p in normal cells when the FSD motif was mutated (Fig. 4 C). Higher resolution of the membranes in the *gga1/2* mutant was obtained by adjusting the density profile of the gradient (Fig. 8 C). Under these conditions, Tlg1p could easily be separated from a plasma

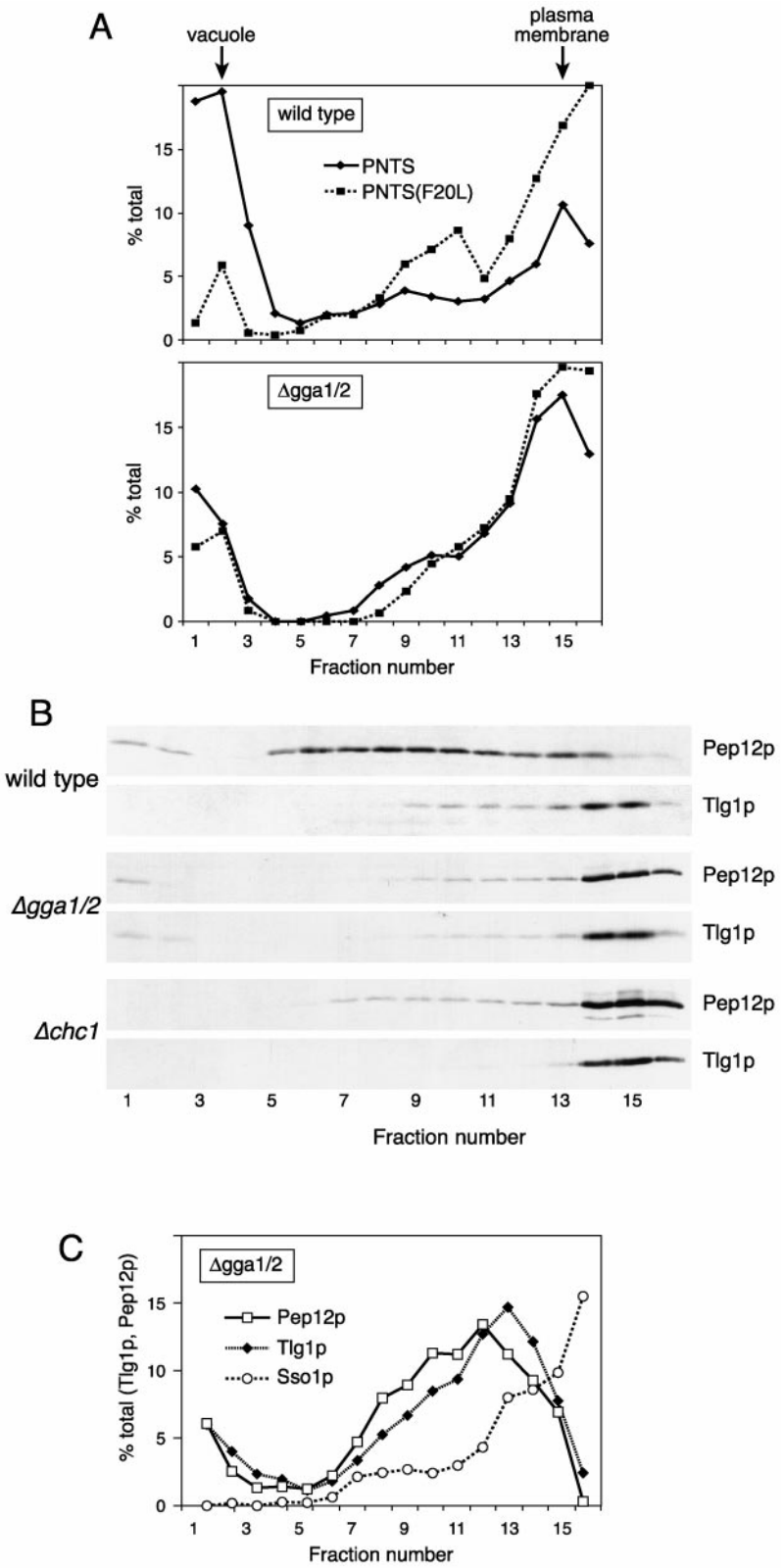


Figure 8. Subcellular fractionation of PNTS and Pep12p in mutants. (A) Wild-type and *gga1/2* mutant cells expressing GFP-tagged PNTS or its F20L mutant variant were fractionated on sucrose gradients as in Fig. 4 C and the expressed proteins detected by immunoblotting. The peak positions of markers for the vacuole (Vam3p) and plasma membrane (Sso1p) are indicated at the top. (B) Extracts of wild-type, *gga1/2,* and *chc1* cells were fractionated as in A and analyzed by immunoblotting for endogenous Pep12p and Tlg1p. (C) The *gga1/2* cells were fractionated on a gradient of higher density (see Materials and Methods) to resolve the early endosome region, and the distributions of Tlg1p, Pep12p, and Sso1p were measured by immunoblotting. The Sso1p profile has been scaled by a factor of 0.6 for clarity.

membrane marker (Sso1p), but still overlapped extensively with Pep12p, though slight differences in the profiles suggest some segregation of Tlg1p and Pep12p.

As a further test of the *gga* null phenotype, we also examined the distribution of GFP-Pep12p in these cells (Fig. 9 A). Unlike in wild-type cells, GFP-Pep12p did not reach the vacuoles (revealed by FM4-64 staining), but accumulated in punctate structures very similar to those seen with the F20L mutant in wild-type cells (Fig. 4 B). Some of the structures were perivacuolar, suggestive of late endosomes. However, delivery of GFP-Pep12p to these was completely independent of the FSD motif (Fig.

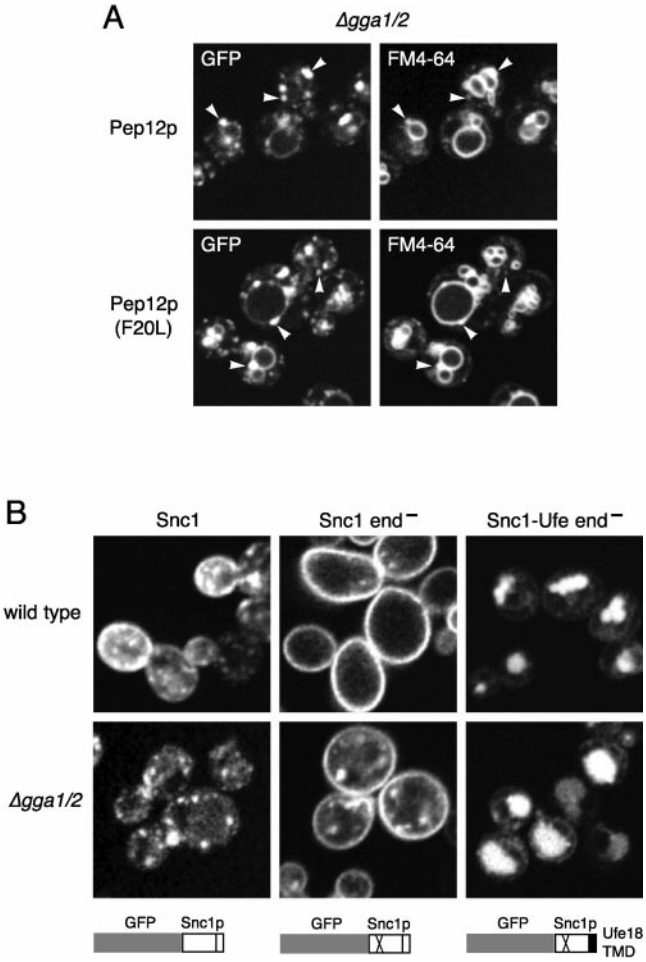


Figure 9. Sorting properties of the *gga 1/2* mutant. (A) Distribution of GFP-tagged Pep12p and the F20L mutant in *gga1/2* cells. Cells were double labeled with FM4-64 (chased for 30 min) to reveal vacuoles. (B) GFP-tagged versions of Snc1p, a double point mutant Snc1p that is endocytosis-defective (end$^-$) and a derivative of this bearing a modified Ufe1p TMD that targets it from the Golgi to the vacuole (ufe18; Lewis et al., 2000) were expressed in wild-type and *gga1/2* double mutant cells.

9 A). All the data are thus consistent with an essential role for the GGA proteins, as well as clathrin, in FSD-dependent sorting.

Sorting of Other Proteins in gga Mutants

Our findings suggest that the observed phenotypes of *gga* mutants may arise from a primary defect in the targeting of Pep12p and perhaps other late endosomal components. Erroneous delivery of these components to the early endosome might well disrupt the normal function of this compartment.

To test this, we examined the distribution of derivatives of GFP-Snc1p whose trafficking we have previously characterized (Lewis et al., 2000). Snc1p is an exocytic v-SNARE that normally cycles from the plasma membrane through early endosomes to the Golgi and back to the surface; in the steady state, it can be observed both on the plasma membrane and in internal structures (Lewis et al., 2000; Fig. 9 B). In the *gga1/2* mutant, however, it was completely internal, indicating that cycling through early endosomes and the Golgi is affected in these cells. An endocytosis-defective version of Snc1p (Lewis et al., 2000) still accumulated on the plasma membrane, confirming that the *gga* defect is postendocytic (Fig. 9 B). The small amount of mutant Snc1p visible in internal structures in *gga1/2* cells likely reflects a low level of residual endocytosis, coupled with inefficient recycling.

Finally, we have shown that addition of a TMD derived from the ER SNARE Ufe1p to the endocytosis-defective Snc1p causes it to pass directly from Golgi to endosomes, enter the internal vesicles of multivesicular bodies, and be delivered to the interior of the vacuole (Lewis et al., 2000). This process was not blocked in the *gga* null cells (Fig. 9 B), confirming that transport through endosomes is still possible in these cells.

Discussion

A Signal-mediated Pathway to Late Endosomes

We have shown that Pep12p contains a signal, the FSD motif, that directs its transport to late endosomes. This signal can divert a protein from the exocytic pathway, and thus must be recognized within this pathway.

The initial sorting event is dependent on the presence of at least one of the GGA proteins, together with the clathrin heavy and light chains—without these, no effect of the FSD motif can be detected. Although it is conceivable that this effect is indirect, resulting from perturbation of the secretory pathway in some general way, this seems unlikely. All our assays were done with viable yeast strains, which must by definition have a functional exocytic pathway; moreover, sorting of proteins to the vacuole via the AP-3 pathway was unaffected in these mutants, and sorting of CPY is also quite efficient in clathrin and *gga* null cells (Seeger and Payne, 1992a; Dell'Angelica et al., 2000; Hirst et al., 2000). Furthermore, FSD sorting was not prevented by a number of other mutations that have very substantial effects on the endosomal system, including *vps1*, *vps45*, *pep12*, and *tlg1* (Conibear and Stevens, 1998; Lewis et al., 2000).

The simplest interpretation of our data is that the GGA proteins, which have homology to the clathrin-binding ear domain of the Golgi-localized gamma adaptin, interact with clathrin to form a coat that selectively incorporates Pep12p into vesicles. Previous studies have shown that in mammalian cells the GGA proteins are found on Golgi membranes, are recruited there by ADP-ribosylation factor, and perturb traffic through the TGN when overexpressed (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000; Poussu et al., 2000; Takatsu et al., 2000). However, overexpressed GGA proteins did not colocalize precisely with the AP-1 adaptor and endogenous ones were not found in isolated clathrin-coated vesicles (Dell'Angelica et al., 2000; Hirst et al., 2000). It is possible, therefore, that they form a distinct coat that does not include the conventional AP-1 subunits and that may not readily survive biochemical isolation. Elucidation of the role of clathrin by genetics is complicated by its pleiotropic effects on cells, but direct studies of GGA-clathrin interactions could address this issue.

The requirement for the GGA proteins suggests that these might bind directly to the FSD motif, but we have been unable to detect this either by coprecipitation or by

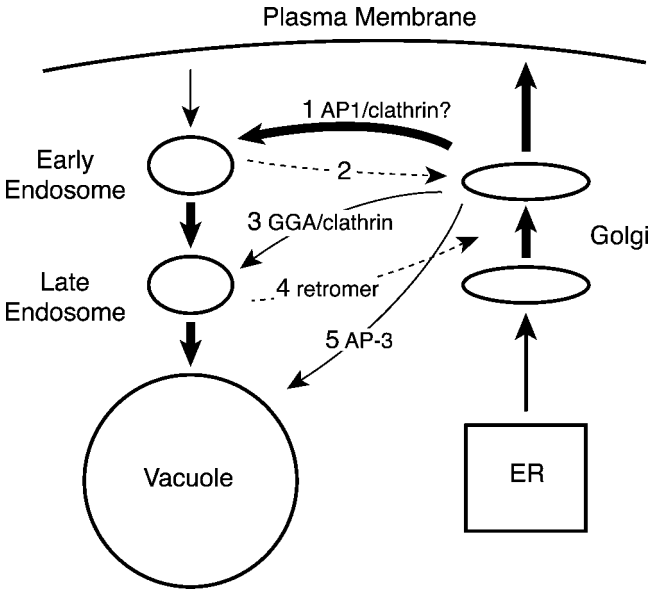


Figure 10. Summary of the proposed connections between the exocytic and endocytic pathways in yeast. The known or postulated coat proteins involved in some of the steps are indicated. See text for details.

two-hybrid analysis. Hence, an additional protein may be required for this recognition. We are currently undertaking genetic screens based on the Pep12-Ste18 chimeras that link FSD-dependent sorting to activation of the pheromone response pathway. These have the potential to identify new components of the sorting machinery as well as more specific mutations in known ones.

Two Different Ways from the Golgi to Late Endosomes

A striking finding was that disruption of the FSD motif resulted in the accumulation of Pep12p in early endosomes, which are distinguishable from late endosomes by their insensitivity to the effects of *vps4* and by their distinct density on sucrose gradients. In fact, a portion of wild-type Pep12p can also be observed to fractionate with the early endosome marker Tlg1p, and also to colocalize with it by immuno-EM (Lewis et al., 2000). We had previously assumed that this represented material on its way to late endosomes, but this may not be the case. Rather, it seems that Pep12p, like its closest relative, Tlg2p, has features that cause it to be retained in the early endosome/late Golgi region, whether it is delivered by endocytosis (when it bears the Sso1p TMD) or directly from the Golgi (with its own TMD). Since all known membrane proteins of early endosomes are retained by cycling through the Golgi, this is presumably also the case for Pep12p. Recently, we have obtained point mutants (in Pep12p derivatives that lack FSD) that fail to be retained in early endosomes, supporting the idea that this is a specific process (our unpublished observations).

The retention of Pep12p in early endosomes allows a distinction to be made between the two different Golgi-endosome transport mechanisms (Fig. 10). As we have previously shown, for a membrane protein exiting the Golgi, the choice between exocytosis and delivery to endosomes is strongly influenced by the nature of its TMD, short TMDs in general favoring endosomes (Rayner and Pelham, 1997; Lewis et al., 2000). In the absence of the FSD signal, the Pep12p TMD performs this function. However, it evidently directs the protein to early rather than late endosomes (Fig. 10, route 1), from which recycling presumably occurs (route 2). In contrast, FSD-dependent sorting avoids retention in early endosomes. We favor the view that this involves a pathway directly from Golgi to late endosomes (route 3). We cannot, however, exclude other possibilities: the FSD signal may allow sorting from early to late endosomes as well as from Golgi to late endosomes, or even take proteins sequentially from the Golgi to early endosomes and thence to late endosomes.

An extreme possibility might be that the FSD signal acts only in early endosomes, but this does not fit with the Golgi/TGN location of the mammalian GGA proteins and is implausible for other reasons. It would require that all the FSD-responsive constructs, including those with the Sso1p TMD, be exocytosed via early endosomes. But the Sso1p TMD does not direct proteins there, and in yeast even the exocytic SNARE Snc1p appears unable to pass directly from early endosomes to the cell surface (Lewis et al., 2000). Hence, it would be necessary to postulate additional signals and routes for which there is no evidence. Rather, the data strongly suggests that the initial sorting of FSD-containing proteins occurs in the Golgi, on the normal exocytic pathway.

Though Pep12p spills into the vacuole when it is overexpressed, it normally does not and thus must be retrieved before the maturing endosome fuses with the vacuole. Possibly, it follows the retromer-dependent pathway back to the Golgi, which is followed by other proteins that cycle through late endosomes (Fig. 10, route 4; Seaman et al., 1998). In agreement with this, some colocalization of Pep12p with the Golgi marker Sed5p can be observed by immuno-EM (H. Riezman, personal communication). In addition, we have shown that should Pep12p ever reach the plasma membrane, it will contain a signal for endocytosis. Thus, the localization of Pep12p is a complex dynamic process, involving as many as five separate signals.

If the GGA proteins and clathrin mediate a direct route from Golgi to late endosomes, what coat is responsible for the route via early endosomes? The most obvious candidate is again clathrin, since temperature-sensitive clathrin mutants missort Kex2p to the plasma membrane rather than to early endosomes (Seeger and Payne, 1992b). This activity of clathrin appears to be aided by the AP-1 complex (Stepp et al., 1995). However, even in a clathrin null mutant, a protein bearing the Pep12p TMD was not observed on the cell surface (our unpublished observations). The explanation for this may be that other coat components substitute for clathrin (Stepp et al., 1995), but the apparent tolerance of this pathway to mutation may in part reflect the nature of the sorting event. The late Golgi in yeast consists of networks of tubules containing swollen nodules, which apparently fragment to yield exocytic vesicles corresponding to the nodules and, presumably, small vesicles that fuse with or become early endosomes (Morin-Ganet et al., 2000). Exocytic vesicles are very rich in sterols (Zinser et al., 1993), and the morphological differentiation of the tubular networks may correspond to the seg-

regation of membrane domains into sterol-rich nodules and relatively sterol-poor tubules. Proteins with short TMDs may simply be excluded from the nodules because they are incompatible with a sterol-rich bilayer (Bretscher and Munro, 1993), and thus require no specific coat protein interaction to be sorted. Nevertheless, segregation may normally be enhanced and regulated by a clathrin coat. Taking all the evidence into account, we suggest that there are two routes from Golgi to endosomes involving clathrin, and that the GGA proteins are specific to the late endosomal pathway.

Multiple Pathways Maintain Functionally Distinct Endosomes

Given that proteins that lack specific retention/retrieval signals pass naturally from early to late endosomes, why should the cell need to have a specific mechanism to transport proteins from Golgi to late endosomes? An answer is suggested by the properties of the *gga1 gga2* double mutant. These cells are still capable of reasonably efficient sorting of the vacuolar protease CPY, although it reaches the vacuole slowly (Dell'Angelica et al., 2000; Hirst et al., 2000). They can also deliver at least one protein (Snc1p with an altered TMD) to the interior of the vacuole, implying that they can form multivesicular bodies. In contrast to these late endosomal functions, the cells do not efficiently recycle endocytosed wild-type Snc1p, a symptom of defective early endosome function (Holthuis et al., 1998a; Lewis et al., 2000). A likely explanation is that when the GGA pathway is disrupted, proteins that normally take this route are redirected to early endosomes and interfere with their function. For example, we have previously noted that in cells that lack all endosomal syntaxins (Vam3p, Pep12p, Tlg1p, and Tlg2p), and hence endocytic structures, the vacuolar $[H^+]$ATPase and Kex2p accumulate in vesicles of different densities, suggesting that they leave the Golgi by different routes (Holthuis et al., 1998b). If the vacuolar ATPase normally uses the GGA pathway and is rerouted to early endosomes in the *gga* mutant, it could substantially alter their properties.

Fig. 10 summarizes our current view of the endosome–Golgi relationships in yeast, based on this and previous work. We envisage three functionally distinct stations on the endocytic pathway, early endosomes, late endosomes, and vacuoles, each served by a direct transport route from the Golgi. The thick arrows represent the paths taken by membrane proteins that lack cytoplasmic signals, these may consist largely of maturation or direct fusion events that do not have an absolute requirement for coat proteins. The remaining routes require specific signals and coats. The apparent complexity is, we suggest, the price that has to be paid to maintain compartments of discrete identity in a constantly flowing membrane system.

We thank J. Hirst, M.S. Robinson, and M. Seaman for helpful discussions, S. Siniossoglou, M. Lewis, and F. Reggiori for comments on the manuscript, and R. Arkowitz for reagents and advice with the Ste18p assay.

M.W. Black is the recipient of a Burroughs Wellcome Fund Hitchings-Elion Fellowship.

Submitted: 14 July 2000
Revised: 23 August 2000
Accepted: 21 September 2000

References

Abeliovich, H., T. Darsow, and S.D. Emr. 1999. Cytoplasm to vacuole trafficking of aminopeptidase I requires a t-SNARE-Sec1p complex composed of Tlg2p and Vps45p. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:6005–6016.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1995. Current Protocols in Molecular Biology. Vol. 1. J. Wiley and Sons, Inc., Boston, MA. 13.6.2–13.6.3.

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

Becherer, K.A., S.E. Rieder, S.D. Emr, and E.W. Jones. 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.

Boman, A.L., C. Zhang, X. Zhu, and R.A. Kahn. 2000. A family of ADP-ribosylation factor effectors that can alter membrane transport through the trans-Golgi. *Mol. Biol. Cell.* 11:1241–1255.

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

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

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

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

Dell'Angelica, E.C., R. Puertollano, C. Mullins, R.C. Aguilar, J.D. Vargas, L.M. Hartnell, and J.S. Bonifacino. 2000. GGAs: a family of ADP ribosylation factor-binding proteins related to adaptors and associated with the Golgi complex. *J. Cell Biol.* 149:81–94.

Finegold, A.A., W.R. Schafer, J. Rine, M. Whiteway, and F. Tamanoi. 1990. Common modifications of trimeric G proteins and ras protein: involvement of polyisoprenylation. *Science.* 249:165–169.

Hirst, J., W.W. Lui, N.A. Bright, N. Totty, M.N. Seaman, and M.S. Robinson. 2000. A family of proteins with gamma-adaptin and VHS domains that facilitate trafficking between the trans-Golgi network and the vacuole/lysosome. *J. Cell Biol.* 149:67–80.

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

Holthuis, J.C.M., B.J. Nichols, and H.R.B. Pelham. 1998b. The syntaxin Tlg1p mediates trafficking of chitin synthase III to polarized growth sites in yeast. *Mol. Biol. Cell.* 9:3383–3397.

Huang, K.M., K. D'Hondt, H. Riezman, and S.K. Lemmon. 1999. Clathrin functions in the absence of heterotetrameric adaptors and AP180-related proteins in yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:3897–3908.

Lewis, M.J., B.J. Nichols, C. Prescianotto-Baschong, H. Riezman, and H.R. Pelham. 2000. Specific retrieval of the exocytic SNARE Snc1p from early yeast endosomes. *Mol. Biol. Cell.* 11:23–38.

McNiven, M.A., H. Cao, K.R. Pitts, and Y. Yoon. 2000. The dynamin family of mechanoenzymes: pinching in new places. *Trends Biochem. Sci.* 25:115–120.

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

Morin-Ganet, M.-N., A. Rambourg, S.B. Deitz, A. Franzusoff, and F. Képès. 2000. Morphogenesis and dynamics of the yeast Golgi apparatus. *Traffic.* 1:56–68.

Nichols, B.J., J.C. Holthuis, and H.R. Pelham. 1998. The Sec1p homologue Vps45p binds to the syntaxin Tlg2p. *Eur. J. Cell Biol.* 77:263–268.

Nichols, B.J., and H.R. Pelham. 1998. SNAREs and membrane fusion in the Golgi apparatus. *Biochim. Biophys. Acta.* 1404:9–31.

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

Nothwehr, S.F., E. Conibear, and T.H. Stevens. 1995. Golgi and vacuolar membrane proteins reach the vacuole in *vps1* mutant yeast cells via the plasma membrane. *J. Cell Biol.* 129:35–46.

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

Odorizzi, G., C.R. Cowles, and S.D. Emr. 1998b. The AP-3 complex: a coat of many colours. *Trends Cell Biol.* 8:282–288.

Pelham, H.R. 1999. SNAREs and the secretory pathway-lessons from yeast. *Exp. Cell Res.* 247:1–8.

Poussu, A., O. Lohi, and V.P. Lehto. 2000. Vear, a novel Golgi-associated protein with VHS and gamma-adaptin "ear" domains. *J. Biol. Chem.* 275:7176–7183.

Pryciak, P.M., and F.A. Huntress. 1998. Membrane recruitment of the kinase cascade scaffold protein Ste5 by the Gβγ complex underlies activation of the yeast pheromone response pathway. *Genes Dev.* 12:2684–2697.

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

Reggiori, F., M.W. Black, and H.R. Pelham. 2000. Polar transmembrane domains target proteins to the interior of the yeast vacuole. *Mol. Biol. Cell.* In press.

Roberts, C.J., C.K. Raymond, C.T. Yamashiro, and T.H. Stevens. 1991. Methods for studying the yeast vacuole. *Methods Enzymol.* 194:644–661.

Rothman, J.E. 1994. Mechanisms of intracellular protein transport. *Nature.* 372:55–63.

Seaman, M.N., J.M. McCaffery, and S.D. Emr. 1998. A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. *J. Cell*

Biol. 142:665–681.
Seeger, M., and G.S. Payne. 1992a. A role for clathrin in the sorting of vacuolar proteins in the Golgi complex of yeast. *EMBO (Eur. Mol. Biol. Organ.) J*. 11:2811–2818.
Seeger, M., and G.S. Payne. 1992b. Selective and immediate effects of clathrin heavy chain mutations on Golgi membrane protein retention in *Saccharomyces cerevisiae*. *J. Cell Biol*. 118:531–540.
Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae. Genetics*. 122:19–27.
Söllner, T., S.W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst, and J.E. Rothman. 1993. SNAP receptors implicated in vesicle targeting and fusion. *Nature*. 362:318–324.
Stepp, J.D., A. Pellicena-Palle, S. Hamilton, T. Kirchhausen, and S.K. Lemmon. 1995. A late Golgi sorting function for *Saccharomyces cerevisiae* Apm1p, but not for Apm2p, a second yeast clathrin AP medium chain-related protein. *Mol. Biol. Cell*. 6:41–58.
Sutton, R.B., D. Fasshauer, R. Jahn, and A.T. Brunger. 1998. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature*. 395:347–353.
Takatsu, H., K. Yoshino, and K. Nakayama. 2000. Adaptor gamma ear homology domain conserved in gamma-adaptin and GGA proteins that interact with gamma-synergin. *Biochem. Biophys. Res. Commun*. 271:719–725.
Vida, T.A., and S.D. Emr. 1995. A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol*. 128:779–792.
Weber, T., B.V. Zemelman, J.A. McNew, B. Westermann, M. Gmachl, F. Parlati, T.H. Sollner, and J.E. Rothman. 1998. SNAREpins: minimal machinery for membrane fusion. *Cell*. 92:759–772.
Wesp, A., L. Hicke, J. Palecek, R. Lombardi, T. Aust, A.L. Munn, and H. Riezman. 1997. End4p/Sla2p interacts with actin-associated proteins for endocytosis in *Saccharomyces cerevisiae*. *Mol. Biol. Cell*. 8:2291–2306.
Wooding, S., and H.R. Pelham. 1998. The dynamics of golgi protein traffic visualized in living yeast cells. *Mol. Biol. Cell*. 9:2667–2680.
Zinser, E., F. Paltauf, and G. Daum. 1993. Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism. *J. Bacteriol*. 175:2853–2858.