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Vps1p, a member of the dynamin GTPase family, is necessary for Golgi membrane protein retention in *Saccharomyces cerevisiae*

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The *KEX2*-encoded endoprotease of *Saccharomyces cerevisiae* resides in the Golgi complex where it participates in the maturation of α-factor mating pheromone precursor. Clathrin heavy chain gene disruptions cause mislocalization of Kex2p to the cell surface and reduce maturation of the α-factor precursor. Based on these findings, a genetic screen has been devised to isolate mutations that affect retention of Kex2p in the Golgi complex. Two alleles of a single genetic locus, *lam1* (*l*owered *a*lpha-factor *m*aturation), have been isolated, which result in inefficient maturation of α-factor precursor. In *lam1* cells, Kex2p is not mislocalized to the cell surface but is abnormally unstable. Normal stability is restored by the *pep4* mutation which reduces the activity of vacuolar proteases. In contrast, the pheromone maturation defect is not corrected by *pep4*. Organelle fractionation by sucrose density gradient centrifugation shows that Kex2p is not retained in the Golgi complex of *lam1* cells. Vacuolar protein precursors are secreted by *lam1* mutants, revealing another sorting defect in the Golgi complex. Genetic complementation reveals that *lam1* is allelic to the *VPS1* gene, which encodes a dynamin-related GTPase. These results indicate that Vps1p is necessary for membrane protein retention in a late Golgi compartment.

Key words: α-factor/Golgi/retention/vacuole/yeast

Introduction

The compartmental organization of the secretory pathway in eukaryotic cells poses a fundamental protein sorting problem. As newly synthesized proteins reach each organelle, a distinction must be made between those that will reside in the organelle and those that will travel to a subsequent compartment. This type of sorting has been characterized for proteins resident in the lumen of the endoplasmic reticulum (ER) (Pelham, 1990). The cell distinguishes resident lumenal proteins from proteins destined for subsequent secretory organelles by a four amino acid sequence at the carboxy-terminus, usually KDEL in animal cells and HDEL in proteins from the yeast *Saccharomyces cerevisiae*. This sequence does not ensure that a protein remains fixed in the ER, but instead acts as a signal (both necessary and sufficient) for retrieval of proteins which reach the *cis* Golgi. These observations led to the hypothesis that a retention signal receptor, located in a *cis*-Golgi compartment, recognizes ER proteins bearing the retention signal and returns the escaped proteins to the ER via retrograde vesicular traffic. KDEL and HDEL signal receptors have been identified, but the mechanism by which they act is unclear (Lewis and Pelham, 1990; Semenza *et al.*, 1990). The recycling paradigm may also apply in the case of ER membrane proteins, but different retention signals may be operative (reviewed in Machamer, 1991).

Although recent studies of Golgi membrane proteins in mammalian cells have implicated transmembrane and/or lumenal domains as retention signals (reviewed in Machamer, 1991), the mechanism of retention remains unknown. In yeast, the retention of a resident Golgi membrane protein, the *KEX2*-encoded endoprotease (Kex2p), has been the focus of similar studies. Kex2p is responsible for initiating proteolytic maturation of the secreted peptide mating pheromone α-factor in the Golgi complex (Fuller *et al.*, 1988). The protease has three domains: a large lumenal amino-terminal segment which harbors the protease activity, a short hydrophobic sequence capable of spanning a lipid bilayer and a carboxy-terminal cytoplasmic tail (Mizuno *et al.*, 1987; Fuller *et al.*, 1989b). Deletion analysis indicates that the cytoplasmic domain is necessary for proper retention of Kex2p in the Golgi complex (Wilcox *et al.*, 1992). Investigations of mutant yeast cells lacking the heavy chain of clathrin suggest that retention of Kex2p might be mediated by clathrin coats. This suggestion is based on the observation that Kex2p is predominantly located at the plasma membrane rather than the Golgi complex in cells carrying mutations in the clathrin heavy chain gene *(CHC1)* (Seeger and Payne, 1992a). Clathrin coats play a role in selective endocytosis of transmembrane proteins in mammalian cells. Through interactions with the cytoplasmic domains of transmembrane receptors, clathrin coats located on the cytoplasmic surface of the plasma membrane collect the receptors in regions of the membrane undergoing vesiculation (Brodsky, 1988; Keen, 1990; Pearse and Robinson, 1990). By analogy it has been proposed that clathrin acts to retain Kex2p in the Golgi complex of yeast via interactions between coat proteins and the cytoplasmic tail of Kex2p (Seeger and Payne, 1992a; Wilcox *et al.*, 1992). It has not been determined whether retention of Kex2p by clathrin occurs by recycling.

The mislocalization of Kex2p in *chc1* mutants results in inefficient maturation of α-factor precursor. Consequently, the mutant cells secrete a glycosylated form of the pheromone precursor. By searching for other mutants which secrete α-factor precursor we have identified mutations in a gene, *lam1* (*l*owered *a*lpha-factor *m*aturation), which do not efficiently retain Kex2p in the Golgi complex. Genetic complementation demonstrates that *lam1* is an allele of *VPS1*, which was isolated based on its phenotype of missorting vacuolar proteases (Rothman and Stevens, 1986). Vps1p, a high molecular weight GTPase, shares homology with a family of GTPases which includes the *Drosophila shibire* protein, a protein thought to be involved in endocytosis (van der Bliek

and Meyerowitz, 1991) and its mammalian counterpart, bovine dynamin (Chen *et al.*, 1991). Our results suggest that in addition to clathrin coats, Vps1p also participates in retention of membrane proteins in the Golgi complex.

Results

Mutants that secrete the precursor form of α-factor

Export of α-factor via the secretory pathway is accompanied by a number of post-translational modifications including glycosylation and proteolytic cleavage (Fuller *et al.*, 1988). α-Factor is synthesized as part of a 165 amino acid (aa) precursor that contains four copies of the 13 aa peptide. In the Golgi complex, the highly glycosylated form of the precursor (apparent molecular weight ~125 kDa) is cleaved by Kex2p after lysine–arginine dipeptides located amino-terminal to each α-factor cassette. The peptides released by Kex2p scission consist of α-factor flanked by six amino-terminal and two carboxy-terminal amino acids. The extra residues are removed from the amino-terminal side by the *STE13*-encoded dipeptidyl aminopeptidase A (DPAP A) and from the carboxy-terminal side by the carboxypeptidase product of the *KEX1* gene. After proteolytic maturation, the biologically active α-factor is secreted.

Since mislocalization of Kex2p to the plasma membrane of clathrin heavy chain deficient cells is accompanied by the secretion of the highly glycosylated form of α-factor precursor (Payne and Schekman, 1989), we reasoned that secretion of the precursor would be a convenient indicator of defects in other proteins that affect retention of Kex2p. In order to identify mutants which secrete α-factor precursor, a nitrocellulose filter overlay technique was developed to screen colonies of mutagenized cells (Figure 1). The technique is based on the observation that the 146 aa α-factor precursor (lacking the 19 aa signal sequence) secreted from cells on solid agar plates can be detected after absorption onto nitrocellulose by applying α-factor-specific antibodies and conventional protocols for antibody visualization (secondary antibodies coupled to horseradish peroxidase or alkaline phosphatase). In contrast, mature α-factor does not yield a signal, probably because it does not efficiently adhere to the filter and is eluted during processing of the filter. In Figure 1, highly glycosylated α-factor precursor secreted by cells lacking the Kex2 endoprotease (*kex2Δ*) and cells deficient in clathrin heavy chain (*chc1Δ*) was readily visualized, whereas mature α-factor secreted by wild-type cells (WT) was undetectable. Neither *MAT***a** *kex2Δ* cells nor *MATα ste13Δ* cells produced signals by the overlay assay, suggesting that the assay is specific for secretion of the 146 aa precursor.

The nitrocellulose overlay assay was applied to 200 000 colonies derived from cells mutagenized with ethyl methanesulfonic acid (EMS); 89 colonies yielded detectable signal (for example see Figure 1). To determine whether the putative mutants export highly glycosylated α-factor precursor, cells from each positive colony were metabolically labelled with [^{35}S]methionine and [^{35}S]cysteine for 20 min and secreted α-factor species were immunoprecipitated from the culture medium and separated by SDS–PAGE. An analysis of a representative sample of seven potential mutants is shown in Figure 2. Two of the strains, mutants #22 and #28 (Figure 2) secreted highly glycosylated α-factor precursor. Similar to the *chc1Δ* strain (Figure 2), mutant #22 also secreted significant levels of mature α-factor, whereas mutant #28 was nearly completely unable to produce the mature species. In total, 11 strains were identified which secrete significant levels of α-factor precursor.

Mutations in at least three known genes, *CHC1*, *CLC1* (clathrin light chain) and *KEX2*, could result in α-factor precursor secretion. Transformation of the newly generated mutant strains with yeast centromere plasmids carrying either *CHC1* or *CLC1* failed to restore α-factor maturation, thus eliminating the possibility that mutations in these genes were responsible for the defect (data not shown). In order to identify *kex2* mutations, complementation tests were performed by crossing the mutants to a *kex2* strain. The genetic background of the strains (see Materials and methods) allowed the diploids to express α-factor so that precursor maturation could be evaluated. Diploids formed between the

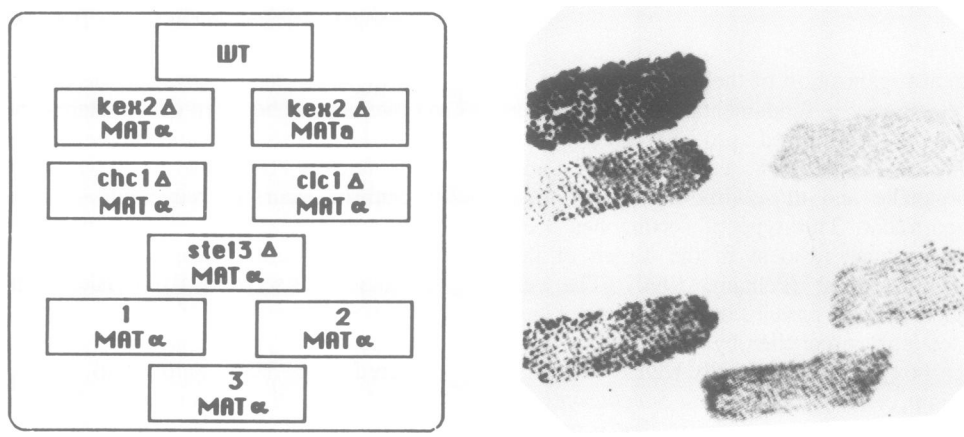

Fig. 1. Nitrocellulose filter overlay assay specifically detects precursor α-factor. WT (GPY148-5D), *kex2Δ* MATα (BFY101-10C), *kex2Δ* MAT**a** (BFY101-11C), *chc1Δ* (GPY163), *clc1Δ* (LSY89.2), *ste13Δ* (GPY 256), 1 (GPY 470), 2 (GPY471) and 3 (GPY472) were grown overnight on solid YPD. The cells were replica plated onto solid YPD, overlayed with a nitrocellulose filter and grown overnight at 30°C. The filter was removed and adherent cells washed off with distilled water. α-factor was detected using polyclonal antiserum and a secondary antibody conjugated to alkaline phosphatase.

known *kex2* strain and eight of the mutants secreted precursor α-factor revealing that these strains harbored mutations in the *KEX2* gene (mutant #28 in Figure 2 is *kex2*). The remaining three mutant strains were backcrossed to a WT strain (SEY6211) and the resulting *MATα* segregants were tested for the α-factor maturation defect. Two of the strains yielded tetrads where approximately half of the *MATα* segregants secreted α-factor precursor, suggesting that the defects in the original mutants are at a single genetic locus. Complementation analysis (see Materials and methods) indicated that the mutations in the original strains are recessive and are alleles of the same gene. The mutations were designated *lam1-1* and *1-2* (mutant #22 in Figure 2 is *lam1-2*). The phenotype of a third strain appeared to be caused by mutations in more than one gene and was not studied further. All of the phenotypic characterizations presented below were carried out on *lam1-1* and *lam1-2* strains derived from either the third or fourth backcross to a WT strain.

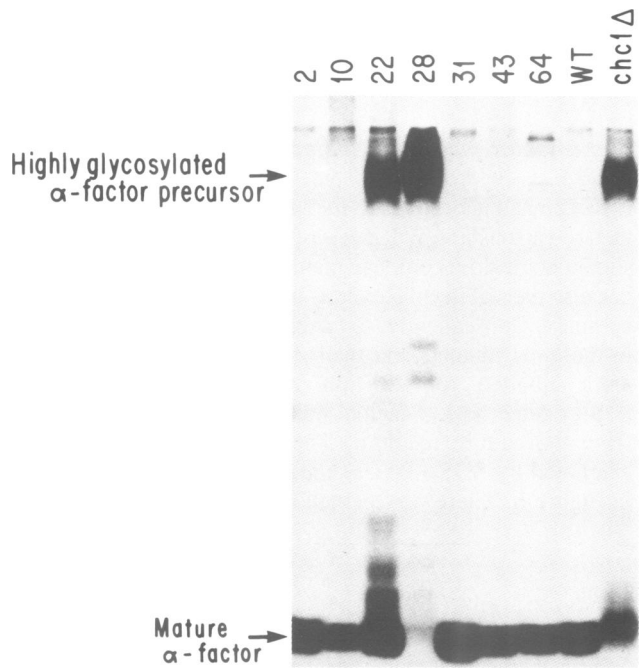

Fig. 2. α-Factor precursor is secreted by a subset of the potential mutant strains. Seven of the potential mutant strains (numbered), a WT strain (GPY148-5D), and a *chc1*Δ strain (GPY163) were labelled with [^{35}S]methionine and [^{35}S]cysteine for 20 min at 30°C. The culture supernatant was collected and α-factor was immunoprecipitated and analyzed by SDS−PAGE. The positions of uncleaved, highly glycosylated α-factor precursor and mature α-factor are shown.

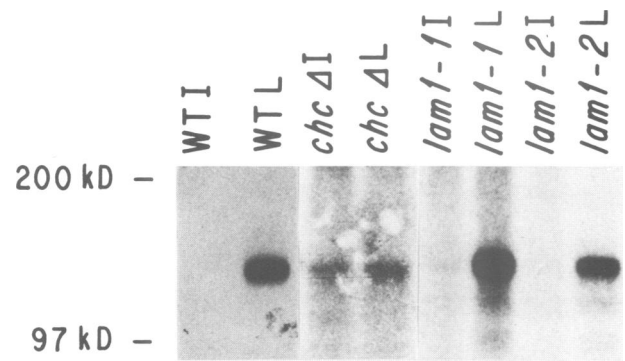

Fig. 3. Kex2p is not exported to the cell surface in *lam1* mutants. WT (GPY148-5D), *chc1*Δ (GPY163), *lam1-1* (GPY594) and *lam1-2* (GPY597) cells were labelled with ^{125}I under conditions that preferentially iodinate surface-exposed proteins (I). For comparison, cell extracts were also iodinated to label proteins irrespective of their location in the cell (L). Following iodination, the intact cells were lysed and Kex2p was immunoprecipitated from surface-labelled (I) and extract-labelled (L) samples. The immunoprecipitates were analyzed by SDS−PAGE and autoradiography.

Kex2p is not exported to the cell surface in lam1 mutants

To examine whether Kex2p is mislocalized to the cell surface in the *lam1* mutant, we applied the same solid phase surface radioiodination procedure used to detect surface-exposed Kex2p in *chc1*Δ cells (Payne and Schekman, 1989). In this protocol, either intact cells or cell lysates were subjected to radioiodination, the intact cells were then lysed and Kex2p was immunoprecipitated from each sample. Labelling of Kex2p in the intact cells indicates that the protein is present at the cell surface. The amount of Kex2p at the surface can be estimated by comparing the level of labelled protein from the intact cells with the level of Kex2p immunoprecipitated from cell lysates where all proteins are labelled regardless of their location in the cell. In contrast to *chc1*Δ cells where Kex2p was readily labelled in intact cells (Figure 3, *chc1*ΔI) as well as lysates (Figure 3, *chc1*ΔL), the *lam1* alleles displayed little or no Kex2p at the cell surface (Figure 3, *lam1-1* and *lam1-2*). In this regard the *lam1* alleles are similar to WT cells (Figure 3, WT lanes) where Kex2p was only labelled in the lysate. Two additional proteins, cytosolic glucose-6-phosphate dehydrogenase (G6PD) and a 33 kDa cell wall protein (Sanz *et al.*, 1987) were immunoprecipitated from the samples to assess the labelling procedure (data not shown). In all cases, G6PD was only labelled in the lysate samples indicating that the cells were not permeable to the label. The 33 kDa cell wall protein was labelled to approximately equal levels in both intact cells and lysates from each strain indicating that the lack of Kex2p labelling in WT and *lam1* cells was not due to a problem with the iodination procedure. Therefore, although *lam1* alleles secrete precursor α-factor, they do not mislocalize Kex2p to the cell surface.

Kex2p levels in the lam1 mutants are PEP4-dependent

The α-factor processing phenotype in *lam1* strains could be caused by reduced levels of Kex2p. Accordingly, the steady state levels of Kex2p were measured in *lam1* strains and compared with levels in the WT strain from which they were derived. Extracts were prepared from each strain and analyzed by immunoblotting using antibodies specific for

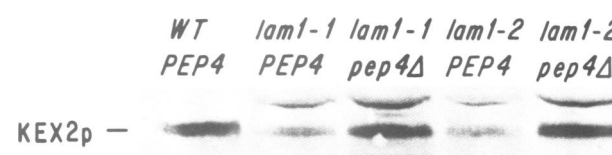

Fig. 4 Kex2p steady state levels in the *lam1* mutants are *PEP4*-dependent. Cell extracts were prepared from cultures of WT (GPY148-5D), *PEP4* and *pep4*Δ versions of *lam1-1* (GPY594 and 595) and *lam1-2* (GPY597 and 598) strains and Kex2p was analyzed by SDS−PAGE and immunoblotting. The more slowly migrating band is not specific and not reproducibly detected in other immunoblots.

Kex2p (Figure 4). Levels of Kex2p were markedly reduced in both *lam1* alleles compared with the WT strain (Figure 4, compare lanes 1, 2 and 4). However, Kex2p could be restored to WT levels in the *lam1* cells if they also carried the *pep4Δ* mutation (Figure 4, lanes 3 and 5). *PEP4*-encoded proteinase A is responsible for activation of vacuolar protease precursors when the precursors reach the vacuole. In *pep4Δ* cells, the proteolytic activity of the vacuole is greatly diminished due to the accumulation of inactive vacuolar protease precursors (Jones, 1991). Given that *PEP4* exerts its effects on the vacuole, the *pep4Δ*-dependent stabilization of Kex2p in the *lam1* strains suggests that Kex2p is accessible to the vacuolar proteases.

We examined the stabilization of Kex2p by *PEP4* in more detail by measuring the half-life of Kex2p in *PEP4* and *pep4Δ* versions of WT and *lam1* strains. Cells were labelled with [^{35}S]methionine and [^{35}S]cysteine for 20 min at which point labelling was terminated by adding excess non-radioactive amino acids. Aliquots of cells were harvested at intervals after termination of labelling. Cell extracts were prepared and Kex2p was immunoprecipitated and subjected to SDS−PAGE (Figure 5). Kex2p was stable over the course of the experiment in *PEP4* and *pep4Δ* versions of the WT strain (Figure 5A and B). In the *PEP4 lam1* strains (Figure 5A) Kex2p was unstable; Kex2p was completely degraded in *lam1-1* by 60 min and only a trace remained in *lam1-2* at this time-point. In contrast, Kex2p in *pep4Δ lam1* strains displayed almost WT stability (Figure 5B). Using densitometry we measured the relative amounts of Kex2p in both *PEP4* and *pep4Δ lam1* strains (data not shown). Although there is some inconsistent fluctuation in the signals, the half-life of Kex2p in the *lam1 PEP4* strains can be estimated as 20 min compared with a half-life of at least 120 min in *lam1 pep4* strains. It therefore appears that disposal of Kex2p in the vacuole reduces levels of the protein in *lam1* mutants. This raises the possibility that the *lam1* mutations interfere with retention of Kex2p in the Golgi complex which leads to transport of Kex2p to the vacuole where it is degraded.

Compared with the WT strain, less Kex2p was synthesized by the *lam1 pep4Δ* cells (Figure 5B). Since *lam1* cells grow more slowly than WT cells, the synthesis of Kex2p may be coordinately reduced. This would account for the equivalent steady state levels of Kex2p in the *lam1 pep4* and WT cells shown in Figure 4. Immediate glycosylation of Kex2p upon arrival in the Golgi complex results in a 6 kDa increase in apparent molecular weight (Wilcox and Fuller, 1991). Twenty minutes of labelling and 5 min of chase is sufficient time for most newly made Kex2p to reach the Golgi complex from the ER (Wilcox and Fuller, 1991). The similar mobilities of Kex2p from WT, *lam1-1* and *lam1-2* cells at this time-point suggests that transport of Kex2p to the Golgi complex is normal in the mutant cells.

The α-factor maturation defect is not affected by pep4Δ

If Kex2p is mislocalized to the vacuole in the *lam1* mutants, then re-establishing WT levels of Kex2p by introducing *pep4* should not restore complete maturation of α-factor precursor because the stabilized Kex2p is in the vacuole, not the Golgi complex. Consequently, α-factor maturation was compared in the *lam1* mutants and their *pep4Δ* counterparts. Strains were labelled for 20 min with [^{35}S]methionine and [^{35}S]cysteine and α-factor was then immunoprecipitated from the culture medium. The immunoprecipitates were examined by SDS−PAGE and autoradiography as shown in Figure 6. There was a small difference in the amount of

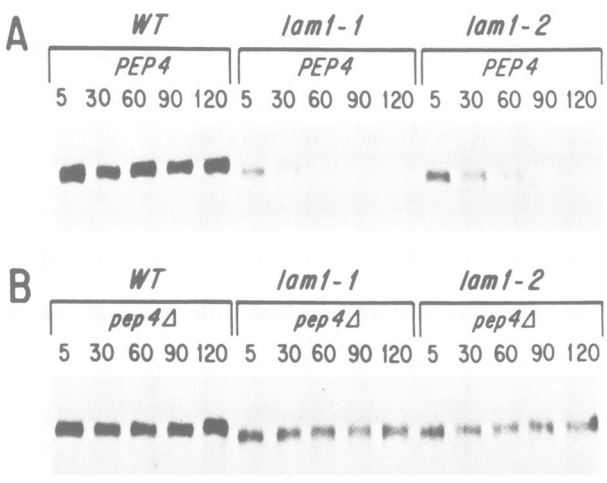

Fig. 5. Kex2p turnover is *PEP4*-dependent in *lam1* mutants. (**A**) *PEP4* WT (GPY675), *lam1-1* (GPY678) and *lam1-2* (GPY673) strains and (**B**) *pep4Δ* WT (GPY676), *lam1-1 pep4Δ* (GPY677) and *lam1-2 pep4Δ* (GPY674) strains were labelled with [^{35}S]methionine and [^{35}S]cysteine for 20 min at 30°C. After labelling was stopped, samples were collected after 5 min and at 30 min intervals for 2 h. Kex2p was immunoprecipitated from cell extracts and analyzed by SDS−PAGE and autoradiography.

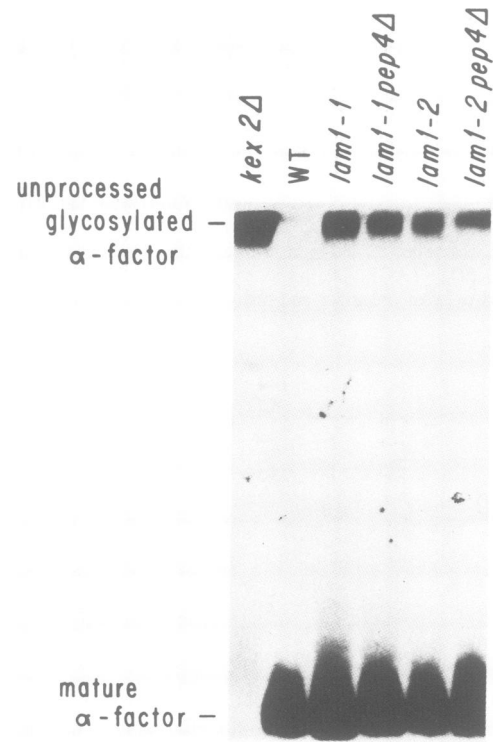

Fig. 6. The α-factor maturation defect is not affected by *pep4Δ*. WT (GPY148-5D) and *PEP4* and *pep4Δ* versions of *lam1-1* (GPY594 and 595) and *lam1-2* (GPY597 and 598) strains were labelled with [^{35}S]methionine and [^{35}S]cysteine for 20 min at 30°C. Culture supernatants were collected and α-factor was immunoprecipitated and analyzed by SDS−PAGE and autoradiography.

precursor α-factor secreted by *lam1-1* and *lam1-2* when compared with *lam1-1 pep4Δ* and *lam1-2 pep4Δ* strains (Figure 6). Densitometry analysis of a shorter exposure of the gel showed that the amount of unprocessed α-factor was between 20 and 35% of the mature form in all cases.

Organelle fractionation shows that Kex2p is not retained in the Golgi complex of lam1 cells

In order to demonstrate directly that *lam1* cells are defective in Golgi membrane protein retention, we compared the fractionation characteristics of Kex2p in mutant and WT cells that carried the *pep4* mutation. Cell lysates were subjected to a differential centrifugation protocol that yielded two pellet fractions, one obtained by centrifugation at 15 000 *g* for 15 min (P1) and the second obtained by centrifugation of the resulting supernatant at 100 000 *g* for 60 min (P2). Each membrane pellet was solubilized, subjected to SDS–PAGE and immunoblotted with antibodies specific for Golgi membrane proteins Kex2p and DPAP A, and the vacuolar membrane protein alkaline phosphatase (ALP). ALP transport to the vacuole is slightly slower but not significantly altered in the *lam1* strains (data not shown and see Figure 8) so it can serve as a marker for the vacuole. Vacuoles, marked by ALP, were evenly distributed between P1 and P2 in both mutant and WT membrane fractions (Figure 7). In contrast, although very little of Kex2p (6.5%) and DPAP A (7%) was found in the P1 fraction in WT cells (Figure 7, lanes 1 and 3), in *lam1-1* cells a significant quantity of Kex2p (50%) and DPAP A (21%) sedimented in the P1 fraction (Figure 7, lanes 2 and 4). The redistribution of Kex2p and DPAP A in *lam1-1,* into a pattern similar to ALP, is consistent with mislocalization of Golgi membrane proteins to the vacuole.

If *lam1* cells are defective in Kex2p retention, then after synthesis the protein should be transported to the Golgi complex with similar kinetics in mutant and WT cells, however, at later times after synthesis, the Kex2p in mutant cells should move out of the Golgi complex while the Kex2p in WT cells should remain in residence. To test this prediction, the location of newly synthesized Kex2p was followed by applying membrane samples to a sucrose gradient which separates the Golgi complex, vacuoles and a putative endosomal compartment (Vida and Emr, 1993; see Materials and methods). WT and *lam1-1* cells were

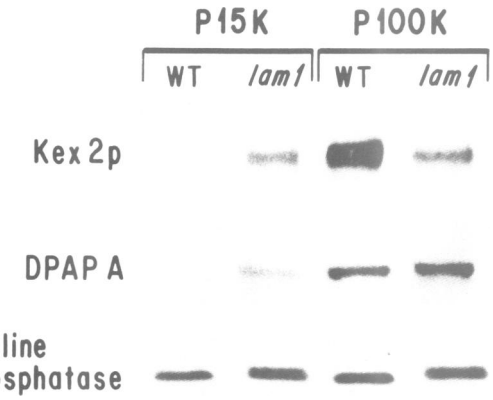

Fig. 7. Kex2p shows altered sedimentation in *lam1-1* cells. Spheroplasts of WT (GPY676), *lam1-1 pep4Δ* (GPY677) and *lam1-2 pep4Δ* (GPY674) were lysed osmotically and separated into 15 000 *g* and 100 000 *g* pellets. These samples were analyzed by SDS–PAGE and immunoblotting with antiserum specific for alkaline phosphatase, Kex2p and DPAP A.

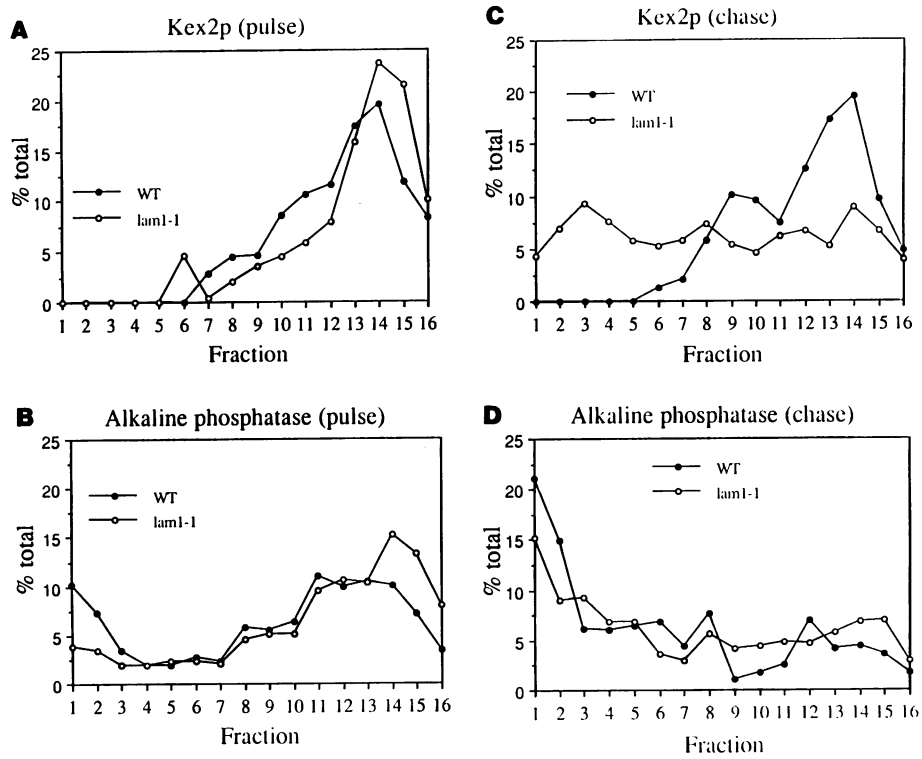

Fig. 8. Kex2p is mislocalized in *lam1-1* cells. WT (GPY676) and *lam1-1* (GPY677) spheroplasts were labelled at 30°C with [^{35}S]methionine for 15 min (pulse), labelling was stopped and the cells incubated for 45 additional min at 30°C (chase). Spheroplasts were osmotically lysed and separated into 15 000 *g* and 150 000 *g* pellets. These pellets were combined and applied to a sucrose density gradient. Proteins from these fractions were TCA precipitated, Kex2p and alkaline phosphatase immunoprecipitated and analyzed by SDS–PAGE autoradiography. Relative amounts were determined by densitometry and graphed as percentage of total. The top of the gradient is fraction 1.

metabolically labelled with [^{35}S]methionine and [^{35}S]cysteine for 15 min and then incubated for an additional 45 min in the presence of excess unlabelled amino acids. Cells were harvested after the labelling and after the chase period, lysed and membrane pellets prepared by centrifugation at 15 000 *g* for 15 min. followed by centrifugation at 150 000 *g* for 30 min. The two membrane pellets from each cell sample were combined and applied to a discontinuous sucrose gradient and centrifuged to equilibrium at 190 000 *g* for 18 h. The distribution of radiolabelled Kex2p and ALP across the gradients was evaluated by immunoprecipitation from gradient fractions, SDS−PAGE, autoradiography and densitometric analysis of the autoradiographs. After the 15 min labelling period, a time-point where Kex2p should be distributed between the Golgi complex and the ER, the bulk of Kex2p in both mutant and WT cells was found at the bottom of the gradient (fractions 12−16) where the Golgi complex and ER fractionate (Figure 8A; Vida and Emr, 1993). This result argues that the Golgi complex in *lam1* and WT cells exhibits the same density. Since ALP reaches the vacuole with a half-time of ~6 min (Klionsky and Emr, 1989), ALP exhibited a bimodal distribution in both cell types, with some at the top of the gradient (Figure 8B, fractions 1−3) where the vacuole fractionates and some at the bottom of the gradient (fractions 10−16). The smaller amount of ALP at the top of the gradient in *lam1* is consistent with the slight delay in transport of ALP to the vacuole in mutant cells observed by monitoring vacuolar maturation of the ALP precursor (data not shown). After the 45 min chase, the fractionation of Kex2p in mutant and WT cells differed markedly (Figure 8C). The Kex2p in WT cells mostly remained at the bottom of the gradient, peaking at fraction 14 with a shoulder at fraction 9, a position where a putative endosomal compartment fractionates (Vida and Emr, 1993). In *lam1* cells, much of the Kex2p had moved from the bottom of the gradient and was found spread throughout the gradient with peaks at fractions 3, 8 and 14. The ALP in both cell types at this time-point was located almost exclusively at the top of the gradient (Figure 8D fractions 1−3). These results suggest that Kex2p reaches the Golgi complex in *lam1* cells but is not efficiently retained in the organelle. The fractionation pattern of Kex2p after the chase period in mutant cells is consistent with movement of the protein into vacuoles.

In order to establish further that the fractionation pattern of Kex2p in mutant cells was not due to an altered density of Golgi membranes, the steady state distribution of guanosine diphosphatase (GDPase), another Golgi membrane protein (Abeijon *et al.*, 1989), was examined using an enzymatic activity assay of gradient fractions (Abeijon *et al.*, 1989). GDPase fractionated identically in WT and *lam1-1* cells, broadly peaking in fractions 11−13 (data not shown). Although this pattern is slightly different from that observed for Kex2p, consistent with data from others that the two proteins reside in separate compartments (Bowser and Novick, 1991), the experiment does indicate that the Golgi complex, as a whole, is not dramatically abnormal in *lam1-1* cells.

Missorting of vacuolar precursors in lam1 mutants

The data described above indicate that the *lam1* mutants fail to retain Kex2p in the Golgi complex. To investigate whether other Golgi functions are perturbed, we monitored the transport and delivery to the vacuole of carboxypeptidase Y (CPY). As with many vacuolar proteases, CPY is synthesized as an inactive precursor which is translocated into the ER where its amino-terminal signal sequence is cleaved and N-linked core oligosaccharides are added (Jones, 1991). The ER form is termed p1 (67 kDa). In the Golgi, limited addition of mannose residues onto the core sugars converts the p1 CPY to the p2 form (69 kDa). p2 CPY is sorted from the secretory pathway in a late Golgi compartment, at or beyond the compartment where Kex2p acts (Stevens *et al.*, 1982; Graham and Emr, 1991). Upon reaching the vacuole, the amino-terminal 'pro' region is excised to generate proteolytically active enzyme (61 kDa). This activation is dependent on the product of the *PEP4* gene (Jones, 1991). The ability of the *lam1* mutants to sort proCPY to the vacuole was determined in a pulse−chase experiment. For comparison, a *chc1*Δ and a *vps16*Δ (vacuolar *p*rotein *s*orting mutant; Rothman and Stevens, 1986; Banta *et al.*, 1988) strain were also analyzed. Cells were labelled with [^{35}S]-methionine and [^{35}S]cysteine for 20 min and then incubated with excess unlabelled amino acids for 30 min. Samples were collected immediately after the 20 min labelling period and also after the 30 min chase and separated into cell and media fractions. The cells were lysed and CPY was immunoprecipitated from cell lysates and media fractions and subjected to SDS−PAGE. In this regimen, even after the initial labelling period, most of the proCPY in WT and *chc1*Δ cells was efficiently sorted, delivered to the vacuole and activated (Figure 9, lanes 1−8). On the other hand, the *lam1* mutant alleles show a dramatic missorting phenotype, similar to that of the *vps16*Δ strain. After the 20 min labelling period, 44% of the newly synthesized CPY was evident in the medium of *lam1-1* cells (Figure 9, lanes 9 and 10), 36% was in the medium of *lam1-2* cells (Figure 9, lanes 13 and

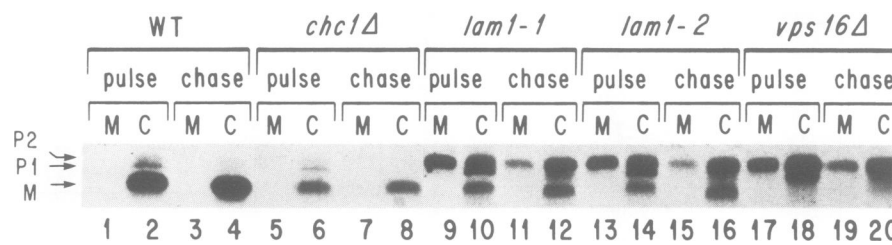

Fig. 9. p2 carboxypeptidase Y is missorted to the cell surface in *lam1* mutants. Wild type cells (GPY148-5D, lanes 1−4), *chc1*Δ cells (GPY163, lanes 5−8), *lam1-1* cells (GPY594, lanes 9−12), *lam1-2* cells (GPY597, lanes 13−16) and *vps16* cells (SEY16Δ, lanes 17−20) were labelled at 30°C with [^{35}S]methionine for 20 min (pulse), labelling was stopped and the cells incubated for 30 additional min at 30°C (chase). Culture supernatant (M) and cell lysate samples (C) were prepared after the pulse and after the chase, CPY was immunoprecipitated and analyzed by SDS−PAGE. The ER form (p1), the Golgi form (p2) and the mature form (M) are indicated.

14) and 36% was in the medium of *vps16Δ* cells (Figure 9, lanes 17 and 18). The cell-associated CPY in these three strains was distributed between p1, p2 and mature forms (Figure 9, lanes 10, 14 and 18). After the chase period, less CPY was present in the medium fractions, probably due to degradation (Figure 9, lanes 11, 15 and 19). All of the p1 CPY was converted to p2 during the chase incubation but 67% (*lam1-1*) and 73% (*lam1-2*) of the cell-associated CPY was still present as p2 (Figure 9, lanes 12 and 16). There was no conversion of p2 to mature CPY in the *vps16Δ* strain (Figure 9, lane 20). We did not convert the cells to spheroplasts because of the possibility that lysis during cell wall removal would confuse the results. Consequently, it cannot be determined whether the cell-associated p2 CPY was secreted into the periplasm or was accumulated within the cell. Nevertheless, the results clearly indicate that *lam1* strains are defective in the sorting of proCPY from the secretory pathway. We noted a similar but less dramatic defect upon examining the sorting of another soluble vacuolar protein precursor, proteinase A (data not shown).

The processing and secretion of CPY also indicates that the *lam1* mutations have a relatively specific effect on the secretory pathway. Conversion of p1 to p2 CPY and secretion of the p2 form indicates that the secretory pathway is intact and functioning efficiently, a conclusion supported by the secretion of α-factor precursor. The presence of mature CPY after the 20 min labelling period (Figure 9) suggests that although the sorting process is perturbed, those proteins which are correctly sorted are delivered to the vacuole and processed.

The co-segregation of the proCPY sorting defect and the α-factor maturation defect was examined in backcrosses of the *lam1* mutants to a WT strain (SEY6211). *MATα* segregants from 10 tetrads were examined for each cross and the two phenotypes cosegregated in every instance (data not shown). By this criterion, the phenotypes are most likely due to a single mutant *lam1* gene.

We also investigated whether the *lam1* mutations affected the delivery of a vacuolar membrane protein, alkaline phosphatase. When steady state levels of alkaline phosphatase were examined by SDS−PAGE and immunoblotting, we observed a slight processing defect in *lam1-1* and *lam1-2* cells (data not shown), however, the majority of alkaline phosphatase was processed to its mature form. Results from pulse−chase experiments are consistent with the immunoblotting data (data not shown). These results are similar to those obtained with *vps* strains (Robinson *et al.*, 1988) which, in general, are not significantly defective in ALP maturation.

lam1 is allelic to VPS1

A large number of mutants (*vps*, *v*acuolar *p*rotein *s*orting) have been identified based on their inability to sort vacuolar protein precursors properly (reviewed in Klionsky *et al.*, 1990). Since *lam1* strains display the same phenotype, we were prompted to carry out complementation tests to determine whether *lam1* is allelic to any of the *vps* mutations. Complementation was determined by using the filter overlay procedure to detect CPY secretion by diploids formed between a collection of *vps* mutants and *lam1-1* or *lam1-2* strains. Diploids formed between the *lam1* strains and *vps1* or *vps32* strains secreted CPY. Since *vps1* but not *vps32* strains secrete precursor α-factor (G.S.Payne, unpublished), we examined whether *lam1* and *vps1* are alleles of the same gene by genetic linkage analysis. The *VPS1* gene was marked with *URA3* by targeted integration (see Materials and methods) and strains carrying the *VPS1::URA3* gene were mated with either *lam1-1* or *lam1-2* strains. After inducing sporulation in the resulting diploids, the meiotic progeny were dissected into tetrads. The segregants were scored for CPY secretion (*lam1*) by the nitrocellulose overlay procedure and for the ability to grow in the absence of uracil (*VPS1::URA3*). In every tetrad (17/17 for diploids carrying *lam1-1* and 22/22 for diploids carrying *lam1-2*), two segregants were *lam1* Ura^- and two segregants were *LAM1* Ura^+. Thus, the combination of complementation tests and genetic linkage analyses demonstrate that the *lam1-1* and *lam1-2* mutations lie in the *VPS1* gene.

Discussion

Based on the properties of cells deficient in clathrin heavy chain, a genetic screen has been developed for mutations that affect the activity of the Golgi membrane protein Kex2p. In addition to mutations in the *KEX2* gene itself, mutations in another complementation group (*lam1*) were identified which reduce retention of Kex2p in the Golgi complex. Our data suggest that in mutant cells the unrestricted Kex2p is delivered to the vacuole. Additionally, missorting of soluble vacuolar precursors demonstrates the pleiotropic effects of the *lam1* mutations on late Golgi compartment function. Complementation tests and genetic linkage analysis demonstrated that *lam1* is allelic to *VPS1*, a gene previously identified by a selection for mutations which cause secretion of soluble vacuolar precursors. This result reveals a novel function for Vps1p in retention of Golgi membrane proteins.

The proteinase A (encoded by *PEP4*)-dependent instability of Kex2p in *lam1/vps1* mutants implies that Kex2p is mislocalized to the vacuole. This interpretation is supported by the sucrose gradient fractionation and by immunofluorescent microscopy of *lam1-1* cells expressing Kex2p under control of the *GAL1* promoter (M.Seeger, personal communication). The rate of Kex2p delivery from the Golgi complex to the vacuole may be estimated from the half-life of Kex2p in *lam1-1 PEP4* cells to be ~20 min. On the other hand, although sucrose gradient fractionation showed that much (62%) of Kex2p has left the Golgi complex in *lam1-1* cells by 45 min after synthesis (Figure 8), the mislocalized protein does not completely co-fractionate with the vacuole. The fractionation data appear to indicate a lower rate of Kex2p delivery to the vacuole than the *PEP4*-dependent turnover measurements. One explanation for the apparent discrepancy is that Kex2p may encounter active proteases in *lam1* cells before reaching the vacuole, perhaps in an endosomal compartment. Recently, sucrose gradient fractionation of newly synthesized CPY and internalized α-factor revealed that both molecules pass through a putative endosomal compartment of intermediate density before reaching the vacuole in WT cells (Vida and Emr, 1993). Thus, the substantial fraction of Kex2p in *lam1-1* cells that was found at intermediate density in the sucrose gradient 45 min after synthesis could represent protein in transit through endosomes. Further characterization of the intermediate density fraction of Kex2p will be needed to assess this possibility. An alternative, but less likely, explanation could account for the rapid proteolysis of Kex2p in *lam1/vps1*

cells: limited proteinase A-mediated activation of the precursors during their passage through the Golgi complex could lead to degradation of properly localized Kex2p. This scenario seems unlikely since stabilization of Kex2p by *pep4Δ* does not alter the α-factor maturation defect. Complete maturation would be anticipated if the defect was due simply to *in situ* degradation of Kex2p. Furthermore, missorting of vacuolar precursors is not sufficient to cause an α-factor maturation defect since some other *vps* strains which exhibit severe sorting defects secrete only mature α-factor (Robinson *et al.*, 1991; G.S.Payne, unpublished).

The partial α-factor maturation defect in the *lam1/vps1* mutants (20−35% precursor) is generally less than that caused by mutations in *KEX2* which were isolated in our screen and also less than deletions of *KEX2* (Fuller *et al.*, 1988), *CHC1* (Payne and Schekman, 1989) or *CLC1* (K.Wilsbach, unpublished). Since *vps1Δ* cells display a level of α-factor maturation commensurate with the *lam1/vps1* strains (K.Wilsbach, unpublished), the incomplete defect in *lam1/vps1* cells cannot be due to mutations which only partially debilitate the gene product. More likely, the Kex2p that reaches the Golgi complex in either *lam1/vps1* or *vps1Δ* cells may reside there long enough to cleave a portion of the α-factor precursor. In fact, even 45 min after synthesis, a significant fraction of Kex2p still fractionates with Golgi organelles (Figure 8; K.Wilsbach, unpublished). A relevant comparison may be made to the retention of *KAR2*-encoded *b*inding *p*rotein (BiP) in the yeast ER. Removal of the carboxy-terminal retention signal results in secretion of BiP but cells expressing this mutant are viable even though a deletion of *KAR2* that completely eliminates BiP expression is lethal (Rose *et al.*, 1989). Presumably cells carrying the retention signal-defective BiP survive because exit from the ER is slow enough to maintain functional levels of BiP.

The finding that *lam1* mutations are allelic to *VPS1* reveals that the *VPS1* gene product, Vps1p, is necessary both for sorting of soluble vacuolar proteins and retention of certain Golgi membrane proteins. Vps1p is a member of a family of high molecular weight GTPases (Rothman *et al.*, 1990; Vater *et al.*, 1992) which now includes dynamin and its *Drosophila* homologue, the *shibire* protein (Obar *et al.*, 1990; Chen *et al.*, 1991; van der Bliek and Meyerowitz, 1991) which is involved in endocytosis, the yeast *MGM1* protein which plays a role in maintenance of mitochondrial DNA (Jones and Fangman, 1992), and the vertebrate Mx proteins which confer resistance to certain viruses (Arnheiter and Meier, 1990). The only member of the family, except for Vps1p, which is known to participate in membrane traffic, is the *shibire* protein. When cells carrying temperature-sensitive mutations in *shibire* are transferred to the non-permissive temperature, a rapid inhibition of endocytosis in several cell types is observed (Kosaka and Ikeda, 1983; Kessel *et al.*, 1989). The defect in endocytosis is accompanied by an increase in the surface area of the plasma membrane and the formation of extensive plasma membrane invaginations which often exhibit clathrin coats. These results suggest that *shibire*, and by analogy dynamin, participate in an early stage of endocytosis, formation of endocytic vesicles. It is tempting to hypothesize that based on homology, Vps1p may also exert its effects on vacuolar protein sorting and Golgi membrane retention through a role in vesicle formation (see below). However, given the absence of data addressing specific functions of Vps1p and the apparent disparity in function among members of the dynamin GTPase family, this model remains speculative.

Recently, it has been reported that four *vps* mutant strains which lack coherent vacuoles also secrete significant levels of precursor α-factor (Robinson *et al.*, 1991). Like *lam1/vps1* mutants, these 'class C' mutants do not mislocalize Kex2p to the cell surface (K.Wilsbach, unpublished). The basis for the maturation defects in these mutants has not been established.

The basis of Golgi membrane protein mislocalization in lam cells

It has been proposed that Kex2p action occurs in the same late Golgi compartment as vacuolar precursor sorting (Graham and Emr, 1991). The pleiotropic phenotypes of *vps1* mutants are consistent with this proposal and suggest that the functional integrity of this compartment is perturbed. We presume that the phenotypes of *vps1* cells are direct results of a loss of Vps1p function. Recently, a strain carrying a temperature-sensitive allele of *VPS1* has been generated and the vacuolar sorting defect has been shown to occur immediately upon shifting cells to the non-permissive temperature (Vater *et al.*, 1992). It will be necessary to examine Kex2p retention in these cells to establish that this defect is also an immediate consequence of a loss of Vps1p function.

One model describing the possible basis of the defects in *vps1* mutants is presented in Figure 10. Although other models can be envisioned (for example see Robinson *et al.*, 1991 and Raymond *et al.*, 1992), we present this hypothesis because it accommodates not only the phenotypes of the *vps1* mutants but also the differences that exist between *vps1* and *chc1* strains. By analogy with the mechanism of lysosomal precursor sorting in mammalian cells, we propose that in WT yeast cells, the putative vacuolar sorting receptors bind vacuolar precursors in a late Golgi compartment and deliver the precursors to the intermediate compartment (IC) or endosome. The precursors dissociate from the receptor in the intermediate compartment and are transferred to the vacuole while the receptors return to the Golgi complex for additional rounds of sorting (Figure 10A). Additionally we suggest that the recycling route is followed by other resident late Golgi membrane proteins, including Kex2p and DPAP A. This suggestion relies on results which indicate that Kex2p retention is not static (Wilcox and Fuller, 1992) but does not involve cycling via the plasma membrane (Redding *et al.*, 1991; Seeger and Payne, 1992a; Wilcox and Fuller, 1992). Any membrane proteins not retrieved from the intermediate compartment would travel to the vacuole. (This pathway could generally be used for turnover of Golgi membrane proteins.)

If the *vps1* mutations interfere with the retrieval of membrane proteins from the intermediate compartment as shown in Figure 10B, then both the Kex2p and the sorting receptor would continue to the vacuole. The ensuing reduced levels of membrane proteins would allow α-factor and vacuolar precursors to proceed through the Golgi complex to the cell surface without encountering Kex2p or the sorting receptor.

In *chc1* cells, Kex2p is mislocalized to the cell surface. In contrast, iodination of *lam1/vps1* (Figure 3) failed to detect Kex2p at the cell surface. The difference between the effects of *vps1* mutations and *chc1* on Kex2p can be

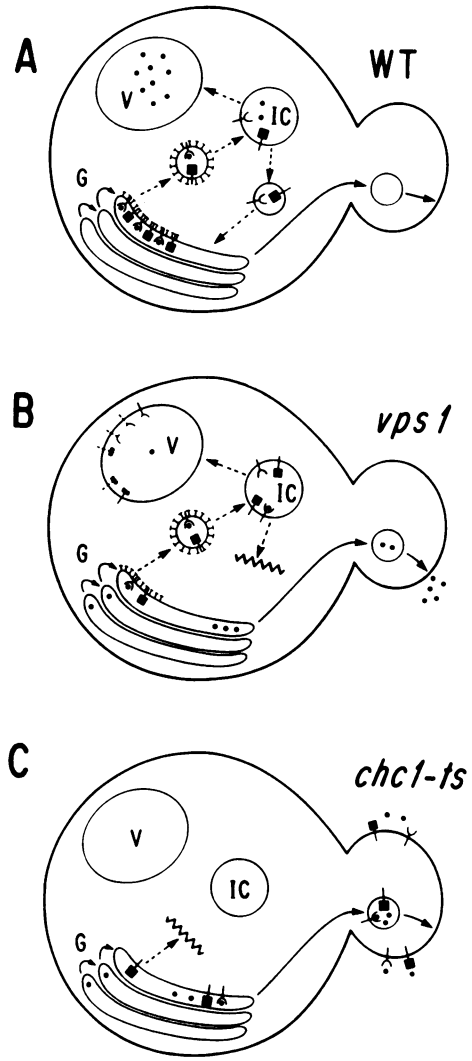

Fig. 10. Model for *lam1/vps1* cell phenotypes. (**A**) Kex2p retention and CPY sorting in WT cells. (**B**) Kex2p mislocalization to the vacuole and CPY missorting in *lam1/vps1* cells. (**C**) Kex2p mislocalization to the cell surface and CPY mislocalization in cells carrying a temperature-sensitive allele of *chc1 (chc1-ts)*. G signifies the Golgi complex, IC signifies the postulated intermediate compartment or endosome, V signifies the vacuole. CPY (●), the putative CPY sorting receptor (Y) and Kex2p (T) are shown. Clathrin-coated Golgi membranes and vesicles are also indicated (T). Proposed transport blocks are indicated with saw-toothed lines in B and C. See text for details.

explained if the effect of *chc1* is on the late Golgi compartment, not the intermediate recycling compartment (Figure 10C). This model also incorporates results of experiments with a temperature-sensitive allele of *chc1* (*chc1-ts*) that suggest a role for clathrin in the sorting of vacuolar precursors (Seeger and Payne, 1992b). In this view, clathrin acts in a late Golgi compartment to collect both Kex2p and the vacuolar precursor sorting receptor for packaging into vesicles destined for the intermediate compartment. In the absence of clathrin, Kex2p and the sorting receptor would disperse throughout the Golgi compartment and ultimately travel to the cell surface.

Our model for Kex2p recycling differs from an emerging view of the retention of TGN38, a resident protein of the *trans*-Golgi network (TGN) in animal cells (Ladinsky and Howell, 1992; Humphrey *et al.*, 1993; Reaves *et al.*, 1993). TGN38 appears to cycle between the plasma membrane and the TGN as part of its normal itinerary. In contrast, neither Kex2p nor DPAP A travels via the plasma membrane in yeast (Redding *et al.*, 1991; Roberts *et al.*, 1992; Seeger and Payne, 1992a; Wilcox and Fuller, 1992). The difference between TGN38 and the yeast Golgi membrane proteins could be due to residence in distinct Golgi compartments. Alternatively, the proteins may reside in analogous Golgi compartments, but upon leaving the Golgi complex, the proteins may be routed differently in animal and yeast cells. In either case, the fundamental retention mechanism in both cell types appears to involve retrieval from a post-Golgi compartment.

In conclusion, the mislocalization of Kex2p to the vacuole and missorting of vacuolar precursors in *vps1* mutants suggests the possibility that Golgi membrane proteins are retained in WT cells by recycling from a pre-vacuole compartment. A recent study of other *vps* mutants led to a similar model as one of several alternatives to account for the accumulation of novel pre-vacuole organelles containing a fusion protein normally located in the Golgi complex (Raymond *et al.*, 1992). Our model suggests that clathrin and the Vps1p GTPase act in opposing directions in the recycling pathway. The role of additional *VPS* gene products in the retention of Golgi membrane proteins awaits further studies.

Materials and methods

Media and strains

Yeast strains were grown in YP medium, which contained 1% yeast extract (Difco Laboratories, Detroit, MI) and 2% bacto-peptone (Difco) supplemented with 2% dextrose (YPD). The medium for *ade2* strains was supplemented with 0.0025% (wt/vol) adenine hydrochloride. SD (synthetic dextrose) minimal medium used for assessing nutritional requirements was prepared using 0.67% yeast nitrogen base, 2% dextrose and appropriate amino acid supplements (Sherman *et al.*, 1974). Minimal proline medium contains 0.67% yeast nitrogen base without amino acids, 1% proline, 2% dextrose and appropriate amino acids. Liquid medium was supplemented with 0.2% yeast extract (SDYE). All solid media included 2% agar. Cell densities in liquid culture were measured in a 1 cm plastic disposable cuvette, using a Beckman Instruments DU-62 spectrophotometer. One A_{500} unit is equivalent to a density of 2.3×10^7 cells/ml. The genotypes of various strains used are shown in Table I.

EMS mutagenesis and mutant screen

Mutagenesis of yeast cells was carried out using ethyl methanesulfonic acid (EMS) (Sigma Chemical Company, St Louis, MO). Cells were grown with aeration in YPD at 30°C to stationary phase. A sample containing 4–5 A_{500} units of cells was dispensed into glass tubes and the cells were washed with 2 ml of phosphate buffer (100 mM sodium phosphate, pH 7). The cells were resuspended in phosphate buffer, divided into two 2 A_{500} (0.75 ml) aliquots and brought to a final volume of 1.75 ml with phosphate buffer. EMS was added to a final concentration of 3.25% to one of the two samples. The cells were incubated with shaking at 30°C for 1 h, at which time the EMS was quenched by the addition of an equal volume of a 12% aqueous solution of sodium thiosulfate. This treatment results in a 85–90% reduction in the number of viable cells. The cells were then sedimented and washed twice with distilled water. Serially diluted samples were spread onto YPD plates and colonies allowed to form at 30°C.

Colony overlay immunoblot

Approximately 250 viable mutagenized cells were plated per YPD plate. Cells were grown at 30°C to form colonies and replica plated onto a second YPD plate. A nitrocellulose filter (45 mm, Schleicher & Schuell, Keene, NH) was placed onto the surface of the replica plate and incubated for ~16 h at 30°C. The filters were then removed, rinsed with distilled water and treated for 20 min with 0.1 M acetic acid to neutralize any endogenous alkaline phosphatase activity. The immunodetection procedure (Burnette, 1981) was carried out with a polyclonal rabbit antiserum raised against a precursor α–factor–β-galactosidase fusion protein (provided by D.Meyer) and goat anti-rabbit IgG coupled to alkaline phosphatase (Bio-Rad,

Table I. Genotypes of strains used

Strain	Genotype	Source
GPY148-5D	*MATα rme1::LEU2 leu2-3,112 ura3-52 his4 trp1 ade2-101*	This study
GPY163	*MATα chc1-Δ12::URA3 rme1::LEU2 leu2-3,112 ura3-52 his4 trp1-Δ901 ade2-101*	This study
GPY256	*MATα dpp2Δ::HIS3 ura3-52 leu2-3,112 his3Δ200 trp1 lys2-801 suc2-Δ9*	M.Seeger
GPY404	*MATa ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	This study
GPY470	*MATα lam1-1 leu2-3,112 rme1::LEU2 ura3-52 his4 trp1 ade2-101*	This study
GPY471	*MATα lam1-2 leu2-3,112 rme1::LEU2 ura3-52 his4 trp1 ade2-101*	This study
GPY553	*MATa1 leu1 ura3-52 ade2-101 rme1 his3-Δ200* and/or *his4*	This study
GPY594	*MATα lam1-1 leu2-3,112 rme1::LEU2 ura3-52 trp1 ade2-101 his3-Δ200* and/or *his4*	This study
GPY595	*MATα lam1-1 leu2-3,112 rme1::LEU2 ura3-52 pep4Δ::URA3 his4 trp1 ade2-101*	This study
GPY596	*MATα leu2-3,112 rme1::LEU2 ura3-52 pep4Δ::URA3 his4 trp1 ade2-101*	This study
GPY597	*MATα lam1-2 leu2-3,112 rme1::LEU2 ura3-52 trp1 ade2-101 his3-Δ200* and/or *his4*	This study
GPY598	*MATα lam1-2 leu2-3,112 rme1::LEU2 ura3-52 pep4Δ::URA3 trp1 ade2-101 his3-Δ200* and/or *his4*	This study
GPY618	*MATa lam1-2 leu2-3,112 trp1 his3-Δ200* and/or *his4*	This study
GPY673	*MATα lam1-2 ura3-52 leu2-3,112 ade2-101 trp1-Δ901 suc2-Δ9 his3-Δ200* and/or *his4*	This study
GPY674	*MATα lam1-2 ura3-52 leu2-3,112 pep4Δ::LEU2 trp1-Δ901 suc2-Δ9 his3-Δ200* and/or *his4*	This study
GPY675	*MATα ura3-52 leu2-3 112 trp1-Δ901 suc2-Δ9 his3-Δ200* and/or *his4*	This study
GPY676	*MATα ura3-52, leu2-3,112 pep4Δ::LEU2 trp1-Δ901 suc2-Δ9 his3-Δ200* and/or *his4*	This study
GPY677	*MATα lam1-1 ura3-52, leu2-3,112 pep4Δ::LEU2 trp1-Δ901 suc2-Δ9 his3-Δ200* and/or *his4*	This study
GPY678	*MATα lam1-1 ura3-52, leu2-3,112 trp1-Δ901 suc2-Δ9 his3-Δ200* and/or *his4*	This study
GPY1100	*MATa ura3-52 leu2-3,112 his4-519 trp1 can1 gal2*	Strain TD4 from G.R.Fink
BFY101-10C	*MATα leu2-3,112 kex2::URA3 ura3-52 his3-Δ200* or *his4-519*	R.Fuller
BFY101-11C	*MATa leu2-3,112 kex2::URA3 ura3-52 his3-Δ200* and *his4-519*	R.Fuller
JRY80	*MATa1 leu1 ura3-52 ade2-101 rme1 cyh2 can1 HMLα HMRα gal2*	J.Rine
LSY89.2-4C	*MATα leu2-3,112 his4 clc1::URA3 trp1 ura3-52*	L.Silveira
SEY6210	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al.* (1988)
SEY6211	*MATa ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 suc2-Δ9 ade2-101*	Robinson *et al.* (1988)
SF838-1D	*MATα ura3-52 leu2-3,112 his4-519 ade6 pep4-3 gal*	R.Schekman
vpt strains	*MATa vpt ade2-101 leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9*	Robinson *et al.* (1988)
vpl strains	*MATa vpl ura3-52 leu2-3,112 lys2 his4-519*	Rothman *et al.* (1986)

Richmond, CA). Bound antibody was visualized with a solution of 330 μl nitroblue tetrazolium (50 mg/ml in 70% DMF; Sigma), 165 μl 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in DMF; Sigma) and 50 ml of alkaline phosphatase buffer (0.1 M Tris−HCl pH 9.5, 0.1 M NaCl and 5 mM $MgCl_2$). This method is based on a similar procedure developed by Rothman *et al.* (1986).

Electroimmunoblotting analysis

Cells were grown in YPD to mid-logarithmic phase at 30°C. Approximately 1.0×10^8 cells were harvested, and cell extracts were prepared by lysis with 2% SDS as described previously (Payne *et al.*, 1987). 6.25×10^7 cell equivalents were loaded onto a 10% SDS−polyacrylamide gel and subjected to electrophoresis as described by Laemmli (1970). Electroblotting to nitrocellulose and visualization of Kex2p with specific antiserum were carried out essentially as described by Burnette (1981). Prior to use, the polyclonal antiserum to a Kex2p cytoplasmic tail β-galactosidase fusion protein (provided by R.Fuller, Stanford University) was passed through a Sepharose-coupled extract of *MATa kex2Δ* cells. Blots were developed as described above.

Iodination, metabolic labelling and immunoprecipitation

For iodination, strains were grown overnight to mid-logarithmic phase in SDYE. Iodinations were performed as described by Payne and Schekman (1989). Antisera against Kex2p, a 33 kDa cell wall protein (Sanz *et al.*, 1987) or G6PD (Sigma) were used to precipitate antigen from portions of each sample. The Kex2p immunoprecipitates were subjected to a second round of precipitation. Samples were analyzed by SDS−PAGE and autoradiography.

Metabolic labelling and immunoprecipitation of α-factor, Kex2p, CPY and alkaline phosphatase were done essentially as described by Payne and Schekman (1989), except that ^{35}S-Translabel (ICN, Costa Mesa, CA) was used instead of $[^{35}S]NaSO_4$. 5×10^6 cells were labelled for α-factor and CPY immunoprecipitations, 1×10^7 cells were labelled for Kex2p and alkaline phosphatase immunoprecipitions. α-factor samples were applied to a 15% polyacrylamide gel and CPY, alkaline phosphatase and Kex2p were applied to 8% polyacrylamide gels. All gels except those with α-factor samples were treated with a fixative solution containing 25% propanol and 10% acetic acid and all gels were treated with Amplify (Amersham, UK), dried and exposed to X-ray film at −70°C.

Organelle fractionation by differential sedimentation and sucrose density gradient centrifugation

10^9 cells were grown in YPD medium to an OD_{500} of 1.0, spheroplasted, washed three times with 1 ml 20 mM HEPES−KOH pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate and 1.2 M sorbitol at 4°C. After the last wash the cells were resuspended in triethanolamine buffer, containing 0.8 M sorbitol, 10 mM triethanolamine pH 7.2 and 1 mM EDTA, and homogenized with 20 pestle strokes in a Wheaton tissue homogenizer. Cells were centrifuged at 15 000 *g* for 5 min and washed twice by resuspension in 1 ml triethanolamine buffer and centrifugation at 15 000 *g* for 5 min. These supernatants were collected and centrifuged at 15 000 *g* for 15 min. The 15 000 *g* supernatant was then centrifuged at 100 000 *g* for 1 h. 5 ml samples from each pellet and supernatant were subjected to SDS−PAGE and immunoblotted as described above for Kex2p, DPAP A and alkaline phosphatase.

Cells for the sucrose density gradient were prepared as described above with the following changes. 10^9 cells were grown in minimal proline medium with 2% yeast extract. They were spheroplasted and metabolically labelled in minimal proline 1.2 M sorbitol at a density of 10 OD units/ml. Half of the cells were collected after a 15 min labelling and the other half collected after an additional 45 min incubation with unlabelled cysteine and methionine. Each sample was washed three times by resuspension and centrifugation with 1 ml 20 mM HEPES−KOH pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate and 1.2 M sorbitol at 4°C. Cells were then resuspended in 100 μl total volume of HEPES−KOH buffer with 1.2 M sorbitol and suspended 8 cm over N_2 vapor for 15 min. They were stored at −80°C overnight. Cells were thawed in a 25°C water bath for 45 s and placed on ice. Samples were washed by resuspension and centrifugation with the HEPES−KOH buffer described above with these changes: wash 1, 250 mM sorbitol; wash 2, 50 mM sorbitol; wash 3, 2 M potassium acetate and 250 mM sorbitol; wash 4, 250 mM sorbitol. Permeabilized spheroplasts were then resuspended in ice-cold triethanolamine buffer. Lysis was completed by passage through a 26 gauge needle three times. Samples were centrifuged at 15 000 *g* for 5 min and the pellets washed

twice with 1 ml triethanolamine buffer. Each of the supernatants following the complete lysis and centrifugation steps were collected and centrifuged at 15 000 *g* for 15 min. The 15 000 *g* supernatant was then centrifuged at 150 000 *g* for 30 min. The 15 000 *g* and 150 000 *g* pellets were each resuspended in 500 μl triethanolamine buffer, combined and loaded on a discontinuous sucrose gradient containing 0.5 ml of 61%, 1.0 ml of 43%, 1.0 ml of 37%, 1.5 ml of 34%, 2.0 ml of 32%, 2.0 ml of 29%, 1.0 ml of 27% and 1.5 ml of 22% sucrose, which had been allowed to equilibrate for several hours.

Complementation analyses

Complementation of the α-factor maturation defect was assessed in diploids expressing α-factor. To surmount the problem that normal (*MATα*/MAT**a**) diploids do not express mating pheromones, *lam1 mat***a***1* strains were constructed. Haploid cells carrying *mat***a***1* at the mating-type locus behave as **a**-type cells and will mate normally to *MATα* cells. However, the resulting *mat***a***1/MATα* diploids are phenotypically *MATα* and secrete α-factor. Meiotic *lam1 mat***a***1* progeny were obtained from diploids formed by mating *MATα lam1* strains to the *mat***a***1* strain JRY80. The *rme1* mutation, present in both the *lam1* mutants and JRY80, allows sporulation of *mat***a***1/MATα* diploids. *mat***a***1* Segregants which carry *lam1* mutations were identified by mating the segregants to the original *MATα lam1* strains and assessing α-factor maturation by metabolic labelling and immunoprecipitation.

For complementation tests between *lam1* and *vps* strains, GPY 470 (*lam1-1*), GPY677 (*lam1-1*), GPY 471 (*lam1-2*) and GPY674 (*lam1-2*) were mated to *MAT***a** *vpl* and *MAT***a** *vpt* strains and diploids selected by complementation of auxotrophic markers. Resulting diploids were patched onto YPD plates, grown overnight at 30°C, replica plated onto YPD and then overlayed with nitrocellulose. Nitrocellulose was incubated with cells overnight at 24°C after which time it was processed for immunodetection using antibodies specific for carboxypeptidase Y.

To determine genetic linkage between *lam1* and *VPS1*, the *VPS1* locus was marked with *URA3* by targeted integration (Rothstein, 1991) using plasmid pCKR5 (provided by T.Stevens, University of Oregon). pCKR5 has an *Eco*RI to *Sal*I DNA fragment containing the *VPS1* gene cloned into the *Eco*RI and *Sal*I sites of YIp5 (Rothstein, 1991). For integration, the plasmid was cut at the unique site *Bam*HI site in *VPS1* and introduced into GPY1100 or SEY6210 by lithium acetate transformation (Ito *et al.*, 1983). An SEY6210 Ura$^+$ transformant was mated to GPY613 (*lam1-1*) and a GPY1100 Ura$^+$ transformant was mated to GPY673 (*lam1-2*). Diploids were selected by complementation of auxotrophic markers, sporulated and dissected into tetrads. The *lam1* mutations were scored based on secretion of CPY measured by the nitrocellulose overlay described above. *VPS1* was scored by growth on medium lacking uracil. To confirm that pCKR5 had integrated at *VPS1*, the GPY1100 putative *VPS1::URA3* transformant was mated to SF838-1D*vps1*-Δ2 (Rothman *et al.*, 1990) which carries an allele of *VPS1* disrupted by *LEU2*. Diploids were sporulated and dissected into tetrads. In each of 12 tetrads, two segregants were Ura$^+$ Leu$^-$ and two segregants were Ura$^-$ Leu$^+$, indicating that *URA3* in the GPY1100 transformant was linked to *VPS1*. The SEY6210 putative *VPS1::URA3* transformant was mated to the GPY1100 *VPS1::URA3* strain. After sporulation and dissection, 18/18 tetrads showed 4:0 segregation of Ura$^+$ indicating that the *URA3* in the SEY6210 transformant was linked to *VPS1*.

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