Mutation of a Tyrosine Localization Signal in the Cytosolic Tail of Yeast Kex2 Protease Disrupts Golgi Retention and Results in Default Transport to the Vacuole

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Kex2 protease processes pro- α -factor in a late Golgi compartment in *Saccharomyces cerevisiae*. The first ~ 30 residues of the 115 amino acid CO₂H-terminal cytosolic tail (C-tail) of the Kex2 protein (Kex2p) contain a Golgi retention signal that resembles coated-pit localization signals in mammalian cell surface receptors. Mutation of one (Tyr₇₁₃) of two tyrosine residues in the C-tail or deletion of sequences adjacent to Tyr₇₁₃ results in loss of normal Golgi localization. Surprisingly, loss of the Golgi retention signal resulted in transport of C-tail mutant Kex2p to the vacuole (yeast lysosome), as judged by kinetics of degradation and by indirect immunofluorescence. Analysis of the loss of Kex2 function in vivo after shutting off expression of wild-type or mutant forms proved that mutations that cause rapid vacuolar turnover do so by increasing the rate of exit of the enzyme from the pro- α -factor processing compartment. The most likely explanation for these results is that mutation of the Golgi retention signal in the C-tail results in transport of Kex2p to the vacuole by default. Wild-type Kex2p also was transported to the vacuole at an increased rate when overproduced, although apparently not due to saturation of a Golgi-retention mechanism. Instead, the wild-type and C-tail mutant forms of Kex2p may follow distinct paths to the vacuole.

INTRODUCTION

Mutations in the yeast (*Saccharomyces cerevisiae*) *KEX2* gene block production of K1 killer toxin and the peptide pheromone α -factor, which is required for mating of *MAT α* haploid cells (Liebowitz and Wickner, 1976). Kex2 protein (Kex2p)¹ is an integral membrane protein

and a Ca²⁺-dependent serine protease that processes pro-killer toxin and pro- α -factor in the secretory pathway (for review, see Julius *et al.*, 1984a; Fuller *et al.*, 1988). The recent characterization of a family of Kex2 homologues in metazoans suggests that Kex2 protease is prototypical of enzymes that process a variety of secretory precursors by cleavage at the carboxyl side of pairs of basic residues and other related sites in eukaryotes (Barr, 1991; Steiner, 1991).

The precision and efficiency exhibited in the selective processing of precursors likely is due to not only the remarkable substrate specificity and catalytic rate of the processing enzymes (Brenner and Fuller, 1992) but also to the cellular mechanisms that transiently co-concentrate enzymes and their substrates in the correct secretory compartments. Thus, the organization of the processing compartments and the signals that localize enzymes and precursors to them are fundamental to

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¹ Abbreviations used: C-tail, cytosolic tail; ER, endoplasmic reticulum; IPB, immunoprecipitation buffer; Kex2p, Kex2 protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST, Tris-buffered saline plus Tween-20; TBSTB, Tris-buffered saline plus Tween-20 containing 1% bovine serum albumin; TGN, *trans* Golgi network; TMD, transmembrane domain; Tris, tris(hydroxymethyl)aminomethane.

understanding the molecular basis of processing specificity and more generally provide an attractive model for cellular targeting events.

Several lines of evidence indicate that Kex2 protease is located in a late Golgi compartment in yeast. Very little Kex2p is detectable at the surface of cells expressing the enzyme either at the wild-type level or at a 150-fold elevated level (Fuller *et al.*, 1989b; Payne and Schekman, 1989). By indirect immunofluorescence, Kex2p is localized in a punctate cytoplasmic distribution (Redding *et al.*, 1991) and largely colocalizes with the SEC7 and SEC14 gene products, which are required for secretory transport through and out of the Golgi complex (Cleves *et al.*, 1991; Franzusoff *et al.*, 1991). Kex2 cleavage of pro- α -factor appears to occur in a compartment distal to that in which α -1,3-linked mannose is added to N-linked oligosaccharides, the "latest" oligosaccharyl modification known in yeast Golgi (Graham and Emr, 1991). Consistent with this, Kex2 proteolytic activity partially separates from α -1,3-mannosyl transferase activity in Percoll density gradients (Cunningham and Wickner, 1989). After transport to the Golgi and for the remainder of its lifetime ($t_{1/2} \sim 80$ min), Kex2p itself undergoes progressive modification of its N-linked and O-linked oligosaccharides that consist, in part, of addition of α -1,3-linked mannose to both kinds of chains (Wilcox and Fuller, 1991). Continued or intermittent exposure of mature Kex2p to late Golgi glycosyl transferases may occur, therefore, by periodic cycling from a more distal compartment to a late Golgi compartment containing the transferases. Such recycling is probably intracellular, because Kex2p does not accumulate in secretory vesicles in the vesicle-accumulating *sec1* mutant (Redding *et al.*, 1991).

A single transmembrane domain (TMD) in Kex2p separates a larger NH₂-terminal luminal domain having enzymatic activity from a highly charged 115 residue CO₂H-terminal cytosolic tail (C-tail). A deletion, KEX2 Δ 4, that removed the 99 CO₂H-terminal residues of the C-tail resulted in appearance of 10–20% of total enzyme at the cell surface when expressed at a high level (Fuller *et al.*, 1989b). Deletion of the entire TMD and C-tail in KEX2 Δ 6 resulted in efficient secretion of a soluble form of the protein (Fuller *et al.*, 1989b; Glushankof and Fuller, unpublished data). In addition, cells lacking clathrin heavy chain were found to be defective in processing pro- α -factor and to export a large proportion (75–91%) of Kex2 protease to the plasma membrane (Payne and Schekman, 1989). Thus, intracellular retention of Kex2p was proposed to involve interaction between the Kex2p C-tail and a clathrin-dependent structure (Fuller *et al.*, 1989b; Payne and Schekman, 1989), analogous to the localization of mammalian cell surface receptors to clathrin-coated pits (Pearse and Robinson, 1990). Failure of Kex2p to appear at the cell surface when highly overproduced suggested, surprisingly, that its retention mechanism was not easily sat-

urable. Conversely, appearance of the protein at the cell surface both on deletion of C-tail sequences and in clathrin-deficient strains suggested that the "default" pathway for Kex2p was to the cell surface.

In contrast to expectations, experiments described here demonstrate that overproduction does indeed result in mislocalization of Kex2p, but the apparent default destination of the protein is the vacuole (yeast lysosome) rather than the plasma membrane. Moreover, mutations in the Kex2p C-tail both disrupt retention in the Golgi and cause rapid transport of the protein to the vacuole rather than accumulation at the plasma membrane. These results may have general implications for the means by which the localization and lifetime of membrane proteins are regulated.

MATERIALS AND METHODS

Reagents and Antibodies

Specialized reagents were adjuvant (MPL + TDM emulsion) from RIBI Immunochem Research (Hamilton, MT), Protein A-Sepharose from Pharmacia (Piscataway, NJ), electron microscopy supplies from Ted Pella (Redding, CA), and fluorescent antibodies from Jackson ImmunoResearch Laboratories (West Grove, PA). The anti-Kex2 tail antiserum and affinity-purified antibody were as described (Fuller *et al.*, 1989a; Redding *et al.*, 1991). The anti-Vat2p monoclonal antibody (13D11) was generously donated by T. Stevens (University of Oregon, Eugene) and has been described (Kane *et al.*, 1992). Rabbit antiserum directed against the luminal domain of Kex2 (anti-Kex2 luminal antiserum) was initially raised against a LacZ fusion protein, LacZ-KX1 Δ 6, consisting of 495 residues of the luminal domain of Kex2p (residues 120–614) fused to *Escherichia coli* β -galactosidase. The LacZ fusion was created by inserting the 1.5 kb *Hind*III-*Bam*HI fragment of KEX2 Δ 6 (Fuller *et al.*, 1989a) into pUR291 (Ruther and Muller-Hill, 1983). Preparation of antigen and immunizations were performed as described (Fuller *et al.*, 1989b) and resulted in low titer sera after 15 monthly boosts of 100–200 μ g each. Subsequent boosts, performed with 30–80 μ g of secreted soluble Kex2 protease (Brenner and Fuller, 1992) in 1 ml of RIBI adjuvant, resulted in higher titer sera.

Strains, Plasmids, and Growth Conditions

Strain DC14 (*MATa his1*) was from J. Thorner (University of California, Berkeley) and strain W303-1A (*MATa can1-100 ade2-1 his3-11,15 leu2-3,-112 trp1-1 ura3-1*) was from R. Rothstein (Columbia University, NY). All other strains used were generated during this study and are identical to W303-1A except as indicated: BFY106-4D (*MATa kex2 Δ ::HIS3*), CB007-1D (*MATa pep4::HIS3 prb1::LEU2*), CB017 (*MATa kex2 Δ ::TRP1 pep4::HIS3 prb1::hisG prc1::hisG*), CB018 (*MATa pep4::HIS3 prb1::hisG prc1::hisG*), KRY108-5D (*MATa kex2 Δ ::TRP1 sec1-1*), and KRY108-7D (*MATa kex2 Δ ::TRP1 pep4::HIS3 sec1-1*). Synthetic minimal, synthetic complete, and synthetic dropout media are as described (Rose *et al.*, 1990). Unless indicated, cells were grown in synthetic complete media lacking uracil to select for plasmid maintenance.

Plasmid pAB-KX22, overproducing Kex2 protease about 150-fold, places the KEX2 structural gene under transcriptional control of the TDH3 promoter within the multicopy episomal vector pAB23 (Fuller *et al.*, 1989a). Plasmid YCp-KX22, which elevates expression of Kex2 protease $\sim$ 25-fold relative to wild-type cells as measured by enzyme assay, consists of the KEX2 structural gene under control of the TDH3 promoter inserted into the *Bam*HI site of yeast centromere vector YCp50 (Rose *et al.*, 1987). In pBM-KX22, the wild-type KEX2 structural gene is under *GAL1* promoter control (Johnston and Davis, 1984) in vector pBM258 (*CEN4 ARS1 URA3*) (Redding *et al.*, 1991). Similarly,

plasmids pBM-KX212Δ4 and pBM-KX212Δ6 consist of the *KEX2Δ4* and *KEX2Δ6* genes inserted into pBM258 but contain slightly longer 5' untranslated sequences than found in pBM-KX22 (Fuller *et al.*, 1989a). Plasmid mYcPn3·KEX2, kindly provided by Charles Brenner, was created by addition of both the M13 origin of replication and *KEX2* structural gene to vector YcPn3 (*CEN4 ARS1 TRP1*), which was derived from the single copy yeast centromere plasmid YcPn1 (Nakayama *et al.*, 1985) lacking *EcoRI* sites. Plasmids pCWKX10 and pCWKX2 were derived from YcPn3 (Fuller *et al.*, 1989a) and pBM-KX22 as follows. Plasmids pBM-KX22 and YcPn3 were digested with *Nar I* and the overhanging ends were filled in with DNA polymerase I large fragment and ligated in the presence of *Sal I* linkers. The resulting plasmids were digested with *Xba I* and *Sal I*, the vector fragment was isolated, and the *KEX2* structural gene was re-created by combining the vector *Xba I-Sal I* fragment with a *Xba I-Sal I* fragment containing the 3' coding region of the *KEX2* structural gene from plasmid pKX2 (Fuller *et al.*, 1989a). Therefore, plasmid pCWKX10 contains the *KEX2* structural gene under control of the wild-type promoter, and plasmid pCWKX20 contains the *KEX2* structural gene under control of the yeast *GAL1* promoter. Plasmids were introduced into yeast strains by lithium acetate transformation (Ito *et al.*, 1983).

Site-Directed Mutagenesis

To facilitate subsequent cloning steps, plasmid mYcPn3·KEX2 was modified by introducing a *Sal I* site ~270 nucleotides beyond the translational termination codon of the *KEX2* gene, resulting in plasmid pCWKX1. Single-stranded uracil-containing DNA was produced by propagating pCWKX1 in either *E. coli dut- ung-* strain CJ236 or BW313 with M13K07 helper phage (Kunkel *et al.*, 1987; Vieira and Messing, 1987). Site-directed mutagenesis using *E. coli* DNA polymerase III holoenzyme was performed essentially as described (Tsurushita *et al.*, 1988), except that T4 DNA ligase was present throughout the reaction. Mutant clones were identified by restriction site polymorphisms introduced by the mutagenic primers. *Nar I-Sal I* fragments containing the mutations were sequenced in their entirety (Sanger *et al.*, 1977) and then subcloned into both pCWKX10 and pCWKX20. Sequences of oligonucleotides used for site-directed mutagenesis were AGAGCGGAAACGGCTGAGTTCGATATCA-TTG (Y₇₁₃A), ATACAGACTCTGAAGCCGATTCTATT (Y₇₂₄A), GGTCAAGAGCGGAAACGTATAATGGAACCTCCGGAATTAC (AblockΔ), TCATGTTTTTATGAAATCAGAGTTCGATATCAT-TGATACAG (B-blockΔ), TCATGTTTTTATGAAATCAAATGGA-ACCTCCGGAATTAC (BA-blockΔ), GTTTTTATGAAATCAT-GAGTGAGTGAAGGAGAAGGATCAGA (C-tailΔ), CTGAGTA-CGATTCTACTTTGAATAAATTACAAGAATTACA (*K. lactis*-like), AACGCAGAATCTGCTAGCTGAAAATTACAAGAATTA (N-blockΔ), GAGTACGATTCTACTCTAGAAATGGAACCTCCGGA (BA-tail). The Y_{713,724}A mutant was created by using both the oligonucleotides for Y₇₁₃A and Y₇₂₄A.

Radiolabeling and Immunoprecipitation

Labeling with [³⁵S]-H₂SO₄ was performed as described (Wilcox and Fuller, 1991). Cells were depleted of sulfate for 30 min, 300 μCi/ml of [³⁵S]-H₂SO₄ was added for a 10-min labeling, and a chase was initiated. Cells were harvested, washed, lysed, and Kex2p was immunoprecipitated from the lysate with the anti-Kex2 tail antiserum as described (Wilcox and Fuller, 1991). The C-tailΔ, *K. lactis*-like tail, and BA-tail mutants were isolated by serial immunoprecipitations using the anti-Kex2 luminal antiserum by a modification of the procedure used with the anti-Kex2 tail antiserum (Wilcox and Fuller, 1991). Cell pellets were resuspended in 25 μl of lysis buffer, vortexed with 0.18 g 0.5-mm glass beads and then diluted with 0.25 ml immunoprecipitation buffer (IPB). The lysate was clarified, 20 μl of anti-Kex2 luminal antiserum and 60 μl of Pansorbin were added, and samples were incubated at 4°C for ≥5 h. Pansorbin pellets were washed sequentially

with 0.8 ml of IPB, IPB plus 2 M urea, and IPB plus 1% 2-mercaptoethanol. The second round of immunoprecipitation was performed with 20 μl of anti-Kex2 luminal antiserum and 20 μl Protein A-Sepharose. The data in Table 4 were obtained with a Molecular Dynamics PhosphorImager (Sunnyvale, CA). Analysis of immunoprecipitated proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) relative to size standards confirmed that deletion mutations in the Kex2p C-tail caused expected shifts in apparent molecular weight.

Indirect Immunofluorescence and Immunoelectron Microscopy

Cells were fixed and processed for immunofluorescence as described (Redding *et al.*, 1991). The primary incubation contained a 1:50 dilution of affinity-purified anti-Kex2 tail antibody and a 1:50 dilution of anti-Vat2p antibody. The secondary incubation contained 1.5 μg/ml Texas-Red conjugated goat anti-rabbit IgG and 1.7 μg/ml fluorescein isothiocyanate-conjugated goat anti-mouse IgG. In Figure 6, wild-type and C-tail mutant proteins were expressed at a 15-fold elevated level under *GAL1* promoter control on a single copy plasmid, conditions that did not significantly alter localization of wild-type Kex2p. At this expression level, the half-life of wild-type Kex2p (Table 1) and punctate localization of the protein was similar to that found with the wild-type expression level (Table 1) (Redding *et al.*, 1991).

Electron microscopy was performed essentially as described (Wright and Rine, 1989). Log phase cells (100 ml at OD₆₀₀ = 1) grown in synthetic glucose media at 30°C were fixed with 1% (wt/vol) glutaraldehyde, 1% formaldehyde, 50 mM potassium phosphate (pH 6.7) and embedded in LR White resin (Ted Pella, Redding, CA). Sections (~50 nm) were cut with a Sorvall MT2 ultramicrotome and applied to 200 mesh nickel grids. Grids were blocked in tris-(hydroxymethyl)aminomethane (Tris)-buffered saline plus Tween-20 (TBST; 20 mM Tris, pH 7.0, 500 mM NaCl, 0.1% Tween-20) containing 1% bovine serum albumin (TBSTB) for 20 min at room temperature, incubated for 1 h with a 1/100 dilution of affinity-purified anti-Kex2 tail antibody in TBSTB, washed four times for 5 min in 3 ml of TBST, reblocked for 20 min with TBSTB, and finally incubated for 30 min in TBSTB containing affinity-purified goat anti-rabbit IgG complexed with 10-nm gold particles. Sections were poststained with 1% uranyl acetate to enhance contrast and were examined using a Philips (Eindhoven, The Netherlands) EM300 electron microscope.

To characterize the morphology of Kex2p-containing vesicle clusters, photographs were taken of 57 randomly chosen cell sections of the *kex2Δ* strain and 83 of the Pep⁺ overproducing strain. Prints were analyzed by overlaying a grid, with squares corresponding to 0.5 by 0.5 μm, and by counting the gold particles in the squares. A total of 1544 squares of the *kex2Δ* strain and 1308 squares of overproducing strain were analyzed. All squares having ≥10 gold particles (only 1 square from the micrographs of the *kex2Δ* strain had >5 gold particles) were examined further. This resulted in the examination of 48 different structures found in 72 squares from 31 different cells.

Kex2 Protease Assays

One unit of Kex2 activity (Fuller *et al.*, 1989a) is defined as Ca⁺⁺-dependent release of 1 pmol of 7-amino-4-methylcoumarin from t-boc-Gln-Arg-Arg-MCA per min at 37°C in a volume of 50 μl (values from reactions containing 20 mM EDTA were subtracted from those containing 1 mM CaCl₂). The fraction of total active Kex2 protease at the cell surface (Table 2) was determined as described (Fuller *et al.*, 1989b), except that freeze-thaw was performed in 1% Na·deoxycholate and 1% Triton-X-100. Assays in Figures 7 and 8 used clarified glass bead lysates (Fuller *et al.*, 1989a). Total protein content was determined with bicinchoninic acid (Pierce, Rockford, IL). Clarified lysates of *kex2Δ* cells exhibited a specific activity of 2.5 ± 1.5 U/mg, corresponding to ~1% the specific activity of wild-type cells.

Kex2 Depletion Experiments

Strains BFY106-4D and CB017 harboring pCWKX20, pCWKX21, or pCWKX27 were grown in synthetic galactose medium into log phase at 30°C. Cells were filtered and resuspended in synthetic glucose medium and maintained thereafter in log phase by periodic dilution into synthetic glucose medium. Aliquots of cells were removed at various times for biochemical and mating assays. Cells (10^8) were washed in 150 mM NaCl, 1 mM EDTA and frozen on dry ice for determining Kex2 specific activity. Mating assays were performed essentially as described (Hartwell, 1980). DC14 cells (1.5×10^7) were used as mating partners for 5×10^6 MAT α cells. Mating proceeded for 4 h at 30°C. Serial dilutions of matings were plated on synthetic minimal and synthetic complete minus histidine. The number of colonies on the minus histidine plates represented the total number of diploids and MAT α cells; the number on the minimal plates represented total diploids. The mating efficiency of the MAT α strain was calculated as the number of diploids divided by the number of diploids plus MAT α cells.

RESULTS

Degradation of Kex2p is Accelerated by Elevated Expression and Temperature

Previous studies showed that when a haploid strain with a single copy of the wild-type KEX2 gene was grown at 30°C, Kex2p was relatively stable, having a half-life of 83 min (Wilcox and Fuller, 1991) (Figure 1A, wild-type, lanes 1–6; B; and Table 1). Two conditions dramatically affected the lifetime of Kex2p. Overproduction markedly decreased the half-life of Kex2p. With expression at a level ~ 25 -fold greater than wild-type, the half-life of Kex2p decreased to 39 min (Figure 1A, 25 $\times$ wild-type, lanes 1–6; B; and Table 1). At an expression level

Table 1. Half-life of Kex2 protein

Plasmid	Expression level ^a	Half-life (min) ^b		
		30°C	37°C	Pep ⁻ , 30°C
YCp50	1	80 $\pm$ 20	42 $\pm$ 1	>180
YCp-KX22	25	39 $\pm$ 6	26 $\pm$ 4	>180
pAB-KX22	150	22	7	>180
pBM-KX22 ^c	15	65 $\pm$ 21	ND	>180

^a The level of Kex2p, expressed relative to that in wild-type cells, was determined by assaying Kex2 protease specific activity as described (Fuller *et al.*, 1989a).

^b Half-lives were determined from experiments in Figure 1 and from similar experiments as described in Wilcox and Fuller (1991).

^c Strains BFY106-4D (Pep⁺) and CB017 (Pep⁻), containing pBM-KX22, were grown in synthetic galactose medium.

ND, not determined.

150-fold greater than wild-type, the half-life of the protein decreased to 22 min (Figure 1A, 150 $\times$ wild-type, lanes 1–6; B; and Table 1). Increasing growth temperature also caused more rapid turnover. When cells expressing Kex2p at the wild-type level were pulse-labeled at 30°C and shifted to 37°C at the start of a chase, the half-life of Kex2p dropped to 42 min (Figure 1A, wild-type, lanes 7–12; B; and Table 1). The effects of elevated expression and temperature were additive (Figure 1A, 25 $\times$ wt and 150 $\times$ wt, lanes 7–12, and Table 1). When wild-type cells were grown for >6 gen-

Figure 1. Effects of expression level, growth temperature, and vacuolar proteases on the lifetime of Kex2p. Strains were grown at 30°C, labeled with [³⁵S]-H₂SO₄, subjected to chase at 30 or 37°C, and samples were harvested at the indicated times. Immunoprecipitated Kex2p was analyzed by SDS-PAGE as described (Wilcox and Fuller, 1991). As previously described, the gradual increase in apparent molecular weight is due to progressive modification of N- and O-linked oligosaccharides of Kex2p in the Golgi complex (Wilcox and Fuller, 1991). (A) W303-1A transformants containing plasmid YCp50 (vector), YCp-KX22 (25-fold overexpression), or pAB-KX22 (150-fold overexpression). (B) The data in A and similar experiments were quantified as described (Wilcox and Fuller, 1991). (C) Pep⁻ strain CB007-1D containing YCp50. (D) Pep⁻ strain CB007-1D containing pAB-KX22 or YCp-KX22 grown at 30°C.

Figure 2. Indirect immunofluorescence localization of Kex2p to vacuoles in Pep^- cells. Strains CB017[pAB23] ($\text{kex2}\Delta$ Pep^- ; A–C), W303-1A[pAB-KX22] (Pep^+ Kex2p-overproducer; D–F), and CB018[pAB-KX22] (Pep^- Kex2p-overproducer; G–I) were fixed, processed for indirect immunofluorescence, and double-labeled for Vat2p (B, E, and H) and Kex2p (C, F, and I). Corresponding differential interference contrast micrographs are displayed in A, D, and G. Bar equals 5 μm .

erations at 37°C before labeling, Kex2p still exhibited a reduced half-life (30 min), indicating that the accelerated turnover of the protein at 37°C did not display adaptation, a feature typical of heat-shock phenomena.

The Vacuole is the Site of Turnover of Wild-Type Kex2p

Analogous to the mammalian lysosome, the yeast vacuole is an acidic organelle containing hydrolytic enzymes (Klionsky *et al.*, 1990). To determine whether degradation of Kex2p occurred in the vacuole, pulse-chase experiments were performed in an isogenic strain lacking vacuolar proteases A and B. The vacuoles of such Pep^- strains are largely devoid of hydrolytic activities (Jones, 1991). Expressed at the wild-type level in the Pep^- background, Kex2p exhibited markedly increased stability when labeled at 30°C and chased at either 30 or 37°C (Figure 1C and Table 1). Even when expressed at moderate and high levels in the Pep^- strain, Kex2p was stabilized substantially (Figure 1D and Table 1). Therefore, regardless of expression level, or temperature, degradation of wild-type Kex2p required active vacuolar hydrolases.

Were the vacuole the site of Kex2p degradation in Pep^+ cells, Kex2p would accumulate in the vacuoles of Pep^- cells. Localization of Kex2p was examined by indirect immunofluorescence microscopy in isogenic Pep^+ and Pep^- strains expressing Kex2p at a 150-fold elevated level. Fixed cells were doubly labeled with antibodies specific for Kex2p and Vat2p, a vacuolar membrane marker (Kane *et al.*, 1992). In the Pep^+ cells, anti-Kex2p antibody revealed a punctate distribution reminiscent of Golgi staining of Kex2p seen in cells expressing Kex2p at the wild-type or at a moderately elevated level (Redding *et al.*, 1991), although with greater variation in the size and staining intensity of individual spots of fluorescence (Figure 2, D–F). Frequently, large concentrations of Kex2 antigen were found next to vacuoles, but staining was not found within vacuoles or in vacuolar membranes, as identified by anti-Vat2p staining. In Pep^- cells, in addition to punctate labeling, dramatic labeling of vacuoles was observed with the anti-Kex2p antibody (Figure 2, G–I). Unlike Vat2 staining, which was restricted to the vacuolar membrane, Kex2 staining frequently appeared to fill the volume of the vacuole and sometimes appeared as punctate staining within the vacuole.

Immunogold electron microscopy was used to analyze Kex2p localization at higher resolution. Little gold labeling was seen in sections of $\text{Pep}^- \text{kex2}\Delta$ cells (Figure 3A) or, in corroborative experiments, $\text{Pep}^+ \text{kex2}\Delta$ cells. In sections of the Pep^+ cells overexpressing Kex2p, most gold particles labeled clusters of vesicles, frequently adjacent to vacuoles (Figure 3B). Similar vesicle clusters were not observed in $\text{kex2}\Delta$ cells (Figure 3A) or by way of confirmation in either wild-type cells or cells expressing Kex2p at only a 15-fold elevated level, sug-

gesting that the vesicle clusters form in response to high-level expression of Kex2p. This phenomenon might be analogous to formation of "karmellae" in response to overproduction of [3-hydroxy-3-methylglutaryl] (HMG)-CoA reductase (Wright and Rine, 1989). Gold particles were also associated with cytoplasmic tubules and vesicles that may correspond to endoplasmic reticulum (ER) and Golgi structures (Wright and Rine, 1989; Preuss *et al.*, 1991, 1992). Consistent with immunofluorescence results, little labeling of vacuolar structures

Figure 3. Immunogold electron microscopy localization of Kex2p to the vacuolar lumen in Pep^- cells. Strains CB017[pAB23] ($\text{kex2}\Delta \text{Pep}^-$; A), W303-1A[pAB-KX22] (Pep^+ Kex2p-overproducer; B), and CB018[pAB-KX22] (Pep^- Kex2p-overproducer; C-E) were processed for immunogold electron microscopy as described in MATERIALS AND METHODS. N, nucleus; V, vacuole. Arrowheads point to clusters of vesicles labeled by anti-Kex2 antibody. Labeling of the vesicle clusters was statistically significant; the probability of the observed labeling occurring by chance was $<10^{-6}$ (Student's *t*-test). Vesicles labeled by anti-Kex2 antibody were of two types. Oval vesicles (average dimensions of 113 ± 27 by 89 ± 20 nm) were more abundant. These usually occurred in large closely juxtaposed clusters (97% of the ovals were within 40 nm of another vesicle; clusters had 6.3 ± 4.3 ovals), often near vacuoles (65% of the clusters were within 40 nm of a vacuolar membrane). Circular vesicles (average diameter of 57 ± 12 nm) were less frequently observed and were found among groups of oval vesicles or in more dispersed groups composed solely of circular vesicles.

was observed. In contrast, sections of Pep⁻ cells over-producing Kex2p exhibited heavy labeling of both vacuolar membranes and interiors in addition to extensive labeling of vesicle clusters (Figure 3, C-E). Internal labeling of vacuoles was associated with electron-lucent structures that appear to be vesicles within the vacuolar lumen (Figure 3E). Kinetic analysis of posttranslational modifications of Kex2p has shown that at both wild-type and elevated levels of expression, wild-type Kex2p is transported from the ER to the Golgi complex (Wilcox and Fuller, 1991). Taken together, these results strongly support the conclusion that wild-type Kex2p is degraded in the vacuole after transport from the Golgi complex.

Mutagenesis of the Kex2p C-Tail

The KEX1 gene product of *Kluyveromyces lactis*, a functional homologue of *S. cerevisiae* Kex2p, is 63% identical to Kex2p over its entire length (Tanguy-Rougeau *et al.*, 1988). Comparison of the two revealed that the *K. lactis* Kex1p C-tail represents a naturally occurring deletion of 70 residues internal to the *S. cerevisiae* Kex2p C-tail, leaving two conserved regions, one adjacent to the TMD and the other near the very C-terminus (Figure 4). The first has a polarized charge distribution with a basic region (7 of 14 residues in the B-block are Arg or Lys) followed by an acidic region (6 of 15 residues in the A-block are Asp and Glu, with a seventh Glu residue in the B-block). Two Tyr residues found in the B- and A-blocks, Tyr₇₁₃ and Tyr₇₂₄, were of particular note, given the requirement for specific Tyr residues along with contextual sequences in directing numerous cell surface receptors to clathrin-coated pits (Davis *et al.*, 1987; Lazarovitis and Roth, 1988; Ktistakis *et al.*, 1990; Pearse and Robinson, 1990). Mutations created to probe the role of specific sequence elements within the Kex2 C-tail are diagrammed in Figure 4 (see MATERIALS AND METHODS for details). The two Tyr residues were

changed to Ala both individually (Y₇₁₃A and Y₇₂₄A) and together (Y_{713,724}A). The B- and A-blocks were deleted individually (B-blockΔ and A-blockΔ) and together (BA-blockΔ). Insertion of a termination codon just beyond codon 701 left only two residues of the tail (C-tailΔ). Insertion of a termination codon just beyond codon 728 created a tail comprising the B- and A-blocks (BA-tail). The conserved sequence near the CO₂H-terminus began with Asn and was designated the N-block. Insertion of a termination codon just beyond codon 797 deleted the N-block along with distal residues (N-blockΔ). Deletion of codons 729–797 created a C-tail similar to that of *K. lactis* Kex1p (*K. lactis*-like tail).

C-tail Mutant Proteins are Active and do not Accumulate at the Cell Surface

Cells expressing the wild-type and C-tail mutant genes contained similar levels of Kex2 activity (Table 2). Therefore, none of the C-tail mutations reduced formation of active enzyme. To determine whether the mutant genes complemented a *kex2* null mutation, the genes were expressed under control of the wt KEX2 promoter on a single copy centromeric plasmid in MATα *kex2*Δ strain BFY106-4D. All of the C-tail mutant genes restored mating competence and α-factor production (Wilcox, 1991), as measured by the α-factor halo assay (Julius *et al.*, 1984a). However, decreased halo size was observed with the C-tailΔ, suggesting reduced accumulation of the C-tailΔ enzyme in the pro-α-factor processing compartment (Wilcox, 1991).

To determine whether mutant proteins were exported to the plasma membrane, cells expressing wild-type and C-tail mutant Kex2p under GAL1 promoter control were assayed without permeabilization to measure cell surface enzyme (Fuller *et al.*, 1989b). Less than 0.3% of total activity was measurable in intact cells expressing

Figure 4. Comparison of C-tails of *S. cerevisiae* Kex2p and *K. lactis* Kex1p. Mutations created in the Kex2 C-tail are indicated. C-terminal deletions KEX2Δ4 and KEX2Δ6, respectively, end with the sequences: -Phe₇₁₅-Asn-Arg-CO₂H and -Pro₆₁₄-Val-Ile-Gly-Asn-Arg-CO₂H, where the residues in boldface indicate residues appended by translation reading into a terminator-linker (Fuller *et al.*, 1989a,b).

Table 2. Cell-surface activity of wild-type and mutant Kex2 proteins

Form of Kex2p on plasmid	Total Kex2 activity ^a	Cell-surface Kex2 activity ^a	% Kex2 activity at cell surface
None (vector)	−0.3 ± 0.8 ^b	−0.01 ± 0.02 ^b	—
Wild-type	159 ± 37	0.36 ± 0.04	0.23 ± 0.06
Y ₇₁₃ A	189 ± 52	0.29 ± 0.03	0.16 ± 0.04
Y ₇₂₄ A	168 ± 36	0.48 ± 0.11	0.29 ± 0.09
Y _{713,724} A	162 ± 38	0.28 ± 0.07	0.17 ± 0.06
A-blockΔ	149 ± 26	0.45 ± 0.10	0.30 ± 0.08
B-blockΔ	140 ± 25	0.28 ± 0.02	0.20 ± 0.04
BA-blockΔ	142 ± 24	0.52 ± 0.17	0.37 ± 0.14
C-tailΔ	125 ± 20	1.38 ± 0.11	1.10 ± 0.20
<i>K. lactis</i> -like	157 ± 34	0.30 ± 0.04	0.19 ± 0.05
N-blockΔ	153 ± 25	0.30 ± 0.03	0.20 ± 0.04
BA-tail	156 ± 28	0.23 ± 0.04	0.15 ± 0.04
Kex2Δ4 ^c	56.3 ± 7.6	0.51 ± 0.01	0.90 ± 0.12
Kex2Δ6 ^c	10.7 ± 7.1	5.51 ± 0.95	51.6 ± 35.6

^a Kex2 protease activities: units/10⁶ cells. Strain CB017, harboring plasmids expressing the indicated forms of Kex2p via the *GAL1* promoter, was grown at 30°C in synthetic galactose medium and harvested in log phase. (n = 5 except for Kex2Δ4, where n = 3). Under *GAL1* promoter control on a single copy centromeric vector, total Kex2 activity is elevated 15-fold relative to the level found in cells with a single copy of the wild-type *KEX2* gene (Redding *et al.*, 1991).

^b Activities measured in the presence of EDTA were higher than in Ca²⁺.

^c Expression of Kex2Δ4p and Kex2Δ6p is about three-fold lower than for other forms of Kex2p due to a difference in the 5' untranslated sequences (Fuller *et al.*, 1989a). Because Kex2Δ6p is largely secreted into the medium, the fraction at the cell surface probably represents periplasmic enzyme (Gluschkankof and Fuller, unpublished data).

either wild-type Kex2p or any of the C-tail mutants that retain the TMD, with two exceptions (Table 2). Approximately 1.2–1.4% of the activity of the C-tailΔ was consistently measured at the cell surface. Previously, high level expression of *KEX2Δ4*, which is truncated after Tyr₇₁₃, resulted in significant accumulation of enzyme at the cell surface (Fuller *et al.*, 1989b). In contrast, when *KEX2Δ4* was expressed at a level elevated five-fold relative to wild-type, only ~0.9% of total activity was measurable in intact cells (Table 2). However, in concert with previous results (Fuller *et al.*, 1989b), Kex2Δ6p, which lacks the TMD and C-tail, was readily detectable at the cell surface (Table 2). Thus, at moderate expression levels, the vast majority of wild-type Kex2p and all C-tail mutant proteins was intracellular.

Mutation of Tyr₇₁₃ Results in Rapid Transport of Kex2p to the Vacuole

To assess whether C-tail mutations affected intracellular localization of Kex2p, the half-lives of the wild-type and C-tail mutant proteins expressed from the *KEX2* promoter on a single copy centromeric plasmid were

determined. Wild-type Kex2p expressed from the plasmid exhibited the expected half-life of ~80 min at 30°C, and shifting cells to 37°C before pulse-labeling resulted in an increased turnover rate (Figure 5 and Table 3). In contrast, the Y₇₁₃A mutant turned over at a 3.8-fold increased rate relative to wild-type at 30°C and, after shifting to 37°C, at a 2.7-fold increased rate (Figure 5 and Table 3). The Y₇₂₄A mutant was degraded at the same rate as wild-type protein at both temperatures, but the double mutant, Y_{713,724}A, behaved like the Y₇₁₃A mutant. When the same plasmids were introduced into *Pep[−] kex2Δ* strain CB017, both the mutant and wild-type proteins were stabilized indefinitely (Figure 5 and Table 3), indicating that degradation depended on vacuolar proteolysis.

To determine whether the accelerated degradation of the Y₇₁₃A and Y_{713,724}A proteins reflected an increased rate of transport to the vacuole, localization of the mutant proteins and controls was determined by indirect immunofluorescence in a *Pep[−]* strain. As shown in Figure 6 (B and D), cells expressing the wild-type and the Y₇₂₄A proteins exhibited the dispersed punctate fluorescence typical of wild-type Kex2p. In contrast, cells expressing the Y₇₁₃A or Y_{713,724}A proteins exhibited little punctate fluorescence with the anti-Kex2 antibody. Instead, most of the fluorescence corresponded precisely to the vacuolar membrane as indicated both by anti-Vat2 staining and differential interference contrast (Figure 6, C and E). Localization of the mutant proteins to the vacuolar membrane in *Pep[−]* cells was in sharp contrast to the localization of overexpressed wild-type Kex2p to the vacuolar lumen. Additional fields of cells expressing the Y₇₁₃A and Y₇₂₄A Kex2p mutants are presented in Figure 7.

The Membrane-Proximal Portion of the Tail is Required for Golgi Retention

Deletion of the B-block, which removed Tyr₇₁₃, resulted in a dramatically increased rate of degradation (Figure 5 and Table 3). However, deletion of the A-block, which left Tyr₇₁₃ intact, also reduced the half-life, as did deletion of the entire BA region (Figure 5 and Table 3). In each case, stability was decreased further when cells were shifted to 37°C before labeling, and expression in the *Pep[−]* background blocked degradation completely. Indirect immunofluorescence confirmed localization of these proteins to the vacuolar membrane (Figure 6, F–H). These results suggested that contextual sequences in the A-block in addition to Tyr₇₁₃ are required for effective retention of Kex2p in the Golgi. Deletion of sequences distal to the A-block, creating the BA-tail mutant, resulted in a protein nearly as stable as wild-type Kex2p, with a half-life of 59 min at 30°C (Figure 5 and Table 3). Thus, the BA-block appears to constitute the entire portion of the C-tail required for Golgi retention.

A trivial explanation for the instability of the C-tail mutants might be that disrupting the structure of the C-tail causes the proteins to be recognized as "abnormal" and to be transported to the vacuole. If so, then the C-tail Δ mutant, lacking any tail structure, should be stable. However, the C-tail Δ protein was the least stable form analyzed, with a half-life of 11 min at 30°C (Figure 5 and Table 3). Unfortunately, the antibody used for immunoprecipitation of C-tail Δ Kex2p (and the BA-tail protein), which was directed against the luminal domain of Kex2p, was not suitable for immunofluorescence. However, like the other forms of Kex2p, the C-tail Δ protein was completely stabilized in the Pep⁻ strain (Figure 5 and Table 3). The half-life of the C-tail Δ protein was only slightly longer than the half-time for transport of pro-carboxypeptidase Y and pro-alkaline phosphatase to the vacuole (~6 min) (Hasilik and Tanner, 1978; Klionsky and Emr, 1989), suggesting that the C-tail Δ protein may follow a relatively unimpeded route to the vacuole.

Wild-Type and Mutant Kex2ps Travel from the ER to the Golgi but not to the Plasma Membrane Before Reaching the Vacuole

The possibility that the mutant proteins were recognized as abnormal in the ER and were transported directly to the vacuole was unlikely because all of the mutants supported processing of pro- α -factor (Wilcox, 1991) (also evident from Figures 7 and 8), an event that ordinarily occurs in the Golgi (Julius *et al.*, 1984b; Franzusoff and Schekman, 1990; Graham and Emr, 1991). Corroborative rapid pulse-chase analysis of the Y₇₁₃A and C-tail Δ proteins demonstrated that these molecules underwent the same posttranslational modifications as wild-type Kex2p (Wilcox and Fuller, 1991) with similar kinetics, indicating that the C-tail mutant proteins are transported first to the Golgi and then to the vacuole.

If either wild-type or mutant Kex2ps must first reach the plasma membrane before arriving at the vacuole through some endocytotic mechanism, then a block in transport between the Golgi and the plasma membrane should prevent the proteins from reaching the vacuole. To test this, we constructed congenic *sec1 kex2* strains expressing the wild-type or mutant Kex2ps from the *KEX2* promoter using a single copy centromeric vector. The *sec1-ts* mutation blocks fusion of secretory vesicles with the plasma membrane at the restrictive temperature of 37°C (Novick and Schekman, 1979). The half-lives of wild-type Kex2p and Y₇₁₃A and C-tail Δ mutant Kex2ps in the *sec1* strain at 37°C were not significantly different from those determined at 37°C in the Sec⁺ background (Table 4). Moreover, degradation of all three proteins in the *sec1* background at 37°C required vacuolar proteolysis as evidenced by the stabilizing effect of the *pep4* mutation in each case (Table 4). These results indicate that fusion of secretory vesicles with the plasma

membrane was not required for delivery of either wild-type or mutant Kex2ps to the vacuole. Therefore, we conclude that vacuolar transport of neither the wild-type nor mutant proteins involves transit to the plasma membrane followed by endocytotic uptake.

Deletion of the N-block Decreases the Rate of Transport to the Vacuole

The N-block Δ was the only C-tail mutant form of Kex2p that was transported to the vacuole more slowly than wild-type Kex2p ($t_{1/2}$ = 110 min; Figure 5 and Table 3). Like wild-type Kex2p, indirect immunofluorescence revealed its localization in a punctate distribution (Figure 6J). Thus, the N-block sequence may play a role, although clearly not an essential one, in targeting wild-type Kex2p to the vacuole for degradation. Consistent with this interpretation, the *K. lactis*-like tail mutant, equivalent to the BA-tail with the N-block added (except for deletion of Asp₇₂₉), was significantly less stable than the BA-tail mutant, having a half-life of 25 min at 30°C (Figure 5 and Table 3). Surprisingly, the *K. lactis*-like tail mutant was localized in a punctate distribution (Figure 6I), the only rapidly degraded form of Kex2p that gave such a result. However, careful examination of the staining revealed that much of the *K. lactis*-like tail mutant protein may lie inside vacuoles.

A Direct Biological Assay for Defective Golgi Retention of Kex2p C-Tail Mutants

The preceding results showed that turnover of all wild-type and mutant forms of Kex2p occurred in the vacuole, although at widely different rates. Immunolocalization in the Pep⁻ strain showed that degradation rates reflected rates of transport to the vacuole, rather than the intrinsic susceptibilities of the wild-type and mutant proteins to vacuolar proteolysis. We reasoned that it should be possible to measure the relative rates of transport of wild-type and mutant Kex2p out of the pro- α -factor processing compartment by measuring the rates of loss of mating competence of a MAT α strain after shutting off synthesis of wild-type and mutant proteins. This "Kex2p depletion experiment" was performed as follows. MAT α *kex2* Δ strains containing the wild-type, Y₇₁₃A, and C-tail Δ *KEX2* genes under *GAL1* promoter control were grown in 2% galactose and then shifted to media containing 2% glucose to repress transcription. Samples removed at intervals after the shift were assayed for mating competence (by a quantitative mating assay) and Kex2 specific activity. After shutting off synthesis of Kex2p, the level of α -factor production should begin to drop when the concentration of Kex2 protease in the pro- α -factor processing compartment decreases below the level at which the enzyme becomes limiting for pro- α -factor cleavage. In turn, mating competence should begin to drop when the rate of α -factor production decreases below a threshold at which α -fac-

tor becomes limiting for mating. After shutting off synthesis, the rate of decrease of enzyme concentration in the processing compartment should depend on 1) dilution due to growth of the cells (with a $t_{1/2}$ = doubling time of the cells), 2) transport of Kex2p out of the processing compartment, and 3) inactivation of enzyme within the processing compartment. The rate of decrease of enzyme specific activity should depend only on the rate of dilution due to growth and the overall rate of inactivation of the enzyme, whether by vacuolar degradation or another process.

After shifting Pep^+ cells expressing wild-type Kex2p to glucose, a 4-h lag elapsed before mating competence decreased. The $Y_{713}A$ mutant exhibited only a slight lag of <2 h, whereas no lag was observed with the C-tail Δ mutant (Figure 8A). This difference in lag times demonstrated that wild-type Kex2p populates the processing compartment at a much higher steady-state level than the mutant forms. After the lag (if any), the rates of decrease of mating competence paralleled the rates of degradation of the form of Kex2p expressed, consistent with the interpretation that increased rates of vacuolar-protease dependent degradation reflected increased rates of transport out of the processing compartment. By 16 h, mating efficiencies plateaued at levels characteristic of each form of Kex2p, with wild-type ($5 \pm 2 \times 10^{-3}$) > $Y_{713}A$ ($2 \pm 0.4 \times 10^{-3}$) > C-tail Δ ($3 \pm 0.7 \times 10^{-4}$). This mating depended on low level transcription from the *GAL1* promoter in the presence of 2% glucose, because the same *MAT α kex2 Δ* strain transformed with vector alone exhibited a mating efficiency of 3×10^{-6} (4×10^{-6} for Pep^-). Thus, under conditions of exceedingly low level expression, wild-type and mutant forms of Kex2p still displayed characteristic efficiencies of retention in the Golgi.

Unlike mating, enzyme specific activity decreased without a lag for both wild-type and mutant Kex2ps (Figure 8B). The rate of decrease was substantially greater for the C-tail mutant proteins than for wild-type, consistent with more rapid transport of the mutant forms to the vacuole. Inactivation by processes other than vacuolar protease-dependent degradation appeared to be negligible. When expressed in a Pep^- background, the specific activity of wild-type and mutant Kex2p decreased at identical rates after imposition of glucose repression (Figure 9, A–C). The half-time for decrease in specific activity was ~ 1.6 h. This was close to the doubling time, which declined from 2.0 to 1.6–1.7 h during the experiment. From this, a minimum half-time for enzyme inactivation in the Pep^- strain of

Table 3. Half-lives of C-tail mutants of Kex2p

Form of Kex2p on plasmid	Half-lives (min) ^a		
	30°C	37°C	Pep^- , 30°C
Wild-type	83 $\pm$ 9	30 $\pm$ 2	>180
$Y_{713}A$	22 $\pm$ 1	11	>180
$Y_{724}A$	86 $\pm$ 2	31	>180
$Y_{713,724}A$	23 $\pm$ 3	17 $\pm$ 5	>180
A-block Δ	18 $\pm$ 1	13	>180
B-block Δ	12 $\pm$ 3	8	>180
BA-block Δ	19 $\pm$ 5	10 $\pm$ 1	>180
C-tail Δ	11 $\pm$ 1	7.6	>180
<i>K. lactis</i> -like	25 $\pm$ 1	ND	>180
N-block Δ	110 $\pm$ 15	36 $\pm$ 2	>180
BA-tail	59 $\pm$ 1	ND	>180

^a Cells were grown at 30°C before the pulse-labeling and chasing at 30 or 37°C. The Pep^+ strain is BFY106-4D and the Pep^- strain is CB017. ND, not determined.

6 h can be calculated. Therefore, in the absence of vacuolar proteolysis, enzyme specific activity decreased almost exclusively by dilution and vacuolar transport of neither wild-type nor mutant Kex2p depended on prior inactivation in the Golgi.

Mating was surprisingly efficient with low levels of wild-type Kex2 protease. At 4 h after shutting off wild-type Kex2p, Kex2 specific activity was ~ 10 -fold less than that found in cells with a single chromosomal *KEX2* gene, yet mating was unaffected. By the time Kex2 specific activity had dropped to 2–4 U/mg (at 8 h for wild-type and 4 h for both mutants), mating competence of wild-type was reduced only 4-fold but that of the $Y_{713}A$ and C-tail Δ mutants had decreased 60-fold and 580-fold, respectively.

Differential Effects of Pep^+ and Pep^- on Rates of Loss of Mating Competence for Wild-Type and C-Tail Mutant Forms of Kex2p

In the Pep^+ background, transport of Kex2p out of the pro- α -factor processing compartment to the vacuole resulted in degradation of the enzyme. In the Pep^- background, active Kex2p persisted in the vacuole. If there existed a retrograde pathway from the vacuole to the pro- α -factor processing compartment, then loss of mating competence would be faster in a Pep^+ than in a Pep^- strain. Although the rates of decay of enzyme spe-

Figure 5. Pulse-chase immunoprecipitation analysis of wild-type and mutant Kex2ps. Transformants of BFY106-4D (*kex2 Δ Pep $^+$*) harboring the indicated plasmids (pCWKX10–pCWKX110) were grown at 30°C, harvested, and split. One half was kept at 30°C and the other was shifted to 37°C during the 30-min sulfate depletion before labeling each with [³⁵S]-H₂SO₄ for 10 min. A chase was initiated at t_0 , and samples were harvested at the indicated times. For the Pep^- series, transformants of CB017 (*kex2 Δ Pep $^-$*) harboring the indicated plasmids (pCWKX10–pCWKX110) were grown, labeled, and chased at 30°C. The anti-Kex2 luminal antiserum was used to immunoprecipitate the C-tail Δ , *K. lactis*-like and BA-tail forms of Kex2p; the anti-Kex2 tail antibody was used in all other cases.

Figure 6. Steady-state vacuolar localization of mutant Kex2ps. Wild-type and mutant Kex2ps were expressed under control of the *GAL1* promoter in strain CB017 (*kex2Δ Pep⁻*). Cells were processed for double-label immunofluorescence as in Figure 2. Left, differential interference contrast; middle, Vat2 immunofluorescence; right, Kex2 immunofluorescence. Forms of Kex2p expressed were as follows: A, none (cells contained vector pBM258); B, wild-type; C, Y₇₁₃A; D, Y₇₂₄A; E, Y_{713,724}A; F, A-blockΔ; G, B-blockΔ; H, BA-blockΔ; I, *K. lactis*-like; J, N-blockΔ. Bar equals 5 μ m.

cific activity in Pep^+ and Pep^- cells differed markedly after shutting off expression of wild-type Kex2p, the rates of loss of mating competence were identical in the two strains (Figure 9A). Thus, transport of wild-type Kex2p from the pro- α -factor processing compartment to the vacuole appeared to be irreversible. However, with both Y_{713}A Kex2p (Figure 9B), and to a lesser extent the C-tail Δ Kex2p (Figure 9C), loss of mating competence was reproducibly slower in the Pep^- than in the Pep^+ background.

DISCUSSION

Although long-lived, wild-type Kex2p is eventually transported from a late Golgi compartment to the vacuole, where it is degraded. Mutations in the membrane proximal sequences in the Kex2p C-tail result in accelerated transport to the vacuole and appear to define a Golgi-retention signal that resembles mammalian coated pit localization signals. Depletion experiments showed that, in comparison to wild-type Kex2p, the Y_{713}A and C-tail Δ proteins exhibit both higher rates of exit from and decreased steady-state concentrations in the pro- α -factor processing compartment. Loss of mating competence during depletion provides a powerful biological assay that can be used to identify genes involved in retention of Kex2p in the Golgi and in transport of both mutant and wild-type Kex2p to the vacuole. We have

Table 4. Half-lives of wild-type and mutant Kex2ps in a *sec1* mutant at 37°C

Form of Kex2p on plasmid	Half-lives (min) at 37°C ^a		
	$\text{Sec}^+ \text{Pep}^+$	<i>sec1</i> Pep^+	<i>sec1 pep4</i>
Wild-type	30 ± 2	34	>180
Y_{713}A	11	11	>180
C-tail Δ	7.6	8.4	>180

^a Cells were grown at 25°C before sulfate-depletion, pulse-labeling, and chase at 37°C. The *sec1* strains are KRY108-5D (Pep^+) and KRY108-7D (*pep4*). The Sec^+ strain is BFY106-4D, and the data are taken from Table 3 for facile comparison.

exploited this system to isolate mutants that exhibit a decreased rate of loss of mating competence after shutting off expression of Y_{713}A Kex2p (Redding and Fuller, unpublished results).

The Golgi Retention Signal of Kex2p and the Mechanism of Retention

The effects of mutations in the TMD-proximal residues of the C-tail and the near wild-type stability of BA-tail Kex2p strongly support the conclusion that the first ~30 residues of the C-tail contain a signal necessary for re-

Figure 7. Differential localization of Kex2p mutants Y_{713}A and Y_{724}A . Strain CB017 expressing either the Y_{713}A (A–C) or the Y_{724}A (D–F) form of Kex2p was processed and double-labeled for Vat2p (B and E) and Kex2p (C and F) as in Figures 2 and 6. Corresponding differential interference contrast micrographs are displayed in A and D. Bar equals 5 μm .

Figure 8. Decay of enzyme activity and mating competence in the Pep⁺ background. Strain BFY106-4D (*kex2Δ* Pep⁺) expressing the indicated forms of Kex2p from the *GAL1* promoter were grown in synthetic galactose medium. After shifting to glucose, aliquots were taken at the indicated times. For each aliquot, mating competence (A) and Kex2 specific activity (B) were determined. ■, wild-type; ▲, Y₇₁₃A; ●, C-tailΔ.

tention in the pro- α -factor processing compartment. Containing an essential Tyr residue along with contextual sequences, the signal in Kex2p resembles signals in the C-tails of mammalian receptors that direct their localization to and internalization by clathrin-coated pits. Sequences surrounding Tyr₇₁₃ in Kex2p fit the criteria proposed for a "tyrosine internalization signal" significantly better than do the sequences surrounding Tyr₇₂₄. Thirteen C-tails examined had basic or polar residues at 5.6 ± 0.9 of nine positions surrounding an essential tyrosine (Ktistakis *et al.*, 1990). Eight C-tails had such residues at five out of nine positions, whereas none had fewer. Sequences surrounding Tyr₇₁₃ in Kex2p conformed to this consensus at five of nine positions, whereas those surrounding Tyr₇₂₄ did at only two of

nine positions. Nuclear magnetic resonance (NMR) structures of the coated pit localization signals of the (low density lipoprotein) LDL-receptor and lysosomal acid phosphatase locate the essential Tyr residue in the fourth position of a type I β turn (Bansal and Gierasch, 1991; Eberle *et al.*, 1991). In Kex2p, a type I β turn having Tyr₇₁₃ as the fourth residue might appose the B- and A-blocks as antiparallel β strands stabilized by multiple interstrand salt bridges. Such structural features of the C-tail might account for greater retention of Y₇₁₃A Kex2p in comparison to the C-tailΔ mutant.

Mutations in the Golgi retention signal in Kex2p resulted in rapid transport to the vacuole; however, deletion of the *CHC1* gene encoding clathrin heavy chain caused transport of Kex2p to the plasma membrane (Payne and Schekman, 1989). Models invoking clathrin-dependent intracellular retention of Kex2p must resolve this apparent contradiction. Loss of clathrin heavy chain might have an indirect effect on retention of Kex2p by disrupting Golgi function. However, inactivation of a Ts⁻ *CHC1* allele has selective effects on Golgi membrane proteins, resulting in rapid appearance at the plasma membrane of Kex2p and Ste13 dipeptidyl aminopeptidase but not guanosine diphosphatase (Seeger and Payne, 1992a). Alternatively, clathrin might form part of a stable structural element of the Golgi that binds statically to the Kex2p C-tail. Although formally possible, this model fails to invoke the dynamic aspects of known roles of clathrin in mammalian cells, and neither this nor the previous model accounts for the differential effects of C-tail and clathrin mutations in an obvious way. Net retention in the Golgi might result from constitutive transport of Kex2p to the plasma membrane followed by rapid clathrin-dependent retrieval. However, immunolocalization and fractionation experiments indicate that the bulk of Kex2p does not follow an obligatory pathway to the cell surface in secretory vesicles (Redding *et al.*, 1991). This model also predicts that C-tail mutant Kex2p should accumulate at the plasma membrane, which does not occur except at very high levels of expression (Fuller *et al.*, 1989b).

We favor a fourth possibility, in which Kex2p follows an intracellular recycling pathway (Figure 10A). In this model, interaction of the C-tail of Kex2p with a clathrin-coated pit in the (hypothetical) yeast *trans* Golgi network (TGN) incorporates the enzyme along with substrates into a nascent "processing vesicle." Maturation of the processing vesicle gives rise to a secretory vesicle that delivers processed products to the cell exterior by fusion with the plasma membrane and a coated "return vesicle" that recycles Kex2p and possibly other molecules to the TGN. This cycling pathway may explain the continual addition of α -1,3-linked mannose to wild-type Kex2p in the Golgi (Wilcox and Fuller, 1991), despite evidence for presence of α -1,3-mannosyl transferase and Kex2p in distinct compartments (Cunningham and Wickner, 1989; Graham and Emr, 1991). Mutation of

the C-tail blocks entry of Kex2p into (or retention in) the processing vesicle (Figure 10B). Failing to enter the processing vesicle, C-tail mutant forms of Kex2p are transported to the vacuole. Possible reasons for this vacuolar transport will be discussed later.

With certain assumptions, the intracellular cycling model can explain transport of wild-type Kex2p to the plasma membrane in clathrin-deficient cells. First, interaction of the C-tail of Kex2p with a clathrin-coated pit is probably mediated not by clathrin but by "adaptors." Candidate adaptors are the hetero-oligomeric "AP" complexes of "adaptors," distinct classes of which are found at the plasma membrane and TGN (Pearse and Robinson, 1990). APs underlie the clathrin coats of pits and vesicles and are thought to mediate specific interactions with receptor C-tails (Pearse and Robinson, 1990). Yeast homologues of AP subunits have been identified, although no function has been attributed to any of these (Kirchhausen, 1990; Kirchhausen *et al.*, 1991; Nakayama *et al.*, 1991). Second, the adaptors that recognize the C-tail of Kex2p are presumed to assemble on the processing vesicle in the absence of clathrin. Indeed, APs have been shown to bind to plasma membranes with high affinity at presumptive coated pit assembly sites (Mahaffey, *et al.*, 1990). Given these assumptions, Kex2p may enter the processing vesicle due to association with an adaptor lattice that forms even in the absence of clathrin (Figure 10C). Without clathrin, no return vesicle forms, and the defective processing vesicle proceeds to the cell surface by virtue of targeting information present in the secretory vesicle portion of the structure and delivers Kex2p and other molecules to the plasma membrane. This model predicts that the C-tailΔ and Y₇₁₃A mutants expressed in a *chc1* mutant would be transported to the vacuole rather than the plasma membrane. Interpretation of such experiments may be complicated if clathrin is also involved in transport from the Golgi to the vacuole (Seeger and Payne, 1992b).

This model for intracellular recycling of Kex2p recalls features of maturation of secretory granules in neu-

Figure 9. Differential effects of Pep⁺ and Pep⁻ backgrounds on depletion of wild-type and mutant Kex2ps. Strains BFY106-4D (Pep⁺, ■, ●) and CB017 (Pep⁻, □, ○) expressing the indicated forms of Kex2p from the *GAL1* promoter were grown in synthetic galactose medium. After shifting to glucose, aliquots were taken at the indicated times. For each aliquot, mating competence (●, ○) and Kex2 specific activity (S. A., ■, □) were determined. A, wild-type; B, Y₇₁₃A; C, C-tailΔ. The *t*_{1/2} for decay of Kex2 enzyme specific activity in the Pep⁻ strain was determined from linear-regression analysis to be 1.6 h (wild-type), 1.5 h (Y₇₁₃A), and 1.6 h (C-tailΔ). The doubling times of the strains decreased gradually from 2.0 h at *t* = 0 to 1.6–1.7 h at *t* = 24 h. Thus, the half-time for loss of Kex2 activity by processes other than dilution was ≥6 h. At *t* = 24 h, Kex2 specific activities were in all cases ≤2% of normal wild-type level and ≤0.2% the level achieved with full induction in 2% galactose. These levels were so low that the values at late times are not significant.

Figure 10. Models for localization of wild-type and mutant Kex2ps. Major pathways of Kex2p transport are indicated by bold arrows. (A) Retention of Kex2p by intracellular recycling. In the TGN, Kex2p (represented as “pac-man on a stick”) is bound to a coated pit (CP) consisting of an underlying adaptin lattice (stippled) and an outer clathrin coat (cross-hatched) and is incorporated along with pro- α -factor (p α F) into a nascent processing vesicle (PV). The PV matures into a secretory vesicle (SV) that delivers α -factor (α F) to the plasma membrane (PM) and a recycling vesicle (RV) that delivers Kex2p to the TGN. (B) Default transport to the vacuole of C-tail mutant Kex2p. Lacking a CP localization signal, mutant Kex2p fails to associate with the CP and is transported to the vacuole (V). The PV lacks Kex2p, but some processing of pro- α -factor occurs due to Kex2p in the TGN. (C) Transport of wild-type Kex2p to the PM in a clathrin-deficient mutant. In the absence of clathrin, Kex2p still associates with the adaptin lattice at the TGN in a defective coated pit (dCP). Due to absence of clathrin, the defective PV (dPV) cannot give rise to an RV and therefore delivers Kex2p to the PM. Inefficient processing of pro- α -factor results from failure to concentrate Kex2p in the late Golgi (Wilcox and Fuller, 1991). (D) Sorting of Kex2p to the Endo-TGN. Kex2p, Kex1p, Ste13p and substrates are sorted into the Endo-TGN. Bulk secretory proteins, such as invertase (Inv), that are destined for the PM or for secretion, enter the Exo-TGN, perhaps by default. The Endo- and Exo-TGN may be distinct vesicular structures or subdomains of the TGN. This ambiguity is indicated by the question mark and dashed structure connecting the Endo- and Exo-TGN. The default pathway from the Endo-TGN is to the vacuole. In this model, the return vesicle might be targeted either to the Endo-TGN or to an earlier Golgi compartment. In the latter case, the Endo-TGN might be viewed as an immature endosome.

roendocrine cells. Processing of precursors in the regulated secretory pathway occurs mostly in condensing secretory granules, although it may begin in the *trans*-most Golgi cisternae (Orci *et al.*, 1987; Tooze *et al.*, 1987; Fisher *et al.*, 1988). In pancreatic β cells and anterior pituitary corticotrophs, nascent secretory granules containing proinsulin or proopiomelanocortin bud from the TGN with partial clathrin coats, but clathrin is absent from dense core granules in which processing is complete (Orci *et al.*, 1987; Tooze *et al.*, 1987). Possibly, the partial clathrin coat is a coated pit involved in recycling processing machinery to the TGN. Unlike secretory granules, whose fusion with the plasma membrane is regulated, secretory vesicles containing mature α -factor constitutively fuse with the plasma membrane. It is intriguing that the mammalian Kex2p homologue furin, localized to the Golgi in cells thought to possess only a constitutive secretory pathway, has, like Kex2p, both a TMD and a cytosolic tail containing two Tyr residues (Roebroek *et al.*, 1986; Fuller *et al.*, 1989b; Bresnahan *et al.*, 1990).

Vacuolar Targeting of Kex2p: Signal-Dependent or Default?

Accelerated transport to the vacuole on overexpression of wild-type Kex2p or mutation of the C-tail suggests that on saturation of the retention mechanism or loss of the retention signal, Kex2p travels to the vacuole by default. Vacuolar targeting of Kex2p requires the TMD, because deletion of the TMD results in rapid secretion

of soluble Kex2p (Fuller *et al.*, 1989b; Gluschkof and Fuller, unpublished data). Transport of Kex2p to the vacuole by a true default path (i.e., one requiring no further decoding of targeting information) would challenge the widely held idea that the plasma membrane is the default destination for membrane proteins and imply that specific signals are required for transport to the plasma membrane (Pfeffer and Rothman, 1987). Although signals have been defined for localization of membrane proteins to discrete domains of the plasma membrane (Casanova *et al.*, 1991) and for transcytosis in polarized epithelial cells (Casanova *et al.*, 1990), no signals have yet been identified in membrane proteins that specify transport from the Golgi to the plasma membrane. Conceivably, default transport of Kex2p from the Golgi to the vacuole could involve transport first to the plasma membrane, which would be accelerated by mutation of the Golgi retention signal. However, the failure of the *sec1* mutation to disrupt delivery of wild-type and mutant Kex2ps to the vacuole seems to rule out this possibility.

Alternatively, Kex2p may contain a cryptic or recessive vacuolar targeting signal to which the Golgi retention signal is dominant. Rapid vacuolar degradation of C-tail Δ Kex2p argues that any hypothetical vacuolar targeting signal lies outside of the C-tail. The TMD alone or in combination with the luminal domain might contain a vacuolar targeting signal, or a signal in the luminal domain might require membrane association. Some evidence has been obtained for sorting signals within TMDs of Golgi glycosyl transferases (Munro, 1991;

Nilsson *et al.*, 1991; Wong *et al.*, 1992). Vacuolar targeting of C-tail mutant Kex2p might instead be a fortuitous consequence of perturbing as yet unknown quarternary interactions of Kex2p. By analogy, trimerization of VSV G protein and influenza hemagglutinin is required for progression through the secretory pathway. However, unlike C-tail Δ Kex2p that exits the ER and travels through Golgi compartments to the vacuole, mutant forms of VSV G and flu hemagglutinin that fail to trimerize are retained in the ER (Gething *et al.*, 1986; Kreis and Lodish, 1986; Copeland *et al.*, 1988; Doms *et al.*, 1988).

Another possibility is that the TMD of Kex2p alone or in combination with the lumenal domain "pre-sorts" the protein to a specialized part of the Golgi, designated the "Endo-TGN" in Figure 10D, from which the default pathway is to the vacuole. By default, most molecules destined for secretion or the plasma membrane enter the "Exo-TGN," which gives rise to the bulk of secretory vesicles. This model is consistent with the fact that soluble proteins targeted to the vacuole pass through a compartment containing active Kex2 protease (Graham and Emr, 1991) and with the finding that novel mutants identified as defective in pro- α -factor processing were found to have defects in vacuolar protein sorting (Wilsbach and Payne, personal communication). This model predicts that pro- α -factor should be sorted into the Endo-TGN along with Kex2p and that α -factor should be found in secretory vesicles distinct from those containing bulk secretory proteins such as invertase.

Interestingly, mutation of the Golgi retention signals in the C-tails of the Ste13 dipeptidyl aminopeptidase (Roberts, *et al.*, 1992) and the Kex1 carboxypeptidase (Cooper and Bussey, 1992), transmembrane peptidases that act after Kex2p in α -factor biosynthesis, results in transport of these proteins to the vacuole. Whether vacuolar transport of mutant forms of these three proteins is signal driven or default, it seems likely that the same mechanism is at work in each case.

Two Pathways to the Vacuole?

Surprisingly, more rapid loss of mating competence was observed on depletion of the Y₇₁₃A and C-tail Δ forms of Kex2p in Pep⁺ than in Pep⁻ cells, whereas the rate of loss of mating on depletion of wild-type Kex2p was indistinguishable in the two backgrounds. One explanation is that C-tail mutant forms but not wild-type Kex2p have access to a retrograde pathway from a degradative compartment. Alternatively, transport of the C-tail mutant proteins but not wild-type Kex2p to the vacuole might depend on a factor that becomes limiting in Pep⁻ cells. In either case, these results indicate that the pathways of vacuolar transport differ for the wild-type and mutant forms of Kex2p, which calls into question the notion that overproduction causes rapid vacuolar transport simply by saturation of the retention mechanism.

With a single exception, all of the C-tail mutant forms of Kex2p that exhibited rapid vacuolar turnover were localized to the vacuolar membrane in Pep⁻ cells, suggesting delivery of these proteins by fusion of a carrier vesicle with the vacuolar membrane. In contrast, after shutting off *GAL1* promoter-dependent expression of wild-type Kex2p in Pep⁻ cells, vacuolar membrane localization was never observed. Instead, the wild-type protein continued to localize in a punctate pattern at times at which the bulk of the protein was known to have exited the pro- α -factor processing compartment and ought to have reached the vacuole based on turnover rates measured in Pep⁺ cells (Redding and Fuller, unpublished data). Significantly, the only rapidly degraded form of Kex2p that did not localize to the vacuolar membrane was the *K. lactis*-like tail protein, which retains the putative tyrosine localization signal. The punctate distribution of the *K. lactis*-like tail mutant at steady state and of wild-type Kex2p at extended times after shutting off expression may reflect localization within the vacuolar lumen. An intriguing possibility is that turnover of wild-type Kex2p reflects a normal pathway of organellar turnover by vacuolar engulfment. The N-block might facilitate entry of Kex2p into this pathway.

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