Allele-Specific Suppression of a Defective *trans*-Golgi Network (TGN) Localization Signal in Kex2p Identifies Three Genes Involved in Localization of TGN Transmembrane Proteins

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Kex2 protease (Kex2p) and Ste13 dipeptidyl aminopeptidase (Ste13p) are required in *Saccharomyces cerevisiae* for maturation of the α -mating factor in a late Golgi compartment, most likely the yeast *trans*-Golgi network (TGN). Previous studies identified a TGN localization signal (TLS) in the C-terminal cytosolic tail of Kex2p consisting of Tyr-713 and contextual sequences. Further analysis of the Kex2p TLS revealed similarity to the Ste13p TLS. Mutation of the Kex2p TLS results in transport of Kex2p to the vacuole by default. When expression of a *GAL1* promoter-driven *KEX2* gene is shut off in *MAT α* cells, the TGN becomes depleted of Kex2p, resulting in a gradual decline in mating competence which is greatly accelerated by TLS mutations. To identify the genes involved in localization of Kex2p, we isolated second-site suppressors of the rapid loss of mating competence observed upon shutting off expression of a TLS mutant form of Kex2p (*Y₇₁₃A*). Seven of 58 suppressors were allele specific, suppressing point mutations at Tyr-713 but not deletions of the TLS or entire C-terminal cytosolic tail. By linkage analysis, the allele-specific suppressors defined three genetic loci, *SOI1*, *SOI2*, and *SOI3*. Pulse-chase analysis demonstrated that these suppressors increased net TGN retention of both *Y₇₁₃A* Kex2p and a Ste13p-Pho8p fusion protein containing a point mutation in the Ste13p TLS. *SOI1* suppressor alleles reduced the efficiency of localization of wild-type Kex2p to the TGN, implying an impaired ability to discriminate between the normal TLS and a mutant TLS. *soi1* mutants also exhibited a recessive defect in vacuolar protein sorting. Suppressor alleles of *SOI2* were dominant. These results suggest that the *SOI1* and *SOI2* genes encode regulators or components of the TLS recognition machinery.

Signals that operate through several distinct mechanisms are thought to be involved in localization of proteins to specific compartments of the eukaryotic secretory pathway (36, 39, 43, 57). Signal sequences and mannose-6-phosphate tags are examples of signals that mediate the selective diversion of proteins into specific compartments (31, 57). Retrieval signals, such as the C-terminal HDEL or KDEL signals or KKXX motifs found on resident endoplasmic reticulum proteins, permit the recovery of proteins from more distal compartments via retrograde transport (36, 43). Features of the transmembrane domains of *cis* and medial Golgi proteins may prevent or inhibit exit from Golgi compartments by promoting the assembly of aggregates too large to be incorporated into transport vesicles or by preferential interactions with membranes of certain lipid compositions (33, 36, 43). The localization of integral membrane proteins to the *trans*-Golgi network (TGN) may require both cytosolic and transmembrane sequences and may be achieved through retrieval, retention, or a combination of the two (2, 38, 39, 44, 59). Because of the central role of the

TGN as a site for sorting in both anterograde and retrograde protein transport, the mechanisms of localization of transmembrane proteins to this compartment are of particular interest.

The *Saccharomyces cerevisiae* Kex2 protease (Kex2p) cleaves precursors of secreted peptides, including the α -mating pheromone and K1 killer toxin in a late Golgi compartment (18, 28, 29) that has been identified by indirect immunofluorescence as a set of dispersed, punctate structures in the yeast cytoplasm (15, 47). Proteolytic maturation of α -factor is completed by the action of the Ste13 dipeptidyl aminopeptidase (Ste13p) and the Kex1 carboxypeptidase. All three enzymes appear to be localized to the same compartment, as shown by indirect immunofluorescence (38) and coimmunoprecipitation (5). Proteolytic maturation of pro- α -factor occurs after its modification by the latest acting yeast Golgi carbohydrate modification enzyme, the *MNN1*-encoded α -1,3-mannosyl transferase (20). This, together with the fact that Kex2p is also modified by α -1,3-mannosyl residues (58) even though Kex2p and α -1,3-mannosyl transferase activities can be separated from Kex2p by fractionation (5, 9, 21), supports the classification of the Kex2p-containing compartment as the yeast TGN (21, 59), although the morphology of this organelle has not yet been described at the ultrastructural level.

Newly synthesized Kex2p is transported rapidly (half-time [$t_{1/2}$], ~ 3 min) to the TGN and then is shunted slowly ($t_{1/2}$, ~ 80 min) to the vacuole where it is degraded (58, 59). A signal in the first ~ 30 residues of the C-terminal cytosolic tail (C-tail) promotes net retention of the protein in the TGN (59). This TGN localization signal (TLS) consists of an essential Tyr residue, Tyr-713, along with contextual sequences (59). The substitution of Ala for Tyr-713 (*Y₇₁₃A*) or deletions that re-

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move the TLS (e.g., the BA-block Δ) or entire C-tail (C-tail Δ) result in decreased efficiency for retention of the protein (59). TLS mutant proteins reach the Golgi but are transported at an accelerated rate to the vacuole by a presumed default pathway that does not traverse the plasma membrane (59). The half-life of Kex2p thus serves as a measure of the efficiency of localization of the protein in the TGN. Deletions of the C-tails of Ste13p and Kex1p also result in decreased efficiency of localization of these molecules in the TGN and transport to the vacuole by the default pathway (8, 38, 48). A TLS has been identified for the N-terminal cytosolic tail of Ste13p that contains a Phe-Gln-Phe motif (residues 85 to 87), reminiscent of the Tyr-Glu-Phe sequence (residues 713 to 715) in the TLS of Kex2p (38). The Ste13p tail can function as an autonomous localization signal when substituted for the cytosolic tail of vacuolar alkaline phosphatase (ALP), preventing its transport to and proteolytic maturation in the vacuole (38). The substitution of an alanine for either of the phenylalanines in the TLS (Phe-85 or Phe-87) results in inefficient TGN localization of this fusion protein and transport to the vacuole (38).

When Kex2p expression under *GAL1* promoter control is shut off in *MAT α* cells, depletion of the enzyme from the TGN results in an exponential decay in mating competence after a lag of ~ 4 h (59). After shutting off expression of TLS mutant forms of Kex2p, cells exhibit a more rapid loss of mating competence with little or no lag (59). This results in a window between ~ 4 and 8 h after a shift to glucose medium during which the mating efficiency of cells being depleted of wild-type (WT) Kex2p is elevated 100- to 1,000-fold relative to that of cells being depleted of Y₇₁₃A Kex2p (59). We used this difference in the rate of decay of mating competence, or onset of impotence, to identify second-site suppressors of the Y₇₁₃A point mutation. Seven allele-specific suppressors of the Y₇₁₃A substitution that defined three *SOI* genes were isolated. The products of these genes are candidates for factors involved in TLS recognition.

MATERIALS AND METHODS

Reagents and antibodies. Polyclonal anti-carboxypeptidase Y (CPY) antiserum was the generous gift of L. Westehuebe and R. Schekman (University of California, Berkeley), monoclonal anti-CPY antibody was obtained from Molecular Probes (Eugene, Oreg.), and anti-ALP antiserum was generously provided by S. Nothwehr, N. Bryant, and T. Stevens (University of Oregon). Microtiter plates were from Becton Dickinson Labware (Lincoln Park, N.J.). Nitrocellulose filters were from Schleicher and Schuell (Keene, N.H.). Zymolyase 100T was from ICN Biomedicals (Costa Mesa, Calif.). All chemicals, unless otherwise indicated, were from Sigma Chemical Co. (St. Louis, Mo.).

Strains, plasmids, and growth conditions. The strains used were BFY106-4D (*MAT α kex2 Δ 2::HIS3*), BFY126-35A (*MAT α kex2 Δ 2::HIS3*), KRY18-1A (*MAT α kex2 Δ 2::TRP1*), KRY18-1B (*MAT α kex2 Δ 2::TRP1*), JBY10 (KRY18-1B $\times$ BFY106-4D), CB017 (*MAT α kex2 Δ 2::TRP1 pep4::HIS3 prb1::hisG prc1::hisG*), CB018 (*MAT α kex2 Δ 2::TRP1 pep4::HIS3 prb1::hisG prc1::hisG*), and DC14 (*MAT α his1*). All strains except DC14 were from the W303 background and had the following additional genetic markers: *can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*. Yeast rich medium (YPD), synthetic minimal 2% (wt/vol) glucose medium (SD), synthetic complete 2% (wt/vol) glucose medium (SDC), synthetic complete 2% (wt/vol) galactose medium (SGC), and synthetic complete dropout media, such as SDC-uracil (SDC-Ura), were prepared as previously described (51). Unless indicated, cells were grown in SDC-Ura at 30°C. Sporulation plates (51) contained 0.1% galactose instead of glucose.

Plasmids pRY131 (60), pRCTC-MacI (7), and pSN98 (38) were as previously described. Plasmid pCWKX10 (59) contains the WT *KEX2* gene under its own promoter on a yeast single-copy replicating plasmid (*CEN4 ARS1 URA3*). Vector pBM258 is a single-copy yeast replicating plasmid containing the *GAL1* promoter (47). pCWKX20, pCWKX21, and pCWKX27 are single-copy yeast replicating plasmids in which the *GAL1* promoter controls expression of WT Kex2p, Y₇₁₃A Kex2p, and C-tail Δ Kex2p, respectively (59). Plasmids were introduced into yeast strains by lithium acetate transformation (14, 23).

Site-directed mutagenesis. Substitutions at position 713 were generated by PCR. Plasmid pKX2 (16), linearized with *HindIII*, was used as the template. The 5' primer included the *NarI* site upstream of the Tyr-713 codon and a silent mutation removing a *BstXI* site. The 3' primers included the *EcoRI* site directly

after the Tyr-713 codon and appropriate substitutions in codon 713. PCR products were digested with *NarI* and *EcoRI* and used to replace the same sequence in plasmid pKRKX10. pKRKX10 was derived from pCWKX10 by filling in the *EcoRI* site in the pBR322 sequences, making the site after the Tyr-713 codon unique. Recombinant plasmids were screened for loss of the *BstXI* site and then sequenced to confirm the mutation.

Mutation of positions 712, 715, and 719 was performed as previously described (59). Mutagenesis was performed on single-stranded uracil-containing DNA from pKEXSCRIPT. This plasmid consists of a *KEX2* fragment from the *XbaI* site in the coding sequence to the downstream *BamHI* site inserted into pBABYBLUE, a variant of pBLUESCRIPT KS⁺, in which sequences between the *BamHI* and *KpnI* sites have been replaced by the following sequence containing a unique *SalI* site: GATCCGTCGACGGTAC. Mutagenic oligonucleotides were (5' to 3') GTCAAGAGCGGAACCGTATGAGTTCGATATCATTG (T₇₁₂P), AACGTATGAAGCCGATATCATTGA (F₇₁₅A), AGCGGAAACGGCTGAAGCCGATATCATTGA (Y₇₁₃A F₇₁₅A), and GTATGAATTCGATATAATTAAACA GATCTGAGTAC (D₇₁₉Amb). Mutants were identified by loss of diagnostic restriction sites. After sequencing to confirm the proper substitutions, mutant *NarI-SalI* fragments were subcloned in place of the WT sequences in either pCWKX10 or pCWKX20 (59) and introduced into BFY106-4D or KRY18-1A, respectively.

Plate-based onset-of-impotence assay. Patches grown on galactose were replica plated to nitrocellulose filters (82-mm diameter; 0.45- μ m pore size) (BA85; Schleicher and Schuell) on SGC-Ura plates. After overnight incubation (10 to 14 h) at 30°C, filters were shifted to SDC-Ura plates for various times before replica plating to mating plates, SD plates spread with a late logarithmic DC14 culture. DC14 cells were grown in YPD to an optical density at 600 nm of 5 to 6, harvested by centrifugation, and resuspended in SD to an optical density at 600 nm of 20. One hundred twenty-five milliliters of the resuspension was spread onto plates (100-mm diameter). Mating plates were scored after 2 days. As controls, each replica contained patches of KRY18-1A harboring the WT and mutant Kex2p expression plasmids.

Chemical mutagenesis and screen for mutants. Fifty milliliters of an logarithmic culture of KRY18-1A(pCWKX21) grown in SGC-Ura was filtered and resuspended in 4 ml of SGC-Ura plus 12 ml of 150 mM sodium phosphate (pH 6.7). Two milliliters of this suspension was added to 60 μ l of ethyl methanesulfonate (EMS) in each of five 50-ml Falcon tubes. After vigorous mixing (1 min on a vortex mixer), the tubes were shaken in a 30°C water bath for various times (30 to 60 min). EMS was inactivated with 2 ml of sterile 10% sodium thiosulfate. Cells were collected by filtration, washed with 30 ml of sterile water, resuspended in 10 ml of SGC-Ura, and allowed to recover from mutagenesis by shaking for 2 h at 30°C. Negligible growth, as measured by the optical density at 600 nm, occurred during this time.

The culture treated with EMS for 60 min exhibited 55% killing, relative to that of unmutagenized cells, and was used in the subsequent screen. Mutagenized cells were thawed and diluted into sterile water, and 500 to 1,000 cells were filtered onto 135-mm-diameter nitrocellulose filters (0.45- μ m pore size), which were placed on 150-mm-diameter SGC-Ura plates. After 2 to 3 days, filters were shifted to SDC-Ura master plates for 7 h before replica plating onto mating plates. A total of 25,000 to 30,000 colonies from the mutagenized culture were screened. Approximately 3,000 colonies from an unmutagenized culture were analyzed as a control; $\sim 5\%$ produced one or two mating events (i.e., 1 or 2 colonies on the SD plate), and only 1 produced greater than two mating events. Therefore, we picked all colonies from the master plates that had produced greater than two mating events.

After retesting patches for the ability to mate at 7 h but not at 24 h, two colonies were purified from each patch. Unless the two were phenotypically distinguishable (as in the case of isolates 79 and 130), only one was retained and designated s- followed by the original isolate number. Isolates 130-A and 130-B were renamed s-1301 and s-1302 because temperature-sensitive growth (Ts⁻; inability to grow at 37°C) was found in 130-B, not in 130-A. Subsequent analysis demonstrated that the suppressors in s-1301 and s-1302 were tightly linked, suggesting that these alleles were not independently isolated; moreover, the Ts⁻ phenotype in s-1302 was unlinked to the *Soi*⁺ phenotype.

Genetic analysis. Isolates were tested for vacuolar peptidase deficiency (Pep⁻ phenotype) by assaying CPY activity in microtiter wells (27) and by assessing complementation of the sporulation defect of *pep4* or *prb1* mutations (26) by crossing *Soi*⁺ mutants to strain CB018 (*pep4 prb1*) and testing for the ability to sporulate (50). Defects in vacuolar protein sorting (Vps⁻ phenotype) were tested by performing the CPY immunoblot assay (52).

For random spore analysis, spores were prepared as previously described (50), except that diploids were sporulated on plates (51) and were digested in 200 μ g of Zymolyase 100T per ml for 7 to 8 min at room temperature. Single colonies were purified by restreaking before subsequent analysis.

For segregation analysis, original mutant isolates were mated to BFY126-35A (*MAT α kex2 Δ 2::HIS3*) and sporulated on galactose sporulation medium, and random spores were generated. Segregation of the *MAT* locus and His⁺ and Trp⁺ traits was verified, and *MAT α* segregants were analyzed for inheritance of the *Soi*⁺ phenotype. Ts⁻ and Vps⁻ phenotypes were also checked. Although 7 of the original 58 *Soi*⁺ mutants displayed poor or no growth at higher temperatures ($>33^\circ\text{C}$), only in 1 case (s-131) were the *Soi*⁺ and Ts⁻ phenotypes tightly linked. In the case of the eight allele-specific suppressors, tetrad analysis was

performed after an additional backcross to check directly for 2:2 segregation. *MATa* segregants were tested for the suppressor mutation by backcrossing to a *MATa* strain, sporulating, and screening *MATa* progeny for the *Soi*⁺ phenotype.

β-Galactosidase assays. Cells were transformed with pRY131, a multicopy plasmid that places the *Escherichia coli lacZ* gene under control of the *GAL1* promoter. Culture growth and freezing of aliquots for assays were done as described for the Kex2 depletion assay (59). β-Galactosidase assays were performed essentially as previously described (51). Total protein was measured by bicinchoninic acid assay (Pierce Chemical Co.).

Indirect immunofluorescence, quantitative onset-of-impotence experiments, radiolabeling, and immunoprecipitation. Indirect-immunofluorescence and quantitative onset-of-impotence experiments were performed as previously described (59). Radiolabeling and immunoprecipitation experiments were performed as previously described (58). Some Kex2p immunoprecipitations were performed by using the following modification of this protocol: after glass bead lysis and boiling, 0.5 ml of immunobuffer (0.1 M Tris-HCl [pH 8.0], 0.1% sodium dodecyl sulfate [SDS], 0.1% Triton X-100, 20 mM EDTA) was added. Immunoprecipitates were washed as previously described (38) and not precipitated a second time. All data were obtained with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) and analyzed by using either ImageQuant or IPLabGel software. Half-lives were determined by linear regression using data from five or six time points.

Immunoprecipitation of the Ste13-Pho8p-F₈₇A fusion protein (A-ALP-F₈₇A) was performed as previously described (59), with the following modifications. Cell pellets were thawed and resuspended in 50 μl of lysis buffer containing 1% SDS and 8 M urea. After being boiled, lysates were diluted into 950 μl of immunobuffer (lacking SDS). Immunoprecipitates were washed either as previously described (38) or twice with 1 ml of immunobuffer by vortexing for either 10 s (first wash) or 3 min (second wash). With our strains, unlike what has been observed previously (38), a band migrating at the same position as that of mature A-ALP-F₈₇A was present even at zero time of chase (data not shown). The production of this species depended on the presence of both the plasmid and functional vacuolar protease genes (data not shown). Because of this, our values for rates of delivery of A-ALP-F₈₇A to the vacuole were based solely on measurements of precursor disappearance.

Invertase assays to assess mislocalization of Suc2 fusion proteins. To assess the Gem phenotype (Golgi enzyme maintenance [7]), plasmid pRCTC-MacI was introduced into *Soi*⁺ segregants from the first backcross after curing plasmid pCWKX21. Plasmid pCWKX11 was reintroduced into pRCTC-MacI transformants for determinations of Kex2p-dependent invertase secretion. Cultures of four independent transformants of each suppressor, grown in SDC to repress the endogenous *SUC2* gene (54), were harvested by centrifugation, washed with water, and subjected to one-step invertase assays (19). The *A*₆₅₀ was measured in a microtiter plate spectrophotometer (Vmax kinetic microplate reader; Molecular Devices, Menlo Park, Calif.) to normalize assay values to cell density. Cell-associated Δ*A*₆₅₀/Δ*t* for each transformant, normalized to cell density, was determined by subtracting the no-cell background.

RESULTS

Further definition of the Kex2p TLS. Previously, either substitution of Ala for Tyr-713 or deletion of sequences including or adjacent to Tyr-713 was found to impair localization of Kex2p to the TGN (59). To examine further the resemblance of the Kex2p TLS to mammalian tyrosine internalization signals and to the TLS of Ste13p, additional mutations were created in the Kex2p C-tail (Fig. 1). The effects of these mutations on the localization of Kex2p were analyzed by two assays for TGN localization, (i) determination of the half-life of Kex2p by pulse-chase immunoprecipitation to measure the rate of delivery of Kex2p to the vacuole (58, 59) and (ii) the rate of loss of mating competence after shutting off expression of mutant Kex2p under *GAL1* promoter control in a *MATa* *kex2Δ* strain (59). The latter assay was adapted so that mating efficiency could be monitored on plates (see Materials and Methods).

A series of substitutions was made for Tyr-713 to probe the specificity of the localization apparatus for the chemical nature of the side chain. In Table 1, the half-lives of Tyr-713 substitution mutants have been expressed as retention efficiencies relative to that of WT Kex2p. Phe substituted well for Tyr-713 (75% retention efficiency), whereas the substitution of the bulkier but aromatic Trp resulted in lower-level but significant retention (42%). The retention efficiencies of the His-713 (37%) and Leu-713 (33%) mutants were only slightly better

FIG. 1. Comparison of the Kex2p and Ste13p TLSs and mutagenesis of the Kex2p TLS. The C-tail of Kex2p (residues 700 to 719) and the N-terminal cytosolic tail of Ste13p (residues 72 to 91) are paired to maximize the alignment of residues in their TLS sequences. An asterisk indicates identity; a dot indicates similarity. Annotations above the Kex2p C-tail sequence indicate the sites and natures of mutations discussed in the text. The substitution of Ala for Tyr-713 has been previously described (59). TMD, transmembrane domain.

than that of the Ala-713 mutant (27%). Substitution of Asn at position 713 led to even poorer retention (20%), only slightly better than that observed with the C-tailΔ (13% [59]). The preference for an aromatic residue and the rank order of the effects of the substitutions at position 713 strikingly paralleled the effects of a similar series of substitutions made for Tyr-807 in the human low-density lipoprotein receptor on the efficiency of its internalization by clathrin-coated pits (Table 1) (10).

The TLS of Ste13p corresponds to residues 81 to 90 in the N-terminal tail. A mutation of either Phe-85 or Phe-87 in the Ste13p TLS impairs TGN retention (38). These Phe residues can be aligned with sequences surrounding Tyr-713 in Kex2p (39) (Fig. 1). To determine whether Phe-715 in Kex2p is important for TLS function, it was changed to Ala alone or in combination with the Y₇₁₃A substitution. As indicated by half-life measurements, F₇₁₅A Kex2p was retained threefold less well than was WT Kex2p and slightly better than was the Y₇₁₃A mutant. The combination of the two mutations (Y₇₁₃A F₇₁₅A) resulted in a phenotype like that of Y₇₁₃A alone (Table 1),

TABLE 1. Further mutational analysis of the Kex2p TLS

Kex2p C-tail	Half-life (min) ^a	Retention efficiency (%) ^b	Internalization index ^c
WT	83 ± 9	100	100
Y ₇₁₃ F	62 ± 3 ^d	75	96
Y ₇₁₃ W	35 ± 6 ^d	42	61
Y ₇₁₃ H	30 ± 2 ^d	37	25
Y ₇₁₃ L	27 ± 1 ^d	33	27
Y ₇₁₃ A	22 ± 1	27	19
Y ₇₁₃ N	16 ± 1 ^d	20	ND ^e
F ₇₁₅ A	27 ± 3 ^d	33	— ^f
Y ₇₁₃ A F ₇₁₅ A	20 ^g	24	— ^f
T ₇₁₂ P	25 ^g	30	— ^f
D ₇₁₉ Amb	111 ^g	134	— ^f

^a Determined at 30°C by pulse-chase analysis, as described in Materials and Methods. The strain was BFY106-4D. The data for WT Kex2p and Y₇₁₃A Kex2p were taken from reference 59.

^b Normalized to the half-life of WT Kex2p (Tyr at position 713).

^c AT position 807 in the low-density lipoprotein receptor; data were taken from reference 9 and normalized to the WT receptor (Tyr at position 807).

^d Half-life values were determined by two independent pulse-chase immunoprecipitation experiments, each involving linear regression analysis of four or five time points. The stated error represents the standard error of the mean.

^e ND, not determined.

^f —, no relevant comparison.

^g Half-life values were determined by a single pulse-chase immunoprecipitation experiment, involving linear regression analysis of four or five time points.

FIG. 2. TLS mutants exhibit characteristic rates of loss of mating competence. The onset-of-impotence experiment was performed as described in Materials and Methods. KRY18-1A transformants expressing *KEX2* TLS mutants under the control of the *GAL1* promoter were assayed for mating competence at the indicated times after the shift to glucose. One of two duplicate patches is shown.

suggesting that Tyr-713 and Phe-715 are part of one signal and that Kex2p and Ste13p utilize similar TLSs.

Certain Tyr-containing internalization motifs have been shown to adopt a type-1 β -turn structure with Tyr as the fourth residue (1, 12). Such structures should not accommodate Pro at the third position. The substitution of Pro for Thr-712 resulted in a severe retention defect (Table 1).

Previous deletion analysis limited the Kex2p TLS to the first 29 residues of the C-tail (with Tyr-713 as residue 14 [Fig. 1]). A deletion of 16 residues immediately after Tyr-713 abrogated retention (59). Truncation of the Kex2p C-tail after Ile-718, however, resulted in a molecule that was retained slightly better than was either WT Kex2p (Table 1) or the form of Kex2p having the 29-residue tail (59). These results limit the Kex2p TLS to the first 19 residues of the C-tail and indicate that an extended sequence of acidic residues beyond Tyr-713 is not required for TLS function in Kex2p, in contrast to the results obtained with mammalian furin (56).

Isolation and initial characterization of *Soi*⁺ mutants. To create an assay for isolating second-site suppressors of the localization defect of Y₇₁₃A Kex2p, the onset-of-impotence assay was adapted to a replica plating format. As shown in Fig. 2, an analysis of several TLS mutants by the replica mating onset-of-impotence assay not only gave the same rank order of retention efficiency as that determined by half-life determination but also was capable of clearly distinguishing among mutants that differed only slightly in half-life (compare Table 1 and Fig. 2).

Figure 3 illustrates the rationale behind the screen for second-site suppressors of the Y₇₁₃A Kex2p localization defect. Strain KRY18-1A, containing Y₇₁₃A Kex2p under *GAL1* promoter control on *CEN* plasmid pCWKX21, was mutagenized with EMS, and *Soi*⁺ suppressor strains (suppression of the rapid onset of impotence; the WT phenotype is *Soi*⁻) were identified by replica mating 7 h after shifting colonies grown on filters from glucose-containing plates to galactose-containing plates (see Materials and Methods for details). Mating-competent colonies (*Soi*⁺) were picked into microtiter plate arrays and retested. Of 470 initial isolates, 99 displayed the *Soi*⁺ phenotype reproducibly. These 99 isolates were further screened to eliminate uninteresting mutants. These included 17 that still mated after 24 h on glucose; 3 that were plasmid dependent; 2 with defects in either *PEP4* or *PRB1*, which

FIG. 3. The *Soi* screen. (Left) WT cells exhibit rapid transport of Y₇₁₃A Kex2p from the TGN to the vacuole (V). Within 7 h after shutting off expression of *KEX2*, essentially all of the Kex2 enzyme has been depleted from the TGN and the cells no longer process sufficient amounts of pro- α -factor to mate. (Right) *Soi*⁺ mutants exhibit a slower rate of exit of Y₇₁₃A Kex2p from the TGN after the shift to glucose. After 7 h of depletion, *Soi*⁺ cells retain sufficient Kex2p in the TGN to produce adequate amounts of mature α -factor for mating. Empty arrows represent transcription and translation of the *KEX2* gene, while solid arrows represent protein transport steps. The thicknesses of curved arrows reflect the relative rates of transport of Y₇₁₃A Kex2p from the TGN to the vacuole. N, nucleus; ER, endoplasmic reticulum.

FIG. 4. The *SoI*⁺ phenotype. Patches of the parental strain (KRY18-1A) and representative *SoI*⁺ strains were shifted from galactose to glucose 7 h before replica mating (as described in Materials and Methods). The selective mating plate is shown; each colony is the result of one mating event. The parental strains contained (left to right) vector plasmid pBM258, pCWKX21 (*Y*₇₁₃A *Kex2p* under *GAL1* promoter control), and pCWKX20 (WT *Kex2p* under *GAL1* promoter control), respectively. The top two rows represent mating by six *SoI*⁺ isolates (left to right and top to bottom: s-1302, s-131, s-172, s-227, s-284, and s-291), each containing pCWKX21.

encode major vacuolar proteases (expected because *pep4* mutants exhibit a weak *SoI*⁺ phenotype [see Fig. 9 in reference 59]); and 19 weak suppressors, leaving 58 mutant strains. These 58 mutant strains are hereafter referred to by the designation of the original isolate (for example, s-291). The *SoI*⁺ phenotype is illustrated in Fig. 4. After 7 h on glucose, a WT *MATα kex2Δ* strain expressing *Y*₇₁₃A *Kex2p* lost the ability to mate while the same strain expressing WT *Kex2p* mated well. A control strain containing the vector alone was unable to mate at any time. The suppression of *Y*₇₁₃A *Kex2p* by six independent *SoI*⁺ isolates is shown in the top two rows of Fig. 4.

Random spore segregation analysis was used to eliminate strains in which the *SoI*⁺ phenotype was caused by the mutation of multiple loci. Suppressor mutants were crossed to a *MATa* strain congenic to the parental *MATα* strain. *MATα* spores from diploids were tested for the *SoI*⁺ phenotype, with the expected frequency being 0.5 for 2:2 segregation of a single mutation. Spores derived from crosses of 8 of the 58 *SoI*⁺ mutants exhibited a frequency of the *SoI*⁺ phenotype of <0.1 and were not studied further. Allele-specific suppressors were subjected to more stringent segregation analysis (see below).

Allele specificity of suppression. Suppressor strains derived from the first backcross were transformed with plasmid pCWKX27 (*C-tailΔ Kex2p* under *GAL1* promoter control) and tested for suppression of the *C-tailΔ* mutation by the onset-of-impotence assay. Most exhibited significant suppression of the *C-tailΔ* mutation (data not shown) and thus bypassed the need for the TLS, possibly by inhibiting the default vacuolar transport pathway. Twenty-one *SoI*⁺ isolates, including all that failed to suppress the *C-tailΔ*, were examined more closely by transforming with a set of plasmids encoding various point and deletion mutant forms of *Kex2p* under *GAL1* promoter control and then analyzing the transformants by the onset-of-impotence assay. Examples of the assay results with these various forms of *Kex2p* are presented in Fig. 5. All of the suppressors that suppressed the *C-tailΔ* also suppressed the *BA-blockΔ* (Fig. 5 and data not shown), which lacks residues 702 to 729, a

FIG. 5. Allele specificity of *SoI*⁺ mutants. The replica mating test for the onset of impotence was performed, as described in the legend to Fig. 4, 7 h after the shift to glucose. Each column corresponds to a *SoI*⁺ mutant or the parental strain transformed with one of a bank of *GAL1-KEX2* expression plasmids that differ only in the nature of the *C-tail*.

region that includes the entire TLS (59). As shown in Fig. 5, s-333 is an example of the bypass suppressor class in that it suppressed both deletions of the TLS and substitutions for Tyr-713. Eight suppressors, exemplified by s-227 and s-291 (Fig. 5), exhibited allele specificity in that they suppressed point mutations at Tyr-713 (*Y*₇₁₃A and *Y*₇₁₃L) but not the *C-tail* or the *BA-blockΔ* deletions. The allele-specific suppressors were analyzed further.

Genetic analysis of allele-specific suppressors. The eight allele-specific suppressors were backcrossed again, and tetrad analysis was performed to determine whether the *SoI*⁺ phenotype segregated strictly 2:2, as expected for single genetic loci. *MATα* spores were scored directly for the *SoI*⁺ phenotype. *MATa* spores were tested for *SoI*⁺ by crossing to the parental strain, sporulating, and scoring *MATα* progeny for the *SoI*⁺ phenotype. Seven of the allele-specific suppressors displayed 2:2 segregation. The *SoI*⁺ phenotype of s-412 segregated as a consequence of two or more unlinked mutations. None of the suppressors exhibited linkage to the *MAT* locus (data not shown).

Complementation and dominance analyses were complicated by the fact that the *SoI*⁺ phenotype cannot be observed in *MATa/MATα* diploids. Attempts to score the phenotype in *MATα/matΔ* and *MATα/MATα* diploids were frustrated by the reduced initial mating efficiencies of *MATα/MATα* diploids and by more rapid loss of mating competence in both cases, problems that compromised the sensitivity of the assay in *α*-mating diploids (data not shown). Therefore, linkage analysis was used to establish the number of loci represented by the allele-specific suppressor mutants. Allele-specific mutants were crossed to one another, and *MATα* progeny were scored

TABLE 2. Linkage analysis of allele-specific *Soi*⁺ suppressors

<i>MAT</i> α suppressor ^a	<i>MAT</i> α suppressor ^a	Progeny ^b	<i>P</i> ^c	Linkage ^d	Alleles ^e
s-291	s-172	42:0	<0.001	+	<i>soi1-3/soi1-1</i>
s-291	s-227	39:1	<0.002	+	<i>soi1-3/soi1-2</i>
s-291	s-1302	24:5	>0.5	—	<i>soi1-3/SOI2-3f</i>
s-284	s-227	48:27	<0.025 ^g	—	<i>SOI2-1/soi1-2</i>
s-291	s-284	24:3	<0.1	—	<i>soi1-3/SOI2-1</i>
s-227	s-1302	44:24	0.05 ^g	—	<i>soi1-2/SOI2-3</i>
s-1301	s-284	24:0	<0.01	+	<i>SOI2-2/SOI2-1</i>
s-284	s-1302	42:0	<0.001	+	<i>SOI2-1/SOI2-3</i>
s-1301	s-1302	40:0	<0.001	+	<i>SOI2-2/SOI2-3</i>
s-291	s-400	26:8	>0.9	—	<i>soi1-3/soi3-1</i>
s-284	s-400	15:6	>0.7	—	<i>SOI2-1/soi3-1</i>

^a The *Soi*⁺ isolate from which the *MAT* α or *MAT* α mating partner was derived.

^b The ratio of *Soi*⁺ progeny to *Soi*[−] progeny (note that *Soi*⁺ indicates suppression of *KEX2-Y₇₁₃A* and therefore a mutant phenotype).

^c Based upon χ^2 analysis; the probability that the observed distribution has arisen from two unlinked genes.

^d +, linkage; −, no linkage.

^e The allele present in the *MAT* α partner/the allele present in the *MAT* α partner.

^f Subsequent analysis (discussed later) indicated that s-284 and s-1302 were dominant; hence, these alleles are denoted in capital letters. s-1301 was tentatively assigned as dominant as well.

^g The low *P* value was due to the high number of *Soi*[−] spores. The cause of this is unknown (see Results), but these mutations are obviously not linked.

for the *Soi* phenotype. All progeny derived from diploids possessing two tightly linked suppressor alleles should be *Soi*⁺. In contrast, 25% of the progeny derived from diploids possessing two unlinked suppressor alleles should be *Soi*[−]. By these criteria, the mutant alleles represented by the seven allele-specific suppressor mutants fell into three linkage groups, *SOI1* with three members (s-172, s-227, and s-291), *SOI2* with 3 members (s-284, s-1301, and s-1302), and *SOI3* with one member (s-400). Each suppressor isolate was assigned an allele number (Table 2). An elevated number of *Soi*[−] *MAT* α progeny derived from crosses between *soi1* mutants and *SOI2-3* mutants was observed. Tetrad analysis showed that this was not due to poor spore viability of *soi1 SOI2-3* double mutants per se. The most likely explanation is that the double-mutant spores are more sensitive than are normal spores to the harsh conditions employed in the preparation of spores for random spore analysis (50).

Mutations in *SOI1* cause a defect in vacuolar protein sorting. As judged by secretion of both CPY and a CPY-invertase fusion protein (25), 4 of the original 58 mutants (s-172, s-227, s-164, and s-467) exhibited a recessive *Vps*[−] phenotype (vacuolar protein sorting defect) that was linked to the *Soi*⁺ phenotype (data not shown). *soi1-1/soi1-2* diploids (s-172 $\times$ s-227) exhibited a *Vps*[−] phenotype, consistent with the assignment of these as alleles of *SOI1*. After backcrossing, *soi1-3* mutants were also found to be *Vps*[−]. Complementation analysis of the *Vps*[−] phenotype of s-164 and s-467 indicated that the *vps* mutations in these strains were not linked to one another or to the *SOI1* gene. Indirect immunofluorescence using antibody against the Vat2 protein, a subunit of the vacuolar H⁺-ATPase, was used to characterize vacuolar morphology in the s-172, s-227, s-164, and s-467 mutants (data not shown). All displayed normal vacuolar morphology, with Vat2p-specific fluorescence coinciding perfectly with vacuolar structures seen by differential interference contrast optics. Vacuolar segregation structures (45) were frequently observed. By these criteria, the *soi1* (s-164 and s-467) mutants were designated class A *vps* mutants (45, 49). The sorting of soluble vacuolar proteins from secreted proteins is thought to occur in the compartment containing Kex2p (20, 55). Thus, it is significant that *soi1* mutations, which alter the retention of a TGN protein, also affect the sorting of a vacuolar precursor (see Discussion).

Mutations in *SOI1* interfere with retention of WT Kex2p. By the replica mating assay, *soi1-2* and *soi1-3* strains lost mating competence more rapidly than did the parental strain during depletion of WT Kex2p (data not shown). This result suggested that the retention of WT Kex2p was impaired in these strains, a phenotype we refer to as *Rek*[−] (retention of Kex2p). The *Rek*[−] phenotype was seen at ~10 to 12 h after the shift to glucose and was variable, possibly because reduced growth rates of cells at late times also affected mating competence. However, quantitative mating-depletion assays with *soi1-2* and *soi1-3* strains containing WT Kex2p under *GAL1* promoter control clearly indicated that both mutant strains lost mating competence more rapidly than did the parental control strain (Fig. 6). The suppression of *Y₇₁₃A* Kex2p by *soi1-3* appeared to be weak by this assay (Fig. 6C).

Allele-specific *Soi*⁺ mutations slow transport of *Y₇₁₃A* Kex2p to the vacuole. Several additional experiments were done to exclude trivial explanations of *Soi*⁺ suppression. First,

FIG. 6. Depletion of WT Kex2p and Kex2p mutants in parental, *soi1-2*, and *soi1-3* strains. The parental strain (BFY106-4D [A]) and *soi1-2* (B) and *soi1-3* (C) mutants harboring the indicated single-copy *GAL1-KEX2* expression plasmids were tested by quantitative onset-of-impotence assay (59). At time zero, cultures were shifted from galactose to glucose and maintained in exponential growth phase by periodic dilution, and the mating efficiency of each culture was measured by quantitative mating assays at various points in the time course. Data for the parental strain were taken from reference 59.

FIG. 7. Repression of *GAL1-lacZ* in parental and *SoI*⁺ strains. The parental strain and various *SoI*⁺ strains containing plasmid pRY131 were grown in galactose-containing media and shifted to glucose-containing media at time zero. The shifted cultures were maintained in exponential growth phase by periodic dilution, and aliquots were taken at various points to determine β -galactosidase specific activities, as described in Materials and Methods).

allele-specific suppressors were examined for their effects on repression of a *GAL1* promoter-driven *lacZ* construct (Fig. 7). None exhibited a rate of loss of β -galactosidase activity that was significantly different from that of the parent strain. Thus, these mutants do not suppress *Y*₇₁₃A Kex2p by affecting the rate of the onset of glucose repression.

Second, because TLS mutations in the Kex2p C-tail cause rapid transport to the vacuole (59), suppressors of TLS mutations should slow vacuolar delivery. Likewise, mutations that cause the *Rek*[−] phenotype should increase the rate of delivery of WT Kex2p to the vacuole. The half-lives of WT and *Y*₇₁₃A Kex2p were measured by pulse-chase immunoprecipitation of strains derived from the second backcross of allele-specific suppressors. These strains contained WT or *Y*₇₁₃A Kex2p expressed from the WT *KEX2* promoter on single-copy centromere plasmids (59). With all alleles of *SOI1*, *SOI2*, and *SOI3* tested, the half-life of *Y*₇₁₃A Kex2p was increased, although in the case of the *soi1-1* strain, this increase was slight (Table 3).

TABLE 3. Half-lives of Kex2p and *Y*₇₁₃A Kex2p in *SoI*⁺ mutants

Strain	Half-life (min) ^a	
	WT Kex2p	<i>Y</i> ₇₁₃ A Kex2p
Parent	83 ± 9	22 ± 1
<i>soi1-1</i>	33	25
<i>soi1-2</i>	39	33
<i>soi1-3</i>	41	30
<i>SOI2-1</i>	ND ^b	30
<i>SOI2-3</i>	80	37
<i>soi3-1</i>	152	36

^a Cells were grown at 30°C prior to a 30-min sulfate depletion, pulse-labeled for 10 min, and chased at 30°C for up to 3 h. The parental data were taken from reference 59. Each half-life was determined by a single pulse-chase immunoprecipitation experiment, involving linear regression analysis of four or five time points.

^b ND, not determined.

TABLE 4. Half-lives for *Y*₇₁₃A Kex2p in homozygous and heterozygous diploids

Alleles	Half-life (min) ^a
WT/WT ^b	17 ± 5 ^c
<i>soi1-2/soi1-2</i>	34 ± 3 ^c
<i>soi1-2</i> /WT	16 ± 5 ^c
<i>soi1-3/soi1-3</i>	34 ^d
<i>soi1-3</i> /WT	19 ^d
<i>SOI2-1/SOI2-1</i>	26 ^d
<i>SOI2-1</i> /WT	24 ^d
<i>SOI2-3/SOI2-3</i>	31 ± 4 ^c
<i>SOI2-3</i> /WT ^c	28 ± 8 ^c
<i>soi3-1/soi3-1</i>	32 ^d
<i>soi3-1</i> /WT	21 ^d

^a Cells were grown at 30°C prior to a 30-min sulfate depletion, pulse-labeled for 10 min, and chased at 30°C for 60 to 90 min.

^b The WT diploid strain was JBY10.

^c Half-lives were determined by two to four independent pulse-chase immunoprecipitation experiments, each involving linear regression analysis of four or five time points. The stated error represents the standard error of the mean.

^d Half-lives were determined by a single pulse-chase immunoprecipitation experiment, involving linear regression analysis of five time points.

^e Two diploid strains from independent crosses.

Furthermore, all three of the *soi1* alleles, two of which had been judged to be *Rek*[−] by mating assays, caused more rapid turnover of WT Kex2p. Thus, all *soi1* mutants exhibited a *Rek*[−] phenotype at the molecular level. In contrast, neither *SOI2-3* nor *soi3-1* mutations decreased the half-life of WT Kex2p (Table 3). The *soi3-1* mutation increased the lifetime of WT Kex2p as well as that of *Y*₇₁₃A Kex2p (Table 3). The fact that this mutation did not suppress the effects of the C-tail deletion in the onset-of-impotence assay suggests that this mutation leads to improved TGN localization of all forms of Kex2p that possess the C-tail. Observations of consistent *SoI*⁺ and *Rek*[−] phenotypes by two independent assays, mating and half-life determination, argue strongly that allele-specific suppressors directly affect the localization of *Y*₇₁₃A Kex2p (and WT Kex2p, in the case of *soi1* mutations) in the TGN.

***SOI2* suppressor mutations are dominant; *soi1* and *soi3* suppressor mutations are recessive.** Measurements of the half-life of *Y*₇₁₃A Kex2p in homozygous and heterozygous suppressor mutant strains were used to determine whether the *SoI*⁺ phenotype was dominant or recessive in allele-specific suppressors. Control experiments demonstrated that *Y*₇₁₃A Kex2p turned over more rapidly in diploids than in haploids (Table 4), consistent with the increased rate of loss of mating competence observed with *MAT* α /*MAT* α diploids compared with that of *MAT* α haploids. *soi1-2*, *soi1-3*, and *soi3-1* homozygous diploids displayed an increased half-life of *Y*₇₁₃A Kex2p, whereas diploid strains heterozygous at these loci did not, indicating that the suppression phenotype in *soi1* and *soi3-1* strains is recessive (Table 4). *SOI2-1* and *SOI2-3* mutations decreased the rate of *Y*₇₁₃A Kex2p turnover to an equal degree in both heterozygous and homozygous diploids, indicating that these alleles likely represent dominant, gain-of-function mutations (Table 4).

Suppressor mutations specifically affect transmembrane proteins in the TGN, not earlier Golgi compartments. To test the compartmental specificity of suppression, we examined the effects of allele-specific mutations on the localization of two additional Golgi proteins. The *MNT1*-encoded α -1,2-mannosyltransferase is localized in a medial Golgi compartment (7). A chimeric protein consisting of full-length Mnt1p fused to invertase at a Kex2p cleavage site is efficiently retained in the Golgi (9). One such fusion construction was introduced into the seven allele-specific suppressor strains lacking Kex2p.

TABLE 5. $t_{1/2}$ values for delivery of Ste13p-Pho8p-F₈₇A fusion protein to the vacuole

Strain	$t_{1/2}$ (min) ^a
Pep ⁺ parent.....	53 ± 11
Pep ⁻ parent ^b	>>300
<i>soi1-2</i>	176
<i>soi1-3</i>	162
<i>SOI2-1</i>	104
<i>SOI2-3</i>	164
<i>soi3-1</i>	182

^a Cells were grown at 30°C prior to a 30-min sulfate depletion, pulse-labeled for 10 min, and chased at 30°C for 60 to 90 min. Except for the $t_{1/2}$ in the Pep⁺ parent, which is the mean ± standard error of the mean for three trials, the $t_{1/2}$ value was determined once from analysis of five or six time points of chase.

^b CB018 (*pep4 prb1 prc1*); this strain lacks PrA, PrB, and PrC, the major vacuolar proteases (59).

None of these strains exhibited a significant increase in extracellular invertase activity, indicating that the fusion protein was not mislocalized to the plasma membrane (data not shown). In *gem* mutants, transport to the TGN allows cleavage of the fusion protein by active Kex2p, releasing invertase for export to the periplasm (7). *Soi*⁺ strains expressing both Y₇₁₃A Kex2p under *KEX2* promoter control and the fusion protein exhibited no significant amount of extracellular invertase (data not shown). We conclude both that the Mnt1p-invertase fusion protein is properly retained in an early Golgi compartment in *Soi*⁺ strains and that *Soi*⁺ mutations do not cause Y₇₁₃A Kex2p to be retained in this compartment. These considerations suggest that the organization of the Golgi is not grossly affected in *Soi*⁺ strains.

Ste13p (18) appears to colocalize with Kex2p, and this localization requires a similar TLS (5, 38) (Fig. 1 and 2 and Table 1). To determine whether *soi1*, *SOI2*, and *soi3* mutations suppressed Ste13p TLS mutations, chimeras that contained the N-terminal C-tail of Ste13p with the F₈₇A mutant TLS fused to the transmembrane domain and luminal domain of the *PHO8*-encoded vacuolar ALP (38) were used. Delivery of this fusion protein, A-ALP, to the vacuole can be detected by *PEP4*-dependent processing of the C-terminal region of the precursor form of A-ALP (30, 38). The WT Ste13p TLS causes the precursor form of A-ALP to be stably retained in the TGN, whereas the F₈₇A substitution, like the Y₇₁₃A mutation in Kex2p, results in decreased TGN retention and more rapid delivery to the vacuole ($t_{1/2}$ for processing, 55 min) (38). The rate of transport of the F₈₇A A-ALP protein to the vacuole was measured in Pep⁺ and Pep⁻ parental strains and in five *Soi*⁺ strains. As judged by the rate of vacuolar protease-dependent disappearance of the A-ALP precursor band, the $t_{1/2}$ of processing of the F₈₇A A-ALP fusion were increased by two- to threefold in *Soi*⁺ strains relative to that of the WT *Soi*⁻ strain (Table 5). Thus, suppressor alleles of *SOI1*, *SOI2*, and *SOI3* suppress the effects of similar mutations in the TLSs of two TGN transmembrane proteins, Kex2p and Ste13p.

DISCUSSION

The suppressor alleles of the *SOI1*, *SOI2*, and *SOI3* genes have several characteristics which indicate that their products play an intimate role in the functioning of TGN localization signals in *S. cerevisiae*. We have shown that mutations in three genes, *SOI1*, *SOI2*, and *SOI3* result in allele-specific suppression of the localization defects caused by mutations in the TLS of Kex2p, suppressing the effects of point mutations at Tyr-713 but not those of deletions that remove the TLS. Moreover,

these suppressor alleles not only prolong mating in the onset-of-impotence assay but also directly affect the retention of TLS mutant Kex2p in the TGN, as measured by pulse-chase immunoprecipitation. We have shown that alleles of *SOI1*, *SOI2*, and *SOI3* suppress not only the effects of mutations in the Kex2p TLS but also the effects of mutations in the Ste13p TLS. Taken together with the observation that overexpression of WT Kex2p causes more rapid delivery of the A-ALP fusion protein to the vacuole (38), our results are consistent with there being a common "receptor" for the TLSs of Kex2p and Ste13p, despite the limited sequence similarity of the two signals (Fig. 1). The fact that mutations in the *SOI1*, *SOI2*, and *SOI3* genes affect the TLS in Ste13p, a type II TGN protein, establishes a general role for the *Soi1*, *Soi2*, and *Soi3* proteins in localization of transmembrane proteins in the TGN. In contrast, we have found that none of the allele-specific suppressors affect the localization of an Mnt1p-Suc2 fusion protein, indicating that mutations in the *SOI1*, *SOI2*, and *SOI3* genes do not perturb localization of transmembrane proteins in earlier Golgi compartments. We have shown that *SOI1* suppressor alleles not only improve the retention of Y₇₁₃A Kex2p but also impair the retention of WT Kex2p. Thus, *Soi1p* may play a role in the fidelity of recognition of TLS sequences. The *SOI1* gene has been cloned and sequenced and is predicted to encode a highly charged, 3,144-residue polypeptide (3a). Disruption of the *SOI1* gene is nonlethal and results in a Vps⁻ phenotype (3a). A more extensive analysis of the *SOI1* gene and its product will appear elsewhere. The sequence of the *SOI1* gene corresponds to open reading frame YLL040c on the left arm of chromosome XII in the Martinsried Institute for Protein Sequences database (3a). Further analysis of the *Soi1* protein will clarify its function in the localization of transmembrane proteins in the TGN and help to identify additional molecules involved in this process. Finally, we have shown that *SOI2* suppressor alleles are dominant, making the *SOI2* gene product a candidate component of the TLS recognition machinery. The fact that the mutations are dominant makes it possible that they represent an alteration of the specificity of a TLS receptor.

Signals within cytosolic tail sequences appear to be involved in the localization of TGN transmembrane proteins both in yeast (6, 8, 38, 59) and mammalian (2, 3, 44, 56) cells. Several lines of evidence point to a model in which proteins such as Kex2p, Ste13p, and Kex1p cycle between the TGN and a prevacuolar (endosomal) compartment. By immunofluorescence, a fraction of Kex2p was localized to sites lacking the Sec7 protein, which might correspond to prevacuolar compartments (15). Class E *vps* mutants, which appear to accumulate vacuolar proteins in a prevacuolar compartment, display significant Ste13p localization in this compartment as well as in the TGN (45). Finally, the Vps10 protein, the sorting receptor for pro (p2)-CPY (34), appears to colocalize with Kex2p and its C-tail is important for proper sorting (6). Both Vps10p and Kex2p are destabilized in class E *vps* mutants, whereas the medial Golgi protein, Mnn1p, is not (6). These results suggest that Vps10p cycles between the TGN and the endosome, like the mannose-6-phosphate receptor in mammalian cells (31), and that recycling to the TGN is carried out by the same system that recycles Kex2p. The Vps⁻ phenotype of *soi1* mutants may reflect a disruption of the Vps10 cycling pathway. In addition, a common apparatus recognizing both the Kex2p and Vps10p C-tails might explain the observation that overexpression of Kex2p results in the secretion of CPY (46a).

This prevacuolar retrieval model has similarities to a model proposed to account for the localization of TGN38 and furin to the mammalian TGN. Both proteins appear to be recycled from the plasma membrane to the TGN by an endocytic path-

way. In both cases, retrieval relies at least partly on a tyrosine-dependent signal in the C-tails of these proteins (2, 3, 22, 35, 44, 46). In *S. cerevisiae*, however, inactivation of Sec1p, which blocks the fusion of secretory vesicles with the plasma membrane (40, 41), does not cause an accumulation of Kex2p in secretory vesicles (47), does not affect the continued processing of Kex2p by the Golgi α -1,3-mannosyltransferase (58), and does not affect the delivery of WT or mutant forms of Kex2p to the vacuole (59). Thus, if localization of Kex2p and other TGN transmembrane proteins in *S. cerevisiae* does involve transport to and retrieval from the endosome, the pathway does not transit the plasma membrane. The transport of TGN transmembrane proteins to the prevacuolar compartment in the first leg of a recycling pathway may correspond to the default vacuolar transport pathway. Some of the non-allele-specific suppressor mutants may affect this default pathway to the prevacuolar compartment.

Kex2p is mislocalized to the cell surface when either clathrin heavy chain or the yeast dynamin/*shibire* homolog Vps1p is inactivated (38, 42, 53). In *chc1* (ts) mutants, clathrin-related functions such as endocytosis are quickly inhibited and Kex2p is detectable at the cell surface within 30 min after the shift to the nonpermissive temperature. This mislocalization and the observation that the Kex2p C-tail is required for proper localization led to the hypothesis that a clathrin-containing structure was responsible for TGN localization of Kex2p (17, 42). Our finding that the Kex2p localization signal shares characteristics with clathrin-mediated endocytosis signals (32, 59) (this work) lends support to this model.

The fact that TLS mutant forms of Kex2p are delivered to the vacuole led to the proposal that clathrin-containing structures selectively return WT but not TLS-defective forms of Kex2p to the TGN from a post-Golgi compartment (59). If this hypothesis were correct, then inactivation of clathrin should not affect the localization of TLS mutant forms of Kex2p. However, we have recently shown that the Y₇₁₃A and C-tail Δ forms of Kex2p are transported to the plasma membrane after clathrin inactivation at a rate indistinguishable from that of WT Kex2p (47a). This suggests that clathrin inactivation causes profound defects in both TGN retention and default transport of transmembrane proteins to the vacuole, making it difficult to assign a direct role for clathrin in TLS-dependent localization of Kex2p.

The isolation of *prl* mutations as suppressors of mutations in a bacterial signal sequence represented the first application of suppressor genetics to the identification of proteins that interact with protein localization signals (13). The success of this approach became widely appreciated when it was found that *prlA* mutations were alleles of the *SecY* gene (24), which encodes an essential component of the protein translocation pore in the *E. coli* plasma membrane (4). Similarly, analysis of the *SOI* genes and their products should add to our understanding of the mechanism of localization of TGN membrane proteins. The method used here for the isolation of *Soi*⁺ mutants utilized a screen for the persistence of mating competence after shutting off expression of an essential but labile component of the mating machinery. A similar screen based on persistence of mating after shutting off expression of the *a*-factor receptor, Ste3p, identified mutants defective in receptor endocytosis (11). In both cases, the yeast mating system has provided an assay sensitive enough to permit the isolation of mutations that might otherwise be difficult to detect but clearly alter the kinetics of transport and targeting of membrane proteins in the secretory pathway.

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