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The Effects of Clathrin Inactivation on Localization of Kex2 Protease Are Independent of the TGN Localization Signal in the Cytosolic Tail of Kex2p

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Localization of Kex2 protease (Kex2p) to the yeast *trans*-Golgi network (TGN) requires a TGN localization signal (TLS) in the Kex2p C-terminal cytosolic tail. Mutation of the TLS accelerates transport of Kex2p to the vacuole by an intracellular (*SEC1*-independent) pathway. In contrast, inactivation of the clathrin heavy-chain gene *CHC1* results in transport of Kex2p and other Golgi membrane proteins to the cell surface. Here, the relationship of the two localization defects was assessed by examining the effects of a temperature-sensitive *CHC1* allele on trafficking of wild-type (WT) and TLS mutant forms of Kex2p. Inactivation of clathrin by shifting *chc1-ts* cells to 37°C caused WT and TLS mutant forms of Kex2p to behave identically. All forms of Kex2p appeared at the plasma membrane within 30–60 min of the temperature shift. TLS mutant forms of Kex2p were stabilized, their half-lives increasing to that of wild-type Kex2p. After inactivation of clathrin heavy chain, vacuolar protease-dependent degradation of all forms of Kex2p was blocked by a *sec1* mutation, which is required for secretory vesicle fusion to the plasma membrane, indicating that transport to the cell surface was required for degradation by vacuolar proteolysis. Finally, after clathrin inactivation, all forms of Kex2p were degraded in part by a vacuolar protease-independent pathway. After inactivation of both *chc1-ts* and *sec1-ts*, Kex2 was degraded exclusively by this pathway. We conclude that the effects of clathrin inactivation on Kex2p localization are independent of the Kex2p C-terminal cytosolic tail. Although these results neither prove nor rule out a direct interaction between the Kex2 TLS and a clathrin-dependent structure, they do imply that clathrin is required for the intracellular transport of Kex2p TLS mutants to the vacuole.

INTRODUCTION

Kex2 protease (Kex2p) is required for maturation of the precursors of several secreted peptides and proteins, including the α-mating factor, in *Saccharomyces cerevisiae* (Julius *et al.*, 1984; Fuller *et al.*, 1988). A type I transmembrane protein, newly synthesized Kex2p is transported rapidly from the endoplasmic reticulum (ER) through early Golgi compartments to a late Golgi compartment, most likely the yeast equivalent of the mammalian *trans*-Golgi network (TGN),[1] where it functions for an extended period of time before ultimate delivery to and degradation in the vacuole (Redding *et al.*, 1991; Wilcox and Fuller, 1991; Wilcox *et al.*, 1992). Mutational analysis of the C-terminal cytosolic tail (C-tail) of Kex2p has defined a TGN localization signal (TLS) that consists of an essential tyrosine res-

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[1] Abbreviations used: C-tail, C-terminal cytosolic tail; TGN, *trans*-Golgi network; TLS, TGN localization signal; WT, wild-type.

idue (Tyr_{713}) within contextually important sequences (Wilcox *et al.*, 1992; Redding *et al.*, 1996). The TLS in the Kex2p C-tail conforms well to a consensus "tyrosine recognition signal" proposed to mediate the localization of numerous cell surface receptors to clathrin-coated pits (Ktistakis *et al.*, 1990; Wilcox *et al.*, 1992). Mutation of the TLS results in accelerated delivery of Kex2p, by default, to the vacuole (Wilcox *et al.*, 1992). Neither the rapid delivery of TLS mutant forms of Kex2p to the vacuole nor the eventual delivery of WT Kex2p to the vacuole is affected by the *sec1* mutation (Wilcox *et al.*, 1992), which prevents fusion of post-Golgi secretory vesicles with the plasma membrane (Novick and Schekman, 1979). Thus, both the WT and TLS mutant forms of Kex2p reach the vacuole by an intracellular pathway (Wilcox *et al.*, 1992). In contrast, soluble forms of Kex2p lacking the transmembrane domain and C-tail are secreted efficiently (Gluschankof and Fuller, 1994). Vacuolar transport seems also to be the default pathway for other transmembrane proteins in yeast (Cooper and Bussey, 1992; Roberts *et al.*, 1992; Gaynor *et al.*, 1994). These include the *STE13*-encoded dipeptidyl aminopeptidase and *KEX1*-encoded carboxypeptidase, which function after Kex2p in pro-α-factor processing and seem to colocalize with Kex2p (Bryant and Boyd, 1993; Nothwehr *et al.*, 1993).

Depending on the genetic background, deletion of the *CHC1* gene encoding clathrin heavy chain results either in an extreme slow growth phenotype (Payne and Schekman, 1985; Payne *et al.*, 1987) or in lethality (Lemmon and Jones, 1987; Munn *et al.*, 1991). Viable *CHC1* disruptants exhibit both drastically reduced pro-α-factor processing and mislocalization of Kex2p to the cell surface (Payne and Schekman, 1989). Cells containing a conditional *CHC1* allele appear normal at the permissive temperature but transport Kex2p to the cell surface within 30 min after a shift to 37°C (Seeger and Payne, 1992a). The fact that mislocalization of Kex2p results both from mutation of a sequence in the Kex2p C-tail that resembles a clathrin-coated pit localization signal and from mutation of the clathrin heavy-chain gene led to the hypothesis that correct localization of Kex2p involves interaction between the C-tail and a clathrin-containing structure (Fuller *et al.*, 1989b; Payne and Schekman, 1989; Seeger and Payne, 1992a; Wilcox *et al.* 1992). Nevertheless, an apparent paradox arises from considering the different consequences of mutations in the Kex2p TLS, which result in transport of Kex2p to the vacuole, and mutations in *CHC1*, which result in transport of Kex2p to the plasma membrane.

One model that accounts for the distinct effects of TLS mutations and clathrin heavy-chain defects, while conserving an interaction between the Kex2p C-tail and clathrin, posits that the Kex2p TLS diverts Kex2p from the vacuolar pathway by interacting with constituents of clathrin coats on the TGN or nascent secretory vesicles (Wilcox *et al.*, 1992). This interaction would result in TGN retention either by static anchoring or by a recycling pathway. In the absence of clathrin heavy chain, the TLS might still divert Kex2p from the vacuolar pathway by associating with remaining coat components (e.g., clathrin adaptors), but the absence of clathrin heavy chain would abolish retention or retrieval of Kex2p, and the protein would be transported via secretory vesicles to the cell surface. In contrast, mutations in the TLS would prevent Kex2p from associating with the remaining coat components, and the mutant protein would follow the default pathway to the vacuole. The model predicts that the TLS and its putative receptor would, in effect, function before clathrin in the Kex2p retrieval pathway. Because TLS mutant Kex2p molecules would be unable to associate with clathrin coats, the TLS mutations would be epistatic to mutations in *CHC1*, and rapid transport of TLS-mutant forms of Kex2p to the vacuole would be unaffected by loss of clathrin function. We have tested this model by expressing wild-type (WT) and TLS-mutant forms of Kex2p in *chc1-ts* cells and determining their cellular itineraries after clathrin inactivation. Remarkably, the effects of clathrin inactivation on the localization of Kex2p are independent of a functional TLS or even the presence of the C-tail. The results do imply that inactivation of clathrin heavy chain blocks the "default" transport of Kex2p TLS mutants to the vacuole.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions

The strains used were created by standard methods (Rose *et al.*, 1990) and are listed in Table 1 along with intermediates in their construction. 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. For cell surface assays, WT and mutant forms of Kex2p were expressed at 15-fold elevated levels under *GAL1* promoter control on single-copy *CEN* plasmids (Redding *et al.*, 1991). For all other experiments, WT and mutant forms of Kex2p were expressed under the control of the WT *KEX2* promoter on single-copy *CEN* plasmids. Specific plasmids are identified in the legends and are described in more detail in Wilcox *et al.* (1992). $Y_{713}W$ Kex2p was expressed under WT *KEX2* promoter control from single-copy *CEN* plasmid pKRKX10-$Y_{713}W$ (Redding *et al.*, 1996). Plasmids were introduced into yeast strains by lithium acetate transformation (Ito *et al.*, 1983) or spheroplast transformation (Burgers and Percival, 1987).

Radiolabeling and Immunoprecipitation

These experiments were performed as described in Wilcox *et al.* (1992). All data were obtained with a Molecular Dynamics PhosphorImager (Sunnyvale, CA). Kex2p half-lives were determined by regression analysis with at least four, and usually five, time points assuming exponential decay.

Table 1. Yeast strains used

Strain	Genotype	Origin
W303-1A	*MATa can-1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein, Columbia University, New York, NY
CRY2	W303-1A *MATα*	Wilcox and Fuller, 1991
BFY106-4D	CRY2 *kex2Δ::HIS3*	Wilcox *et al.*, 1992
KRY18-1B	W303-1A *kex2Δ::TRP1*	This study
KRY36-5B	CRY2 *pep4::HIS3 prb1::LEU2*	This study
CB017	CRY2 *pep4::HIS3 prb1::hisG prc1::hisG*	Wilcox *et al.*, 1992
KRY108-5D	CRY2 *kex2Δ::TRP1 sec1-1*	Wilcox *et al.*, 1992
KRY108-7D	CRY2 *kex2Δ::TRP1 sec1-1 pep4::HIS3*	Wilcox *et al.*, 1992
KRY108-8B	W303-1A *kex2Δ::TRP1 sec1-1 pep4::HIS3*	This study
GPY686	W303-1A *chc1-521*	This study
KRY112	KRY36-5B × GPY686	This study
KRY112-7B	CRY2 *pep4::HIS3 prb1::LEU2 chc1-521*	This study
KRY113	KRY18-1B × KRY112-7B	This study
KRY113-11A	CRY2 *kex2Δ::TRP1 pep4::HIS3 prb1::LEU2 chc1-521*	This study
KRY121	CRY2 × KRY113-19C	This study
KRY121-9D	CRY2 *kex2Δ::TRP1 chc1-521*	This study
KRY130	KRY108-8B × KRY121-9D[pCWKX10]	This study
KRY130-23D	CRY2 *kex2Δ::TRP1 chc1-521 sec1-1*	This study
KRY130-30B	CRY2 *kex2Δ::TRP1 chc1-521 sec1-1 pep4::HIS3*	This study

Cell Surface Iodination

These experiments were performed as described in Seeger and Payne (1992a). In all experiments, lysis efficiency was controlled for by immunoprecipitation of glyceraldehyde-3-phosphate dehydrogenase (Seeger and Payne, 1992a).

Kex2 Protease Assays

One unit of Kex2 activity (Fuller *et al.*, 1989a) is defined as the Ca^{2+}-dependent release of 1 pmol of 7-amino-4-methylcoumarin from t-boc-Gln-Arg-Arg-MCA per minute at 37°C in a volume of 50 μl. Blank values were obtained from reactions containing 20 mM EDTA and were subtracted from those containing 1 mM $CaCl_2$. The fraction of total active Kex2 protease at the cell surface was determined as described (Wilcox *et al.*, 1992).

RESULTS

Creating a System to Study Epistasis

Clathrin heavy chain is encoded by a single gene, *CHC1*, in *S. cerevisiae* (Payne and Schekman, 1985; Lemmon and Jones, 1987). The conditional *CHC1* allele *chc1-521* encodes a protein that seems to be fully functional at 25°C but quickly loses function at the nonpermissive temperature. On shifting to 37°C, *chc1-521* cells rapidly display phenotypes characteristic of *chc1* null mutants: secretion of pro-α-factor, cell surface expression of Kex2p, and inhibition of rapid endocytosis of the **a**-factor receptor Ste3p (Seeger and Payne, 1992a,b; Tan *et al.*, 1993). In addition, immediately after shifting to the restrictive temperature, *chc1-521* cells exhibit a defect in vacuolar sorting and maturation of pro-carboxypeptidase Y (pro-CPY), a phenomenon that exhibits "adaptation" (Seeger and Payne, 1992b). After extended incubation at 37°C, *chc1-521* cells cease secreting pro-CPY, and the precursor undergoes proteolytic maturation in an intracellular compartment. The *chc1-521* allele was used to replace the normal *CHC1* gene in the W303 strain background. Backcrosses against isogenic strains carrying disruptions or deletions of the *KEX2*, *PEP4*, and *PRB1* genes were used to create a set of congenic *kex2* strains with either a WT *CHC1* or *chc1-ts* allele and either having (Pep^+) or lacking (Pep^-) vacuolar proteases (Jones, 1991a,b; Table 1). WT and C-tail mutant forms of Kex2p having a substitution of Ala for Tyr713 (Y713A) or a complete deletion of the C-tail (C-tailΔ) were expressed from the WT *KEX2* promoter or the inducible *GAL1* promoter on centromeric vectors.

Appearance of WT and C-Tail Mutant Forms of Kex2p at the Cell Surface after Clathrin Inactivation

It was demonstrated previously that WT Kex2p is delivered to the plasma membrane after inactivation of clathrin heavy chain (Seeger and Payne, 1992a). To determine how TLS mutant forms of Kex2p would be affected by clathrin inactivation, we grew *CHC1* and *chc1-ts* cells expressing either WT or C-tail mutant forms of Kex2p at 25°C and then shifted them to 37°C. To detect transport of Kex2p to the plasma membrane, we assayed whole cells for Kex2p proteolytic activity with a sensitive fluorogenic peptide substrate either before or after permeabilization with detergent, and the percentage of activity at the cell surface was calculated (Fuller *et al.*, 1989b; Wilcox *et al.*, 1992). Vacuolar protease-deficient (Pep^-) strains were used to reduce background proteolytic activity. As shown in Figure 1, with all forms of Kex2p in both *chc1-ts* and *CHC1* cells, only a low background amount of Kex2-

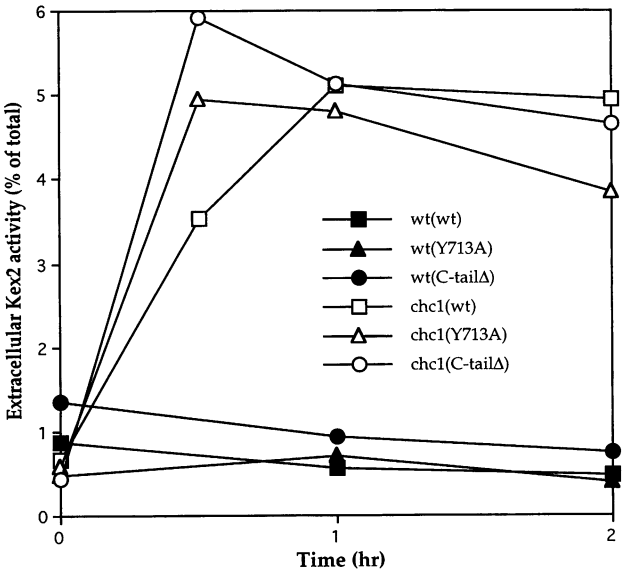

Figure 1. Appearance of cell surface Kex2 activity in a *chc1-ts* Pep$^-$ strain at the restrictive temperature. The WT (pCWKX20), $Y_{713}A$ (pCWKX21), or C-tailΔ (pCWKX27) forms of Kex2p were expressed under *GAL1* promoter control in *chc1-ts kex2*Δ Pep$^-$ strain KRY113-11A and *CHC1 kex2*Δ Pep$^-$ strain CB017. Strains containing the plasmids expressing the indicated forms of Kex2p were grown in synthetic media plus 2% galactose at 25°C and shifted to 37°C. Samples harvested at the time of the temperature shift or at indicated times thereafter were split, and Kex2 activity was measured in intact cells and in detergent-permeabilized cells, as described (Wilcox *et al.*, 1992). Kex2 activity that was detected at the cell surface is expressed as a percentage of total cellular Kex2 activity: % of activity at cell surface = [(activity measured with intact cells)/(activity with permeabilized cells)] × 100.

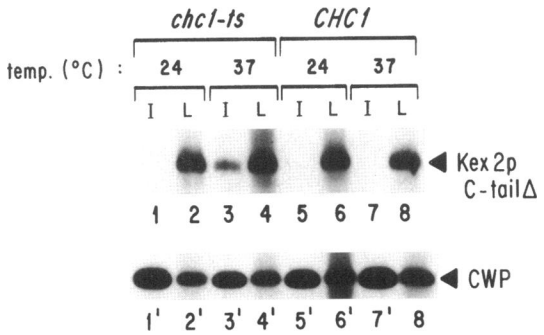

Figure 2. Cell surface iodination of C-tailΔ Kex2p in *chc1-ts* cells. *CHC1 kex2*Δ Pep$^-$ (CB017) and *chc1-ts kex2*Δ Pep$^-$ (KRY113-11A) cells expressing the C-tailΔ form of Kex2p at the WT level of expression from plasmid pCWKX17 were grown at 24°C and shifted to 37°C for 0 min or 2 h. Intact cells (I) and lysates (L) were labeled by solid phase iodination and immunoprecipitated with antibody against Kex2p or CWP, a cell wall protein found accessible to iodination both in intact cells and in lysates (Seeger and Payne, 1992a). Immunoprecipitates were subjected to SDS-PAGE, followed by autoradiography.

like activity was detected in assays of intact cells grown at 25°C before shifting the temperature. Within 30 min of shifting to 37°C, a significant amount of Kex2 proteolytic activity (3–6% of total) was detected at the cell surface for WT, $Y_{713}A$, and C-tailΔ Kex2p in the *chc1-ts* strains, whereas surface activity in the *CHC1* strains remained at the background level in each case. Cell surface activity reached a maximum level (5–6% of total) for all three forms of Kex2p by 30–60 min after shift.

These results were in good agreement, qualitatively, with the demonstration by cell surface iodination of appearance of WT Kex2p at the cell surface in *chc1-ts* cells within 30 min after shifting from 25 to 37°C (Seeger and Payne, 1992a). Therefore, the cell surface iodination assay was used as an alternative approach to detect the appearance of C-tail mutant forms of Kex2p at the cell surface in *chc1-ts* cells at 37°C. Intact WT and *chc1-ts* cells expressing C-tailΔ Kex2p were subjected to radioiodination after growth at 24°C or after a 2 h shift to 37°C (Figure 2, "I" samples). In addition, cell lysates were prepared and iodinated to label total protein (Figure 2, "L" samples). After the labeling, intact cells were lysed, and Kex2p was immunoprecipitated from all samples with the use of antiserum that recognizes the lumenal domain of Kex2p. As an internal control, a 33-kDa cell wall protein (CWP) that should be equally accessible in both intact and lysed cells was immunoprecipitated from all samples. At 24°C, no C-tailΔ Kex2p was labeled in either *CHC1* or *chc1-ts* cells (Figure 2, lanes 1, 2, 5, and 6), consistent with previous findings that mislocalization of C-tailΔ Kex2p to the vacuole does not proceed by way of the plasma membrane (Wilcox *et al.*, 1992). After the shift to 37°C, 20% of the total C-tailΔ Kex2p was labeled at the cell surface in *chc1-ts* cells (Figure 2, lanes 3 and 4), whereas no labeling was detectable with the use of intact *CHC1* cells (Figure 2, lanes 7 and 8).

The fraction of total C-tailΔ Kex2p detected at the cell surface by enzymatic assay (~5%) or by surface iodination (20%) in these Pep$^-$ *chc1*-ts cells at 37°C was considerably smaller than measured previously for WT Kex2p by surface iodination of Pep$^+$ *chc1-ts* cells (60–90%). It seemed likely that the difference was due principally to accumulation of stable C-tailΔ Kex2p in the vacuole before the temperature shift, because the vacuolar pool of Kex2p would not be subject to mislocalization to the cell surface at clathrin inactivation. To assess this possibility, we examined the time course of cell surface appearance of WT, $Y_{713}A$, and C-tailΔ Kex2p in Pep$^+$ *chc1-ts* cells at 37°C by the surface iodination assay (Figure 3). Approximately 70% of each form of Kex2p was detected on the surface of Pep$^+$ *chc1-ts* cells after incubation at 37°C for 1 h. Moreover, the time course of appearance of each form of the protein at the cell surface, as measured by the iodination assay, was identical and par-

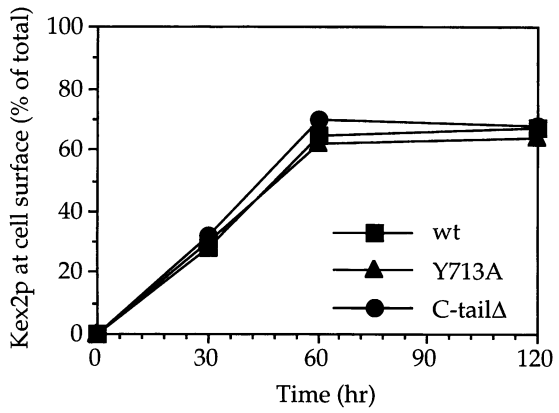

Figure 3. Time course of cell surface appearance of WT and mutant Kex2p after clathrin inactivation in a Pep$^+$ background measured by iodination. Strain KRY121-9D (*kex2Δ chc1-ts* Pep$^+$) expressing WT (pCWKX10), $Y_{713}A$ (pCWKX11), or C-tailΔ (pCWKX17) Kex2p at the WT level was grown at 25°C and shifted to 37°C; samples were harvested at the indicated times. Samples were split and iodinated as intact cells or as total cellular lysates. Kex2 protein was immunoprecipitated with an antibody directed against the entire Kex2 protein. Amounts of total Kex2p (accessible to iodination in the whole-cell lysate) and cell surface Kex2p (accessible to iodination in intact cells) were normalized by the iodination efficiency of CWP (see legend to Figure 2). The amount of Kex2p at the cell surface is expressed as the ratio of the normalized values (intact/total), expressed as a percentage.

alleled the appearance of enzyme activity at the cell surface in that a maximum value was reached after 1 h at 37°C (Figure 2). Measurements of surface Kex2p by activity assays always resulted in lower estimates than did cell surface iodination assays, most likely because of instability of Kex2 enzymatic activity at the cell surface. Kex2 activity is unstable at low pH (Brenner *et al.*, 1994; Gluschankof and Fuller, 1994), such as is found at the cell surface during yeast growth. In contrast, enzyme activity is quite stable inside living cells, even when the enzyme is primarily localized to the vacuole (the half-time of Kex2 enzyme inactivation in Pep$^-$ cells is >6 h; Wilcox *et al.*, 1992). Taken together, measurements of plasma membrane localization by cell surface activity assays and cell surface iodination demonstrate that transport of Kex2p to the cell surface after clathrin inactivation is independent of the nature or presence of the C-tail.

Vacuolar Degradation of WT and Mutant Forms of Kex2p after Clathrin Inactivation

Deletion of the Kex2p C-tail or mutation of Tyr_{713} in the Kex2p C-tail impairs retention in the Golgi and results in transport to the vacuole (Wilcox *et al.*, 1992). Because Kex2p is degraded rapidly in the vacuole, its rate of transport to the vacuole can be measured by determining the half-life of the protein in Pep$^+$ cells (Wilcox *et al.*, 1992). WT, $Y_{713}A$, and C-tailΔ forms of Kex2p exhibit half-lives of 83, 22, and 11 min, respectively, at 30°C in Pep$^+$ cells but are stabilized indefinitely in Pep$^-$ cells ($t_{1/2}$ > 3hr). Substitution of Trp for Tyr_{713} results in a $t_{1/2}$ of 35 min at 30°C, phenotypically intermediate between the WT and $Y_{713}A$ forms of the protein (Redding *et al.*, 1996). At 37°C, the WT and TLS mutant forms of Kex2p all exhibit more rapid rates of degradation (see data in Table 2, taken from Wilcox *et al.*, 1992), but the relative rates of degradation of the three forms remain similar. Degradation at 37°C also occurs in the vacuole, because each form of the protein is stabilized indefinitely when expressed in a Pep$^-$ strain at 37°C (see data in Table 2, taken from Wilcox *et al.*, 1992). To determine the effect of clathrin inactivation on vacuolar degradation of WT and TLS mutant forms of Kex2p, we shifted *chc1-ts* cells expressing WT or TLS mutant Kex2p to 37°C for 30 min; the cells were pulse labeled with ^{35}S-SO_4 and chased for various times with unlabeled SO_4, and Kex2p was immunoprecipitated from cell extracts (Figure 4). As shown in Table 2, all forms of Kex2p examined were now degraded with nearly identical half-times (33–35 min). Comparison of the results with *CHC1* and *chc1-ts* strains indicates that the TLS mutant forms of Kex2p were all substantially stabilized by inactivation of clathrin heavy chain. This result implies that the default pathway to the vacuole is perturbed significantly by inactivation of clathrin heavy chain. Furthermore, the fact that the turnover rates of all four forms of the protein converged after clathrin inactivation suggests that all four proteins are metabolized identically, rather than differentially, after the shift to 37°C. After inactivation of clathrin heavy chain, degradation of the WT and TLS mutant forms of Kex2p became partially independent of vacuolar proteolysis. Although rates of degradation at 37°C for all forms of Kex2p

Table 2. Half-lives of wt Kex2p and C-tail mutants in *chc1-ts* strains

Form of Kex2p on plasmid	Half-lives (min at 37°C)[a]			
	CHC1 Pep$^+$	*CHC1* Pep$^-$	*chc1-ts* Pep$^+$	*chc1-ts* Pep$^-$
wt	30 ± 2	>180	33 ± 4	125
$Y_{713}W$	ND	ND	33 ± 2	135
$Y_{713}A$	11	>180	36 ± 1	135
C-tailΔ	7.6	>180	34 ± 5	150

[a] Cells were grown at 25°C before a shift to 37°C. The cells were subjected to a 30-min sulfate depletion before a 10-min pulse labeling and chase at 37°C (Wilcox and Fuller, 1991). For comparative purposes, the *CHC1* Pep$^+$ (strain BFY106-4D) and *CHC1* Pep$^-$ (strain CB017) data were taken from Wilcox *et al.* (1992). The *chc1-ts* Pep$^+$ strain was KRY121-9D. The *chc1-ts* Pep$^-$ strain was KRY113-11A.

ND, not determined.

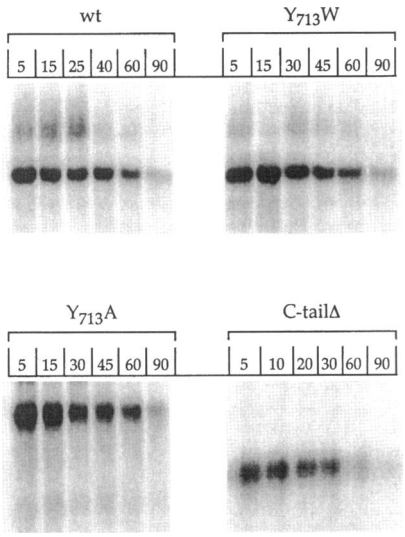

Figure 4. Pulse–chase immunoprecipitation analysis of WT and mutant Kex2p in *chc1-ts* strains. The WT (pCWKX10), $Y_{713}A$ (pCWKX11), $Y_{713}W$ (pKRKX10-$Y_{713}W$), and C-tailΔ (pCWKX17) forms of Kex2p were expressed from the WT *KEX2* promoter on single-copy *CEN* plasmids (Wilcox *et al.*, 1992; Redding *et al.*, 1996). The *chc1-ts kex2Δ* Pep^+ strain KRY121-9D expressing the indicated forms of Kex2p were grown at 25°C, harvested, and shifted to 37°C during a 30-min sulfate depletion before labeling with [^{35}S]-SO_4 for 10 min (Wilcox and Fuller, 1991). Unlabeled sulfate, methionine, and cysteine were added as a chase, and samples were harvested at the indicated times after initiation of the chase (Wilcox *et al.*, 1992). The anti-Kex2 lumenal antiserum was used to immunoprecipitate the C-tailΔ form of Kex2p (Wilcox *et al.*, 1992). The anti-Kex2 tail antibody (Fuller *et al.*, 1989b) was used in all other cases. Immunoprecipitates were subjected to SDS-PAGE (Wilcox and Fuller, 1991), and images were obtained with a Molecular Dynamics PhosphorImager.

were decreased in *chc1-ts* Pep^- strains relative to *chc1-ts* Pep^+ cells, a measurable component of vacuolar protease-independent degradation was observed in the *chc1-ts* Pep^+ cells (Table 2; $t_{1/2}$ ~125-150 min). In contrast, vacuolar protease-independent degradation of WT and TLS mutant forms of Kex2p was never observed in *CHC1* cells (Wilcox *et al.*, 1992).

After Clathrin Inactivation, SEC1 Function Is Required both for Transport of Kex2p to the Cell Surface and for Its Degradation by Vacuolar Proteases

The data presented thus far indicate that, after inactivation of clathrin heavy chain, an equal fraction of each form of Kex2p was transported to the plasma membrane and that each form of Kex2p was degraded by vacuolar proteases at the same rate. These results raised the possibility that, after loss of clathrin function, separate populations of Kex2p were transported to the vacuole and to the plasma membrane. Alternatively, it was possible that, after clathrin inactivation, delivery of all Kex2p to the vacuole required transport to the plasma membrane. In *CHC1* cells, transport of WT and C-tail mutant forms of Kex2p to the vacuole was shown to be independent of the *SEC1* gene (Wilcox *et al.*, 1992), which is required for fusion of post-Golgi secretory vesicles with the plasma membrane (Novick and Schekman, 1979). In contrast, delivery of WT Kex2p to the plasma membrane after inactivation of clathrin heavy chain was found to require *SEC1* (Seeger and Payne, 1992a).

To determine whether *SEC1* function is involved in trafficking of TLS mutant forms of Kex2p after inactivation of clathrin function, we constructed congenic Pep^+ and Pep^- *chc1-ts sec1-ts* strains and transformed them with vectors expressing WT and C-tailΔ Kex2p. Here, the Pep^- phenotype was due solely to the disruption of the *PEP4* gene, which results in a pleiotropic reduction of vacuolar proteolytic activity (Jones, 1991a,b). Pep^- *SEC1 chc1-ts* and *sec1-ts chc1-ts* cells expressing C-tailΔ Kex2p were shifted to 37°C for 1 h and then subjected to the iodination regime (Figure 5). Although 15% (normalized to the labeling of a cell wall protein) of the C-tailΔ Kex2p was detected at the surface after 1 h in the case of *SEC1 chc1-ts* cells (Figure 4, lanes 1 and 2), appearance of the protein at the cell surface was blocked in the *sec1-ts chc1-ts* cells (Figure 5, lanes 3 and 4). Thus, like WT Kex2p (Seeger and Payne, 1992a), C-tail mutant forms of Kex2p seem to be delivered to the plasma membrane in secretory vesicles.

To determine whether delivery to the cell surface was required for vacuolar protease-dependent degradation of WT and TLS mutant forms of Kex2p after clathrin inactivation, we subjected Pep^+ *chc1-ts sec1-ts* strains to the pulse–chase/immunoprecipitation regimen used previously with the *chc1-ts SEC1* strains (Figure 6 and Table 3). The presence of the *sec1-ts* mutation resulted in a marked stabilization of both WT and $Y_{713}A$ Kex2p, with the half-lives of each protein increasing from ~30 to 80 min (Figure 6A and Table 3). Thus, once again the fates of the WT and the C-tail mutant proteins were indistinguishable after

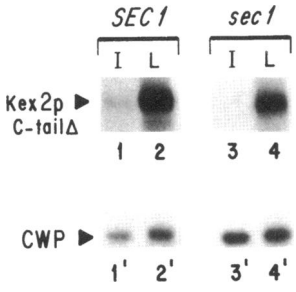

Figure 5. Appearance of the C-tailΔ form of Kex2p at the cell surface on clathrin inactivation is blocked by the *sec1* mutation. The *chc1-ts kex2Δ SEC1* Pep^- strain KRY113-11A and the *chc1-ts kex2Δ sec1-ts* Pep^- strain KRY130-30B expressing the C-tailΔ form of Kex2p at wild-type levels from plasmid pCWKX17 were grown at 24°C and shifted to 37°C for 1 h. Cells were then labeled by surface iodination and immunoprecipitated as described in the legend to Figure 2 and in Seeger and Payne (1992a).

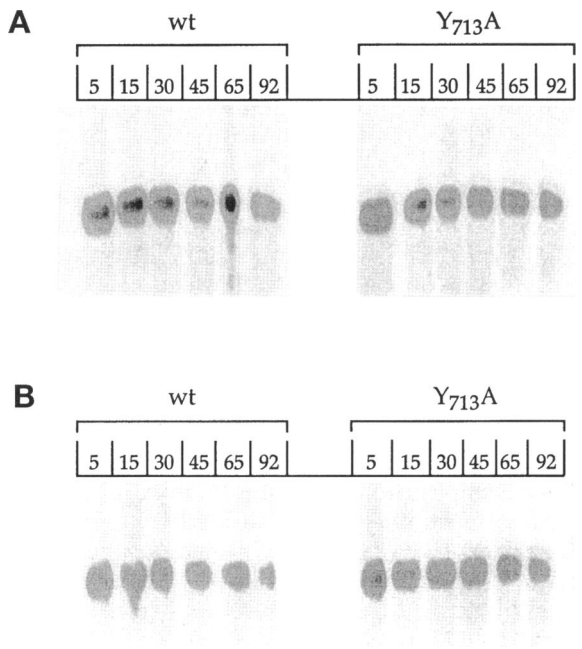

Figure 6. After inactivation of clathrin heavy chain, the *sec1* mutation blocks transport of both WT and $Y_{713}A$ Kex2p to the vacuole. Strains expressing WT (pCWKX10) or $Y_{713}A$ (pCWKX11) Kex2p from the WT *KEX2* promoter on single-copy *CEN* plasmids were grown at 25°C, harvested, and shifted to 37°C during the 30-min sulfate depletion before labeling with [^{35}S]-H_2SO_4 for 10 min. A chase was initiated by adding unlabeled Met, Cys, and SO_4, samples were harvested at the indicated times (min), and immunoprecipitates obtained by using anti-Kex2p C-tail antiserum were subjected to SDS-PAGE (Wilcox and Fuller, 1991). Images were obtained with a Molecular Dynamics PhosphorImager. (A) KRY130-23D (*chc1-ts kex2Δ PEP4 sec1-ts*); (B) KRY130-30B (*chc1-ts kex2Δ pep4 sec1-ts*).

inactivation of clathrin heavy chain. However, when the turnover rates in the Pep$^-$ *chc1-ts sec1-ts* strains were examined, two striking features emerged (Figure 6B and Table 3). First, the *pep4* mutation had no effect on the turnover rates of either WT or $Y_{713}A$ Kex2p in the *chc1-ts sec1-ts* background. Therefore, after inactivation of clathrin heavy chain, vacuolar protease-dependent degradation of both WT and C-tail mutant forms of Kex2p requires delivery to the plasma membrane. Second, it is clear from this experiment as well as from the data in Table 2 that, after inactivation of clathrin heavy chain, both WT and C-tail mutant forms of Kex2p undergo degradation at an appreciable rate by a vacuolar protease-independent pathway. Turnover of Kex2p was faster in the *chc1-ts sec1-ts* strains ($t_{1/2}$ ~ 80 min; Table 3) than in the *chc1-ts SEC1* strains ($t_{1/2}$ ~125 min; Table 2), suggesting that Kex2p may be degraded by this pathway more rapidly when accumulated in secretory vesicles. This effect required inactivation of clathrin heavy chain, because the *sec1-ts* mutation had no effect on rates of Kex2p turnover in cells having the WT *CHC1* gene (Wilcox *et al.*, 1992; Table 3). Furthermore, it is clear that vacuolar protease-independent degradation of Kex2p in the *chc1-ts sec1-ts* strain occurs in a late secretory compartment, because Kex2p has undergone the shift up in SDS-PAGE mobility that results from modification of the protein's Asn-linked and Ser-/Thr-linked oligosaccharide in the Golgi (Figure 6; Wilcox and Fuller, 1991).

DISCUSSION

Consequences of Clathrin Inactivation for Localization of Kex2p

Mutations in the Kex2p TLS have profound effects on localization of the protein to the TGN in cells having a WT *CHC1* gene (Wilcox *et al.*, 1992). Likewise, loss of clathrin function alters the localization of WT Kex2p (Payne and Schekman, 1989; Seeger and Payne, 1992a). Contrary to the expectation that clathrin inactivation should only affect forms of Kex2p containing a functional TLS, we have shown here that clathrin inactivation affects all forms of Kex2p, causing them to behave identically. Clathrin inactivation dramatically alters transport and localization of TLS mutant forms of Kex2p, including a form completely lacking the C-terminal cytosolic tail. The default intracellular transport of TLS mutant forms of Kex2p to the vacuole is

Table 3. Half-lives of wt and mutant Kex2p in *sec1 chc1* mutant at 37°C

Form of Kex2p on plasmid	Half-lives (min at 37°C)[a]				
	SEC1 PEP4 CHC1	*sec1 PEP4 CHC1*	*sec1 pep4 CHC1*	*sec1 PEP4 chc1*	*sec1 pep4 chc1*
wt	30	34	>180	83	84
$Y_{713}A$	11	11	>180	82	90

[a] Cells were grown at 37°C and labeled as in the legend to Table 2. Data for the *CHC1 PEP4 SEC1* (strain CRY2), *CHC1 PEP4 sec1-ts* (strain BFY106-4D) and *CHC1 pep4 sec1-ts* (strain CB017) were taken from Wilcox *et al.* (1992) and are provided for comparison. The *chc1-ts PEP4 sec1-ts* strain was KRY130-23D, and the *chc1-ts pep4 sec1-ts* strain was KRY130-30B.

blocked, and all forms of Kex2p are delivered to the cell surface. Inactivation of clathrin causes all forms of Kex2p to be degraded at identical rates. Degradation now occurs both by a vacuolar protease-dependent pathway (presumably in the vacuole) and also by a nonvacuolar pathway. Finally, unlike the situation in *CHC1* cells, vacuolar protease-dependent degradation of WT and TLS mutant forms of Kex2p after clathrin inactivation requires initial delivery of the proteins to the cell surface.

It is of interest that mutation of *VPS1*, another gene that affects TGN protein localization (Wilsbach and Payne, 1993), also seems to reroute TGN membrane proteins to the plasma membrane (Nothwehr *et al.*, 1995). Vps1p is related to dynamin (Vater *et al.*, 1992), a GTP-binding protein implicated in the formation of clathrin-coated pits (Herskovits *et al.*, 1993; Van der Bliek *et al.*, 1993; Damke *et al.*, 1994). These relationships raise the possibility that Vps1p and clathrin act together in normal TGN function. The results with *vps1* mutants reinforce the conclusion that there is a basic difference between the transport routes followed by TGN membrane proteins lacking the TLS in WT cells and TGN membrane proteins in cells with defects in cytosolic components of the TGN.

Why Does Inactivation of Clathrin Affect Localization of Kex2p?

Because Kex2p TLS mutants ($Y_{713}A$ and C-tailΔ) are also diverted to the cell surface after clathrin inactivation, specific recognition of the Kex2p TLS is not required for trafficking of Kex2p to the cell surface in the absence of clathrin function. Thus mutation of *CHC1* is epistatic to mutations in the Kex2p TLS, and the model presented in the INTRODUCTION cannot be correct. Here, we consider two possible interpretations. The first is that the effects of clathrin inactivation on Kex2p localization may be indirect, caused, for example, by global alterations of TGN function. Indeed, clathrin can be viewed as a major peripheral membrane protein of the TGN (Ladinsky *et al.*, 1994). The second is that clathrin may participate directly in steps in the transport or localization of Kex2p in a way that does not require the TLS or, indeed, any part of the C-tail.

In support of the "indirect" model is the well-known pleiotropy of clathrin mutations in yeast. Clathrin mutations affect not only localization of Golgi membrane proteins but also endocytosis and the sorting of soluble vacuolar protein precursors. As we have shown here, inactivation of clathrin induces a nonvacuolar protease-dependent pathway of membrane protein degradation. The pleiotropic effects of clathrin inactivation might reflect a generalized disturbance of TGN function that could cause both the rerouting of Kex2p TLS mutants and the mislocalization of WT Kex2p to the cell surface. Such a general effect on Golgi function could account for the observation that inactivation of *CHC1* results in mislocalization of the *MNN1*-encoded α-1,3 mannosyl transferase, a type 2 transmembrane protein whose Golgi localization is independent of its N-terminal cytosolic tail (Graham *et al.*, 1994). However, it should be noted that some aspects of Golgi function are unaffected by clathrin inactivation. There is no effect on rates of secretory transport through the Golgi (Payne and Schekman, 1985, 1989), on transport of pro-alkaline phosphatase to the vacuole (Seeger and Payne, 1992b), or on retention of guanosine diphosphatase in the Golgi (Graham *et al.*, 1994; Vowels and Payne, unpublished data). These observations argue against a complete disruption of Golgi function after inactivation of clathrin heavy chain.

The alternative view is that clathrin is involved directly in a step common to both the transport of Kex2p TLS mutants to the vacuole and the mechanism of localization of WT Kex2p in the TGN. Proteins targeted to the vacuole from the Golgi pass through a prevacuolar or endosome-like compartment (Vida *et al.*, 1990, 1993; Raymond *et al.*, 1992; Piper *et al.*, 1995). It is possible that both WT and TLS mutant forms of Kex2p are transported to the prevacuolar compartment from the TGN via clathrin-coated vesicles. TGN localization of WT Kex2p could be achieved by TLS-dependent retrieval from the prevacuolar compartment to the TGN or to an earlier secretory compartment. Lacking a retrieval signal, TLS mutant forms of Kex2p would be delivered to the vacuole. The transmembrane pro-CPY receptor encoded by the *VPS10* gene is thought to follow a similar recycling pathway (Marcusson *et al.*, 1994; Cereghino, *et al.*, 1995; Piper *et al.*, 1995). Collection of some transmembrane cargo proteins into clathrin-coated pits at the TGN might require interaction between cytosolic sequences of the cargo proteins and components of the clathrin coats. However, recognition of cytosolic sequences could not be an absolute requirement for incorporation of all transmembrane proteins into these clathrin-coated vesicles, because C-tailΔ forms of Kex2p, Kex1p, Ste13p, and Vps10p are all transported to the vacuole. Transport of membrane proteins to the prevacuolar compartment and thence to the vacuole would represent a signal-independent or default pathway. Inactivation of clathrin or *VPS1* would block transport from the TGN to the prevacuolar compartment, and membrane proteins would be transported—in a distinct default mode—to the plasma membrane.

At its core, this model has the unprecedented feature that it postulates clathrin-dependent transport of membrane proteins without recognition of cytosolic localization information. However, it is entirely consistent with the present data. Some evidence against a direct interaction of a clathrin-dependent structure with the Kex2p TLS at the TGN can be taken from the genetic analysis of yeast adaptor protein subunits.

Were clathrin involved in a TLS-dependent localization event, the candidate receptor for the tail signal would be yeast homologues of the adaptor protein complexes that have been implicated in binding tyrosine internalization signals (Glickman *et al.*, 1989; Beltzer and Spiess, 1991; Sorkin and Carpenter, 1993; Sosa *et al.*, 1993; Ohno *et al.*, 1995). Homologues of several adaptor subunits have been identified in yeast. Although disruption of certain of these genes (*APL2*, *APM1*, and *APS2*) has synthetic effects in combination with *chc1* mutations both on viability and on the efficiency of pro-α-factor processing, disruption of none of them has by itself any effect on localization of Kex2p (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995). It is possible, however, that these results reflect redundancies in the adaptor subunit genes in yeast.

The data presented here do not rule out an interaction between a clathrin-dependent structure and the tyrosine-containing TLS of Kex2p at a later step in the hypothetical cycling of Kex2p between the TGN and the endosome. With the recent identification of abundant clathrin-coated buds on mammalian endosomes that largely lack both α- and γ-adaptin (Stoorvogel *et al.*, 1996), a role for clathrin in the TLS-dependent retrieval of Kex2p and other transmembrane proteins from the endosome to the TGN is conceivable. However, the current experiments provide no evidence for interaction between a clathrin-dependent structure and the TLS of Kex2p, because the effects of clathrin inactivation are indifferent to the presence of the Kex2p TLS or C-tail. Recognition of new classes of Tyr-containing localization motifs involved in basolateral targeting (Brewer and Roth, 1991; Casanova *et al.*, 1991; Prill *et al.*, 1993; Thomas *et al.*, 1993; Thomas and Roth, 1994) or both basolateral targeting and endocytosis (Prill *et al.*, 1993) indicate that not all Tyr-containing signals operate by a common mechanism. In TGN38, a Tyr-containing signal confers both TGN localization and efficient internalization on heterologous proteins (Bos *et al.*, 1993; Humphrey *et al.*, 1993). Mutational analysis of this signal indicates that TGN localization and efficient internalization are separable functions. Finally, a signal implicated in recycling from the late endosome to the TGN was identified in the C-tail of the 46-kDa Man-6-P receptor (Schulze-Garg *et al.*, 1993). This signal contains a Tyr residue previously shown to be part of a signal required for efficient endocytosis (Johnson *et al.*, 1990).

Vacuolar Protease-dependent Degradation of Kex2p after Clathrin Inactivation

Because clathrin inactivation results in transient secretion of pro-CPY (Seeger and Payne, 1992b), the vacuolar protease-dependent degradation of Kex2p observed might be due to delivery of vacuolar protease precursors to the periplasm. This scenario seems unlikely, however, because vacuolar protease precursors are not matured efficiently at the cell surface, and maturation is required for activity (Stevens *et al.*, 1986). A more likely alternative is that, after clathrin inactivation, WT and C-tail mutant forms of Kex2p are first transported to plasma membrane and then internalized and delivered to the vacuole. Internalization would obviously be independent of clathrin function. Precedents for clathrin-independent endocytosis exist in both yeast and other eukaryotes. In yeast *chc1-ts* mutants, endocytosis of the **a**-factor receptor Ste3p was still observed after shift to 37°C, although at an ~2.5-fold reduced rate (Tan *et al.*, 1993). The $t_{1/2}$ for Ste3p internalization under these conditions was 25–30 min, similar to the $t_{1/2}$ for degradation of Kex2p in the *chc1-ts* mutant (Table 2). The *chc1-ts* mutation also caused no significant decrease in endocytic internalization by yeast of a fluorescent lipid probe at 37°C (Kean *et al.*, 1993). In clathrin heavy-chain null mutants of *Dictyostelium discoideum*, pinocytotic internalization was observed at 20–25% the WT rate (Ruscetti *et al.*, 1994). Likewise, inhibition of clathrin-coated pit and vesicle formation in rat fetal fibroblasts by either potassium depletion or hypotonic medium did not affect bulk phase internalization (Cupers *et al.*, 1994). Indeed, inactivation of dynamin in HeLa cells was found to stimulate clathrin-independent pinocytosis (Damke *et al.*, 1995). Additional support for the interpretation that Kex2p is transported to the vacuole after transit through the plasma membrane comes from the observation that *vps1* mutations cause both Kex2p and Ste13p to be transported to the vacuole after transit through the plasma membrane (Nothwehr *et al.*, 1995).

Living without Clathrin

The critical difference between strains that die or live without clathrin function is unknown, but differences in protein degradation may play a major role. Using a strain in which loss of clathrin function was lethal, Nelson and Lemmon (1993) found that overexpression of the *UBI4* gene, which encodes polyubiquitin, suppressed lethality. Moreover, clathrin inactivation was found to result in an increase in the steady-state level of protein–ubiquitin conjugates. In the W303 background, in which inactivation of clathrin heavy chain is lethal (Munn *et al.*, 1991), vacuolar protease-independent degradation of WT Kex2p was relatively slow ($t_{1/2}$ = 125 min; Table 2). However, in strain GPY1100, which is viable after clathrin inactivation, WT Kex2p exhibited a significantly higher rate of vacuolar protease-independent degradation at the restrictive temperature ($t_{1/2}$ = 50 min; our unpublished observations). An interesting possibility is that the vacuolar protease-independent degradation of Kex2p after clathrin inactivation is mediated directly or indirectly by ubiquitin. The ability to induce a robust nonvacu-

olar pathway for membrane protein degradation may be required for cells to survive the loss of clathrin.

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