Clathrin: A Role in the Intracellular Retention of a Golgi Membrane Protein

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Yeast mutants deficient in the clathrin heavy chain secrete a precursor form of the α-factor, a peptide-mating pheromone. Analysis of this defect indicates that the endoprotease Kex2p, which is responsible for initiating proteolytic maturation of the α-factor precursor in the Golgi apparatus, is unexpectedly present at the plasma membrane in mutant cells. This result suggests that clathrin is required for the retention of Kex2p in the Golgi apparatus.

ELABORATION AND MAINTENANCE OF THE COMPARTMENTAL organization of eukaryotic cells requires delivery and retention of components specific for each compartment. The secretory pathway is responsible for transporting newly synthesized proteins to the plasma membrane and cell exterior, and also plays a role in localizing lysosomal proteins (*1*). Unlike proteins targeted for these destinations, resident proteins of secretory pathway organelles must be retained within their respective organelles. Although progress has been made toward identifying retention signals on endoplasmic reticulum (ER) and Golgi apparatus components (*2*), little is known about factors that recognize these signals and effect compartmentalization.

In mammalian cells, polyhedral lattices of clathrin adorn the plasma membrane and Golgi apparatus membranes (*3*). These clathrin coats, composed of clathrin heavy chain, clathrin light chain, and distinct sets of associated proteins, are found as patches along the cytoplasmic faces of the membranes, often at sites undergoing vesiculation. Clathrin-coated vesicles derived from these sites have been proposed to be precursors of transport vesicles that mediate intercompartmental protein transport from the plasma membrane and the Golgi apparatus (*4*). Clathrin has been postulated to perform two functions in the process of transport vesicle biogenesis. First, polymerization of clathrin subunits into polyhedral cages could drive formation of coated vesicles from coated membrane regions (*4, 5*). Second, the clathrin coat could serve as a scaffold that selectively concentrates receptors and their ligands in newly forming transport vesicles (*4*). In this fashion, clathrin coats could collect cargo for newly forming transport vesicles.

In order to determine the function of clathrin in intracellular protein transport, we have obtained mutants of the yeast *Saccharomyces cerevisiae* that lack the clathrin heavy chain subunit gene (*CHC1*) (*6, 7*). Such mutants (*chc1*), devoid of clathrin heavy chain, grow slowly (*6–8*) or, in certain genetic contexts, are inviable (*8*). The *chc1* strains used in the experiments reported below are viable. Analysis of intercompartmental protein traffic routes in these viable mutants provides a test of clathrin's biological function.

Here, we describe the biosynthesis of mating pheromones in *chc1* yeast strains. Haploid yeast cells exhibit one of two mating types, mating type α (*MAT*α) or mating type **a** (*MAT***a**). Cells of opposite mating type can conjugate and form diploids (*9*). Each haploid cell type secretes a peptide pheromone, α-factor or **a**-factor, respectively, which interacts with specific cell surface receptors on cells of the opposite mating type to elicit physiological responses necessary for mating. Production of both pheromones requires proteolytic excision of the active peptide from a larger precursor. We have observed that disruption of *CHC1* perturbs production of α-factor but not **a**-factor. Investigation of this anomaly has led to the finding that a resident secretory pathway protease involved in α-factor maturation is aberrantly localized to the plasma membrane. Our results suggest that clathrin is required for retention of the protease within an intracellular organelle of the secretory pathway.

A mating type–specific conjugation defect in clathrin-deficient cells. We have adopted a general strategy to compare intracellular transport pathways in clathrin-deficient and wild-type cells (*7,*

Table 1. A conjugation defect displayed by clathrin-deficient *MAT*α cells. Sets of strains (*13*) indicated as strain 1 and strain 2 were allowed to conjugate for 6 hours before dispersal and assessment of diploid formation. The mating type is indicated after the number of each strain. Strains GPY1100 and GPY1101 are isogenic except at *CHC1*. Strains GPY2200 and GPY2201 are similarly congenic. Diploid formation was measured as described in (*41*) except that cells were allowed to conjugate on solid YPD medium (*42*) for 6 hours before dispersal. Efficiency is expressed as the number of diploids divided by the total number of cells.

Strain 1	Strain 2	Diploid formation	
		Frequency	Ratio of *CHC1* to *chc1*
1100α *CHC1*	2200**a** *CHC1*	5.5×10^{-1}	
1100**a** *CHC1*	2200α *CHC1*	3.6×10^{-1}	
1101α *chc1*	2200**a** *CHC1*	1.8×10^{-2}	31
1101**a** *chc1*	2200α *CHC1*	8.0×10^{-2}	4.5
2201α *chc1*	1100**a** *CHC1*	1.0×10^{-3}	3.5×10^{2}
2201**a** *chc1*	1100α *CHC1*	1.4×10^{-1}	3.9
1101α *chc1*	2201**a** *chc1*	2.3×10^{-4}	2.4×10^{3}
1101**a** *chc1*	2201α *chc1*	1.8×10^{-5}	1.9×10^{4}

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Table 2. Reduced secretion of biologically active mating pheromone by clathrin-deficient *MAT*α cells. Cells were grown overnight in liquid YPD medium to midlogarithmic phase, harvested by centrifugation, and resuspended in fresh YPD. After 3 hours, pheromone production was measured by halo assays (*43*) and standardized with respect to cell density (*44*).

α-Factor production per 10^7 cells			**a**-Factor production per 10^7 cells		
Strain	Amount (nmol)	Ratio of *CHC1* to *chc1*	Strain	Amount (limiting dilution)	Ratio of *CHC1* to *chc1*
1100α *CHC1*	4.3		1100a *CHC1*	10.4	
1101α *chc1*	0.3	14	1101a *chc1*	7.3	1.4
2200α *CHC1*	3.9		2200a *CHC1*	9.5	
2201α *chc1*	0.2	18	2201a *chc1*	4.3	2.2

10). We used single-step gene transplacement to disrupt *CHC1* in haploid cells (*11*). The *chc1* transformants obtained by this technique are isogenic with the parental *CHC1* haploid, except at the clathrin heavy chain gene locus. Pairs of congenic strains were subjected to phenotypic analyses.

In order to compare directly the conjugation efficiencies of mutant and wild-type strains, we subjected congenic sets of *chc1* and *CHC1* strains to a procedure that switches the mating type of each strain (*12*). From these manipulations two sets of strains were obtained (*13*), each of which had four congenic members: *CHC1 MAT*α, *chc1 MAT*α, *CHC1 MAT***a**, and *chc1 MAT***a**. The congenic sets allowed the effect of *chc1* on conjugation proficiency to be compared between strains that differed only at *CHC1* or *MAT*. The *chc1* strains conjugated with lower efficiencies than congenic wild-type strains (Table 1), but the presence of *chc1* reduced the mating ability of *MAT*α strains more drastically than congenic *MAT***a** strains. This mating type–specific effect varied by a factor of 7.5 in one genetic background (1100 and 1101) to a factor of 90 in another (2200 and 2201). When both members of a mating pair carried *chc1*, the frequency of diploid formation dropped more than 1000-fold compared to congenic pairs of wild-type strains (Table 1). The basis for this synergistic sterility is not clear.

To investigate the discrepancy between mating abilities of clathrin-deficient *MAT*α and *MAT***a** strains, we used "halo" assays to measure the production of biologically active pheromone by each strain; this method takes advantage of the cell-cycle arrest triggered in response to pheromone (*14*), such that, when a solution of pheromone was spotted onto a sparse lawn of tester cells, cell-cycle arrest was manifested as a zone of growth inhibition. This zone appeared as a clearing or halo in the lawn of cells. The radius of the halo caused by α-factor is proportional to the log of the concentration (*15*); the concentration of **a**-factor is proportional to the limiting dilution of pheromone where no halo is observed (*16*). When normalized for cell density, production of α-factor by mutant cells was diminished 14- or 18-fold after 3 hours of growth when compared to wild-type counterparts (Table 2). In contrast, **a**-factor secretion by *chc1* cells was not less than 45 percent of that of the wild type (Table 2). Thus, the absence of clathrin affects the production of mature α-factor more dramatically than the production of **a**-factor. This result did not completely account for the reduced conjugation abilities of clathrin-deficient *MAT*α cells compared to *MAT***a** cells. Although α-factor production was reduced to nearly the same extent in 1101α and 2201α, the former was ten times more proficient at mating. We have not explored other factors that could account for this difference.

Secretion of α-factor precursor by clathrin-deficient cells. In *S. cerevisiae* the two mating pheromones are apparently matured and exported by separate routes (*17*). The α-factor pheromone follows the conventional secretory pathway, whereas **a**-factor may bypass secretory organelles on its way to the cell surface. Earlier studies indicated that there does not appear to be a general secretory pathway defect in clathrin heavy chain–deficient cells (*6, 7, 10*). However, export of α-factor through the secretory pathway is accompanied by a series of specific processing events that are necessary for production of mature pheromone. It therefore seemed possible that the *MAT*α-specific effect of *chc1* was due to a defect in one or more of these maturation steps. Progress of newly synthesized α-factor through the secretory pathway can be conveniently monitored by SDS–polyacrylamide gel electrophoresis (PAGE), which reveals shifts in size caused by stage-specific modifications (*17, 18*). Like most mammalian peptide hormones, the 13–amino acid α-factor peptide is synthesized as part of a larger precursor polypeptide. The primary translation product, 165 amino acids in length (about 19 kD), contains four copies of the mature pheromone sequence. Once translocated into the ER, the signal sequence is removed, and the precursor is core-glycosylated at three sites to yield a species of 26.5 kD. The core oligosaccharides are heterogeneously extended into long, branched chains in the Golgi apparatus. This highly glycosylated precursor (125 kD on average) then undergoes a series of well-defined proteolytic cleavages that begin in the Golgi apparatus and are thought to proceed as the precursor is packaged into secretory vesicles. The first cleavages are carried out by the *KEX2* product, which cleaves after pairs of basic amino acids to generate peptides with six NH_2-terminal amino acids and two COOH-terminal amino acids flanking the mature sequence. The NH_2-terminal extension is removed by the dipeptidyl aminopeptidase encoded by *STE13*. The extra COOH-terminal amino acids are severed by the *KEX1* carboxypeptidase. The extended and mature peptides migrate together near the bottom of our 15 percent SDS-polyacrylamide gels and we refer to them collectively as 3.5-kD α-factor.

Because complete maturation of α-factor precursor is required for biological activity (*18, 19*), the absence of halo-forming activity in *chc1* cell secretions could indicate either a biosynthetic or maturation defect. These possibilities were addressed by labeling pairs of congenic *chc1* and *CHC1* cells with $^{35}SO_4^{2-}$ and then precipitating α-factor from the medium with specific antiserum. Included in this analysis were *chc1* strain GPY2201 and two additional *chc1 MAT*α strains, GPY64 and GPY68, which displayed conjugation defects similar to those of GPY2201. Although *chc1* strains secreted immunoreactive α-factor species, most of the material precipitated from the medium of each *chc1* strain unexpectedly comigrated with highly glycosylated α-factor precursor (about 125 kD) secreted by a mutant that lacks the Kex2 endoprotease (Fig. 1A, lanes 1, 2, 4, and 6). The congenic *CHC1* strains all displayed in the medium a pheromone of small molecular size (Fig. 1A, lanes 3, 5, and 7). When α-factor secreted by *chc1* and *kex2* strains was incubated with endoglycosidase H to eliminate the carbohydrate moieties, the uncleaved 19-kD polypeptide backbones became exposed (Fig. 1B). Introduction of a yeast centromere plasmid carrying *CHC1* (7) into *chc1* strains GPY2201 and GPY68 reinstated complete α-factor maturation (*20*). Thus, secretion of highly glycosylated precursor is

due to the absence of clathrin heavy chain and is not the result of a secondary genetic alteration that might have occurred in the *chc1* strains. The demonstration that clathrin-deficient yeast secrete the highly glycosylated precursor form of α-factor suggests that the deficit in production of biologically active pheromone by *chc1* strains may be due to a defect in proteolytic maturation.

Rapid secretion of α-factor precursor by *chc1* cells. We used the progressive modifications described above to monitor the biosynthetic pathway of α-factor in *chc1* cells. The *CHC1* and *chc1* strains were labeled for 5 minutes with $^{35}SO_4^{2-}$, after which the labeling was terminated by the addition of unlabeled Na_2SO_4. At designated intervals, samples were collected, and separated into cell and medium fractions. The cells were then lysed and α-factor was immunoprecipitated from each fraction and subjected to SDS-PAGE (Fig. 2A). In both strains, the ER form of α-factor precursor was apparent in the cell lysates immediately after the 5-minute labeling period (Fig. 2A, lanes 1) but disappeared by 2 minutes after incorporation of label was ended (Fig. 2A, lanes 3). After 5 minutes of labeling, *CHC1* cells also contained the 3.5-kD α-factor (Fig. 2A, lane 1, left panel), whereas *chc1* cells displayed highly glycosylated precursor (Fig. 2A, lane 1, right panel). The conversion of ER α-factor to more highly processed forms is diagnostic of transport to the Golgi apparatus (*18*). (In wild-type cells, proteolytic processing of the highly glycosylated precursor occurs so rapidly that this form does not accumulate at observable levels.) The α-factor species (Fig. 2A) were quantified by densitometry (Fig. 2B). About half of the total α-factor synthesized during the 5-minute labeling period had acquired Golgi-specific modifications. The remaining ER form was modified in the subsequent 2 minutes. Thus, transport of the precursor from the ER to the Golgi apparatus occurred with equivalent kinetics in the two strains. An analogous result has been obtained with the secreted protein invertase (7) and provides further evidence that clathrin is not involved in transport from the ER to the Golgi apparatus. Although differently processed forms of α-factor were secreted by wild-type and mutant strains, the rates at which each form appeared in the medium were comparable (Fig. 2B, media), implying that α-factor follows the same route from the Golgi apparatus to the cell surface in both strains.

Two additional experiments support the conclusion that, in *chc1*

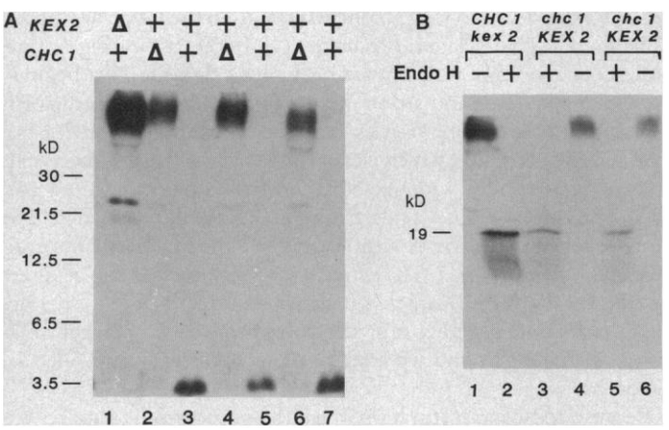


Fig. 1. Secretion of highly glycosylated α-factor precursor by clathrin-deficient cells. Congenic pairs of *chc1* and *CHC1* strains and a *kex2 CHC1* strain were grown at 30°C to midlogarithmic phase in Wickerham's minimal medium (*46*) with 2 percent glucose and 200 μ*M* ammonium sulfate. For labeling, cells were resuspended at 2 × 10^7 cells per milliliter in Wickerham's minimal medium without sulfate salts and with ovalbumin (200 μg/ml). Cells were incubated at 30°C with 500 μCi of $^{35}SO_4^{2-}$ (ICN Biochemical) per 10^7 cells for 25 minutes (*CHC1* cells) or 50 minutes (*chc1* cells). The culture medium was collected, adjusted to 1 percent SDS, heated to 95°C, and subjected to immunoprecipitation (*47*). (**A**) Part of each sample was directly applied to a 15 percent SDS-polyacrylamide gel. After electrophoresis, the gel was treated with Amplify (Amersham), dried, and exposed to x-ray film at −70°C. Δ indicates a gene disruption, + signifies the wild-type allele. The mobilities of the molecular standards are shown on the left side. Secreted α-factor from: lane 1, strain BFY101-10C (*kex2 CHC1*); lane 2, strain GPY2201 (*chc1*); lane 3, strain GPY2200 (*CHC1*); lane 4, strain GPY68 (*chc1*); lane 5, strain GPY55-15B (*CHC1*); lane 6, strain GPY64 (*chc1*); lane 7, strain GPY55-10B (*CHC1*). (**B**) The remainder of each sample was treated with endoglycosidase H (*48*) and then subjected to SDS-PAGE and autoradiography. (Lanes 1 and 2), α-Factor precipitated from the medium of strain BFY101-10C; (lanes 3 and 4), α-factor precipitated from the medium of strain GPY68; (lanes 5 and 6), α-factor precipitated from the medium of strain GPY64. In lanes 2, 3, and 5, α-factor was incubated with endoglycosidase H. Samples in lanes 1, 4, and 6 were untreated.

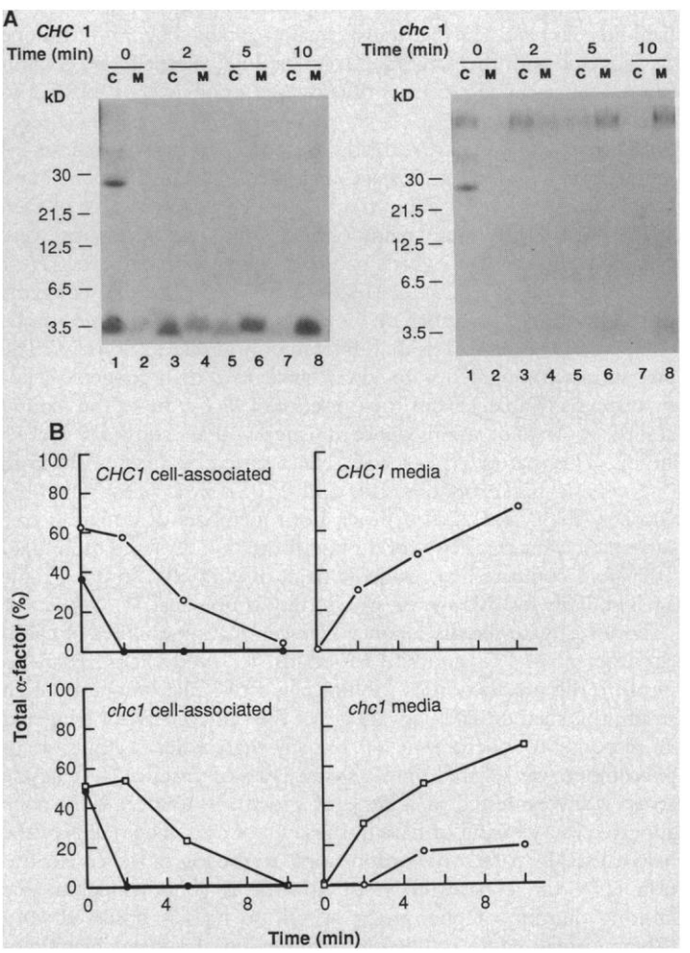


Fig. 2. Rate of α-factor secretion by *chc1* and *CHC1* cells. (**A**) Strains GPY55-15B (*CHC1*) and GPY68 (*chc1*) were grown, harvested, and labeled with $^{35}SO_4^{2-}$ for 5 minutes as described in the legend to Fig. 1. Ammonium sulfate (3 m*M*), cysteine (0.01 percent), and methionine (0.01 percent) were added to prevent further labeling. At the designated times (minutes), 2 × 10^6 cells were harvested, sodium azide was added to 10 m*M*, and the samples were placed on ice. Each sample was separated into cell and culture medium fractions. Cell extracts were prepared by homogenization with glass beads (7). Cell and culture medium fractions were analyzed by immunoprecipitation with antiserum to α-factor, SDS-PAGE, and autoradiography (as in Fig. 1). The times after labeling ceased are indicated by the numbers above each set of lanes. C, cell lysate fraction. M, culture medium fraction. Positions of molecular size markers are displayed to the left of each panel. (Lanes 1 to 8, left panel), α-Factor from GPY55-15B cells (odd-numbered lanes) and culture medium fractions (even-numbered lanes); (lanes 1 to 8, right panel), α-factor from GPY68 cells (odd-numbered lanes) and culture medium fractions (even-numbered lanes). (**B**) Densitometric analysis (Beckman DU-62 spectrophotometer; Gelscan accessory) of α-factor secretion by *CHC1* and *chc1* cells shown in (A). Closed circles represent the 26.5-kD ER form of α-factor precursor. Open circles represent 3.5-kD α-factor. Open squares represent the low mobility, highly glycosylated α-factor precursor.

cells, precursor α-factor follows the conventional secretory pathway to the cell surface (*20*). First, α-factor precursor secreted by *chc1* cells and *CHC1 kex2* cells was equally precipitated by antibody specific for α1→3 linked outer chain mannose. The mannosyl transferase that adds α1→3 linked outer chain mannose to secreted glycoproteins appears to act in a late Golgi apparatus compartment (*21*). The presence of this modification on precursor α-factor secreted by *chc1* cells thus reflects passage of the precursor through this secretory pathway compartment. Second, a temperature-sensitive mutation, *sec1*, that blocks fusion of secretory vesicles to the plasma membrane (*22*) was used to establish that α-factor precursor travels from the Golgi apparatus to the cell surface via secretory vesicles. Imposition of the nonpermissive temperature (37°C) blocked export of newly synthesized α-factor precursor in *sec1 chc1* cells but not *chc1* cells.

A minor amount of 3.5-kD α-factor was present in the medium of *chc1* cells at the 5- and 10-minute points (Fig. 2A, lanes 6 and 8, right panel, and Fig. 2B, *chc1* media). This species was observed in the medium, but not in the cells. In contrast, the 3.5-kD α-factor appears in *CHC1* cells before it is evident in the medium. Endoproteolytic maturation of α-factor precursor therefore occurs at a later secretory stage in *chc1* cells, perhaps at the cell surface.

Synthesis but not retention of Kex2 endoprotease in *chc1* cells. Since severely reduced α-factor precursor maturation in *chc1* strains mimics the phenotype of strains lacking the *KEX2*-encoded endoprotease, Kex2p synthesis in *chc1* cells was investigated. Mutant and wild-type cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 10 minutes and then incubated in the presence of unlabeled amino acids for 30 minutes. Cells were collected and lysed, and Kex2p was immunoprecipitated from the extracts and displayed on an SDS-polyacrylamide gel. In a 10-minute period, equivalent amounts of Kex2p were made by both mutant (Fig. 3, lane 1) and wild-type cells (Fig. 3, lane 3). The size of Kex2p observed in these samples reflects addition of N-linked, and probably O-linked, carbohydrate (*23*). After the 30-minute incubation, a slight decrease in Kex2p mobility was apparent in both samples (Fig. 3, lanes 2 and 4). The basis of this mobility shift is unknown. The coincident behavior of Kex2p in both strains suggests that the initial stages of Kex2p biosynthesis are unaffected in a *chc1* mutant and dispels the possibility that a Kex2p synthesis defect accounts for the absence of α-factor maturation by *chc1* cells.

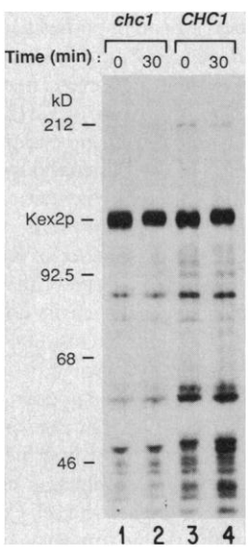


Fig. 3. Synthesis of Kex2p by *chc1* cells. Congenic strains GPY64 (*chc1*) and GPY55-10B (*CHC1*) were grown to midlogarithmic phase in SD medium (*42*) with 0.2 percent Bacto yeast extract, collected, and resuspended in SD medium. "Tran^{35}S-label" (50 μCi per 10^7 cells; ICN Biomedicals, Inc.) was added, and cells were incubated for 10 minutes. Labeling was stopped as in the legend to Fig. 2. At this time and 30 minutes later, samples were removed, placed on ice, and sodium azide was added to 10 m*M*. Cell extracts were prepared by glass bead lysis (*7*) and Kex2p was precipitated with antiserum to Kex2p as described in (*47*), except that an extract of *kex2::URA3* cells (5×10^7 cell equivalents) was included in the antibody incubation. Samples were subjected to electrophoresis through a 7.5 percent SDS-polyacrylamide gel. The gel was fixed in 25 percent isopropanol and 10 percent acetic acid, then soaked in 2*M* sodium salicylate, dried, and exposed to x-ray film. (Lanes 1 and 2) Kex2p from GPY64 cells (*chc1*) after 10 minutes of ^{35}S labeling (lane 1), and 30 minutes after labeling was quenched (lane 2); (lanes 3 and 4) Kex2p from GPY55-10B cells (*CHC1*) after the 10-minute labeling (lane 3) and 30 minutes after labeling was stopped (lane 4).

Julius *et al.* (*24*) described a fluorescent tripeptide substrate that could be used to specifically measure Kex2p activity in cell extracts. Kex2p activity is dependent on the presence of calcium and is absent in extracts of *kex2* strains. We applied the activity assay to extracts of *chc1* and *CHC1* cells (Table 3, column 3). In one set of congenic strains, Kex2p activity detected in the *chc1* member was 50 percent of that in the *CHC1* counterpart. In a second set, both mutant and wild-type cells had the same amount of activity. The specificity of the assay was demonstrated by the low background activity in an extract of *chc1* cells harboring a disruption of *KEX2*. These data indicate that the Kex2p synthesized in *chc1* cells is enzymatically active.

Because *chc1* strains grow more slowly than wild-type strains, the equivalent synthetic rates of Kex2p (Fig. 3) should result in higher quantities of Kex2p per cell in mutant strains. However, similar amounts of Kex2p activity were observed in mutant and wild-type cells (Table 3). When mutant and wild-type strains were metabolically labeled for 30 minutes and then allowed to grow without further label incorporation for 3 hours, we noted a 3.4-fold increase in the turnover rate of Kex2p in *chc1* cells (*20*). The enhanced lability of Kex2p in *chc1* cells could cause a reduction in the amount of Kex2p to that found in wild-type cells. The decreased stability of Kex2p may reflect the altered location of the protease in the mutant cells.

An explanation for the secretion of α-factor precursor by *chc1* cells became apparent when we localized the Kex2 endoprotease. From the nucleotide sequence of *KEX2* it has been predicted that the encoded protein assumes a transmembrane configuration (*25*). In wild-type cells, Kex2p activity is latent unless the cells are permeabilized with detergent (*24*). On the basis of these two observations, it appears that Kex2p normally resides in an intracellular compartment with its active site oriented toward the lumen. If, instead, Kex2p were not retained within the cell, but reached the cell surface, the active site would be exposed, and activity would be evident when intact cells were assayed with a membrane impermeable substrate. We therefore investigated the localization of Kex2p in *chc1* cells by comparing the Kex2p activity of intact cells to that in detergent extracts of the cells. As we expected, there was little or no activity when intact *CHC1* cells were assayed (Table 3, rows 2 and 4). In contrast, intact *chc1* cells exhibited 75 and 91 percent of the total cell-associated activity (Table 3, rows 1 and 3). Disruption of the *KEX2* gene in *chc1* cells eliminated the cell surface activity, confirming that the activity was indeed due to Kex2p (Table 3, row 5). The Kex2p activity detected when intact cells were assayed was not due

Table 3. Kex2p endoprotease at the cell surface of clathrin-deficient cells. Kex2p endoprotease and glucose-6-phosphate dehydrogenase (G6PD) activities were measured in intact cells and cell lysates (*45*) from *chc1* strain GPY64 and its congenic *CHC1* partner GPY55-10B, *chc1* strain GPY68 and its congenic *CHC1* counterpart GPY55-15B, and *kex2 chc1* strain GPY100-9C. The percent activity at the cell surface represents the activity expressed by intact cells divided by the activity expressed in cell extracts.

Strain	Relevant genotype	KEX2p activity		G6PD activity	
		Total*	Cell surface (%)	Total*	Cell surface (%)
GPY64	*chc1*	0.28	75	0.85	0.2
GPY55-10B	*CHC1*	0.58	3.4	0.50	0
GPY68	*chc1*	0.24	91	0.74	0.5
GPY55-10B	*CHC1*	0.23	0	0.55	0
GPY100-9C	*chc1 kex2::URA3*	0.03	0	0.76	2.1

*Activity is expressed as units per 10^7 cells.

to cell lysis or membrane permeability because a cytoplasmic enzyme, glucose-6-phosphate dehydrogenase (G6PD), maintained its latency in all strains (Table 3). Although total Kex2p activity varied up to 50 percent in any given strain from experiment to experiment, intact *chc1* strains always expressed at least 65 percent of the activity detected in cell extracts.

If Kex2p is at the plasma membrane of *chc1* cells, then it should be accessible to other membrane impermeant probes. To test this prediction, we labeled mutant and wild-type strains with ^{125}I under conditions that preferentially iodinate surface-exposed proteins (*26*). For comparison, cell extracts were also iodinated to label proteins regardless of their location in the cell. After iodination, the intact cells were lysed, and three proteins were immunoprecipitated from surface-labeled and extract-labeled samples, namely Kex2p, G6PD, and a 33-kD cell wall protein (*27*). We used G6PD to determine that iodination of intact cells did not result in labeling of internal proteins. The 33-kD cell wall protein provided a means of assessing the relative incorporation of iodine into intact cells and cell lysates. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Whereas nearly equivalent signals were obtained from Kex2p iodinated in both *chc1* and *CHC1* cell lysates (Fig. 4, lanes 3 and 5), Kex2p was detected in mutant but not wild-type cells when intact cells were labeled (Fig. 4, lanes 2 and 4). Some degradation of Kex2p was observed when intact *chc1* cells were iodinated resulting in 130-kD and 93-kD species in addition to the full-length 135-kD polypeptide (the 130-kD and 135-kD forms are not resolved on the gel shown in Fig. 4 but were separated by electrophoresis for longer times). The specificity of the antiserum was established by immunoprecipitation of samples from labeled *chc1 kex2* cell extracts and intact cells. The 135-kD, 130-kD, and 93-kD polypeptides were not observed in either the intact cell or lysate samples (*20*). The proteolysis of Kex2 observed when intact *chc1* cells were iodinated probably occurred during labeling since *chc1* cell extracts prepared by boiling in SDS before labeling contained only full-length Kex2p (Fig. 4, lane 3). Also, the smaller forms of Kex2p were not observed when metabolically active cells were labeled with [^{35}S]methionine and [^{35}S]cysteine (Fig. 3). Attempts to reduce the proteolysis with protease inhibitors or mutations that lower endogenous protease activity were unsuccessful.

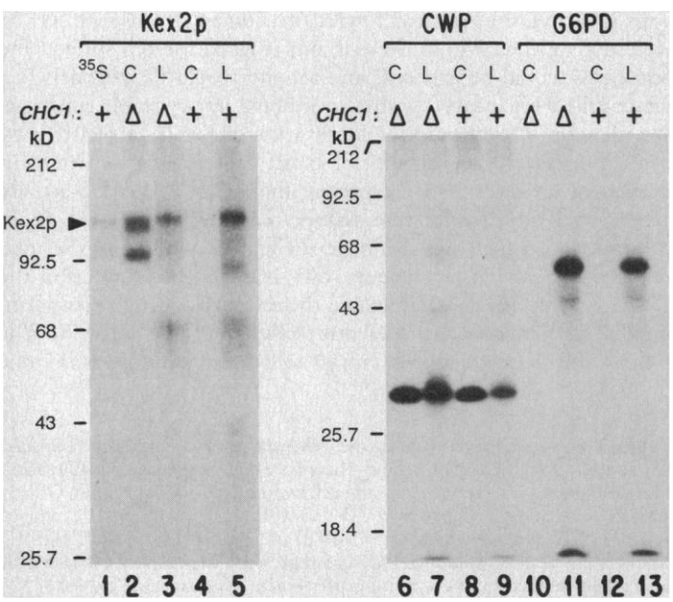


Fig. 4. Labeling of Kex2p by surface iodination of *chc1* cells. GPY64 (*chc1*) and GPY55-10B (*CHC1*) intact cells and cell lysates were labeled with ^{125}I (*49*). After the labeling reaction, the intact cells were lysed and all samples were treated with various antisera. Precipitated proteins were applied to SDS-polyacrylamide gels, subjected to electrophoresis, and visualized by autoradiography. (Lane 1) Kex2p immunoprecipitated from GPY55-10B (*CHC1*) cells metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine; (lanes 2 to 13) iodinated samples treated with antisera specific for the proteins indicated above each panel. Kex2p is the endoprotease encoded by *KEX2*; CWP is a 33-kD cell wall protein (*26*); G6PD is glucose-6-phosphate dehydrogenase. C represents samples iodinated as intact cells and L represents samples iodinated as extracts. *chc1* cell samples are designated by Δ and *CHC1* cell samples by +. Positions of molecular size markers are shown to the left of each panel.

The 33-kD cell wall protein was precipitated from equivalent amounts of labeled intact cells or cell lysates. When intact cells were labeled (Fig. 4, lanes 6 and 8), iodine incorporation into the cell wall protein was greater than that in cell lysates similarly labeled (Fig. 4, lanes 7 and 9). When this result was used to correct for the different labeling conditions, densitometric analysis of the Kex2p bands indicated that 70 percent of Kex2p was at the surface of GPY64 cells. As would be expected, G6PD was labeled only in cell extracts and not in intact cells (Fig. 4, lanes 10 to 13), an indication that the iodination of intact cells resulted in exclusive labeling of cell-surface proteins. Similar results were obtained when another pair of congenic strains, GPY68 and GPY55-15B were iodinated (*20*).

The role of clathrin in Kex2p localization. The above two independent techniques, cell-surface iodinations and enzyme activity assays, show that Kex2 endoprotease is present at the cell surface of yeast mutants deficient in clathrin heavy chain. In contrast, Kex2p is sequestered within wild-type cells. The abnormal residence of Kex2p in *chc1 MATα* cells appears to affect the ability of the enzyme to initiate maturation of α-factor precursor; *chc1* cells secrete highly glycosylated pheromone precursor. This finding can account, at least in part, for the debilitated conjugation ability of *chc1 MATα* strains. Nearly normal levels of **a**-factor are exported by *chc1 MAT***a** cells, and the conjugation efficiency of these mutants is not significantly impaired. Since production of **a**-factor pheromone is not dependent on Kex2p activity (*17*), abnormal localization of Kex2p in *chc1* strains would not be expected to influence **a**-factor maturation and secretion.

The inefficient maturation of α-factor precursor by *chc1* cells can be attributed to the reduced intracellular concentration of Kex2p. We suppose that the residual intracellular enzyme may be overwhelmed by α-factor precursor and the pheromone thereby evades maturation during transit through the secretory pathway. In support of the hypothesis, an independent approach taken by Fuller *et al.* (*23, 28*) indicates that relocation of Kex2p to the cell surface is sufficient to prevent maturation of the α-factor precursor (*23, 28*). Efficient export of Kex2p in otherwise wild-type cells was achieved by deletion of the cytoplasmic tail, the transmembrane anchor, and part of the lumenal domain of Kex2p (*28*). Strains expressing only this secreted, enzymatically active form of Kex2p fail to mature α-factor precursor. It is possible, however, that the correlation between the presence of Kex2p at the plasma membrane and secretion of α-factor precursor may be fortuitous. For example, the absence of clathrin heavy chain could alter the *p*H of the Golgi lumen and thereby inhibit residual Kex2p which otherwise would be able to cleave α-factor precursor efficiently.

Our results support the conclusion that Kex2p maintains an intracellular residence in wild-type yeast (*17, 18, 23, 24*). The α-factor precursor first incurs Kex2p proteolysis in the Golgi apparatus and is completely cleaved before secretory vesicles deliver the pheromone to the cell exterior (*18*). Thus, Kex2p most likely resides in the Golgi apparatus but may also be present in nascent secretory vesicles. On the basis of this localization, we propose that clathrin serves to retain Kex2p in the Golgi apparatus of wild-type yeast. In this capacity, clathrin plays an integral role in the maintenance of a

compartmental boundary. We envision at least two general models consistent with this role (Fig. 5), which differ in the assumption that Kex2p transiently resides at the plasma membrane of wild-type cells (Fig. 5B).

One possibility (Fig. 5A, route 1) is based on a potential analogy with the formation of peptide hormone–containing secretory granules in mammalian endocrine cells. In insulin-secreting pancreatic β cells and in adrenocorticotropic hormone (ACTH)–secreting pituitary cells, proteolytic maturation of the hormone precursors begins as, or immediately after, the precursor polypeptide is packaged into secretory granules that form from the trans Golgi network (TGN) (*29, 30*). The regions of the TGN that give rise to secretory granules, and the nascent granules themselves, display patches of clathrin coats. The nascent granules are converted to mature secretory granules that carry the fully processed, biologically active peptide hormone and are no longer decorated with clathrin. Images obtained by electron microscopy show clathrin-coated vesicles budding from the forming secretory granule (*30*). These observations support the proposition that clathrin functions to remove excess membrane from the newly forming granules and then recycles the membrane back to the Golgi apparatus (*31*). The mature secretory granules reside in the cytoplasm until an extracellular stimulus triggers granule fusion to the plasma membrane and release of hormone.

Although secretory vesicles carrying α-factor are constitutively transported to the cell surface rather than being stored in the cytoplasm, aspects of their formation could mimic the generation of secretory granules (Fig. 5A, route 1). In this proposal, clathrin coats collect Kex2p (shown as scissors) at sites where α-factor precursor (drawn in the Golgi lumen) is being packaged into forming secretory vesicles. The coincident concentration of the enzyme and its substrate would ensure efficient processing. If clathrin-coated vesicles bud from the nascent secretory vesicles in yeast as they apparently bud from secretory granules in mammalian endocrine cells, then they would retrieve Kex2p from the secretory route. Uncoating of the vesicles and fusion with the Golgi cisternae would return Kex2p for another round of processing. In clathrin-deficient cells, the protease would not be retrieved and would instead accompany α-factor to the cell surface in secretory vesicles. If extended to mammalian endocrine cells, this model predicts that clathrin participates in the recycling of the peptide hormone maturation enzymes (as yet unidentified) from the secretory granules back to the TGN.

A different role for clathrin may be that of a stable cytoskeletal element of the Golgi apparatus that tethers Kex2p to the Golgi membrane (Fig. 5A, route 2). This model has clathrin excluding Kex2p from vesiculating membrane regions. Kex2p would proceed to the cell surface in the absence of a clathrin anchor. This view of clathrin function does not account for the documented association of clathrin with newly forming vesicles in mammalian cells (*3, 4*), nor does it exploit the capacity of clathrin to assemble into cages, a property displayed in vitro by both mammalian and yeast clathrin (*4, 5, 8*). Nevertheless, planar sheets of clathrin coats have been observed in vivo (*32*) and the function of such arrays could be different from that of clathrin cages.

Instead of acting at the Golgi apparatus, clathrin may recycle Kex2p by endocytosis (Fig. 5B). Here we refer to the paradigm of clathrin's involvement in mammalian cell receptor-mediated endocytosis (*3, 4, 33*). Kex2p is shown following the secretory pathway in its entirety to the plasma membrane. However, once at the plasma membrane, the enzyme is rapidly collected into clathrin-coated pits, packaged into endocytic clathrin-coated vesicles and delivered back to the Golgi apparatus. If the endocytic retrieval step occurs rapidly, then the steady-state amount of Kex2p at the cell surface could be very low. The route back to the Golgi apparatus could pass through intermediate compartments like mammalian endosomes (*34*), although endosomal compartments have not been identified in yeast.

Finally, a hybrid model suggests that Kex2p is concentrated into nascent secretory vesicles through an association with clathrin coats (Fig 5A, route 1), but instead of immediate recycling Kex2p would travel with the vesicle to the cell surface where rapid internalization would occur through clathrin-coated plasma membrane regions (Fig. 5B).

The above models do not imply that clathrin is required for the formation of secretory vesicles from the Golgi apparatus. The observation that α-factor precursor is efficiently and rapidly secreted by *chc1* cells supports our previous conclusion, based on analysis of invertase secretion (*7*), that generation of secretory vesicles can occur in the absence of clathrin heavy chain.

In addition to Kex2p, the proteases encoded by *KEX1* and *STE13* participate in maturation of the α-factor precursor (*17*). The compartmental locations of Kex1p and *STE13*-encoded DPAP A (dipeptidyl aminopeptidase A) have not been identified, but both enzymes appear to reside intracellularly (*14, 35*). Our models can be extended to include the possibility that clathrin acts to collect all three enzymes together in the Golgi apparatus or nascent secretory vesicles. In this way, the colocalization of each enzyme involved in α-factor maturation would be assured. Consistent with this suggestion, preliminary results suggest that Kex1p activity can be detected at the surface of intact clathrin-deficient cells (*35*). Similar analyses should be possible with DPAP A.

A systematic study of other resident Golgi proteins may define a subset that is mislocalized in *chc1* cells, and may explain why clathrin heavy chain–deficient strains grow slowly or die (*6, 7, 8*). The mannosyl transferases that add outer chain mannose to secreted glycoproteins act in the Golgi apparatus (*21*) and are therefore presumably localized in Golgi cisternae. Precursor α-factor secreted by *chc1* cells and *CHC1 kex2* cells is precipitated with equal efficiency by antibodies specific for α1→3 linked outer chain mannose (*20*), suggesting that the enzymes are properly situated and fully active in the Golgi apparatus. Analysis of mannosyl transferase location by enzyme activity assays on intact cells and cell lysates is difficult

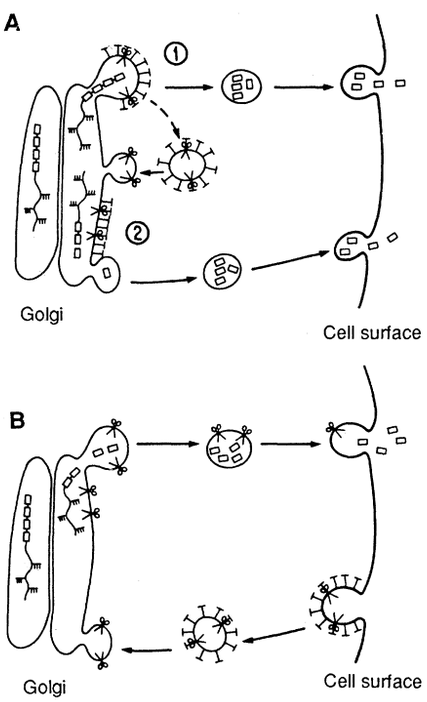


Fig. 5. Possible mechanisms for clathrin-mediated retention of Kex2p in the Golgi apparatus. (**A**) Kex2p is continuously retained intracellularly. (**B**) Kex2p reaches the plasma membrane but is efficiently endocytosed. Glycosylated pro–α-factor is shown within the Golgi cisternae with combs representing asparagine-linked carbohydrate chains and boxes signifying mature peptide units. T, Clathrin coating the cytoplasmic surfaces of the Golgi apparatus and a Golgi apparatus-derived vesicle in (A), and an endocytic pit and endocytic vesicle in (B). Kex2p is drawn as scissors which interact with clathrin coats.

because a cytoplasmic inhibitor precludes accurate determination of total cell-associated activities (*36*), and specific antibodies are not yet available.

While it is clear that Kex2p is mislocalized in the *chc1* mutants, at least one other pathway that involves protein sorting in the Golgi apparatus does not depend on clathrin. Vacuolar proteins are properly localized and processed in *chc1* strains (*10*). Thus, although at least 50 genes are required for sorting of vacuolar proteins (*37, 38*), the clathrin heavy chain gene is not among them.

Our proposals imply a direct interaction between Kex2p and some constituent of clathrin coats. Kex2p, as well as Kex1p and DPAP A, displays a predicted membrane topology (*17*) that is congruent with transmembrane proteins that interact with clathrin coats in mammalian cells (*32*); the polypeptide spans the lipid bilayer once and is divided into lumenal, membrane, and cytoplasmic domains. Deletion of the cytoplasmic tails of the low-density lipoprotein receptor (*33*) and the transferrin receptor (*39*) eliminates association with clathrin coats in vivo, and Pearse (*40*) has recently presented evidence that cytoplasmic tails of several proteins bind to clathrin-associated proteins, which she has designated "adaptins." When most of the predicted cytoplasmic tail of Kex2p is eliminated, a modest amount of the enzyme (10 to 20 percent) reaches the plasma membrane (*28*). Efficient export is not achieved until the entire cytoplasmic tail, the transmembrane domain, and part of the lumenal domain are removed. Several factors could influence the level of mislocalization in these experiments. First, the less severe truncation leaves a short cytoplasmic tail of 16 amino acids (*28*) that could still interact with clathrin coats. Second, the experiments with mammalian cells involved proteins which reside, at steady state, in the plasma membrane. Retention of Kex2p in the Golgi apparatus may involve more complicated interactions than simple association of the cytoplasmic tail with clathrin coats. Perhaps a domain on the "tailless" Kex2p interacts with other Golgi proteins, such as Kex1p, which retain the ability to associate with clathrin coats. Experiments designed to demonstrate direct association of Kex2p and clathrin coats should resolve these issues.

In summary, phenotypic characterization of clathrin heavy chain–deficient yeast has offered evidence that clathrin is required, in vivo, for the intracellular retention of a resident secretory pathway protein, the Kex2p endoprotease. By providing this previously unrecognized function, clathrin exerts a role in orchestrating peptide pheromone precursor maturation. An analogous situation may apply to the biosynthetic pathway of peptide hormones in mammalian endocrine cells.

REFERENCES AND NOTES

1. S. R. Pfeffer and J. E. Rothman, *Annu. Rev. Biochem.* **56**, 829 (1987).
2. J. Brown *et al.*, *J. Cell Biol.* **105**, 1571 (1987); C. E. Machamer and J. K. Rose, *J. Cell Biol.* **105**, 1205 (1987); H. R. B. Pelham, K. G. Hardwick, M. J. Lewis, *EMBO J.* **7**, 1757 (1988).
3. F. M. Brodsky, *Science* **242**, 1396 (1988).
4. B. M. F. Pearse and M. S. Bretscher, *Annu. Rev. Biochem.* **50**, 85 (1981).
5. S. C. Harrison and T. Kirchhausen, *Cell* **33**, 650 (1983).
6. G. S. Payne and R. Schekman, *Science* **230**, 1009 (1985).
7. G. S. Payne, T. B. Hasson, M. S. Hasson, R. Schekman, *Mol. Cell. Biol.* **7**, 3888 (1987).
8. S. K. Lemmon and E. W. Jones, *Science* **238**, 504 (1987).
9. G. F. Sprague, L. C. Blair, J. Thorner, *Annu. Rev. Microbiol.* **37**, 623 (1983).
10. G. S. Payne, D. Baker, E. van Tuinen, R. Schekman, *J. Cell Biol.* **106**, 1453 (1988).
11. R. J. Rothstein, *Methods Enzymol.* **101**, 202 (1983).
12. The mating types of congenic strains GPY1100 (*MAT***a** *CHC1*) and GPY1101 (*MAT***a** *chc1-Δ8*) or GPY2200 (*MATα CHC1*) and GPY2201 (*MATα chc1-Δ8*) were switched with a yeast centromere plasmid carrying the *HO* gene (*50*) as described in (*51*).
13. Strains used in this study: GPY1100**a**: *MAT***a** *leu2-3,112 ura3-52 his4-519 trp1 can1*; GPY1100α: *MATα leu2-3,112 ura3-52 his4-519 trp1 can1*; GYP1101**a**: *MAT***a** *chc1-Δ8::LEU2 leu2-3,112, ura3-52 his4-519 trp1 can1*; GPY1101α: *MATα chc1-Δ8::LEU2 leu2-3,112 ura3-52 his4-519 trp1 can1*; GPY2200**a**:*MAT***a** *leu2-3,112 ura3-52 ade6*; GPY2200α: *MATα leu2-3,112 ura3-52 ade6*; GPY2201**a**: *MAT***a** *chc1-Δ8::LEU2 leu2-3,112 ura3-52 ade6*; GPY2201α: *MATα chc1-Δ8::LEU2 leu2-3,112 ura3-52 ade6*; BFY101-10C: *MATα kex2Δ1::URA3 ura3-52 leu2-3,112*; GPY55-15B: *MATα leu2-3,112 ura3-52 his4-519 trp1 prb1*; GPY68: *chc1-Δ10::LEU2* transformant of 55-15B; GPY55-10B: *MATα leu2-3,112 ura3-52 trp1 prb1*; GPY64: *chc1-Δ10::LEU2* transformant of 55-10B; GPY100-9C: *MATα kex2::URA3 chc1-Δ10::LEU2 ura3-52 leu2-3,112 trp1*; RC687: *MAT***a** *sst2 rme1 ade2 his6 met1 ura1*; RC757: *MATα sst2 met1 his6 can1 cyh1.*
14. D. Julius, L. Blair, A. Brake, G. Sprague, J. Thorner, *Cell* **32**, 839 (1983).
15. J. E. Reneke, K. J. Blumer, W. E. Courchesne, J. Thorner, *ibid.* **55**, 221 (1988).
16. S. Michaelis and I. Herskowitz, *Mol. Cell Biol.* **8**, 1309 (1988).
17. R. S. Fuller, R. E. Sterne, J. Thorner, *Annu. Rev. Physiol.* **50**, 345 (1988).
18. D. Julius, R. Schekman, J. Thorner, *Cell* **36**, 309 (1984).
19. A. Dmochowska, D. Dignard, D. Henning, D. Y. Thomnas, H. Bussey, *ibid.* **50**, 573 (1987).
20. G. Payne, unpublished observations.
21. M. A. Kukuruzinska, M. L. E. Bergh, B. J. Jackson, *Annu. Rev. Biochem.* **56**, 915 (1987); A. Franzuzoff and R. Schekman, *EMBO J.*, in press.
22. P. Novick and R. Schekman, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1858 (1979).
23. R. S. Fuller, A. Brake, J. Thorner, *ibid.* **86**, 1434 (1989).
24. D. Julius, A. Brake, L. Blair, R. Kunisawa, J. Thorner, *Cell* **37**, 1075 (1984).
25. K. Mizuno, T. Nakamura, T. Takada, K. Sakakibara, H. Matsuo, *Biochem. Biophys. Res. Commun.* **4**, 807 (1987).
26. M. A. K. Markwell, *Anal. Biochem.* **125**, 427 (1983).
27. P. Sanz, E. Herrero, R. Sentandreu, *Biochem. Biophys. Acta* **924**, 193 (1987).
28. R. S. Fuller and J. Thorner, personal communication.
29. L. Orci *et al.*, *J. Cell Biol.* **99**, 2187 (1984); L. Orci *et al.*, *Cell* **39**, 39 (1984); *ibid.* **42**, 671 (1985); *ibid.* **49**, 865 (1987); *ibid.* **51**, 1039 (1987); J. Tooze, M. Hollinshead, R. Frank, B. Burke, *J. Cell Biol.* **105**, 155 (1987); H. W. Davidson, C. J. Rhodes, J. C. Hutton, *Nature* **333**, 93 (1988).
30. J. Tooze and S. Tooze, *J. Cell Biol.* **103**, 839 (1986).
31. R. B. Kelly, *Science* **230**, 25 (1985).
32. J. Heuser, *J. Cell Biol.* **84**, 560 (1980).
33. J. L. Goldstein, M. S. Brown, R. G. W. Anderson, D. W. Russell, W. J. Schneider, *Annu. Rev. Cell Biol.* **1**, 1 (1985).
34. A. Helenius, I. Mellman, D. Wall, A. Hubbard, *Trends Biochem. Sci.* **8**, 245 (1983).
35. A. Cooper and H. Bussey, personal communication.
36. P. Esmon, thesis, University of California, Berkeley.
37. J. H. Rothman and T. H. Stevens, *Cell* **47**, 1041 (1986).
38. L. M. Banta, J. S. Robinson, D. J. Klionsky, S. D. Emr, *J. Cell Biol.* **107**, 1369 (1989).
39. B. J. Iacopetta, S. Rothenberger, L. C. Kuhn, *Cell* **54**, 485 (1988).
40. B. M. F. Pearse, *EMBO J.* **7**, 3331 (1988).
41. S. K. Dutcher and L. H. Hartwell, *Cell* **33**, 203 (1983).
42. Unless otherwise indicated, all media are described in F. Sherman, G. R. Fink, and J. B. Hicks, [*Methods in Yeast Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986)].
43. Pheromone production was measured as follows: Cells were sedimented, washed with YPD medium, and resuspended at 10^6 cells per milliliter in fresh YPD (yeast extract, peptones, dextrose) medium and allowed to grow at 30°C. At intervals (0, 1.5, and 3 hours after the resuspension), samples were removed, and a portion was used to determine cell density and the remainder was filtered through a 0.45-μm nitrocellulose filter. The filtrate was serially diluted in YPD medium, and the dilutions were applied to sparse lawns of RC687 (*MAT***a** *sst2*) (*13*) or RC757 (*MATα sst2*) (*16*) tester cells; *sst2* confers supersensitivity to cell cycle arrest by mating pheromones (*52*). Known concentrations of α-factor (J. Thorner, University of California, Berkeley) were used as standards. Pheromone concentration in the culture media of *CHC1 MATα*, *CHC1 MAT***a**, and *chc1 MAT***a** cells doubled between 1.5 and 3 hours. Barely detectable halos from *chc1 MATα* culture supernatants were visible after 1.5 and 3 hours, but the halo at 3 hours could be adequately measured. No halos were observed at the start (0 hour).
44. Cell density in liquid cultures was determined from the absorbance (at 600 nm) of dilute cell suspensions in a 1-cm cuvette. An absorbance of 1 corresponded to approximately 10^7 cells per milliliter
45. Cells were grown in SD (synthetic dextrose) medium plus CA (0.5 percent Bacto vitamin assay Casamino acids, adenine, histidine, methionine, and cysteine at 50 μg/ml) to the midlogarithmic phase of growth. In order to grow in this medium, cells must express *URA3* or uracil must be added to the medium. In this experiment and those described in Figs. 3 and 4, we used GPY64,68,55-10B and 55-15B transformed with YCp50, a yeast centromere vector carrying the *URA3* gene. For preparation of cell extracts, 5×10^8 cells were lysed in 200 μl of buffer A (50 m*M* Hepes-NaOH, *p*H 7.5, 1 m*M* EDTA) plus a protease inhibitor mixture (*23*) by vigorous mixing with 0.3 ml of 0.5-mm glass beads for three 1-minute pulses on a Vortex mixer set at the highest speed. The lysate was removed, the glass beads were washed with 0.3 ml of the lysis buffer, and the lysate and washings were combined. Triton X-100 was added to 1 percent. For assays of intact cells, 10^8 cells were resuspended in 100 μl of buffer A with protease inhibitors. Kex2p endoprotease assays and G6PD assays were conducted with each sample. Kex2p activity was measured as in (*23*) except that Triton X-100 was used as detergent; the final volume of the assay mix was 100 μl, and the substrate, *t*-butoxycarbonyl-glutamyl-arginyl-arginyl-7-amino-4-methyl-coumarin, was present at 200 μ*M*. Assays were performed in the presence of 2 m*M* calcium chloride or 10 m*M* EDTA at two different concentrations of cells or extract. Each concentration was assayed in duplicate. Activity is expressed as the average number of units of activity per 10^7 cells in calcium-containing buffer minus that measured in buffer with EDTA, 1 unit of activity is 1 pmol of 7-amino-4-methyl-coumarin produced per minute at 37°C. G6PD was assayed according to a modification of the procedure described in (*53*). Lysate and cell samples were added to 1.6 ml of 50 m*M* tris-HCl, *p*H 7.5. Nicotinamide adenine dinucleotide phosphate ($NADP^+$) (0.2 ml of a 5 m*M*

solution) was added and the samples were incubated at 24°C for 5 minutes. Addition of 0.2 ml of 6.67 m*M* glucose 6-phosphate initiated the reaction, and the absorbance at 340 nm was determined 15 minutes later. One unit of activity is 1 pmol of NADPH produced in 1 minute at 24°C.

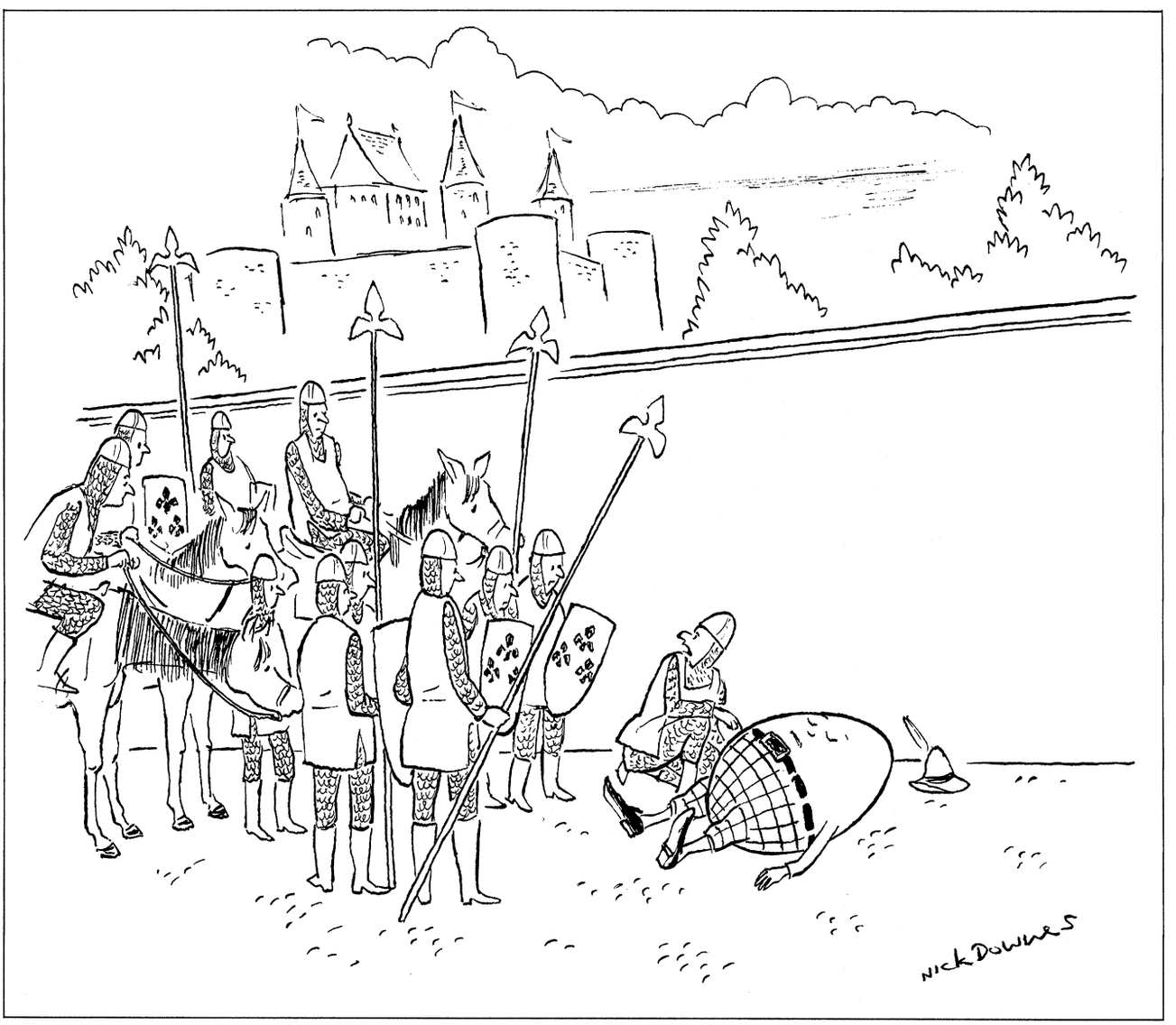
46. L. J. Wickerham, *J. Bacteriol.* **142**, 414 (1946).
47. R. J. Deshaies and R. Schekman, *J. Cell Biol.* **105**, 633 (1987).
48. P. C. Bohni, R. J. Deshaies, R. Schekman, *J. Cell Biol.* **106**, 1035 (1988); endoglycosidase H was provided by P. Robbins.

49. Strains were grown overnight to midlogarithmic phase in synthetic dextrose (SD) plus CA. Cell extracts were prepared by glass bead lysis (7) of 10^8 cells with 50 μl of 2 percent SDS. The resulting lysate was diluted to 0.6 ml with SPE buffer (20 m*M* sodium phosphate, *p*H 7.3, 1 m*M* EDTA). Intact cells (4×10^7) were resuspended in 0.25 ml of phosphate-buffered saline (PBS) and 1 m*M* EDTA. For cell lysate labeling, 0.25 ml (4×10^7 cell equivalents) from each sample was added to 0.25 ml of SPE buffer containing three Lodobeads (Pierce Chemical Co.) and 300 μCi of $Na^{125}I$ (100 mCi/ml) (Amersham). After incubation (20 minutes) on ice, labeling was terminated by removing the liquid from the beads and adding it to an equal volume of 2× PBS and 2 percent Triton X-100. Intact cells were labeled by adding 0.25 ml (4×10^7 cells) of intact cells to 0.25 ml of PBS and 1 m*M* EDTA containing 1 iodobead and 300 μCi of $Na^{125}I$. After 20 minutes on ice, the cell suspension was removed from the beads, washed twice with PBS, 1 m*M* EDTA plus the protease cocktail described in (*23*), and then subjected to glass bead lysis and processed for immunoprecipitation as described in Fig. 1. Antisera to Kex2P, a 33-kD cell wall protein (*27*), or G6PD (Sigma) was used to precipitate antigen from portions of each sample. The Kex2p immunoprecipitates were subjected to a second round of precipitation. Samples were analyzed by SDS-PAGE and autoradiography.

50. R. Jensen, G. F. Sprague, I. Herskowitz, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3935 (1983).
51. P. S. Kayne *et al.*, *Cell* **55**, 27 (1988).
52. R. K. Chan and C. A. Otte, *Mol. Cell. Biol.* **2**, 11 (1982).
53. G. W. Lohr and H. D. Waller, in *Methods of Enzymatic Analysis,* H. U. Bergmeyer, Ed. (Academic Press, New York, 1974), pp. 636–643.

54. We thank R. Fuller and J. Thorner for advice and reagents; D. Meyer, R. Sterne, S. Michaelis, S. Emr, J. Rothman, P. Sanz, K. Cunningham, and P. Herman for materials and advice; R. Deshaies and L. Silveira for helpful comments on the manuscript; and A. Cooper, H. Bussey, R. Fuller, and J. Thorner for communicating unpublished data. During the first stages of this work G.P. was supported as a senior postdoctoral fellow of the California Division of the American Cancer Society. Supported by NIH grants GM 36881 (R.S.) and GM 39040 (G.P.) and a Pew Scholar's award (G.P.).

10 April 1989; accepted 5 July 1989



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