Selective and Immediate Effects of Clathrin Heavy Chain Mutations on Golgi Membrane Protein Retention in Saccharomyces cerevisiae

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Abstract. The role of clathrin in retention of Golgi membrane proteins has been investigated. Prior work showed that a precursor form of the peptide mating pheromone α-factor is secreted by Saccharomyces cerevisiae cells which lack the clathrin heavy chain gene (CHC1). This defect can be accounted for by the observation that the Golgi membrane protein Kex2p, which initiates maturation of α-factor precursor, is mislocalized to the cell surface of mutant cells. We have examined the localization of two additional Golgi membrane proteins, dipeptidyl aminopeptidase A (DPAP A) and guanosine diphosphatase (GDPase) in clathrin-deficient yeast strains. Our findings indicate that DPAP A is aberrantly transported to the cell surface but GDPase is not. In mutant cells carrying a temperature-sensitive allele of CHC1 (chcl-ts), α-factor precursor appears in the culture medium within 15 min, and Kex2p and DPAP A reach the cell surface within 30 min, after imposing the nonpermissive temperature. In contrast to these immediate effects, a growth defect is apparent only after 2 h at the nonpermissive temperature. Also, sorting of the vacuolar membrane protein, alkaline phosphatase, is not affected in chcl-ts cells until 2 h after the temperature shift. A temperature-sensitive mutation which blocks a late stage of the secretory pathway, secl, prevents the appearance of mislocalized Kex2p at the cell surface of chcl-ts cells. We propose that clathrin plays a direct role in the retention of specific proteins in the yeast Golgi apparatus, thereby preventing their transport to the cell surface.

Proteins that enter the secretory pathway are destined for the cell exterior, the plasma membrane, the major degradative organelle (lysosomes or vacuoles), and the secretory organelles themselves (Pfeffer and Rothman, 1987). In the case of several ER or Golgi proteins, domains have been identified which are required to maintain the proteins in residence. Without these sequences, the proteins usually continue along the secretory pathway to the cell surface. In a well-characterized example, retention of ER luminal proteins appears to occur by means of a membrane-bound receptor which recognizes a carboxy-terminal four-amino acid retention signal on ER proteins (Pelham, 1990). The receptor has been proposed to dwell in an early Golgi compartment where it functions to return ER retention signal–bearing proteins back to the ER. The retention signals on several ER and Golgi membrane proteins have been recently identified (Machamer, 1991), but, in most cases, little else is known about other factors which influence membrane protein retention.

In the yeast Saccharomyces cerevisiae, the Golgi membrane protein encoded by the KEX2 gene (Kex2p) requires its cytoplasmic domain for retention in the Golgi apparatus (Fuller et al., 1989). Insight into the mechanism of Kex2p retention has been provided by our studies of yeast mutants lacking the clathrin heavy chain gene (CHC1) (Payne and Schekman, 1989). In these mutants, Kex2p is mislocalized to the cell surface, a finding which suggests a novel role for clathrin as a participant in the retention process. The involvement of clathrin in receptor-mediated endocytosis in mammalian cells (Pearse and Robinson, 1990) provides a paradigm for clathrin’s function in Kex2p retention. Lattices at the plasma membrane composed of clathrin heavy and light chains and associated proteins (APs, also known as adaptins) collect transmembrane receptors at sites undergoing vesiculation, leading to selective incorporation of the receptors into clathrin-coated endocytic vesicles. Collection of receptors at clathrin-coated plasma membrane sites depends on the receptors’ cytoplasmic domains and is thought to occur by recognition of these domains by the clathrin APs. It has also been suggested that clathrin lattices on the trans-Golgi network (TGN) act in a similar fashion to recognize the cytoplasmic domains of mannose-6-phosphate receptors and direct packaging of the receptors carrying lysosomal precursors into vesicles targeted for prelysosomal compartments (Pearse and Robinson, 1990). By analogy, models for clathrin’s role in retention posit interactions between the cytoplasmic tail of Kex2p and clathrin coats (Fuller et al.,

1. Abbreviations used in this paper: AP, associated protein; CHC1, clathrin heavy chain gene; CWP, cell wall protein; DPAP A, dipeptidyl aminopeptidase A; GDPase, guanosine diphosphatase; G6PD, glucose-6-phosphate dehydrogenase.

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Materials and Methods

Materials

Unless noted all reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Strains, Media, and Genetic Methods

E. coli strain used in this study was HB101 (F-, hsdS20(m-, m-), recA13, ara-l4, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, supE44, l-). Yeast mating, sporulation, and tetrad analyses were conducted as described by Sherman et al. (1974). DNA transformations were performed by the lithium acetate procedure (Ito et al., 1982) or by spheroplast transformation (Hinnen et al., 1978).

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Strains carrying chclA and dpplA were made by single step gene replacement (Rothstein, 1983) using plasmid pchcl-A10 (Payne et al., 1987) and plasmid pG6 (obtained from Tom Stevens, University of Oregon).

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Table I. Yeast Strains Used in This Study

| Strain   | Genotype                        | Source                    |
|----------|---------------------------------|---------------------------|
| GPY55-10 | MATa alpha-leu2-3,112 ara3-52 trpl-289 prb1 gai2 | Payne and Schekman (1989) |
| GPY112.1 | MATa leu2-3,112, chcl-521 his4-519 trpl can1 with pCHC102 | This study               |
| GPY146.2 | MATa leu2-3,112, chcl-521 his4-519 trpl his4 can1 GAL2 | This study               |
| GPY176   | MATa leu2-3,112, chcl-521 trpl prb1 gai2 chcl-521 ; LEU2 YCpCHCTRP | This study               |
| SEY6210  | MATa alpha-leu2-3,112 his3-200 trpl-1g01 his2-297 | S. Emr                   |
| GPY154   | MATa dpp2-2A::HIS3 transformant of SEY6210 | This study               |
| GPY155   | MATa dpp2-2A::HIS3 transformant of SEY6210 | This study               |
| GPY242   | MATa alpha-leu2-3,112 ade2-101 his3a200 dpp2-2A::HIS3 leu2-3,112 chcl-521::LEU2 | This study               |
| GPY268   | MATa alpha-leu2-3,112 his3-200 trpl-1g01 his2-297 | This study               |
| GPY382   | pCHC102 transformant of GPY268 cured of YCpCHCTRP | This study               |
| GPY383   | pBP6 transformant of GPY268 cured of YCpCHCTRP | This study               |
| GPY440   | MATa alpha-trp1 ura3-52 pBP6 leu2-3,112 chcl-521::LEU2 sect-1 | This study               |
| GPY441   | MATa alpha-trp1 ura3-52 pBP6 leu2-3,112 chcl-521::LEU2 sect-1 | This study               |
| GPY442   | MATa alpha-trp1 ura3-52 pBP6 leu2-3,112 pBP6 sect-1 | This study               |
| GPY443   | MATa alpha-trp1 ura3-52 pBP6 leu2-3,112 pBP6 sect-1 | This study               |
Radiolabeling and Immunoprecipitations

For labeling with Na$^{35}$S (Amersham, Arlington Heights, IL; New England Nuclear, Wilmington, DE), cells were grown to midlogarithmic phase in YPD at 30°C. Intact cells and cell lysates were prepared and labeled as described previously (Payne and Schekman, 1989) except that iodoacetamide was added to the lysates to a concentration of 10 mM and samples were incubated at 37°C for 10 min before labeling. Temperature-sensitive strains were grown in SD CAA-ura or SD CAA-trp at 24°C to midlogarithmic phase. Cells were shifted to 37°C for 0, 30, 60, or 120 min then harvested and labeled as above.

For metabolic labeling, cells were grown to midlogarithmic phase at 24°C in SD CAA-ura. Cultures were shifted to 30 or 37°C for 0, 15, 30, 60, or 120 min. 5 × 10$^7$ cells were harvested and washed twice in supplemented SD. Cells were resuspended in 250 μl of supplemented SD plus 0.2 mg/ml ovalbumin. Labeling was initiated by addition of 50 μCi of Trcsi$^{35}$S-Label (ICN, Irvine, CA), and incubated at 30 or 37°C for 10 min or at 24°C for the 0-min samples. Na$$_2$$SO$_4$ (10 mM) was added and samples were placed on ice to terminate label incorporation. Labeled cells were sedimented and 200 μl of media was removed for immunoprecipitation of α-factor. 5 μl of 2% SDS and 0.2 mg/ml ovalbumin was added to the sample which was then heated to 100°C for 3 min. Samples were brought to 1 ml with PBS, 1% TX-100 (PT) 0.2 mg/ml ovalbumin.

All samples subjected to immunoprecipitation received 50 μl of 10% S. aureus suspension (IgSorb; The Enzyme Center, Maiden, WA) and were incubated on ice for 15 min. The IgSorb was sedimented by centrifugation at 16,000 x g for 15 min. To the supernatant was added antiserum against α-factor, DPAP A, alkaline phosphatase, or Kex2p. For precipitation of DPAP A and Kex2p, 50 μl of a concentrated extract of cells (200 A$$_260$ unit equivalents/ml) carrying deletions of STE13 or KEX2, respectively, was also added. After an overnight incubation at 4°C, protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) 20% suspension was added to collect antibody. In the case of α-factor and alkaline phosphatase, beads were washed once with PBS, 1% TX-100, 0.1% SDS (PTS), once with 2 M urea, 2 M NaCl, 1% TX-100, 100 mM Tris·HCl, pH 7.4, again with PTS, and finally twice with 10 mM NaCl, 10 mM Tris, pH 6.8. Beads were resuspended in 25 μl Laemmli sample buffer (Laemmli, 1970) and heated at 100°C for 3 min. DPAP A and Kex2p immunoprecipitates collected by protein A-Sepharose were washed with PTS and twice with 10 mM NaCl, 10 mM Tris·HCl, pH 6.8. Beads were resuspended in 50 μl 2% SDS, 0.2% BME and heated at 100°C for 3 min. Immediately thereafter, 1 ml of PT containing 200 μg/ml ovalbumin was added. The beads were sedimented, and the supernatant was collected and subjected to a second round of immunoprecipitation. All samples were subjected to SDS-PAGE. In the case of α-factor, gels were treated with Amplify (Amerham) for 20 min prior to autoradiography. All other gels were incubated in 25% methanol, 10% acetic acid. Gels were exposed to x-ray film (Kodak, X-OMAT AR) at -70°C.

Enzymatic Activity Assays

GDPlase activity was determined according to the method of Abejón et al. (1989). Cells were grown at 30°C in YPD to midlogarithmic phase. 1 × 10$^6$ cells were harvested and washed with water. Cell lysates were prepared by glass bead lysis (Payne and Schekman, 1989) in the absence of detergent. Membranes were sedimented at 100,000 g for 1 h. Intact cells or membranes from an equivalent number of cells were assayed in a reaction mix containing 20 mM imidazole, pH 7.4, 10 mM CaCl$_2$, 7 mM GDP. Samples were incubated at 37°C for 30 min. The reaction was stopped by addition of 0.2 M EDTA and heating at 100°C for 3 min. Immediately thereafter, 1 ml of PT containing 200 μg/ml ovalbumin was added. The beads were sedimented, and the supernatant was collected and subjected to a second round of immunoprecipitation. All samples were subjected to SDS-PAGE. In the case of α-factor, gels were treated with Amplify (Amersham) for 20 min prior to autoradiography. All other gels were incubated in 25% methanol, 10% acetic acid. Gels were exposed to x-ray film (Kodak, X-OMAT AR) at -70°C.

Role of Clathrin in Golgi Membrane Protein Retention

Kex2p normally acts to initiate proteolytic maturation of the α-factor mating pheromone precursor in the Golgi apparatus (Fuller et al., 1988). Cleavage by Kex2p after basic amino acid pairs at four sites in the precursor releases four α-factor cassettes appended by short stretches of amino acids. Two additional proteases, dipeptidyl aminopeptidase A (DPAP A) and Kexlp carboxypeptidase, are necessary to convert the cassettes to the 13-amino acid, biologically active form of α-factor (Fuller et al., 1988). Like Kex2p, each of these proteases has three domains: a large luminal domain which contains the protease activity; a single membrane spanning domain; and a cytoplasmic domain of ~100 amino acids (Fuller et al., 1988, 1989; Cooper et al., 1989). Kex2p and Kexlp are type I membrane proteins with the amino terminus located in the lumen; DPAP A, a type II membrane protein, has the opposite orientation. Since all three proteases appear to reside in the Golgi apparatus (personal communication, T. Stevens and H. Bussey, Redding et al., 1991) and have similar topologies, it seemed possible that clathrin would also be required for proper retention of DPAP A and Kexlp. We have focused on DPAP A and used solid phase radioiodination (Payne and Schekman, 1989) to determine whether the protease is mislocalized to the cell surface of clathrin heavy chain-deficient (chlΔ) cells. In this procedure, chlΔ and wild-type cells were iodinated either as intact cells or as cell lysates. After lysis of the labeled intact cells, DPAP A was precipitated from each sample with specific antiserum, subjected to electrophoresis through SDS-PAGE and visualized by autoradiography. If DPAP A is present at the cell surface then it should be labeled in intact cells. The amount of DPAP A at the surface can be estimated by comparing the amount labeled in intact cells to that labeled in lysates where all the protease should be accessible to iodination. As shown in Fig. 1, DPAP A is clearly labeled in intact chlΔ cells (Fig. 1, lane 3) cells but not wild type cells (Fig. 1, lane 1). Like Kex2p (Payne and Schekman, 1989), DPAP A sometimes undergoes a minor degree of degradation during the labeling period, resulting in a smaller labeled species.

As controls, glucose-6-phosphate dehydrogenase (G6PD) and a 33-kD cell wall protein (CWP) were also immunoprecipitated from each sample and analyzed by SDS-PAGE. G6PD, a cytoplasmic protein, serves as a control for cell lysis in intact cell samples. No labeling of G6PD in either mutant or wild-type intact cells was observed, but G6PD was readily detected when lysates were labeled (data not shown). This result eliminates the possibility that DPAP A is accessi-
Mislcalization of DPAP A to the cell surface in clathrin-deficient cells. CHC1 (GPY155) (lanes 1, 2, 5, and 6) and chclΔ (GPY242) (lanes 3, 4, 7, and 8) intact cells (C) and cell lysates (L) were radiolabeled with 125I as described in Materials and Methods. After labeling, intact cells were lysed and aliquots of all samples were treated with antisera against DPAP A (lanes 1-4) or against the 33-kD cell wall protein (lanes 5-8). Immunoprecipitated proteins were subjected to SDS-PAGE and autoradiography.

Golgi-localized Guanosine Diphosphatase Is Retained in chclΔ Cells

Both proteins shown to be mislocalized to the cell surface of chclΔ cells function in the maturation of α-factor and are probably located in the same late Golgi compartment. Two possibilities could account for the effect of chcl on retention of these proteins. First, in the absence of clathrin the integrity of the entire Golgi apparatus could be compromised resulting in haphazard expulsion of all Golgi membrane proteins. Alternatively, clathrin may play a selective role in the retention of a subset of Golgi membrane proteins.

Like DPAP A, guanosine diphosphatase (GDPase) is a Golgi-localized, type II membrane protein (Abeijon et al., 1989; C. Hirschberg, personal communication). Characterization of GDPase location in chclΔ cells was undertaken to evaluate whether all Golgi membrane proteins are mislocalized to the cell surface. In the absence of antibody, we employed enzyme activity assays to assess cell surface localization. These assays rely on the same principle as iodinations, a membrane-impermeant probe is applied to intact and lysed cells. In this case the probe is guanosine diphosphate, a substrate for GDPase (Abeijon et al., 1989). If GDPase is at the surface of mutant cells then activity should be manifested in intact as well as lysed cell samples. Because of other, soluble nucleoside diphosphatases, total GDPase activity was measured in a membrane fraction obtained from lysed cells rather than in unfractonated extracts. Results of representative GDPase activity assays are shown in Table II. Only 2% of the total cell-associated GDPase activity was measurable in intact chclΔ cells (Table II, row 1) and no activity was present in intact wild-type cells. The slightly higher amount of GDPase detected in intact chclΔ cells compared to wild-type cells (Table II, row 1) can be accounted for by cell lysis, as measured by G6PD activity assays (Table II, row 6). Although the absolute GDPase activity varied up to twofold between separate experiments, the percentage of GDPase activity at the surface was uniformly low. The lack of significant GDPase activity in intact mutant cells stands in contrast to measurements of DPAP A activity using the membrane-impermeant substrate, alanine-proline nitroanilide. In accordance with the results obtained by iodination, activity assays of DPAP A indicate that 25–30% of the protein is at the surface of chclΔ cells (Table II, row 3).

We have also determined by iodination whether an ER membrane protein, Sec63p (Deshaies et al., 1991; Sadler, 1989), can be detected at the surface of chclΔ cells. No evidence for mislocalization was obtained (data not shown). Taken together, our results argue that the absence of clathrin heavy chain does not result in the indiscriminate export of secretory organelle membrane proteins and suggest that the

Table II. GDPase Activity Is Not Found at the Surface of chclΔ Cells

| Enzyme | CHC1 (U/10^7 cells) | Total activity (U/10^7 cells) | % at Surface |
|--------|---------------------|-------------------------------|--------------|
| GDPase | 0                   | 288                           | 0            |
| G6PD   | 0                   | 149                           | 0            |
| DPAP A | 1                   | 25                            | 4.0          |
| G6PD   | 0                   | 118                           | 0            |

GDPase and DPAP A activity at the surface of chclΔ cells. CHC1 (GPY155) and chclΔ (GPY242) cells were grown in YPD at 30°C, cells were washed, and activity at the cell surface (intact cells) or total activity (cell lysates for DPAP A or membrane fraction for GDPase) was measured. Activity is represented as Units/10^7 cells. G6PD activity was measured during each assay to control for cell lysis.
retention of only a subset of Golgi membrane proteins is influenced by clathrin.

**α-Factor Maturation in Cells Expressing a Temperature-sensitive Allele of CHC1**

Cells which have sustained a deletion of CHC1 grow slowly and exhibit accumulation of membranous structures when visualized by electron microscopy (Payne et al., 1987). Although the effect of chclA on Golgi membrane protein retention is selective, it could result as a secondary consequence of slow growth or some other anomaly caused by the cells' continuous growth without clathrin heavy chain. Concern about indirect effects of a gene disruption can be addressed if cells carrying a conditionally defective (e.g., temperature-sensitive) allele of the gene are available. Commonly, the immediate appearance of a phenotype after such cells are transferred to nonpermissive conditions constitutes a reliable indicator of a primary and direct effect of a loss of gene product function. Based on this rationale, the phenotypes of cells carrying a temperature-sensitive allele of CHC1 (chcl-ts; see Materials and Methods) were analyzed after shifting the cells to a nonpermissive temperature.

One of the most dramatic phenotypes which accompanies the mislocalization of Kex2p in chclA cells is inefficient maturation of α-factor (Payne and Schekman, 1989). The highly glycosylated form of the precursor which is secreted by chclΔ cells can be conveniently and easily distinguished from mature α-factor by SDS-PAGE; the precursor migrates with an apparent molecular mass of \( \sim 125 \) kD whereas the mature form migrates as a 3.5-kD species. The secretion of precursor α-factor was monitored before and after shifting chcl-ts or wild-type cells from 24 to 37°C. Cells were labeled for 15 min with \(^{35}\)S-methionine and cysteine and then secreted α-factor forms were collected from the medium by immunoprecipitation, separated by SDS-PAGE, and detected by autoradiography (Fig. 2a). At 24°C both strains secreted only mature α-factor (Fig. 2a, lanes 1 and 2). However, when cells were shifted to 37°C and immediately labeled for 15 min, glycosylated precursor is apparent in the chcl-ts culture medium (Fig. 2a, lane 4) but not the wild-type culture medium (Fig. 2a, lane 3). Synthesis of α-factor is repressed by the heat shock which occurs when cells are shifted from 24 to 37°C (J. Finlay, unpublished observation) which precludes detecting maturation defects in samples harvested at 30 and 45 min after the temperature shift (Fig. 2a, lanes 5–8). When expression of α-factor begins to return at the 60-min time point, the maturation defect in the chcl-ts cells has worsened (compare the ratios of precursor to mature α-factor in Fig. 2a, lanes 4 and 10); and by the 2-h time point the defect is comparable to that observed in chclA cells (Fig. 2a, lane 12).

In an attempt to circumvent the decrease in α-factor synthesis caused by shifting cells from 24 to 37°C, we conducted a parallel experiment in which cells were transferred to 30°C instead of 37°C (Fig. 2b). The effect of the temperature shift on α-factor expression is significantly diminished using this protocol (Fig. 2b, lanes 5–8). Although the extent of the α-factor maturation defect is not as extreme when mutant cells are shifted to 30°C rather than 37°C (comparing ratios of mature to precursor forms), secretion of highly glycosylated precursor again occurs within the first 15 min after temperature shift (Fig. 2b, lane 4). Furthermore, this experiment more clearly reveals the progressive increase in precursor secretion by chcl-ts cells after imposition of the nonpermissive temperature (Fig. 2b, even-numbered lanes). These results indicate that the α-factor maturation defect is an immediate consequence of reducing clathrin function.

As a means to evaluate the relationship between the α-factor maturation phenotype and cell growth, the effect of temperature shifts on growth rates of the chcl-ts and wild-type
retention of Kex2p and DPAP A. Mislocalization of Kex2p and DPAP A to the cell surface was determined directly by solid phase iodination of intact chcl-ts cells at various times after a shift from 24 to 37°C (Fig. 4). At each time point, Kex2p, DPAP A, and CWP were immunoprecipitated from lysates of the labeled cells. Both Kex2p (Fig. 4, lanes 1–4) and DPAP A (Fig. 4, lanes 5–8) appeared at the cell surface within 30 min of the temperature shift and reached maximal levels (70% of total for Kex2p and 30% of total for DPAP A) at the plasma membrane between 1 and 2 h after incubation at 37°C was initiated. Analysis of CWP (Fig. 3, lanes 9–12) indicates that the efficiency of surface iodination was commensurate in all samples. The temperature shift did not lead to cell surface mislocalization of Kex2p or DPAP A in wild-type cells (data not shown). These data show that mislocalization of Kex2p and DPAP A is also an immediate consequence of clathrin malfunction.

**Sorting of a Vacuolar Membrane Protein in chcl-ts Cells**

Sorting of a vacuolar integral membrane protein, alkaline phosphatase (ALP, encoded by PHO8) (Kaneko et al., 1987), provides an additional diagnostic for Golgi apparatus function in chcl mutant cells. ALP is synthesized as a precursor which is translocated into the ER as a type II membrane protein, like DPAP A, and transported to the Golgi apparatus (Klionsky and Emr, 1989). ProALP is transferred from the Golgi apparatus to the vacuole where proteolytic maturation occurs. If chcl affects the ability of the Golgi apparatus to divert proALP from the secretory pathway then proALP might be expected to accumulate at the cell surface. Using the same iodination procedures used to detect cell surface Kex2p and DPAP A, mislocalization of proALP to the cell surface was evaluated in both chclΔ cells and in chcl-ts cells incubated at 37°C. Immunoprecipitation of ALP from iodinated samples of chclΔ cells (Fig. 5, lanes 1 and 2) revealed a fraction of ALP, mostly the precursor form (Fig. 5, open arrowhead), which was accessible to the reactive iodide in intact cells (Fig. 5, lane 1). The precursor form is not visible in the lysate sample, probably due to the high background. Using CWP to normalize labeling efficiencies, 20% of the total cell-associated ALP was labeled as the precursor form and 10% as the mature form in the intact cell sample. The mature ALP which is accessible to iodination (Fig. 5, lane 1, closed arrowhead) could result from a low level of cell lysis or from limited proteolytic maturation of the precursor which reaches the cell surface.

To determine whether ALP missorting rapidly occurs upon a loss of clathrin function, we examined this phenomenon in the temperature-sensitive strain. ALP appearance at the cell surface was determined directly by solid phase iodination of intact chcl-ts cells at various times after a shift from 24 to 37°C (Fig. 4). At each time point, Kex2p, DPAP A, and CWP were immunoprecipitated from lysates of the labeled cells. Both Kex2p (Fig. 4, lanes 1–4) and DPAP A (Fig. 4, lanes 5–8) appeared at the cell surface within 30 min of the temperature shift and reached maximal levels (70% of total for Kex2p and 30% of total for DPAP A) at the plasma membrane between 1 and 2 h after incubation at 37°C was initiated. Analysis of CWP (Fig. 3, lanes 9–12) indicates that the efficiency of surface iodination was commensurate in all samples. The temperature shift did not lead to cell surface mislocalization of Kex2p or DPAP A in wild-type cells (data not shown). These data show that mislocalization of Kex2p and DPAP A is also an immediate consequence of clathrin malfunction.

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In chclΔ cells, inefficient α-factor maturation is correlated with, and can be accounted for by, mislocalization of Kex2p to the cell surface (Payne and Schekman, 1989). The rapid appearance of an α-factor maturation defect in chcl-ts cells incubated at 37°C implies a similarly immediate effect on retention of Kex2p and DPAP A. Mislocalization of Kex2p and DPAP A to the cell surface was determined directly by solid phase iodination of intact chcl-ts cells at various times after a shift from 24 to 37°C (Fig. 4). At each time point, Kex2p, DPAP A, and CWP were immunoprecipitated from lysates of the labeled cells. Both Kex2p (Fig. 4, lanes 1–4) and DPAP A (Fig. 4, lanes 5–8) appeared at the cell surface within 30 min of the temperature shift and reached maximal levels (70% of total for Kex2p and 30% of total for DPAP A) at the plasma membrane between 1 and 2 h after incubation at 37°C was initiated. Analysis of CWP (Fig. 3, lanes 9–12) indicates that the efficiency of surface iodination was commensurate in all samples. The temperature shift did not lead to cell surface mislocalization of Kex2p or DPAP A in wild-type cells (data not shown). These data show that mislocalization of Kex2p and DPAP A is also an immediate consequence of clathrin malfunction.

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In chclΔ cells, inefficient α-factor maturation is correlated with, and can be accounted for by, mislocalization of Kex2p to the cell surface (Payne and Schekman, 1989). The rapid appearance of an α-factor maturation defect in chcl-ts cells incubated at 37°C implies a similarly immediate effect on
Figure 4. Kex2p and DPAP A are mislocalized to the cell surface rapidly after shifting to the nonpermissive temperature in chcl-ts cells. chcl-ts (GPY268) cells were grown at 24°C in SD CA A-trp to early log phase. Cells were shifted to 37°C for 30, 60 or 120 min. At each time point cells were harvested and intact cells radiolabeled with 125I as described in Materials and Methods. After lysis, Kex2p (lanes 1–4), DPAP A (lanes 5–8), and CWP (lanes 9–12) were immunoprecipitated and analyzed as described in the legend to Fig. 1. Arrow indicates the migration position of the relevant protein. The faster migrating band in the DPAP A panel is a nonspecifically precipitated protein.

Golgi, the protein moves to the surface via conventional secretory vesicles. A prediction of this hypothesis is that cell surface delivery of Kex2p should depend on gene products which are necessary for fusion of secretory vesicles to the plasma membrane (Novick et al., 1981). The inducible mislocalization of Kex2p in chcl-ts cells allowed us to test this prediction by introducing a second temperature-sensitive mutation, secl, which prevents fusion of secretory vesicles to the plasma membrane at 37°C (Novick and Schekman, 1979). A wild-type strain and strains carrying either chcl-ts or secl alone or in combination were shifted to 37°C for 1 h to eliminate clathrin heavy chain function and impose the secl block to secretory vesicle fusion. The cells were then radiiodinated either as intact cells or extracts and, after lysing the labeled intact cells, Kex2p was immunoprecipitated. As anticipated, Kex2p was mislocalized to the surface in chcl-ts cells (Fig. 6, lanes 3 and 4) but not in wild-type (Fig. 6, lanes 1 and 2) or secl cells (Fig. 6, lanes 5 and 6). No Kex2p was iodinated in intact secl chcl-ts double mutant cells (Fig. 6, lane 7). Thus, the secl-imposed block of secretory vesicle fusion to the plasma membrane prevents cell surface appearance of mislocalized Kex2p, suggesting that mislocalized Kex2p travels to the plasma membrane in secretory vesicles. It could be argued that the reason Kex2p is not mislocalized in the double mutant cells is that secl somehow suppresses the effect of chcl-ts on the retention of Kex2p so that Kex2p remains in the Golgi apparatus. However, in contrast to secl CHC1 cells which accumulate mature α-factor (data not shown), the secl chcl-ts cells accumulate precursor α-factor indicating that chcl-ts still exerts an effect on Kex2p localization.

Discussion

We have investigated clathrin's role in the retention of Golgi membrane proteins by analyzing selected properties of cells carrying mutations in the clathrin heavy chain gene. Prior work indicated that the Golgi membrane protein Kex2p is mislocalized to the cell surface in yeast cells devoid of clathrin heavy chain (Payne and Schekman, 1989). Here we...
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If interactions between clathrin coats and the cytoplasmic tails of Kex2p and DPAP A are responsible for proper Golgi retention then mutations in the tails might be expected to prevent retention. Recently, mutations have been introduced into the cytoplasmic domains of Kex2p, DPAP A, and Kex1p.
Indeed, many of the mutations affect the Golgi localization of the proteases, but, contrary to our observations with chcl cells, the mutant proteases appear to travel to the vacuole rather than the cell surface (C. Wilcox and R. Fuller; T. Stevens; and H. Bussey, personal communications). We can envision several ways to reconcile the different consequences of chcl and cytoplasmic tail mutations. At the outset, it should be noted that different assays have been used for assessing localization. We have used cell surface iodination while the other groups have used methods including immunofluorescence techniques, cell surface enzyme activity assays, and vacuole-dependent enzyme turnover measurements. Our iodination approach only distinguishes between cell surface and internal populations, the location of the internal pool cannot be evaluated. We have considered the possibility that much of the mislocalized Kex2p in chcl cells travels to the vacuole where it is degraded. This would lower the internal pool at the expense of cell surface Kex2p and skew upward our estimates of the amount mislocalized to the plasma membrane. This could also explain the different percentages of cell surface Kex2p and DPAP A since DPAP A is related to a vacuolar membrane dipeptidyl aminopeptidase (DPAP B) (Roberts et al., 1989) and might be more stable in the vacuole. To address this possibility, we examined Kex2p mislocalization in chcl cells also carrying the pep4 and prbl mutations which drastically reduce the level of active vacuolar proteases (Jones, 1991). Such mutations stabilize Kex2p in mutants where the protein reaches the vacuole (K. Wilsbach and G. Payne, unpublished observations). The reduction in vacuolar protease activity does not, however, alter the level of cell surface Kex2p in chcl cells; we consistently measure 60-70% at the cell surface of these strains (data not shown). Yet another consideration is the possibility that the mutations in the Golgi membrane cytoplasmic domains alter the protein structure sufficiently to target (by unknown means) the abnormal protein to the vacuole for degradation. This possibility is reasonable for deletions that potentially change the protein structure in dramatic fashion but seems less credible for single amino acid changes which lead to vacuolar mislocalization (K. Redding and R. Fuller, personal communication).

Other explanations for the difference between cells expressing mutant Golgi membrane proteins and chcl cells could reflect more interesting aspects of intracellular traffic. It is evident from characterization of various transport pathways in chcl cells that clathrin acts at multiple points. For example, receptor-mediated endocytosis of α-factor is reduced two to threefold in chclΔ cells (Payne et al., 1988) and this effect occurs immediately in chcl-ts cells at the nonpermissive temperature (P. Tan and G. Payne, unpublished observations). Such pleiotropic effects of the mutation could alter an organelle(s) which is an intermediate between the Golgi apparatus and vacuoles. In this scenario, Kex2p retention would be disrupted in chcl cells and the protein would move to the intermediate organelle en route to the vacuole. Perturbation of the intermediate organelles in chcl cells could result in export of the Kex2p rather than delivery to the vacuole. On the other hand, mutant forms of Kex2p which leave the Golgi apparatus in otherwise wild-type cells would continue on to the vacuole. One finding which is inconsistent with this idea is that sec1 blocks cell surface appearance of Kex2p in chcl-ts cells. The simple interpretation of this result is that the unrestrained Kex2p in chcl-ts cells is packaged into secretory vesicles as it leaves the Golgi apparatus, since sec1 is known to block fusion of secretory vesicles to the plasma membrane (Novick et al., 1981).

However, it could be proposed that the SEC1 gene product also functions in vesicular traffic from the putative intermediate compartment to the cell surface in which case it would still prevent delivery of Kex2p to the cell surface of the chcl-ts sec1 cells. A different explanation suggests that transport to the vacuole is a default pathway and some interaction must occur to keep Kex2p from moving to the vacuole. The important interaction may be binding of the cytoplasmic tail to other clathrin coat proteins (e.g., APs). Thus, in chcl cells, where Kex2p is not properly retained in the Golgi apparatus, interaction with these proteins might still act to exclude Kex2p from the vacuole pathway, in the absence of clathrin heavy chain, leading to transport to the plasma membrane. Mutations in the cytoplasmic tail of Kex2p would eliminate interaction with the clathrin APs and the mutant Kex2p would not be able to avoid delivery to the vacuole via the default pathway.

Analysis of the fate of mutant forms of Kex2p and DPAP A in chcl cells may aid in distinguishing among these numerous proposals. For example, if cell surface delivery is a consequence of an abnormal organelle between the Golgi apparatus and the vacuole in chcl cells, then mutant forms of Kex2p should be delivered to the cell surface rather than the vacuole. If, on the other hand, interaction of Kex2p with other clathrin coat proteins dictates cell surface delivery then the cytoplasmic tail mutants should travel to the vacuole in chcl cells. As other clathrin coat proteins are identified, their role in retention can also be investigated. Such analyses, based on the work described here, should allow definition of the potentially complex interactions which are necessary for the retention of membrane proteins in the Golgi apparatus.

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