Clathrin Facilitates the Internalization of Seven Transmembrane Segment Receptors for Mating Pheromones in Yeast

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Abstract. The role of clathrin in endocytosis of the yeast pheromone receptors was examined using strains expressing a temperature-sensitive clathrin heavy chain. The yeast pheromone receptors belong to the family of seven transmembrane segment, G-protein-coupled receptors. A rapid and reversible defect in uptake of radiolabeled α-factor pheromone occurred when the cells were transferred to the nonpermissive temperature. Constitutive, pheromone-independent internalization of newly synthesized α-factor pheromone receptor was also rapidly inhibited in mutant strains at the nonpermissive temperature. In both cases residual endocytosis, 30–50% of wild-type levels, was detected in the absence of functional clathrin heavy chain.

Once internalized, the α-factor receptor was delivered to the vacuole at comparable rates in chel-ts and wild-type cells at the nonpermissive temperature. Clathrin heavy chain was also required for maximal uptake of a mutant α-factor receptor which is dependent on pheromone for internalization. In the presence of α-factor, the internalization rate of the mutant receptor in chel-ts cells at the nonpermissive temperature was 2.5 times slower than the rate observed for endocytosis of the mutant receptor in wild-type cells. These experiments provide in vivo evidence that clathrin plays an important role in the endocytosis of the seven transmembrane segment pheromone receptors in yeast.

Receptor-mediated endocytosis of many extracellular ligands proceeds through clathrin-coated domains of the plasma membrane known as clathrin-coated pits (Brodsky, 1988; Pearse and Robinson, 1990; Anderson, 1993; Schmid, 1993). The clathrin coats are assembled onto the plasma membrane as polyhedral lattices from trimers of clathrin heavy chains and associated light chains. Assembly is thought to require complexes of associated proteins that bridge the clathrin lattices to the membrane. The clathrin-coated pits collect receptors, invaginate, and pinch off, thereby selectively packaging the receptors into endocytic clathrin-coated vesicles. Receptors known to be internalized through clathrin-coated pits generally share common structural motifs, a large extracellular ligand-binding domain, a single transmembrane sequence, and a cytoplasmic domain (Pears and Robinson, 1990). In the case of several receptors, the cytoplasmic domains have been shown to contain sequences which mediate interaction with clathrin coats and are necessary for efficient uptake (Chen et al., 1990; Collawn et al., 1990; Kistakiss et al., 1990; Miettinen et al., 1992).

A structurally distinct class of cell surface receptors is characterized by seven transmembrane segments (7-TMS) and coupling to trimeric G-proteins (Dohlman et al., 1991). Several members of this receptor class have been shown to undergo endocytosis, and internalization may lead to long-term desensitization (down-regulation) to the effects of the corresponding ligand (Ascoli and Segaloff, 1987; Raposo et al., 1987, 1989; von Zastrow and Kobilka, 1992). The role of clathrin in the endocytosis of 7-TMS receptors has not been resolved.

In the yeast Saccharomyces cerevisiae, 7-TMS receptors are involved in the process of mating. The two haploid mating types of yeast, MATα and MATα, each secrete peptide pheromones, α-factor and α-factor respectively, which bind to specific 7-TMS receptors on the surface of cells of the opposite mating type (Cross et al., 1988; Marsh et al., 1991). Pheromone binding initiates a trimeric G-protein-mediated signal which triggers a program of physiological changes necessary for conjugation (Cross et al., 1988; Marsh et al., 1991). Both α-factor and its receptor are internalized and degraded in the vacuole (Chvatchko et al., 1986; Singer and Patrick, 1990; Davis et al., 1993) and recently it has been shown that endocytosis of the

1. Abbreviations used in this paper: 7-TMS, seven transmembrane segments.
a-factor receptor also occurs (Davis et al., 1993). Although some degree of cellular desensitization to α-factor does not require endocytosis of the receptor, internalization and degradation in the vacuole may enhance recovery from the effects of the pheromone (Reneke et al., 1988; Rohrer et al., 1993).

The role of clathrin in endocytosis of the yeast mating pheromone receptors has been studied previously (Payne et al., 1988). In yeast cells carrying a deletion of the clathrin heavy chain gene (chcl Δ), endocytosis of α-factor was not completely blocked, but was reduced two- to threefold. The importance of clathrin in this process was not clear, however, because the defective uptake could be attributed to the slow growth rates and morphological abnormalities of chcl Δ cells.

Here we report analysis of endocytosis in strains expressing a temperature-sensitive allele of the S. cerevisiae clathrin heavy chain gene (chcl-ts) (Munn et al., 1991; Seeger and Payne, 1992a,b). The chcl-ts allele provides a more direct means to test the involvement of clathrin in endocytosis since, in cells that harbor this allele, clathrin function is perturbed immediately while cell growth continues at normal rates for 1.5–2 h (Seeger and Payne, 1992a,b). The endocytosis assays applied to the mutant cells have been extended to include a newly developed method to monitor uptake of the α-factor pheromone receptor directly (Davis et al., 1993). Characterization of the effects of chcl-ts on endocytosis of wild type and mutant forms of the a-factor receptor has allowed us to investigate the role of clathrin in both constitutive and pheromone-stimulated uptake. We find that shifting chcl-ts cells to the nonpermissive temperature results in an immediate, reversible but incomplete block in endocytosis of mating pheromone receptors. The loss of clathrin function in chcl-ts cells affects both constitutive and pheromone-stimulated uptake. In all cases, the endocytosis defects occur long before the cells exhibit growth anomalies. Our results argue that clathrin acts at the plasma membrane to selectively internalize the 7-TMS pheromone receptors.

**Materials and Methods**

**Materials**

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

**Strains, Media, and Genetic Methods**

The genotypes of the strains used in this work are shown in Table 1.

YP medium is 1 % Bacto-Yeast Extract, 2 % Bactopeptone (Difco Laboratories, Inc., Detroit, MI). YPD medium is YP with 2 % dextrose. SD medium is 0.67 % yeast nitrogen base without amino acids (Difco Laboratories, Inc.), with 2 % dextrose. SG medium is 0.67 % yeast nitrogen base without amino acids with 2 % galactose. As needed, nutritional supplements were added to SD medium as described by Sherman et al. (1974). SDYE medium is SD medium with 0.2 % Bacto-Yeast extract. SRYE medium contains 2 % raffinose instead of glucose. DNA transformations were performed by the lithium acetate procedure (Ito et al., 1982).

YCpehcl-521TRP (Seeger and Payne, 1992a) carries chcl-521, a temperature-sensitive allele of CHCL, which was generated by hydroxylamine mutagenesis. The 8.4-kb BamHI to SalI fragment from YCpehcl-521TRP was inserted into Ylp5e (Struhl et al., 1979) to generate Ylpchcl-521. The 5' end of chcl-521 was then deleted by treating the Ylpchcl-521 with Clal and then circularizing the cleaved plasmid by ligation to generate Ypchcl-521ACla.

GPY 400 and 401 were generated by two consecutive DNA transformations. First, GPY 74-2IC was transformed with YCpehcl-521TRP. A resulting Trp + transformant was then transformed with a linearized version of pchcl-A1::LEU2 (Payne et al., 1987). Leu + transformants were screened for temperature-sensitive growth. GPY400 exhibits temperature-sensitive growth and GPY401 exhibits wild-type growth rates at all temperatures tested. The structure of the plasmid in GPY401 is inferred from the genotype and has not been physically tested. GPY418 was constructed by "Pop-in/Pop-out" replacement procedure as described by Rothstein (1991). Briefly, GPY100a was transformed with Ylpchcl-521ACla linearized with XbaI to target integration to the chromosomal CHCL locus. Ura + transformants were tested for temperature-sensitive growth. A temperature-sensitive transformant was then plated on medium containing 5-fluoroorotic (5-FOA) acid to select for cells where homologous recombination had taken place between the duplicated CHCL sequences. 5-FOA-resistant colonies were tested for temperature-sensitive growth to identify cells where recombination had resulted in replacement of the wild-type sequences with the sequences carrying the temperature-sensitive mutations. To obtain GPY449, the PEP4 gene was disrupted in GPY100a using pTS17 (pep4::LEU2) (a gift from Tom Stevens, University of Oregon, Eugene, Oregon).

| Strain       | Genotype                        | Source                  |
|--------------|---------------------------------|-------------------------|
| GPY74-2IC    | MATa leu2-3,112 his4 or 6 trp1-289 ura3-52 pep4::URA3 prb1 sst1-3 | This study              |
| GPY400       | YCpehcl-521TRP: pep4::LEU2 chcl::LEU2 | This study              |
| GPY400       | MATa leu2-3,112 his4 or 6 trp1-289 pep4::URA3 ura3-52 prb1 sst1-3 | This study              |
| GPY423       | MATa leu2-3,112 his4 or 6 trp1-289 pep4::URA3 ura3-52 prb1 sst1-3 chcl::LEU2 | This study              |
| GPY1100a     | MATalpha leu2-3,112 ura3-52 his4-519 trp1 can1 | This study              |
| GPY1100b     | MATalpha leu2-3,112 ura3-52 his4-519 trp1 can1 chcl-521 | This study              |
| GPY418       | MATalpha leu2-3,112 ura3-52 his4-519 trp1 can1 chcl-521 | This study              |
| GPY449       | MATalpha pep4::LEU2 leu2-3,112 ura3-52 his4-519 trp1 can1 | This study              |
| GPY731       | MATalpha pep4::LEU2 leu2-3,112 ura3-52 his4-519 trp1 can1 pSL1922 | This study              |
| GPY735       | MATalpha leu2-3,112 ura3-52 his4-519 trp1 can1 chcl-521 pSL1922 | This study              |
| SM1581       | MATa leu2 ura3 his4 trp1 can1 pSM219 | This study              |
Oregon). GPY419 was generated by disrupting PEP4 in GPY418 using pSL1922 expresses a truncated form of the α-factor receptor which lacks the carboxy-terminal 105 amino acids under the control of the GAL1 promoter (Davis et al., 1993). The plasmid was introduced into GPY449 and GPY418 to yield GPY731 and GPY735, respectively. SM1581 contains pSM219, a multicopy plasmid carrying Mfa1 (a gift from Dr. Susan Michaelis, Johns Hopkins University School of Medicine, Baltimore, MD).

**Production and Purification of 35S-labeled α-factor**

Production and purification of 35S-labeled α-factor was carried out as described by Blumer et al. (1988) using an α-factor overproducing strain harboring the plasmid pDA6300 (a gift from Dr. Jeremy Thorner, University of California, Berkeley, CA). The purified 35S-labeled α-factor comigrated with synthetic cold α-factor during reverse-phase HPLC chromatography. The labeled α-factor bound to MATA cells but not to MATa cells, and this binding was prevented by the addition of excess synthetic cold α-factor. The specific activity, determined by bioassay using synthetic α-factor as standard, was 50-100 Ci/mmol.

**Assay for Internalization of 35S-labeled α-factor and Reversibility of the Internalization Defect**

The assay for binding and internalization of α-factor is a modification of published procedures (Dulic et al., 1991). CHCI (GPY400), chcl-ts (GPY400), and chclA (GPY423) were grown to mid-log phase in YPD medium at 24°C. Cells were collected by centrifugation and resuspended at 1-2 × 10^7 cells/ml in ice cold KPO4 buffer (50 mM KPO4, pH 6, containing 1% BSA, 1 mM PMSF, and 10 mM p-tosyl-L-arginine methyl ester (TAME)). 35S-labeled α-factor was added at 1-2 × 10^7 cpm/10^7 cells and allowed to bind to cells on ice for 30 min. Following the incubation, the cells were sedimented by centrifugation and the supernatant was aspirated to remove unbound α-factor. The cell pellet was resuspended in an equal volume of ice cold KPO4 buffer and 100-μl aliquots were then incubated at 24 or 37°C for various times (the preshift time). Under these conditions α-factor remains bound to the cells but is not internalized (Chvatchko et al., 1986; Tan, E, unpublished observations). Glucose was then added to 2% to stimulate internalization, and the incubation at 24 or 37°C continued for 30 min. At this point, cells were diluted in ice cold 50 mM sodium citrate, pH 1.1, and incubated for 15 min to remove surface bound α-factor. The low pH-treated cells were collected by vacuum filtration on a Whatman GF/A filter disc (Whatman Inc., Clifton, NJ). The filters were washed with 2 × 5 ml ice cold 50 mM KPO4, pH 6, and 10 mM NaF. After washing the samples once with ice cold pronase buffer containing 1 mM EDTA and a protease inhibitor cocktail (1 mM PMSF, 1 mM benzamidine-HCl, 1 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 μg/ml chymostatin, 1 μg/ml antipain, 1 μg/ml antipain, diluted from a 1,000 x stock solution in DMSO). The cell pellet was lysed with glass beads in 50 μl 8 M urea, 5% SDS, 40 mM Tris–HCl, pH 6.8, and 0.1 mM EDTA (Blumer et al., 1988). The receptor was then immunoprecipitated from the lysate as described above. Samples were analyzed on 11% SDS-polyacrylamide gels. The amount of receptor was quantified by scanning densitometry of the autoradiographs using a LKB Ultrascan XL (Pharmacia Diagnostics, Inc., Fairfield, NJ).

For labeling and immunoprecipitation of the truncated α-factor receptor, CHCI pep4Δ (GPY731) and chcl-ts (GPY735) cells were grown in SKYE media at 24°C and then washed and resuspended in SK media at 2 × 10^7 cells/ml. After a 5-min incubation at 24°C, the cells were labeled as described above for 45 min. The labeling was terminated by addition of unlabeled methionine and cysteine, yeast extract, and glucose to final concentrations of 0.006, 0.2, and 3%, respectively. The cells were incubated for another hour at 24°C to accumulate the labeled truncated receptors at the plasma membrane. The cells were then placed at 37°C for 5 min prior to addition of an equal volume of exhausted YPD media from a stationary culture of SM1581 cells which overproduce α-factor. This media was supplemented with unlabeled cysteine and methionine, yeast extract, and glucose as described above and prewarmed to 37°C. A control sample received an equal volume of the same media from a stationary culture of GPY1100a cells which do not produce α-factor. At various time intervals after addition of α-factor, 1 × 10^7 cells were removed, pronase treated, lysed, and the truncated α-factor receptor immunoprecipitated as described above, except that 1 × 10^7 SM1581 cells were added as carrier to the samples prior to the removal of pronase.

**Results**

**Uptake of α-factor in chcl-ts Cells Is Rapidly Impaired After Shift to the Nonpermissive Temperature**

We have assessed the role of clathrin in internalization of α-factor receptors by measuring receptor-mediated uptake of radiolabeled pheromone (Dulic et al., 1991) in chcl-ts cells shifted to the nonpermissive temperature (Fig. 1A). Labeled α-factor was bound to either chcl-ts cells or congenic wild-type (CHCI) cells at 0°C in the absence of glucose. Following removal of unbound pheromone, the cells were shifted to the permissive (24°C) or nonpermissive temperature (37°C) for various periods of time (preshift) in the absence of glucose. Without glucose, the cells lack sufficient energy stores for intracellular membrane transport processes including endocytosis (Chvatchko et al., 1986). Thus, when the preshift protocol is carried out at the nonpermissive temperature, it provides a means to eliminate tempera-
The a-factor Internalization Defect in chcl-ts Cells Is Reversible

To further evaluate the endocytosis defect in chcl-ts cells, we determined whether internalization could be reestablished by returning the cells to the permissive temperature. Following a-factor binding and a 5-min preshift, mutant and wild-type cells were incubated at 24 or 37°C for 30 min as described above and a portion was tested for endocytosis. At this point, the chcl-ts cells incubated at 37°C indicates that a-factor internalization can occur in the absence of functional clathrin heavy chain, albeit with reduced efficiency.

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Figure 2. Reversibility of the α-factor internalization defect in chcl-ts cells. Same as Fig. 1, except that cells were in YP media with 50 mM KPO4, pH 6. All samples were preshifted for 5 min and allowed to internalize α-factor for 30 min. Samples at 37°C were then either harvested and analyzed as described in the legend to Fig. 1 (37°C), transferred to 24°C for another 30-min incubation with a second addition of glucose (37→24), or maintained at 37°C for another 30 min with a second addition of glucose (37→37). Results are the mean ± standard error for three experiments.

37°C bars). The remainder of the 37°C-cell samples were divided; one part was incubated at 37°C while the second part was shifted to 24°C for an additional 30 min before measuring internalization. When the chcl-ts cells were shifted to 24°C (Fig. 2, 37→24 bars), the level of α-factor internalization reached 71% of the wild-type level. In contrast, the slower clathrin-independent internalization in the chcl-ts cells maintained at 37°C resulted in only 52% uptake relative to CHCI cells (Fig. 2, 37→37 bars). The substantial recovery of endocytosis in chcl-ts cells returned to 24°C suggests that the endocytosis defect is due to a reversible, temperature-induced impairment of clathrin heavy chain function.

The Rate of α-factor Receptor Uptake Is Reduced in chcl-ts Cells

Recent studies on the biosynthesis of the α-factor receptor in MATα cells allowed us to examine whether clathrin plays a role in endocytosis of this receptor (Davis et al., 1993). The transport itinerary of the receptor was examined using pulse-chase regimens followed by immunoprecipitation or immunoblotting. With these approaches, it was possible to monitor the receptor directly in the absence of radiolabeled pheromone. The results indicated that newly synthesized α-factor receptors (and α-factor receptors) in wild-type cells are transported to the cell surface and then internalized, even in the absence of pheromone, and delivered to the vacuole where they are degraded.

Since degradation of α-factor receptors depends on delivery to the vacuole and occurs in the absence of pheromone, turnover of newly synthesized receptor can be used as a convenient diagnostic assay for constitutive endocytosis of the receptor (Davis et al., 1993). To follow turnover of α-factor receptors, chcl-ts and CHCI cells were labeled with [35S]-methionine and cysteine for 30 min at 24°C. Labeling was quenched by addition of excess unlabeled amino acids and then one half of each sample was transferred to 37°C while the other half was maintained at 24°C. At time intervals, a-factor receptor was immunoprecipitated with polyclonal antiserum specific for the receptor's carboxy-terminal cytoplasmic domain (Clark et al., 1988). Precipitated receptor was visualized by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 3). At 24°C, the rate of α-factor receptor turnover was identical in mutant and wild-type cells (Fig. 3, lanes 1–4). At 37°C, the receptors in the chcl-ts cells were clearly more stable than those in the CHCI cells (Fig. 3, lanes 5–7). This result is consistent with reduced endocytosis of the receptors in the mutant cells. However, at the later times, the receptor was degraded in mutant cells (Fig. 3, compare lanes 5 and 7) suggesting that, like α-factor endocytosis, internalization of the α-factor receptor can occur in the absence of clathrin. We observed a similarly delayed turnover of α-factor receptor in chclΔ cells (Tan, P., and G. Payne, unpublished observations). The properties of the α-factor receptor are not universally shared with other plasma membrane proteins; the plasma membrane ATPase remained stable in both strains at both temperatures over the time course of the experiment shown in Fig. 3 (Tan, P., and G. Payne, unpublished observations). Furthermore, in the absence of pheromone, a truncated version of the α-factor receptor lacking the carboxy-terminal 105 amino acids (Davis et al., 1993) remains at the plasma membrane as measured by its susceptibility to exogenous proteases (see below).

If stabilization of α-factor receptors in chcl-ts cells reflects defective endocytosis then the receptors should accumulate at the cell surface. Accordingly, we used the 24°C pulse, 37°C chase protocol described above and determined the sensitivity of receptors to exogenously added pronase. To obtain a more synchronous population of radiolabeled receptors, the labeling in these experiments was carried out for only 5 min. Since the receptors are unstable even in the absence of exogenous protease (see Fig. 3), we introduced the pep4 mutation into both CHCI and chcl-ts cells. The pep4 mutation eliminates activation of vacuolar proteases (Hemmings et al., 1981), and consequently prevents degradation of receptors that are delivered to the vacuole (Davis et al., 1993). After the 5-min labeling period at 24°C, pronase treatment did not affect the levels of receptors in either cell type.

Figure 3. Stability of newly synthesized α-factor receptor in congenic CHCI (GPY1100a) and chcl-ts (GPY418) cells. Cells were labeled at 24°C and chased at 24 or 37°C for the indicated times, followed by immunoprecipitation of the receptor as described in Materials and Methods. Results are from one experiment and have been reproduced in two other experiments.
Results are from one experiment and are representative of a total of three experiments. (C) Time course of the pronase sensitivity of intact receptors from A and B as measured by scanning densitometry and calculated as the percent of receptor degraded after pronase treatment relative to mock treated.

Figure 4. Pronase sensitivity of newly synthesized α-factor receptor in congenic (A) CHC1 pep4Δ (GPY449) and (B) chcl-ts pep4Δ (GPY419) cells. Cells were labeled at 24°C for 5 min and then shifted to 37°C for the indicated chase times. At each time point cells were harvested and either treated with pronase (+) or mock-treated (−) prior to immunoprecipitation of the receptor as described in Materials and Methods. The arrows mark pronase-resistant carboxy-terminal receptor fragments. The portions of the gels containing the pronase-resistant fragments were exposed for longer periods of time to facilitate visualization of the fragments. Results are from one experiment and are representative of a total of three experiments. (C) Time course of the pronase sensitivity of intact receptors from A and B as measured by scanning densitometry and calculated as the percent of receptor degraded after pronase treatment relative to mock treated.

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Internalize a-factor Receptor Is Delivered to the Vacuole at Similar Rates in chcl-ts and Wild-type Cells

The reduced rate of pheromone receptor uptake in chcl-ts cells shifted to 37°C suggests that clathrin acts directly at the plasma membrane to facilitate internalization. An alternative interpretation is that clathrin acts at a subsequent stage along the endocytic pathway. In this scenario, severe inhibition of a later stage of endocytosis in chcl-ts cells at 37°C would lead to an indirect delay in transport from the cell surface. The PEP4-dependent turnover of the a-factor receptor shown in Fig. 3 suggests that transport of receptors to the vacuole via the endocytic pathway is not completely blocked in chcl-ts cells. To examine the effects of chcl-ts on later endocytic stages more directly, we used the pulse-chase regimen to monitor a-factor receptor uptake in chcl-ts and CHC1 strains carrying the wild-type PEP4 allele. Because receptors that reach the vacuole are degraded in these strains, pronase-resistant receptors detected at time points after the receptors arrive at the cell surface (10–15 min of chase, see Fig. 4) should represent molecules that have left the cell surface but not yet gained access to vacuolar proteases. Therefore, we reasoned that a strong inhibition of receptor transport to the vacuole at stages subsequent to internalization in chcl-ts cells should result in accumulation of pronase-resistant receptors at later time points when compared to wild-type cells.

Fig. 5 presents the results of a pulse-chase experiment where cells were metabolically labeled for 5 min, then harvested at the designated time intervals and subjected to pronase treatment (note that the chase times for the two strains are different). Consistent with the measurements of a-factor receptor turnover shown in Fig. 3, the PEP4-dependent degradation of receptor in CHC1 cells not treated with pronase (Fig. 5 A, odd-numbered lanes) occurred more rapidly at 37°C than in chcl-ts cells (Fig. 5 B, odd-numbered lanes). By the 30-min time point, 30% of the labeled receptors remained in CHC1 cells (Fig. 5 A, lane 7) compared to 80% in the mutant cells (Fig. 5 B, lane 5). Pronase treatment of the cells revealed the internal pool of receptors (Fig. 5, even-numbered lanes). Similar to the results in Fig. 4, the majority of the receptors reached the cell surface and became pronase sensitive by 10–15 min (Fig. 5, A and B, lanes 3 and 4). Importantly, at later points the levels of pronase-resistant receptors in the two strains were comparable (Fig. 5, A and B, lanes 5–10). For example, after 30 min at the non-permissive temperature, 30% of the receptors were pronase resistant in both chcl-ts and wild-type cells. Thus, the chcl-ts allele does not cause conspicuous accumulation of receptors in an intracellular pre-vacuolar compartment. These results argue that the partial endocytic defect in chcl-ts cells cannot be attributed to a more complete block at a transport step subsequent to the internalization of cell-surface a-factor receptors.

The Rate of Ligand-induced Uptake of a Truncated a-factor Receptor Is Reduced in chcl-ts Cells

Recently, a truncated a-factor receptor was engineered which is missing the carboxy-terminal 105-amino acid residues (Davis et al., 1993). This mutant receptor (ste3-Δ365) remains at the cell surface in the absence a-factor but is internalized upon addition of the pheromone. The properties of the truncated receptor allowed us to monitor the role of clathrin in pheromone-induced endocytosis.

We assayed ligand-induced endocytosis using CHC1 pep4Δ and chcl-ts cells harboring a plasmid which expresses ste3-Δ365 under the control of the inducible GAL promoter (Davis et al., 1993). Expression of the mutant receptor was induced at 24°C by the addition of galactose. Concurrent with growth in galactose, cells were labeled for 45 min after which time glucose was added to repress receptor gene expression, and excess unlabeled amino acids were added to quench the labeling. Cells were incubated under these conditions for an additional hour in order to ensure that all receptors reached the cell surface. Following the 1-h incubation at 24°C in glucose medium, the cells were transferred to 37°C for 5 min prior to addition of media containing a-fac tor. Samples were removed various times after addition of pheromone, subjected to pronase, and immunoprecipitated as already described.

At the time of a-factor addition, most of the mutant receptor was at the surface in both cell types as shown by the virtually complete pronase-sensitivity of the intact receptor (Fig. 6, A and B, lanes / and 2) and presence of a pronase-resistant fragment (arrows). However, after addition of a-factor a dramatic difference in receptor pronase sensitivity between CHC1 and chcl-ts cells was detected. In CHC1 cells, the receptors acquired complete pronase resistance by 30 min (Fig. 6 A, lanes 3–12) while in chcl-ts cells, significant amounts of the intact mutant receptor remain pronase sensitive for at least 60 min (Fig. 6 B, lanes 3–12). The difference between levels of pronase-resistant receptor in mutant and wild-type cells is detectable within 5 min after addition of a-fac tor (compare lanes 3 and 4 in Fig. 6, A and B) which
treated samples the amount of resistant fragments (arrowheads) generally varied in reciprocal fashion to the amount of intact receptor.

Densitometric analysis of the data in Fig. 6, A and B is presented in Fig. 6 C. In comparison to CHC1 cells, internalization of mutant receptors in chcl-ts cells proceeds at a reduced rate after a slight lag. Half-times for the ligand-induced internalization are approximately 8 min for CHC1 cells and 20 min for chcl-ts cells. This 2.5-fold reduction in the rate of internalization is in agreement with the previous results, and argues that clathrin is also required to facilitate ligand-induced endocytosis of this truncated receptor. The rate of wild-type receptor uptake in the presence of pheromone was similarly affected by chcl-ts (data not shown).

Discussion

The role of clathrin in endocytosis of mating pheromone receptors has been examined by monitoring uptake in cells expressing a temperature-sensitive allele of clathrin heavy chain. Upon shift to the nonpermissive temperature, a dramatic and immediate reduction in endocytosis of α-factor receptor and α-factor ensued. In prior work, internalization of α-factor was shown to be reduced in chclA cells to 35-50% of wild-type levels (Payne et al., 1988). Because chclA cells grow slowly and form multi-cell aggregates, the partial endocytosis defect was not interpreted as a direct consequence of a loss of clathrin function. Two findings presented here argue that eliminating clathrin function has a direct effect on the first step of the endocytic pathway, removal of pheromone receptors from the cell surface. First, at 37°C a defect in internalization was apparent in chcl-ts cells within 2 min after endocytosis was initiated by provision of glucose. Thus, the endocytic defect occurs significantly faster than the half-time for α-factor uptake (5-7 min). This observation makes it unlikely that the uptake defect in chcl-ts cells is due to effects on later endocytic steps such as recycling of endocytic machinery components from endosomes to the cell surface after a round of internalization. Second, the efficient degradation of internalized α-factor receptors in chcl-ts cells also provides evidence that the partial internalization defect cannot be due to a block in transport at a subsequent step in the endocytic pathway. These results offer genetic evidence that clathrin acts directly at the plasma membrane to facilitate endocytosis of the pheromone receptors, and thereby represent the first in vivo demonstration of clathrin-mediated uptake of 7-TMS receptors. It should be noted that our results do not exclude the possibility that clathrin also facilitates later endocytic steps.

The immediate effect of the chcl-ts mutation on both constitutive and pheromone-stimulated endocytosis provides evidence that clathrin plays a role in both processes. Our results are consistent with a model in which clathrin facilitates pheromone receptor endocytosis by clustering the receptors at plasma membrane sites undergoing vesiculation. We envision that receptors are collected at these sites through interactions of the receptor cytoplasmic domains and components of the clathrin coats. Based on our findings, we suggest that membrane vesiculation still proceeds in the absence of clathrin but receptors are not rapidly incorporated into the newly forming vesicles, thereby reducing the rate of receptor up-
take. Immunocytochemical studies will be necessary to test this interpretation and confirm the clustering of pheromone receptors in clathrin-coated pits.

Consistent with our hypothesis, the cytoplasmic domains of both the α-factor and α-factor receptors are important for internalization. In the case of the α-factor receptor, a small region in the carboxy-terminal cytoplasmic domain has been identified which plays a key role in pheromone-stimulated endocytosis (Rohrer et al., 1993). This sequence does not display the characteristics of sequences in plasma membrane proteins which mediate clustering in clathrin-coated pits in mammalian cells (Chen et al., 1990; Collawn et al., 1990; Kistakiss et al., 1990; Letourneau and Klausner, 1992; Miettinen et al., 1992). As suggested by Rohrer et al. (1993), this difference may indicate that the α-factor receptor sequences play a role in regulating endocytosis in response to pheromone, or that the sequences represent a new motif capable of interacting with clathrin coats. In the case of the α-factor receptor, a 105-amino acid truncation (ste3-A365) of the cytoplasmic tail results in a receptor that remains at the cell surface unless pheromone is present (Davis et al., 1993). In the context of our model, the truncation may cause an altered structure which occludes internalization signals unless pheromone is bound. Alternatively, there may be both pheromone-dependent and -independent signals in the wild-type receptor and the Δ365 mutation may remove the pheromone-independent signal. Although this possibility has not been addressed in the case of the α-factor receptor, there may be multiple internalization signals in the α-factor receptor (Rohrer et al., 1993).

Endocytosis of pheromone or pheromone receptors continued at the nonpermissive temperature in chcl-ts cells at significant rates, with half-times of 20–30 min. This internalization is most likely not due to residual activity of the temperature-sensitive clathrin heavy chain at 37°C since the uptake rate is commensurate with that observed in chelΔ cells. Thus, in cells devoid of functional clathrin, receptors are still internalized, but with reduced rates compared to wild-type cells. We cannot distinguish at present between the possibility that residual uptake occurs through a second clathrin-independent pathway, perhaps analogous to that described in mammalian cells (Hansen et al., 1991, and references therein), or the possibility that other elements of clathrin coats are still capable of limited vesiculation in the absence of clathrin heavy chain.

**Possible Roles for Clathrin-mediated Pheromone Receptor Endocytosis**

Why has a mechanism evolved to enhance endocytosis of yeast pheromone receptors? By analogy to down-regulation of mammalian 7-TMS receptors, uptake could play a role in clearing the surface of receptor-bound pheromone and contribute to the recovery of the responding cell to the effects of the pheromone. In addition, perhaps constitutive endocytosis is necessary during the process of mating-type switching. Homothallic yeast strains are able to switch mating types through a gene conversion process which replaces the master regulatory sequences at the mating-type (MAT) locus (Herskowitz, 1988). Mating-type switching in these strains occurs at high frequency. The gene conversion occurs prior to replication of MAT during the cell cycle, and by the time cytokinesis occurs the two resulting cells have acquired the phenotypic properties of the new cell type. Clathrin-mediated endocytosis may play a role in constantly clearing the surface of pheromone receptors so that, after a mating-type switch, the old receptors (which are no longer expressed) can be replaced by newly synthesized receptors for the opposite mating-type pheromone.

Hartwell and his colleagues have defined an early step in the mating process which involves orienting towards the mating partner (Jackson and Hartwell, 1990a,b; Jackson et al., 1991). If several partners are available, the cell producing the highest level of pheromone is chosen, and pheromone receptors become concentrated at the region of the cell surface facing the chosen partner. Clathrin-deficient mutants are partly defective in this process of mating partner discrimination (Jackson et al., 1991). Our results suggest that this defect could be due to reduced endocytosis of the pheromone receptor.

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