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 **a**-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 **a**-factor receptor was delivered to the vacuole at comparable rates in *chcl-ts* and wild-type cells at the nonpermissive temperature. Clathrin heavy chain was also required for maximal uptake of a mutant **a**-factor receptor which is dependent on pheromone for internalization. In the presence of **a**-factor, the internalization rate of the mutant receptor in *chcl-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 (Pearse 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; Ktistakis et al., 1990; Miettinen et al., 1992).

A structurally distinct class of cell surface receptors is characterized by seven transmembrane segments (7-TMS)[1] 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 Koblika, 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, *MAT*α and *MAT***a**, each secrete peptide pheromones, α-factor and **a**-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; Jenness and Spatrick, 1986; Singer and Riezman, 1990; Davis et al., 1993) and recently it has been shown that endocytosis of the

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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 (*chc1*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 *chc1*Δ cells.

Here we report analysis of endocytosis in strains expressing a temperature-sensitive allele of the *S. cerevisiae* clathrin heavy chain gene (*chc1-ts*) (Munn et al., 1991; Seeger and Payne, 1992*a*,*b*). The *chc1-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, 1992*a*,*b*). The endocytosis assays applied to the mutant cells have been extended to include a newly developed method to monitor uptake of the a-factor pheromone receptor directly (Davis et al., 1993). Characterization of the effects of *chc1-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 *chc1-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 *chc1-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 I.

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).

YCpchc1-521TRP (Seeger and Payne, 1992*a*) carries *chc1-521*, a temperature-sensitive allele of *CHC1*, which was generated by hydroxylamine mutagenesis. The 8.4-kb BamHI to SalI fragment from YCpchc1-521TRP was inserted into YIp5 (Struhl et al., 1979) to generate YIpchc1-521. The 5′ end of *chc1-521* was then deleted by treating the YIpchc1-521 with ClaI and then circularizing the cleaved plasmid by ligation to generate YIpchc1-521ΔCla.

GPY 400 and 401 were generated by two consecutive DNA transformations. First, GPY 74-21C was transformed with YCpchc1-521TRP. A resulting Trp^+ transformant was then transformed with a linearized version of pchc1-Δ10::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, GPY1100α was transformed with YIpchc1-521ΔCla linearized with XbaI to target integration to the chromosomal *CHC1* 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 *CHC1* 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 GPY1100α using pTS17 (*pep4::LEU2*) (a gift from Tom Stevens, University of Oregon, Eugene,

Table I. Yeast Strains Used in This Study

Strain	Genotype	Source
GPY74-21C	*MATa leu2-3,112 his4 or 6 trp1-289 ura3-52 pep4::URA3 prb1 sst1-3*	This study
GPY401	*MATa leu2-3,112 his4 or 6 trp1-289 pep4::URA3 ura3-52 prb1 sst1-3* YCpchc1-521TRP::chc1::LEU2	This study
GPY400	*MATa leu2-3,112-his4 or 6 trp1-289 pep4::URA3 ura3-52 prb1 sst1-3 chc1::LEU2* YCpchc1-521TRP	This study
GPY423	GPY400 cured of YCpchc1-521TRP	This study
GPY1100α	*MATalpha leu2-3,112 ura3-52 his4-519 trp1 can1*	Payne and Schekman, 1989
GPY1100a	*MATa leu2-3,112 ura3-52 his4-519 trp1 can1*	Payne and Schekman, 1989
GPY418	*MATalpha leu2-3,112 ura3-52 his4-519 trp1 can1 chc1-521*	This study
GPY419	*MATalpha pep4::URA3 leu2-3,112 ura3-52 his4-519 trp1 can1 chc1-521*	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 chc1-521* pSL1922	This study
SM1581	*MATa leu2 ura3 his4 trp1 can1* pSM219	Susan Michaelis (Johns Hopkins University School of Medicine, Baltimore, MD)

Oregon). GPY419 was generated by disrupting *PEP4* in GPY418 using pTS15 (*pep4::URA3*) (a gift from Tom Stevens).

pSL1922 expresses a truncated form of the a-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 *MFal* (a gift from Dr. Susan Michaelis, Johns Hopkins University School of Medicine, Baltimore, MD).

Production and Purification of ^{35}S-labeled α-factor

Production and purification of ^{35}S-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 ^{35}S-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 *MATα* 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 ^{35}S-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). *CHC1* (GPY401), *chc1-ts* (GPY400), and *chc1*Δ (GPY423) were grown to mid-log phase in YPD medium at 24°C. Cells were collected by centrifugation and resuspended at 1–2 × 10^9 cells/ml in ice cold KPO_4 buffer (50 mM KPO_4, pH 6, containing 1% BSA, 1 mM PMSF, and 10 mM *p*-tosyl-L-arginine methyl ester [TAME]). ^{35}S-labeled α-factor was added at 1–2×10^5 cpm/10^9 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 KPO_4 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, P., 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 KPO_4, pH 6, and internalized α-factor was measured by scintillation counting of the filters. Total bound α-factor was assessed by washing and filtering cells in ice cold 50 mM KPO_4, pH 6, after the binding step. Typically 30–90% of the radioactivity bound to the cells and 80–90% of the bound radioactivity was internalized after 1 h at 24°C.

Time courses for internalization of labeled α-factor at 37°C were conducted after a 5-min preshift at 37°C as stated above. Internalization was initiated with addition of 5% glucose and terminated at various times up to 20 min. Identical results were obtained in experiments using 2% glucose.

For measuring the reversibility of the temperature-sensitive α-factor internalization defect, *CHC1* and *chc1-ts* cells were allowed to bind and internalize α-factor for 30 min after a 5-min preshift as described, except in YP media with 50 mM KPO_4 adjusted to pH 6. Samples at 37°C were then either harvested and analyzed as described, transferred to 24°C for another 30-min incubation with a second addition of glucose, or maintained at 37°C for another 30 min with a second addition of glucose before being harvested and analyzed as described.

Metabolic Labeling, Immunoprecipitation, and Protease Sensitivity of the a-factor Receptor

CHC1 (GPY1100α), *chc1-ts* (GPY418), and *CHC1 pep4*Δ (GPY449) cells (2 × 10^7 cells/ml) were labeled in supplemented SD medium at 24°C for 30 min with 100 μCi/ml *trans*-^{35}S-label (ICN Biomedicals, Irvine, CA). Labeling was terminated by adding an equal volume of SD containing 4% yeast extract and 0.06% unlabeled cysteine and methionine (chase), and the samples were incubated at 24 or 37°C for various times. The chase was terminated by removing 2 × 10^6 cells and diluting the aliquot into 1 ml ice cold spheroplast buffer (50 mM Tris-HCl, pH 7.5, 1.4 M sorbitol, 10 mM NaN_3, and 40 mM β-mercaptoethanol). Cells were then converted to spheroplasts with 50 U oxalyticase (Enzymogenetics, Corvalis, OR) for 15 min at 30°C and then lysed in 50 μl 8 M urea, 5% SDS, 40 mM Tris-HCl, pH 6.8, and 0.1 mM EDTA (Blummer et al., 1988). After incubation at 70°C for 5 min, the receptor was immunoprecipitated from the lysate (Seeger and Payne, 1992*b*) using 5 μl 348-la anti-a-factor receptor antiserum (Clark et al., 1988). The precipitate was collected with protein A-Sepharose, solubilized in Laemmli sample buffer (Laemmli, 1970) at 70°C for 5 min, and subjected to electrophoresis through a 10% SDS-polyacrylamide gel. The gel was fixed, incubated in Entensify aqueous fluor (New England Nuclear, Boston, MA), dried, and exposed to X-ray film.

For protease sensitivity of newly synthesized a-factor receptor, *CHC1* (GPY1100α), *CHC1 pep4*Δ (GPY449), *chc1-ts* (GPY418), and *chc1-ts pep4*Δ cells (GPY419) were labeled at 24°C for 5 min and then subjected to the chase regimen described above, except the chase was terminated by transferring 1 × 10^7 cells on ice into tubes containing 10 mM NaN_3 and 10 mM NaF. After washing the samples once with ice cold pronase buffer (spheroplast buffer with 2 mM $MgCl_2$) the intact cells were divided in half with one part mock-treated and the other part treated with pronase according to Truehart and Fink (1989) with the following modifications. 50 μl of a 30 mg/ml pronase (Calbiochem-Novabiochem, La Jolla, CA) solution or buffer alone was added to the samples in 1 ml of pronase buffer and incubated with agitation at 37°C for 1–1.5 h. Prior to removal of pronase, 2 × 10^7 cells in pronase buffer of strain 1100a, which does not express the a-factor receptor, were added as carrier. The cells were then pelleted and washed twice with 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 aprotinin, and 1 μg/ml antipain, diluted from a 1,000 × 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 Ultroscan XL (Pharmacia Diagnostics, Inc., Fairfield, NJ).

For labeling and immunoprecipitation of the truncated a-factor receptor, *CHC1 pep4*Δ (GPY731) and *chc1-ts* (GPY735) cells were grown in SRYE media at 24°C and then washed once and resuspended in SG 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 a-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 GPY1100α cells which do not produce a-factor. At various time intervals after addition of a-factor, 1 × 10^7 cells were removed, pronase treated, lysed, and the truncated a-factor receptor immunoprecipitated as described above, except that 2 × 10^7 SM1581 cells were added as carrier to the samples prior to the removal of pronase.

Results

Uptake of α-factor in chc1-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 *chc1-ts* cells shifted to the nonpermissive temperature (Fig. 1 *A*). Labeled α-factor was bound to either *chc1-ts* cells or congenic wild-type (*CHC1*) 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-

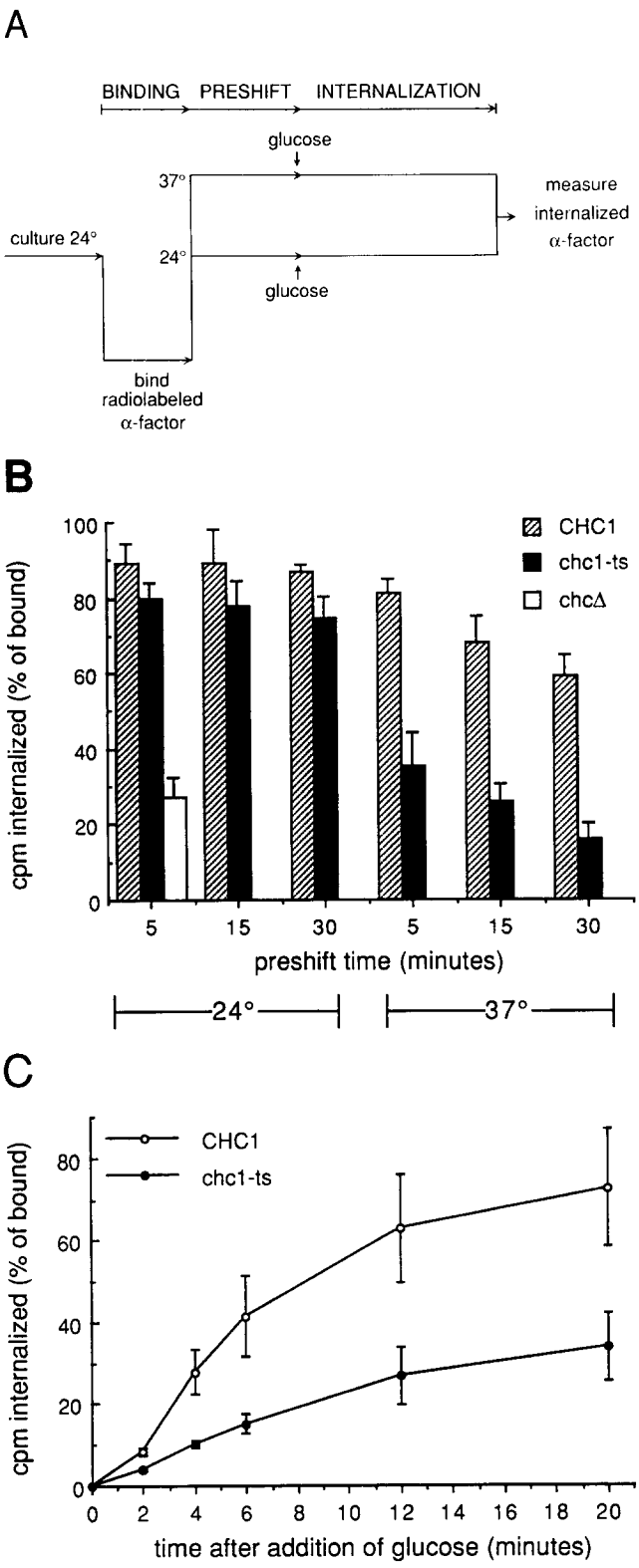

Figure 1. Internalization of radiolabeled α-factor in isogenic *CHC1* (GPY401), *chcl-ts* (GPY400), and *chcl*Δ (GPY423) cells. (*A*) Flow chart of the procedure used to assay internalization of radiolabeled α-factor. Preshift occurs in the absence of an energy source to prevent endocytosis and other membrane transport processes. (*B*) Internalization of α-factor at 24 and 37°C after various preshift times, expressed as the percentage of bound counts. Internalized counts were measured 30 min after initiation of internalization by addition of 2% glucose (see Materials and Methods). For each time point, duplicate samples were analyzed and the results averaged. Data are the mean ± standard error for three experiments. (*C*) Time course of internalization at 37°C after a 5-min preshift for *CHC1* and *chcl-ts* cells. Same as *B*, except that internalization was initiated with addition of 5% glucose and terminated at the indicated times. For each time point, duplicate samples were analyzed and the results averaged. Data are the mean ± standard error for two experiments.

ture-sensitive clathrin heavy chain function in the absence of membrane traffic. Following the preshift, glucose was added and uptake was determined after 30 min by treating the cells with a low pH buffer to remove surface-bound α-factor (Dulic et al., 1991). As shown in Fig. 1 *B*, at 24°C the uptake of α-factor by *chcl-ts* cells (*solid bars*) was virtually the same as wild-type cells (*stippled bars*). In contrast, at 37°C the *chcl-ts* cells displayed an immediate defect in endocytosis; with a 5 min preshift, mutant cell uptake was only 44% of wild-type levels. This level was similar to that observed in *chcl*Δ cells devoid of clathrin heavy chain due to a deletion of *CHC1* (30%; Fig. 1 *B*, *open bar*). After longer preshift times, compared to *CHC1* cells, the ratio of uptake by *chcl-ts* cells remained relatively constant (27% after a 30-min preshift), although both mutant and wild-type strains showed a progressive decline in internalization at the elevated temperature. Similar results have been obtained with another pair of congenic *chcl-ts* and *CHC1* strains. We previously reported that *chcl*Δ cells internalize α-factor at 35–50% of wild-type levels (Payne et al., 1988). Since *chcl*Δ cells grow more slowly than wild-type cells, it was not clear whether the endocytosis defect was a consequence of slow growth. The results shown in Fig. 1 *B* do not support this possibility because the growth rate of *chcl-ts* cells does not decline for at least 90 min following shift to 37°C (Seeger and Payne, 1992*a*).

A time-course of α-factor uptake in cells preshifted to 37°C for 5 min is plotted in Fig. 1 *C*. The temperature-induced endocytosis defect in *chcl-ts* cells was apparent at the first time point (2 min) after addition of glucose. From 2–6 min after addition of glucose, when internalization of α-factor was linear for both cell types, the rate of internalization in *chcl-ts* cells was approximately threefold lower relative to *CHC1* cells. Internalization continued at a slower rate in *chcl-ts* cells throughout the course of the experiment. The immediate onset of the endocytosis defect after shifting *chcl-ts* cells to 37°C suggests that clathrin plays a direct role in facilitating endocytosis of the receptor-bound pheromone. However, the residual endocytosis of α-factor in *chcl-ts* cells incubated at 37°C and in *chcl*Δ cells at 24°C indicates that α-factor internalization can occur in the absence of functional clathrin heavy chain, albeit with reduced efficiency.

The α-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 α-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 internalized 40% of the pheromone compared to wild-type cells (Fig. 2,

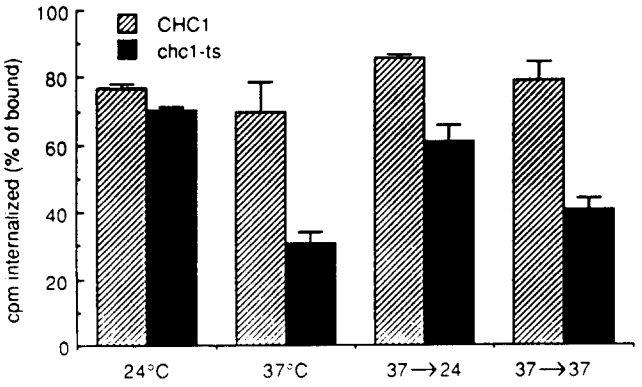

Figure 2. Reversibility of the α-factor internalization defect in *chcl-ts* cells. Same as Fig. 1, except that cells were in YP media with 50 mM KPO_4, pH 6. All samples were preshifted for 5 min and allowed to internalize α-factor for 30 min. Samples at 37°C were then either harvested and analyzed as 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 *CHCl* 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 a-factor Receptor Uptake Is Reduced in chcl-ts Cells

Recent studies on the biosynthesis of the a-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 a-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 a-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 a-factor receptors, *chcl-ts* and *CHCl* cells were labeled with [^{35}S]-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 a-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 *CHCl* 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 a-factor receptor can occur in the absence of clathrin. We observed a similarly delayed turnover of a-factor receptor in *chclΔ* cells (Tan, P., and G. Payne, unpublished observations). The properties of the a-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 a-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 a-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 *CHCl* 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

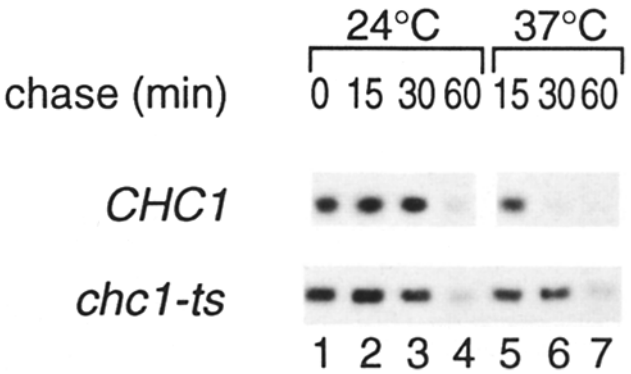

Figure 3. Stability of newly synthesized a-factor receptor in congenic *CHCl* (GPY1100α) and *chcl-ts* (GPY418) cells. Cells were labeled at 24°C and chased at 24 or 37°C for the indicated times, followed by immunoprecipitation of the receptor as described in Materials and Methods. Results are from one experiment and have been reproduced in two other experiments.

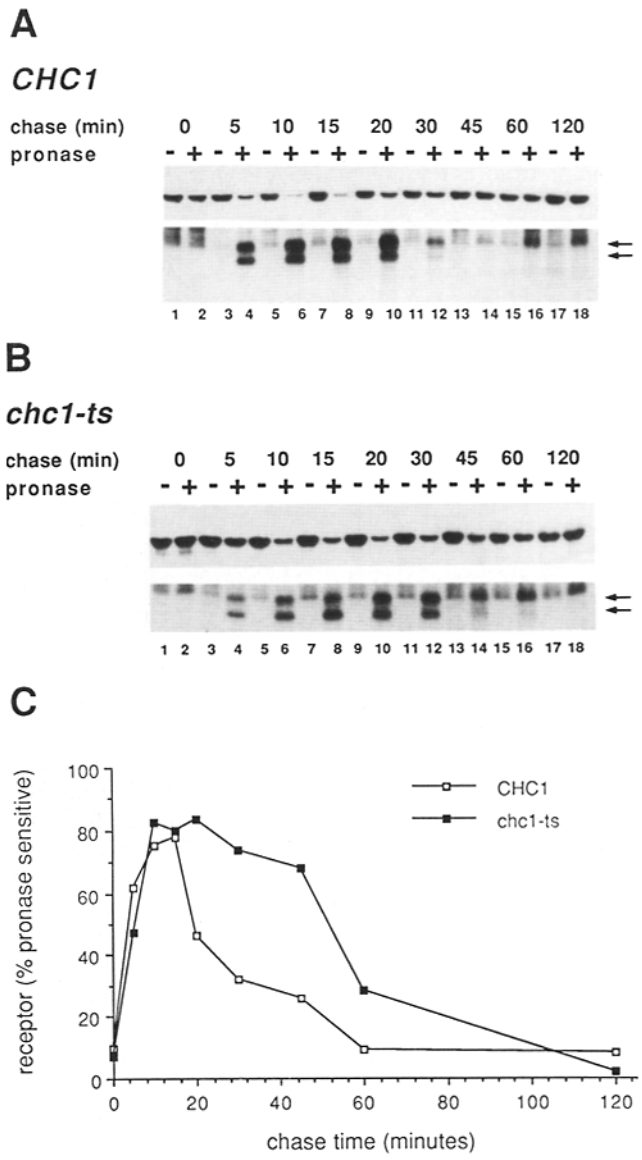

Figure 4. Pronase sensitivity of newly synthesized a-factor receptor in congenic (*A*) *CHCl pep4Δ* (GPY449) and (*B*) *chcl-ts pep4Δ* (GPY419) cells. Cells were labeled at 24°C for 5 min and then shifted to 37°C for the indicated chase times. At each time point cells were harvested and either treated 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.

(Fig. 4 *A* and *B*, lanes *1* and *2*, *upper panels*). At this time point the newly synthesized receptors are still within the secretory pathway in transit to the cell surface and consequently are not accessible to the exogenous pronase. Upon a further 5-min incubation at 37°C, the amount of intact receptor was slightly reduced in both cell types by pronase treatment (Fig. 4, *A* and *B*, lanes *3* and *4*, *upper panels*) and products of the proteolysis (*arrowheads*, *lower panels*) appeared in both cell types. Since the antibodies used in the immunoprecipitations are specific for the cytoplasmic domain, these pronase-resistant receptor fragments most likely encompass the cytoplasmic domain that is inaccessible to the exogenous pronase. By the 10-min chase time (Fig. 4, *A* and *B*, lanes *5* and *6*, *upper panels*), the bulk of the receptors (~80%) were accessible to pronase in both cell types, demonstrating that they had reached the plasma membrane by this time.

When the labeled cells were incubated at 37°C for longer times, a difference in receptor pronase-sensitivity between *chcl-ts* and *CHCl* cells was apparent. Pronase treatment of *chcl-ts* cells severely reduced the amount of intact receptor up to the 45-min time point (Fig. 4 *B*, lanes *5–14*, *upper panel*), but in contrast, significant amounts of receptor in *CHCl* cells were resistant to pronase at the 20- and 30-min time points (Fig. 4 *A*, lanes *9–12*, *upper panel*) and by 45 min most of the receptor was resistant to pronase (Fig. 4 *A*, lanes *13–18*, *upper panel*). In accordance with the prolonged pronase-sensitivity of the intact receptor in *chcl-ts* cells, the pronase-resistant fragments are apparent for up to 45 min, while in *CHCl* cells the fragments are absent after 30 min (Fig. 4 *A* and *B lower panels*, compare lanes *11–14*). We interpret these results as evidence that efficient endocytosis of the receptor occurred in the *CHCl* cells so that, after 30 min at 37°C, most of the newly made receptor was internalized and thereby sequestered from the exogenously added pronase. On the other hand, the prolonged pronase-sensitivity of the receptor in *chcl-ts* cells demonstrates that newly made receptors remain at the surface for longer times and provides direct in vivo evidence that clathrin facilitates uptake of the a-factor receptor.

Significant pronase resistance of the intact receptor in *chcl-ts* cells was observed after 60 min (Fig. 4 *B*, lanes *15–18*, *upper panel*). This results reveals the existence of a slower, clathrin-independent internalization process that is consistent with the results for internalization of radiolabeled α-factor. The possibility that the receptors in *chcl-ts* cells remain accessible to pronase due to lysis of the cells after shift to 37°C is unlikely based on the presence of the resistant fragments in *chcl-ts* cells, the pronase-resistance of the intact receptor by 60 min, and the pronase-resistance of cytoplasmic glucose 6-phosphate dehydrogenase at all time points (Tan, P., and G. Payne, unpublished observations).

The amount of receptor immunoprecipitated from untreated and pronase-treated samples was quantified by densitometry and the percent of intact receptor that was pronase-sensitive (i.e., at the cell surface) relative to the chase time is plotted in Fig. 4 *C*. The coincidence of the curves at early time points illustrates that receptors reach the cell surface at the same rates in both cell types; 80% of the receptors are present at the cell surface by 15 min. After this time, the pronase sensitivity in *CHCl* decreases rapidly, while in *chcl-ts* cells the peak pronase sensitivity of the receptor persists for up to 20 min and then declines slowly. From these results we estimate that the half times for internalization of the receptors is *11–15* min for *CHCl* cells and 25–30 min for *chcl-ts* cells, corresponding to a two- to threefold decrease in the rate of endocytosis of the a-factor receptor in *chcl-ts* cells.

Internalized 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 *CHCl* 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 *CHCl* 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 *CHCl* 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 nonpermissive 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.

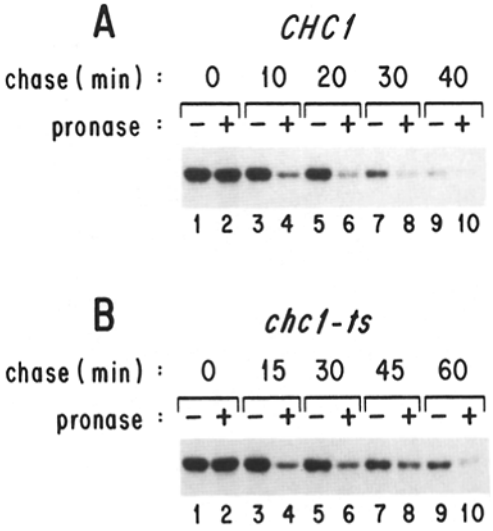

Figure 5. Pronase sensitivity of a-factor receptors in congenic *PEP4* (*A*) *CHCl* (GPY1100α) and (*B*) *chcl-ts* cells (GPY418). Cells were labeled at 24°C for 5 min and then shifted to 37°C for the indicated chase times. At each time point cells were harvested and either treated with pronase (+) or mock treated (−) prior to immunoprecipitation of the receptor as described in Materials and Methods. Results are from one experiment and are representative three experiments.

We assayed ligand-induced endocytosis using *CHCl pep4Δ* and *chcl-ts* cells harboring a plasmid which expresses *ste3-Δ365* under the control of the inducible *GAL1* 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-factor. 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 *1* and *2*) and presence of a pronase-resistant fragment (*arrows*). However, after addition of a-factor a dramatic difference in receptor pronase sensitivity between *CHCl* and *chcl-ts* cells was detected. In *CHCl* 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-factor (compare lanes *3* and *4* in Fig. 6, *A* and *B*) which

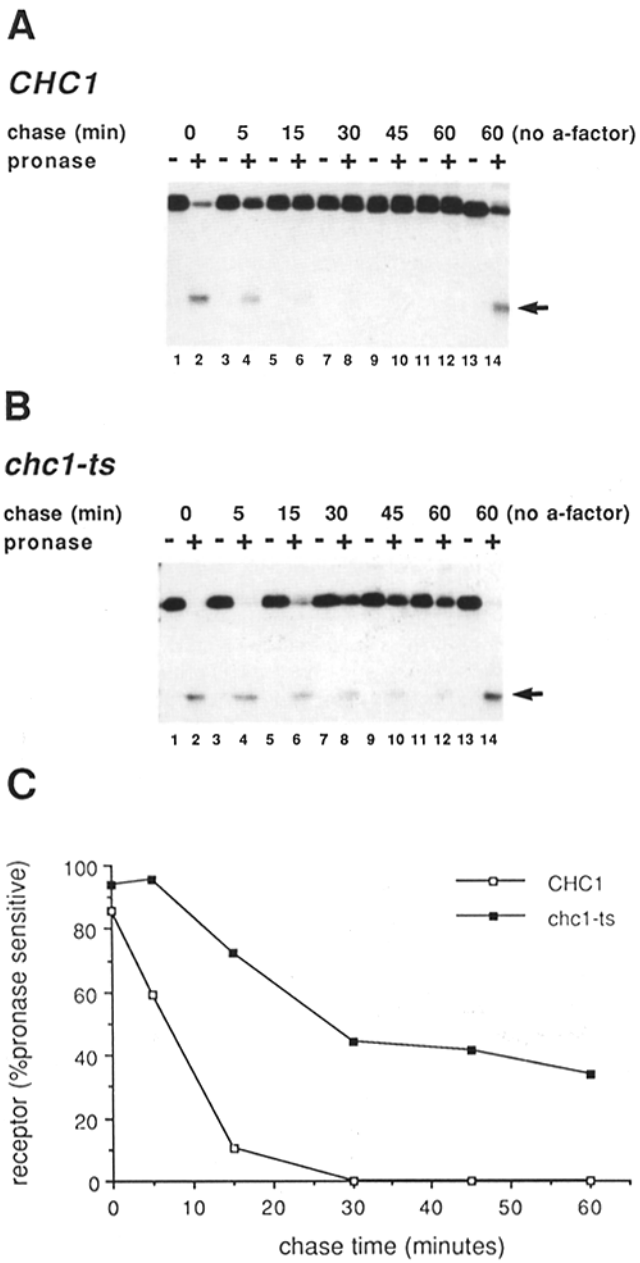

Figure 6. Pronase-sensitivity of truncated a-factor receptors in congenic (*A*) *CHC1 pep4Δ* cells (GPY731) and (*B*) *chcl-ts* cells (GPY735). Labeled receptors were accumulated at the plasma membrane without a-factor as described in Materials and Methods. The cells were then shifted to 37°C for 5 min before addition of the pheromone to induce endocytosis. Cells were collected at the time points indicated and the receptors examined for pronase sensitivity as described in Fig. 4. The experiment has been repeated three times with similar results. (*C*) Plot of the pronase sensitivity as described in Fig. 4 *C*.

corresponds to 10 min at 37°C. Thus, the onset of the *chcl-ts* effect after shift to 37°C is rapid, similar to the effect of *chcl-ts* on uptake of α-factor (Fig. 1 *C*). The resistance to pronase is ligand dependent: in the absence of a-factor, most of the receptors remain pronase-sensitive even after 60 min (Fig. 6, *A* and *B*, lanes *13* and *14*). As expected, in the pronase-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 *CHCl* 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 *CHCl* 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 a-factor receptor and α-factor ensued. In prior work, internalization of α-factor was shown to be reduced in *chclΔ* cells to 35–50% of wild-type levels (Payne et al., 1988). Because *chclΔ* 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 a-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 a-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; Ktistakis et al., 1990; Letourneur 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 a-factor receptor, a 105-amino acid truncation (*ste3*-Δ365) 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 a-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 *chc1*Δ 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, 1990*a*,*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. If receptors diffusely distributed along the plasma membrane are constantly endocytosed and replaced by new receptors, then excluding the receptors facing the mating partner from endocytosis would establish an orientation. Exclusion of receptors from endocytosis could occur by attachment to the underlying cytoskeleton in the same manner that the FcRII-B1 isoform of the Fc receptor is excluded from endocytosis in B-lymphocytes and macrophages (Miettinen, 1992) and the Na^+-K^+ ATPase is sequestered to the basal-lateral membrane of the kidney epithelial cells (Hammerton et al., 1991). Alternatively, polarized secretion (Field and Schekman, 1980) towards the mating partner combined with endocytosis could lead to oriented receptor localization without the need to invoke any mechanism for endocytic exclusion. It remains to be established which of these models applies to mating partner discrimination. However, in both cases, reduced endocytosis caused by defective clathrin would diminish the polarized distribution of receptors and thus reduce the discrimination capacity of the responding cell.

We have shown that clathrin is required for efficient endocytosis of the 7-TMS pheromone receptors in yeast. This is the first direct, in vivo evidence for clathrin-dependent uptake of receptors of the 7-TMS family. Our results raise the possibility that down-regulation of 7-TMS receptors in mammalian cells may similarly occur by clathrin-mediated endocytosis.

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