

Direct Evidence for Ligand-Induced Internalization of the Yeast α -Factor Pheromone Receptor

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When *Saccharomyces cerevisiae* cells bind α -factor pheromone, the ligand is internalized and its binding sites are lost from the cell surface in a time-, energy-, and temperature-dependent manner. This report presents direct evidence for α -factor-induced internalization of cell surface receptors. First, membrane fractionation on Renografin density gradients indicated that the α -factor receptors were predominantly found in the plasma membrane peak before α -factor treatment and then appeared in membranes of lesser buoyant density after α -factor exposure. Second, receptors were susceptible to cleavage by extracellular proteases before α -factor treatment and then became resistant to proteolysis after exposure to pheromone, consistent with the transit of receptors from the cell surface to an internal compartment. The median transit time in both assays was approximately 8 min. The ultimate target of the internalized receptors was identified as the vacuole, since the membranes containing internalized receptors cofractionated with vacuolar membranes, since the turnover of receptors was stimulated by α -factor exposure, and since receptor degradation was blocked in a *pep4* mutant that is deficient for vacuolar proteases. The carboxy-terminal domain of the receptor that is required for ligand internalization was also found to be essential for endocytosis of the receptor. A receptor mutant, *ste2-L236H*, which is defective for pheromone response but capable of ligand internalization, was found to be proficient for receptor endocytosis. Hence, separate structural features of the receptor appear to specify its signal transduction and internalization activities.

For *Saccharomyces cerevisiae*, the induction of mating-specific processes is mediated by the exchange of the peptide mating factors that are produced by the two haploid cell types, **a** and α cells (reviewed in references 39 and 57). **a** cells secrete the pheromone **a**-factor, whereas α cells secrete α -factor pheromone. Receptors specific for these pheromones are found on the surface of cells of the opposite cell type, i.e., the α -factor receptors, encoded by the *STE2* gene, are found on **a** cells (28), whereas the **a**-factor receptors, encoded by *STE3*, are found on α cells (24). The binding of either pheromone to its receptor initiates a signal transduction pathway that includes the action of a heterotrimeric G protein (21, 42, 62) and a protein kinase cascade related to the mitogen-activated protein kinase cascade found in mammalian systems (44). This signal facilitates mating by blocking cell division at G₁, stimulating the transcription of several genes that encode mating-specific activities, and inducing morphological changes of the cells. In the absence of mating, the haploid cells adapt to the action of α -factor by degrading the pheromone extracellularly (11, 15) and by regulating postreceptor events in the response pathway (4, 13, 20, 34, 41, 47).

DNA sequence analysis of the *STE2* and *STE3* genes (8, 24, 43) as well as topological studies of the gene products (10, 18) suggest that both receptors contain seven transmembrane domains, an extracellular amino terminus, and a carboxy terminus exposed to the cytoplasm. This structure is characteristic of G-protein-coupled receptors such as rhodopsin and the β -adrenergic receptors (22). Although the **a**-factor and α -factor receptors activate the same signalling pathway (3),

presumably by interacting with a common G protein (6, 29), they share no obvious sequence homology. Mutational studies of the α -factor receptor (17, 61) and other receptors with a similar structure (23, 32) implicate the third cytoplasmic loop in the coupling of these receptors to their respective G proteins. Other mutational analyses indicate that the carboxy-terminal cytoplasmic domain of the pheromone receptor is important for receptor down regulation, pheromone internalization, adaptation to the pheromone-induced signal, and pheromone-induced morphological changes (33, 34, 47, 49).

A large body of experimental evidence suggests that pheromones are internalized by receptor-mediated endocytosis. Radioactive α -factor becomes associated irreversibly with **a** cells in an energy- and receptor-dependent manner (14, 30). This association is accompanied by a disappearance of ligand-binding sites from the cell surface (30). Clathrin, which plays a major role in receptor-mediated endocytosis in mammalian cells, has recently been shown to facilitate the internalization of α -factor (58). Following internalization, the pheromone appears to be translocated to the vacuole via vesicular intermediates (54), which may represent early and late endosomes (55). Although there is no direct evidence that the α -factor receptor leaves the cell surface upon binding pheromone, **a**-factor receptors have been shown to pass from the plasma membrane to the vacuole, and this process is accelerated when α cells are treated with **a**-factor (19). There is as yet no direct evidence for the internalization of the **a**-factor ligand.

We sought physical evidence for the ligand-induced endocytosis of the α -factor receptor. Three independent criteria were used to establish the movement of α -factor receptors from the plasma membrane to internal compartments of the cell. First, Renografin density gradient centrifugation was used to fractionate the cellular membranes, allowing us to follow the ligand-induced exit of the receptor from the plasma membrane upon α -factor exposure. Second, protease susceptibility assays

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showed that the receptor became resistant to external protease upon pheromone addition. Third, the destination for the internalized receptors was identified as the vacuole by examining the half-life of the receptor in the presence and absence of vacuolar proteases. The relationship between endocytosis and signal transduction was explored by using receptor mutants exhibiting defects in one of these functions.

MATERIALS AND METHODS

Strains and plasmids. All strains are congenic to strain 381G (27). The genotype of strain DJ211-5-3 is *MAT α cry1 bar1-1 ade2-1 his4-580 leu2 lys2 trp1 tyr1 ura3 SUP4-3*. Strain DJ213-7-3 is *MAT α cry1 bar1-1 ste2-10::LEU2 ade2-1 his4-580 leu2 lys2 trp1 tyr1 ura3 SUP4-3*. Strain DJ900-A, which contains the *pep4::URA3* mutation, is a derivative of strain DJ211-5-3. It was constructed by a single-step gene replacement procedure (52) in which strain DJ211-5-3 was transformed with the *XhoI-EcoRI* fragment from plasmid pTS15 (51) carrying *pep4::URA3*. The presence of the *pep4::URA3* allele was confirmed by PCR analysis. Yeast centromere plasmid pJBK008, which contains *STE2* and *URA3*, was described previously (34). Plasmid pDJ252 was constructed by subcloning the *ste2-T326* allele from pJBK023 (34) into the yeast integrating vector pDJ251, which contains the *URA3* gene. Strain DJ903-A-1 containing *ste2-T326* was derived from strain DJ211-5-3 by the two-step gene replacement method (7) with plasmid pDJ252; the presence of the *ste2-T326* allele was confirmed by Southern blot analysis. Strains DJ900-B and DJ903-C, both of which contain the *mfa1::URA3* allele, were constructed by transforming strains DJ211-5-3 and DJ903-A-1, respectively, with the *EcoRI-SalI* fragment from plasmid pBH3 (36); PCR analysis was used to confirm the presence of the *mfa1::URA3* allele.

Antisera and reagents. Rabbit polyclonal antisera used in immunoblotting procedures were specific for the amino-terminal or the carboxy-terminal portion of the α -factor receptor (34); an integral membrane subunit of the vacuolar ATPase, Vph1 (38); a marker of the endoplasmic reticulum, Kar2 (53); the Golgi apparatus lumenal guanosine diphosphatase (GD-Pase) (1); or the cytosolic marker phosphoglycerate kinase. Mouse monoclonal antibodies C56 (2) were shown to be specific for the plasma membrane ATPase, Pma1, since they could specifically bind a Pma1- β -lactamase fusion protein (obtained from D. J. Tipper, University of Massachusetts, Worcester). Peroxidase-conjugated secondary antibodies, goat anti-mouse and goat anti-rabbit, were from Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md. Renografin-76 (76% Renografin) is a product of Squibb Diagnostics, New Brunswick, N.J. Synthetic α -factor was purchased from Sigma Chemical Co., St. Louis, Mo. ^{35}S -labelled α -factor (approximately 50 Ci/mmol) was prepared by culturing strain FY70 (*MAT α leu2*) containing plasmid pDA6300 (5) in low-sulfate minimal medium with carrier-free [^{35}S]sulfuric acid, and subsequent Bio-Rex 70 chromatography (16) of the resulting culture supernatant.

Membrane fractionation. Cells were cultured overnight at 30°C in YM-1 medium (26) to 10^7 cells per ml. Cycloheximide was added to 10 $\mu\text{g/ml}$. After 5 min, α -factor was added to 10^{-8} M. At the times indicated, samples containing 5×10^8 cells were removed to prechilled flasks and poisoned by adding NaN_3 and KF to 10 mM. Cells were collected by centrifugation, washed with 10 ml of ice-cold sorbitol buffer (50 mM Tris [pH 7.6], 0.8 M sorbitol, 10 mM NaN_3 , 10 mM KF), washed twice with 1 ml of sorbitol buffer, once with 1 ml of sorbitol buffer containing 100 μg of phenylmethylsulfonyl fluoride (PMSF) and 2 μg of pepstatin A and once with 1 ml of TE (50

mM Tris [pH 7.5], 1 mM EDTA) containing the same protease inhibitors. The cells were suspended in 0.5 ml of TE containing protease inhibitors and then lysed by mechanical disruption with glass beads. Unbroken cells were removed from the lysate by centrifugation for 5 min at $330 \times g$ in a 1.5-ml microcentrifuge tube in a Sorvall SS-34 rotor. Of the supernatant, 0.5 ml was combined with 0.5 ml of Renografin-76 and placed in the bottom of a centrifuge tube. Flotation gradients were prepared by successively layering 1 ml of 34, 30, 26, and 22% Renografin solutions that had been prepared by diluting Renografin-76 with TE. The gradients were centrifuged in an SW50.1 rotor at $150,000 \times g$ for 20 h at 4°C. Fractions (350 μl) were collected from the top of each gradient. Samples were diluted 1:3 with sample buffer (1 g of urea dissolved in 1 ml of 17.5 mM Tris-HCl [pH 6.8] containing 1.75% sodium dodecyl sulfate [SDS], 1% β -mercaptoethanol, bromophenol blue), heated at 37°C for 10 min, and loaded on 10% polyacrylamide gels for SDS electrophoretic analysis (37). Proteins were transferred to an Immobilon membrane (Millipore Corp, Bedford, Mass.). The membrane was probed with antiserum, and the immune complexes were detected by using the ECL chemiluminescence detection system (Amersham Life Science, Arlington Heights, Ill.). The resulting signal was quantified by using a model SLR-1D/2D Zeineh soft laser scanning densitometer. The density of the fractions was estimated from the refractive index (9).

Determination of receptor half-life. Strains DJ211-5-3 and DJ900-A were grown to mid-log phase and treated with cycloheximide and pheromone (as indicated), and lysates were prepared as for membrane fractionation. After the $330 \times g$ centrifugation step, the protein content of the supernatant was determined by the bicinchoninic acid assay (Pierce, Rockford, Ill.). Samples were diluted to 0.2 mg of protein per ml with sample buffer, heated at 37°C for 10 min, and analyzed by the electrophoretic and immunoblotting methods detailed above.

Protease sensitivity assays. Cells were grown to 10^7 cells per ml in YM-1 medium at 30°C. When present, cycloheximide was added to 10 $\mu\text{g/ml}$. The culture was divided into seven 20-ml cultures. α -Factor (10^{-8} M final concentration) was added to the cultures at staggered intervals so as to give different durations of α -factor exposure and identical durations (20 min) of cycloheximide exposure. After pheromone treatment, the cultures were chilled rapidly and NaN_3 and KF were added to 10 mM. The cells were collected by centrifugation, washed with 10 ml of sorbitol buffer, and suspended in 1 ml of sorbitol buffer. Each sample was divided into two 500- μl aliquots. After centrifugation, one cell pellet was suspended in 150 μl of sorbitol buffer and the other was suspended in 150 μl of sorbitol buffer containing 750 μg of chymotrypsin A_4 (Boehringer Mannheim, Indianapolis, Ind.). These suspensions were incubated with agitation at 30°C for 16 h. Proteolysis was stopped by adding PMSF and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) to 100 $\mu\text{g/ml}$. The cells were collected, washed three times with sorbitol buffer containing PMSF and TPCK, and then suspended in 150 μl of sample buffer lacking β -mercaptoethanol and bromophenol blue and lysed by mechanical disruption with glass beads. Lysates were transferred to fresh tubes and heated at 37°C for 10 min, and particulate matter was removed by centrifugation. The protein concentration of the cleared lysates was determined with bicinchoninic acid method and adjusted to 1 mg of protein per ml with sample buffer (containing β -mercaptoethanol) and then heated to 37°C for 10 min. The proteins were resolved electrophoretically and analyzed by immunoblotting as for membrane fractionation.

Isolation of the *ste2-L236H* mutant. Plasmid pJBK008 (34)

containing the *STE2* gene was subjected to hydroxylamine mutagenesis (50) and used for the transformation of strain DJ213-7-3. A total of 800 isolates were screened for growth on minimal medium containing 2×10^{-8} M α -factor. The 65 α -factor-resistant isolates were tested for pheromone-binding activity (30). Only one mutant retained the ability to bind pheromone to the same extent as wild-type cells. The site of the mutation was identified by subcloning various restriction fragments into an otherwise wild-type copy of the *STE2* gene contained in plasmid pDJ251. The 274-bp *AatII*-*ClaI* fragment that conferred the mutant phenotype was sequenced by using the Sequenase kit (United States Biochemicals, Cleveland, Ohio) and a double-stranded template. Two mutations were found—a G-to-A transition at position 615 and a T-to-A transversion at position 706. The Altered Sites *in vitro* Mutagenesis System (Promega, Madison, Wis.) was used to create a single T-to-A mutation at position 706 in the *STE2* gene. The presence of the mutation was confirmed by DNA sequencing, and the *AatII*-*ClaI* fragment was subcloned into plasmid pDJ251. Two-step gene replacement was used to replace the chromosomal copy of *STE2* in strain DJ211-5-3 with the mutant gene, creating strain DJ901-A-1. The presence of the mutant allele, which carries a unique *NcoI* restriction site, was confirmed by PCR analysis followed by restriction digestion. Mutant cells containing the single-base-pair substitution (designated *ste2-L236H*) exhibited a pheromone response phenotype that was indistinguishable from that of the original isolate.

Pheromone response assays. α -Factor halo assays were conducted by moistening sterile filter disks (0.64-cm diameter) with 20 μ l of various dilutions of α -factor (1×10^{-6} to 5×10^{-5} M). The disks were then placed onto yeast extract-peptone-dextrose YEPD solid medium that had been spread with 5×10^6 cells of the strain to be tested. The size and clarity of the zones of growth inhibition (haloes) were noted after 16, 24, and 48 h at 30°C, and the diameters of the zones were measured after 48 h. Agglutination was assayed by the method of Hartwell (27). The time course and dose response for arrest of cell division in the presence of pheromone was determined by the method of Moore (40). The strains used in this work were *bar1* mutants defective for α -factor destruction, which obviated the need to work at low cell concentrations (30). Cultures of strain DJ211-5-3 (*MATa bar1 STE2*) and strain DJ901-A-1 (*MATa bar1 ste2-L236H*) growing exponentially in YM-1 medium at 30°C were diluted to 5×10^5 cells per ml, and α -factor was added to between 5×10^{-10} and 1×10^{-8} M. Samples were removed periodically, fixed with formaldehyde (3.7%), and sonicated lightly to disperse cell clumps. At least 200 cells from each time point were examined by phase-contrast microscopy, and the number of budded and unbudded cells was noted. The untreated control cultures gave roughly 40% unbudded cells, whereas maximally induced *Ste*⁺ cultures reached unbudded levels of nearly 100%.

RESULTS

Previous studies have used indirect techniques to monitor the ligand-induced movement of α -factor receptors from the cell surface to internal compartments of the cell. When a cell is exposed to α -factor, the pheromone-binding sites disappear from the cell surface in a time- and energy-dependent manner with a concomitant internalization of the ligand (30). Furthermore, α -factor receptors are periodically exposed to internal compartments, since the basal rate of receptor turnover is dependent on vacuolar proteases (19). In this study, we have used antibodies to monitor directly the movement and degradation of receptor protein. We provide three lines of

evidence for ligand-induced endocytosis of α -factor receptors. Membrane fractionation experiments demonstrated that receptors exit the plasma membrane and appear in membranes of lighter buoyant density. Protease sensitivity assays show a reduction in the susceptibility of receptors to extracellular proteolysis upon pheromone addition. The destination of the endocytosed receptors was inferred by comparing the half-lives of the receptors in strains containing or lacking vacuolar proteases.

Membrane fractionation. Centrifugation in Renografin density gradients was used to resolve the plasma membrane from the other membranes of the cell. Previous workers (60) have shown that the major plasma membrane species from *S. cerevisiae* bands at a buoyant density of 1.17 g/ml in Renografin gradients. We found that membranes carrying the plasma membrane ATPase band at a similar density of 1.18 g/ml and are clearly resolved from membranes containing a vacuolar ATPase subunit, Vph1 (38), which were distributed from a buoyant density of 1.09 to 1.15 g/ml (Fig. 1A). Under the conditions used, markers for the endoplasmic reticulum (Kar2) and the Golgi apparatus (GDPase) were also separated from the plasma membrane marker, but they were not resolved from the vacuolar membrane marker (data not shown). Figure 1B shows the buoyant density profiles obtained for the membranes that contain the α -factor receptor after the cells had been exposed to α -factor for various periods of time. A growing culture of a cell was treated with cycloheximide to block new protein synthesis, and a saturating concentration of α -factor was added at time zero. As expected for a cell surface protein, essentially all of the receptor was initially found in a peak corresponding to the density of the plasma membrane. As time progressed, the receptor protein gradually disappeared from these fractions and accumulated in fractions of lighter buoyant density that coincided with the vacuolar ATPase. By 20 min, essentially all of the receptor was in these fractions of lighter density. The positions of the marker proteins within the gradient did not change with the addition of α -factor (data not shown). Receptors from control cultures that received cycloheximide and no α -factor did not show this shift in density (not shown), indicating that cycloheximide did not cause the redistribution of receptors. Moreover, a similar density shift was obtained with cultures that had been treated with α -factor for 12 min in the absence of cycloheximide (not shown), indicating that for this shorter time course the receptors disappeared from the plasma membrane faster than they were replaced by new synthesis. This result parallels our earlier finding that α -factor exposure causes initial down regulation of the cell surface pheromone-binding sites which is followed by a period of reaccumulation (30).

By comparing the relative amounts of receptor protein in the plasma membrane and the vacuolar membrane fractions, we were able to estimate the time required for the transit of receptors between the plasma membrane and the internal compartments of the cell. Figure 1C shows that with increasing time after pheromone addition an increasing proportion of the receptor left the plasma membrane fractions and appeared in the fractions of lesser density. After an 8-min exposure to α -factor, equal amounts of receptor protein were partitioned between the plasma membrane and vacuolar fractions. We were concerned that this period may be an underestimate of the actual transit time, since the degradation of receptors was likely to be more rapid in the vacuolar compartment than in the plasma membrane. However, as described below, the relative degradation rates of the two receptor species does not significantly affect the apparent transit time, since very few receptors, regardless of location, have been degraded during

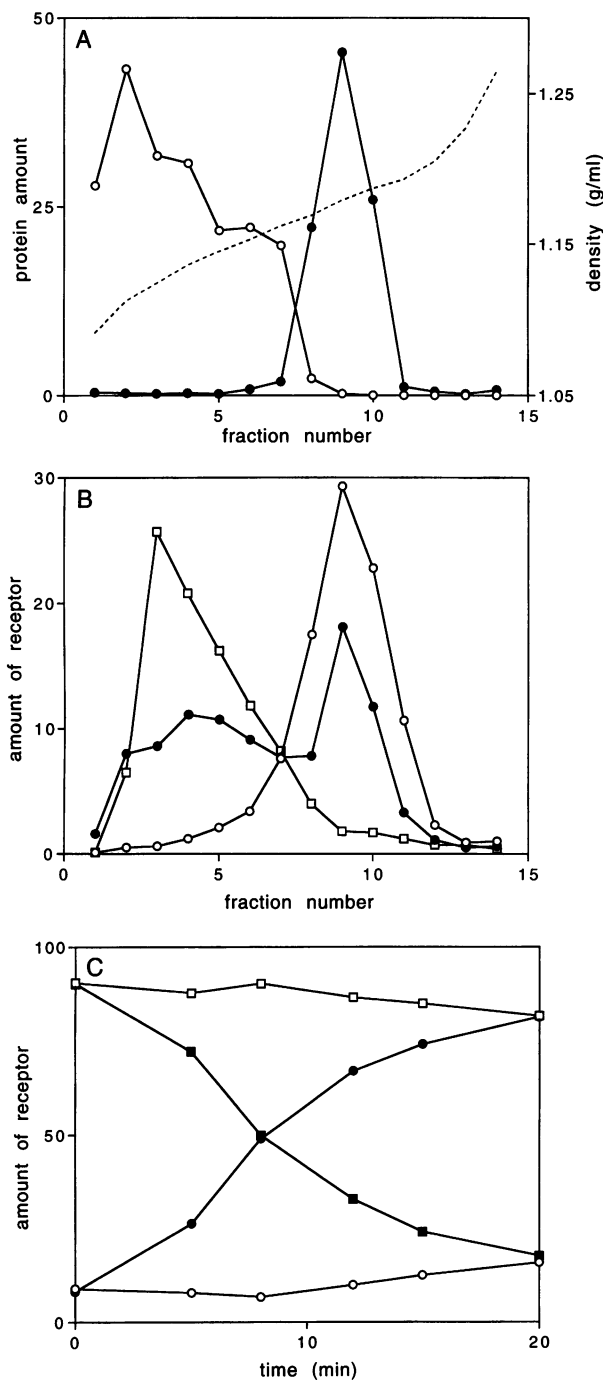


FIG. 1. Membrane fractionation and localization of α -factor receptors. (A) Membranes from strain DJ211-5-3 were resolved on Renografin density gradients, and the fractions were assayed for plasma membrane ATPase (●) and an integral membrane subunit of the vacuolar H⁺ ATPase (○). Densities of the fractions are indicated by the dashed line. (B) Strain DJ211-5-3 was treated with cycloheximide 5 min before the addition of α -factor at time zero. At the times indicated, membranes were resolved on Renografin gradients and the fractions were analyzed for α -factor receptor by using antibodies directed to the carboxy terminus. The amount of receptor is the percentage found in each fraction. Exposure to α -factor was for 0 min (○), 8 min (●), and 20 min (□). (C) The percentage of receptor molecules found in the plasma membrane peak (fractions 7 to 12) and the percentage of receptors found in the vacuolar peak (fractions 1 to 6) are plotted as a function of the duration of α -factor exposure. Receptor found in the plasma membrane peak: ■, culture that

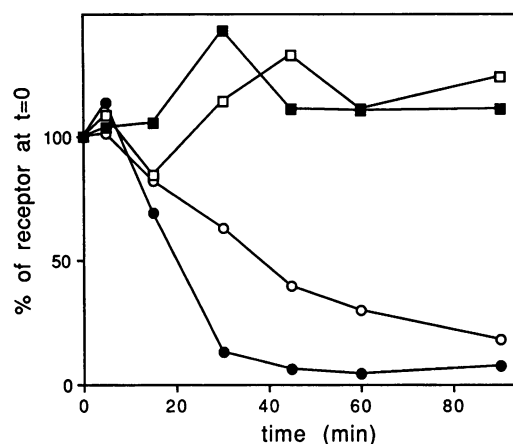


FIG. 2. Turnover of α -factor receptors. Strains DJ211-5-3 (*PEP4*) and DJ900-A (*pep4::URA3*) were treated with cycloheximide 5 min before the addition of α -factor at time zero. Shown is a time course depicting the decay of receptor protein for strain DJ211-5-3 that had been incubated in the presence (●) and in the absence (○) of α -factor. No significant decay was observed for strain DJ900-A incubated in the presence (■) or in the absence (□) of pheromone.

this 8-min period (Fig. 2). Hence, 8 min is an accurate estimate for the median transit time between the plasma membrane and the internal compartments. This value obtained at 30°C agrees with our previous determination of 10 min for the half-life of cell-surface α -factor binding sites at 34°C (30).

Degradation of α -factor receptors is dependent on vacuolar proteases. The shift in density of the membranes carrying α -factor receptors following pheromone treatment indicates that the receptors exit the plasma membrane. Although these internalized receptors colocalized with a vacuolar marker protein, an unequivocal assignment of the vacuole as the final destination cannot be made by using these data, as other internal cellular membranes also migrate to this position in the Renografin gradients. As a criterion for determining whether the internalized receptors are directed to the vacuole, we asked whether receptor degradation was stimulated by α -factor and whether this degradation was dependent on the proteolytic activities that reside in the vacuole. Since the product of the *PEP4* gene is required for the processing of vacuolar proteases, vacuoles in *pep4* mutants are almost completely devoid of proteolytic activities (31). Thus, the exposure of a protein to the vacuolar lumen is suggested by differences in its turnover rates in *PEP4*⁺ and *pep4* strains.

Figure 2 shows the effect of α -factor on receptor turnover in a wild-type strain that expresses the normal complement of vacuolar proteases and in a *pep4* mutant that lacks the activities of the major vacuolar proteases. Cells were treated with cycloheximide to block new receptor synthesis, and the subsequent decay of receptor protein was monitored. In the *PEP4*⁺ strain, the half-life of the receptor was approximately 35 min, whereas in a parallel culture that received α -factor, the half-life was reduced to 20 min. In contrast, when the same measurements were made with an isogenic *pep4* mutant, we found that the receptors were stable over the entire length of the 90-min time course, regardless of α -factor treatment. Davis et al. (19) have reported a similar *PEP4* dependence on the

received α -factor; □, untreated control. Receptor found in the vacuolar peak: ●, culture that received α -factor; ○, untreated control.

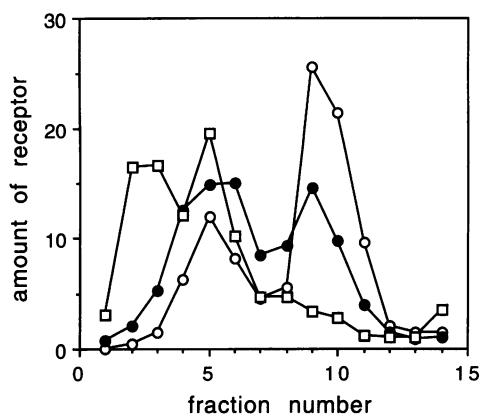


FIG. 3. Membrane fractionation and localization of α -factor receptors in strain DJ900-A (*pep4::URA3*). The culture was treated with cycloheximide 5 min before the addition of α -factor at time zero. After 0 min (○), 8 min (●), and 20 min (□) of α -factor exposure, the membranes were resolved by Renografin density gradient centrifugation as for Fig. 1B, and the fractions were assayed for α -factor receptor protein by using antibodies directed to its carboxy-terminal domain.

turnover rate of the α -factor receptor in the absence of pheromone. Our results indicate that α -factor stimulates the movement of the receptors to the vacuole, where they are degraded in a *PEP4*-dependent manner. The degradation of the receptors in the absence of pheromone presumably reflects basal protein turnover, which also occurs in the vacuole. Since the accumulation of receptors in the vacuole was not detectable in the absence of α -factor (Fig. 1B), the rate-determining step in receptor turnover is apparently delivery to the vacuole rather than degradation of material that has reached the vacuole. In contrast, since a vacuolar pool of receptors accumulates in the presence of α -factor, it appears that the faster internalization rate causes degradation in the vacuole to become rate limiting.

Renografin density gradient centrifugation was used to ensure that the stability of the receptors in the *pep4* mutant was not a consequence of failure to exit the plasma membrane. Figure 3 shows that the majority of receptors are present in the plasma membrane fractions in the absence of α -factor and that they appear in the vacuolar fractions after the cells have been exposed to α -factor. However, a number of differences can be noted in comparing the gradient profile of the *pep4* mutant (Fig. 3) with that of the *PEP4*⁺ strain (Fig. 1B), even though the localization of the marker proteins is not affected by the *pep4* mutation (data not shown). Before α -factor treatment, a significant proportion of receptor in the *pep4* strain was found in fraction 5, a fraction that contains a portion of the vacuolar marker protein (Fig. 1A). We expected some of the receptor to be initially present in the vacuole in the *pep4* mutant, since the receptors that would have migrated to the vacuole as part of the basal receptor turnover would fail to be degraded in this protease-deficient strain. With increasing time after pheromone addition, the receptor left the plasma membrane peak and shifted to fraction 5 and eventually appeared in fraction 2, illustrating that the receptors in the *pep4* mutant are capable of exiting the plasma membrane. Whether the material in fraction 5 represents a transport intermediate is unclear, since the vacuolar marker protein Vph1 is found in a broad peak spanning fractions 1 through 7 (Fig. 1A).

Proteolytic cleavage of cell surface receptors. Further evidence supporting the exit of receptors from the plasma mem-

brane in response to α -factor was obtained by examining the susceptibility of receptors to extracellular proteases. Under the appropriate conditions, the receptors present at the cell surface are expected to be cleaved by exogenously added protease, while the internal receptors remain protected from proteolysis. Optimal assay conditions were sought by exposing intact cells to a number of different proteases under a variety of digestion conditions. Following electrophoretic analysis of whole-cell extracts, antibodies specific for the carboxy-terminal cytoplasmic domain of the receptor were used to detect intact receptors as well as the proteolytic fragments generated by cleavages in the exposed amino-terminal extension and in the extracellular loops of the receptor. Antibodies to phosphoglycerate kinase (PGK) were used to monitor proteolysis of cytoplasmic proteins. Trypsin digestion proved to be unsuitable, as it gave insufficient cleavage of the receptor whereas pronase and proteinase K, even though they cleaved the receptor efficiently, were also unsuitable since they resulted in nonspecific degradation of other cellular proteins as evidenced by the proteolysis of PGK and by a general loss of high-molecular-weight proteins on Coomassie blue-stained gels (data not shown). Chymotrypsin was chosen for our assay as it gave nearly complete cleavage of the receptor without affecting the levels of intact PGK (Fig. 4) or bulk protein (not shown). The cycloheximide that was added to the cultures to prevent new protein synthesis, however, appeared to inhibit the action of chymotrypsin, since a slightly reduced extent of receptor cleavage was observed for the cultures that had been treated with cycloheximide (compare lane 8 in Fig. 4A with lane 8 in Fig. 4B). Moreover, cultures treated with cycloheximide for a longer duration showed a greater reduction in chymotrypsin susceptibility (not shown). For this reason, the results depicted in Fig. 4A were obtained with multiple identical cultures in which the addition of α -factor was staggered so that the period of cycloheximide exposure would remain constant for each of the time points.

As depicted in Fig. 4, pheromone receptors showed a reduced susceptibility to proteolytic cleavage when the cultures were exposed to α -factor. Cells of the *pep4* strain that had been exposed to α -factor for various lengths of time were poisoned with NaN_3 and KF to prevent further internalization of the receptor and then treated with chymotrypsin to cleave the receptors at the cell surface. The intact receptor species were defined as the forms that were present in controls that did not receive chymotrypsin—full-length receptors are known to be glycosylated and give multiple electrophoretic bands (5, 34). The proteolyzed species were defined as the forms that were present only in the reaction mixtures that contained chymotrypsin. Nearly all of the receptors were susceptible to chymotrypsin at early times after α -factor addition, since the majority of the immunoreactive material was recovered as proteolytic fragments ranging in size from 20 to 30 kDa, corresponding to cleavage in the extracellular loops. With increasing duration of α -factor exposure, recovery of the proteolytic fragments decreased as recovery of the intact receptor molecules increased, consistent with the receptors leaving the cell surface and moving to an internal compartment where they were protected from proteolysis. By 20 min, essentially all of the receptor protein was recovered as the intact, protected species.

The rate at which the cell surface receptors were protected from proteolysis was the same for the cells that had been cultured in the presence (Fig. 4A) or in the absence (Fig. 4B) of cycloheximide. Thus, it appears that the loss of protease sensitivity is not a result of cycloheximide treatment, nor are the kinetics of acquired resistance to chymotrypsin altered by cycloheximide treatment. Moreover, the rate of appearance of

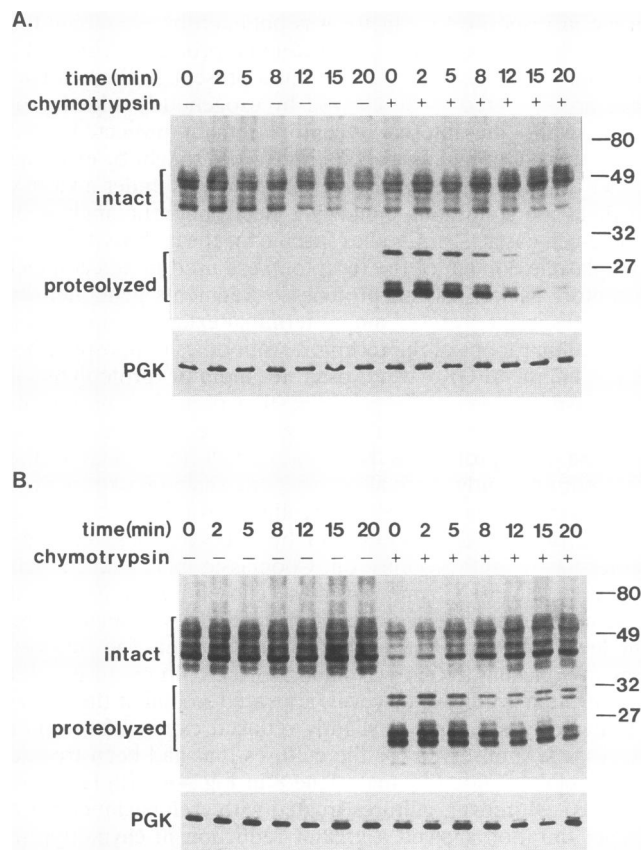


FIG. 4. Sensitivity of α -factor receptors to exogenous protease. Strain DJ900-A (*pep4::URA3*) was exposed to α -factor for the duration indicated in the presence (A) or absence (B) of cycloheximide. Cells were poisoned with NaN_3 and KF and then subjected to chymotrypsin digestion (+) or mock protease treatment (-). Proteolytic products of the receptor were resolved electrophoretically and detected with antibodies directed to the intracellular carboxy-terminal domain. The positions of the intact forms and the proteolyzed forms of the receptor are indicated at the left and molecular mass markers (in kilodaltons) are shown at the right of each panel. To monitor nonspecific proteolysis of cytoplasmic proteins, the immunoblots were reprobbed with antibodies directed to the cytoplasmic protein PGK (bottom of each panel).

chymotrypsin-resistant receptors mirrors the rate at which receptors were removed from the plasma membrane as determined by Renografin density gradient centrifugation (Fig. 1C). However, minor differences between the cycloheximide-treated and untreated cultures were observed. As mentioned above, cycloheximide reduced the efficiency of receptor cleavage that was apparent in the samples from shorter periods of α -factor exposure, since more of the receptor remained intact in the presence of cycloheximide even though the majority of these receptors were at the cell surface (Fig. 3). In the absence of cycloheximide, the persistence of some chymotrypsin-sensitive receptors after the longest α -factor exposures was anticipated because of the continued synthesis of new receptors in the absence of cycloheximide. For the cycloheximide-treated cultures, α -factor exposure also reduced the recovery of receptors in the samples that did not receive chymotrypsin during the control incubation (Fig. 4A, first seven lanes). This twofold loss is likely to be a consequence of slow degradation of intracellular receptors by the endogenous proteolytic activities present even in a *pep4* strain, since there was no loss of

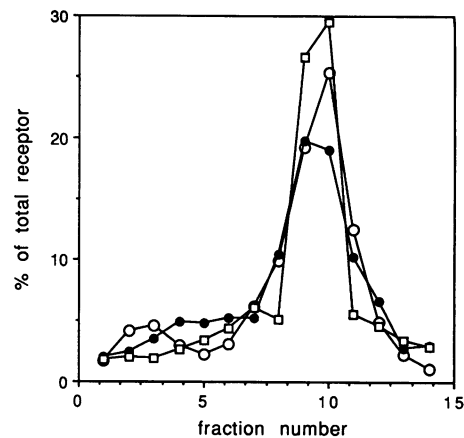


FIG. 5. Membrane fractionation and localization of truncated receptors during α -factor treatment. Strain DJ903-C (*MATa ste2-T326 mfa1::URA3*) was treated with cycloheximide 5 min before the addition of α -factor at time zero. After 0 min (○), 8 min (●), and 20 min (□) of α -factor exposure, the membranes were resolved by Renografin density gradient centrifugation as for Fig. 1B, and the fractions were assayed for α -factor receptor protein by using antibodies directed to its amino-terminal domain.

receptors in the *pep4* strain when this second incubation was omitted (Fig. 2). In the absence of cycloheximide, this reduction in receptor level was probably offset by the increased rate of receptor synthesis that is induced by α -factor (25, 30).

Internalization defects of truncated α -factor receptors. The carboxy-terminal cytoplasmic domains of many cell surface receptors have been shown to contain sequences necessary for receptor internalization (59). The carboxy-terminal cytoplasmic domain of the α -factor receptor appears to consist of 133 amino acids. Truncation mutants that remove part or all of this domain lead to α -factor hypersensitivity and a failure to recover from α -factor exposure (34, 47). The role of this domain in receptor down regulation and pheromone internalization has been explored in several laboratories (34, 47, 49). To look directly at the ability of truncated receptors to leave the plasma membrane in response to pheromone, we employed our membrane fractionation and chymotrypsin susceptibility assays. The truncation allele *ste2-T326* was analyzed in this study for the purpose of clarifying conflicting reports in the literature concerning the capacity of these mutant receptors to undergo ligand-stimulated internalization (34, 49). Cells carrying *MATa bar1 ste2-T326* are supersensitive to pheromone because of the receptor truncation (34) and because of the *bar1* mutation, which blocks α -factor degradation (13, 56). Thus, many of these cells in culture are large and distended, presumably because they respond to the low level of α -factor produced by the *a* cells that have switched mating type. We found that the *MATa bar1 ste2-T326 mfa1::URA3* strain used in our receptor internalization assays did not exhibit this phenotype, consistent with the *MFA1* gene being the major source of α -factor in α cells (36).

The *MATa bar1 ste2-T326 mfa1::URA3* strain was subjected to the Renografin density gradient centrifugation analysis described in the legend to Fig. 1. As shown in Fig. 5, the truncated receptors failed to leave the plasma membrane peak, even after a 20-min exposure to pheromone. The presence of the *mfa1::URA3* allele did not affect the endocytosis of wild-type receptors or the position of the marker proteins within the gradient (data not shown). As further evidence for the failure

of the truncated receptors to leave the plasma membrane in response to α -factor, we looked at the susceptibility of the truncated receptors to chymotrypsin cleavage (not shown). In this test, cells were exposed to pheromone and then treated with chymotrypsin as described in the legend to Fig. 4, except that antiserum directed against the amino-terminal domain of the receptor was used for the Western blot (immunoblot) analysis. We found that none of the receptors was protected from chymotrypsin degradation even after a 20-min exposure to α -factor; however, the use of the amino terminus-specific antibodies precluded the detection of the proteolytic fragments generated by cleavage of cell surface receptors.

These data support the view that the truncated receptors fail to exit the plasma membrane in response to pheromone. This is consistent with the inability of these truncated receptors to internalize pheromone and to undergo down regulation (49). The ability of the truncated receptor sites to undergo α -factor-induced down regulation in the earlier reports (34) was apparently due to blockage of the cell surface binding sites by the initial α -factor exposure, since longer washing periods following α -factor treatment revealed that the receptor sites were not lost from the cell surface (49, and unpublished data). Although the truncated receptors show a normal affinity for α -factor (34, 47), the rates of association and dissociation of the ligand are reduced significantly (46, and unpublished data). These observations underscore the importance of using a direct assay for the internalization of receptor protein, since irreversible association of α -factor with its cell surface receptor sites can result in the down regulation of cell surface receptor sites and apparent α -factor internalization.

α -Factor-induced internalization of receptors defective for pheromone response. The results of the preceding section and other published reports (47, 49) suggest that the intracellular factors controlling receptor internalization recognize structural features of the receptor contained in its carboxy-terminal cytoplasmic domain. Apparently, different structural features are recognized during the signal transduction events that lead to the arrest of cell division and the transcriptional induction of pheromone-responsive genes. In order to determine whether the structural features that specify signal transduction also participate in receptor internalization, we wished to examine the internalization properties of receptor mutants that block pheromone-induced transcription and cell division arrest. By using in vitro mutagenesis of the entire *STE2* gene, we obtained mutation *ste2-L236H*, which encoded receptors that retained normal pheromone-binding activity and yet failed to arrest cell division in response to α -factor. This substitution of histidine for leucine in the third cytoplasmic loop of the receptor was also isolated by Weiner et al. (61) after exhaustive PCR mutagenesis of the sequence encoding the third intracellular loop. Weiner et al. (61) found that this mutant receptor is partially defective for G protein coupling in that α -factor affinity is only marginally influenced by the binding of nucleotides to the G protein. The mutant cells require six times more α -factor to give comparable transcriptional induction of a pheromone-responsive gene (*FUS1-lacZ*) and four times more α -factor to give a comparable zone of growth inhibition in an α -factor halo assay. We found a comparable fivefold reduction in α -factor sensitivity for the mutant in the halo assay, but the ability of the mutant to induce the synthesis of cell surface agglutinins was reduced by 100-fold (50% effective concentration = 2×10^{-8} M α -factor for the mutant versus 2×10^{-10} M for the wild-type control). As shown in Fig. 6, the mutant cells arrest division and recover from the effects of 1×10^{-7} M α -factor in a manner that is indistinguishable from wild-type cells that had been exposed to 2.5×10^{-9} M; hence,

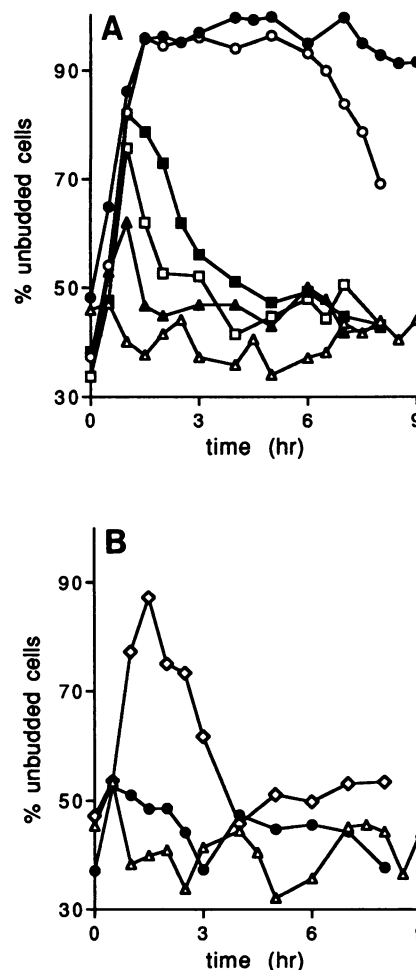


FIG. 6. Pheromone response of the *ste2-L236H* signalling-defective mutant. Strain DJ211-5-3 (*STE2*) and strain DJ901-A-1 (*ste2-L236H*) were cultured with various concentrations of α -factor. The percentage of unbudded cells in these cultures is plotted as a function of the duration of α -factor exposure. (A) Strain DJ211-5-3 (*STE2*) received 1×10^{-8} M (\bullet), 5×10^{-9} M (\circ), 2.5×10^{-9} M (\blacksquare), 1×10^{-9} M (\square), or 5×10^{-10} M α -factor (\blacktriangle) or no pheromone (\triangle). (B) Strain DJ901-A-1 (*ste2-L236H*) received 10^{-7} M (\diamond) or 10^{-8} M (\bullet) α -factor or no pheromone (\triangle).

this behavior of the mutant also indicates a weaker response to α -factor.

The pheromone response defect of the *ste2-L236H* mutant does not block its ability to internalize α -factor (61). We wished to determine directly whether the *ste2-L236H* mutant blocks the ability of α -factor receptors to exit the plasma membrane and move to an internal compartment. The Renografin density gradient fractionation technique was used to examine movement of the *ste2-L236H* mutant receptors in response to α -factor. With increasing duration of α -factor exposure, the mutant receptors shifted from the plasma membrane fractions to a position of lesser buoyant density in the gradient (Fig. 7). A similar shift was observed for the wild-type receptors (Fig. 1B). The rate of this process is also very similar to the wild-type case. Thus, the *ste2-L236H* mutation, which decreases pheromone responsiveness, has no detectable effect on the internalization of receptors.

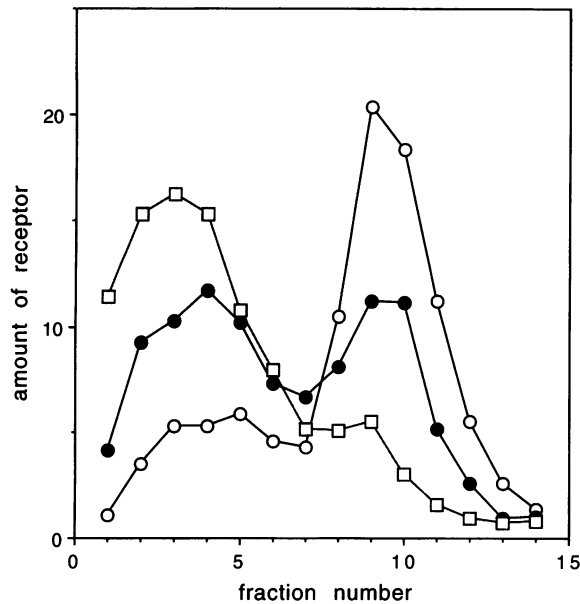


FIG. 7. Membrane fractionation of cells expressing signalling-defective receptors. Strain DJ901-A-1 (*ste2-L236H*) was treated with cycloheximide and presented with α -factor at time zero. After 0 min (○), 8 min (●), and 20 min (□) of α -factor exposure, the membranes were resolved by Renografin density gradient centrifugation as for Fig. 1B, and the fractions were assayed for α -factor receptor protein by using antibodies directed to its carboxy-terminal domain.

DISCUSSION

We report the first direct evidence for ligand-stimulated internalization of the α -factor receptor, and we have tested whether specific mutant receptors are internalized in response to ligand. Previous evidence supporting internalization was indirect in that it was based on the down regulation of cell surface binding sites that accompanied pheromone internalization (14, 30). Although these results provided preliminary evidence for ligand-mediated endocytosis of the α -factor receptor, this interpretation was limited by the possibility that the receptors were inactivated by α -factor binding or that α -factor bound irreversibly to receptor sites that remained at the cell surface. Indeed, we have found that the down regulation of surface receptor sites observed for truncated forms of the receptor (34, 47) appears to be a consequence of stable binding of α -factor to the receptor sites rather than ligand-mediated endocytosis. We report methods that directly measure the ligand-induced removal of the α -factor receptor from the plasma membrane. The membrane fractionation studies showed that the receptors exited the plasma membrane when the cells had been exposed to pheromone and that these receptors could be recovered in fractions of lighter buoyant density containing vacuolar membrane proteins. Moreover, α -factor exposure led to a sequestered form of the receptor that was no longer susceptible to cleavage by chymotrypsin. Davis et al. (19) have recently reported that the basal turnover of α -factor receptors is dependent on the activity of vacuolar proteases. We find that receptor turnover is stimulated by α -factor exposure and that the more rapid turnover rate is also dependent on vacuolar protease activity. Thus, it appears that the final destination for both occupied and unoccupied receptors is the vacuole. Whether basal and ligand-induced endocytoses follow the same pathway from the plasma membrane to the vacuole is not known.

The kinetics at which α -factor receptors exited the plasma membrane allowed us to estimate the time required for the receptors to be translocated from the plasma membrane to the internal compartments of the cell. For cells that had been growing in the absence of α -factor, all of the receptors were found in the plasma membrane fractions and were susceptible to chymotrypsin cleavage in whole cells. We were able to follow the fate of these receptors after α -factor addition, since the synthesis of new receptors had been blocked with cycloheximide. After 8 min, half of the receptors had left the cell surface. Since the half-life of total receptor protein was 20 min in the presence of α -factor and since receptors accumulated in the vacuolar fractions during this period, we concluded that the clearance of receptors in the presence of α -factor was limited by the rate of their degradation in the vacuole. Our inability to detect receptors in vacuolar fractions in the absence of pheromone suggests that the basal rate of turnover is limited by exit from the plasma membrane. Therefore, the presence of pheromone stimulates the removal of the receptors from the plasma membrane, perhaps by altering the conformation of the receptor so that it is more efficiently recognized by the endocytic machinery of the cell. The presence of cycloheximide in these experiments did not appear to affect the initial kinetics of receptor movement, since identical results were obtained for the early time points when cycloheximide was omitted. Previous studies also showed that cycloheximide had no effect on the initial rate at which receptor binding sites disappeared from the cell surface (30).

The characteristics of α -factor receptor internalization reported here are similar to those reported by Singer and Riezman for the internalization of α -factor pheromone (54). Half of the pheromone is internalized after 4 min at 30°C (54). Singer and Riezman used Nycodenz density gradients to show that pheromone internalized for 20 min at 15°C was resolved into two peaks corresponding to buoyant densities of 1.12 and 1.14 g/ml (54). After 20 min at 30°C, we found the receptor in a similar range of 1.09 to 1.15 g/ml in Renografin gradients. The association of the internalized α -factor with two different membrane fractions may represent intermediates in the endocytic pathway such as early and late endosomes (55) or possibly the vacuolar compartment. Whether the pheromone and the receptor remain in a stable complex as they are translocated through the pathway is not known; however, it is clear that both reach the same final destination, as the degradation of the pheromone, as well as the receptor, is dependent on vacuolar proteases (54).

Our findings concerning the internalization of the α -factor receptor are similar to recent reports (19, 58) concerning α -factor receptor internalization. Turnover of α -factor receptors is dependent on vacuolar proteases (half-life = 20 min at 30°C in the absence of pheromone) (19). In the presence of α -factor, receptors were found to move into a protease-resistant compartment within 60 min at 30°C (19). Further protease susceptibility studies of the α -factor receptor that were conducted at 37°C showed that the half-life for receptor internalization was between 11 and 15 min (58), a range that is similar to that observed for the α -factor receptor at 30°C. Immunofluorescence studies using cells overexpressing the α -factor receptor provided evidence supporting the movement of receptors from the plasma membrane to the vacuole; however, because the vacuole contained a significant number of receptors before the cells had been exposed to pheromone, it is unclear whether additional receptors were delivered to the vacuole or were simply lost from the cell surface. Davis et al. (19) also reported the isolation of a *trans*-acting mutation, *ren1-1* (allelic to *vps2*), that affects receptor internalization.

This mutation also blocks the turnover of both pheromone receptors, thereby suggesting that a common mechanism is used for the endocytosis of both receptors. These authors also found that mutation *ren1-1* blocked the ligand-induced as well as the basal turnover of truncated α -factor receptors (19), suggesting that the basal and the ligand-induced endocytic pathways also share common steps. Even though an intracellular compartment is the major site of α -factor receptor accumulation in the *ren1-1* mutant, the overall rate at which receptors are lost from the cell surface is reduced. This raises the possibility that endocytosed receptors recycle from this intermediate compartment to the cell surface (19). Our results with the α -factor receptor do not address the issue of receptor recycling. The overall rate at which α -factor receptors are lost from the plasma membrane may be a composite of the rates of internalization and recycling.

The endocytic signal carried by cell surface receptors is commonly contained in the cytoplasmic, carboxy-terminal tail. The cytoplasmic tail of the α -factor receptor is thought to include residues 298 through 431 of the polypeptide backbone (8, 10, 43). Conflicting reports regarding the role of this domain in endocytosis have been based on indirect assays that measure the abilities of truncated receptors to promote α -factor internalization and to undergo ligand-stimulated down regulation of the cell surface α -factor binding sites. Reneke et al. (47) found that truncation at position 296 blocked down regulation, whereas truncation at position 313 resulted in a reduced capacity for down regulation. Konopka et al. (34) found that truncation at position 326 blocked the basal rate at which surface receptor sites were lost but failed to block ligand-stimulated down regulation of the sites. In contrast, Rohrer et al. (49) found that a truncation at this position completely blocked down regulation. Further studies of the dissociation rates for bound α -factor conducted under the same assay conditions used in the down regulation assays (30) reveal that the k_{off} rate is reduced 3-fold for the *ste2-T326* mutant (our unpublished results) and 3- to 10-fold for internal deletions of the carboxy-terminal domain (46), even though the K_d values are unaffected (34, 47). Thus, the standard indirect assays for endocytosis do not reliably measure receptor internalization because they depend on the dissociation of α -factor bound to cell surface receptors. The direct assays reported here indicate that receptors truncated at position 326 remain at the plasma membrane after α -factor exposure, in agreement with the down regulation and α -factor internalization assays of Rohrer et al. (49).

By making point mutations and small internal deletions in the cytoplasmic tail of the α -factor receptor, Rohrer et al. (49) determined that the peptide sequence DAKSS spanning residues 335 to 339 is required for pheromone internalization and receptor down regulation. Thus, cellular factors that regulate receptor internalization require, at least in part, this portion of the cytoplasmic tail. Truncated receptors are found in greater numbers on the cell surface (34, 47), presumably because of their reduced basal turnover (34). Hence, the endocytic signal or signals found in the carboxy-terminal tail of the α -factor receptor appear to control both basal and ligand-induced internalization. The α -factor receptor also appears to carry an internalization signal in its cytoplasmic tail, but this signal is probably required only for basal receptor turnover (19). Deletion of the final 105 amino acid residues of the α -factor receptor (truncation at position 365, leaving a 79-residue cytoplasmic tail) results in receptors that show increased stability in the absence of α -factor but that remain subject to ligand-induced endocytosis. A consensus endocytic sequence, DXKXS, as defined by Rohrer et al. (49), spans residues 397 to

401 and thus is eliminated by truncation at position 365. In the case of the α -factor receptors, this presumptive endocytic signal is apparently required only for constitutive endocytosis. Perhaps a second endocytic signal, one that is eliminated in the *ste2-T326* mutant but not in the *ste2-T365* mutant, is required for ligand-induced internalization.

Binding of α -factor to its receptor initiates the signal transduction events controlling pheromone response in addition to its role in promoting receptor endocytosis. As a consequence of its association with pheromone, the receptor is likely to assume one or more conformational states that allow intracellular factors to recognize the ligand-occupied state. Two types of information suggest that the factors responsible for receptor internalization differ from the factors controlling signal transduction. First, *trans*-acting mutations that alter signal transduction do not affect receptor-mediated endocytosis, and other *trans*-acting mutations that block endocytosis do not affect signal transduction. Second, truncated receptors are specifically defective for endocytosis, whereas amino acid substitutions in the third cytoplasmic loop of the receptor are specifically defective for signal transduction. Transacting mutations in the *END3*, *END4*, and *REN1* genes block α -factor internalization, yet these mutations do not affect mating or signal transduction (19, 45). Similarly, mutations in the actin gene *ACT1*, the clathrin heavy chain gene *CHC1*, and the gene for the yeast fimbrin homolog, *SAC6*, lead to defects in pheromone internalization but have little effect on signal transduction (35, 48). Furthermore, an intact signal transduction pathway is not required for receptor endocytosis. Mutations in the *STE4* gene, encoding the β subunit of the heterotrimeric G protein, or in the *STE5* gene block signal transduction events but have no effect on the down regulation of the receptor (30, 63), whereas mutations in the *GPA1* gene, encoding the G protein α subunit, cause constitutive activation of pheromone response without affecting the ability of receptors to undergo down regulation (4). Pheromone receptors expressed from a plasmid under the control of the GAL promoter in diploid cells were competent for endocytosis, even though diploid cells do not have a functional pheromone response pathway (19, 63).

To test whether signal transduction plays a role in the endocytosis of the receptor, we examined the capacity of *ste2-L236H* mutant receptors to undergo ligand-induced endocytosis. Weiner et al. (61), who originally identified this mutation, showed that it blocks the ability of the receptor to couple with the G protein in cell-free extracts, that it partially blocks the ability of cells to respond to α -factor, and that it does not block α -factor internalization as judged by the stable association of α -factor with metabolically active cells. Our results show that this coupling-deficient receptor has a reduced capacity to mediate cellular responses to α -factor, yet it is able to undergo ligand-induced endocytosis, in agreement with the α -factor internalization results of Weiner et al. (61). The weaker G protein-mediated response observed for this mutant is anticipated from the analysis of hybrid adrenergic receptors in which the third cytoplasmic loop of the receptor was found to specify G protein interaction (22). Taken together, the results with the *ste2-L236H* mutant and the truncation mutants indicate that different structural features of the receptor specify its interaction with the endocytic and signal transduction pathways.

Although the *ste2-L236H* mutant cells show reduced responsiveness to α -factor, it is difficult to establish whether this defect reflects a reduced capacity to generate the initial signal or an increased sensitivity to the regulatory processes that dampen the signal. Since the length of time required for cells to recover from α -factor is proportional to the α -factor con-

centration used (41), mutant cells that generate a weaker signal are expected to recover more rapidly. α -Factor halo assays are often used as a qualitative measure for adaptation, since the cells within the zone of growth inhibition that surrounds a source of α -factor eventually grow to fill the zone. Weiner et al. (61) propose that the *ste2-L236H* mutant receptors promote an adaptation pathway, since halo assays of the mutant initially give a clear zone that fills in more rapidly than the wild-type control and since the *sst2* mutation, which blocks adaptation (12, 20), also blocks the ability of the *ste2-L236H* mutant to fill the zone. In contrast, we believe that the transient arrest of cell division of the mutant cells within the zone and the effects of the *sst2* mutation could be simple consequences of a weaker signal in the *ste2-L236H* mutant and the profound sensitivity of *sst2* mutants to weak signals. Moreover, after the 2-day incubation period required for this assay, it is difficult to distinguish the inability to adapt from an inability to survive prolonged α -factor exposure. The adaptation phenotype of the *ste2-L236H* mutant could simply result from the generation of a weaker signal, since our results show the same arrest and recovery profiles for the mutant cells at 10^{-7} M α -factor as for the wild-type cells at 2.5×10^{-9} M α -factor (Fig. 6). Furthermore, we found zones of α -factor inhibition that were turbid and remained turbid throughout the course of observation when halo assays were conducted with the mutant cells. Thus, the reduced pheromone responsiveness of the *ste2-L236H* mutant is consistent with the generation of a weaker signal or with an increased ability to adapt to the signal.

In conclusion, we have examined the ability of this response-defective mutant to internalize the pheromone receptor. Using the direct internalization assays described here, we have shown that cells expressing *ste2-L236H* are competent for receptor endocytosis. This provides further evidence that the signals for receptor internalization and signal transduction are separable at the level of the receptor, suggesting that different domains of the receptor participate in these two functions. Different structural features of the receptor appear to be recognized by the endocytic machinery and the signal transduction apparatus within the cell. The carboxy-terminal cytoplasmic tail is required for the endocytosis of the receptor, while the third cytoplasmic loop plays a role in signal transduction. However, we cannot rule out the possibility that some structural features of the receptor are required for both functions. A more detailed mutational analysis of the receptor is required to further define the functional domains of the receptor.

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