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Identification of a Novel Sequence Mediating Regulated Endocytosis of the G Protein–Coupled α-Pheromone Receptor in Yeast

Jack Rohrer,* Hélène Bénédetti, Bettina Zanolari, and Howard Riezman†

Biocenter of the University of Basel, CH-4056 Basel, Switzerland

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The *Saccharomyces cerevisiae* α-pheromone receptor, a polytopic, G protein–coupled, membrane protein, is internalized after binding of α-factor. Mutational analysis suggested that the first 39 residues of the receptor's cytoplasmic tail carries sufficient information for internalization. A point mutation in one of these 39 residues, K^{337} to R^{337}, renders the receptor nonfunctional for endocytosis. Other residues, D^{335} and S^{338}, contribute to the efficiency of internalization. When the sequence DAKSS is added onto a severely truncated receptor, endocytosis of the receptor is restored, showing that this sequence functions to mediate or to signal interaction with the endocytic machinery. Analysis of pheromone response and recovery in strains expressing mutant receptors suggests that receptor internalization is not important for response but contributes to recovery from pheromone.

INTRODUCTION

The initiation of the mating program in *Saccharomyces cerevisiae* involves the exchange of peptide mating pheromones between haploid cells of the opposite mating type. These pheromones bind to haploid cell type-specific receptors (Jenness *et al.*, 1983; Burkholder and Hartwell, 1985; Nakayama *et al.*, 1985; Hagen *et al.*, 1986) that are polytopic membrane proteins responsible for transmission of the pheromone signal via a tripartite G protein and a protein kinase cascade (Dietzel and Kurjan, 1987; Miyajima *et al.*, 1987; Whiteway *et al.*, 1989; Marsh *et al.*, 1991). Binding of the pheromones to their target receptors induces several genes, the expression of cell-surface agglutinins, arrests cells in the G1 phase of the cell-cycle, and causes a change in cell shape (shmoo formation). All of these processes play a role in the mating process (Cross *et al.*, 1988; Marsh *et al.*, 1991). It has been shown that when α-factor binds to its receptor, Ste2p (Jenness *et al.*, 1983), the pheromone is internalized in a time-, temperature-, and energy-dependent manner (Chvatchko *et al.*, 1986). α-factor binding sites are cleared from the cell surface only upon addition of α-factor, showing that α-factor–receptor complexes enter the cell in a regulated fashion (Jenness and Spatrick, 1986). α-factor internalization and the regulation of α-factor–receptor uptake can be completely uncoupled from the G protein–mediated pheromone signal transduction pathway, showing that pheromone signaling is not the regulator of receptor trafficking. It was proposed, therefore, that the regulation of receptor uptake may be controlled through a conformational change of the receptor induced by ligand binding (Zanolari *et al.*, 1992). Once internalized, the α-factor is transported via vesicular intermediates to the vacuole where the pheromone is degraded (Chvatchko *et al.*, 1986; Singer and Riezman, 1990).

There are many similar G protein–coupled receptors in animal cells, but for the most part their endocytic routes are still poorly defined. Some evidence suggests that the β_2-adrenergic receptor may enter cells through surface invaginations that are not clathrin coated (Raposo *et al.*, 1989). Very little information is available concerning possible sequences or signals on these receptors that may be necessary for their regulated internalization. *S. cerevisiae* offers an advantageous system to search for such signals because the α-factor receptor is well characterized concerning its function and topology and because its endocytosis can be uncoupled from the signal transduction pathway (Marsh *et al.*,

* Present address: Division of Hematology/Oncology, Washington University School of Medicine, 660 S. Euclid, Saint Louis, MO 63110.
† Corresponding author.

1991; Zanolari *et al.*, 1992). Recent studies of the α-factor receptor (Konopka *et al.*, 1988; Reneke *et al.*, 1988) have demonstrated that sequences in the cytoplasmic tail mediate receptor internalization and desensitization but are not essential for pheromone binding or signaling. To define regions within the α-factor receptor that are essential for internalization, we have created a series of truncations and small deletions in the cytoplasmic tail. With these mutants we show that a sequence of five amino acids contains information for internalization of α-factor. Point mutations within this region reveal that a single amino acid, Lys^{337}, is an essential feature of the internalization sequence. When this sequence is added onto a severely truncated receptor that cannot undergo endocytosis, the process is restored, providing positive evidence for the endocytic function of this sequence. Experiments measuring α-factor sensitivity and recovery in receptor mutants suggest that endocytosis does not play a role in the pheromone response but does play a role in the process of recovery from pheromone.

MATERIALS AND METHODS

Materials and Media

Escherichia coli media and yeast media have been described elsewhere (Maniatis *et al.*, 1982; Dulic *et al.*, 1991). YPUAD medium contains 1% yeast extract, 2% peptone, 2% glucose, and 30 mg/l each of uracil and adenine sulphate. ^{35}S-labeled α-factor was prepared from biosynthetically labeled yeast cells overproducing the pheromone (Dulic *et al.*, 1991). α-factor was synthesized by the Biochemistry Department of the University of Lausanne (Lausanne, Switzerland). Vent Polymerase (New England Biolabs, Beverly, MA) was purchased from Flow Laboratories (Basel, Switzerland). DNA-modifying enzymes were from Biofinex (Praromen, Switzerland) or Boehringer Mannheim (Rotkreuz, Switzerland). DNA sequencing was performed according to the method of Sanger *et al.* (1977) using the Sequenase 2.0 kit from United States Biochemical (LucernaChem, Switzerland).

Plasmids and Strains

Plasmid pJR3-wt was constructed by inserting a 1.6-kilobase *Hin*dIII fragment carrying STE2 into YlpLac211 (Giez and Sugino, 1988). The resulting plasmid was cut with *Xba* I and *Sph* I; the ends were converted to blunt ends by using T_4 polymerase and ligated to remove the *Pst* I and *Sal* I site of the polylinker. The plasmid was then opened with *Sma* I and *Cla* I, and a *Sal* I- (converted to blunt ends by using the Klenow fragment of DNA polymerase) *Cla* I fragment of pAB510 (Burkholder and Hartwell, 1985) was ligated into the plasmid resulting in the plasmid pJR3-wt. The relevant features of pJR3-wt are as follows: the *STE2* gene is under the control of its own promoter, the restriction endonuclease sites of *Cla* I, *Pst* I, and *Sal* I within the *STE2* gene are unique in the plasmid, and the plasmid can be integrated into the yeast chromosome at the *ura3* locus after linearization with StuI. The mutant forms of the *STE2* gene were created using standard polymerase chain reaction (PCR) protocols (Ho *et al.*, 1989). To avoid undesired changes in the sequence, we used Vent Polymerase and subcloned only the PCR-amplified *Cla* I-*Sal* I or *Cla* I-*Pst* I fragments into pJR3-wt, respectively. The entire subcloned fragments were sequenced (Sanger *et al.*, 1977) after subcloning into pJR3-wt. The *STE2* gene was deleted and disrupted in the yeast strain RH144-3D (MAT**a** *ura3 leu2 his4 bar1-1*) using the plasmid pUSTE203 (Nakayama *et al.*, 1988) to create strain RH1298 (*Mat***a** *ura3 leu2 his4 bar1-1 Δste2::LEU2*). All other strains were created by integrating wild-type or mutant alleles of *STE2* cloned in pJR3-wt at the *ura3* locus of RH1298. Strains with single integration events were selected after analysis by Southern blotting (Southern, 1975). Table 1 contains a list of the strains used in this study.

α-Factor Uptake Assay

The α-factor uptake assay was performed as described in Dulic *et al.* (1991). Briefly, cells were grown overnight in YPUAD to ~1×10^7 cells/ml, collected by centrifugation, washed in YPUAD, and resuspended to 1×10^9 cells/ml in YPUAD on ice. The cells were then incubated with ^{35}S-labeled α-factor for 1 h on ice, washed, and resuspended in prewarmed (30°C) media. After various incubation times samples of 100 μl were taken in duplicates and diluted into either pH 6 or pH 1.1 buffer at 0°C. Samples in pH 1.1 buffer were incubated for 20 min on ice to allow dissociation of surface bound α-factor. The cells were collected on GF/C filters (Whatman, Basel, Switzerland) that were dried and counted in a liquid scintillation counter (1900 TR, Packard, Groningen, Netherlands) using 5 ml of Emulsifier Safe (Packard). The total input of ^{35}S-labeled α-factor was between 5000 and 10 000 cpm per sample (100 μl) with the values for bound α-factor found at pH 6 being ≥2000 cpm.

Receptor Clearance Assay

The assay was performed essentially according to Jenness and Spatrick (1986). Cells were grown overnight in YPUAD to a final density of 5×10^6 cells/ml. Cultures were then centrifuged; diluted to 2×10^6 cells/ml in YPUAD containing 10 mM Nα-*p*-tosyl-L-arginine methyl

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype
RH765	*MATa/MATα ura3/ura3 leu2/leu2 his4/his4*
RH151-7B	*MATa ura3 leu2 his4*
RH449	*MATα ura3 leu2 his4 lys2 bar1-1*
RH144-3D	*MATa ura3, leu2, his4, bar1-1*
RH1298	RH144-3D except *Δste2::LEU2*
RH1722	RH1298 except *STE2::URA3*
RH1860	RH1298 except *ste2-345Stop::URA3*
RH1870	RH1298 except *ste2-339Stop::URA3*
RH1871	RH1298 except *ste2-334Stop::URA3*
RH1900	RH1298 except *ste2-326Stop::URA3*
RH1728	RH1298 except *ste2-320Stop::URA3*
RH1907	RH1298 except *ste2-Δ310-314,345Stop::URA3*
RH1732	RH1298 except *ste2-Δ315-325,345Stop::URA3*
RH1908	RH1298 except *ste2-Δ325-329,345Stop::URA3*
RH1862	RH1298 except *ste2-Δ328-331,345Stop::URA3*
RH1863	RH1298 except *ste2-Δ331-335,345Stop::URA3*
RH1872	RH1298 except *ste2-Δ335-339,345Stop::URA3*
RH1864	RH1298 except *ste2-331A,345Stop::URA3*
RH1902	RH1298 except *ste2-331D,345Stop::URA3*
RH1865	RH1298 except *ste2-333A,345Stop::URA3*
RH1903	RH1298 except *ste2-333D,345Stop::URA3*
RH1904	RH1298 except *ste2-334R,345Stop::URA3*
RH1867	RH1298 except *ste2-336Q,345Stop::URA3*
RH1905	RH1298 except *ste2-335A,345Stop::URA3*
RH1866	RH1298 except *ste2-335R,345Stop::URA3*
RH1868	RH1298 except *ste2-338A,345Stop::URA3*
RH1969	RH1298 except *ste2-338D,345Stop::URA3*
RH1874	RH1298 except *ste2-338A,339A,345Stop::URA3*
RH1906	RH1298 except *ste2-337A,345Stop::URA3*
RH1873	RH1298 except *ste2-337R,345Stop::URA3*
RH2002	RH1298 except *ste2-312Stop::URA3*
RH1998	RH1298 except *ste2-312DAKSS-Stop::URA3*
RH2003	RH1298 except *ste2-312SINNDAKSS-Stop::URA3*
RH2096	RH1298 except *ste2-312SINNDARSS-Stop::URA3*

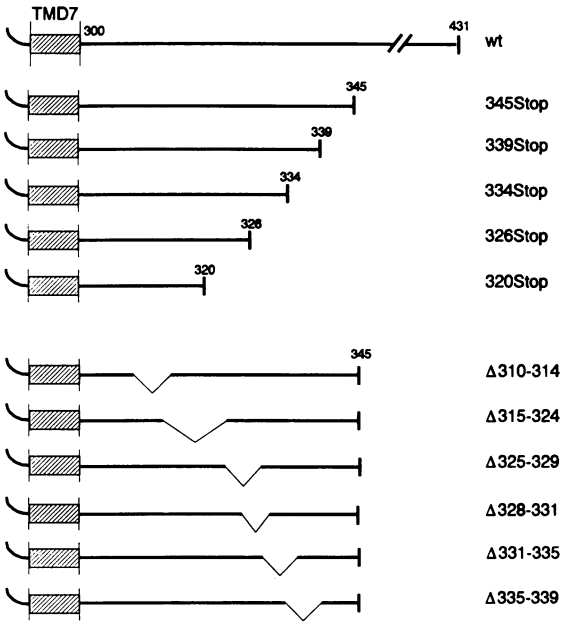

Figure 1. Ste2p cytoplasmic tail mutants. TMD7 indicates the last transmembrane domain of Ste2p, and the lines represent the cytoplasmic tails of the wild-type and of the various mutants. The numbers indicate the position of the stop-codons and the deletions, respectively. The mutant forms of Ste2p were created by site directed mutagenesis using PCR.

ester (TAME), 1.4×10^{-4} M cycloheximide; and treated with unlabeled α-factor (10^{-7} M final concentration). Control cells were mock-treated omitting α-factor. After various times 20-ml samples were taken, collected on nitrocellulose filters (HA, 0.45 μm pore size, Millipore, Bedford, MA), and washed with inhibitor media (YPUAD containing 10 mM NaN_3, 10 mM NaF, 10 mM TAME). The cells were then resuspended in 20 ml inhibitor media and incubated at 30°C for 3 h to allow the dissociation of surface-bound α-factor. After this incubation the cells were centrifuged, resuspended in inhibitor media, and stored on ice for up to 3 h. Samples of those cells were then incubated either with ^{35}S-labeled α-factor alone or with ^{35}S-labeled α-factor plus an excess of unlabeled α-factor (2×10^{-5} M) to determine background binding. After a 30-min incubation at 30°C, the cells were filtered on glass fiber filters (Whatman, GF/C) and washed twice with inhibitor media. The filters were dried and counted in a liquid scintillation counter as above.

α-Factor Response and Recovery Assays

Cells were grown overnight at 30°C in YPUAD to log phase, washed with YPUAD, resuspended to 5 (shmoo and recovery assays) or 15 (*FUS1* induction) $\times 10^6$ cells/ml, and incubated with the indicated amounts of synthetic α-factor at 30°C. After 30 min the cells were harvested, extracted, and RNA was isolated for Northern blotting and detection of FUS1 gene expression as described in Dulic and Riezman (1990) except that total RNA was separated rather than poly A^+ RNA. After 3.5 h of incubation with α-factor, shmoo formation was quantified by determining the fraction of shmoo-like cells by visualization in a hemocytometer.

For the recovery assay, cells were grown overnight at 30°C in YPUAD to a final concentration of 5×10^6 cells/ml and diluted to 2×10^6 cells/ml. These cultures were then incubated with 2.5×10^{-8} M α-factor for 90 or 120 min (separate experiments) at 30°C until all cells were unbudded. The cells were washed twice in conditioned medium from a *BAR1, MAT***a** strain that contained Bar1p, a secreted protease that degrades external α-factor. This ensured that all unbound α-factor is either removed or degraded and resulted in a more synchronous recovery of *bar1-1* mutants from α-factor treatment. Finally, the cells were resuspended in YPUAD and incubated at 30°C. Every 15 min samples were taken, and the percentage of budded cells was quantified by visualization in a hemocytometer. Conditioned medium was obtained by growing strain RH151-7B (*MAT***a**, *ura3, leu2, his4*) to $\sim 1 \times 10^7$ cells/ml, and the culture supernatant was harvested by centrifugation ($2 \times 12\,000 \times g$, 5 min).

RESULTS

Localization of Sequences Essential for STE2p Internalization

To localize the features within the cytoplasmic tail of Ste2p that play a role in receptor internalization, a series of truncations and small internal deletions were created as indicated in Figure 1. Stop codons were introduced by PCR mutagenesis at various positions along the DNA coding for the C-terminal tail of Ste2p. Mutant *ste2* alleles under control of the wild-type *STE2* promoter were integrated into the genome of yeast cells (Δ*ste2::LEU2*) at the *ura3* locus. Cells expressing mutant Ste2p, as detected by an α-factor binding assay, were then analyzed for their ability to internalize α-factor. ^{35}S-labeled α-factor was bound to cells on ice; unbound α-factor was removed and internalization was allowed to occur by shifting the cells to 30°C. The percentage of internalized α-factor was calculated as the percentage of bound pheromone that was resistant to treatment of the cells with pH 1.1 buffer.

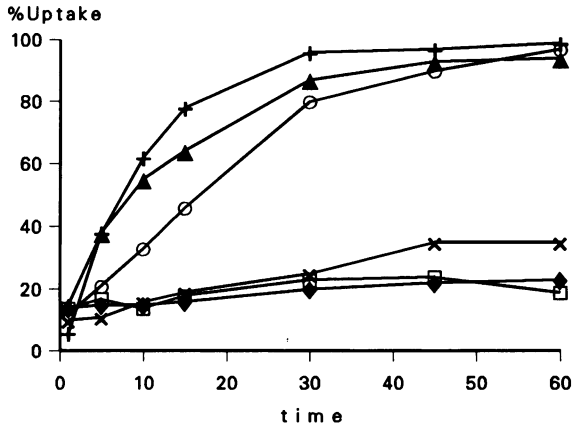

Figure 2. Information for α-factor internalization is found upstream of aa 339. Cells expressing mutant forms of Ste2p were incubated with ^{35}S-labeled α-factor, and samples were withdrawn in duplicate at the times indicated. The samples were subsequently washed in pH 6 or pH 1.1 buffer to remove unbound or unbound and surface-bound α-factor, respectively. The percentage of internalized α-factor was then determined by scintillation counting (cpm pH 1.1/cpm pH 6 × 100). The values represent averages of at least three independent experiments. +, strain RH1722 (*STE2*); ▲, strain RH1860 (*ste2-345Stop*); ○, strain RH1870 (*ste2-339Stop*); ×, strain RH1871 (*ste2-334Stop*); □, strain RH1900 (*ste2-326Stop*); ♦, strain RH1728 (*ste2-320Stop*).

Results of α-factor uptake experiments are graphed in Figure 2. Strain RH1722 (*STE2*), expressing wild-type Ste2p (Figure 2), internalized almost 100% of the bound α-factor with a $\tau_{1/2}$ = 7.5 min, as did strain RH1860 (*ste2-345Stop*) with slightly slower kinetics and strain RH1870 (*ste2-339Stop*) with still somewhat slower kinetics (Table 2). In contrast to these mutant strains, internalization by RH1871 (*ste2-334Stop*) cells was only detectable at late time points with a maximum uptake of 25%. The mutant strains RH1900 (*ste2-326Stop*) and RH1728 (*ste2-320Stop*) showed no significant uptake of α-factor. These results suggested not only that the region NH_2-terminal to position 339 in the cytoplasmic tail plays an important role but, more specifically, that residues between 334 and 339 may contain important information for internalization of α-factor, as is confirmed below.

In the following experiments we constructed deletion mutations in the vector that encodes the Ste2p, which is truncated at position 345 (Figure 1), to simplify our analysis by excluding the possibility of interference by redundant information contained downstream of this position. Deletions in the cytoplasmic tail of the receptor between amino acids (aa) 310–314 (strain RH1907, *ste2-Δ310-314,345Stop*), aa 315–325 (strain RH1732, *ste2-Δ315-325,345Stop*), or aa 325–329 (strain RH1908, *Δ325-329,345Stop*) did not strongly affect the internalization kinetics as compared with strain RH1860 (*ste2-345Stop*) (Figure 3 and Table 2). However, removal of aa 331–335 (RH1863, *ste2-Δ331-335,345Stop*) or aa 328–331 (RH1862, *ste2-Δ328-331,345Stop*) created a mutant receptor that reached ~75% internalization after 60 min and showed a distinctive lag at the initial phase of internalization.

Strikingly, the mutant strain RH1872 (*ste2-Δ335-339,345Stop*) was almost completely incapable of α-factor uptake. These results suggest that at least part of the sequence from aa 335 to aa 339 is essential for the internalization of α-factor in the truncated receptor. The sequence upstream of aa 335, although not essential, might be important for rapid internalization as indicated by the kinetics of α-factor uptake in the mutant strains RH1862 (*ste2-Δ328-331,345Stop*) and RH1863 (*ste2-Δ331-335,345Stop*). Comparison of the new sequences of the deletion mutants (Figure 4) suggested that the DAKS (AspAlaLysSer) sequence, in common between all strains capable of α-factor internalization and removed in the mutant strain RH1872 (*ste2-Δ335-339,345Stop*), is essential for internalization of the truncated receptor.

Table 2. α-factor internalization kinetics of *ste2* truncation and internal deletion mutant strains

STE2 allele	Half time of α-factor internalization (min)
wild-type	7.5
345Stop	8.5
339Stop	17
334Stop	>60
326Stop	>60
320Stop	>60
Δ310-314, 345Stop	8.5
Δ315-325, 345Stop	11
Δ325-329, 345Stop	13.5
Δ328-331, 345Stop	26
Δ331-334, 345Stop	20
Δ335-339, 345Stop	>60

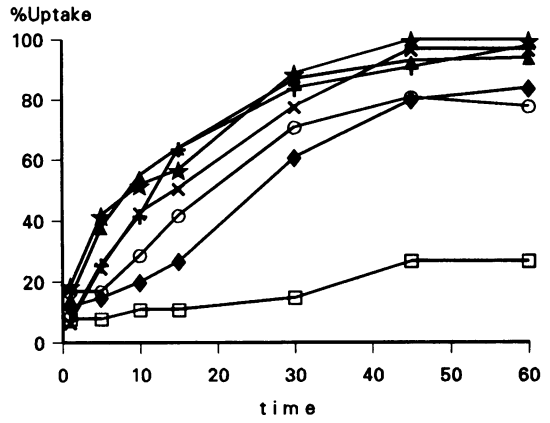

Figure 3. Internalization of α-factor by the deletion mutants reveals that aa 335–339 are essential. The cells were treated as described in Figure 2. ▲, strain RH1860 (*ste2-345Stop*); ★, strain RH1907 (*ste2-Δ310-314,345Stop*); +, strain RH1732 (*ste2-Δ315-325,345Stop*); ×, strain RH1908 (*ste2-Δ325-329,345Stop*); ♦, strain RH1862 (*ste2-Δ328-331,345Stop*); ○, strain RH1863 (*ste2-Δ331-335,345Stop*); □, strain RH1872 (*ste2-Δ335-339, 345Stop*).

Lys^{337} *Is Essential for α-Factor Internalization in the Receptor Truncated at Position 345*

To determine the contribution of individual amino acid residues to this putative internalization sequence, a series of point mutations in the 345Stop mutant receptor gene was created (Figure 4). Several amino acids upstream of position 335 were mutated. Ser^{331} was changed to alanine (strain RH1864, *ste2-331A,345Stop*) and to aspartic acid (strain RH1902, *ste2-331D,345Stop*), because it has been shown (Casanova *et al.*, 1990) that aspartic acid mimics a phosphorylated serine in at least one transport signal. Neither mutation strongly affected the internalization of α-factor (Figure 5A), although strain RH1864 (*ste2-331A,345Stop*) internalized α-factor with slower kinetics ($\tau_{1/2}$ = 20 min) than the 345Stop mutant strain carrying *ste2-345Stop*. The mutant strain RH1902 (*ste2-331D,345Stop*) did not differ significantly from the 345Stop. The amino acid Asn^{333} was changed to alanine (strain RH1865, *ste2-333A,345Stop*) or to aspartic acid (strain RH1903, *ste2-333D,345Stop*). Aspartic acid was chosen to introduce a negative charge. Corroborative uptake experiments revealed that both mu-

345Stop Allele	327												339	Internalization
	Sequence													
wt	F	Q	T	D	S	I	N	N	D	A	K	S	S	+++
Δ328-331	T	L	S	S	F	I	N	N	D	A	K	S	S	+
Δ331-335	G	T	L	S	S	F	Q	T	D	A	K	S	S	++
Δ335-339	F	Q	T	D	S	I	N	N	S	L	R	S	R	-
	327												339	
wt	F	Q	T	D	S	I	N	N	D	A	K	S	S	
331A	-	-	-	-	A	-	-	-	-	-	-	-	-	++
331D	-	-	-	-	D	-	-	-	-	-	-	-	-	+++
333A	-	-	-	-	-	-	A	-	-	-	-	-	-	+++
333D	-	-	-	-	-	-	D	-	-	-	-	-	-	+++
334R	-	-	-	-	-	-	-	R	-	-	-	-	-	+++
335A	-	-	-	-	-	-	-	-	A	-	-	-	-	+
335R	-	-	-	-	-	-	-	-	R	-	-	-	-	+
335E	-	-	-	-	-	-	-	-	E	-	-	-	-	+++
336Q	-	-	-	-	-	-	-	-	-	Q	-	-	-	+++
337A	-	-	-	-	-	-	-	-	-	-	A	-	-	-
337R	-	-	-	-	-	-	-	-	-	-	R	-	-	-
338A	-	-	-	-	-	-	-	-	-	-	-	A	-	+++
338D	-	-	-	-	-	-	-	-	-	-	-	D	-	+++
338AA	-	-	-	-	-	-	-	-	-	-	-	A	A	+

Figure 4. Point mutations within the cytoplasmic tail of Ste2p. A partial amino acid sequence (position 327–339) of the cytoplasmic tail of wild-type Ste2p and the sequence generated in some deletion mutants are shown (single letter code). The amino acids changed in the various point mutants are indicated in the lower part of the figure. The + and − symbols represent relative internalization efficiencies taking into account both the initial rates of α-factor internalization and the percent uptake after 60 min. 327 and 339 refer to the respective positions in the wild-type receptor.

tants internalized α-factor even slightly faster than the 345Stop control. This indicated that Asn^{333} was not part of the internalization sequence. To check whether the introduction of a positive charge adjacent to the Asp^{335} would disturb the ability of the receptor to internalize α-factor, Asn^{334} was changed to arginine (strain

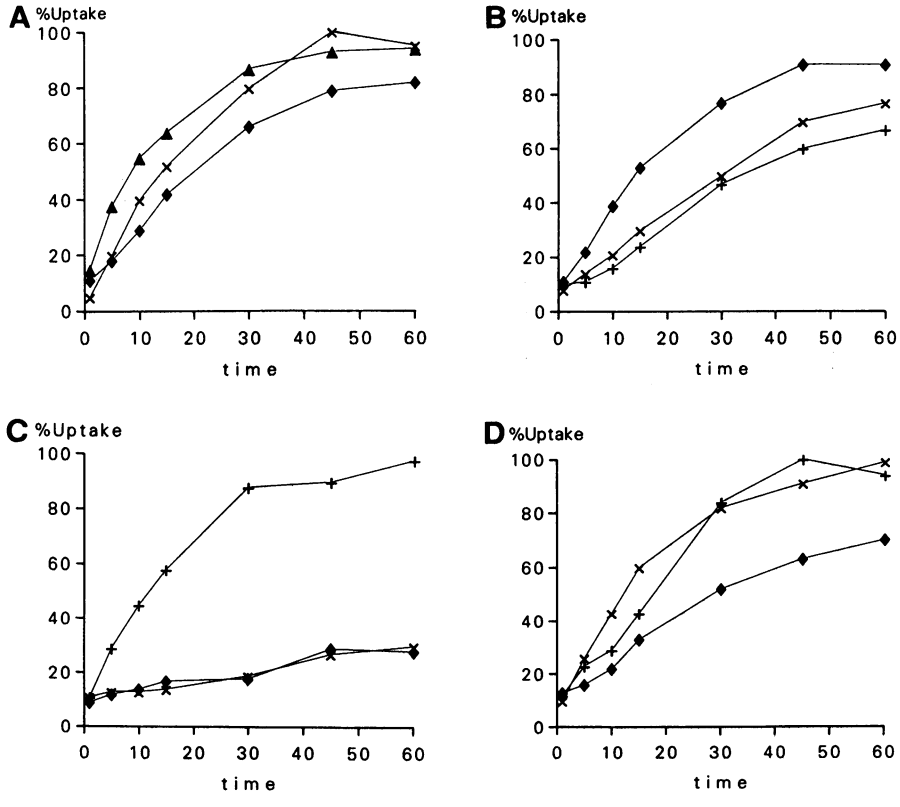

Figure 5. Internalization of α-factor by point mutants reveals that K^{337} is an essential feature. The cells were treated as described in Figure 2. (A) ▲, strain RH1860 (*ste2-345Stop*); ×, strain RH1902 (*ste2-331D,345Stop*); ♦, strain RH1864 (*ste2-331A,345Stop*). (B) ♦, strain RH1904 (*ste2-334R,345Stop*); ×, strain RH1905 (*ste2-335A,345Stop*); +, strain RH1866 (*ste2-335R,345Stop*). (C) +, strain RH1867 (*ste2-336Q, 345Stop*); ×, strain RH1906 (*ste2-337A,345Stop*); ♦, strain RH1873 (*ste2-337R,345Stop*). (D) +, strain RH1868 (*ste2-338A,345Stop*); ×, strain RH1869 (*ste2-338D,345Stop*); ♦, strain RH1874 (*ste2-338A,339A,-345Stop*).

RH1904, *ste2-334R,345Stop*). Figure 5B shows that this mutation had only a minor effect on internalization ($\tau_{1/2}$ = 13 min).

Next, we created single amino acid changes in residues 335–339 that we suggested are required for internalization. A change of Ala^{336} into the bulky nonaromatic amino acid glutamine (strain RH1867, *ste2-336Q,345Stop*) was created to see if a small amino acid in position 336 is required. This change did not affect α-factor internalization (Figure 5C). The first amino acid of the DAKS sequence, the Asp^{335}, was then changed to alanine (strain RH1905, *ste2-335A,345Stop*) and to arginine (strain RH1866, *ste2-335R,345Stop*) to replace the negative charge of Asp^{335} with a positive charge. Both mutants had a fairly strong and equivalent affect on the initial rate of α-factor uptake (Figure 5B). Internalization of 50% of the α-factor was reached after ~30 min compared with 8.5 min for the 345Stop receptor. Neither the change of the Ser^{338} to an alanine (strain RH1868, *ste2-338A,345Stop*) nor to an aspartate (strain RH1969, *ste2-338D,345Stop*) blocked internalization of α-factor (Figure 5D). However, both mutants were affected in their internalization kinetics ($\tau_{1/2}$ = 17 min for 338A and $\tau_{1/2}$ = 12 min for 338D). Because S^{339} might be able to replace a missing S^{338}, we changed both serines to alanines (strain RH1874, *ste2-338A,339A, 345Stop*). The internalization of α-factor by this mutant was significantly reduced ($\tau_{1/2}$ = 27 min), a rate comparable with that observed for the 335A and 335R mutants. Therefore, it is likely that at least one serine around position 338 is required for rapid internalization.

To evaluate the importance of position 337 in the DAKS sequence, we changed the Lys^{337} to an alanine (strain RH1906, *ste2-337A,345Stop*). This mutation completely blocked internalization of α-factor (Figure 5C). To determine if a positive charge is the essential feature of Lys^{337}, we changed the Lys^{337} to an arginine. This mutant (strain RH1873, *ste2-337R,345Stop*) was also defective for internalization, suggesting that a lysine is specifically required at position 337.

The DAKSS Sequence Is Sufficient to Restore Internalization Competence to a Severely Truncated Receptor

The above experiments showed a loss of endocytic function that correlated with the mutation of the DAKS sequence located at aa 335–339 of Ste2p. If this sequence is important for the endocytic process, then it should also be possible to show a gain of function that correlates with the presence of this sequence. To demonstrate this, we started with an α-factor receptor that was truncated at residue 312, which is well upstream of the truncations that we showed completely blocked α-factor internalization. To this receptor we added the sequences DAKSS, SINNDAKSS, and SINNDARSS and then measured α-factor uptake by yeast strains carrying the mutant receptors (Figure 6). Strains carrying the DAKSS or SINNDAKSS sequence were capable of α-factor internalization with the SINNDAKSS sequence being better than the DAKSS sequence. The SINNDARSS sequence with the essential K replaced with R was incapable of restoring internalization function to the truncated receptor. The α-factor internalization curve was not the same shape as the curves derived from strains carrying wild-type receptors, but the internalization capacity was clearly partially restored and dependent on the lysine that is normally found at position 337.

α-Factor Binding Sites Remain at the Cell Surface when Pheromone Internalization Is Blocked

In the α-factor uptake experiments described above, we followed the fate of the ligand. To confirm our conclusions regarding receptor internalization, we also quantified cell surface receptors and their subsequent disappearance after α-factor treatment. For receptor clearance assays (Jenness and Spatrick, 1986), cells were incubated with cycloheximide to prevent new receptor synthesis. α-factor (1×10^{-7} M final concentration) was added, and the cells were further incubated for various times at 30°C. Thereafter, the cells were washed, and the bound α-factor was dissociated from the receptors at the surface by incubating in the presence of NaN_3 and NaF for 3 h at 30°C. The remaining cell surface receptors were quantified by a binding assay using ^{35}S-labeled α-factor.

Figure 7 shows that ~80% of the α-factor binding sites disappeared from the surface upon addition of α-

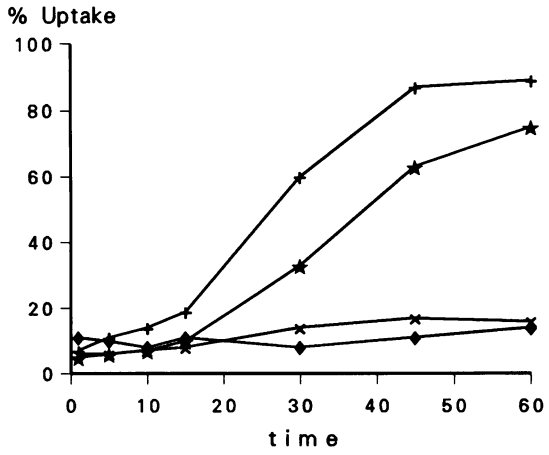

Figure 6. SINNDAKSS restores internalization competence to a severely truncated receptor. Yeast strains were cultured and uptake assays were performed as described in the legend to Figure 2. +, strain RH2003 (*ste2-312SINNDAKSS-Stop*); ★, strain RH1998 (*ste2-312DAKSS-Stop*); ◆, strain RH2096 (*ste2-312SINNDARSS-Stop*); ×, strain RH2002 (*ste2-312Stop*).

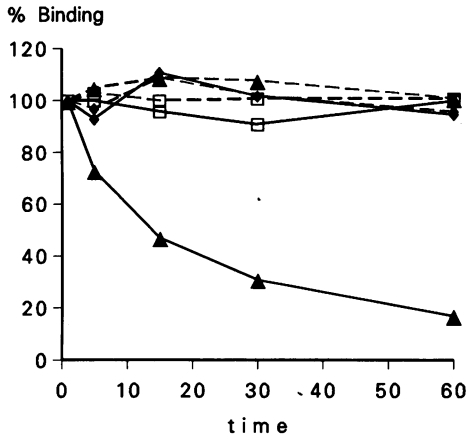

Figure 7. Mutant Ste2p receptors that do not mediate α-factor uptake remain at the cell surface. Cultures were treated with cycloheximide to inhibit protein synthesis and with 2×10^{-7} M α-factor (solid lines) or without α-factor (broken lines) starting at 0 min. Samples were withdrawn at the times indicated, washed, and incubated for 3 h in inhibitor media (YPUAD containing 10 mM NaN_3, 10 mM NaF, 10 mM TAME) to allow dissociation of surface-bound α-factor. The cells were then assayed for their capacity to bind ^{35}S-labeled α-factor. The values represent averages of two independent experiments. The experiments were standardized by setting the cpm found at 1 min to 100%. ▲, strain RH1860 (*ste2-345Stop*); □, strain RH1900 (*ste2-326Stop*); ♦, strain RH1873 (*ste2-337R,345Stop*).

factor in case of the mutant strain RH1860 (*ste2-345Stop*). In contrast, receptors remained at the surface after α-factor addition to the mutant strains RH1900 (*ste2-326Stop*) and RH1873 (*ste2-337R,345Stop*). Receptor clearance of several of the other mutant strains was measured using this assay, and the results were always consistent with the α-factor internalization results. These data show that there is a good correlation between the two assays, implying strongly that they measure the same event, receptor internalization, and show again that K^{337} can play an important role in receptor internalization.

Internalization of the α-Factor Receptor Contributes to the Recovery Process After Removal of Pheromone

To determine the effect of the internalization of the Ste2p on pheromone response, the shmoo assay was performed. This assay measures the dose of α-factor at which the cells become committed to the mating pathway. The strain RH1860 (*ste2-345Stop*) was about twofold more sensitive to α-factor treatment than the strain RH1722 (*STE2*) (Figure 8A). The strain deficient for endocytosis, RH1873 (*ste2-337R,345Stop*), showed a sensitivity to α-factor equal to that of RH1860 (*ste2-345Stop*) (Figure 8A). To examine a more sensitive and early response to pheromone, we measured the dose-dependent induction of the FUS1 gene. This gene is induced by α-factor (Trueheart *et al.*, 1987) in a rapid and direct manner (McCaffrey *et al.*, 1987). Cells were treated with various concentrations of α-factor for 30 min, total RNA was extracted, and the *FUS1* and *URA3* transcripts were detected by Northern blotting (Figure 8, B and C).

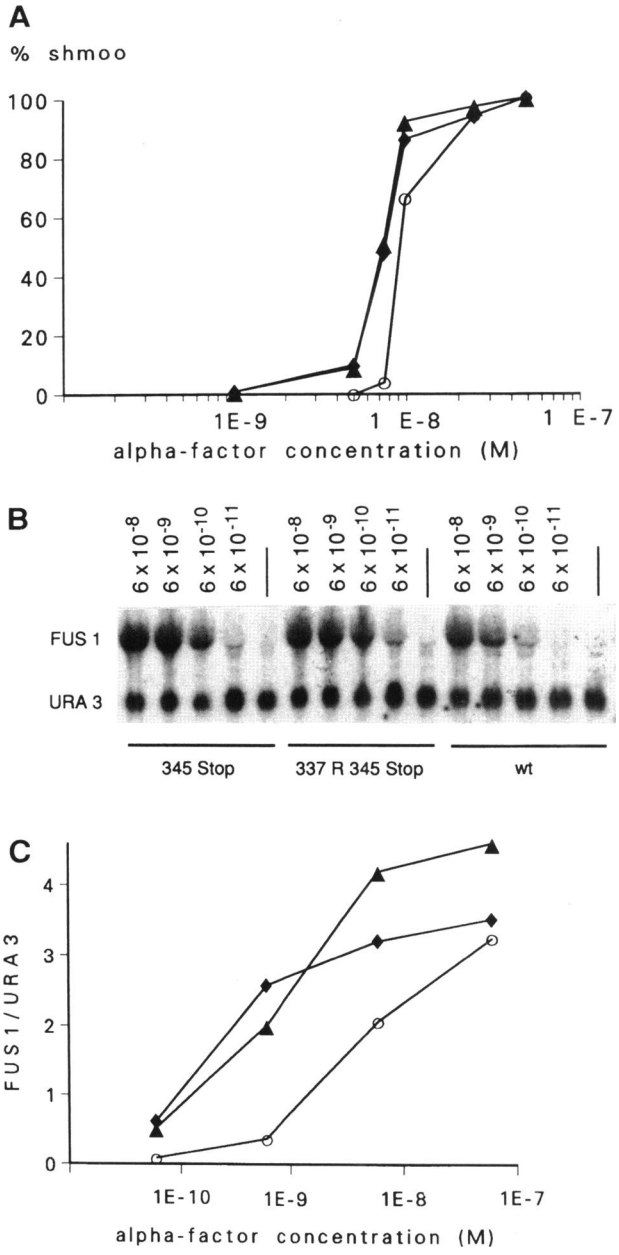

Figure 8. Response of cells to α-factor is independent of Ste2p internalization. (A) Cultures were treated with various amounts of α-factor as indicated. After 3.5 h incubation, pheromone response was quantified by determining the fraction of shmoo-like cells by visualization in a hemocytometer. The values represent averages of three independent experiments. ○, strain RH1722 (*STE2*); ▲, strain RH1860 (*ste2-345Stop*); ♦, strain RH1873 (*ste2-337R,345Stop*). (B) As in A, but cells were harvested after 30 min of α-factor treatment, RNA was extracted and analysed by Northern blotting using *FUS1* and *URA3* as probes. An autoradiograph of the Northern blot is shown. (C) Quantitation of FUS1 induction. Lighter exposures from experiments performed as in B were quantified using a scanning densitometer. Symbols as in A.

Strains RH1860 (*ste2-345Stop*) and RH1873 (*ste2-337R,345Stop*) responded similarly to α-factor for *FUS1* induction and were about an order of magnitude more sensitive than strain RH1722 (*STE2*). These results are consistent with the results from the shmoo assay and show that receptor endocytosis has no detectable influence on pheromone response.

To compare the influence of receptor internalization on the ability of cells to recover from α-factor treatment, recovery assays were performed. Cells were incubated with 2.5×10^{-8} M α-factor until arrested in G1. α-factor was then removed and the cells were allowed to recover and grow in rich media (see MATERIALS AND METHODS for details). Recovery was assessed by determining the percentage of budded cells upon visualization in a hemocytometer. After 40 min of incubation in rich media, 50% of the cells of strain RH1722 (*STE2*) were budded (Figure 9). Fifty percent of the cells of the strain RH1860 (*ste2-345Stop*) were budded after 50 min, and for the strain RH1873 (*ste2-337R,345Stop*), 50% of the cells were budded only after 140 min (Figure 9). Therefore, a single amino acid change that blocks receptor endocytosis specifically causes a delay in recovery after α-factor treatment.

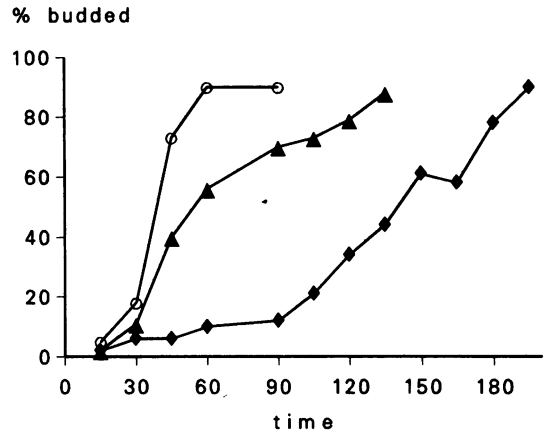

Figure 9. Ste2p internalization is required for rapid recovery of cells after exposure to α-factor. Cells were arrested in G1 by the addition of 2.5×10^{-8} M α-factor for 90 or 120 min (separate experiments). The cells were then washed in Bar1 p-conditioned media, resuspended in YPUAD, and incubated at 30°C. Samples were taken at the times indicated, and the fraction of budded cells was quantified by visualization in a hemocytometer. The values represent averages of two independent experiments. ○, strain RH1722 (*STE2*); ▲, strain RH1860 (*ste2-345Stop*); ♦, strain RH1873 (*ste2-337R,345Stop*).

DISCUSSION

In this study we have shown that a small region of four to eight amino acids of the cytoplasmic tail of Ste2p can play a role in the targeting of the protein into the endocytic pathway. A single amino acid (K^{337}) within this region is essential for the activity of this signal, whereas other nearby residues can also have an influence on the efficiency of internalization. Using an endocytosis-deficient receptor point mutant, we probed the relationships between receptor endocytosis and response and recovery from pheromone. The capacity of the receptor to undergo ligand-mediated internalization does not influence its capacity to transmit the pheromone signal, but receptor internalization seems to be one of the mechanisms that allows rapid recovery from pheromone.

Truncations of the cytoplasmic tail of Ste2p defined the region upstream of aa 339 as one region that is sufficient for receptor internalization, because the strain RH1870 (*ste2-339Stop*) was still functional, whereas the strain RH1871 (*ste2-334Stop*) was not. With internal deletions in the cytoplasmic tail of Ste2p truncated at position 345, we were able to show that there is a small region of 5 amino acids downstream of aa 334 (position 335–339) that is essential for internalization and receptor clearance. Two other deletions of sequences upstream of position 335 (Δ328–331 and Δ331–335) had fairly strong effects on the initial rate of α-factor internalization. The effect of the Δ328–331 mutation was greater than for the Δ331–335 mutation. This could be explained in two ways. First, the upstream information, especially the serine at position 331, could be part of the sequence information that is necessary for efficient endocytosis. In the Δ328–331 mutation this serine is replaced by a phenylalanine. Changing this serine to alanine (A^{331}) had less effect and a change to aspartate (D^{331}) even less (Figure 4). It is possible that this serine could be a phosphorylation target as the receptor tail is heavily phosphorylated (Reneke *et al.*, 1988; Zanolari *et al.*, 1992). In this event an aspartic residue would resemble a phosphoserine more than an alanine or a bulky phenylalanine (Casanova *et al.*, 1990). A second explanation of these results is that these deletions prevent the sequence between 335 and 339 from assuming its correct conformation.

Further analysis of point mutations revealed that there is only one amino acid within the truncated receptor that is essential for internalization of α-factor and receptor clearance, K^{337}. The requirement for a lysine at position 337 is highly specific because replacement by an arginine is nonfunctional. Mutations changing D^{335} and $S^{338}S^{339}$ strongly reduced the initial rate of internalization but did not completely block the process as mutations of K^{337} did. Either one of the two serines at positions 338 and 339 seemed to be sufficient to give the receptor near wild-type uptake characteristics. At position 335 it seems that the negative charge is important, because replacement of D with E gave a receptor that was fully functional for endocytosis, whereas replacement with an A or R did not. The finding that all of the receptor mutants transduced the pheromone signal and bound normal amounts of α-factor at the cell surface indicates that the biosynthesis, transport to the cell surface, ligand binding, and interaction with the G protein of the mutant receptors were intact.

Therefore, it is unlikely that the mutant receptors are grossly misfolded.

The strongest argument that the DAKSS sequence has a function for endocytosis is the experiment where it was added back onto the severely truncated receptor. Only a sequence with K, not with R, was capable of driving the receptor into the endocytic pathway. The kinetics of uptake of α-factor by these mutant receptors was, however, somewhat different than by wild-type receptors. A lag period was seen before a period of high efficiency uptake. This abnormal behavior could be due to a disturbance of the mechanism of regulation of receptor internalization that would introduce a new rate-limiting step into the internalization process. It could also be due to difficulties for the receptor to undergo the ligand-induced conformational change that has been proposed to be necessary for receptor internalization (Zanolari *et al.*, 1992). In any event the clear difference between the behavior of the 312-SINNDARSS-Stop and 312-SINNDAKSS-Stop alleles allows us to conclude that the sequence DXKS (AspXLysSer) is a sequence motif that contains information that promotes the internalization of Ste2p (*ste2-345Stop*) with the lysine being the major determinant.

Our results are in apparent contradiction to the data published by Konopka *et al.* (1988). They described a frameshift mutant Ste2p (*ste2-T326*) modified at position 326 by insertion of a linker, which was still capable of internalization and receptor clearance. However, in our hands, strain RH1298 (*Δste2::LEU2*) transformed with a CEN plasmid carrying the *ste2-326T* allele (plasmids kindly provided by J.B. Konopka, State University of New York, Stony Brook) bound, but did not internalize, α-factor, nor did it clear receptors from the surface. Our results do not appear to be subject to differences in temperature or genetic background because we obtained identical results when the test was performed at 24°C using a diploid strain. Diploid cells control the internalization of Ste2p normally, even though they do not respond to pheromones (Zanolari *et al.*, 1992). The results we obtained with the mutants under these conditions corresponded perfectly with our results presented above.

Reneke *et al.* (1988) reported that a receptor frameshift mutation at position 313 was partially capable of receptor clearance from the cell surface. Even though position 313 is upstream of the sequence we have identified, 35 random amino acids were added and these amino acids could play a role in internalization. In fact, there is a KXS sequence within this stretch of amino acids that could lead to the described phenotype. Because some of the point mutations we created (*ste2-335A,345Stop; ste2-335R,345Stop*) also had partial defects, we do not consider the data as inconsistent with our results.

Similar sequences to the DXKS sequence are also found in proteins homologous to Ste2p. There is a DXKXS sequence present in the cytoplasmic tail of Ste3p; the receptor for **a**-factor; and in the cytoplasmic tail of Mam2p (DXK), a homolog of Ste2p in *Schizosaccharomyces pombe* (Kitamura and Shimoda, 1991). The distance of those sequences relative to the predicted end of the last transmembrane domain varies from 29 amino acids (Mam2p) to 100 amino acids (Ste3p).

There are at least two possible functions for this novel sequence determinant. First, it could be involved in the regulation of receptor internalization. One way that this sequence could affect the regulation of receptor endocytosis is if it affects hyperphosphorylation. The receptor is hyperphosphorylated under conditions where it is internalized (Reneke *et al.*, 1988; Zanolari *et al.*, 1992). It has not, however, been demonstrated that hyperphosphorylation plays any role in the internalization process. If this sequence is involved only in regulation of the uptake, then it is likely that one of the cytoplasmically exposed loops of the receptor has the complementary information that would be necessary for interaction with the endocytic machinery.

Second, the SINNDAKSS sequence could be an internalization signal directing interaction with the endocytic machinery. In this case it would be significantly different from the tyrosine-containing signals found thus far in animal systems that are believed to carry out this function (Lazarovits and Roth, 1988; Chen *et al.*, 1990; Glickman *et al.*, 1989; Beltzer and Spiess, 1991), because these contain an essential aromatic residue. The 312-SINNDAKSS-Stop allele of the receptor has no aromatic residues in its cytoplasmic tail. Recently, a novel dileucine motif has been found directing endocytosis (Letourneur and Klausner, 1992) of a single pass membrane protein. Therefore, it is possible that we have not yet discovered all of the possible endocytic signals, especially due to the fact that some classes of proteins (type III) and most species have hardly been explored. There have been few systematic searches for sequences required for endocytosis of a polytopic G protein–coupled membrane proteins even though a small deletion in the cytoplasmic tail of the β_2-adrenergic receptor has been shown to affect its sequestration (Hausdorff *et al.*, 1991).

Another possible explanation for the difference between this sequence and Y-signals would be that Ste2p is internalized via a pathway that does not require clathrin-coated pits. The tyrosine internalization signal directs accumulation in clathrin-coated pits (Anderson *et al.*, 1977; Davis *et al.*, 1986; Lazarovits and Roth, 1988). We consider this unlikely because a clathrin heavy chain mutant (*chc1*) is substantially defective in α-factor internalization (Payne *et al.*, 1988). However, some differences in internalization structures may exist between yeast and vertebrates as well as within vertebrate cells. In yeast, actin is essential for lucifer yellow and α-factor uptake (E. Kübler and H. Riezman, 1993), whereas this is the case for clathrin-dependent endo-

cytosis only from the apical surface of Madin-Darby canine kidney cells (Gottlieb *et al.*, 1993).

Other possibilities are that yeast have different targeting sequences than mammalian cells or that they use these targeting sequences for different targeting steps. Some protein-targeting signals, such as the mannose-6-phosphate signal, do not seem to be conserved between yeast and mammals (Valls *et al.*, 1990). Recently, it was shown that mutation of a canonical tyrosine-containing signal in the cytoplasmic tail of the late Golgi enzyme, Kex2p, disrupts its retention in the Golgi and results in its transport to the vacuole without prior passage to the plasma membrane (Wilcox *et al.*, 1992). Therefore, yeast may use this mammalian endocytic signal for other purposes.

Using our mutants, we could define the functions of receptor internalization for pheromone response and recovery. Cells with receptors that are or are not endocytosed showed no differences in pheromone response. These results agree well with the data published by Reneke *et al.* (1988), who reported that truncated receptors that are not internalized were able to transduce the pheromone signal. Their assay for pheromone response, the halo assay, measured both response and recovery or adaptation to pheromone. Using two assays, one that measures response and the other recovery, we could show that mutations that block receptor endocytosis do not cause a supersensitivity to pheromone but result in a delay in recovery from α-factor. Therefore, we hypothesize that receptors with bound α-factor have to be cleared from the surface to allow efficient recovery. Noninternalized receptors with bound α-factor may continue signaling, thus increasing the time it takes for the cells to recover. If this were the only mechanism for desensitization, then the cells would not recover until α-factor dissociates from the receptor. However, at least two other avenues for desensitization may be used: hyperphosphorylation of the receptor tail (Reneke *et al.*, 1988; Zanolari *et al.*, 1992) and phosphorylation of the β-subunit of the trimeric G protein involved in mating (Cole and Reed, 1991). Interestingly, two mutations that caused adaptation defects, a mutation in the β-subunit of the trimeric G protein that prevents its phosphorylation and a *ste2* mutation (*ste2-326T*) causing a frameshift at position 326, showed a synergistic effect when combined (Cole and Reed, 1991). From our data, we now know that this mutant receptor was not internalized. In conclusion, it seems that the cell has adopted multiple synergistic mechanisms to downregulate the pheromone receptor activity efficiently once the receptor has performed its task and that receptor endocytosis is one of these mechanisms.

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