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# Regulation of the G-Protein-Coupled α-Factor Pheromone Receptor by Phosphorylation

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**The α-factor pheromone receptor activates a G protein signaling cascade that stimulates *MAT*a yeast cells to undergo conjugation. The cytoplasmic C terminus of the receptor is not necessary for G protein activation but instead acts as a regulatory domain that promotes adaptation to α-factor. The role of phosphorylation in regulating the α-factor receptor was examined by mutating potential phosphorylation sites. Mutation of the four most distal serine and threonine residues in the receptor C terminus to alanine caused increased sensitivity to α-factor and a delay in recovering from a pulse of α-factor. $^{32}PO_4$ labeling experiments demonstrated that the alanine substitution mutations decreased the in vivo phosphorylation of the receptor. Phosphorylation apparently alters the regulation of G protein activation, since neither receptor number nor affinity for ligand was significantly altered by mutation of the distal phosphorylation sites. Furthermore, mutation of the distal phosphorylation sites in a receptor mutant that fails to undergo ligand-stimulated endocytosis caused increased sensitivity to α-factor, which suggests that regulation by phosphorylation can occur at the cell surface and is independent of endocytosis. Mutation of the distal serine and threonine residues of the receptor also caused a slight defect in α-factor-induced morphogenesis, but the defect was not as severe as the morphogenesis defect caused by truncation of the cytoplasmic C terminus of the receptor. These distal residues in the C terminus play a special role in receptor regulation, since mutation of the next five adjacent serine and threonine residues to alanine did not affect the sensitivity to α-factor. Altogether, these results indicate that phosphorylation plays an important role in regulating α-factor receptor function.**

Peptide mating pheromones stimulate conjugation between opposite mating type cells of the budding yeast *Saccharomyces cerevisiae*. *MAT***a** cells secrete **a**-factor pheromone, and *MAT*α cells secrete α-factor pheromone, which bind to cell surface receptors on cells of the opposite mating type and activate a signaling pathway that stimulates the cells to undergo conjugation and fuse to form *MAT***a**/α diploid cells (30, 33, 44). The pheromone signal transduction components in yeast cells show remarkable similarity to the signaling cascades present in other eukaryotic organisms. The pheromone receptors for α-factor, encoded by *STE2* (4, 36), and **a**-factor, encoded by *STE3* (15, 36), contain seven transmembrane domains, which make them members of a large family of receptors that includes rhodopsin and the β-adrenergic receptor (11). Receptors in this family function by stimulating the α subunit of a trimeric G protein to exchange GTP for GDP and dissociate from the $G_{\beta\gamma}$ subunits (17). During pheromone stimulation, free $G_{\beta\gamma}$ activates the downstream signaling components which include homologs of MEKK, MEK, and mitogen-activated protein kinases (18). A pheromone-responsive transcription factor, Ste12p, stimulates transcription of mating-specific genes (13, 43).

The pheromone signal pathway must be properly regulated for cells to mate efficiently. Mutants which are hypersensitive to pheromone mate inefficiently and are defective in choosing appropriate mating partners (21, 22). The functions that regulate signal transduction during conjugation also act to promote adaptation in cells that have been exposed to pheromone but fail to mate. The *SST2* gene plays an important role in regulating signal transduction because *sst2* mutants are ≥100-fold more sensitive to pheromone (5, 9). The *MSG5* and *AFR1* genes are also thought to regulate pheromone signaling because their overexpression causes cells to become resistant to α-factor. *MSG5* encodes a protein phosphatase that acts on the mitogen-activated protein kinase homolog *FUS3* (12), and *AFR1* may act on the pheromone receptors (27). Genetic evidence suggests that other genes that regulate pheromone signal transduction are likely to exist (8, 10, 19, 32).

The pheromone receptors are regulated in a complex manner. The N terminus of the pheromone receptors, which contains the seven transmembrane domains, carries out ligand binding and G protein activation. The cytoplasmic C terminus regulates receptor signaling; truncation of the C-terminal domain causes increased pheromone sensitivity and a defect in adaptation (2, 28, 37). One function of the α-factor receptor C terminus is to mediate pheromone-stimulated receptor endocytosis, which downregulates receptors by removing them from the cell surface (40, 46). It has also been suggested that the α-factor receptor may be regulated by phosphorylation, since other G-protein-coupled receptors are regulated by phosphorylation (28, 37, 46). In particular, biochemical analysis of the β-adrenergic receptor and rhodopsin indicates that activated receptors are desensitized because they are phosphorylated on C-terminal residues (11, 31). The C-terminal sequences of the α-factor receptor are phosphorylated in vivo (37, 46), but the significance of this has been unclear since the yeast receptor kinase has not yet been identified. Therefore, the role of phosphorylation in the regulation of the α-factor receptor was examined in this study by analyzing the effects of mutating po-

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TABLE 1. Yeast strains and plasmids

| Strain or plasmid | Description | Source |
|---|---|---|
| Strains | | |
| JK7441-4-2 | *MAT***a** *ade2-1$^o$ cry1 his4-580$^a$ leu2 lys2$^o$ trp1$^a$ ura3 SUP4-3$^{ts}$ bar1-1 ste2-T326* | J. Konopka |
| DJ211-5-3 | *MAT***a** *ade2-1$^o$ cry1 his4-580$^a$ leu2 lys2$^o$ trp1$^a$ tyr1$^o$ ura3 SUP4-3$^{ts}$ bar1-1* | D. Jenness |
| DJ213-6-3 | *ste2*::*LEU2 MAT***a** *ade2-1$^o$ cry1 his4-580$^a$ leu2 lys2$^o$ trp1$^a$ tyr1$^o$ ura3 SUP4-3$^{ts}$* | D. Jenness |
| DJ213-7-3 | *ste2*::*LEU2 MAT***a** *ade2-1$^o$ cry1 his4-580$^a$ leu2 lys2$^o$ trp1$^a$ tyr1$^o$ ura3 SUP4-3$^{ts}$ bar1-1* | D. Jenness |
| QCY1 | As DJ211-5-3 except *ste2*::*URA3* | This study |
| QCY2 | As QCY1 except *ste2-D297-391* | This study |
| QCY3 | As QCY1 except *ste2-D4ala* | This study |
| QCY4 | As QCY1 except *STE2(R1)* | This study |
| QCY5 | As QCY1 except *ste2-4ala* | This study |
| Plasmids | | |
| pQC4 | *CEN3 ARS1 URA3 STE2(R1)* | This study |
| pQC2 | As PQC4 except *ste2-D297-391* | This study |
| pQC3 | As PQC4 except *ste2-D4ala* (*ste2-D297-391 S398A T411A T414A T425A*) | This study |
| pQC5 | As PQC4 except *ste2-4ala* [*ste2-(R1) S398A T411A T414A T425A*] | This study |
| pQC6 | As PQC4 except *ste2-5ala* [*ste2(R1) T382A T384A S385A S386A T389A*] | This study |
| pQC7 | As PQC4 except *ste2-9ala* [*ste2(R1) T382A T384A S385A S386A T389A S398A T411A T414A T425A*] | This study |
| pQC8 | As PQC4 except *ste2(R1) T425A* | This study |
| pQC9 | As PQC4 except *ste2(R1) S398A T425A* | This study |
| pQC10 | As PQC4 except *ste2(R1) T411A T414A T425A* | This study |
| pQC391 | As PQC4 except *ste2-T391* | This study |

tential sites of phosphorylation in the C terminus of the α-factor receptor.

## MATERIALS AND METHODS

**Strains and media.** Cells were grown either in rich YPD medium or in synthetic medium containing adenine and amino acids but lacking uracil to select for plasmid maintenance (42). Plasmids were transformed into yeast strains by using lithium acetate (20). The yeast strains used in this study were derived from the 381G strain (16) and are shown in Table 1.

**Construction of *STE2* C-terminal mutants.** The *ste2-D4ala* mutation was constructed as a derivative of the *ste2-D297-391* mutation (28). A *Bam*HI site 5′ of the *ste2-D297-391* gene in pJBK-063-16 was destroyed by filling in the overhang with the Klenow fragment of DNA polymerase to create pQC2. The only remaining *Bam*HI site in pQC2 is at the 3′ end of the *ste2-D297-391* gene. This made it possible to insert the *Eco*RI-*Bam*HI fragments containing the alanine substitution mutations in frame with *STE2* codons 1 to 297 in this vector. The oligonucleotide-directed site-specific mutagenesis method developed by Kunkel et al. (29) was used to mutate serine and threonine codons to alanine in *STE2*. The *Eco*RI-*Bam*HI fragment from plasmid pQC2 was cloned into phagemid pBluescript II to create plasmid pX2. pX2 was transformed into *Escherichia coli* XL1-Blue, which was then infected with helper phage M13KO7 (VWR Scientific), and then the single-stranded DNA was isolated from the supernatant. Oligonucleotides complementary to the single-stranded template but which with the indicated serine or threonine codon mutated to alanine were synthesized and then used to prime in vitro DNA synthesis. Mutation of Ser-398 (AGT→GCT), Thr-411 (ACT→GCT), Thr-414 (ACG→GCG), and Thr-425 (ACT→GCT) to alanine was confirmed by DNA sequence analysis with a dideoxy DNA sequencing kit from United States Biochemical. The *Eco*RI-*Bam*HI fragment containing the four alanine substitution mutations was cloned back to pQC2 to replace the wild-type C terminus to create plasmid pQC3 (*ste2-D4ala*).

In order to construct mutations in the full-length receptor, PCR was used to insert an *Eco*RI restriction site after codon 391 of the wild-type *STE2* gene to create the *STE2(R1)* allele. An *Hpa*I-*Eco*RI fragment of *STE2(R1)* was used to replace the *Hpa*I-*Eco*RI of pQC2 to create *STE2(R1)* plasmid pQC4. The same *Hpa*I-*Eco*RI fragment was used to replace the *Hpa*I-*Eco*RI fragment of pQC3 to create pQC5 (*ste2-4ala*). Five additional alanine substitutions were introduced by using a PCR primer that introduced the *Eco*RI site after codon 391 and also mutated serine and threonines to alanine at codons Thr-382 (ACA→GCA), Thr-384 (ACG→GCG), Ser-385 (AGT→GCT), Ser-386 (TCA→GCA), and Thr-389 (ACT→GCT). The *Hpa*I-*Eco*RI fragment resulting from the PCR product was cloned into pQC2 to create pQC6 (*ste2-5ala*) and cloned into pQC3 to create pQC7 (*ste2-9ala*). The *ste2-T391* truncation mutant was constructed by introduction of a stop codon prior to the *Eco*RI site in a PCR primer. All of the receptor mutations were confirmed by DNA sequence analysis. The mutant receptor genes were integrated into the genome by using homologous recombination to replace the *ste2*::*URA3* gene in strain QCY1. All of the mutations were recessive to wild-type *STE2*.

**α-Factor-induced responses.** Halo assays were performed by adding 20 μl of α-factor to a sterile filter disk (Difco, Detroit, Mich.), which was then applied to the surface of an agar plate that had been spread with a lawn of $10^5$ stationary-phase cells. Plates were incubated at 30°C for 2 days. The halo size was determined as the diameter of the zone of growth inhibition. Values reported represent the average of four independent sets of halo assays. The standard deviation of the halo measurements was always less than 10% except for the data points obtained with the lowest concentrations of α-factor, for which the standard deviation was less than 15%. α-Factor induction of *AFR1-lacZ* was assayed in cells carrying plasmid pJK33 (27), a derivative of YEplac195 (14) carrying an *AFR1-lacZ* fusion gene. Cells were grown to logarithmic phase in selective medium and then diluted to $2 \times 10^6$ cells per ml with rich YPD medium. After 2.5 h of growth, these cultures were split into aliquots and incubated with various concentrations of α-factor for 1.5 h. Inductions were stopped by adding 10 μg of cycloheximide per ml, and then β-galactosidase assays were performed with the colorimetric substrate ONPG (*o*-nitrophenyl-β-D-galactopyranoside) (34). The assays were performed in duplicate, and the average value was reported. α-Factor-induced morphogenesis was investigated by incubating *MAT***a** cells in YPD medium with $10^{-6}$ M α-factor for 0, 3, 6, or 9 h at 30°C. Cells were fixed with formaldehyde, and then 400 cells of each sample were examined for the formation of acute projections of new cell growth that are induced by α-factor (35). Cell morphology was photographed with Kodak TMAX film in an Olympus BH2 microscope equipped with differential interference (Nomarski) optics.

Recovery from α-factor-induced cell division arrest was examined in liquid culture. An overnight culture of cells was diluted to $3 \times 10^6$ cells per ml and then grown to about $1 \times 10^7$ cells per ml in YPD medium at 30°C, diluted to $2 \times 10^6$ cells per ml, and then incubated for 1.5 h in the presence of α-factor ($10^{-7}$ M) to synchronize cells at the unbudded $G_1$ phase. Synchronized cells were collected by centrifugation and washed twice with conditioned medium from a *MAT***a***-BAR1* strain, which contains a protease that degrades α-factor and helps to remove it from the medium. The cells were then resuspended in fresh YPD medium and incubated for 3 h at 30°C. Every 15 min, a 1-ml sample was withdrawn, and formaldehyde was added to 5% to block further cell growth. A total of 400 cells from each time point were then examined microscopically to determine the fraction of cells containing buds. Conditioned medium was prepared by filter sterilizing the supernatant of strain DJ213-6-3.

**Radiolabeling of the Ste2p protein.** Cells were metabolically labeled with $^{32}PO_4$ or $^{35}S$ essentially as described before (37, 46). Strains QCY4 and QCY5 were grown to a cell density of $10^7$ cells per ml in YPD medium at 30°C, harvested by centrifugation, and resuspended in low-phosphate medium at a density of $2 \times 10^6$ cells per ml. After a 4-h incubation, the cells were collected by centrifugation, washed with medium lacking phosphate, and resuspended in two 1-ml aliquots of medium lacking phosphate at a final density of $5 \times 10^7$ cells per ml. $^{32}PO_4$ (1 mCi; ICN, Costa Mesa, Calif.) was added to each aliquot, the cells were incubated for 30 min at 30°C, and then α-factor ($10^{-6}$ M) was added to one set of samples. The incubation was continued for an additional 15 min, and then the labeling period was stopped by the addition of 0.1 ml of 10× stop solution (0.1 M sodium pyrophosphate, 0.1 M $NaNO_3$, 0.1 M NaF, 4 mM EDTA, 4 mM $Na_3VO_4$). A similar procedure was used to label cells with 100 μCi of a mixture

of [$^{35}$S]methionine and [$^{35}$S]cysteine (Tran$^{35}$S-Label; ICN). The labeled cells were recovered by centrifugation, and the cell pellet was frozen at −70°C. Cells were lysed by resuspension in 100 μl of lysis buffer (8 M urea, 40 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA, 2% sodium dodecyl sulfate [SDS]) and vortexing four times for 1 min each in the presence of glass beads. The extracts were diluted 20-fold with immunoprecipitation buffer (1% Triton X-100, 20 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA) containing the protease inhibitors phenylmethyl sulfonyl fluoride (0.5 mM), AEBSF (1 mM), benzamidine (1 mM), leupeptin (1 μg/ml), pepstatin (1 μg/ml), and aprotinin (1 μg/ml) and stop solution. The extracts were centrifuged (11,000 × *g*) at 4°C for 15 min, and then the supernatant was transferred to clean tubes. Equal portions of the extract were incubated with preimmune serum or with a mixture of anti-N- and anti-C-terminal Ste2p antibodies (28) on ice for 3 h, and then the immune complexes were allowed to bind to protein A-Sepharose beads for 1 h. The immunoprecipitated proteins were eluted from the beads with lysis buffer and by heating to 42°C for 10 min. The samples were diluted 20-fold with immunoprecipitation buffer and then reprecipitated in order to eliminate a background band that migrated near the ste2-D297-391p protein. The labeled proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and were then detected by autoradiography with Kodak X-Omatar film at −70°C with a screen for 7 days. Some gels were reexposed for 1 month to attempt to detect weaker signals.

**α-Factor binding assays.** α-Factor binding assays were carried out as described previously (24, 40). Cells were grown overnight to logarithmic phase, collected by centrifugation, washed twice with ice-cold inhibitor medium (IM; YPD medium containing 10 mM KF and 10 mM $NaN_3$), and then resuspended at a density of $10^9$/ml. Cells (50 μl) were mixed with 50 μl of $^{35}$S-α-factor and incubated for 30 min, the cells were collected on a Whatman GF/C filter, and the unbound α-factor was removed by washing. Nonspecific binding was determined by performing parallel reactions in the presence of a 100-fold excess of unlabeled synthetic α-factor. All assay points were performed in duplicate, and the results presented represent the average of three independent assays. $^{35}$S-labeled α-factor was purified as described previously (40). Essentially, *MAT*α cells were labeled with $^{35}SO_4$, and the radiolabeled α-factor was purified from the supernatant by chromatography on a Bio-Rex 70 column. Radiolabeled α-factor was adjusted to a specific activity of ~2 Ci/mmol with unlabeled synthetic α-factor prior to the assay.

**Western immunoblotting and receptor stability assays.** For Western immunoblot assays, $2 \times 10^8$ log-phase cells were harvested by centrifugation, washed once in cold water, and vortexed with 0.2 ml of lysis buffer and glass beads as described above. Extracts were centrifuged at 11,000 × *g* for 3 min, and the supernatants were resolved by electrophoresis on a 10% polyacrylamide–SDS gel. The gels were transferred to nitrocellulose, and the blot was incubated with a mixture of anti-N-terminal and anti-C-terminal anti-Ste2p antibodies (28) followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibodies. The Ste2p proteins were detected by chemiluminescence with an Amersham ECL kit. The stability of the Ste2p proteins was assayed essentially as described before (40). Logarithmic-phase cells were adjusted to $10^7$ cells per ml, and then cycloheximide was added to 20 μg/ml. After a 10-min incubation, α-factor was added to $10^{-7}$ M final concentration, and samples were withdrawn at various times for analysis. Cell extracts were prepared as described above, and the protein concentration was determined with a Pierce (Rockford, Ill.) BCA Protein Assay kit. Equal amounts of protein (~50 μg) were loaded in each lane of an SDS-polyacrylamide gel, and the Ste2p proteins were detected by immunoblotting as described above. The chemiluminescent signal was detected with Kodak X-ray film, and the bands were quantified with an UltraScan XL scanning laser densitometer.

## RESULTS

**Identification of C-terminal serine and threonine residues that are required for normal α-factor sensitivity.** The observation that mutants with C-terminal truncations of the α-factor receptor show ≥10-fold increased sensitivity suggested that this receptor may be regulated by phosphorylation on C-terminal sequences, similar to the β-adrenergic receptor and rhodopsin (28, 37). However, the biochemical approaches used to examine these other receptors are not readily applicable to the α-factor receptor at this time because there are no convenient assays to monitor receptor activity in vitro since the receptor has not been purified and the target of the pheromone-stimulated G protein is not defined. Therefore, a genetic approach was used to investigate the effects of mutating potential sites of phosphorylation in the C terminus. One problem in implementing this genetic strategy is that the 134-residue C terminus of the wild-type receptor contains a combined total of 34 serine and threonine residues (4, 36). In order to focus on the residues that are important for receptor regulation, we identified

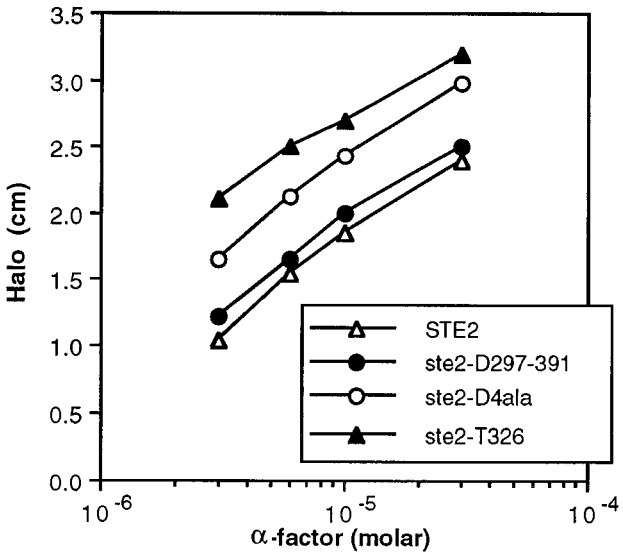


FIG. 1. α-Factor sensitivity of *ste2-D297-391* and *ste2-D4ala* strains. Sensitivity to α-factor was determined in a halo assay for cell division arrest. The *y* axis shows the diameter of the halo, a zone of growth inhibition, caused by adding 20 μl of the concentration of α-factor indicated on the *x* axis to a lawn of cells on an agar plate. Plasmids carrying the *STE2*, *ste2-D297-391*, *ste2-D4ala*, and *ste2-T326* genes were introduced into *ste2*::*LEU2* strain DJ213-7-3 for this analysis.

an internal deletion mutation, *ste2-D297-391*; the mutant carrying this mutation shows nearly normal α-factor sensitivity yet contains only one serine and three threonine residues in its C terminus. The *ste2-D297-391* mutant contains an in-frame deletion that removes codons 297 to 391, which results in the distal 40 amino acids of the C terminus being fused to the end of the seventh transmembrane domain. The remaining serine and three threonine residues in the C terminus of this receptor were mutated to the nonphosphorylatable residue alanine by oligonucleotide-directed mutagenesis to construct mutation *ste2-D4ala* (see Materials and Methods). The receptor genes were inserted into yeast-*E. coli* shuttle plasmid YCplac195 and transformed into a receptor deletion strain (*ste2*::*LEU2* strain DJ213-7-3) for analysis of receptor activity.

The pheromone sensitivity of wild-type and mutant strains was quantified in a halo assay which measures α-factor-induced cell division arrest (Fig. 1). The *ste2-D297-391* strain displayed a slight (<2-fold) increase in sensitivity relative to the wild type, as shown previously (28). Mutation of the C-terminal serine and three threonine codons to alanine to create the *ste2-D4ala* strain caused an additional 2.5-fold increased sensitivity compared with *ste2-D297-391*. Thus, the distal C-terminal serine and threonine residues are important for normal α-factor sensitivity. However, it is interesting that although the *ste2-D4ala* mutant lacks C-terminal phosphorylation sites, it was slightly less sensitive to α-factor than the *ste2-T326* C-terminal truncation mutant (Fig. 1).

**ste2-D297-391p but not ste2-D4alap protein is phosphorylated in vivo.** The increased sensitivity of the *ste2-D4ala* mutant suggests that the receptor is phosphorylated on the distal region of the C terminus. Therefore, we examined the ability of the proteins encoded by *ste2-D297-391* and *ste2-D4ala* (ste2-D297-391p and ste2-D4alap, respectively) to be phosphorylated in vivo. Cells were metabolically labeled with $^{32}PO_4$, and the mutant proteins were detected by immunoprecipitation with anti-Ste2p antiserum. As shown in Fig. 2A, the ste2-D297-391p protein was phosphorylated in vivo. In contrast, the ste2-

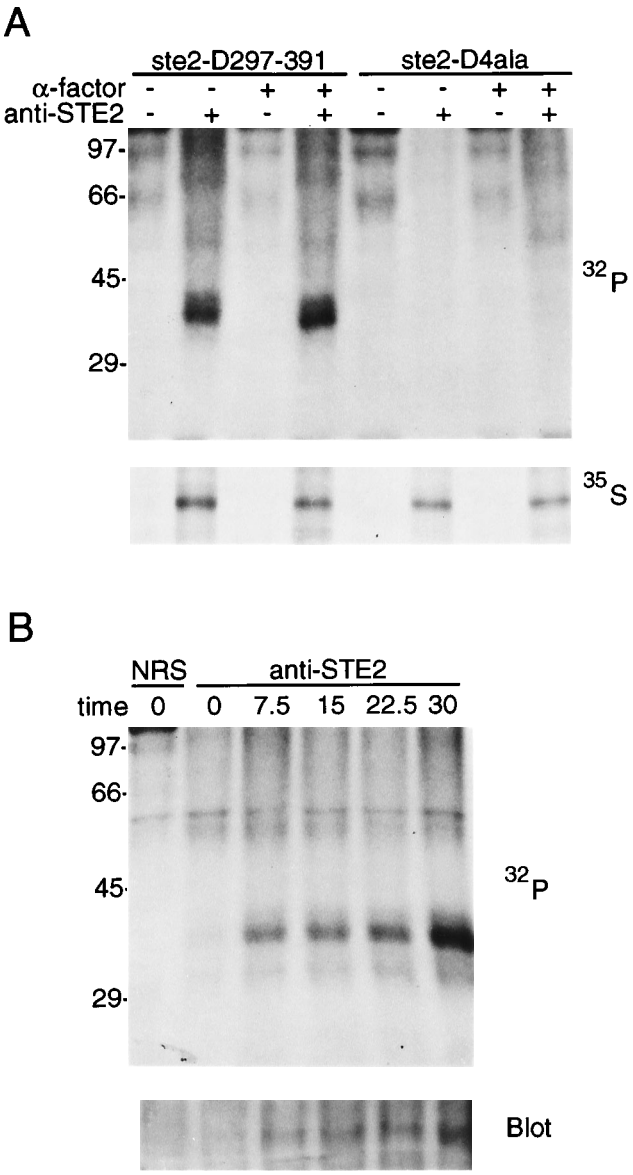


FIG. 2. In vivo phosphorylation of ste2-D297-391p and ste2-D4alap proteins. (A) *ste2-D297-391* strain QCY2 and *ste2-D4ala* strain QCY3 were metabolically labeled with $^{32}PO_4$ for 30 min and then incubated in the presence or absence of $10^{-6}$ M α-factor for an additional 15 min prior to immunoprecipitation analysis. Sizes are shown at the left (in kilodaltons). $^{35}S$, $^{35}S$-labeled cells analyzed in the manner described for the $^{32}P$ blot. (B) *ste2-D297-391* strain QCY2 was incubated for a total of 1 h with $^{32}PO_4$. During the course of the labeling, the cells were also treated with α-factor for the indicated number of minutes. Cells metabolically labeled with $^{32}PO_4$ were extracted and immunoprecipitated with normal rabbit serum (NRS) or anti-Ste2p antibodies as indicated. Immune complexes were separated by electrophoresis on an 10% polyacrylamide–SDS gel, and then the $^{32}P$-labeled proteins were detected by autoradiography with an intensifying screen. Blot, portion of the immune complex that was analyzed by Western blotting.

D4alap protein, which lacks C-terminal phosphorylation sites, was not detectably phosphorylated in vivo in four independent experiments (Fig. 2A and data not shown). This indicates that the distal residues of the C terminus are phosphorylated in vivo. These results are in agreement with a previous study which showed that phosphorylation occurs only on serine and threonine residues in the C terminus of Ste2p (37).

We also examined the ability of α-factor to induce receptor phosphorylation, since it has been shown that activation of the β-adrenergic receptor and rhodopsin stimulates their phosphorylation (11, 31). α-Factor apparently caused a slight increase in phosphorylation but was not required to observe phosphorylation of ste2-D297-391p (Fig. 2A). Therefore, we examined the phosphorylation of ste2-D297-391p during a time course of α-factor stimulation and compared this with the amount of ste2-D297-391p recovered by immunoblotting (Fig. 2B). Although the autoradiogram indicates that there is apparently greater stimulation of phosphorylation in this experiment, the immunoblot shows that this is because less ste2-D297-391p was recovered by immunoprecipitation for the sample incubated in the absence of α-factor. We also observed an increase in receptor phosphorylation between 15 and 30 min, which is probably due to increased receptor production caused by α-factor stimulation (25). Attempts to quantify the slight increase in phosphorylation did not lead to reliable estimates because of variation caused by changes in receptor production and variation in the level of nonspecific background between lanes. However, the increase in phosphorylation was less than twofold, which is consistent with previous studies which showed that phosphorylation of the wild-type Ste2p protein is only induced about 50 to 75% by α-factor (37, 46).

**Analyses of full-length C-terminal mutants.** The effects of mutating the four distal serine and threonine residues in the full-length receptor were examined because these residues were found to be important for normal sensitivity with the *ste2-D4ala* deletion mutant. First, it was necessary to insert a six-base *Eco*RI restriction site after codon 391 of the full-length *STE2* so that the C-terminal coding sequences could be joined in frame with the full-length receptor. The *STE2* gene carrying the six-base *Eco*RI site insertion is designated *STE2(R1)* to distinguish it from the true wild-type *STE2*. The C-terminal coding sequence from *ste2-D4ala* was then used to replace the C-terminal coding sequences in *STE2(R1)* to create *ste2-4ala*, encoding a full-length receptor with alanine substituted for the distal four serine and threonine codons.

Halo assays showed that the relative sensitivities of the *STE2* and *STE2(R1)* strains were indistinguishable, which indicates that the *Eco*RI site inserted into *STE2(R1)* did not affect the sensitivity to α-factor (Fig. 3A). However, a 2.5-fold increase in sensitivity was obtained for the *ste2-4ala* strain containing a full-length receptor with the four alanine substitutions (Fig. 3A). This indicates that these specific four serine and threonine residues are also important for regulation of full-length receptors. We also observed that the sensitivity increased proportionally to the number of alanine substitutions (Table 2). The T425A mutant was 1.5-fold more sensitive, the S398A T425A double mutant was 1.7-fold more sensitive, the T411A T414A T425A triple mutant was 2.1-fold more sensitive, and the S398A T411A T414A T425A quadruple mutant was 2.5-fold more sensitive (numbered according to wild-type *STE2* codons). The synergistic effects of each additional alanine mutation are consistent with these residues' acting as sites of phosphorylation that regulate the receptor. Mutation of the last four phosphorylation sites was equivalent to deleting the last 40 residues of the receptor, since the *ste2-T391* truncation mutant showed a 2.5-fold increase in sensitivity similar to that of the *ste2-4ala* mutant.

Mutation of the terminal four phosphorylation sites to alanine still leaves 30 serine and threonine residues in the full-length C terminus that could act as phosphorylation sites. Therefore, wild-type *STE2* and mutant *ste2-4ala* cells were examined for the ability to be radiolabeled with $^{32}PO_4$ in vivo.

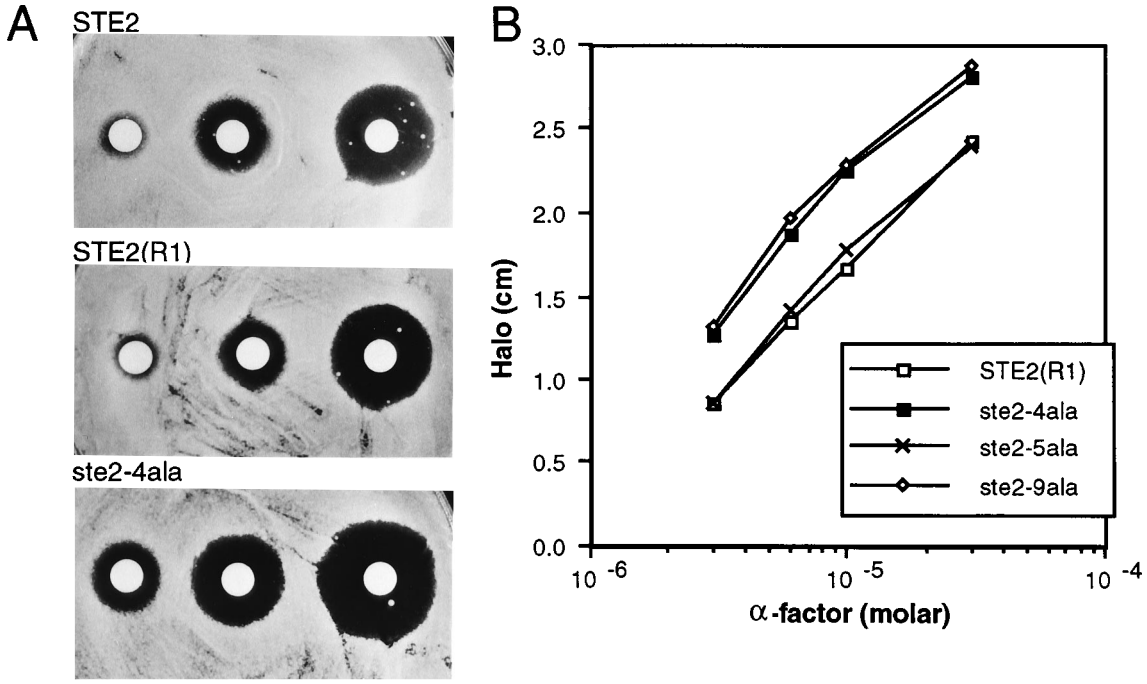


FIG. 3. Effects of mutating C-terminal serine and threonine residues in the full-length receptor. (A) Halo assays demonstrating α-factor sensitivity for *STE2* strain DJ211-5-3, *STE2(R1)* strain QCY4, and *ste2-4ala* strain QCY5. Filter disks containing 20 μl of $10^{-5}$ M, $3 \times 10^{-6}$ M, and $10^{-6}$ M α-factor were applied to a lawn of the indicated cell type and then incubated to observe the zone of growth inhibition caused by α-factor. (B) Results of halo assays for *STE2(R1)* strain QCY4, *ste2-4ala* strain QCY5, *ste2-5ala* strain QCY6, and *ste2-9ala* strain QCY7.

The receptors were isolated by immunoprecipitation from extracts of cells grown in the presence of $^{32}PO_4$, separated by SDS-PAGE, and detected by autoradiography (Fig. 4). A phosphorylated species of about 50 kDa can be seen for both wild-type Ste2p and mutant ste2-4alap before and after α-factor addition. In addition, a proteolytic fragment of about 29 kDa was detected, as previously described (37). The ability to detect ste2-4alap in this assay indicates that the four serine and threonine residues in the distal C terminus are not the only phosphorylation sites in the full-length receptor.

**Mutation of the next five adjacent potential phosphorylation sites has no effect on sensitivity.** The role of the next proximal five serine and threonine residues was examined, since the full-length ste2-4alap was still phosphorylated in vivo. Mutation of the next five serine and threonine residues to alanine (*ste2-5ala* mutation) gave the same sensitivity to α-factor as in the wild type (Fig. 3B), and mutation of all nine of the most distal serine and threonine residues to alanine (*ste2-9ala* mutation) gave sensitivity similar to that of the *ste2-4ala* mutation. These data provide additional evidence that the four distal C-terminal serine and threonine residues play a special role in regulating receptor function.

**Analysis of receptor production and ligand binding.** A Western immunoblot was performed to monitor the produc-

TABLE 2. Pheromone sensitivity of *STE2* mutants

| Plasmid | *STE2* allele[a] | Relative pheromone sensitivity[b] |
|---|---|---|
| pQC4 | *STE2* | 1.0 |
| pQC8 | *ste2 T425A* | 1.5 ± 0.20 |
| pQC9 | *ste2 S398A T425A* | 1.7 ± 0.18 |
| pQC10 | *ste2 T411A T414A T425A* | 2.0 ± 0.06 |
| pQC5 | *ste2 S398A T411A T414A T425A* | 2.5 ± 0.28 |
| pQC391 | *ste2 T391* | 2.5 ± 0.19 |

[a] *STE2* allele designations indicate the sites at which serine or threonine codons were mutated to alanine as described in Materials and Methods.

[b] *ste2*::*LEU2* strain DJ213-7-3 carrying the indicated *STE2* plasmid was analyzed for pheromone sensitivity by a halo assay. The sensitivity of the *STE2* strain was defined as 1 in each assay.

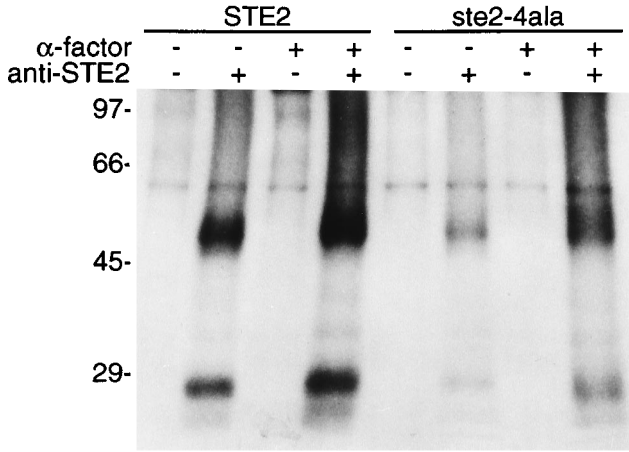


FIG. 4. In vivo phosphorylation of the ste2-4alap protein. *STE2(R1)* strain QCY4 and *ste2-4ala* strain QCY5 were metabolically labeled with $^{32}PO_4$ for 30 min and then incubated in the presence or absence of $10^{-6}$ M α-factor for an additional 15 min. Cell extracts were immunoprecipitated with normal rabbit serum or anti-Ste2p antibodies. Immune complexes were separated by electrophoresis on a 10% polyacrylamide–SDS gel, and then the $^{32}P$-labeled proteins were detected by autoradiography with an intensifying screen. Sizes are shown on the left (in kilodaltons).

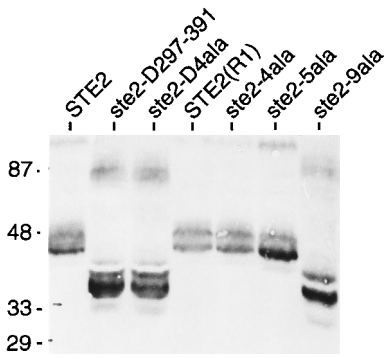


FIG. 5. Western immunoblot analysis of *STE2* C-terminal mutants. Anti-Ste2p antiserum was used to probe a Western immunoblot containing extracts of cells carrying the following *STE2* genes on a plasmid: *STE2* (pJBK-008), *ste2-D297-391* (pQC2), *ste2-D4ala* (pQC3), *STE2(R1)* (pQC4), *ste2-4ala* (pQC5), *ste2-5ala* (pQC6), and *ste2-9ala* (pQC7). The *STE2* genes were carried on YCp plasmids in *ste2*::*LEU2* strain DJ213-7-3. The Ste2p protein bands were detected by chemiluminescence with horseradish peroxidase-conjugated anti-rabbit IgG antibodies. Sizes are shown on the left (in kilodaltons).

tion of the mutant receptor proteins, since their altered structure could affect their synthesis or stability. Extracts from the various *STE2* mutants were separated on SDS gels, transferred to nitrocellulose, and probed with anti-Ste2p antibodies (Fig. 5). All of the receptor mutants showed the expected heterogeneity due to N-glycosylation in the receptor N terminus, which indicates that they can efficiently enter the secretory pathway. As expected, the internal deletion *ste2-D297-391* mutant and the alanine mutant version of this deletion mutant (*ste2-D4ala* mutant) produced proteins with faster gel mobilities, consistent with the extents of their deletions. The full-length Ste2p proteins from the *STE2(R1)*, the *ste2-4ala*, and the *ste2-5ala* strains showed the same gel mobility as the wild-type Ste2p protein. In contrast, only a small amount of the nine-alanine-substituted receptor protein (ste2-9alap) could be seen at the expected position; the majority was detected in a lower-molecular-weight portion of the gel. The altered gel mobility of the ste2-9alap protein was probably due to proteolytic degradation, since the faster gel mobility forms of ste2-9alap cross-reacted very poorly with antibodies directed against the receptor C terminus (data not shown). Apparently, the multiple alanine substitutions caused a change in protein stability.

The possibility that the increased sensitivity of the *ste2-4ala* mutant was due to a change in receptor number or affinity for α-factor was investigated by performing ligand-binding assays with $^{35}$S-labeled α-factor. Scatchard plot analysis of the data demonstrated that the *STE2(R1)* and *ste2-4ala* strains had similar binding properties (Fig. 6). The number of cell surface receptors, derived from the value of the x axis intercept, was estimated to be 3,420 per cell for the *STE2(R1)* and 3,960 per cell for the *ste2-4ala* mutant. The apparent equilibrium dissociation constant ($K_d$), derived from the slope of the line, was 4.5 nM for the *STE2(R1)* and 5.4 nM for the *ste2-4ala* mutant. The $K_d$ values for these strains are also similar to the values obtained by other investigators for wild-type and C-terminally truncated receptors (1, 23, 28). Thus, the increased sensitivity caused by mutation of the phosphorylation sites in the receptor C terminus is not due to changes in ligand binding.

α-Factor stimulates the endocytosis and degradation of receptors, so we also investigated the stability of the receptors after the exposure of cells to α-factor. Cells were incubated with cycloheximide to block protein synthesis and treated with α-factor for various times, and immunoblots were performed to determine the stability of the receptor proteins. Comparison of the amounts of Ste2(R1)p and ste2-4alap proteins detected at each time point shows that they have a half-life of about 20 min in the presence of α-factor (Fig. 7A and C). This is in agreement with the results of Schandel and Jenness for Ste2p (40). Interestingly, the ste2-D297-391p and ste2-D4alap proteins were stable during the 1-h time course. Increased stability of these deletion mutant proteins and the ste2-T326p protein is expected because they all lack the portion of the C terminus that is required for ligand-stimulated endocytosis (39). The alanine substitution mutations also had no significant effect on receptor stability in the absence of α-factor (Fig. 7B). Altogether, these data demonstrate that the increased sensitivity caused by mutation of the phosphorylation sites is independent of receptor endocytosis and degradation.

**Alanine substitution mutations show altered pheromone responses.** The cell division arrest assay that was used to detect the increased sensitivity of *ste2-4ala* cells is a long-term assay (48 h). Therefore, this mutant was also analyzed in a short-term assay that measures the induction of a reporter gene. A plasmid carrying a pheromone-inducible *AFR1-lacZ* fusion

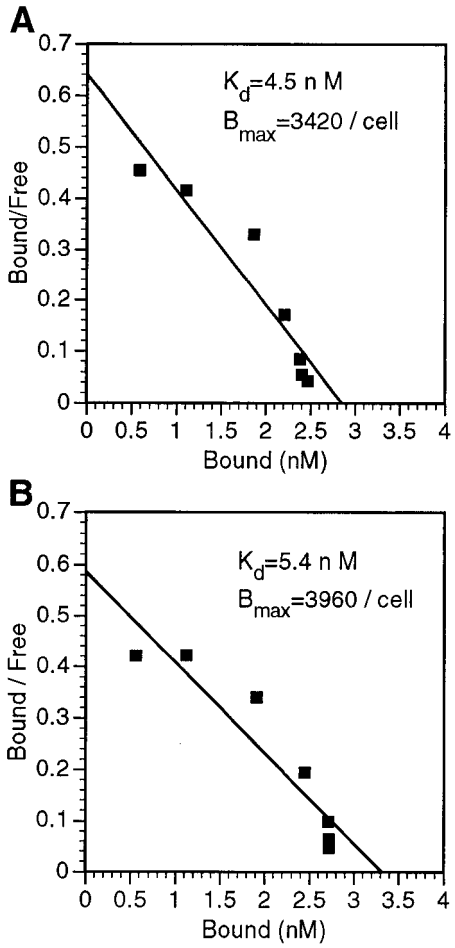


FIG. 6. α-Factor-binding properties of *STE2(R1)* and *ste2-4ala* cells. The binding of $^{35}$S-labeled α-factor to *STE2(R1)* strain QCY4 (A) and *ste2-4ala* strain QCY5 (B) was quantified in equilibrium binding assays as described in Materials and Methods. The average values from three independent experiments are displayed on Scatchard plots. The lines drawn are the best fit of the data obtained by the least-squares method. The concentration of cells used in the assay was $5 \times 10^8$/ml. $B_{max}$, maximum number of cell surface receptors for α-factor.

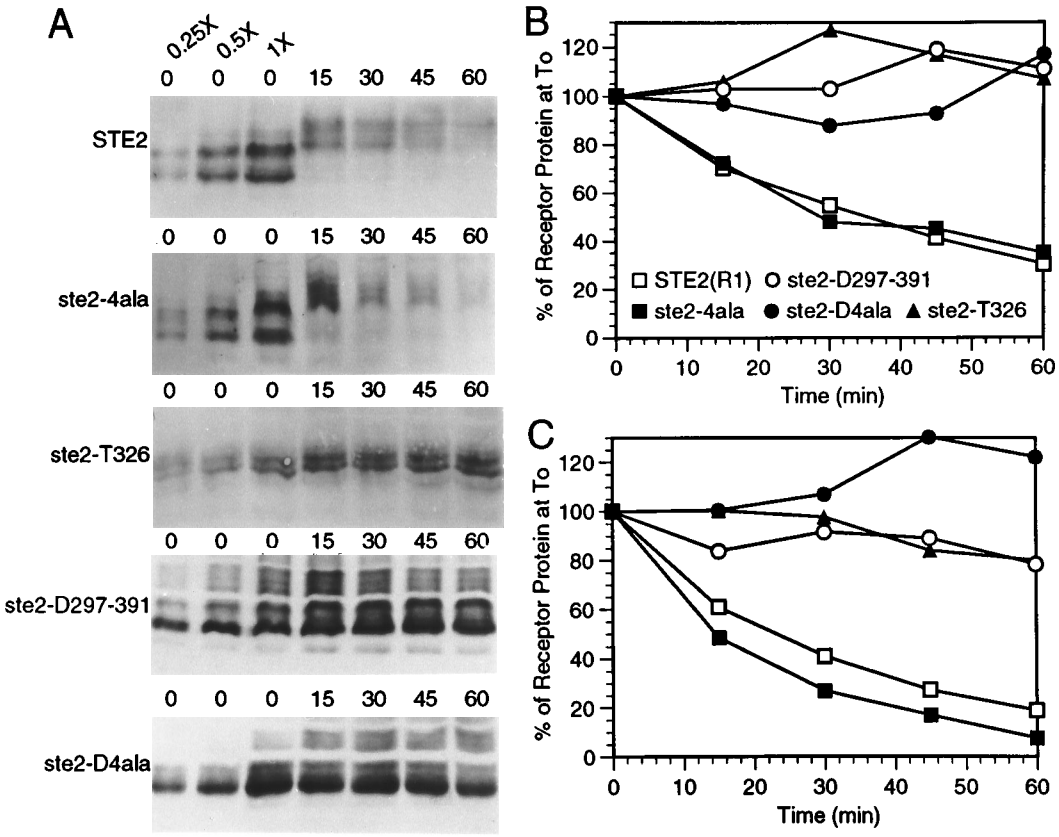


FIG. 7. α-Factor receptor stability assay. (A) Western immunoblot assay showing the stability of receptor proteins in strains carrying the following genes: *STE2(R1)* (QCY4), *ste2-4ala* (QCY5), *ste2-T326* (JKY7441-4-2), *ste2-D297-391* (QCY2), and *ste2-D4ala* (QCY3). New receptor synthesis was blocked with cycloheximide (20 μg/ml), and then cells were exposed to α-factor ($10^{-7}$ M) for the time indicated above each lane (in minutes). Cell extracts were separated by SDS gel electrophoresis and transferred to nitrocellulose, the blot was probed with anti-Ste2p antibodies, and the receptor proteins were detected by chemiluminescence as described in Materials and Methods. The first two lanes contain the indicated dilutions of the time zero sample to facilitate quantitative analysis. (B and C) The stability of the receptor proteins in the absence of α-factor (B) and in the presence of α-factor (C) was quantified by laser scanning densitometry. The results represent the average of two independent experiments.

gene (27) was introduced into the cells, and the ability of α-factor to induce the *lacZ*-encoded β-galactosidase activity was examined by incubating the cells in the presence of various concentrations of α-factor for 90 min. The *STE2(R1)* strain showed the expected result that β-galactosidase activity was induced by α-factor in a dose-dependent manner (Fig. 8A). Cells expressing the *ste2-4ala* mutant receptors induced β-galactosidase activity to about the same maximum level. However, the α-factor concentration required to induce the half-maximal level of β-galactosidase was about 3-fold lower for the *ste2-4ala* strain. Half-maximal induction occurred at $6 \times 10^{-9}$ M for the wild type and at $2 \times 10^{-9}$ M for the *ste2-4ala* mutant. This closely parallels the results obtained from the halo assay (Fig. 3) and confirms that the alanine substitutions affect sensitivity to α-factor.

We also investigated the ability of *ste2-4ala* cells to induce acute projections of morphogenesis (shmoos) in response to α-factor. During mating, pheromone-induced projections are thought to form a conjugation bridge that connects the cells (35). Wild-type and *ste2-4ala* cells were incubated with α-factor ($10^{-6}$ M) for 6 h at 30°C, and the proportion of cells showing acute projections of morphogenesis in each sample was determined by microscopic analysis (Fig. 8B). More than 95% of *STE2(R1)* cells produced projections, and 62% produced more than one projection under these conditions. However, only 54% of the *ste2-4ala* mutant cells produced projections, and only 3% produced more than one projection under the same conditions. Similar results were obtained at lower concentrations of α-factor and at different times of induction (data not shown). Thus, instead of showing increased sensitivity for projection formation, the *ste2-4ala* mutant showed a defect in α-factor-induced morphogenesis. A similar morphogenesis defect was observed previously for α-factor receptor C-terminal truncation mutants (26–28). Analysis of C-terminal truncation *ste2-T326* mutant in this assay showed that it had a stronger defect, since at most only 10% of the cells formed projections and almost none of them gave more than one projection (Fig. 8B). The *ste2-4ala* and *ste2-T326* cells were also similar in that they attained a larger cell size in the presence of α-factor than wild-type cells. Therefore, the serine and threonines in the C terminus of the receptor contribute, at least in part, to the ability of the cells to undergo α-factor-stimulated morphogenesis.

**Recovery from α-factor-induced division arrest.** The increased sensitivity of *ste2-4ala* cells suggested that the mutant receptors are not properly regulated. Therefore, the ability of the mutant cells to recover from a pulse of α-factor was examined. Cells were incubated with α-factor ($10^{-7}$ M) for 90 min to arrest their cell cycle at the unbudded $G_1$ phase, the α-factor was washed out, and then the cells were resuspended in fresh medium and incubated in the absence of α-factor. At 15-min intervals, samples were withdrawn, fixed with formaldehyde,

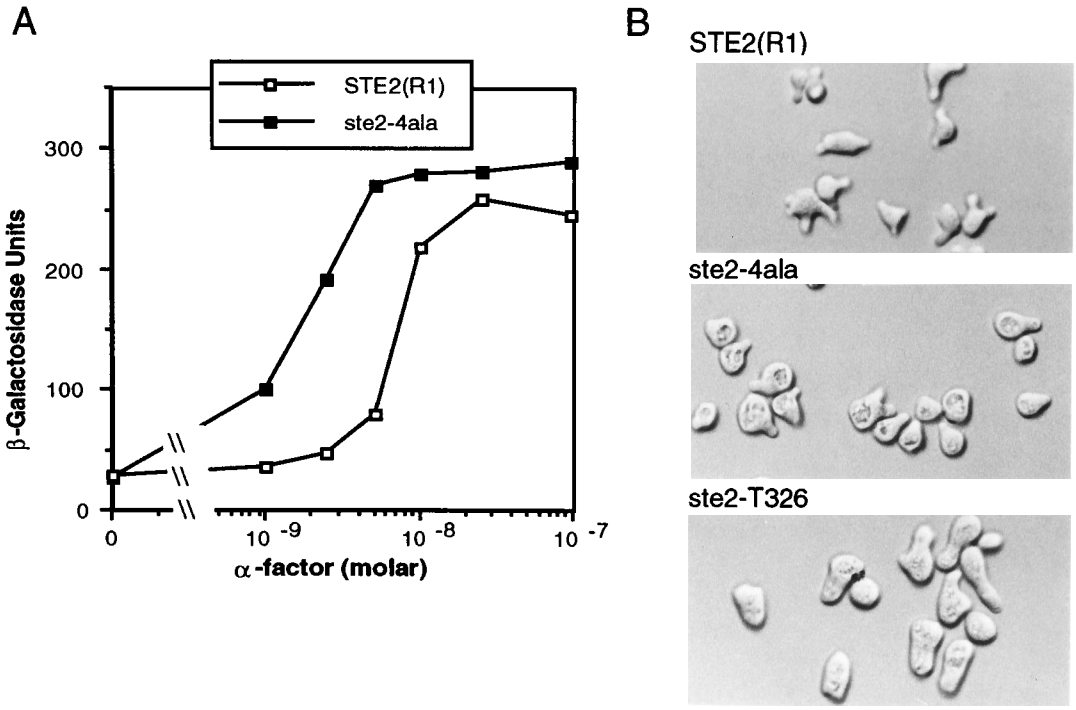


FIG. 8. *ste2-4ala* mutant shows altered reporter gene induction and morphological responses to α-factor. (A) α-Factor induction of an *AFR1-lacZ* reporter gene. *STE2(R1)* strain QCY4 and *ste2-4ala* strain QCY5 carrying plasmid pJK33 (*AFR1-lacZ*) were induced with the indicated concentration of α-factor for 90 min at 30°C, and then β-galactosidase activity was assayed as described in Materials and Methods. (B) α-Factor-induced morphogenesis. *STE2(R1)* strain QCY4, *ste2-4ala* strain QCY5, and *ste2-T326* strain 7441-4-2 were incubated with α-factor ($10^{-6}$ M) for 6 h. The cells were photographed through a 40× objective with differential interference contrast (Nomarski) optics.

and then examined microscopically for the appearance of buds, which indicate that the cells have recovered from cell division arrest and have initiated a new cell cycle.

The *STE2(R1)* cells recovered within 60 min after α-factor was removed (Fig. 9). In contrast, the *ste2-4ala* mutant strain lagged in recovery and took about 90 min. The *STE2(R1)* cells showed half-maximal recovery in 35 min, whereas the half-maximal recovery time for the *ste2-4ala* mutant was 52 min. Thus, the *ste2-4ala* mutant lagged in recovery by 17 min (49% increase). These results indicate that the distal C-terminal four serine and threonine residues are important for recovery from a pulse of α-factor. Surprisingly, the *ste2-T326* truncation mutant cells delayed in recovery much longer than *ste2-4ala* cells even though they are only an additional fourfold more sensitive to α-factor in other assays. This indicates that other C-terminal sequences that are absent in ste2-T326p, such as those that mediate receptor endocytosis (39), act together with the distal serine and threonine residues to promote recovery.

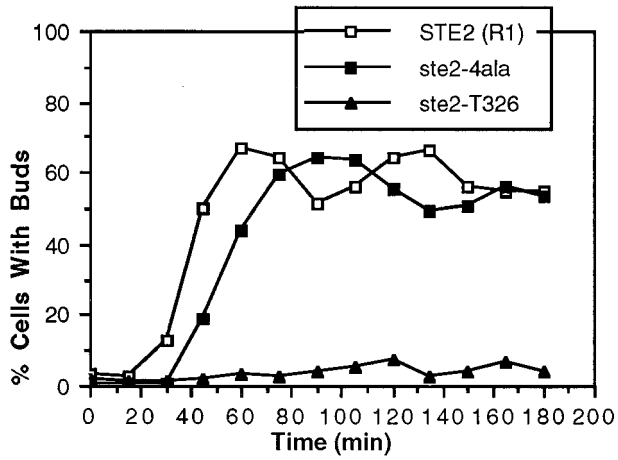


FIG. 9. *ste2-4ala* mutant is defective in recovery from α-factor-induced cell division arrest. *STE2(R1)* strain QCY4, *ste2-4ala* strain QCY5, and *ste2-T326* strain JKY7441-4-2 were incubated with α-factor ($10^{-7}$ M) for 1.5 h to promote cell division arrest, and then α-factor was washed out as described in Materials and Methods. Samples taken every 15 min were analyzed microscopically to determine the fraction of cells that had initiated bud formation, indicating recovery from cell division arrest. The *x* axis represents the time after removal of α-factor, and the *y* axis represents the percentage of budding cells at each time point examined.

## DISCUSSION

**Regulation of the α-factor receptor.** The role of phosphorylation in the regulation of the α-factor receptor was investigated by mutating potential phosphorylation sites to the non-phosphorylatable residue alanine. The α-factor receptor C terminus contains 34 potential phosphorylation sites, so initial studies focused on the distal 40 residues of the C terminus because this region is sufficient to confer nearly normal sensitivity to α-factor and contains only four potential phosphorylation sites (summarized in Fig. 10). Mutation of the four potential phosphorylation sites (*ste2-D4ala*, which encodes only the distal 40 residues of the C terminus) caused 2.5-fold-increased sensitivity to α-factor, demonstrating that these sites are important for receptor regulation. It is possible that the alanine substitution mutations could be affecting receptor function independently of phosphorylation. However, it seems likely that the distal sequences of the C terminus are a target

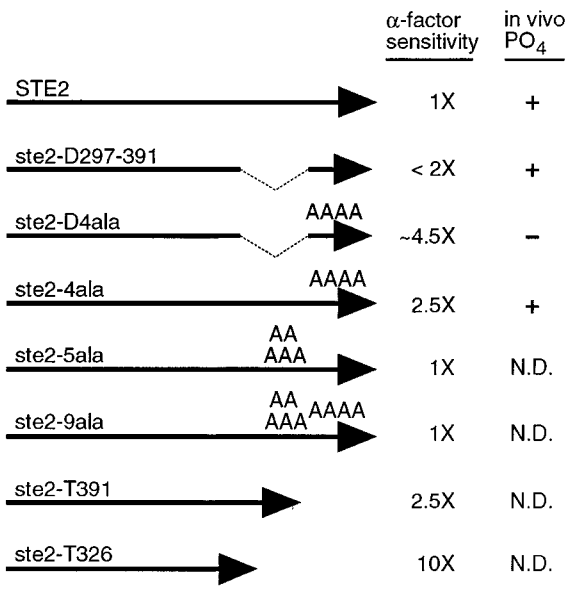


FIG. 10. Summary of receptor mutants. The structures of the mutant receptor proteins described in this study are indicated on the left. Mutation of a potential phosphorylation site to the nonphosphorylatable residue alanine is indicated by an A positioned above the receptor protein. The relative sensitivity to α-factor shown for each mutant was normalized to that of a wild-type *STE2* strain. In vivo $PO_4$, ability of the proteins to be metabolically labeled with $^{32}PO_4$. N.D., not determined.

for a protein kinase that negatively regulates receptor function, since the distal region of the receptor is phosphorylated in vivo and mutation of these sites prevented the phosphorylation of ste2-D4alap.

The distal region of the C terminus is not the only region that can contribute to receptor regulation, because previous studies indicated that there are redundant domains in the C terminus that function in receptor regulation. For example, C-terminal truncation mutants show a progressive increase in α-factor sensitivity corresponding to the extent of truncation, yet the distal 40 residues are sufficient for nearly normal receptor regulation (28, 37). Perhaps some of the other 30 potential phosphorylation sites can function, albeit less efficiently, to mediate receptor regulation in the truncation mutants. Alternatively, there could be other regulatory mechanisms that act via the C terminus, because the *ste2-D4ala* mutant, which lacks C-terminal serine and threonine residues, is not quite as sensitive to α-factor as is the receptor truncation *ste2-T326* mutant (see Fig. 10). Nonetheless, the distal sequences of the full-length C terminus play a special role in the regulation of the receptor, because mutation of the distal phosphorylation sites to create the full-length *ste2-4ala* mutant caused 2.5-fold-increased sensitivity to α-factor. A similar increase in α-factor sensitivity was observed for the *ste2-T391* truncation mutant, which lacks the last 40 amino acids, which shows that phosphorylation can account for the entire contribution of this region to receptor regulation. In addition, all four serine and threonine residues in this distal region may be important for receptor regulation, since mutation of one, two, three, or all four of the sites showed that each additional mutation apparently caused a further increase in α-factor sensitivity (Table 2). In contrast, mutation of the next five adjacent potential phosphorylation sites did not alter α-factor sensitivity.

The mechanism by which phosphorylation regulates the α-factor receptor was investigated by analyzing the production and ligand-binding properties of the mutant receptors. Ligand-binding assays showed that mutation of the distal phosphorylation sites in the *ste2-4ala* mutant did not significantly alter the affinity for α-factor. This is in agreement with the previous result that the *ste2-T326* truncation mutant did not display increased affinity for α-factor (28). Increased receptor number does not account for the increased sensitivity of the mutants, since the steady-state number of receptors was not significantly altered (Fig. 5 and 6) and since overproduction of either wild-type or mutant Ste2p proteins does not cause increased sensitivity (28 and unpublished data). We also investigated the stability of the mutant receptors in the presence of α-factor, since a sequence surrounding Lysine 337 in the C terminus (39) mediates endocytosis of ligand-bound α-factor receptors, leading to their degradation in the vacuole (6, 25, 40, 46). However, mutation of the distal phosphorylation sites did not cause a significant change in receptor stability in the presence or absence of α-factor. Phosphorylation apparently acts independently of endocytosis, since mutation of the phosphorylation sites in the *ste2-D297-391* mutant caused increased α-factor sensitivity, even though this mutant lacks the domain that mediates endocytosis and α-factor did not stimulate degradation of either the ste2-D297-391p or the ste2-D4alap receptor protein. Therefore, in view of the observations that mutation of the distal phosphorylation sites leads to greater activation of G protein signaling in yeast cells and that phosphorylation regulates G protein activation by other receptors (see below), we conclude that the most likely function of phosphorylation is to regulate G protein stimulation by the α-factor receptors.

**Regulation of G-protein-coupled receptors by phosphorylation.** Several G-protein-coupled receptors appear to be regulated by phosphorylation, but the sites of the regulatory phosphorylations can differ. Biochemical analysis indicates that phosphorylation of the β-adrenergic receptor and photoreceptor rhodopsin occurs on C-terminal sequences, which are then bound by a protein termed arrestin that desensitizes the receptors and blocks their ability to activate G proteins (11). The importance of phosphorylation in the regulation of the β-adrenergic receptor was confirmed by demonstrating that mutation of 11 potential phosphorylation sites in the C terminus caused increased sensitivity (3). Other G-protein-coupled receptors, such as the muscarinic cholinergic receptor, appear to be regulated by phosphorylation of the third intracellular loop instead of the C terminus (11, 38). Phosphorylation of the third intracellular loop does not play a significant role in the regulation of the α-factor receptor because mutation of the serine residues in the third loop did not cause a change in sensitivity (7, 45).

Studies on the protein kinases that phosphorylate the $\beta_2$-adrenergic receptor have identified at least two pathways for receptor regulation by phosphorylation (11, 31). The homologous pathway is mediated by a receptor-specific kinase that phosphorylates ligand-activated receptors, and the heterologous pathway is mediated by the cyclic AMP-dependent kinase, which can be activated by $\beta_2$-adrenergic receptor signaling or by other signaling pathways. Identification of the α-factor receptor kinase(s) will similarly be required to determine the mechanism by which phosphorylation regulates this receptor. The α-factor receptors may be regulated by a feedback pathway in which one of the five protein kinases that function in transducing the pheromone signal also phosphorylates the receptor. However, this possibility seems unlikely in view of the observation by Zanolari et al. (46) that receptor phosphorylation occurs in *MAT***a**/α diploid cells in which the pheromone signal pathway is not active.

In contrast to the β-adrenergic receptor and rhodopsin,

phosphorylation of the α-factor receptor is not highly stimulated by α-factor. Furthermore, phosphopeptide analysis indicates that α-factor does not cause a significant change in the pattern of phosphorylation (37, 46). This raises the possibility that constitutive receptor phosphorylation may be important. One potential advantage of constitutive receptor phosphorylation is that signaling would occur predominantly from newly synthesized receptors that have not yet been phosphorylated. This mechanism of receptor regulation could contribute to intracellular polarization of the α-factor signal, since older receptors are scattered throughout the cell and new receptors become polarized to the site of projection formation (22). Thus, temporal regulation of the receptors by phosphorylation could reinforce the ability of yeast cells to detect a gradient of pheromone emanating from a mating partner (41).

**Pheromone-induced morphogenesis.** α-Factor induces cells to form projections of new cell growth in a dose-dependent manner (35). These projections form the conjugation bridge that connects cells during mating. Receptor regulation and projection formation may be interrelated, because truncation of the α-factor receptor C terminus causes both a defect in projection formation and increased sensitivity to α-factor (27, 28). The analysis of the *AFR1* gene also indicates that regulation of signaling and morphogenesis are interrelated processes (27). *AFR1* overexpression acts in a pathway with the receptor C terminus to cause cells to produce longer projections and confers resistance to α-factor; deletion of *AFR1* causes a defect in the formation of projections and a slight increase in pheromone sensitivity. Receptor phosphorylation also appears to be important for both morphogenesis and regulation of signaling, since a partial defect in projection formation was detected in the *ste2-4ala* mutant. However, the four distal serine and threonine residues were apparently not required to observe the effects of *AFR1* overexpression (unpublished data).

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