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Substitutions in the Hydrophobic Core of the α-Factor Receptor of *Saccharomyces cerevisiae* Permit Response to *Saccharomyces kluyveri* α-Factor and to Antagonist

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Mutations in the *Saccharomyces cerevisiae* α-factor receptor that lead to improved response to *Saccharomyces kluyveri* α-factor were identified and sequenced. Mutants were isolated from cells bearing randomly mutagenized receptor gene (*STE2*) plasmids by an in vivo screen. Five mutations lead to substitutions in hydrophobic segments in the core of the receptor (M54I, S145L, S145L-S219L, A229V, L255S-S288P). Remarkably, strains expressing these mutant receptors exhibited positive pheromone responses to desTrp1,Ala3-α-factor, an analog that normally blocks these responses. The M54I mutation appeared to affect only ligand specificity. The other mutations conferred additional effects on signaling or recovery. Two mutants were more sensitive to α-factor than wild type (S145L, A229V). One mutant was more sensitive to α-factor-induced cell cycle arrest initially, but then recovered more efficiently (S145L-S219L). One mutant (L255S-S288P) conferred positive pheromone responses to α-factor as assayed by *FUS1-lacZ* reporter induction, but did not display growth arrest. The hydrophobic receptor core thus appears to control activation by some ligands and to play roles in aspects of signal transduction and recovery.

Haploid budding yeast cells of **a** and α mating types make and respond to peptide mating factors during the mating process that culminates in the formation of an **a**/α diploid (reviewed in references 5, 14, 22, and 24). *Saccharomyces cerevisiae* α-factor (c-α-f) is a peptide of 13 amino acid residues (39). The α-factor receptor of *S. cerevisiae* is encoded by the *STE2* gene (1, 17, 30) and is an integral membrane protein coupled to a heterotrimeric G protein (6, 28, 43). Signal transduction and cellular responses require a number of other gene products as well (reviewed in reference 24). Responses to α-factor include cell cycle arrest, morphological changes, and induction of transcription of specific genes, such as *FUS1*, which is involved in cell fusion.

The G protein-coupled receptor family is very large and exhibits conserved structural and topological features, most notably seven membrane-spanning domains (reviewed in reference 8). However, the detailed tertiary structure of these receptors is not known. Members of this family include the β-adrenergic receptor, rhodopsin, and the receptors for many neurotransmitters and neurohormones. Current models suggest a core domain that includes seven hydrophobic segments spanning the membrane and interacting with the ligand in an external pocket and with G protein on the cytoplasmic side (7, 11, 13). Signal transduction is thought to involve a conformational change propagated across the membrane and sensed by G protein on the cytoplasmic face rather than formation of a pore as with the regulated ion-channel family of receptors.

Much effort has been devoted to characterizing agonist and antagonist ligands for receptors of this family, especially those that represent potential pharmaceutical drugs. However, only a few of the receptors themselves have been genetically analyzed (8). Understanding the mechanism by which ligand binding activates the receptor which in turn activates cellular responses requires the identification of residues that control receptor function.

The budding yeast *Saccharomyces kluyveri*, only distantly related to *S. cerevisiae*, makes an α-factor peptide (k-α-f) that, like c-α-f, is 13 amino acid residues long but that differs in sequence at five positions (9, 34). The k-α-f receptor (k-*STE2*) has been cloned and sequenced and is 50% identical to *STE2* on an amino acid level (23). *STE2* and k-*STE2* confer partial species specificity in response to the c-α-f and k-α-f peptides, suggesting differences in ligand binding and/or activation (23, 25, 34).

Analogs of c-α-f have been synthesized (29). The 12-residue peptide analog desTrp1,Ala3-α-factor (dTA-α-f) is a c-α-f antagonist (37). dTA-α-f binds efficiently to the α-factor receptor as determined by competition assays (31). However, it does not elicit pheromone responses and in fact blocks responses to α-factor, suggesting that it is unable to stabilize the conformational state required to activate G protein.

I report here the identification of mutations in *STE2* that lead to improved response to k-α-f. Five of these mutations affect residues in hydrophobic segments in the core of the receptor. Remarkably, strains expressing these core mutant receptors exhibit positive pheromone responses to the antagonist dTA-α-f. Several mutants also confer other specific effects on signaling or desensitization. Unexpectedly, one mutant retained the ability to respond to α-factor as assayed by induction of a *FUS1-lacZ* reporter gene, but was resistant to the cell cycle arrest by α-factor.

MATERIALS AND METHODS

Strains and media. Yeast strains used in this study are described in Table 1. All strains in this study were *MAT***a** *bar1* unless otherwise indicated. Standard synthetic medium (SD) with or without supplements or rich medium (yeast

TABLE 1. Yeast strains used in this study

Strain	Genotype[a]	Source or reference
LM100	**a** *ste2*-i4 *trp1 leu2 ura3 his4 can1*	This study
LM23-16az	**a** *ste2*-i4 *bar1* [*FUS1-lacZ::URA3*] *leu2 ura3 his4 lys5 met1*	This study
LM23-3az	**a** *STE2*$^+$ [*FUS1-lacZ::URA3*] *bar1 ura3 leu2 his4 trp1 met1*	This study
LM102	Isogenic to LM23-3az but *ste2*-d1	This study
IH1207	α *ura1*	I. Herskowitz
EG123	**a** *trp1 leu2 ura3 his4 can1*	P. Siliciano
FC139	α *HMR***a** *HML***a** *ura3-52 met1 lys5 bar1-1*	F. Chang
XM12-7	*S. kluyveri* α Thr$^-$	25

[a] All strains are *S. cerevisiae* unless otherwise noted.

extract-peptone-dextrose [YPD]) was used for all experiments (33).

Mating assays. Mating assays were as previously described (23). Briefly, 100 μl of an appropriate dilution of overnight culture of LM100 bearing various plasmids in SD medium was added to 100 μl of the α mating partner IH1207 suspended in YPD (approximate A_{600} of 0.4). The mixture was plated on SD agar plates, and the number of prototrophic (diploid) colonies was determined. Control dilutions of LM100-derived strains were plated on SD medium with supplements lacking leucine to determine the number of cells participating in the assay. The mating frequency was calculated as the ratio of the number of mating cells per milliliter to the number of CFU per milliliter and was normalized to the frequency of mating of LM100 with the wild-type (wt) *STE2* plasmid pAB539 (0.02 under these conditions).

α-Factor analogs. Synthetic c-α-f and k-α-f were purchased from Sigma Chemical Co. and gave single major peaks on reverse-phase high-pressure liquid chromatography (31). dTA-α-f was synthesized and analyzed by the Laboratory for Macromolecular Analysis at Albert Einstein College of Medicine. Structure was confirmed by mass spectrometry.

DNA manipulations. General DNA manipulations including DNA preparation, restriction digests, ligations, etc., followed standard procedures (33, 35) or manufacturers' instructions.

DNA sequencing. A Sequenase kit (United States Biochemical) was used for DNA sequencing. Mutations in *STE2* were identified by direct double-stranded DNA sequencing (20) of the entire coding region of *STE2* in each of the mutant plasmids by using appropriately spaced primers.

Insertion and deletion mutagenesis of *STE2*. Insertion mutagenesis of the *STE2* gene was performed by a mini-Tn*3* method (36). A 4.4-kb *Bam*HI DNA fragment containing the *STE2* gene was subcloned into the vector pHSS6 and subjected to in vivo transposon mutagenesis with a mini-Tn*3* derivative that contains the yeast *TRP1* gene. An insertion, *STE2*-i4, with the insertion located approximately between the segments coding for hydrophobic segments four and five, was recovered as well as another, *STE2*-i11, with an insertion at codon 297 in the region encoding the carboxyl terminus of Ste2p. The mutated *STE2* genes were transferred to the yeast chromosome by homologous gene replacement. The *trp1* yeast strain EG123 was transformed with a linear *Not*I DNA fragment carrying *STE2*-i4 or *STE2*-i11. Trp$^+$ colonies were tested for the ability to mate and sensitivity to α-factor. Strains with *STE2*-i4 were α-factor resistant and unable to mate. Strains with *STE2*-i11 were hypersensitive to α-factor and were able to mate at near-normal levels. EG123 *STE2*-i4 (LM100) was transformed with pAB539, and the resulting strain was mated to FC139. The resulting diploid was sporulated, and tetrads were dissected. Strain LM23-16a was isolated, which has the genotype **a** *STE2*-i4 *bar1 leu2 ura3-52 lys5 met1*. This strain was transformed with the chromosomal integration plasmid pSB286 (42) which carries *FUS1-lacZ* and *URA3* to yield the strain LM23-16az.

STE2 was deleted from the chromosome by a two-step process that did not leave any markers at the *STE2* locus. An *STE2* deletion was first engineered in a plasmid (pOS-15) bearing a 4.4-kb *Bam*HI fragment of *STE2*-region DNA by partial digestion with the restriction endonuclease *Sal*I and then religation. The resulting construct, pOSd2-3, contained regions flanking the *STE2* gene (about 1.1 kb upstream and 1.1 kb downstream) as well as the last 23 codons of *STE2*, but lacked the 2.2-kb *Sal*I fragment which contains full *STE2*-complementing activity (12). This *STE2* deletion is designated *ste2*-d1. The *ste2*-d1 allele was introduced into the genome. First, strain LM23-3az was transformed with *STE2*-i11 to mark *STE2* with *TRP1*. The resulting strain was cotransformed with circular vector YEp13 DNA and with pOSd2-3 DNA that had been digested with the endonuclease *Bam*HI. Leu$^+$ colonies were pooled and exposed to α-factor. An α-factor-resistant, Trp$^-$ strain was identified as a candidate *ste2*-d1 strain. Southern blot analysis (35) of chromosomal DNA derived from this strain, LM102, confirmed that *STE2*-i11 had been replaced with *ste2*-d1.

Zone of inhibition assay for responsiveness to α-factor. A lawn of the appropriate yeast strain was prepared by using an overlay of soft SD agar medium (synthetic medium lacking leucine for most of the plasmid-bearing strains). Various amounts of either c-α-f or k-α-f were spotted, and the plates were incubated for about 20 h at 30°C. After preliminary experiments to determine the range of sensitivity, twofold increments of each factor were used. The diameter of the zones of inhibited growth was plotted on a semilog scale (19, 32), and the amount of α-factor or analog required to produce a 1-cm zone of growth inhibition was estimated by interpolation. Analysis of outlying points suggests that these approximations are accurate to about ±20%. For some experiments, plates were incubated for an additional period to assess the ability of the strain to recover from α-factor-induced arrest.

***FUS1-lacZ* induction.** Strain LM102 bearing a *FUS1-lacZ* plasmid integrated into the chromosome and an episomal plasmid bearing *STE2* was grown overnight in SD minimal dropout medium and then subcultured into YPD medium. Various amounts of α-factor or analogs were added to 2-ml samples of log-phase cultures, and the cultures were incubated at 30°C on a roller drum. After 1.5 h, cultures were harvested by centrifugation and processed to determine β-galactosidase levels (33). β-Galactosidase levels were normalized for cell density by using the A_{600} of a sample not treated with α-factor.

Mutagenesis of *STE2* plasmid. The plasmid pAB539, which contains a 4.4-kb fragment of *STE2*-region DNA cloned into YEp13 (1), was mutagenized by two procedures. Plasmid DNA was mutagenized in vitro by treatment with hydroxylamine (33) or by passage through an *Escherichia coli mutD* mutator strain (4). Mutagenesis conditions were chosen to yield about 1×10^{-3} to 2×10^{-3} mutations per nucleotide. In both cases, independent pools of mutated DNA were subsequently transformed into a nonmutator *E. coli* strain to

TABLE 2. Relative mating frequency of strains expressing mutant receptors

Plasmid[a]	*STE2* mutation	Relative mating[b]
YEp13	Vector only	$<1.2 \times 10^{-5}$
pAB539	wt	1.0
pKS-1	M54I	1.5
pKS-2	S145L	0.8
pKS-3	S145L-S219L	0.25
pKS-4	A229V	1.7
pKS-5	L255S-S288P	1.5×10^{-5}
pKS-6	Q328Stop	2.5

[a] LM100 carried the indicated plasmids.
[b] Mating to tester strain IH1207 under semipermissive conditions.

permit segregation of the mutated strands of plasmid DNA and to amplify the mutant plasmids. Pools of mutagenized plasmid DNA were prepared from these transformants.

Identification of mutant plasmids conferring response to k-α-f. A plate assay was used to identify strains with mutant plasmids conferring increased *FUS1-lacZ* induction by k-α-f. Strain LM23-16az was transformed with mutagenized pAB539 and plated on supplemented minimal medium lacking leucine. Transformant colonies were replicated to a cellulose filter (Whatman 50) overlying a lawn of *S. kluyveri* α mating-type cells (XM12-7) (25) on YPD agar medium. The *S. kluyveri* lawn served as a source of k-α-f. Colonies were also replicated to filters on petri plates lacking lawns to test for constitutive induction of pheromone responses. The plates were incubated for about 6 h at 30°C, and then the filter was removed and frozen in liquid nitrogen. To detect colonies with increased β-galactosidase activity due to induction of the *FUS1-lacZ* reporter, the colony-bearing filter was transferred to a filter (Whatman 3) soaked with a 0.03% solution of the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in 2.5 ml of Z-buffer (150 mM Na phosphate buffer [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$) (26) and incubated at 30°C until most colonies had turned pale blue (about 1 to 3 h). Dark blue colonies represented from <0.1 to 0.4% of the colonies on filters that had been exposed to k-α-f. Plasmids from the k-α-f-sensitive strains were recovered and transformed into *E. coli* (15).

RESULTS

Receptor mutations conferring better response to k-α-f. Receptor mutations that partially or completely inactivate receptor function (18) or that block desensitization (19, 32) have been described. However, I wished to isolate mutations affecting other aspects of function and identified mutations that improve response to k-α-f. k-α-f appears to be a partial agonist that does not fully activate the α-factor receptor of *S. cerevisiae* (23). In a formal genetic sense, receptor mutations that confer improved response to k-α-f have a gain of function rather than a loss of function.

An in vivo screen was developed to isolate strains with mutant receptors. An autonomously replicating 2μ plasmid carrying *STE2* (pAB539) (1) was randomly mutagenized by hydroxylamine treatment or passage through an *E. coli mutD* strain under conditions predicted to result in an average of about one substitution in the *STE2* gene (see Materials and Methods). Pools of mutagenized plasmid DNA were transformed into an *ste2* defective yeast strain, LM23-16az, that carried a pheromone-inducible *FUS1-lacZ* reporter gene. Transformant colonies were screened for the ability to respond to k-α-f as manifested by increased expression of β-galactosidase in a replica filter assay. Approximately 18,000 yeast colonies representing 17 independent pools of mutagenized plasmid DNA were screened. Six independent strains were isolated that conferred the k-α-f-sensitive (KS) phenotype. The *STE2* plasmids were recovered from these yeast cells, transformed into *E. coli*, and purified. Yeast strain LM23-16az was transformed with the recovered plasmids to determine whether the plasmids were responsible for the mutant phenotype. In each case, the plasmid conferred the original mutant phenotype, demonstrating that the mutation was plasmid borne. I refer to strains bearing these mutant plasmids as KS-1, KS-2, etc. KS-2, KS-5, and KS-6 were derived from *mutD* mutagenesis. KS-1, KS-3, and KS-4 were derived from hydroxylamine mutagenesis. Although these plasmids conferred increased sensitivity to k-α-f, the strains remained more sensitive to c-α-f than to k-α-f. All the mutants except for KS-5 were capable of promoting mating at frequencies similar to or even greater than those of the parent (Table 2). Thus, most of the mutant receptors retain functions required for their natural role in mating.

DNA sequence analysis of mutants. The mutant receptor genes were sequenced (Table 3). Five of the mutants (KS-1, KS-2, KS-3, KS-4, KS-5) contained substitutions in residues found in hydrophobic core segments thought to span the membrane (Fig. 1). I refer to this group as core mutants. One mutant (KS-6) contained a stop codon causing truncation after residue 327 of the receptor carboxyl-terminal intracellular domain (wt receptor is 431 amino acids). Four of the mutants (KS-6, KS-2, KS-4, KS-1) contained single nucleotide changes which resulted in amino acid substitutions. Mutants KS-5 and KS-3 contained double amino acid substitutions. One of the two substitutions in KS-3 was the same

TABLE 3. Substitutions found in coding region of *STE2* from plasmids conferring increased sensitivity to k-α-f

Designation	Affected codon	Substitution	Phenotype(s)[a]
KS-1	Met-54 (ATG)	Ile (ATA)	No novel phenotypes observed
	Tyr-399 (TAC)	Silent (TAT)	
KS-2	Ser-145 (TCA)	Leu (TTA)	Modest increase in sensitivity to α-factor
KS-3	Ser-145 (TCA)	Leu (TTA)	Increased initial sensitivity to α-factor; improved recovery from arrest
	Ser-219 (TCA)	Leu (TTA)	
KS-4	Ala-229 (GCT)	Val (GTT)	Increased sensitivity to α-factor
KS-5	Leu-255 (TTG)	Ser (TCG)	Defect in cell cycle arrest
	Ser-288 (TCT)	Pro (CCT)	
	Val-109 (GTG)	Silent (GTA)	
KS-6	Gln-328 (CAA)	Stop (TAA)	Large increase in sensitivity to α-factor

[a] Phenotypes conferred in addition to response to foreign peptides.

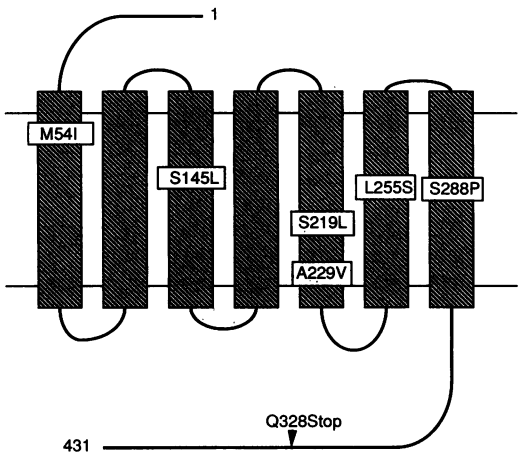

FIG. 1. Positions of substitutions relative to predicted topology of the α-factor receptor. The amino terminus is believed to be extracellular. Substitutions are given in single-letter amino acid code. Mutations are KS-1, M54I; KS-2, S145L; KS-3, S145L-S219L; KS-4, A229V; KS-5, L255S-S288P; KS-6, Q328Stop. See Table 3 for details.

as the substitution found in KS-2 (which was, however, created by a different mutagenic protocol). The KS-3 mutant confers effects not observed with KS-2 (see below), demonstrating that both substitutions in KS-3 affect function.

Response to k-α-f. I confirmed that the *STE2* mutants conferred greater sensitivity to k-α-f by measuring induction of the *FUS1-lacZ* reporter construct more quantitatively in liquid culture. To rule out any possibility of interference with the genomic copy of *STE2*, these experiments were performed with plasmids in a strain with *STE2* deleted (LM102). As expected, the mutant strains were more effectively induced by k-α-f than was the wt parent (Table 4). Some of the induction effects are modest (although reproducible) in liquid culture under these conditions, which may reflect the sensitivity of the original plate assay.

Response to dTA-α-f. Since the core mutant receptors conferred improved response to k-α-f, an apparent partial agonist, I wished to examine response to a known antagonist. The analog dTA-α-f has been previously described as an α-factor antagonist (37). The dTA-α-f analog and the k-α-f analog contain substitutions in different regions of c-α-f. The sequences (in single-letter code) are compared below, with substitutions in boldface and overall regions of difference underlined.

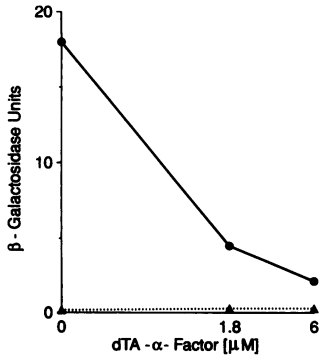

FIG. 2. Inhibition of *FUS1-lacZ* expression by dTA-α-f. Strain LM23-3az was incubated for 1 h with dTA-α-f in the presence (●) or absence (▲) of c-α-f (6×10^{-9} M), and β-galactosidase activity was determined.

c-α-f (39): = W H W L Q L K P G Q P M Y

k-α-f (34): = W H W L **S F S K G E** P M Y

dTA-α-f (37): = **H A** L Q L K P G Q P M Y

Thus, dTA-α-f and k-α-f each resemble c-α-f more than each other. In a strain expressing the wt receptor, the dTA-α-f analog failed to induce a *FUS1-lacZ* reporter gene and inhibited *FUS1-lacZ* induction by c-α-f (Fig. 2). A greater than 100-fold excess of dTA-α-f was required for 50% inhibition of *FUS1-lacZ* induction by c-α-f in this assay. The reported affinity of the receptor for dTA-α-f is 10- to 40-fold less than that for c-α-f (31).

Surprisingly, the core mutants were all highly inducible by dTA-α-f (Table 4). The truncated receptor mutant, KS-6, was weakly induced by dTA-α-f. dTA-α-f did not activate a strain [LM102(YpK2-16)] (23) expressing the k-α-f receptor, suggesting that dTA-α-f does not somehow mimic k-α-f structure (data not shown). dTA-α-f binds to the wt receptor but fails to activate responses (31, 37). Thus, the mutant receptors that permit positive responses to dTA-α-f presumably are altered in their ability to be activated by dTA-α-f.

Growth arrest characterization of mutants. The k-α-f-sensitive mutants were identified by *FUS1-lacZ* reporter gene induction. I also quantitated a different aspect of pheromone response, growth arrest, using a growth inhibition assay (Table 5). The amount of c-α-f or k-α-f required to produce a 1-cm zone of growth inhibition of a lawn of cells on solid medium was determined (see Materials and Methods). The ratio of the amount of k-α-f to the amount of c-α-f

TABLE 4. Effect of *STE2* mutations on induction of *FUS1-lacZ* by α-factor-related peptides

Plasmid[a]	β-Galactosidase (units)[b]			
	No addition	c-α-f (6×10^{-8} M)	k-α-f (6×10^{-8} M)	dTA-α-f (6×10^{-7} M)
YEp13 (vector)	0.7 ± 0.2	1.1 ± 0	1.3 ± 0.1	1 ± 0
pAB539 (*STE2*$^+$)	1 ± 0.4	23 ± 3	5 ± 3	1 ± 0.6
pKS-1	1.6 ± 0.4	27 ± 1	24 ± 3	13 ± 1
pKS-2	1.2 ± 0.3	17 ± 1	17 ± 1	13 ± 0
pKS-3	1.9 ± 0.8	31 ± 6	23 ± 1	23 ± 2
pKS-4	2.3 ± 1.2	31 ± 3	25 ± 4	21 ± 1
pKS-5	1.9 ± 1.5	30 ± 1	24 ± 1	28 ± 1
pKS-6	2.6 ± 1	28 ± 3	9 ± 1	8 ± 0

[a] All assays were performed on strain LM102 bearing *STE2* plasmids.
[b] Mean ± SD.

TABLE 5. Growth arrest by c-α-f and k-α-f

Plasmid	Amt of peptide (ng) required for 1-cm zone of growth inhibition		Ratio k/c[a]	c-α-f sensitivity[b]
	c-α-f	k-α-f		
pAB539 (*STE2*$^+$)	20	1,200	60	1
pKS-1	20	140	7	1.0
pKS-2	14	100	7	1.4
pKS-3	10	20	2	2.0
pKS-4	10	80	8	2.0
pKS-5	>10,000	>10,000		
pKS-6	3	80	27	6.7

[a] Relative sensitivity to k-α-f versus c-α-f. Ratio of amount of k-α-f (k) to c-α-f (c) required for 1-cm zone of inhibition. Strain LM102 was used.

[b] Sensitivity to c-α-f relative to wt. Ratio of the amount of c-α-f required to produce 1-cm zone of inhibition on lawn of wt strain to the amount required on mutant lawn.

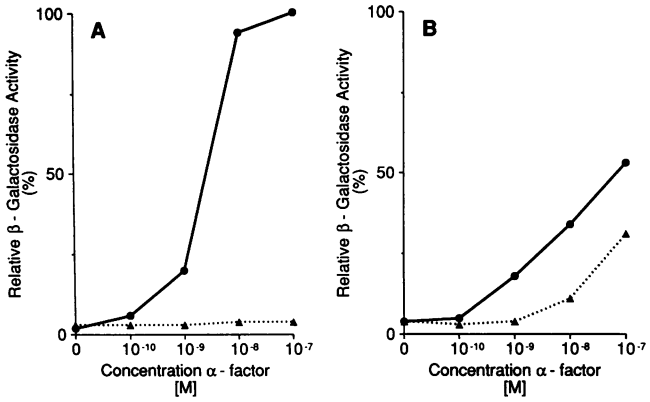

FIG. 3. Induction of *FUS1-lacZ* reporter in strain with KS-5 mutant receptor. β-Galactosidase levels after treatment for 1 h with α-factor. Symbols: ●, c-α-f; ▲, k-α-f. (A) LM102(pAB539) (wt); (B) LM102(pKS-5) (KS-5 mutant).

required to produce the same amount of inhibition provides a measure of the relative specificity for the two peptides. The ratio of the amount of c-α-f required to produce inhibition of a mutant relative to the parent strain indicates the relative increase or decrease in overall sensitivity to α-factor. These assays are highly reproducible with strains and conditions that produce clear zones of complete growth inhibition which are sharply delineated, but less accurate for turbid zones produced when cell growth is only temporarily inhibited (such as KS-3). The parent wt receptor confers a clear-zone phenotype with c-α-f and a turbid zone with k-α-f.

As expected, most of the mutant strains were clearly more sensitive to k-α-f than the parent strain in this assay, consistent with the *FUS1-lacZ* assay results. The mutant strains KS-1, KS-2, KS-4, and KS-6 gave clear zones of inhibition with both c-α-f and k-α-f. None of the mutant strains was more sensitive to k-α-f than to c-α-f in the zone of inhibition assay. However, all were relatively more sensitive than the parent as indicated by the ratio of the amounts of c-α-f and k-α-f required to produce a 1-cm zone of growth inhibition (Table 5). Strain KS-1 showed an unaltered response to c-α-f but was more sensitive to k-α-f. Thus, KS-1 represented an expected class of mutants, which has only experienced a change in specificity. Strains KS-2, KS-4, and KS-6 were more sensitive to k-α-f but also more sensitive to c-α-f than was the parent strain. The KS-3 and KS-5 mutants behaved anomalously in this zone of inhibition assay (see below). Unlike the k-α-f analog, dTA-α-f produced only turbid zones of inhibition (not shown), suggesting that it has only partial agonist activity for the mutant strains. The turbid zone of inhibition phenotype which results from initial arrest and then recovery may result from incomplete receptor activation (see Discussion).

Mutant receptor conferring a defect in cell cycle control but not transcriptional control. The KS-5 mutant (L255S-S288P) was completely resistant to the growth-inhibitory effects of c-α-f and k-α-f (Table 5). c-α-f (10 μg) spotted on a lawn of the KS-5 mutant produced only a barely visible effect on growth. In contrast, 10 μg of c-α-f produced a 31-mm zone of inhibition when spotted on a lawn of the parent strain and represented 1,000 times the minimal amount of c-α-f required to produce a clear zone of growth inhibition on the parent strain. I compared the dose-response characteristics of the wt and KS-5 strain by monitoring induction of the *FUS1-lacZ* reporter gene. The response of the KS-5 strain to c-α-f in this assay was reduced only about 50% relative to that of the parent strain at the concentration of c-α-f that led to maximal induction of the parent strain (Fig. 3). However, the response of KS-5 to k-α-f was better than that of the parent strain, as expected since this was the basis for the original screen. Microscopic observation revealed that KS-5 cells exposed to c-α-f or k-α-f underwent morphological deformation (shmooing) similar to that of the parent strain but less extreme. Unlike the parent, many of the deformed KS-5 cells were budded (Fig. 4). We found that 19% (17 of 90) of deformed cells with the wt receptor were budded, whereas 67% (64 of 96) of the KS-5 deformed cells were budded. With strains such as these bearing the receptor on a 2μm-based plasmid, some cells are always observed that do not exhibit growth arrest or morphological changes after exposure to α-factor. This culture heterogeneity presumably reflects plasmid copy number fluctuation and loss.

Mutant receptor conferring initial hypersensitivity and facilitated recovery from cell cycle arrest. The KS-3 mutant conferred a phenotype which also points to the complexity of the role of the receptor in response. After 18 h of incubation on agar at 30°C, inhibition of growth by α-factor was visible. The size of these zones of growth inhibition indicated that strains expressing the KS-3 mutant were initially more sensitive to α-factor than the wt control (Table 5), although because of the turbidity of the inhibited growth zone with KS-3, this difference should be interpreted cautiously. Also, microscopic examination demonstrated that the KS-3 mutant exposed to low concentrations of α-factor exhibited full morphological changes and stopped budding (data not shown). However, growth arrest for the KS-3 mutant was not permanent. The zones of growth inhibition by both c-α-f and k-α-f appeared turbid after 18 h and were almost completely filled in by 2 days of incubation (Fig. 5). Relative to the parent, the KS-3 mutant appears to signal more effectively early in response and recover more efficiently late in response.

DISCUSSION

The α-factor receptor of *S. cerevisiae* belongs to a superfamily of G protein-coupled receptors abundant in eukaryotes. Members of this receptor family appear to have seven hydrophobic membrane-spanning domains and are coupled to heterotrimeric guanine nucleotide-binding proteins. The α-factor receptor appears to have the same topological

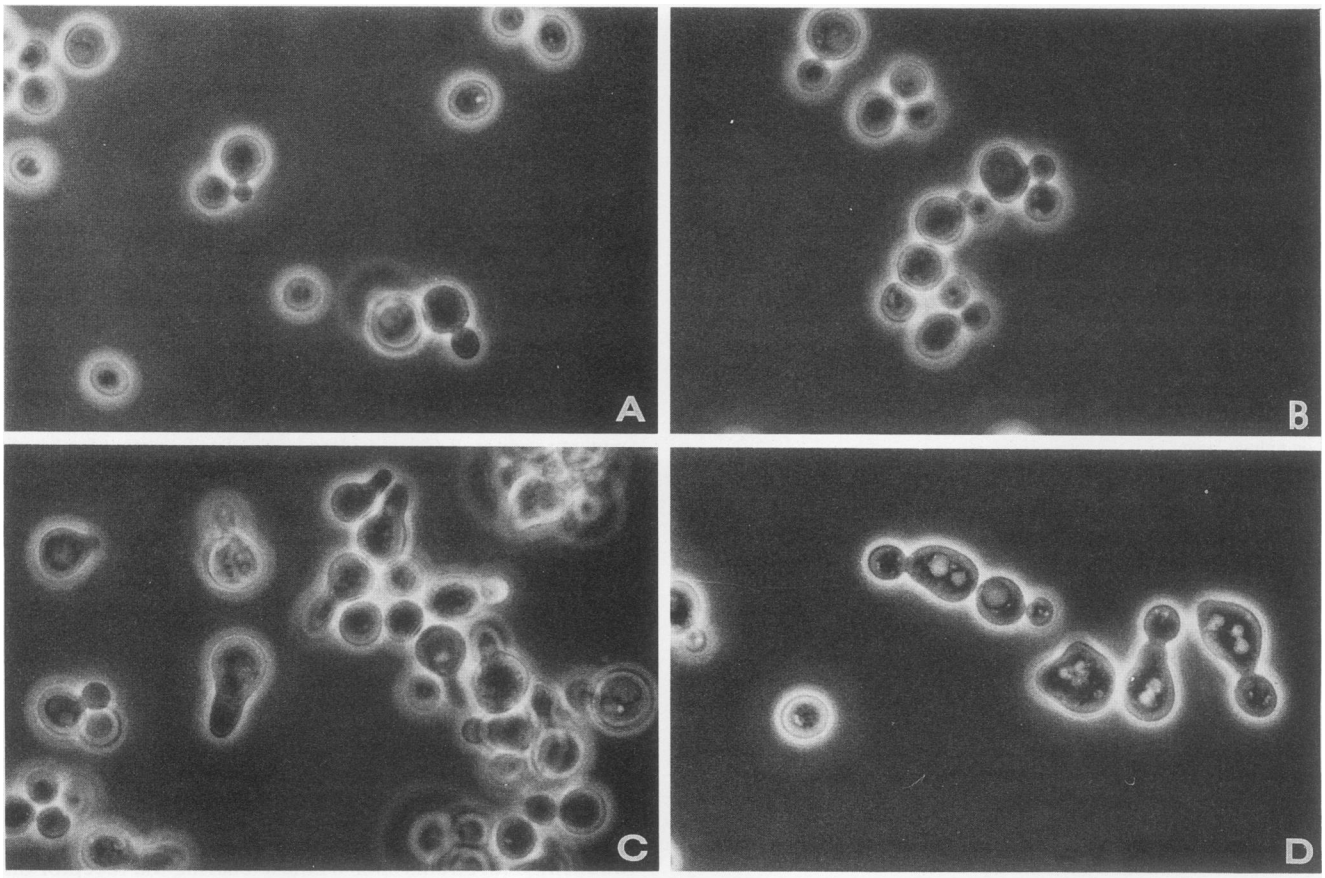

FIG. 4. Effect of α-factor on morphology and bud emergence in a receptor mutant. Photomicrographs of yeast strains with or without α-factor addition after incubation for 7 h at 30°C. (A and C) LM102(pAB539) *STE2*$^+$; (B and D) LM102(pKS-5) *STE2* (L255S-S288P). (A and B) No addition; (C and D) plus c-α-f (6×10^{-6} M).

orientation as mammalian receptors that have been studied previously (2, 32). Although the genes for many of these receptors have now been cloned, the details of the mechanism of receptor activation remain unclear. In particular, the important issue of why some ligands bind and activate receptors (agonists) whereas others bind without activating (antagonists) or only partially activate (partial agonists) is central to understanding the mechanism of receptor activation. I used random mutagenesis of the receptor to identify residues important for function. I screened for improved response to k-α-f and identified five mutations of the α-factor receptor gene mapping to the hydrophobic core. Strikingly, all five mutants also conferred strong responsiveness to an α-factor antagonist peptide, dTA-α-f.

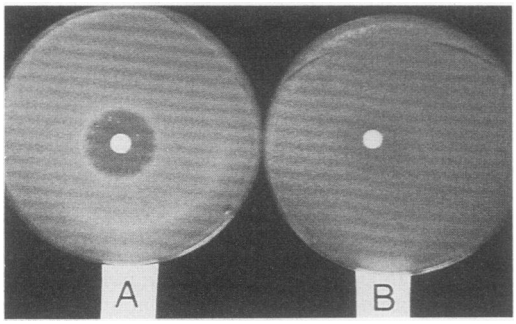

FIG. 5. Growth arrest and recovery of wt and KS-3 mutant. Videophotograph of lawns of yeast cells with wt or KS-3 mutant receptor spotted with 1 μg of c-α-f and incubated at 30°C for 2 days. (A) LM102(pAB539) (wt); (B) LM102(pKS-3) (KS-3 mutant).

There is a previous report of a mutation in a G protein-coupled receptor that leads to activation by an antagonist. The D113E substitution in the β_2-adrenergic receptor third membrane span allows several antagonists to act as partial agonists (40). However, unlike the mutations reported here, the D113E mutation reduces the ability of agonists to activate the β_2-receptor by 10^2- to 10^4-fold (40). Residue 113 in the β_2-receptor has previously been identified as a counterion for the amino group of epinephrine, a natural ligand for the receptor (41), and so may be directly involved in receptor activation. The Met-54 residue of the α-factor receptor that is substituted in the KS-1 mutant lies toward the outer face of the receptor and may form part of the ligand-binding pocket, but other substitutions are predicted to lie deeper in the membrane and are less obvious candidates for direct contact with the ligand.

Most structure-function studies of this receptor family have relied on site-directed mutagenesis. The obvious limitation is that one only finds what one looks for. A reasonable question to ask of my study is what fraction of residues identified would have been identified by a program of site-directed mutagenesis. Several of my random mutations

result in substitutions in hydrophobic residues that are not the sort usually chosen in directed mutagenesis studies. Clearly, one function of a random mutagenesis study such as this is to draw attention to regions of the receptor that should be subjected to more-detailed study by site-directed mutagenesis techniques.

Mechanism of activation by α-factor analogs. How do substitutions in the hydrophobic core of the receptor lead to the ability to respond better to α-factor analogs? α-Factor binding is likely to induce an activated conformation of the receptor. However, it is as yet unclear whether this conformational transition would be limited by kinetic or thermodynamic constraints. It is also not clear how efficient the process of G-protein activation is after the receptor has been activated. Thus, three explanations for the mutant phenotypes present themselves. (i) Activation of the receptor by α-factor analogs may be energetically more favorable in the mutants. (ii) If the α-factor analogs fail to activate for kinetic reasons (e.g., an activation energy barrier between the unactivated and activated receptor-analog complex), the mutant receptors may have a lower energy barrier and an increased rate of transition to the activated state. (iii) The active form of the mutant receptor may have a greater potency for producing the intracellular signal. The phenotype of the mutants I isolated cannot be explained by a model in which the sole change in the receptor is an increased affinity for the α-factor analogs, since the dTA-α-f analog binds the wt receptor efficiently but fails to activate (31, 37).

Model i or ii seems likely to explain the phenotype of the KS-1 mutant, which was no more sensitive to c-α-factor than the parent although it was nine times more sensitive to k-α-f. The phenotypes of the other core mutants (KS-2, KS-3, KS-4, KS-5) suggest that several aspects of function are simultaneously altered. Model iii probably explains the phenotype of KS-6, which encodes a truncated receptor. The KS-6 mutant was extremely sensitive to c-α-f and only weakly activated by dTA-α-f. It has been previously noted that truncated α-factor receptors confer hypersensitivity to the effects of α-factor without altering affinity for ligand (19, 32).

Novel phenotypes conferred by mutant receptors. Several of the mutants exhibited alterations in aspects of signaling other than ligand specificity. These effects may be incidental side effects of mutations that alter the conformation of the receptor. One mutant (KS-1) behaved in a fashion indistinguishable from that of wt except for its ability to be activated by the α-factor analogs. This observation suggests that it is possible to alter response to the foreign peptides without altering other responses. Two of the mutants with the most novel phenotypes have double substitutions (KS-5, KS-3). In one instance (KS-3), it is clear that both substitutions affect function.

Important gaps exist in our understanding of growth arrest and recovery in *S. cerevisiae* (reviewed in references 5, 8, and 24). My observations are consistent with the model (21) that turbid zones of inhibition in response to α-factor need not imply a specific activation of a recovery pathway. Rather, an intermediate level of activation may lead to transitory arrest. For example, the appropriate mixture of c-α-f and dTA-α-f will produce turbid zones of growth inhibition when spotted on a lawn of wt cells (unpublished data). It is possible that the KS-3 mutant which produces larger than normal but turbid zones of growth inhibition in response to α-factor is capable of activating more G protein than wt in response to a weak signal, but less G protein than wt in response to a strong signal. In this regard, the phenotype of KS-3 is reminiscent of certain alleles of *GPA1* (e.g., *GPA1*-Val-50) (21, 27, 38) that encodes the Gα subunit in *S. cerevisiae* (6, 28).

The KS-5 mutant confers a much greater defect in cell cycle arrest than in *FUS1-lacZ* induction. The KS-5 mutant is >1,000-fold less sensitive to α-factor than wt in the zone of inhibition assay, but expresses only 2-fold less *FUS1-lacZ*. Some threshold for arrest may be involved, with KS-5 only able to activate a subset of responses. However, this striking phenotype does resemble that of the *fus3-1* (10) and *far1* (3) mutants that are thought to act at or downstream of a split in the signaling pathway. Unlike *fus3-1* or *far1* strains (3, 10), KS-5 confers a substantial mating defect. Other mutations in *STE2* have been reported to have a greater effect on the arrest phenotype than gene induction (18), but it is not clear whether the phenotypes are as extreme as KS-5. Alteration of temporal aspects of response could explain the KS-5 phenotype. The KS-5 strain might respond to α-factor for only a brief period. *FUS1-lacZ* induction was measured over 1.5 h, whereas the growth inhibition assay required a minimum of 18 h. It has been suggested that the α-factor receptor has a role in mating independent of G protein (16). A defect in this other function might also explain this apparently anomalous phenotype.

We are still far from understanding the mechanism by which ligand binding to the α-factor receptor leads to G-protein activation and cellular responses. Biochemical characterization of the substituted receptors described here may lead to insight into the mechanism of activation of the α-factor receptor and other receptors of this large family.

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