A Limited Spectrum of Mutations Causes Constitutive Activation of the Yeast α-Factor Receptor†

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ABSTRACT: Activation of G protein coupled receptors (GPCRs) by binding of ligand is the initial event in diverse cellular signaling pathways. To examine the frequency and diversity of mutations that cause constitutive activation of one particular GPCR, the yeast α-factor receptor, we screened libraries of random mutations for constitutive alleles. In initial screens for mutant receptor alleles that exhibit signaling in the absence of added ligand, 14 different point mutations were isolated. All of these 14 mutants could be further activated by α-factor. Ten of the mutants also acquired the ability to signal in response to binding of desTrp[1][Ala[3]]α-factor, a peptide that acts as an antagonist toward normal α-factor receptors. Of these 10 mutants, at least eight alleles residing in the third, fifth, sixth, and seventh transmembrane segments exhibit *bona fide* constitutive signaling. The remaining alleles are hypersensitive to α-factor rather than constitutive. They can be activated by low concentrations of endogenous α-factor present in *MAT***a** cells. The strongest constitutively active receptor alleles were recovered multiple times from the mutational libraries, and extensive mutagenesis of certain regions of the α-factor receptor did not lead to recovery of any additional constitutive alleles. Thus, only a limited number of mutations is capable of causing constitutive activation of this receptor. Constitutive and hypersensitive signaling by the mutant receptors is partially suppressed by coexpression of normal receptors, consistent with preferential association of the G protein with unactivated receptors.

Signaling mediated by G protein coupled receptors (GPCRs)[1] is responsible for cellular responses to a wide variety of physical and chemical stimuli. Upon activation by bound ligand, GPCRs interact with cytoplasmic heterotrimeric G proteins, resulting in release of GDP from G protein α subunits and binding of GTP. This, in turn, leads to dissociation of G protein α subunits from β and γ subunits and to activation of downstream effectors either by GTP-bound α subunits or by the released β and γ subunits. GPCRs each contain seven hydrophobic segments of amino acids that appear to correspond to transmembrane α-helices arranged so that the amino terminus of the receptor faces the extracellular medium and the carboxyl terminus faces the cytoplasm (see refs *1* and *2*).

A number of previously identified mutations in GPCRs lead to ligand-independent activation of receptors and constitutive activation of signaling pathways (for review, see refs *3* and *4*). Such mutations are of interest for the following reasons: (1) Amino acid residues that can be mutated to cause constitutive activation of receptors are likely to be involved in switching between the active and inactive states. (2) Analysis of the effects of various agonists and antagonists on constitutive receptors has provided new insights into the mechanisms of drug and hormone actions on receptors (*5*−*7*). (3) Several pathological conditions affecting humans have been linked to constitutive activity of aberrant receptors (*4*, *8*, *9*). (4) Some GPCRs exhibit significant levels of ligand-independent signaling that may be important for maintaining basal activation of downstream effectors (*5*, *6*, *10*).

The pheromone response of yeast provides a convenient system for using genetics to study GPCR function (*11*, *12*). Haploid yeast cells detect potential mating partners by sensing the presence of the peptide pheromones α-factor and **a**-factor that are secreted, respectively, by cells of the *MAT*α and *MAT***a** mating types. The pheromones secreted by cells of one mating type act by binding to receptors on the surface of cells of the opposite mating type. These receptors contain the seven hydrophobic segments typical of GPCRs and they signal via a cytoplasmic G protein composed of α, β, and γ subunits encoded by the *GPA1*, *STE4*, and *STE18* genes, respectively. The downstream target of this G protein is a

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[1] Abbreviations: dTA, desTrp[1][Ala[3]]α-factor; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; FOA, 5-fluororotic acid; GPCR, G protein coupled receptor; TM, transmembrane segment; (i.e., first transmembrane segment, TM1); IC, intracellular loop (i.e., first intracellular loop, IC1).

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Table 1: Plasmids Bearing *STE2* Alleles

STE2 allele	integrating plasmid	CEN plasmid	multicopy plasmid
STE2$^+$	pMD381	pMD149	pMD240
ste2-Δ	pMD147		pMD228
Gly56→Arg	pMD387	pMD283	
Cys59→Arg	pMD388	pMD248	
Asn84→Ser	pMD316	pMD489	pMD420
Leu103→Pro	pMD385	pMD272	pMD419
Ile142→Thr	pMD321	pMD282	
Glu143→Gly	pMD313		
Ser145→Leu	pMD314		
Gln149→Arg	pMD392	pMD274	pMD452
Leu226→Trp	pMD443		
Pro258→Leu	pMD384	pMD251	pMD416
Gln253→Leu	pMD386	pMD252	pMD449
Ser259→Pro	pMD389	pMD253	pMD450
Ser288→Pro	pMD390	pMD255	pMD451
Leu289→Ser	pMD413	pMD278	

MAP kinase pathway that leads to cell cycle arrest, changes in cell morphology, and transcriptional activity of pheromone-responsive genes (*13*−*15*). The yeast receptors and G protein subunits are in some cases functionally interchangeable with components of mammalian G protein signaling systems (*16*, *17*).

We have screened for constitutive mutations of the α-factor receptor encoded by the yeast *STE2* gene. The ease of genetic manipulation of yeast and the availability of assays that allow screening or selection for mutant pheromone receptors make it possible to search through large random mutational libraries to study the spectrum of mutations capable of causing constitutive activation. We report here that, despite extensive mutagenesis, we have recovered only a limited set of constitutively active mutations in the α-factor receptor residing in the third, fifth, sixth, and seventh transmembrane segments. Most of these constitutive mutants have simultaneously acquired a new ability to signal in response to the pheromone analogue desTrp^1[Ala^3]α-factor (dTA) that acts as an antagonist toward normal α-factor receptors. Constitutive signaling by each mutant receptor could be partially suppressed by coexpression of normal receptors.

EXPERIMENTAL PROCEDURES

Plasmids. Plasmids used in this work are presented in Table 1.

Random mutagenesis of the α-factor receptor was conducted using the *CEN* plasmid pMD149 encoding the *STE2* gene with a *URA3* marker (*18*).

Potentially constitutive *ste2* alleles were recreated by site-directed mutagenesis of the *STE2* gene essentially as described previously (*12*, *19*). The sequences of the particular oligonucleotides used for mutagenesis are available on request. The template used for site-directed mutagenesis was either the integrating plasmid pMD310 or the *CEN* plasmid pMD149 (*18*). Plasmid pMD310 was created by ligating a 4.3 kb *Sph*I−*Eco*RI fragment containing the *STE2* gene from plasmid pMD104 (*19*) into *Sph*I−*Eco*RI-cut integrating vector pMD297. This vector was derived from the previously described pMD141 (*20*) by cutting pMD141 with *Bam*HI and religating to remove the *STE2*-encoding insert to create pMD207, then cutting pMD207 with *Cla*I and re-ligating to remove the yeast origin of replication. For chromosomal integration of *ste2* alleles recreated in the *CEN* plasmid pMD149, the mutated *ste2* alleles were transferred to the integrating plasmid pMD364 as *Bsp*E1−*Eco*RI fragments extending from the *Bsp*E1 site 481 bp upstream from the *STE2* coding region to the *Eco*RI site in the multiple cloning region 3′ to the *STE2* gene in pMD149. Plasmid pMD364 was derived from pMD310 by excising a 354 bp *Hpa*I−*Stu*I fragment from the *STE2* gene and religating the blunt ends. Deletion of this *Hpa*I−*Stu*I fragment from this parent plasmid allowed unambiguous identification of plasmids in which the full-length mutated *ste2* allele had subsequently been ligated.

Multicopy plasmids containing various mutant *ste2* alleles were created by transferring the *Sac*I−*Sph*I fragments containing the altered *ste2* genes from integrating plasmids (see above) to the multicopy *URA3*$^+$ vector pMD228 (*20*). The multicopy plasmid containing the HA-tagged *STE2*$^+$ gene was pMD240 (*20*).

To create the *CEN LEU2* plasmid pMD357 containing a c-myc-tagged version of *STE2*, the *Not*I fragment containing the HA tag was excised from the carboxyl terminus of the tagged version of *STE2* in pMD240 (*20*) and replaced by a *Not*I fragment containing three tandem copies of the c-myc tag (*18*). The c-myc-tagged allele was then transferred as a *Sac*I−*Sph*I fragment to a similarly cut, *LEU2*-marked, *CEN* plasmid pMD292. pMD292 was, in turn, created by inserting a *Bgl*II fragment containing the *LEU2* gene from pFL46L (*21*) as a replacement for the *Bgl*II fragment containing *URA3* in pMD149.

Yeast Strains. A summary of the yeast strains used in this study is presented in Table 2. Screening for constitutive mutations was conducted in *far1*-Δ *BAR1*$^+$ host strain A266. This strain was derived from strain A170 (20) *MAT***a** *ste2-10*::*LEU2 cry1*^{R} *ade2-1 his4-580 lys2*$_{oc}$ *trp1*$_{am}$ *tyr1*$_{oc}$ *SUP4-3*^{ts} *leu2 ura3 FUS1*::p[*FUS1*−*lacZ TRP1*]) by use of plasmid pMD150 (*20*), containing flanking regions from *FAR1* with an inserted hisG−*URA3*−hisG cassette (*22*). The deletion cassette was integrated and then removed by recombination, using medium containing 5-fluororotic acid (FOA) to screen for the desired clones as described previously (*20*). Characterization and retesting of constitutive mutations was conducted by integrating the mutant allele into the chromosome of the *far1*-Δ *bar1*$^-$ host strain A268 (*20*). Integration was accomplished by cleaving *ste2*-encoding plasmids with *Pml*I just 3′ to the *STE2* coding region. Ura$^+$ transformants were selected, and then the unwanted allele was popped out by plating to medium containing FOA. Retention of the mutant allele was confirmed by testing for colonies that had lost the *LEU2* gene inserted into the original chromosomal *STE2* locus, by verifying that the colonies were at least partially pheromone-responsive, and by PCR analysis of the *STE2* locus with primers outside the region encoded by the integrated plasmid.

The *KEX2* gene was deleted from strains containing various mutations by one-step gene replacement using a plasmid pKX11::hisG−*URA3*−hisG kindly provided by Alison Bevan and Robert S. Fuller of the University of Michigan (see ref *23*). The *URA3* marker was recovered by plating on FOA. The resulting *kex2*-Δ alleles were confirmed by their cold sensitivity and by PCR amplification of the *KEX2* locus.

Random Mutagenesis of STE2. Random mutagenesis targeted to particular regions of the *STE2* gene was conducted

Table 2: Yeast Strains Containing Various *STE2* Alleles

	strains containing the indicated *STE2* allele on the chromosome				strains containing a chromosomal *STE2*$^+$		strains containing a chromosomal *ste2*-Δ	
STE2 allele	with no plasmid[a]	with the indicated *STE2* allele on a multicopy plasmid[b]	with *STE2*$^+$ on *CEN* plasmid[c]	with a *URA3* vector without insert[d]	with the indicated *STE2* allele on *CEN* plasmid[e]	with the indicated *STE2* allele on multicopy plasmid[f]	with the indicated *STE2* allele on *CEN* plasmid[g]	with the indicated *STE2* allele on multicopy plasmid[h]
STE2$^+$	A529	A742		A740		A742	A1238	A1143
ste2-Δ	A575		A1238	A1239				A1239
Gly56→Arg	A535							
Cys59→Arg	A536							
Asn84→Ser	A508	A585	A586	A1242	A875	A739	A876	A866
Leu103→Pro	A533	A583	A584					
Ile142→Thr	A515		A976					
Glu143→Gly	A505		A974					
Ser145→Leu	A506		A975	A1116				
Gln149→Arg	A540	A734	A735		A1129	A1126	A1135	A1132
Leu226→Trp	A712		A980					
Pro258→Leu	A532	A631	A632	A1118	A860	A736	A862	A863
Gln253→Leu	A534	A728	A729	A1243	A1139	A1140	A1141	A1142
Ser259→Pro	A537	A730	A731	A1120	A1127	A1124	A1133	A1130
Ser288→Pro	A538	A732	A733	A1122	A1128	A1125	A1134	A1131
Leu289→Ser	A635		A979					

[a] The host strain for transformation of the relevant integrating plasmids bearing constitutive *ste2* alleles (see Table 1) was A268. [b] These strains were created by transforming the relevant multicopy plasmids (see Table 1) into yeast strains containing integrated copies of the same mutant *STE2* allele (see previous column of this table). [c] These strains were created by transforming the *CEN* plasmid pMD149 into yeast strains containing integrated copies of the indicated *STE2* allele. [d] These strains were created by transforming the multicopy plasmid pMD228 containing no insert into yeast strains containing integrated copies of the indicated *STE2* allele. [e] These strains were created by transforming a *CEN* plasmid containing the relevant *STE2* allele (see Table 1) into strain A529. [f] These strains were created by transforming a multicopy plasmid containing the relevant *STE2* allele (see Table 1) into strain A529. [g] These strains were created by transforming a *CEN* plasmid containing the relevant *STE2* allele (see Table 1) into strain A575. [h] These strains were created by transforming a multicopy plasmid containing the relevant *STE2* allele (see Table 1) into strain A575.

Table 3: Mutagenesis of the α-factor Receptor

region mutagenized	technique	oligonucleotides used
Asn10−Val86	PCR + recombination with *Hpa*I-cut pMD149	ON32(5′) + ON26(3′)
Ile80−Asn157	PCR + recombination with *Xba*I-cut pMD149	ON56(5′) + ON12(3′)
Glu143−Ile230	PCR + recombination with *Nhe*I-cut pMD149	ON34(5′) + ON29(3′)
Ser214−Asp317	PCR + recombination with *Kpn*I-cut pMD149	ON57(5′) + ON37(3′)
Ile169−Val186 (TM4)	oligonucleotide-directed mutagenesis	ON16
Ser207−Ala229 (TM5)	oligonucleotide-directed mutagenesis	ON95
Val224−Leu238 (IC3, NH_2)	oligonucleotide-directed mutagenesis	ON133
Leu236−Ile249 (IC3, COOH)	oligonucleotide-directed mutagenesis	ON135
Val68−Ile82 (IC1)	oligonucleotide-directed mutagenesis	ON134
Ile150−Thr167 (IC2)	oligonucleotide-directed mutagenesis	ON132

essentially as described previously (*12*, *19*) by error-prone PCR and oligonucleotide-directed mutagenesis with oligonucleotides containing a low percentage of incorrect base at each position. Initial mutagenesis of the gene was performed by the PCR technique over four regions of the gene (see Table 3). Additional searches for mutants were conducted by oligonucleotide-directed mutagenesis of intracellular loops and of the fourth and fifth transmembrane segments (see Table 3), with "spiked" oligonucleotides as described previously (*12*, *19*).

Other Methods. Assays of *FUS1*−*lacZ* induction were performed as described by Sommers and Dumont (*12*, *19*). As indicated in the figures, the effects of mutations on receptor signaling were assayed simultaneously with control strains because of a day-to-day variability in the assays. Immunoblotting with anti-HA antibodies was performed as described by Leavitt et al. (*20*). Yeast transformations were conducted as described by Chen et al. (*24*) for replicating plasmids and by Gietz and Woods (*25*) for integrating plasmids and introduction of mutant libraries into yeast.

The ligand binding properties of mutant receptors were determined with ^{3}H-[Nle^{12}]α-factor (specific activity 30 Ci/mmol) as described previously (*26*). To obtain adequate numbers of receptors expressed on the cell surface, assays were performed on cells expressing relevant *ste2* alleles both from the chromosome and from multicopy plasmids. In each assay, the amount of ligand bound to the strain expressing a particular receptor was corrected by subtracting the binding to an isogenic *ste2*-Δ strain measured at the same time.

RESULTS

Mutagenesis and Screening for Constitutively Active Receptors. The initial strategy for isolation of constitutive mutations in the *STE2* gene, encoding the α-factor receptor, was to randomly mutagenize the receptor by a combination of low-fidelity PCR and in vivo recombination (*27*). Mutagenesis was targeted to the amino-terminal and transmembrane regions, comprising approximately the first 300 out of the 431 residues of the wild-type sequence, because the carboxyl-terminal tail has been shown to be dispensable for

signaling (*28*, *29*). Linear products of low-fidelity PCR amplification corresponding to regions of the *STE2* gene were cotransformed into yeast with *STE2*-encoding centromere-based plasmids that had been linearized at unique restriction sites within the region targeted for mutagenesis (see Table 3). Recombination of the PCR product with the linearized plasmids leads to introduction of the altered region of *STE2* into the replicating plasmid (*12*, *19*, *30*).

The host strain A266 used for screening contains a deletion of the chromosomal *STE2* gene, making it dependent on a plasmid-encoded *STE2* receptor for activation of the pheromone response. This strain also contains a chromosomal copy of a fusion of the *Escherichia coli* β-galactosidase gene (*lacZ*), to *FUS1*, a yeast gene that is transcriptionally induced by activation of the pheromone response pathway. This allows the detection of activating receptor mutations as blue colonies on plates that lack α-factor but contain the β-galactosidase substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal). Expression of the normal α-factor receptor in this genetic background leads to blue colonies on this culture medium only in the presence of added α-factor.

Strain A266 also contains a deletion of the *FAR1* gene, which mediates induction of cell cycle arrest in response to activation of the pheromone response pathway (see refs *14*, and *15*). This deletion was introduced into the strain to prevent strong constitutive mutations from causing permanent cell cycle arrest and lethality. A266 also contains a normal copy of the *BAR1* gene, which encodes a protease that degrades α-factor (see refs *13*, *14*). The normal *BAR1*$^+$ allele was used in the initial screen for constitutive mutants to provide discrimination between true constitutive receptor mutations and hypersensitive alleles, since the *BAR1* protease should reduce levels of endogenously produced α-factor in culture medium. Although α-factor is normally secreted only by *MAT*α cells, low levels of α-factor in a culture of *MAT***a** cells can arise from rare spontaneous mating-type switching or low-level secretion by *MAT***a** cells (see below). A *bar1*$^-$ background was used for testing and characterization of recreated putative constitutive *ste2* alleles, since the absence of the protease allows for more reproducible assays of receptor signaling function.

Two initial rounds of screening were conducted. In each round, at least 12 000 colonies were screened from each of four PCR-generated mutant libraries encompassing the seven transmembrane segments of the receptor (see Table 3). Plasmids were isolated from colonies that exhibited blue color on X-gal plates lacking α-factor and were then retransformed into the host to eliminate the possibility that the phenotype arose from a spontaneous chromosomal mutation in a gene other than *STE2*. Typically, more than half the initial isolates exhibiting signaling in the absence of added ligand were found to have resulted from such chromosomal mutations. Plasmids that gave rise to a reproducible phenotype following retransformation were sequenced over the region targeted for mutagenesis. The alleles sequenced from each of the PCR-generated libraries all contained averages of 1−1.5 nucleotide substitutions per 100 sequenced base pairs or about 3 substitutions per 250 base pair region targeted for mutagenesis. This implies that the activating mutation in each isolated plasmid was, on average, accompanied by two additional silent substitutions and that the average frequency of unselected mutation rate was greater than 0.5 substitution per 100 base pairs. At this mutation rate, assuming that the mutational libraries are completely random, one would expect to find any of the 750 possible single-base substitutions at least once in every 1000 PCR products, which corresponds to one in every 1000 colonies. Thus, the 24 000 colonies screened for each library should represent a significant excess over the number required to achieve complete coverage of all possible mutations. This is consistent with the recovery of the strongest constitutive alleles up to seven times (Table 4).

Table 4: Isolation of Mutations Exhibiting Increased Signaling in the Absence of Added α-Factor

mutation	no. of isolates	mutation	no. of isolates
Pro258→Leu	7	Gly56→Arg[a]	1
Gln253→Leu	4	Cys59→Arg[a]	1
Ser259→Pro	4	Asn84→Ser[b]	1
Leu226→Trp	2	Ile142→Thr	1
Gln149→Arg	2	Ser145→Leu	1
Leu103→Pro[a]	2	Ser288→Pro	1
Glu143→Gly[a]	2	Leu289→Ser[c]	1

[a] These mutations could not be confirmed as constitutive because of the low level of constitutive signaling and their failure to respond to antagonist. [b] This mutation appears to be predominantly hypersensitive rather than constitutive. [c] This mutation may be constitutive or hypersensitive.

Mutations identified by screening of the PCR-generated libraries were recreated by site-directed mutagenesis to separate the multiple substitutions that were present in most alleles and to confirm that the identified sequence alterations were responsible for the altered phenotype. Recreated *ste2* alleles were integrated into the *STE2* locus of the host chromosome to reduce variations in signaling that could be caused by different plasmid copy numbers. Signaling by strains with the integrated site-directed alleles was then assayed by quantitative liquid assays of *FUS1*−*lacZ* expression.

Table 4 includes 13 activating mutations that were isolated in the initial rounds of screening. Six of these alleles were isolated multiple times as alleles that usually also contained various additional mutations, confirming that the mutant clones arose from independent events in which PCR products recombined with linearized plasmids. In addition to the mutations indicated in Table 4, several other alleles causing slight increases in ligand-independent *FUS1*−*lacZ* expression were isolated. However, because constitutive signaling by these other mutants was no greater than the levels of *FUS1*−*lacZ* expression seen in *ste2*-Δ strains, and because these mutant receptors did not respond to α-factor or cause additional phenotypes (see below), they were not considered further.

The limited number of alleles recovered in the initial screens was surprising in view of reports that constitutive mutations are common in some other GPCRs. Thus, more extensive mutagenesis was targeted to certain regions of the receptor in which no alterations had been recovered, including the fourth and fifth transmembrane segments and each of the intracellular loops (see Table 3). This was accomplished by site-directed mutagenesis with "spiked" oligonucleotides that had been synthesized so as to contain an average of one incorrect base per molecule as described previously (*12*, *19*). After screening of approximately 12 000 colonies from each of these oligonucleotide-directed mu-

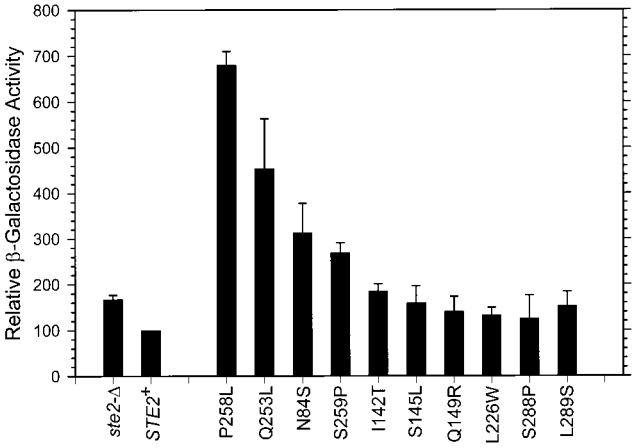

FIGURE 1: *FUS1−lacZ* expression in the absence of added α-factor by cells expressing receptors from a single chromosomal *STE2* allele. The results are normalized to the level of *FUS1−lacZ* expression in the absence of added α-factor in strain A529 expressing a chromosomal copy of the normal *STE2*$^+$ gene.

tagenesis reactions, only one additional allele, Leu226→Trp, that conferred a reproducible weak constitutive phenotype was recovered from a library encompassing the amino-terminal portion of the third intracellular loop (see Tables 3 and 4). In addition, two amino acid substitutions in the third intracellular loop (Gly237→Ala and Phe241→Ala) that had been previously reported to cause weak constitutive phenotypes (*31*) were recreated by site-directed mutagenesis. Neither of these substitutions caused constitutive signaling in our host strain at a level that was significantly different from that of the normal receptor (results not shown).

Signaling Activity of the Mutant Receptors. Levels of activation of receptors were determined in liquid assays of *β*-galactosidase to quantitate induction of the *FUS1−lacZ* reporter construct (see Figure 1). In the absence of added ligand, the levels of signaling by the strongest two mutant alleles, Pro258→Leu and Gln253→Leu, were approximately 7-fold and 5-fold greater, respectively, than the basal level of signaling by the normal receptor. Other mutations that gave clear phenotypes when assayed on X-gal plates exhibited levels of activation that were barely above basal levels in the liquid assay. The amino acid substitutions causing the strongest phenotype were also the substitutions that were the most frequently recovered as multiple isolates from the screens of the mutational libraries (Table 4). Even these strongest alleles do not activate the pheromone response at saturating levels, since larger responses are detected in the presence of α-factor (Figure 2). Furthermore, stronger ligand-independent signaling can be obtained by combining two mutations conferring constitutive activation in the same *STE2* allele or by combining constitutive mutants with a carboxyl-terminal truncation of the receptor after Ser303, which renders the receptor hypersensitive to pheromone (results not shown).

In comparing ligand-independent signaling by a yeast strain containing normal receptors with signaling by an isogenic strain containing a deletion of the entire *STE2* coding region, the strain with the deletion reproducibly exhibits basal *FUS1−lacZ* expression that is 1.7-fold greater than that of the strain with normal receptors (Figure 1). This suggests that the presence of normal receptors may inhibit low-level spontaneous activation of the G protein, at least

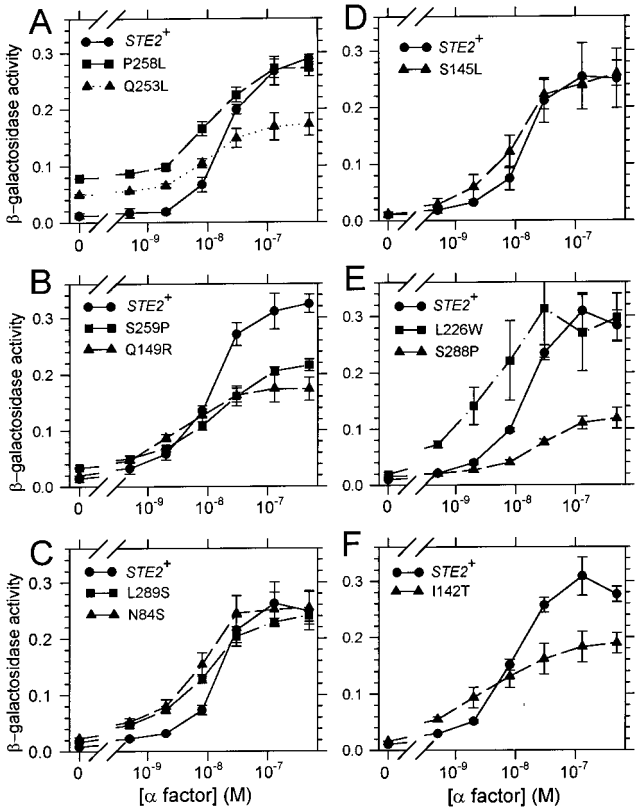

FIGURE 2: Response of strains expressing mutant α-factor receptors to various concentrations of α-factor. Strains shown in each panel of the figure were assayed simultaneously. The assayed yeast strains contained a single chromosomal copy of the indicated *ste2* alleles (see Table 2).

in strains with this particular genetic background. It also raises the question of whether any strains that exhibit a level of ligand-independent activity that is less than 1.7 times that of the *ste2*-Δ control could contain a receptor allele that simply fails to be expressed. However, 10 of the 14 *ste2* alleles we isolated exhibit an additional phenotype, responsiveness to the α-factor analogue dTA (see below), indicating that their constitutive phenotype did not simply result from poor expression. The positions of these 10 mutations in a topological model of the receptor are indicated in Figure 3. The four additional alleles that were recovered, Gly56→Arg, Cys59→Arg, Leu103→Pro, and Glu143→Gly, exhibited slightly elevated levels of ligand-independent signaling and significant responses to α-factor but no response to dTA. Because of the possibility that the apparent constitutive activity of these four alleles might be the result of low levels of receptor expression, they were not studied further. The use of X-gal-containing plates appears to be an extremely sensitive way of detecting low levels of signaling activity, since it allowed identification of mutants that exhibit an increase in basal signaling that is less than 2-fold when quantitated in liquid assays. The reliability of the method is confirmed by the observation that most of the mutants that exhibited ligand-independent signaling on X-gal plates simultaneously acquired a second phenotype, the ability to respond to dTA, an α-factor analogue that acts as an antagonist toward the wild type α-factor receptor.

The 10 mutant *ste2* alleles selected for further study retained the ability to respond to added α-factor (see Figure 2). At saturating concentrations of α-factor, some of the

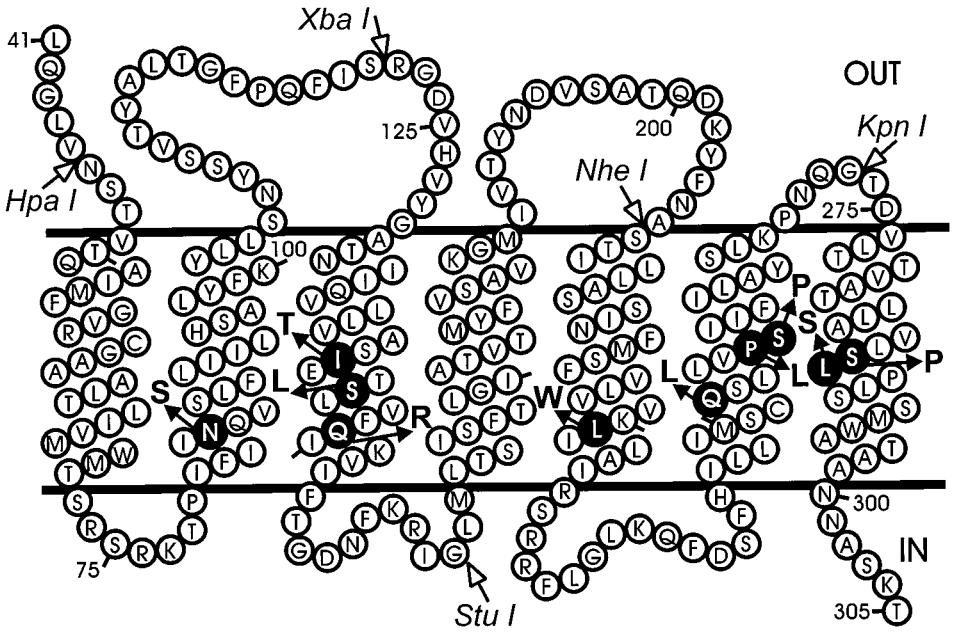

FIGURE 3: Schematic diagram of a proposed topological arrangement of the transmembrane regions of the yeast α-factor receptor. The positions of mutations identified in this study are indicated. The indicated restriction sites refer to unique cutting sites in the *STE2* gene that were used to linearize plasmids for recombinational insertion of mutagenized PCR products (see Experimental Procedures and Table 3).

mutant receptors induced levels of expression of *FUS1*−*lacZ* that were similar to maximal levels induced by normal receptors (Figure 2). However, five of the mutants, Ile142→Thr, Gln149→Arg, Gln 253→Leu, Ser259→Pro, and Ser288→Pro, exhibited diminished maximal responses to α-factor. Several of the mutants, Asn84→Ser, Leu226→Trp, Pro258→Leu, and Leu289→Ser, exhibited decreased EC_{50}s (concentrations of agonist required for 50% activation) for α-factor, compared with normal receptors.

To determine whether the level of expression of the mutant receptors affected levels of ligand-independent signaling, some of the mutant alleles were transferred to multicopy plasmids and expressed in a host strain that also contained the same mutant allele integrated into the chromosome. The increased gene dosage had little effect on the constitutive signaling of most of the mutations (results not shown. Also, compare the constitutive signaling by single-copy chromosomal *ste2* alleles in Figure 1 with the level of signaling by the same alleles on multicopy plasmids in a *ste2*-Δ host strain; open bars, Figure 7).

Effects of Deletion of the KEX2 Gene Responsible for Maturation of α*-Factor on Signaling by Mutant Receptors.* Production of α-factor is normally repressed in *MAT***a** cells, but low levels of α-factor can be present in cultures of *MAT***a** strains, either because of infrequent mating-type switching or because of low-level basal production of α-factor by normal *MAT***a** cells. In some hypersensitive strains, these low levels of pheromone are sufficient to activate the pheromone response pathway (*32*; L. M. Leavitt, C. M. Sommers, and M. E. Dumont, unpublished results). For mutant receptors that exhibit near-normal EC_{50}s and normal or reduced maximal responses to α-factor, autocrine stimulation by endogenous α-factor is not a plausible explanation for the ability to signal in the absence of added ligand. However, for mutants with decreased EC_{50}s, such as Asn 84→Ser, Leu226→Trp, and Leu289→Ser, and for those with the strongest constitutive phenotype (Gln253→Leu and Pro258→Leu), we determined whether the apparent constitutive behavior could actually be the result of autocrine stimulation. To resolve this question, the *KEX2* gene was deleted from strains expressing some of the mutant receptors (Figure 4). *KEX2* encodes a protease involved in processing the polyprotein precursor to α-factor. Deletion of this gene leads to a severe reduction in the level of secreted α-factor (*23*). In the absence of added ligand, deletion of *KEX2* did not cause any significant reduction in most of the strains tested (Figure 4A,C,D). However, signaling by the Asn84→Ser allele was substantially decreased in the *kex2*$^-$ background (see Figure 4B). One additional mutant, Leu289→Ser, that showed slight hypersensitivity to α-factor was not tested in a *kex2*$^-$ background, so signaling by this mutant in the absence of added ligand could also be the result of hypersensitivity rather than constitutive activation.

The ability of *kex2* deletions to deplete endogenous α-factor from cultures of *MAT***a** cells has been confirmed in our system by use of a carboxyl-terminal truncation of the α-factor receptor. It has previously been reported that cells expressing such truncations are hypersensitive to pheromone (*28*, *29*). Consistent with this, we found that introduction of a stop codon into the *STE2* gene at amino acid residue 304, just following the seventh transmembrane segment, caused cells to be hypersensitive to added α-factor, reducing the EC_{50} for pheromone approximately 20-fold. In the absence of added α-factor, the *bar1*$^-$ strain expressing the truncated receptor exhibited levels of the *FUS1*−*lacZ* activity that were higher than those observed for any of the constitutive receptors. However, deletion of the *KEX2* gene reduced this signaling to a level that was the same as the basal level in cells expressing normal receptors (results not shown). Thus, deletion of *KEX2* is sufficient to eliminate the response to endogenous α-factor by a receptor that makes cells much more sensitive to α-factor than any of the constitutive mutations we have isolated, confirming that deletion of *KEX2* provides a rigorous test for distinguishing hypersensitive from constitutive alleles.

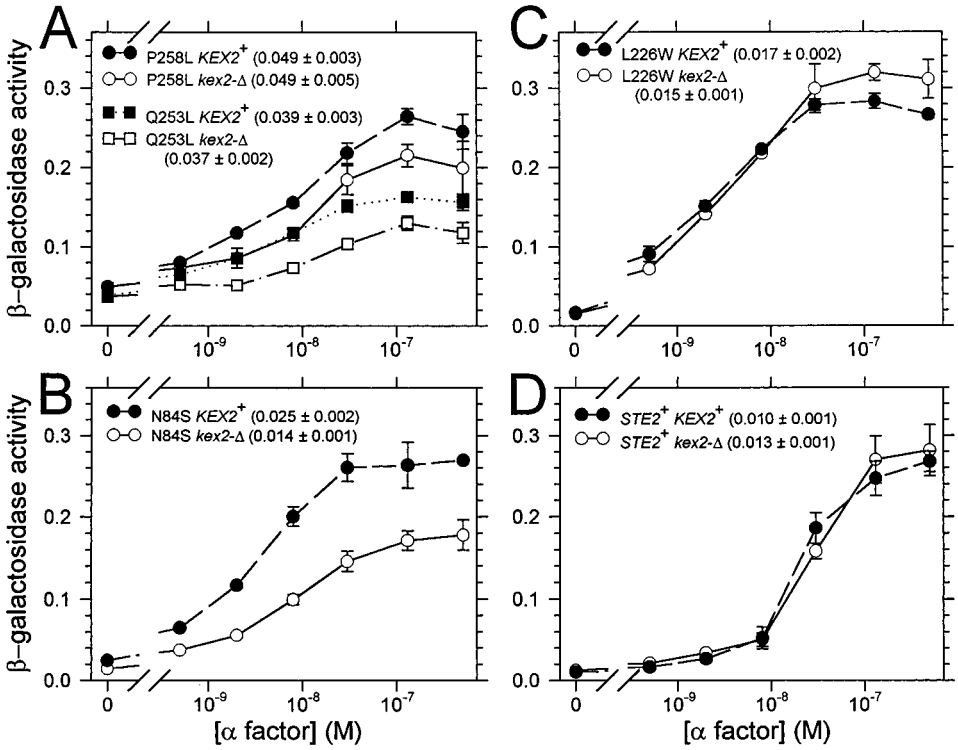

FIGURE 4: Effects of deleting the *KEX2* gene on expression of the *FUS1*−*lacZ* reporter gene in strains containing either normal or mutant *STE2* alleles. Strains shown in each panel of the figure were assayed simultaneously. The assayed yeast strains contained a single chromosomal copy of the indicated *ste2* alleles (see Table 2). The magnitudes of the *FUS1*−*lacZ* activities detected in the absence of added α-factor are indicated in each panel. The *KEX2*$^+$ strains are listed in Table 2. The *kex2*$^-$ strains used for the assays were A895 (*STE2*$^+$), A1224 (Pro258→Leu), A1225 (Gln253→Leu), and A1109 (Leu226→Trp).

While deletion of the *KEX2* gene did not affect signaling of most of the mutants in the absence of added ligand, deletion of this gene did significantly reduce the response to added α-factor of some of the strongest constitutive receptors (Figure 4). This effect of *KEX2* on the response to ligand cannot be directly explained by the low levels of α-factor secreted by *MAT***a** cells, as the reduced signaling persists at micromolar concentrations of added α-factor, representing several orders of magnitude excess over endogenous levels. No such reduction in the response to added α-factor was observed in strains expressing normal *STE2*$^+$ receptors (Figure 4D). One explanation for this effect might be that unprocessed α-factor precursors that accumulate in the absence of the *KEX2* protease inhibit the response to α-factor by the mutant receptors. Thus, in the *kex2*-Δ strains, constitutive receptors (but not normal receptors) may nonproductively bind the unprocessed precursors, rendering the cells less able to be activated by mature α-factor added to the medium.

Expression Levels and Ligand Affinities of Constitutively Active Receptors. All the tested mutant receptors were present in crude cell extracts at near-normal levels. This was determined by immunoblotting with antibodies directed against the influenza hemagglutinin HA epitope fused to the carboxyl termini of the various *ste2* alleles (see Figure 5A). Immunoblotting was also used to determine whether expression of *ste2* alleles from multicopy plasmids in fact led to increased expression of receptors in cells (Figure 5B). While expression of the Gln253→Leu and Asn84→Ser mutants from multicopy plasmids clearly increased cellular levels of these mutant receptors, little or no increase was seen in the case of the Pro258→Leu mutation, implying that some factor other than gene copy number is limiting synthesis of this mutant.

To determine the relative abundances of mutant receptors at the cell surface, we assayed the binding of radiolabeled α-factor to selected mutant receptors. The number of receptors expressed on the cell surface was severely diminished for all the mutants tested (see Table 5). However, for each of the mutant alleles listed in Table 4, the existence of some population of mutant receptors on the cell surface was demonstrated by the sensitivity of strains expressing these receptors to exogenous α-factor. In addition, as discussed below, 10 of the mutants also respond to externally added dTA. There was no correlation between the numbers of ligand binding sites at the cell surface and the constitutive activity of the mutant receptors. For example, the strongest constitutive allele, Pro258→Leu, yielded a relatively low number of binding sites for α-factor.

In cases where the number of binding sites was sufficient to allow calculation of a dissociation constant, the affinities of the constitutive receptors were higher than that of the normal allele. The measured dissociation constant of the *STE2*$^+$ allele was somewhat higher than has been reported in some studies (*33*). However, as discussed previously (*34*), this may be the result of conducting the assay on a strain that expresses abnormally high levels of receptor. Since association with G protein has been reported to lead to increased ligand affinity, overexpression may reduce the apparent affinity by increasing the fraction of cell-surface receptors that cannot associate with G proteins. Conversely, the observed decrease in the level of cell-surface expression of the constitutive receptors may explain why they exhibit increased apparent affinities for α-factor, since the fraction

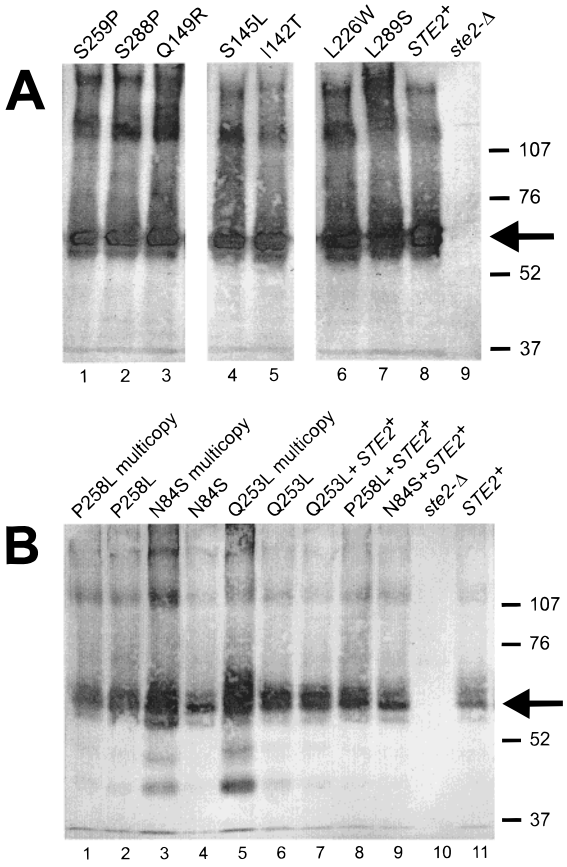

FIGURE 5: Immunoblot analysis of total cellular levels of normal and mutant α-factor receptors by use of antibody against the HA epitope tag. The arrow indicates the mobility of the family of bands comprising of monomeric α-factor receptor. Note that the α-factor receptor has previously been reported to migrate as differentially glycosylated bands in various oligomeric states (see refs *18*, *20*, and *62*). (A) Cellular levels of receptors expressed from chromosomal *STE2* alleles. Each lane of the gel was loaded with extract derived from 8×10^5 cells of each strain. (B) Lanes, 1, 3, and 5: The indicated *ste2* alleles were expressed both from a chromosomal copy of the gene and from a multicopy plasmid. (Note that less extract was loaded on these lanes than on the other lanes in this figure.) Lanes 2, 4, and 6: The indicated constitutive alleles were expressed only from a chromosomal copy of the gene. Lanes 7−11: The indicated constitutive alleles were coexpressed with a normal *STE2*$^+$ allele that was tagged with a c-myc epitope and lacked an HA epitope. Thus only the constitutive allele is detected on the blot. For samples derived from strains containing only a chromosomal copy of the particular *STE2* allele, the gel was loaded with extract obtained from 10^6 cells. For samples derived from strains containing the *STE2* allele on a multicopy plasmid, the gel was loaded with extract obtained from 2×10^5 cells. The strains used in this figure are listed in Table 2, except for lanes 7−11 of panel B, which contained extract from strains A867, A877, A878, A868, and A869, respectively. Each of these strains was constructed by transforming the *CEN* plasmid pMD357, encoding the c-myc-tagged *STE2*$^+$ gene (see Experimental Procedures) into a host with an integrated copy of the indicated *ste2* allele.

of the mutant cell-surface receptors that can associate with G proteins may be increased.

Constitutive and Hypersensitive Mutants Can Be Partially Activated by the α-Factor Analogue dTA, Which Acts as an Antagonist toward Normal Receptors. In a *STE2*$^+$ strain, the α-factor antagonist dTA elicits no detectable pheromone response and inhibits the response to agonist (see Figures 6 and 7). However, most of the constitutive and hypersensitive mutant alleles we isolated acquired the ability to signal in response to this α-factor analogue (Figure 6). Such signaling occurred at concentrations of dTA that were similar to those at which this peptide exhibits antagonist behavior toward normal *STE2*$^+$ receptors. Maximal responses to dTA binding ranged from approximately 10% to 60% of the maximal response of normal receptors to α-factor and from approximately 15% to 100% of the maximal response of the particular mutant allele to α-factor (compare Figure 6 with Figure 2; note that the concentration of α-factor applied to the *STE2*$^+$ strain in Figure 6A is subsaturating). The strength of the response to the dTA was not correlated with the level of constitutive signaling by a particular mutant receptor but did appear to be related to the level of signaling in response to α-factor by each receptor. For example, two mutants, Gln253→Leu and Ile142→Thr, that are the weak responders to α-factor exhibited the lowest absolute levels of signaling in response to dTA, although one of these mutants, Gln253→Leu, exhibits one of the strongest constitutive phenotypes.

Table 5: Binding of α-Factor to Mutant and Normal Receptors

mutation[a]	K_D (nM)	no. of binding sites[b]
STE2$^+$	21 ± 7	50000 ± 7000
Asn84→Ser	5 ± 7	8600 ± 900
Leu103→Pro	8 ± 3	6800 ± 800
Gln149→Arg	ND[c]	<5000
Gln253→Leu	2 ± 2	5200 ± 1400
Pro258→Leu	ND[c]	<5000
Ser259→Pro	ND[c]	<5000
Ser288→Pro	ND[c]	<5000

[a] Binding assays were conducted with strains expressing the indicated mutant or normal *STE2* alleles both from the chromosomal *STE2* locus and from multicopy plasmids (see Table 2). [b] The number of binding sites per cell is indicated. Binding assays for the *STE2*$^+$ strain were conducted with 1×10^7 cells/assay. Binding assays for the Pro258→Leu and Ser259→Pro alleles were conducted with 3×10^7 cells/assay. Binding assays for the Asn84→Ser, Leu103→Pro, Gln149→Arg, and Ser288→Pro, mutants were conducted with 3.2×10^7 cells/ assay. The binding assay for the Gln253→Pro allele was conducted with 3×10^7 cells/assay. [c] ND, not determined; low levels of binding made it impossible to calculate a reliable dissociation constant for these mutants.

Suppression of Mutant Phenotypes by Coexpression of STE2$^+$ *Receptors.* Expression of a normal α-factor receptor from a centromere-based plasmid in cells that also contained constitutive or hypersensitive mutant receptors substantially reduced both basal and dTA-stimulated signaling by the mutant receptors (Figures 6 and 7). To test the dependence of this recessive behavior of the mutant receptors on the relative gene dosages of the normal and mutant alleles, we also examined signaling by mutant alleles expressed from multicopy plasmids in a host strain containing a single chromosomal copy of a normal α-factor receptor. Even at this high ratio of constitutive to normal alleles, ligand-dependent signaling was suppressed (Figure 7). An increase in the overall cellular levels of the mutant receptors expressed from multicopy plasmids was demonstrated by immunoblotting (Figure 5B); however, low numbers of binding sites detected for the mutant receptors (Table 5) make it difficult to confirm that overexpression in fact leads to increased numbers of receptors on the cell surface.

The constitutive and hypersensitive mutants were not completely recessive. Coexpression of normal receptors with the strongest constitutive mutant, Pro258→Leu, resulted in

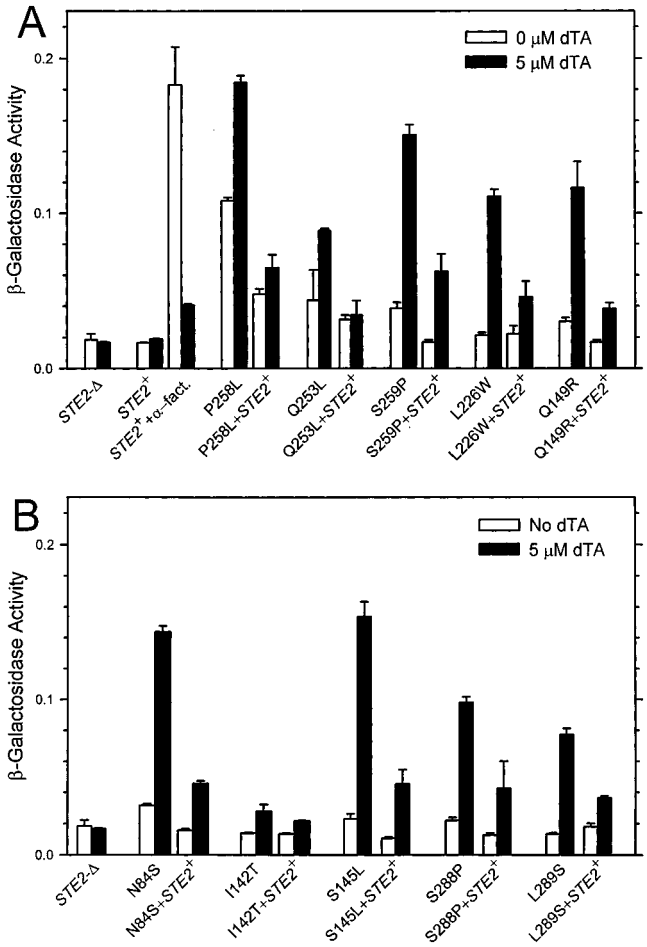

FIGURE 6: Mutant constitutive and hypersensitive receptors are capable of responding to the α-factor antagonist dTA. The graph shows levels of induction of the *FUS1−lacZ* reporter in response to the α-factor antagonist dTA. Where indicated, signaling by the indicated mutant receptors was tested in a strain that coexpressed a normal *STE2*$^+$ allele from the *CEN* plasmid pMD149 in the presence and absence of 5 μM dTA (in the absence of α-factor). Also shown is the response of a normal *STE2*$^+$ strain (A529) to dTA, to a subsaturating level of α-factor (10 nM), and to dTA in the presence of 10 nM α-factor. The indicated mutant alleles were present as single chromosomal copies in strains listed in Table 2.

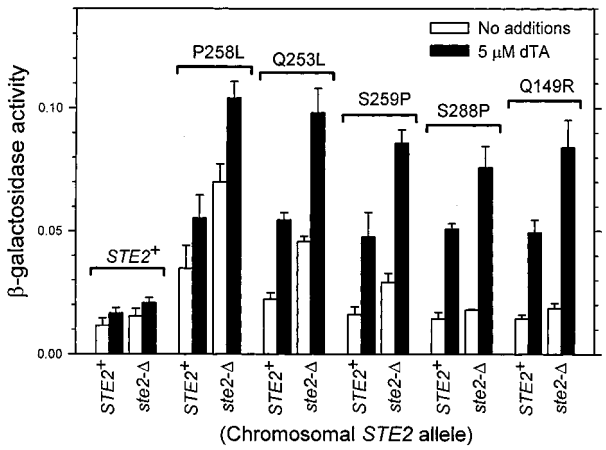

FIGURE 7: Expression of a single copy of *STE2*$^+$ can suppress signaling by constitutive receptors expressed from multicopy plasmids. The mutant alleles indicated above the bars were present on multicopy plasmids. Labels below the bars indicate whether the host strain contained a chromosomal *STE2*$^+$ or a deletion of the chromosomal *STE2* gene (see Table 2). Assays of β-galactosidase activity were performed with no additions (open bars) or in the presence of 5 μM dTA (solid bars).

residual levels of constitutive signaling that were more than twice the level of basal signaling of an isogenic strain expressing normal receptors alone (Figures 6 and 7). Similarly, responses of the mutant receptors to dTA in the presence of a coexpressed *STE2*$^+$ gene were as much as 5 times greater than the response of normal receptors to similar concentrations of dTA. This incomplete recessiveness was dependent on the gene dosage of the coexpressed normal *STE2*$^+$ receptor. Residual signaling by the mutant receptors was greatest in strains expressing only a single chromosomal copy of the normal *STE2*$^+$ gene (see Figure 7), was somewhat weaker in strains expressing *STE2*$^+$ from a *CEN* plasmid (Figure 6), and was weakest in strains expressing *STE2*$^+$ from a multicopy plasmid (results not shown).

DISCUSSION

Screening of libraries of random mutations in the transmembrane regions and loops of the α-factor receptor yielded only three constitutive mutations, Pro258→Leu, Gln253→Leu, and Ser259→Pro, that activated signaling more than 2-fold over the basal level seen with normal receptors. Five additional alleles exhibited weaker, though unambiguous, constitutive phenotypes. Of this total of eight constitutive mutants, five, including the three alleles with the highest constitutive activity, were recovered multiple times from the random mutational libraries. These statistics indicate that the screen for single-base mutations that cause strong constitutive activation of the α-factor receptor is nearly saturated. Recovery of mutations causing weak constitutive activation of the α-factor receptor, on the other hand, did not reach saturation in the screens we conducted, since several alleles were recovered only a single time. This is most likely explained by the weakness of the phenotype, leading to inefficient detection on X-gal plates. However, only a limited number of mutations appears to be capable of causing weak constitutive activation of the receptor, since the total number of weak alleles recovered was low and several were recovered more than once.

These results indicate that the spectrum of single-base mutations capable of causing constitutive activation of the α-factor receptor is severely restricted. The failure to recover additional constitutive alleles is striking in view of the fact that the number of colonies screened in each PCR-generated mutational library should have been sufficient to include multiple examples of every single-base mutation in the targeted regions of the receptor (see Results). Furthermore, we were unable to recover more than a single additional allele by using oligonucleotide-directed mutagenesis to intensively mutagenize particular regions of the receptor in which no constitutive mutations were recovered from the initial PCR-generated libraries. Still more evidence for the scarcity of mutations capable of causing constitutive activation of this receptor is provided by overlap between mutations that we recovered and alleles identified in independent mutational studies conducted by other laboratories:

(1) The two strongest constitutive mutations we recovered, Pro258→Leu and Gln253→Leu (recovered in seven and four different isolates, respectively) have also been identified by other groups using different methods for screening and

mutagenesis (*35*−*37*). These other groups have also used site-directed mutagenesis to create two weaker alleles, Pro258→Ala (*37*) and Ser254→Phe (*36*) that were not recovered in the present screen. We would not expect to recover any of the additional site-directed mutants created by these groups, as they are detected only in supersensitive *sst2*$^-$ strains or they require multiple base substitutions, which would be rare in random mutational libraries.

(2) The substitution Ser259→Pro, the third strongest constitutive mutations we recovered, was previously identified in a screen for *STE2* mutations capable of conferring responsiveness to dTA (*38*). This same screen recovered three additional candidates for dTA-sensitive mutants, Asn84→Asp, Asp, Gln149→Arg, and Ile142→Asn, that are the same as, or are mutated at the same residue as, mutations that we recovered by screening for constitutive activation (*38*).

(3) Two weak constitutive mutations we recovered, Ser145→Leu and Ser288→Pro, were previously identified in a screen for mutant *Saccharomyces cerevisiae* α-factor receptors that had acquired the ability to respond to α-factor from the related species *Saccharomyces kluyveri*. These mutations were also previously shown to confer responsiveness to dTA (*39*).

While it has not been possible to conduct complete screens of mutant libraries to isolate constitutive mutations in mammalian receptors, the α-factor receptor appears to be less likely to be activated by mutations than other GPCRs, such as the thyrotropin receptor, in which more than a dozen constitutive alleles have been identified from studies of human thyroid abnormalities (*9*). This resistance of the α-factor receptor to constitutive mutations is particularly striking in examining the third intracellular loop of the receptor. Constitutive mutations in this loop have been found in the α_{1B}-adrenergic receptor (*40*), in the β_2-adrenergic receptor (*5*), in luteinizing hormone receptors (*41*), in thyrotropin receptors (9), and in yeast **a**-factor receptors (*42*). In the thyrotropin (*43*) and **a**-factor (*42*) receptors, the constitutive alleles in this loop include deletions. In α_{1B}-adrenergic receptors, substitution of each of 19 different amino acids at Ala293 in the third loop leads to increased ligand-independent activation of these receptors (*40*). The ease of generating activating mutations in the third intracellular loop suggests that GPCRs act as negative regulatory elements preventing inappropriate association of activating regions of intracellular loops with G proteins. Since there should be many ways of interfering with a negative regulatory element, this leads to the prediction that constitutive mutations should be common. The scarcity of constitutive *ste2* mutations argues against the applicability of this picture to the α-factor receptor. Both the present study and a previous screen of the third intracellular loop of the α-factor receptor by Clark et al. (*31*) failed to find any strongly constitutive mutations in this region, though Clark et al. uncovered loss-of-function alleles. The mutations that we and others (*9*, *41*, *44*−*47*) have recovered in the fifth and sixth transmembrane segments could, however, be acting indirectly to affect the properties of the third intracellular loop.

These results suggest that different receptors may have different stringencies of regulation of activation. Mutations that cause constitutive activation of one type of GPCR have previously been found to produce different effects when introduced at the homologous position in a different, related receptor (*48*, *49*). In endocrine signaling systems, low levels of constitutive activity of some receptors may play important physiological roles in maintaining basal levels of response. In contrast, inappropriate activation of yeast receptors would be expected to be detrimental to yeast cells, since the pheromone response induces growth arrest. In the **a**-factor receptor of yeast, which interacts with the same trimeric G protein as the α-factor receptor, constitutive mutations in the third intracellular loop appear to be common (*42*), but the frequency of such mutations in the rest of the molecule is not known.

Aside from the lack of constitutively activating alleles in the third intracellular loop, the clustering of constitutive mutations in the third, sixth, and seventh transmembrane segments of the α-factor receptor matches the locations of constitutive mutations found in other receptors. Activating mutations in the third transmembrane segment have been found in angiotensin II receptors (*50*), α_{1B}-adrenergic receptors (*51*), β_2-adrenergic receptors (*52*), bradykinin B_2 receptors (*44*), δ-opioid receptors (*53*), thyrotropin receptors (*9*), and rhodopsin (*4*). Constitutive mutations in the sixth transmembrane segment have been found in the bradykinin B_2 receptors (*44*), luteinizing hormone receptors (*41*), m5 muscarinic acetylcholine receptors (*45*), parathyroid hormone receptors (*46*), thyrotropin receptors (*9*), and rhodopsin (*47*). The existence of activating mutations in the third and sixth transmembrane segments is consistent with independent evidence that relative motion of these transmembrane segments is important for receptor activation (*54*, *55*). Constitutive mutations have been found in the seventh transmembrane segments of the luteinizing hormone receptor (*41*), δ-opioid receptor (*53*), rhodopsin (*4*), and the thyrotropin receptor (*9*).

The constitutive, hypersensitive, and dTA-sensitive phenotypes of the mutant receptors we have isolated are all at least partially recessive. In addition, deletion of the *STE2* gene encoding the α-factor receptor causes a small but reproducible increase in *FUS1*−*lacZ* expression in the absence of α-factor. Taken together, these results indicate that the presence of unactivated receptors can inhibit either basal levels of G protein signaling or signaling caused by coexpressed activated receptors. Such inhibition of signaling by unactivated receptors is also consistent with previous demonstrations of recessive properties of hypersensitive and constitutive receptors (*28*, *29*, *35*, *37*, *42*) and dominant negative effects of certain receptors in yeast (*20*, *56*).

In the case of dominant negative receptors, the most likely explanation for the dominant effects appears to be sequestration of inactive G proteins by association with unactivated receptors (*20*, *56*). In theory, this single mechanism can also explain the recessive behaviors of the hypersensitive, constitutive, and dTA-sensitive mutant receptors, since in each of these cases, expression of a less active receptor inhibits signaling by a more active one. Dominant decreases in signaling caused by expression of a particular class of receptors could also conceivably be caused by interference in the biosynthesis or targeting of the more recessive class of receptors or by co-oligomerization of normal and mutant receptors. However, at least in the case of hypersensitive mutant receptors, this is unlikely because expression of substoichiometric numbers of normal receptor are capable of suppressing signaling by large numbers of hypersensitive receptors at the cell surface (*28*, *29*).

Most of the mutations that we isolated on the basis of their ability to signal in the absence of added ligand also simultaneously acquired the ability to signal in response to the α-factor analogue, dTA, which acts as an antagonist toward normal α-factor receptors. Several previous studies have also identified activating mutations that allow α-factor receptors to signal in response to dTA (*32*, *38*, *57*, *58*); thus, mutations rendering the α-factor receptor sensitive to dTA represent a common class of alleles. Deletion of the *SST2* gene, encoding an RGS protein that stimulates the GTPase activity of the yeast G protein and decreases the potency of α-factor signaling (*59*), also allows the α-factor receptor to respond to dTA (*60*).

There are several possible explanations for the fact that so many activating mutations make the receptor sensitive to dTA: (1) Some of the conditions that make it easier to activate the receptor may also lower a kinetic or thermodynamic barrier that prevents dTA from activating normal α-factor receptors. (2) The same portions of the receptor that are responsible for the specificity of ligand discrimination may also be involved in the conformational switch regulating receptor activation so that mutations that affect one function necessarily also affect the other. (3) Conditions that favor receptor activation may destabilize a particular nonsignaling state of the receptor that is induced by binding of the antagonist dTA binding (see ref *61*).

In summary, following extensive screening, we have identified a set of mutations in transmembrane segments that confer constitutive signaling on the GPCR encoded by the yeast *STE2* gene. The number of mutations capable of causing constitutive activation of the α-factor receptor appears to be very limited, and it is likely that no single-base mutations in the fourth transmembrane segment or the intracellular loops are capable of causing this phenotype. The constitutively active α-factor receptors are present at the cell surface in diminished numbers compared with the normal abundance of wild-type receptors. In addition to their constitutively active behavior, the mutants we have characterized are also capable of signaling in response to a ligand that acts as an antagonist toward normal α-factor receptors. The effects of these mutations are all partially suppressed by coexpression of normal receptors.

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REFERENCES

1. Strosberg, A. D. (1991) *Eur. J. Biochem. 196*, 1−10.
2. Ji, T. H., Grossmann, M., and Ji, I. (1998) *J. Biol. Chem. 273*, 17299−17302.
3. Lefkowitz, R. J., Cotecchia, S., Samama, P., and Costa, T. (1993) *Trends Pharmacol. Sci. 14*, 303−307.
4. Rao, V. R., and Oprian, D. D. (1996) *Annu. Rev. Biophys. Biomol. Struct. 25*, 287−314.
5. Samama, P., Cotecchia, S., Costa, T., and Lefkowitz, R. J. (1993) *J. Biol. Chem. 268*, 4625−4636.
6. Costa, T., Ogino, Y., Munson, P. J., Onaran, H. O., and Rodbard, D. (1992) *Mol. Pharmacol. 41*, 549−460.
7. Samama, P., Pei, G., Costa, T., Cotecchia, S., and Lefkowitz, R. J. (1994) *Mol. Pharmacol. 45*, 390−394.
8. Dhanasekaran, N., Heasley, L. E., and Johnson, G. L. (1995) *Endocrine Rev. 16*, 259−270.
9. Van Sande, J., Parma, J., Tonacchera, M., Swillens, S., Dumont, J., and Vassart, G. (1995) *J. Clin. Endocrinol. Metab. 80*, 2577−2585.
10. Bond, R. A., Leff, P., Johnson, T. D., Milano, C. A., Rockman, H. A., McMinn, T. R., Apparsundaram, S., Hyek, M. F., Kenakin, T. P., Allen, L. F., and Lefkowitz, R. J. (1995) *Nature 374*, 272−6.
11. Dohlman, H. G., Thorner, J., Caron, M. G., and Lefkowitz, R. J. (1991) *Annu. Rev. Biochem. 60*, 653−688.
12. Sommers, C. M., and Dumont, M. E. (1999) in *Structure-Function Analysis of G-Protein Coupled Receptors* (Wess, J., Ed.) Receptor Biochemistry and Methodology (Strader, C., and Sibley, D., Series Eds.) Vol. 3, John Wiley and Sons, New York.
13. Sprague, G. (1991) *Methods Enzymol. 194*, 77−93.
14. Kurjan, J. (1992) *Annu. Rev. Biochem. 61*, 1097−1129.
15. Bardwell, L., Cook, J. G., Inouye, C. J., and Thorner, J. (1994) *Dev. Biol. 166*, 363−379.
16. Kajkowski, E. M., Price, L. A., Pausch, M. H., Young, K. H., and Ozenberger, B. A. (1997) *J. Recept. Signal Transduct. Res. 17*, 293−303.
17. Erickson, J. R., Wu, J. J., Goddard, J. G., Tigyi, G., Kawanishi, K., Tomei, L. D., and Kiefer, M. C. (1998) *J. Biol. Chem. 273*, 1506−1510.
18. Martin, N. P., Leavitt, L. M., Sommers, C. M., and Dumont, M. E. (1999) *Biochemistry 38*, 682−695.
19. Sommers, C. M., and Dumont, M. E. (1997) *J. Mol. Biol. 266*, 559−575.
20. Leavitt, L. M., Macaluso, C. R., Kim, K. S., Martin, N. P., and Dumont, M. E. (1999) *Mol. Gen. Genet. 261*, 917−932.
21. Bonneaud, N., Ozier-Kalogeropoulos, O., and Li, G. (1991) *Yeast 7*, 609−615.
22. Alani, E. L., Cao, L., and Kleckner, N. (1987) *Genetics 116*, 541−545.
23. Bevan, A., Brenner, C., and Fuller, R. S. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 10384−10389.
24. Chen, D. C., Yang, B. C., and Kuo, T. T. (1992) *Curr. Genet. 21*, 83−84.
25. Gietz, R. D., and Woods, R. A. (1994) in *Molecular Genetics of Yeast: Practical Approaches* (Johnston, J. A., Ed.) pp 124−134, Oxford University Press, Oxford, England.
26. Raths, S. K., Naider, F., and Becker, J. M. (1988) *J. Biol. Chem. 263*, 17333−17341.
27. Cadwell, R. C., and Joyce, G. F. (1994) *PCR Methods Appl. 3*, S136−S140.
28. Konopka, J. B., Jenness, D. D., and Hartwell, L. H. (1988) *Cell 54*, 609−620.
29. Reneke, J. E., Blumer, K. J., Courchesne, W. E., and Thorner, J. (1988), *Cell 55*, 221−234.
30. Brenner, C., Bevan, A., and Fuller, R. S. (1994) *Methods Enzymol. 244*, 152−167.
31. Clark, C. D., Palzkill, T., and Botstein, D. (1994) *J. Biol. Chem. 269*, 8831−8841..
32. Shah, A., and Marsh, L. (1996) *Biochem. Biophys. Res. Commun. 226*, 242−246.
33. Jenness, D. D., Burkholder, A. C., and Hartwell, L. H. (1986) *Mol. Cell. Biol. 6*, 318−20.
34. Blumer, K. J., and Thorner, J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4363−4367.
35. Konopka, J. B., Margarit, S. M., and Dube, P. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 6764−6769.
36. Dube, P., and Konopka, J. B. (1998) *Mol. Cell. Biol. 18*, 7205−7215.
37. Stefan, C. J., Overton, M. C., and Blumer, K. J. (1998) *Mol. Biol. Cell 9*, 885−899.
38. Abel, M. G., Lee, B.-K., Naider, F., and Becker, J. M. (1998) *Biochim. Biophys. Acta 1448*, 12−36.
39. Marsh, L. (1992) *Mol. Cell. Biol. 12*, 3959−3966.

40. Kjelsberg, M. A., Cotecchia, S., Ostrowski, J., Caron, M. G., and Lefkowitz, R. (1992) *J. Biol. Chem. 267*, 1430−1433.
41. Dufau, M. L. (1998) *Annu. Rev. Physiol. 60*, 461−496.
42. Boone, C., Davis, N. G., and Sprague, G. F. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9921−9925.
43. Wonerow, P., Schoneberg, T., Schultz, G., Gudermann, T., and Paschke, R. (1998) *J. Biol. Chem. 273*, 7900−7905.
44. Marie, J., Koch, C., Pruneau, D., Paquet, J.-L., Groblewski, T., Larguier, R., Lombard, C., Deslauriers, B., Maigret, B., and Bonnafous, J.-C. (1999) *Mol. Pharmacol. 55*, 92−101.
45. Spalding, T. A., Burstein, E. S., Henderson, S. C., Ducote, K. R., and Brann, M. R. (1998) *J. Biol. Chem. 273*, 21563−21568.
46. Schipani, E., Kruse, K., and Juppner, H. (1995) *Science 268*, 98−100.
47. Han, M., Smith, S. O., and Sakmar, T. P. (1998) *Biochemistry 37*, 8253−8261.
48. Kudo, M., Osuga, Y., Kobilka, B. K., and Hsueh, A. J. W. (1996) *J. Biol. Chem. 271*, 22470−22478.
49. Myburgh, D. B., Millar, R. P., and Hapgood, J. P. (1998) *Biochem. J. 331*, 893−896.
50. Feng, Y.-H., Miura, S., Husain, A., and Karnik, S. S. (1998) *Biochemistry 37*, 15791−15798.
51. Porter, J. E., Hwa, J., and Perez, D. M. (1996) *J. Biol. Chem. 271*, 28318−28323.
52. Zuscik, M. J., Porter, J. E., Gaivin, R., and Perez, D. M. (1998) *J. Biol. Chem. 273*, 3401−3407.
53. Befort, K., Zilliox, C., Filliol, D., Yue, S., and Kieffer, B. L. (1999) *J. Biol. Chem. 274*, 18574−18581.
54. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) *Science 274*, 768−770.
55. Sheikh, S. P., Zvyaga, T. A., Lichtarge, O., Sakmar, T. P., and Bourne, H. (1996) *Nature 383*, 347−350.
56. Dosil, M., Giot, L., Davis, C., and Konopka, J. B. (1998) *Mol. Cell. Biol. 18*, 5981−5991.
57. Sen, M., and Marsh, L. (1994) *J. Biol. Chem. 269*, 968−973.
58. Stefan, C. J., and Blumer, K. J. (1994) *Mol. Cell. Biol. 14*, 3339−3349.
59. Apanovitch, D. M., Slep, K. C., Sigler, P. B., and Dohlman, H. G. (1998) *Biochemistry 37*, 4815−4822.
60. Eriotou-Bargiota, E., Xue, C. B., Naider, F., and Becker, J. M. (1992) *Biochemistry 31*, 551−557.
61. Bukusoglu, G., and Jenness, D. D. (1996) *Mol. Cell. Biol. 16*, 4818−4823.
62. Blumer, K. J., Reneke, J. E., and Thorner, J. (1988) *J. Biol. Chem. 263*, 10836−10842.

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