Identification of Residues that Contribute to Receptor Activation through the Analysis of Compensatory Mutations in the G Protein-Coupled α-Factor Receptor†

Jennifer C. Lin,‡,§ Ken Duell,|| Misty Saracino,|| and James B. Konopka*,||

Graduate Program in Molecular and Cellular Biology and Department of Molecular Genetics and Microbiology, State University of New York, Stony Brook, New York 11794-5222

Received September 9, 2004; Revised Manuscript Received October 14, 2004

ABSTRACT: The α-factor receptor (Ste2p) stimulates mating of the yeast *Saccharomyces cerevisiae*. Ste2p belongs to the large family of G protein-coupled receptors that are characterized by seven transmembrane α-helices. Receptor activation is thought to involve changes in the packing of the transmembrane helix bundle. To identify residues that contribute to Ste2p activation, second-site suppressor mutations were isolated that restored function to defective receptors carrying either an F204S or Y266C substitution which affect residues at the extracellular ends of transmembrane domains 5 and 6, respectively. Thirty-five different suppressor mutations were identified. On their own, these mutations caused a range of phenotypes, including hypersensitivity, constitutive activity, altered ligand binding, and loss of function. The majority of the mutations affected residues in the transmembrane segments that are predicted to face the helix bundle. Many of the suppressor mutations caused constitutive receptor activity, suggesting they improved receptor function by partially restoring the balance between the active and inactive states. Analysis of mutations in transmembrane domain 7 implicated residues Ala281 and Thr282 in receptor activation. The A281T and T282A mutants were supersensitive to *S. cerevisiae* α-factor, but were defective in responding to a variant of α-factor produced by another species, *Saccharomyces kluyveri*. The A281T mutant also displayed 8.7-fold enhanced basal signaling. Interestingly, Ala281 and Thr282 are situated in approximately the same position as Lys296 in rhodopsin, which is covalently linked to retinal. These results suggest that transmembrane domain 7 plays a role in receptor activation in a wide range of G protein-coupled receptors from yeast to humans.

The *Saccharomyces cerevisiae* receptor for α-factor mating pheromone (Ste2p) is a member of the large family of G protein-coupled receptors (GPCRs)[1] (*1*, *2*). Other GPCRs transduce the signals for light, taste, olfaction, and a broad range of medically important hormones and neurotransmitters (*3*). These receptors function in a similar manner by activating the α subunit of a guanine nucleotide binding protein (G protein) to bind GTP. This promotes the dissociation of the α subunit from the $\beta\gamma$ subunits and leads to downstream signaling events. In yeast, the free $\beta\gamma$ subunits then activate a MAP kinase pathway that stimulates conjugation (*4*, *5*). Although the G protein subunits are well conserved in structure and function from yeast to mammals (*1*), Ste2p does not share recognizable amino acid sequence similarity with mammalian GPCRs. Furthermore, the cognate pheromone receptor in yeast, **a**-factor receptor (Ste3p), also does not share recognizable sequence similarity with Ste2p or with mammalian receptors. Although this could suggest that these GPCRs act by different mechanisms, the fact that both yeast pheromone receptors and certain mammalian GPCRs can all activate the pheromone-responsive G protein pathway in yeast (*6*, *7*) suggests that the underlying mechanisms of GPCR function may be conserved.

Comparison of Ste2p with other GPCRs shows that there is an overall structural similarity in that they are all composed of seven transmembrane domains (TMDs) connected by extracellular and intracellular loops (*8*). The general functional organization of Ste2p is also similar to that of many other GPCRs in that the core region containing the seven TMDs functions in ligand binding (*9*, *10*) and the third intracellular loop plays a key role in G protein activation (*11*−*13*). Mutational analyses have identified additional similarities between Ste2p and other GPCRs. For example, substitution of Pro258 in TMD6 of Ste2p causes constitutive receptor activity (*14*, *15*), suggesting it functions in receptor activation in a manner similar to that of a highly conserved proline present in TMD6 of many mammalian GPCRs. However, Ste2p lacks many of the other highly conserved residues found in the well-studied class A family of GPCRs that includes rhodopsin and β-adrenergic receptor (*16*, *17*). For example, Ste2p lacks the highly conserved E/DRY motif present at the intracellular end of TMD3 in many mammalian GPCRs. Despite this lack of sequence identity, mutational analyses indicate that the cytoplasmic end of TMD3 regulates

† This work was supported by National Institutes of Health Grant GM55107 awarded to J.B.K.

* To whom correspondence should be addressed. E-mail: james.konopka@sunysb.edu. Phone: (631) 632-8715. Fax: (631) 632-9797.

‡ Graduate Program in Molecular and Cellular Biology.

§ Current address: Department of Cell Biology, The Scripps Research Institute, 10550 North Torrey Pines Rd., ICND-222B, La Jolla, CA 92037-1000.

|| Department of Molecular Genetics and Microbiology.

[1] Abbreviations: TMD, transmembrane domain; GPCR, G protein-coupled receptor.

10.1021/bi048050u CCC: $30.25
Published on Web 01/04/2005

the activation of Ste2p as it does in many mammalian GPCRs (*18*). This suggests that an important role for the intracellular end of TMD3 is conserved in Ste2p.

To gain additional insight into the mechanisms of GPCR activation, a genetic screen for intragenic second-site suppressor mutations was used in this study to identify functionally important residues in Ste2p. The rationale for this approach is that by starting with a strongly defective receptor mutant, second-site mutations that restore signaling activity should define positions in Ste2p that influence critical aspects of receptor structure and function. One of the defective receptor genes was *STE2-F204S*, in which a substitution of Phe204 with Ser at the extracellular end of TMD5 causes a strong defect in ligand binding (*19*). The other defective receptor was *STE2-Y266C*, in which substitution of Tyr266 with Cys near the extracellular end of TMD6 results in a defect in receptor activation (*19*). Screening for compensatory mutations that restore mating ability led to the discovery of 35 different suppressor mutations. The phenotypes caused by the suppressor mutations alone indicated that they affected a range of different properties, including sensitivity, constitutive activity, and ligand binding. The identification of such a wide range of mutations in a simple genetic screen for restoration of function suggests that this approach may be applicable to receptors and other types of multispanning membrane proteins in less genetically tractable organisms.

MATERIALS AND METHODS

Strains and Media. Yeast strains used in this study included yLG123 (*MAT***a** *ade2-1° his4-580*^{a} *lys2° trp1*^{a} *tyr1° leu2 ura3 SUP4-3*^{ts} *bar1-1 mfa2::FUS1-lacZ ste2::LEU2*), PMY1 (*MAT***a** *bar1::hisG far1 ste2*Δ *mfα1::LEU2 mfα2::his5*$^{+}$ *ade2 his3 leu2 ura3 mfa2::FUS1-lacZ*), and lys1α (*MAT*α *lys1*). Plasmids were transformed into yeast, and then the cells were grown in synthetic medium lacking uracil to select for plasmid maintenance (*20*).

Mutagenesis of STE2. The *STE2-F204S* and *STE2-Y266C* genes were randomly mutagenized by performing PCR under error-prone conditions (10-fold lower concentration of either dATP or dGTP). Plasmids pMD45 (YCp-*URA3-STE2-F204S*) and pMD41 (YCp-*URA3-STE2-Y266C*) (*19*) were used as templates. Different sets of PCR primers were used to target three overlapping regions of the *STE2-F204S* and *STE2-Y266C* genes for mutagenesis. The first set of primers amplified the DNA sequence between codons 1 and 146; the second set of primers amplified codons 123−199, and the third set of primers amplified codons 183−295. Mutagenized PCR fragments were then cotransformed into yeast strain yLG123 (*MAT***a** *ste2*Δ) together with either plasmid pMD45 (YCp-URA3-STE2-F204S) or plasmid pMD41 (YCp-URA3-STE2-Y266C) that was linearized at a single site by digestion with either *Hpa*I, *Aat*II, or *Cla*I. Selection for growth on medium lacking uracil enriched for cells carrying plasmids that were repaired by incorporating a mutagenized PCR fragment via homologous recombination since linearized plasmids are not maintained in yeast.

Genetic Screen for Suppressor Mutants. Approximately 35 000 independent colonies carrying mutagenized plasmids were screened for the ability to mate with *MAT*α cells (*MAT*α *lys1*). Cells were allowed to mate on rich medium and replica-plated onto minimal medium to select for the growth of diploids. Cells carrying the parental plasmids (pMD45 or pMD41) were strongly defective and did not exhibit detectable mating under these conditions. More than 100 potential suppressor mutant colonies that exhibited detectable mating with lys1α cells were identified. Plasmids from the mating-positive candidates were recovered and retransformed back into yeast strain yLG123 (*MAT***a** *ste2*Δ) to confirm that the suppression phenotype was plasmid-dependent and not due to an extragenic suppressor mutation in the host cell genome. Plasmids encoding receptors with improved function were subjected to DNA sequence analysis using a Big Dye cycle sequencing kit (Applied Biosystems Inc.) to identify amino acid substitutions. Of 38 independent suppressor mutants of *STE2-F204S*, 36 carried a second-site mutation that caused a single-amino acid substitution, including 18 different substitution mutations that affected 16 different residues (Table 1). In the screen for suppressors of *STE2-Y266C*, 63 independent mutants were isolated, 53 of which carried a second-site change that caused a single-amino acid substitution. There were 24 different substitutions that affected 21 different residues (Table 2).

Table 1: Phenotypes of Intragenic Suppressor Mutants of *STE2-F204S*

STE2 allele	mating ability[a]	cell division arrest[b]	previously identified mutation/phenotype[c]
wild type	+++	+++	
F204S	−	−	ligand biding defect
M54E/F204S	++	+	M54I/binding
N84S/F204S	+++	+	N84S/constitutive
F119L/F204S	++	+	F119S/constitutive
S141P/F204S	++	−	S141P/constitutive
E143K/F204S	++	−	E143K/loss of function
L146Q/F204S	++	+/−	supersensitive (this study)
Q149R/F204S	+	−	Q149R/constitutive
I153N/F204S	+++	+/−	I153F/constitutive
I153F/F204S	+++	−	I153F/constitutive
N216D/F204S	+++	−	N216D/constitutive
N216Y/F204S	+	+/−	N216D/constitutive
L222P/F204S	+++	+	L222P/constitutive
K225R/F204S	+++	−	K225C/loss of function
A229V/F204S	+++	−	A229V/binding A229C/sensitivity
C252G/F204S	+++	+/−	
S259P/F204S	+++	+	S259P/constitutive
L289S/F204S	+++	−	supersensitive (this study)
A296T/F204S	+++	+/−	supersensitive (this study)

[a] Ability of strain yLG123 carrying the indicated receptor gene (*STE2*) to mate on solid agar medium with *MAT*α strain lys1α. Mating efficiency was rated as follows: +++, wild-type level; ++, intermediate; +, weak; −, no detectable mating. [b] Halo assays were used to quantify α-factor-induced cell division arrest: +++, wild-type size; ++, decreased 4−6-fold; +, decreased 10−12-fold; −, no halo. [c] Previously identified mutations that affect binding specificity (*24*, *25*), constitutive receptor activity (*18*, *22*, *23*), and loss of function (*23*, *26*, *40*).

Subcloning of Intragenic Suppressor Mutations into a Wild-Type STE2 Background. A selected set of intragenic suppressor mutations were subcloned into plasmid pDB02 (YCp-*URA3-STE2*) (*14*). To transfer the suppressor mutations, a 276 bp *Aat*II−*Cla*I fragment from *STE2-A212V/Y266C*, a 366 bp *Aat*II−*Hpa*I fragment from *STE2-L146Q/Y266C*, and a 360 bp *Cla*I−*Pst*I fragment from *STE2-L289S/F204S* and *-A296T/F204S* plasmids were ligated into the corresponding restriction sites in pDB02 to create *ste2-A212V*, *-L146Q*, *-L289S*, and *-A296T*, respectively. The

Table 2: Phenotypes of Intragenic Suppressor Mutants of Y266C

STE2 allele	mating ability[a]	cell division arrest[b]	previously identified mutation/phenotype[c]
wild type	+++	+++	
Y266C	−	−	receptor activation defect
M54T/Y266C	++	−	M54I/binding
M54V/Y266C	++	+/−	M54I/binding
F55L/Y266C	+++	+/−	F55V/binding
N84S/Y266C	+++	++	N84S/constitutive
S141P/Y266C	+++	−	S141P/constitutive
S145L/Y266C	+++	++	S141P/constitutive
S145T/Y266C	+++	+/−	S145L/constitutive
L146Q/Y266C	+++	++	supersensitive (this study)
Q149N/Y266C	+++	+/−	Q149R/constitutive
Q149R/Y266C	+	−	Q149R/constitutive
I153F/Y266C	+++	+/−	I153F/constitutive
F154L/Y266C	++	−	
I175V/Y266C	++	+/−	
A212V/Y266C	+++	+	constitutive (this study)
N216D/Y266C	+++	++	N216D/constitutive
L222Q/Y266C	+++	++	L222P/constitutive
K225R/Y266C	+++	+	K225C/loss of function
L255S/Y266C	+++	+/−	
I260T/Y266C	+++	+/−	I260M/loss of function
V276A/Y266C	+++	−	supersensitive (this study)
V280D/Y266C	+++	+/−	supersensitive (this study)
A281T/Y266C	+++	+/−	constitutive (this study)
T282A/Y266C	+++	+/−	supersensitive (this study)
L284H/Y266C	+++	++	constitutive (this study)

[a] Ability of strain yLG123 carrying the indicated receptor gene (*STE2*) to mate on solid agar medium with *MAT*α strain lys1α. Mating efficiency was rated as follows: +++, wild-type level; ++, intermediate; +, weak; −, no detectable mating. [b] Halo assays were used to quantify α-factor-induced cell division arrest: +++, wild-type size; ++, decreased 4−6-fold; +, decreased 10−12-fold; −, no halo. [c] Previously identified mutations that affect binding specificity (*24*, *25*), constitutive receptor activity (*18*, *22*, *23*), and loss of function (*23*, *26*, *40*).

STE2-V276A/Y266C, *-V280D/Y266C*, *-A281T/Y266C*, *-T282A/Y266C*, and *-L284H/Y266C* plasmids were used as a template for PCR amplification using *Pfu* DNA polymerase (Roche), and then DNA fragments containing the suppressor mutations were subcloned into plasmid pDB02 cut with *Pst*I and *Hpa*I to create the *ste2-V276A*, *-V280D*, *-A281T*, *-T282A*, and *-L284H* plasmids.

Ligand-Induced Responses. Mating assays were carried out by replica plating patches of strain yLG123 (*MAT***a** *ste2*Δ) containing the indicated wild-type or mutant version of *STE2* on a plasmid onto a lawn of *MAT*α strain lys1α cells and then selecting for the growth of diploid cells on minimal medium. Halo assays for α-factor-induced cell division arrest were performed by spreading 6×10^5 yeast cells on solid medium agar plates and then placing sterile filter disks containing the indicated amount of α-factor (Bachem) on the lawn of cells. The plates were incubated at 30 °C for 48 h, and then the diameters of the zones of α-factor-induced cell division arrest were measured. *FUS1-lacZ* induction assays were carried out by growing PMY1 cells carrying the indicated *STE2* plasmid to log phase, adjusting the culture to a density of 4×10^6 cells/mL, and then adding the indicated concentrations of α-factor for 2 h. The cells were permeabilized with 0.05% SDS and $CHCl_3$, and then *β*-galactosidase activity was quantified using *O*-nitrophenyl *β*-D-galactopyranoside (Sigma) as a substrate (*21*). The results represent the average of two or three independent experiments, each carried out in duplicate. Assays carried out on different days varied slightly, but the standard deviation was typically less than 15%. The α-factor analogues were synthesized by Invitrogen Corp.

α-*Factor Binding Assays*. Assays for the binding of α-factor to yeast cells were performed essentially as described previously (*12*). Cells grown to logarithmic phase were collected by centrifugation, washed twice with ice-cold inhibitor medium (IM) (YEPD medium containing 10 mM KF and 10 mM NaN_3), and resuspended at a density of 2×10^8 cells/mL. Competition binding assays were carried out by mixing 50 *μ*L of cells with 50 *μ*L of [^{35}S]-α-factor (20 nM) to which variable amounts of nonradioactive *S. cerevisiae* or *Saccharomyces kluyveri* α-factor had been added. Limitations on the solubility of the *S. kluyveri* α-factor prevented it from being tested at higher concentrations in the competition binding assays. Samples were incubated for 1 h; the cells were collected on a Whatman GF/C filter, and the unbound α-factor was removed by washing using a Brandel cell harvester. The bound radioactivity was determined by scintillation counting. Binding data presented in this study represent the average of three independent assays each carried out in triplicate. ^{35}S-labeled α-factor was purified from the supernatant of *MAT*α cells labeled with [^{35}S]-methionine (Translabel from ICN, Irvine, CA) by chromatography on a Bio-Rex 70 (Bio-Rad, Hercules, CA) column as described previously (*12*). The results of the binding assays were analyzed using nonlinear regression analysis using Prism (GraphPad Software, San Diego, CA).

RESULTS

Genetic Screen for Suppressor Mutants. The *STE2-F204S* and *STE2-Y266C* genes were randomly mutagenized by performing PCR under error-prone conditions and then introduced on a plasmid into a yeast strain that lacks the endogenous α-factor receptor gene (*ste2*Δ strain yLG123). The transformants were then screened to identify revertants with improved receptor function as judged by the ability to mate with *MAT*α cells and form diploid cells. One hundred one colonies were identified with improved mating efficiency relative to the parental cells carrying either a *STE2-F204S* or a *STE2-Y266C* plasmid. Plasmids recovered from each candidate were retransformed into a fresh culture of yeast strain yLG123 (*ste2*Δ) to confirm that the suppression phenotype was plasmid-dependent and not caused by a chromosomal mutation. DNA sequence analysis was used to identify the suppressor mutation and to confirm that the original mutation was still present.

Intragenic Suppressor Mutations. The F204S substitution affects a residue near the extracellular end of TMD5 in *STE2* and severely weakens its ability to bind α-factor (*19*). Screening of approximately 17 500 colonies carrying mutagenized *STE2-F204S* identified 38 independent suppressor mutants. DNA sequence analysis of the mutants revealed 18 different second-site suppressor mutations that were due to single-amino acid substitutions (Table 1 and Figure 1). Eleven of the mutants exhibited strongly improved receptor function in mating assays, but none of the mutants was completely restored to wild-type function as they all exhibited a significant defect in their ability to undergo α-factor-induced cell division arrest, which is a more sensitive test of receptor function (Table 1). In comparison, 63 independent

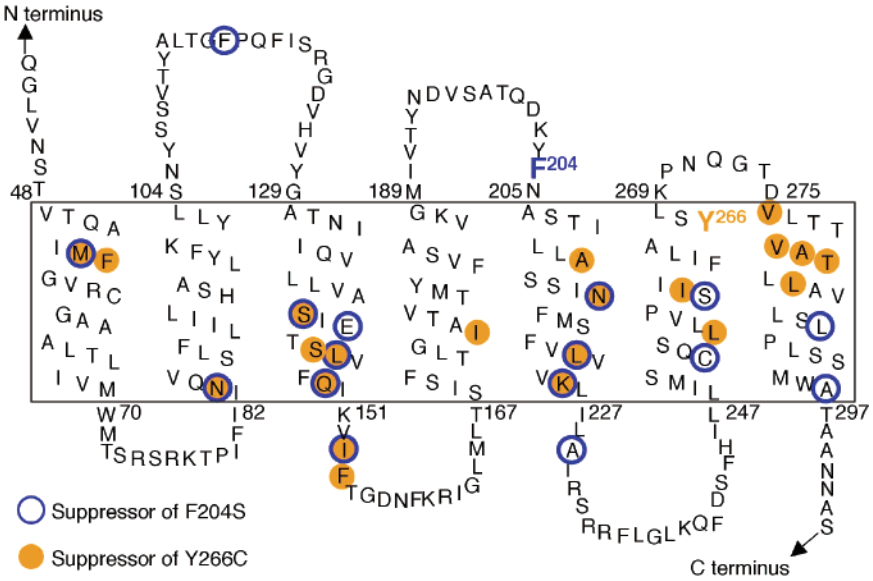

FIGURE 1: Topology model of the α-factor receptor (Ste2p) indicating the residues affected by the intragenic suppressor mutations. Sites of mutations that suppress *STE2-F204S* are marked with blue circles. Sites of mutations that suppress *STE2-Y266C* are marked with circles filled in orange. Phe204 is colored blue, and Tyr266 is colored orange.

revertants of *STE2-Y266C* were identified after approximately 17 500 colonies were screened. The *STE2-Y266C* receptors are strongly defective in activating G protein signaling but still bind α-factor well (*19*). DNA sequencing identified 24 different second-site mutations that were due to a single-amino acid substitution. Many of these second-site mutations compensated well for the Y266C substitution as 19 of the suppressor mutant strains mated efficiently (Table 2). Six intragenic mutants of *STE2-Y266C* appeared to be nearly fully suppressed as they could be stimulated to undergo α-factor-induced cell division similar to that of the wild type (e.g., N84S/Y266C, Table 2). The second-site mutations were interesting in that many of them were identical to the suppressors of *STE2-F204S* described above and included many mutations shown in previous studies to alter ligand binding or to cause constitutive receptor activity (Tables 1 and 2).

Selection of a Subset of Suppressor Mutations for Analysis. In total, 35 different suppressor mutations caused by a single missense substitution were identified that affected 28 different residues (Figure 1). Interestingly, eight of the mutations affected residues (Asn84, Phe119, Ser141, Ser145, Gln149, Ile153, Asn216, Leu222, and Ser259) that were previously shown to be sites of mutations that cause constitutively active signaling (Tables 1 and 2) (*18*, *22*, *23*). In most cases, the amino acid changes caused by the substitutions were the same as those shown previously to cause constitutive signaling. This suggests that mutations causing constitutive activity act as global suppressors of the *STE2-F204S* and *-Y266C* mutations (see the Discussion). Three other mutations affected the same residues identified previously by mutations that altered ligand binding specificity (Met54, Phe55, and Ala229) (*24*, *25*), and two mutations affect the same residues as loss of function mutations (Glu143 and Lys225) (*26*, *27*). Of the remaining mutations, nine were selected for further analysis (Figure 2) because they affected regions of the receptor that have not been studied extensively, and in some cases, they affect residues that are highly conserved in the Ste2p homologues from other species of yeast. The chosen intragenic suppressor mutations were subcloned into wild-type receptor plasmids and analyzed as single-substitution mutants in the absence of other mutations.

Suppressor Mutations Cause Different Levels of Constitutive Signaling. Cells expressing *STE2* with one of the selected suppressor mutations as the only mutation present were all able to mate well. However, the mutants could be divided into three groups based on the basal level of the pheromone-responsive *FUS1-lacZ* reporter gene in the absence of α-factor. The group I mutants (L146Q, V276A, V280D, and T282A) exhibited low basal levels of *FUS1-lacZ* in the absence of α-factor and were similar to the wild type (Figure 2). The group II mutants (L289S and A296T) exhibited weak 2.7−3.1-fold increased basal levels of *FUS1-lacZ*, and the group III mutants (A212V, A281T, and L284H) exhibited 4.4−9.5-fold higher basal levels of *FUS1-lacZ*.

Suppressor Mutations Cause Increased Sensitivity to α-Factor. Dose−response assays were carried out to examine how the mutant receptors respond to α-factor. Interestingly, all of the mutant strains required less α-factor to give half-maximal activation of the *FUS1-lacZ* reporter gene (Figure 3). They were from 2.1- to 6.6-fold more sensitive to α-factor. The ability of the mutant receptors to bind α-factor was analyzed using radiolabeled [^{35}S]-α-factor. Binding was not readily detectable for the group III mutants, consistent with previous studies in which constitutive mutants often exhibited altered trafficking, resulting in lower levels of cell surface receptors (*14*, *15*). The relative affinity of the mutant receptors was examined in competition binding assays with non-radiolabeled α-factor. These mutants gave an IC_{50} for competition that was within ~2-fold of that of the wild type, indicating that the mutant receptors bound α-factor with essentially wild-type affinity (Figure 4 and Table 3). Thus, the increased sensitivity to α-factor seen in these mutants appears to be due to greater signaling potential and not just due to a change in affinity for α-factor. This is consistent with the ability of these suppressor mutations to counteract the effects of a loss of function mutation.

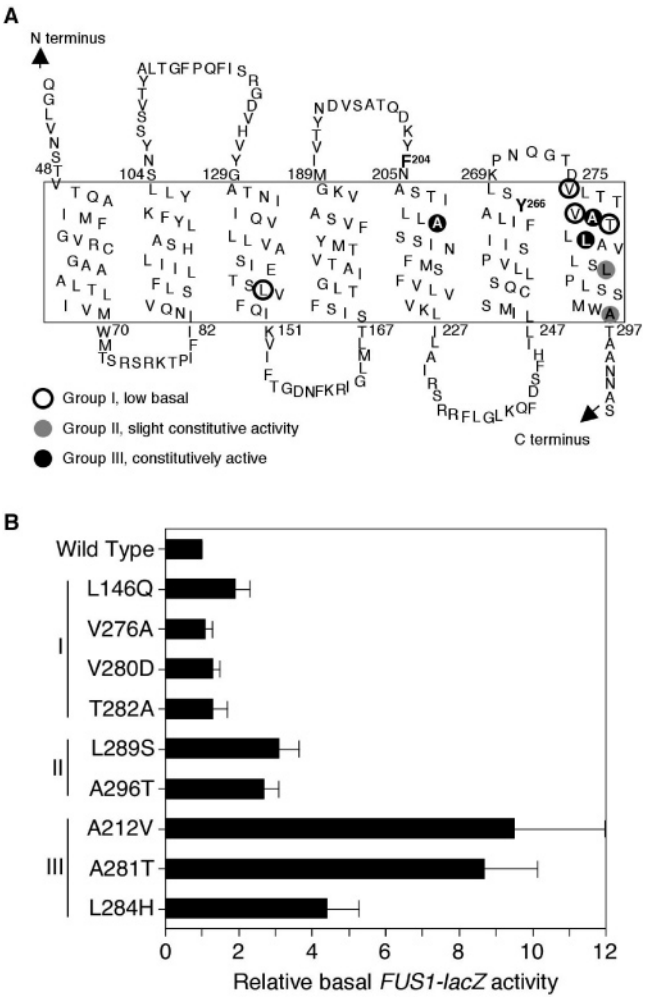

FIGURE 2: Basal levels of the pheromone-responsive *FUS1-lacZ* reporter gene in selected *STE2* mutants. (A) Model of Ste2p indicating positions of residues affected by the selected set of suppressor mutations. (B) Relative levels of basal *FUS1-lacZ*. PMY1 cells carrying either wild-type *STE2* or the indicated substitution mutant on a plasmid were grown to log phase in the absence of α-factor. *FUS1-lacZ* reporter gene activity was determined by assaying β-galactosidase activity as described in Materials and Methods. The data were normalized to the basal level of the wild-type cells. The results represent the average of at least six independent experiments, each carried out in duplicate. Error bars indicate the standard deviation.

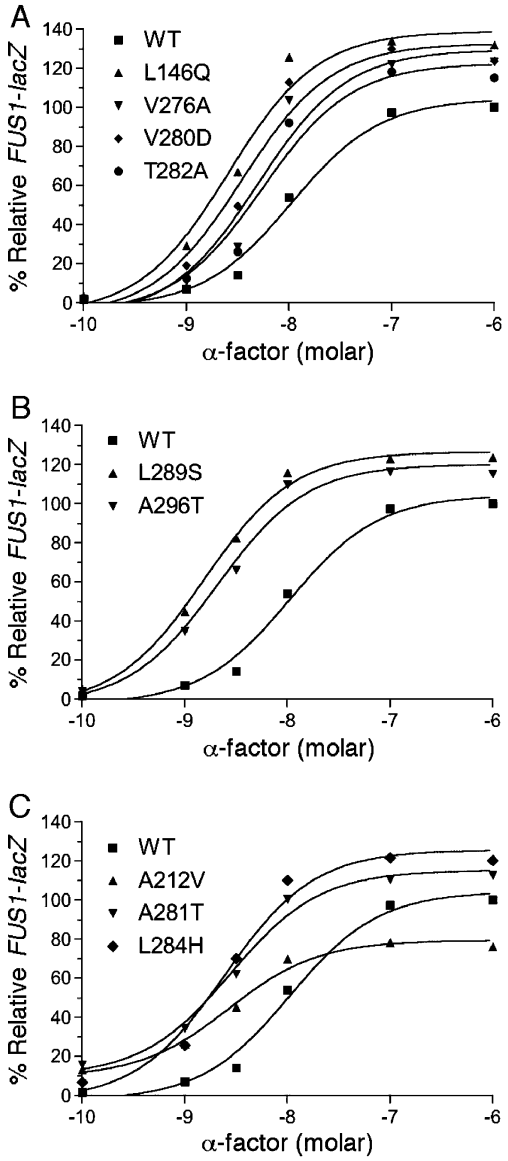

FIGURE 3: Induction of *FUS1-lacZ* in response to *S. cerevisiae* α-factor. Activation of the pheromone-responsive *FUS1-lacZ* reporter gene was assessed by assaying β-galactosidase activity in cells that were treated with the indicated concentrations of *S. cerevisiae* α-factor for 2 h. The group 1 (A), group II (B), and group III (C) mutant receptors were carried on a *STE2* plasmid derived from pDB02 in strain PMY1. The results represent the average of two independent assays, each carried out in duplicate.

The mutants also displayed greater signaling capabilities in that eight of the nine mutants exhibited a 10−30% higher maximum level of *FUS1-lacZ* than the wild-type control (Figure 3). In contrast, the A212V mutant was distinct in that it could only be induced to ~78% of the wild-type level. The lower maximum level of signaling is likely due to low levels of A212V mutant receptors since the cell surface binding site for α-factor were not detectable in binding assays with radiolabeled α-factor (data not shown).

A281T and T282A Mutants Are Defective in Response to α-Factor from S. kluyveri. Since some of the previously studied suppressor mutations were shown to affect ligand specificity, the mutants were tested for their sensitivity to a distinct type of α-factor produced by the yeast *S. kluyveri.* The amino acid sequences of α-factor from *S. cerevisiae* (WHWLQLKPGQPMY) and *S. kluyveri* (WHWLSFSKGEPMY) differ at five residues. As a result, *S. cerevisiae* Ste2p shows greatly weakened ability to respond to α-factor from *S. kluyveri* (Figure 5), in agreement with previous studies (*28*, *29*). For conciseness, the pheromones from *S. cerevisiae* and *S. kluyveri* will be termed c- and k-α-factor, respectively.

The majority of the mutants displayed increased sensitivity to k-α-factor. In contrast, the A281T and T282A mutants stood out because they had the opposite phenotype and exhibited a ≥10-fold decreased sensitivity to k-α-factor. The A281T and T282A mutants affect adjacent residues that are near the extracellular end of TMD7. To examine the basis of their inability to respond to k-α-factor, competition binding studies were carried out using k-α-factor to compete for the binding of radiolabeled c-α-factor to the mutant receptors (Figure 4 and Table 3). The results for binding to the A281T mutant were inconclusive since this mutant produced only low levels of cell surface binding sites for c-α-factor. However, the T282A mutant exhibited essentially

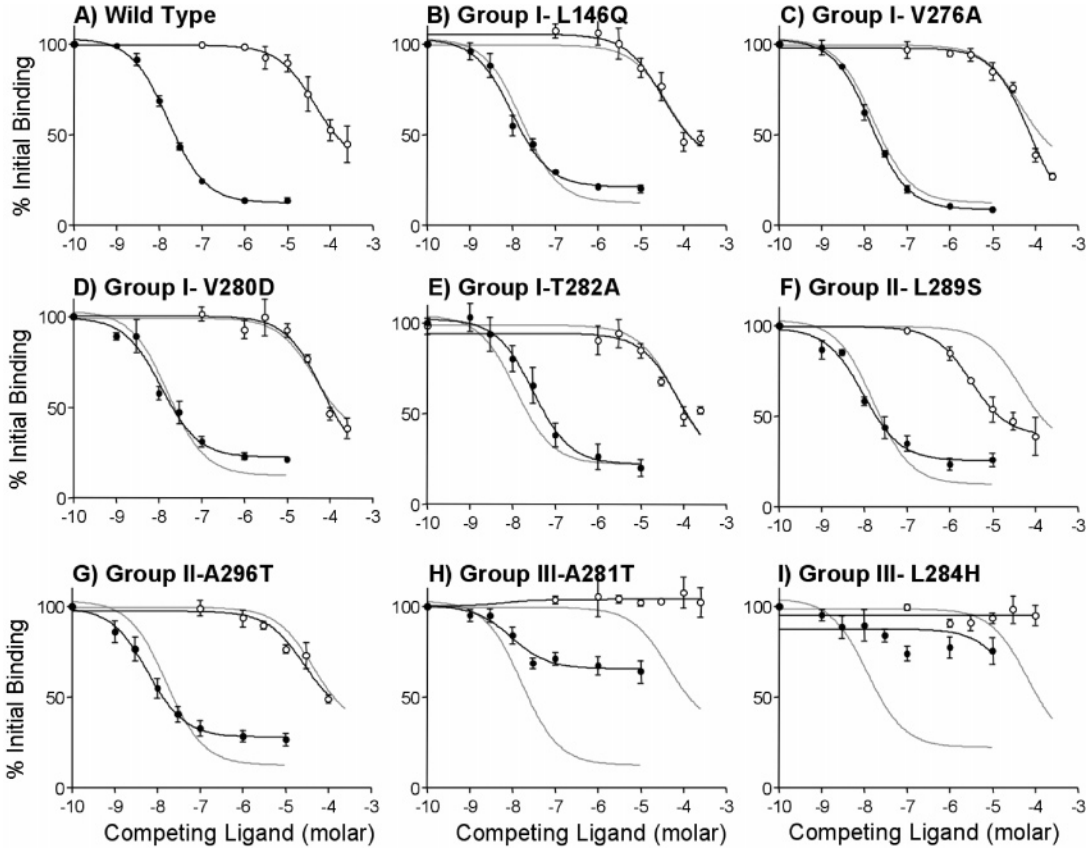

FIGURE 4: Competition ligand binding assays. PMY1 cells carrying the wild-type or a mutant version of *STE2* plasmid pDB02 were analyzed for their relative ability to bind *S. cerevisiae* α-factor [c-α-factor (●)] or *S. kluyveri* α-factor [k-α-factor (○)]. For comparison, the competition curves for the wild type are shown as gray lines. Competition binding assays were carried out by incubating cells with [^{35}S]-α-factor (10 nM) to which the indicated concentration of nonradioactive *S. cerevisiae* or *S. kluyveri* α-factor had been added. The results represent the average of at least three independent assays, each carried out in triplicate. Error bars indicate the standard error.

Table 3: Relative Properties of Selected *STE2* Mutants[a]

STE2 allele	basal *Fus1-lacZ*	FUS1-lac EC_{50} (c-α-factor)	FUS1-lacZ EC_{50} (k-α-factor)	c-α-factor binding affinity	k-α-factor binding affinity
wild type	1.0	1.0	1.0	1.0	1.0
group I					
L146Q	1.9	4.2	1.4	1.5	3.8
V276A	1.1	2.1	2.0	1.2	1.2
V280D	1.3	3.1	3.1	1.3	1.3
T282A	1.3	2.1	0.10	0.6	1.5
group II					
L289S	3.1	6.6	14.2	1.6	34.8
A296T	2.7	5.1	8.9	2.2	4.5
group III					
A212V	9.5	3.9	6.5	ND[b]	ND[b]
A281T	8.7	3.8	0.08	ND[b]	ND[b]
L284H	4.4	4.3	4.4	ND[b]	ND[b]

[a] Relative functional properties of PMY1 cells carrying the wild-type *STE2* plasmid (pDB02) or the indicated mutant allele were assayed for *FUS1-lacZ* activity and ligand binding as indicated in Materials and Methods. To facilitate comparison, values for wild-type STE2 were normalized to 1.0. Values greater than 1 indicate enhanced basal signaling, increased sensitivity to *FUS1-lacZ* induction (EC_{50}), or increased affinity for α-factor (IC_{50}). [b] Not determined due to a low receptor number.

the same affinity for k-α-factor as did wild-type Ste2p (Figure 4E). This indicates that the T282A substitution affects the agonist potency of k-α-factor and not the binding affinity.

Analysis of the other mutants identified interesting differences between the group I and group II mutants. To facilitate comparison, the relative ability of the mutants to function is also summarized in Table 3. Three of the group I mutants (L146Q, V276A, and V280D) exhibited slightly increased sensitivity to k-α-factor (1.4−3.1-fold) (Figure 5) and approximately wild-type affinity for k-α-factor as judged by the ability of k-α-factor to compete for binding of radiolabeled c-α-factor (Figure 4). In contrast, both the group II mutants (L289S and A296T) displayed strongly improved responses to k-α-factor (14.2- and 8.9-fold stronger, respectively), and these mutants also displayed higher affinity for k-α-factor (34.8- and 4.5-fold higher, respectively) as evidenced by a lower IC_{50} for binding of k-α-factor in competition binding assays (Figures 4 and 5). Two of the group III mutants (A212V and L284H) showed increased sensitivity to k-α-factor (6.5- and 4.5-fold, respectively) but displayed too few binding sites for their ability to bind k-α-factor to be compared.

Response to α-Factor Analogues. The surprising inability of the A281T and T282A mutants to respond to k-α-factor was analyzed further by assaying their response to analogues in which portions of k-α-factor were substituted with the corresponding residues from c-α-factor (Figure 6). Three analogues were synthesized, including k56-α-factor (S5Q/F6L), k78-α-factor (S7K/L8P), and k10-α-factor (E10Q). Dose−response assays carried out with wild-type Ste2p showed that all three analogues were at least 10-fold more efficient for induction of the *FUS1-lacZ* reporter gene than the parental version of k-α-factor. In contrast, the A281T and T282A mutants were still defective in responding to all three analogues. These results indicate that the ability of the central region of α-factor to promote receptor activation is

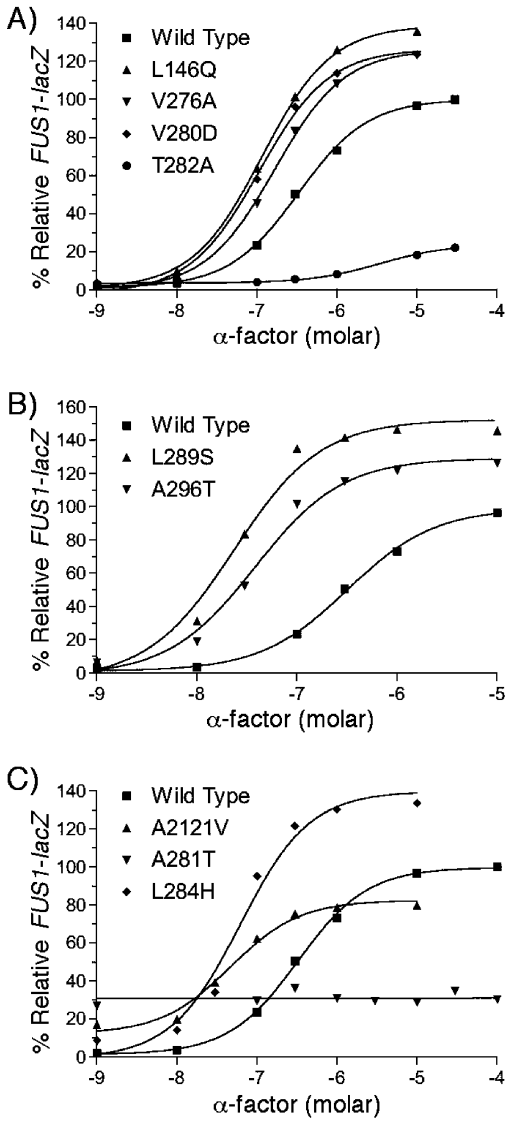

FIGURE 5: Response of mutants to *S. kluyveri* α-factor. The indicated group 1 mutants (A), group II mutants (B), and group III mutants (C) were carried on a plasmid derived from *STE2* plasmid pDB02 in strain PMY1. Cells were induced with the indicated concentration of *S. kluyveri* α-factor (k-α-factor) for 2 h. Activation of the pheromone-responsive *FUS1-lacZ* reporter gene was assessed by assaying *β*-galactosidase activity. The results represent the average of two independent assays, each carried out in duplicate.

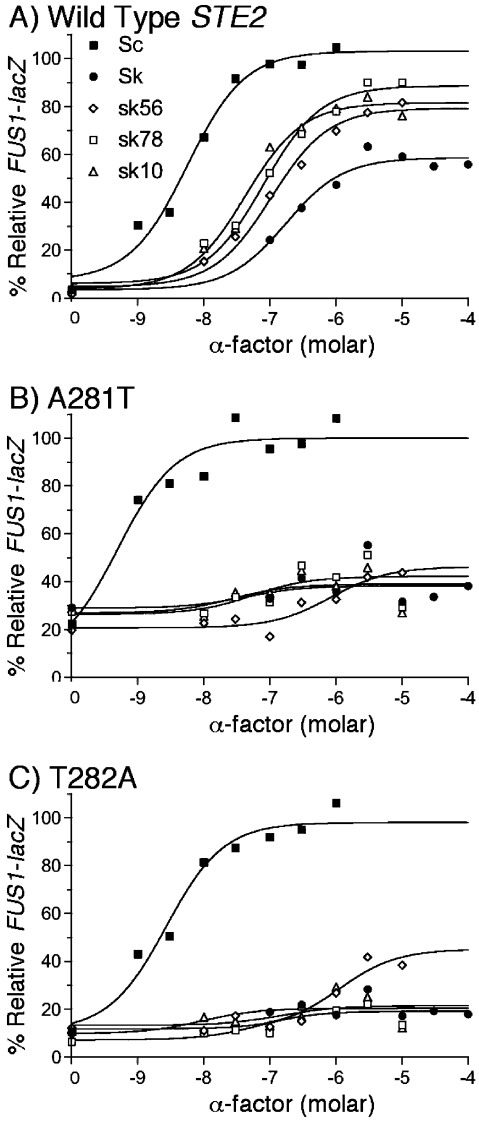

FIGURE 6: Response of A281T and T282A mutants to α-factor analogues. PMY1 cells carrying a plasmid with (A) wild-type *STE2*, (B) the A281T mutant, or (C) the T282A mutant were analyzed for their ability to respond to different forms of α-factor. The amino acid sequences of S. *cerevisiae* α-factor (WHWLQLKPGQPMY) and *S. kluyveri* α-factor (WHWLSFSKGEPMY) differ in the five places that are underlined. The hybrid α-factor analogues that were tested contained residues from *S. cerevisiae* α-factor substituted into k-α-factor as underlined: k56-α-factor (WHWLQLSKGEPMY), k78α-factor (WHWLSFKPGEPMY), and k10-α-factor (WHWLSFSKGQPMY).

strongly influenced by positions 281 and 282, both of which are located close to the extracellular boundary on TMD7. Consistent with this, Ala281 and Thr282 are predicted to face the helix bundle and TMD6 (Figure 7). These results also suggest a relationship between residues in the middle portion of α-factor and TMD7 that is consistent with previous models for the binding of α-factor to Ste2p (*30*).

DISCUSSION

Suppressor mutations were isolated as a way of identifying residues that contribute to the function of the G protein-coupled α-factor receptor (Ste2p). The rationale for this approach is that second-site mutations are likely to compensate for the defects caused by the first mutation by promoting specific conformational changes that modulate receptor signaling activity. Two different starting mutations were utilized that do not affect localization of receptors to the plasma membrane but cause a strong defect in either the affinity for α-factor (F204S) or receptor activation (Y266C). The approach was successful in that 35 different mutations were identified that caused a broad range of phenotypes, including many types of mutants that were previously isolated in specialized yeast genetic screens for constitutive activity, altered ligand binding, and loss of function. This suggests that suppressor mutant analysis could also be a useful general strategy for analysis of GPCRs in mammalian cells and other organisms that are less genetically tractable than yeast.

Suppressor Mutations. Nearly all of the mutations affected residues in the TMDs of Ste2p (Figure 1), consistent with the key role of the transmembrane helix bundle in GPCR function. The cytoplasmic end of TMD3 was a hot spot;

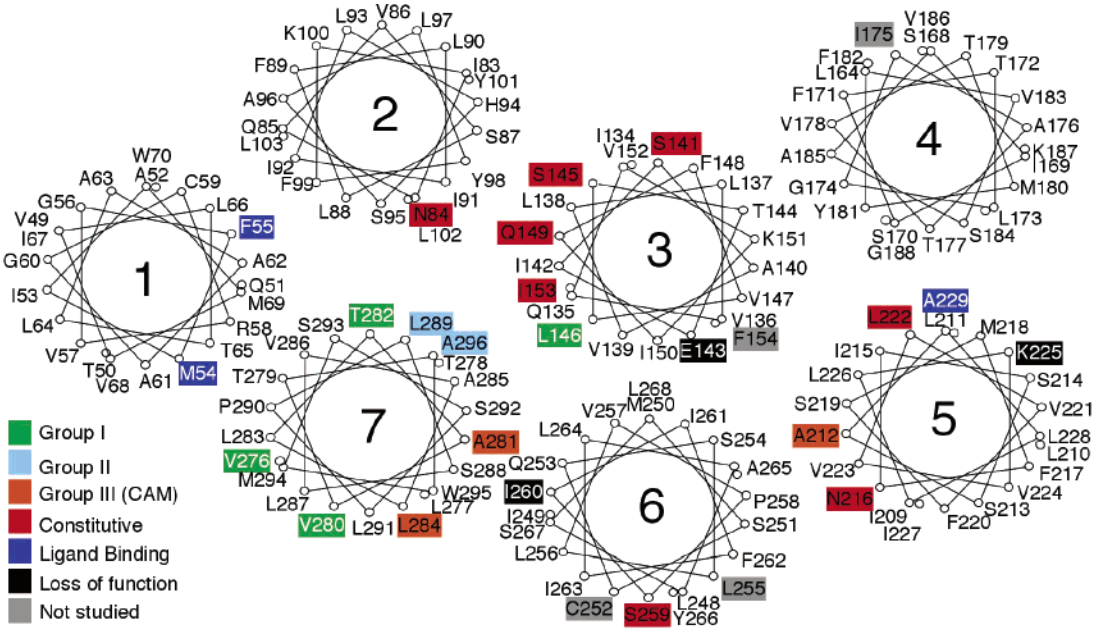

FIGURE 7: Helical wheel plots of TMDs in Ste2p. The relative positions of the amino acids in the TMDs of Ste2p are displayed in helical wheel format as viewed from the cytoplasmic side of the helix bundle. Sites affected by a suppressor mutation are shaded according to phenotype as indicated. The TMDs were oriented as described previously (*8*, *30*) based on shielding polar residues from the membrane, genetic inferences for TMD interaction (*18*, *22*), and a biochemical cross-link between TMD5 and TMD6 (*26*).

suppressor mutations were isolated at seven of 14 residues in this region. This provides additional evidence that the cytoplasmic end of TMD3 plays a key role in regulation of Ste2p as it does in mammalian GPCRs (*16*, *18*). Another hot spot for mutations was TMD7, as described below. Mapping the mutations onto the predicted α-helical structure of the TMDs showed that most mutations affect residues that face the helix bundle and not the lipid domain (Figure 7). The inward-facing residues most likely influence the packing of the helix bundle by direct interaction with the other TMDs. The mutation of residues that appear to face the membrane lipids may have an indirect effect on receptor structure. Most of these substitutions are predicted to alter α-helical structure by introducing proline residues (i.e., S141P or S259P), or by introducing a polar residue into the hydrophobic lipid domain (i.e., V280D). However, some residues that appear to face outward may in fact be involved in interactions with other TMDs. The two-dimensional helical wheel model (Figure 7) is useful for comparison of a large set of residues, but not for predicting local structural features of specific residues.

Although the suppressor mutations were found throughout the TMDs, there was a common theme in that the majority of the mutations caused constitutive receptor activity. Constitutive mutations are thought to make it easier for receptors to enter the activated state (*31*, *32*). Thus, these mutations could suppress the defects in receptor activation by lowering the energy barrier for entering the activated state. In agreement with this, the same conclusion was reached using the opposite approach. Mutations in the M_3 muscarinic receptor that cause constitutive signaling were suppressed by second-site mutations that cause a defect in receptor signaling (*33*). The ability to restore receptor function through so many different compensatory mutations has interesting implications for receptor evolution and suggests a possible basis for the high degree of sequence diversity across the GPCR family.

Intragenic suppressor mutations have often been analyzed in identifying residues that may be capable of interacting (*34*). This type of analysis has been applied effectively to GPCRs in a targeted fashion. For example, interaction between an Asp in TMD2 and an Asn in TMD7 of the 5HT-2A serotonin receptor was analyzed by showing that a double mutant that swaps the Asp and Asn residues at these positions restored proper receptor function, whereas the single mutants were defective (*35*). Interaction between TMD3 and TMD6 in rhodopsin was analyzed by demonstrating that the phenotype caused by substitution of Gly121 with a large leucine could be reverted by substitution of Phe261 with a small alanine (*36*). However, many of the suppressor mutations identified in *STE2* in this study are clearly situated too far from the original mutation to affect an interacting residue. In addition, many mutations failed to show allele specificity as they suppressed both the F204S and Y266C mutations. Allele specificity can also be difficult to establish. A previous analysis of *STE2* mutations found that Y266C and E143K appeared to show a specific suppressor relationship (*27*), but in our study, E143K was also identified as a suppressor of F204S. Thus, caution should be applied in predicting physical interaction based on this type of genetic relationship in GPCRs without direct biochemical confirmation.

Selected Set of Suppressor Mutations. Nine suppressor mutations were selected for detailed study because they affected residues that had not been studied in detail. All of these mutants exhibited an at least modest increase in α-factor sensitivity, and three mutants exhibited significant constitutive activity. One mutation affected a residue in TMD3 (L146Q) and one in TMD5 (A212V), and seven mutations affected residues in TMD7. Interestingly, Leu146, Ala212, and several of the residues mutated in TMD7 are all predicted to face TMD6 (Figure 7). Previous studies have suggested that TMD6 plays a key role in activation of Ste2p as well as in mammalian GPCRs (*14*−*16*, *22*, *37*). TMD6 is

thought to be restrained in the inactive state by interaction with other TMDs and then is thought to move during receptor activation. Thus, mutations of residues that face TMD6 may directly affect Ste2p function by altering the helix packing arrangement of TMD6.

Role of TMD7. The cluster of five mutations in the portion of TMD7 that is closer to the extracellular boundary (V276A, V280D, A281T, T282A, and L284H) implicates this region in receptor activation. The A281T and T282A mutants were particularly interesting because they confer a dramatic defect in responding to k-α-factor from *S. kluyveri* that differs at five residues from *S. cerevisiae* c-α-factor. The T282A mutant exhibited wild-type ability to bind k-α-factor, indicating that it is defective in undergoing an agonist response (A281T produced too few binding sites for conclusive results to be obtained). These mutants were also strongly defective in responding to three hybrids between c-α-factor and k-α-factor. Ala281 and Thr282 are predicted to face TMD6 and the helix bundle so they could play a direct role in receptor activation. Consistent with this, both mutants displayed increased sensitivity to α-factor and A281T displayed 8.7-fold enhanced basal signaling.

Interestingly, Ala281 and Thr282 map approximately one helical turn below Leu277, which contributes to α-factor binding affinity (*8*). Positions 281 and 282 are in a region of TMD7 that was not accessible to a chemically reactive probe, indicating they are buried in the membrane bilayer (*8*). These results expand a trend observed in other regions of Ste2p in which residues near the extracellular ends of the TMDs participate in ligand binding and residues buried beneath them are involved in receptor activations (*8*). Similar trends have been observed for other GPCRs that bind peptide ligands, such as the C5a complement receptor (*38*). It is also interesting that the positions of Ala281 and Thr282 correspond to the same region as Lys296 in TMD7 of rhodopsin, the site at which retinal is attached (*16*). Lys296 plays a key role in promoting the activated state of rhodopsin (*39*). These results suggest a common role for the extracellular half of TMD7 in the activation of many different GPCRs.

The L289S and A296T mutants in the cytoplasmic half of TMD7 showed a strong increase in sensitivity to α-factor (5.1- and 6.6-fold, respectively). An unexpected feature of these mutants is that they both displayed increased binding affinity for k-α-factor. This was particularly true for the L289S mutant receptors, which showed nearly 35-fold improved affinity for k-α-factor. Since these residues are situated too far from the extracellular side to participate directly in binding α-factor, this indicates that the cytoplasmic end of TMD7 can influence helix packing to promote changes in the extracellular ends of the TMDs that form the binding pocket. Both Leu289 and Ala296 bracket a proline-containing region that is highly conserved in the Ste2p family of fungal pheromone receptors (LPLxxxxA). One predicted function of this region is that Ser288 and Ser292 are thought to interact with Gln253 in TMD6 to help restrain Ste2p in the inactive conformation (*22*). This suggests that there may be underlying similarities with the highly conserved proline-containing motif (NPxxY) present in the class A family of GPCRs which is also involved in forming H-bond interactions with polar residues in other TMDs (*16*, *17*).

Altogether, the results of this suppressor mutant analysis reveal new functional features of Ste2p. In particular, clusters of mutations in TMD3 and TMD7 highlight similarities between Ste2p and mammalian GPCRs. The cluster of mutations near the cytoplasmic end of TMD3 in Ste2 suggests that this region functions to regulate signaling similar to the role of the conserved (E/DRY) motif at the cytoplasmic end of TMD3 in mammalian class A GPCRs. In addition, the extracellular half of TMD7 appears to be involved in receptor activation, and the cytoplasmic half of TMD7 contains a conserved proline motif that is similar to the corresponding regions of TMD7 in other GPCRs. This suggests that there are conserved features in GPCR organization, which are not evident from primary sequence analysis.

ACKNOWLEDGMENT

We thank the members of our lab for helpful comments on the manuscript.

REFERENCES

1. Dohlman, H. G., and Thorner, J. W. (2001) Regulation of G protein-initiated signal transduction in yeast: Paradigms and principles, *Annu. Rev. Biochem. 70*, 703−754.
2. Naider, F., Estephan, R., Englander, J., Suresh Babu, V. V., Arevalo, E., Samples, K., and Becker, J. M. (2004) Sexual conjugation in yeast: A paradigm to study G-protein-coupled receptor domain structure, *Biopolymers 76*, 119−128.
3. Watson, S., and Arkinstall, S. (1994) *The G-protein linked receptor FactsBook*, Academic Press Ltd., London.
4. Leberer, E., Thomas, D. Y., and Whiteway, M. (1997) Pheromone signalling and polarized morphogenesis in yeast, *Curr. Opin. Genet. Dev. 7*, 59−66.
5. Elion, E. A. (2000) Pheromone response, mating and cell biology, *Curr. Opin. Microbiol. 3*, 573−581.
6. Pausch, M. H. (1997) G protein-coupled receptors in *Saccharomyces cerevisiae*: High-throughput screening asssays for drug discovery, *Trends Biotechnol. 15*, 487−494.
7. Broach, J. R., and Thorner, J. (1996) High-throughput screening for drug discovery, *Nature 384*, 14−16.
8. Lin, J. C., Duell, K., and Konopka, J. B. (2004) A microdomain formed by the extracellular ends of the transmembrane domains promotes activation of the G protein-coupled α-factor receptor, *Mol. Cell. Biol. 24*, 2041−2051.
9. Konopka, J. B., Jenness, D. D., and Hartwell, L. H. (1988) The C terminus of the *Saccharomyces cerevisiae* α-pheromone receptor mediates an adaptive response to pheromone, *Cell 54*, 609−620.
10. Reneke, J. E., Blumer, K. J., Courchesne, W. E., and Thorner, J. (1988) The carboxyl terminal domain of the α-factor receptor is a regulatory domain, *Cell 55*, 221−234.
11. Stefan, C. J., and Blumer, K. J. (1994) The third cytoplasmic loop of a yeast G-protein-coupled receptor controls pathway activation, ligand discrimination, and receptor internalization, *Mol. Cell. Biol. 14*, 3339−3349.
12. Schandel, K. A., and Jenness, D. D. (1994) Direct evidence for ligand-induced internalization of the yeast α-factor pheromone receptor, *Mol. Cell. Biol. 14*, 7245−7255.
13. Clark, C. D., Palzkill, T., and Botstein, D. (1994) Systematic mutagenesis of the yeast mating pheromone receptor third intracellular loop, *J. Biol. Chem. 269*, 8831−8841.
14. Konopka, J. B., Margarit, M., and Dube, P. (1996) Mutation of pro-258 in transmembrane domain 6 constitutively activates the G protein-coupled α-factor receptor, *Proc. Natl. Acad. Sci. U.S.A. 93*, 6764−6769.
15. Stefan, C. J., Overton, M. C., and Blumer, K. J. (1998) Mechanisms governing the activation and trafficking of yeast G protein-coupled receptors, *Mol. Biol. Cell 9*, 885−899.
16. Sakmar, T. P. (2002) Structure of rhodopsin and the superfamily of seven-helical receptors: The same and not the same, *Curr. Opin. Cell Biol. 14*, 189−195.
17. Okada, T., Ernst, O. P., Palczewski, K., and Hofmann, K. P. (2001) Activation of rhodopsin: New insights from structural and biochemical studies, *Trends Biochem. Sci. 26*, 318−324.
18. Parrish, W., Eilers, M., Ying, W., and Konopka, J. B. (2002) The cytoplasmic end of transmembrane domain 3 regulates the activity

of the *Saccharomyces cerevisiae* G-protein-coupled α-factor receptor, *Genetics 160*, 429−443.
19. Dosil, M., Giot, L., Davis, C., and Konopka, J. B. (1998) Dominant-negative mutations in the G protein-coupled α-factor receptor map to the extracellular ends of the transmembrane segments, *Mol. Cell. Biol. 18*, 5981−5991.
20. Sherman, F. (1991) Getting started with yeast, *Methods Enzymol. 194*, 3−21.
21. Mentesana, P. E., Dosil, M., and Konopka, J. B. (2002) Functional assays for mammalian G-protein-coupled receptors in yeast, *Methods Enzymol. 344*, 92−111.
22. Dube, P., and Konopka, J. B. (1998) Identification of a polar region in transmembrane domain 6 that regulates the function of the G protein-coupled α-factor receptor, *Mol. Cell. Biol. 18*, 7205−7215.
23. Sommers, C. M., Martin, N. P., Akal-Strader, A., Becker, J. M., Naider, F., and Dumont, M. E. (2000) A limited spectrum of mutations causes constitutive activation of the yeast α-factor receptor, *Biochemistry 39*, 6898−6909.
24. Abel, M. G., Lee, B. K., Naider, F., and Becker, J. M. (1998) Mutations affecting ligand specificity of the G protein-coupled receptor for the *Saccharomyces cerevisiae* tridecapeptide pheromone, *Biochim. Biophys. Acta 1448*, 12−26.
25. Marsh, L. (1992) Substitutions in the hydrophobic core of the α-factor receptor of *Saccharomyces cerevisiae* permit response to *Saccharomyces kluyveri* α-factor and to antagonist, *Mol. Cell. Biol. 12*, 3959−3966.
26. Dube, P., DeConstanzo, A., and Konopka, J. B. (2000) Interaction between transmembrane domains 5 and 6 in the α-factor receptor, *J. Biol. Chem. 275*, 26492−26499.
27. Sommers, C. M., and Dumont, M. E. (1997) Genetic interactions among the transmembrane domains of the G protein-coupled receptor encoded by the yeast STE2 gene, *J. Mol. Biol. 266*, 559−575.
28. Lee, B. K., Khare, S., Naider, F., and Becker, J. M. (2001) Identification of residues of the *Saccharomyces cerevisiae* G protein-coupled receptor contributing to α-factor pheromone binding, *J. Biol. Chem. 276*, 37950−37961.
29. Sen, M., Shah, A., and Marsh, L. (1997) Two types of α-factor receptor determinants for pheromone specificity in the mating-incompatible yeasts *S. cerevisiae* and *S. kluyveri*, *Curr. Genet. 31*, 235−240.
30. Lin, J., Parrish, W., Eilers, M., Smith, S. O., and Konopka, J. B. (2003) Aromatic residues at the extracellular ends of transmembrane domains 5 and 6 are important for ligand activation of the G protein-coupled α-factor receptor, *Biochemistry 42*, 293−301.
31. Parnot, C., Miserey-Lenkei, S., Bardin, S., Corvol, P., and Clauser, E. (2002) Lessons from constitutively active mutants of G protein-coupled receptors, *Trends Endocrinol. Metab. 13*, 336−343.
32. Gether, U. (2000) Uncovering molecular mechanisms involved in activation of G protein-coupled receptors, *Endocrin. Rev. 21*, 90−113.
33. Schmidt, C., Li, B., Bloodworth, L., Erlenbach, I., Zeng, F. Y., and Wess, J. (2003) Random mutagenesis of the M3 muscarinic acetylcholine receptor expressed in yeast. Identification of point mutations that "silence" a constitutively active mutant M3 receptor and greatly impair receptor/G protein coupling, *J. Biol. Chem. 278*, 30248−30260.
34. Adams, A. E., Botstein, D., and Drubin, D. G. (1989) A yeast actin-binding protein is encoded by SAC6, a gene found by suppression of an actin mutation, *Science 243*, 231−233.
35. Sealfon, S. C., Chi, L., Ebersole, B. J., Rodic, V., Zhang, D., Ballesteros, J. A., and Weinstein, H. (1995) Related contribution of specific helix 2 and 7 residues to conformational activation of the serotonin 5-HT_{2A} receptor, *J. Biol. Chem. 270*, 16683−16688.
36. Han, M., Lin, S. W., Minkova, M., Smith, S. O., and Sakmar, T. P. (1996) Functional interaction of transmembrane helices 3 and 6 in rhodopsin. Replacement of phenylalanine 261 by alanine causes reversion of phenotype of a glycine 121 replacement mutant, *J. Biol. Chem. 271*, 32337−32342.
37. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) Requirement of rigid body motion of transmembrane domain helices for light activation of rhodopsin, *Science 274*, 768−770.
38. Baranski, T. J., Herzmark, P., Lichtarge, O., Gerber, B. O., Trueheart, J., Meng, E. C., Iiri, T., Sheikh, S. P., and Bourne, H. R. (1999) C5a receptor activation. Genetic identification of critical residues in four transmembrane helices, *J. Biol. Chem. 274*, 15757−15765.
39. Patel, A. B., Crocker, E., Eilers, M., Hirshfeld, A., Sheves, M., and Smith, S. O. (2004) Coupling of retinal isomerization to the activation of rhodopsin, *Proc. Natl. Acad. Sci. U.S.A. 101*, 10048−10053.
40. Martin, N. P., Celic, A., and Dumont, M. E. (2002) Mutagenic mapping of helical structures in the transmembrane segments of the yeast α-factor receptor, *J. Mol. Biol. 317*, 765−788.

BI048050U