The Cytoplasmic End of Transmembrane Domain 3 Regulates the Activity of the *Saccharomyces cerevisiae* G-Protein-Coupled α-Factor Receptor

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ABSTRACT

The binding of α-factor to its receptor (Ste2p) activates a G-protein-signaling pathway leading to conjugation of *MAT***a** cells of the budding yeast *S. cerevisiae*. We conducted a genetic screen to identify constitutively activating mutations in the N-terminal region of the α-factor receptor that includes transmembrane domains 1–5. This approach identified 12 unique constitutively activating mutations, the strongest of which affected polar residues at the cytoplasmic ends of transmembrane domains 2 and 3 (Asn^{84} and Gln^{149}, respectively) that are conserved in the α-factor receptors of divergent yeast species. Targeted mutagenesis, in combination with molecular modeling studies, suggested that Gln^{149} is oriented toward the core of the transmembrane helix bundle where it may be involved in mediating an interaction with Asn^{84}. These residues appear to play specific roles in maintaining the inactive conformation of the protein since a variety of mutations at either position cause constitutive receptor signaling. Interestingly, the activity of many mammalian G-protein-coupled receptors is also regulated by conserved polar residues (the E/DRY motif) at the cytoplasmic end of transmembrane domain 3. Altogether, the results of this study suggest a conserved role for the cytoplasmic end of transmembrane domain 3 in regulating the activity of divergent G-protein-coupled receptors.

THE α-factor receptor (*STE2*) stimulates the conjugation of *MAT***a** cells of the budding yeast *Saccharomyces cerevisiae*. The α-factor receptor is a member of the large family of G-protein-coupled receptors (GPCRs) that respond to a wide range of signals including light, hormones, chemokines, and neurotransmitters (Dohlman *et al.* 1991; Watson and Arkinstall 1994; Ji *et al.* 1998). Receptors in this family function by stimulating the α-subunit of a heterotrimeric guanine-nucleotide-binding protein (G protein) to exchange bound GDP for GTP (Bourne 1997). The GTP-bound Gα-subunit then dissociates from the Gβγ-subunits. Either the GTP-bound Gα-subunit or the free Gβγ-subunits then go on to activate downstream effector molecules. In the α-factor signal pathway, the free Gβγ-complex stimulates a mitogen-activated protein kinase-signaling cascade that triggers the transcriptional induction of pheromone-responsive genes and cell division arrest in the G1 phase of the cell cycle (Herskowitz 1995; Pryciak and Huntress 1998; Mahanty *et al.* 1999). The free Gβγ-complex also leads to the activation of the Rho family GTPase CDC42p, which promotes polarized morphogenesis and mating projection formation (Butty *et al.* 1998; Nern and Arkowitz 1998; Moskow *et al.* 2000).

The receptors in the GPCR family do not share significant sequence similarity (Josefsson 1999) but are structurally similar in that they consist of seven membrane-spanning domains (TMDs) connected by alternating intracellular and extracellular loops (Baldwin *et al.* 1997). The seven TMDs are thought to form a helix bundle in the plasma membrane, as has been observed in the crystal structure of rhodopsin (Unger *et al.* 1997; Palczewski *et al.* 2000). GPCRs also appear to share a similar organization of functional domains. For example, as in many GPCRs, the core region of the α-factor receptor encompassing the seven TMDs carries out ligand binding and G-protein activation (Sen and Marsh 1994; Stefan and Blumer 1994). Mutational analysis of the α-factor receptor indicates that residues near the extracellular ends of the TMDs are involved in ligand binding and in promoting the structural changes that result in receptor activation (Sen and Marsh 1994; Dosil *et al.* 1998; Yesilaltay and Jenness 2000). The intracellular loops of the α-factor receptor, in particular the third intracellular loop, have been implicated in G-protein activation (Clark *et al.* 1994; Schandel and Jenness 1994; Stefan and Blumer 1994). In contrast, the cytoplasmic C terminus is not required for signaling and instead acts as a negative regulatory domain that is a target for desensitization by phosphorylation and ligand-mediated downregulation by receptor endocytosis (Chen and Konopka 1996; Hicke *et al.* 1998).

Analysis of the mechanisms of GPCR activation indicates that receptors are initially held in a restrained

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off state (Bourne 1997; Wess 1997; Gether 2000). For example, the α-factor receptor forms preactivation complexes with the G protein in the absence of ligand without leading to GTP exchange on Gα (Dosil *et al.* 2000). In addition, engineered bivalent metal ion binding sites (Sheikh *et al.* 1996, 1999) as well as engineered disulfide linkages (Farrens *et al.* 1996) between TMDs 3 and 6 prevent ligand-mediated activation of a variety of receptors. Furthermore, studies on constitutively active mutant receptors (CAMs) that activate G-protein signaling in a ligand-independent manner suggest that intramolecular interactions between the TMDs act to restrain the receptor in an inactive conformation (Kjelsberg *et al.* 1992; Robinson *et al.* 1992; Lefkowitz *et al.* 1993; Scheer and Cotecchia 1997; Dube and Konopka 1998). In the case of the α-factor receptor, previous studies indicated that intramolecular interactions between TMDs 5 and 6 and between TMDs 6 and 7 are involved in regulating the activity of this receptor (Dube and Konopka 1998; Dube *et al.* 2000). The function of ligand binding therefore appears to stabilize receptors in the activated state, thereby promoting a change in the third intracellular loop that leads to G-protein activation. Consistent with this, ligand binding causes the third intracellular loop of the α-factor receptor to become hypersensitive to trypsin proteolysis, indicating that this region of the receptor undergoes a conformational change during receptor activation (Bukusoglu and Jenness 1996). Interestingly, TMD 6, which is directly connected to the third intracellular loop, has also been identified as a hotspot for mutations that cause a constitutively active phenotype in many other GPCRs (Shenker *et al.* 1993; Kosugi *et al.* 1994; Scheer and Cotecchia 1997; Spalding *et al.* 1998).

Although there are many similarities between the α-factor receptor and other GPCRs, one interesting difference is that the α-factor receptor lacks the E/DRY motif, a conserved triad of residues found at the cytoplasmic end of TMD 3 in many members of the GPCR family (Baldwin *et al.* 1997). Several lines of evidence indicate that the E/DRY motif functions as a conformational switch that plays a critical role in receptor activation by influencing the packing arrangement of the TMDs (Oliveira *et al.* 1994; Scheer and Cotecchia 1997; Scheer *et al.* 1997, 2000; Ballesteros *et al.* 1998). However, most receptors that do not belong to the largest GPCR family (the rhodopsin/adrenergic or family A receptors), like the α-factor receptor, lack this motif (Josefsson 1999), suggesting that these receptors may use an alternative mechanism for regulating receptor activity. Therefore, to explore this possibility and to extend our analysis of receptor activation to other domains of Ste2p, we carried out a genetic screen for constitutively active mutants in TMDs 1–4. Interestingly, the mutants identified in this screen indicate that the cytoplasmic end of TMD 3 of the α-factor receptor is involved in regulating receptor activity. In particular, the results suggest that an interaction between conserved polar residues at the cytoplasmic ends of TMDs 2 and 3 may help to maintain the receptor in an inactive state. Therefore, the results of this study raise the possibility of an evolutionarily conserved role for sequences at the cytoplasmic end of TMD 3 in regulating the activity of very divergent GPCRs.

MATERIALS AND METHODS

Yeast strains and media: Yeast strains used in this study are described in Table 1. Cells were grown in media as described by Sherman (1991). Yeast cells were transformed by the lithium acetate method (Gietz *et al.* 1995). Cells were grown in synthetic medium containing adenine and amino acids but lacking uracil to select for plasmid maintenance.

Genetic screen for constitutively activating receptor mutations: The *STE2* gene was mutagenized by PCR under error-prone conditions. Basically, PCR was carried out using limiting concentrations of the nucleotide dATP. *Taq* polymerase and all PCR reagents were purchased from Roche (Indianapolis). PCR primers that specifically amplify the DNA sequences between codons 2 and 212 of the *STE2* gene and that code for the N terminus of the receptor through the top of TMD 5 were used. Mutagenized PCR fragments were then cotransformed into yeast strain JKY78 with pDB02, a YCp-*STE2* plasmid (Konopka *et al.* 1996) that had been digested (either with *Hpa*I alone or with *Hpa*I and *Aat*II) at unique sites within the sequence. The intact plasmid was then regenerated by double-strand DNA gap repair in Ura^+ transformants. Approximately 110,000 transformed colonies were generated by this method using DNA prepared from 10 independent PCR reactions. Of these transformants, 130 constitutively signaling mutants were identified as blue colonies 24–48 hr after replica plating onto medium containing 20 μg/ml 5-bromo-4-chloro-3-indoyl β-D-galactoside (X-GAL). The constitutively active receptor signaling phenotype was confirmed by isolating plasmids from these mutants and retransforming them into strain JKY78. Sixty of these mutants reproducibly demonstrated plasmid-dependent elevated basal levels of *FUS1-lacZ* expression. Plasmids from the 23 mutants that displayed at least threefold elevation in basal signaling were subjected to DNA sequence analysis using a dideoxy DNA sequencing kit from United States Biochemical (Cleveland). Individual point mutations were subcloned into *STE2* plasmid pDB02 to confirm that the observed mutations accounted for constitutive receptor activity.

Targeted mutagenesis of the α-factor receptor gene: Site-directed mutations were introduced into the *STE2* sequence by PCR using *Pfu* DNA polymerase (Promega, Madison, WI). The PCR primers used were complementary to the *STE2* sequence, except for the codon change required to introduce the mutation. After PCR, the 365-bp *Hpa*I-*Aat*II fragment containing the change indicated in parentheses was subcloned into plasmid pDB02 to create *ste2-N84A* (AAC → GCC), *ste2-Q85A* (CAA → GCA), *ste2-F148A* (TTT → GCT), *ste2-I150A* (ATT → GCT), *ste2-K151A* (AAA → GCA), *ste2-V152A* (GTT → GCT), and *ste2-F154A* (TTC → GCC). Mutant receptors containing substitutions at positions 149 and 153 were generated using a heterogeneous PCR primer that randomly introduced all four bases at each position of the corresponding codons. The PCR-generated fragment containing the desired mutations was then subcloned into plasmid pDB02 as described above. DNA sequence analysis of 51 plasmids identified 9 different substitution mutations at position 149 (H, G, S, I, A, T, V, P, and R). DNA sequence analysis of 59 plasmids

TABLE 1

Yeast strains used

Strain	Genotype
JKY78	*MAT***a** *far1 bar1::hisG ste2::LEU2 lys2::Fus1-lacZ arg4 his3 leu2 lys2° trp1 ura3*
JKY131	*MAT***a** *bar1::hisG far1 ste2Δ mfα1::LEU2 mfα2::his5*$^{+}$ *ade2 his3 leu2 ura3 mfa2::FUS1-lacZ*
JKY127-36-1	Isogenic to JKY131 except *sst2-1*
YLG123	*MAT***a** *ade2-1 his4-580*^{a} *lys2° trp1*^{a} *tyr1° leu2 ura3 SUP4-3*^{ts} *bar1-1 mfa2::fus1-lacZ ste2-10::LEU2*
lys1α	*MAT*α *lys1*

identified 13 different substitution mutations at position 153 (A, G, T, L, Q, E, P, D, R, S, F, H, and Y). The *ste2-Q149N* (CAG → AAC), *ste2-Q149C* (CAG → TGT), and *ste2-N84Q* (AAC → CAA) mutant plasmids were created using the Quick Change mutagenesis kit (Stratagene, La Jolla, CA). Mutagenic oligonucleotides were designed according to the manufacturer's specifications. The double mutants mutated at both positions 84 and 149 were generated by PCR using *Pfu* DNA polymerase from *STE2* plasmids containing the desired single mutants. DNA fragments containing both substitutions were then subcloned into plasmid pDB02 as described above. A similar strategy was used to subclone the *ste2-I80T* mutation from the plasmid containing the *ste2-I80T/Q149R* double-mutant receptor gene. The *ste2-Q149R-GFP* plasmid was constructed by subcloning the 1714-bp *ste2-Q149R SphI-Cla*I fragment into a pDB02-based *STE2-GFP* plasmid pPK14. All mutations were confirmed by DNA sequence analysis using the Big Dye cycle sequencing kit (ABI, Columbia, MD).

α-Factor receptor analysis: Western immunoblots were carried out essentially as described (Konopka *et al.* 1996). Halo assays for α-factor-induced cell division arrest were performed by spreading ~1 × 10^6 yLG123 yeast cells carrying either a wild-type *STE2* plasmid (pDB02) or the indicated mutant version onto solid medium lacking uracil. Sterile filter disks containing the indicated concentration of α-factor were placed onto the lawn of cells, and the plates were incubated for ~48 hr at 30°. Yeast mating assays were conducted by replica plating patches of yeast strain yLG123 carrying either a mutant or wild-type *STE2* plasmid onto YPD plates containing a lawn of *MAT*α (lys1α) cells. These plates were incubated at 30° for 4 hr to allow mating and were then replica plated to synthetic medium plates lacking amino acids and uracil. The plates were then incubated at 30° for 2 days to select for the growth of diploids. To assay for induction of *FUS1-lacZ* expression, cultures were grown overnight to logarithmic phase in selective medium, diluted to 3 × 10^6 cells/ml, and incubated for 2 hr at 30° prior to induction. The cells were then incubated for an additional 2 hr in the presence of the indicated concentration of synthetic α-factor (Bachem, Torrance, CA). Inductions were stopped by incubating the cells on ice and by adding sodium azide to a final concentration of 6.5 mm. β-Galactosidase assays were performed by using the colorimetric substrate *O*-nitrophenyl-β-d-galactopyranoside as described elsewhere (Miller 1972). Basal levels of *FUS1-lacZ* expression were determined as described above, except that the cells were incubated in the absence of α-factor.

Molecular modeling: The helical wheel model of the transmembrane segments of the α-factor receptor was based on the two-dimensional crystal structure of rhodopsin and on the Baldwin/Schertler model for the organization of its transmembrane helix bundle (Baldwin *et al.* 1997; Unger *et al.* 1997). The transmembrane segments were assigned according to the seven peaks of hydrophobicity in a Kyte/Doolittle hydropathy plot of the α-factor receptor. The relative rotation of the individual helices was initially based on the configuration that optimized the shielding of polar residues. Genetic interactions identified between TMDs 6 and 7 (Dube and Konopka 1998) and between TMDs 5 and 6 (Dube *et al.* 2000) were used to orient these helices with respect to each other. The computer-generated three-dimensional molecular model of Ste2p was based on a predicted structure of the transmembrane region of rhodopsin (Shieh *et al.* 1997). The residues of the α-factor receptor included in this molecular model are as follows: TMD 1 (Ser^{47}–Ser^{73}), TMD 2 (Thr^{78}–Ser^{104}), TMD 3 (Gly^{123}–Asp^{157}), TMD 4 (Ile^{162}–Val^{186}), TMD 5 (Lys^{202}–Lys^{225}), TMD 6 (Asp^{242}–Lys^{269}), and TMD 7 (Asp^{275}–Ala^{298}). These amino acid side chains were then extended from the helical backbone of the rhodopsin model. Hydrogen bonding restraints were applied between the backbone amide and carbonyl groups to allow the TMDs to maintain α-helical character, but provided flexibility for kinks to be introduced at positions of prolines that are unique to the α-factor receptor. The relative rotation of the helices was then set according to the helical wheel model described above, and hydrogen-bonding restraints were introduced between Gln^{253}, Ser^{288}, and Ser^{292}, which have been shown to interact genetically (Dube and Konopka 1998). Restraints were also introduced on the basis of a disulfide bridge identified in crosslinking studies between cysteine residues substituted at positions 223 and 247 of the α-factor receptor (Dube *et al.* 2000). This structure was then energy minimized with the program X-Plor (Brunger 1992), using Powell minimization for 10,000 cycles, and presented using the program INSIGHT II (Molecular Simulations, San Diego).

RESULTS

Identification of new CAMs: A genetic screen was carried out to identify mutations in the sequences for the N-terminal half of the α-factor receptor that cause constitutive receptor signaling in the absence of pheromone. Fragments of the *STE2* gene were mutagenized *in vitro* and then reintroduced into a YCp-*STE2* plasmid by homologous recombination in yeast (see materials and methods). A specially designed yeast strain (JKY78) was used for the screen that contained a pheromone-responsive *FUS1-lacZ* reporter gene to detect receptor signaling and a *far1* mutation to prevent the induction of cell division arrest. Sixty yeast colonies displaying elevated basal expression of *FUS1-lacZ* were identified on medium containing X-GAL, a chromogenic substrate for β-galactosidase. After recovering the receptor plasmids and transforming back into yeast, 23 were found to cause a more than threefold elevation in basal signaling and were reserved for further analysis. All of these

TABLE 2

Constitutive activity of α-factor receptor mutants

STE2 allele	Relative basal *FUS1-lacZ* activity in strain JKY78[a]	Relative basal *Fus1-lacZ* activity in strain JKY131[b]	Relative α-factor-induced *FUS1-lacZ* activity in strain JKY131[c]
Wild type	1 ± 0.1	1 ± 0.2	100%
Y17H	2.9 ± 0.4	2.1 ± 0.2	103.9 ± 3.4
N46S	3.8 ± 0.3	3.5 ± 1.1	102.6 ± 12.9
N84S	4.1 ± 0.2	13 ± 0.2	109.3 ± 6.9
Y98H	10.7 ± 0.7	2.5 ± 0.6	104.1 ± 4.1
N105S	3.6 ± 0.3	4.9 ± 0.6	91.4 ± 2
F119S	3.9 ± 0.8	1.5 ± 0.2	100.9 ± 2.3
S141P	4.5 ± 1.7	4 ± 0.2	103.4 ± 4.8
Q149R	8.1 ± 1.1	14.9 ± 0.4	71.8 ± 3.5
Q149R/I80T	4.9 ± 0.2	11.1 ± 0.9	34.4 ± 3.2
Q149R/I150V	14.7 ± 0.1	14 ± 2.5	77.3 ± 3
Q149R/V152I	3.3 ± 0.5	0.5 ± 0.2	110.8 ± 3.4
I153F	10.9 ± 0.2	10.3 ± 1.4	106.4 ± 4.2
I169K	3.7 ± 0.2	4.1 ± 0.4	94.8 ± 3.8
T199A	6.6 ± 0.4	2.4 ± 0.5	100.6 ± 4.1
L222P	5 ± 0.2	4 ± 0.7	106.3 ± 1.6

[a] Fold elevation of *FUS1-lacZ* activity in yeast strain JKY78. Results are the average of three independent assays each performed in duplicate (±SD).

[b] Fold elevation of *FUS1-lacZ* activity in *mfα1*Δ, *mfα2*Δ yeast strain JKY131. Results are the average of three independent assays each performed in duplicate (±SD).

[c] *FUS1-lacZ* activity of yeast strain JKY131 in response to 1×10^{-7} M α-factor. Results are representative of at least three independent assays performed in duplicate and expressed as percentage of wild type (±SD). The typical response of a wild-type *STE2* strain to this concentration of α-factor causes ~80-fold induction of *FUS1-lacZ* activity over that of the basal.

mutants produced functional cell-surface receptors on the basis of their ability to be further induced by exogenously added α-factor (Table 2 and data not shown). Western immunoblot analysis demonstrated that each of these mutants produced full-length receptors at a level similar to the wild type (data not shown). DNA sequence analysis of the mutant plasmids identified 12 different point mutations (Figure 1). Six of the mutations affected residues toward the extracellular regions of the receptor, including the N terminus and extracellular loops 1 and 2. The remaining six mutations affected residues in the TMDs.

The yeast strain JKY78 was chosen for use in the initial screening because it displays a high degree of sensitivity to the basal activity of the *FUS1-lacZ* reporter gene on plates containing X-GAL. However, since this strain contains the α-factor genes, rare mating-type switching events in the culture could lead to production of low levels of α-factor. Therefore, in addition to constitutively active mutants, we also expected to identify mutants

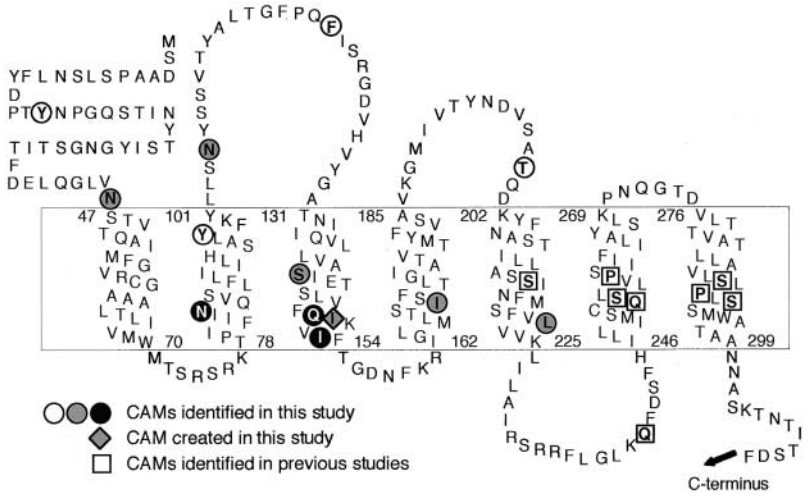

FIGURE 1.—Residues affected by mutations that cause constitutive α-factor receptor signaling. The predicted two-dimensional topology of the α-factor receptor in the plasma membrane is shown with the extracellular region at the top. Residues affected by constitutively activating mutations are highlighted. Circles indicate the positions of new CAMs identified in this study and squares indicate positions of previously characterized constitutively activating mutations. Solid circles denote the positions of the strongest activating mutations, shaded circles signify intermediate activity, and open circles indicate weak activity. The diamond at position 150 signifies that the activating mutation at this position was created by site-directed mutagenesis.

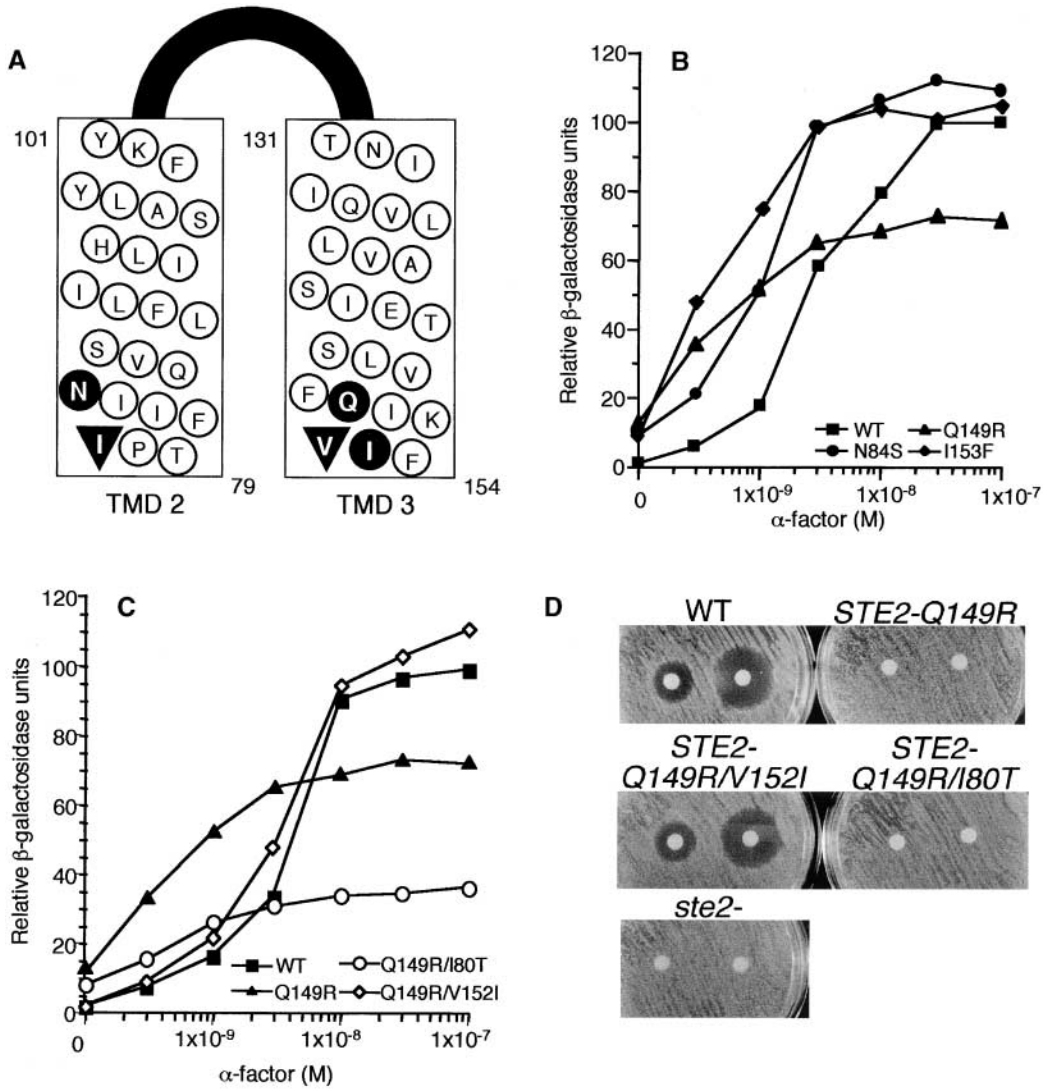

FIGURE 2.—α-Factor-dependent signaling properties of mutant receptors. (A) Relative positions of the strongest constitutively active mutants identified in this study are indicated by solid circles. Positions of the residues affected by intragenic suppressors of *ste2-Q149R* are indicated by solid triangles. (B) Dose-response assays for *FUS1-lacZ* activity in the wild-type, *ste2-N84S*, *ste2-Q149R*, and *ste2-I153F* mutants. (C) Dose-response assays for the I80T and V152I intragenic suppressor mutations of *ste2-Q149R*. (D) Assays for α-factor-induced cell division arrest (halo assay). Filter disks containing either 0.25 or 1.25 μg α-factor were placed onto agar plates spread with a lawn of yeast strain yLG-123 cells carrying the indicated wild-type or mutant version of *STE2* plasmid pDB02. Dose-response assays were conducted in yeast strain JKY131 carrying the indicated wild-type or mutant version of plasmid pDB02. The standard deviation was <15% for all data points.

that are supersensitive to α-factor. To distinguish between these phenotypes, the mutant receptors were retested in a strain in which the α-factor genes were deleted (JKY131). In this strain, four of the six mutants with amino acid substitutions toward the extracellular region of the receptor (Y17H, Y98H, F119S, and T199A) no longer displayed a more than threefold elevation in basal signaling (Table 2). Since this result suggested that the elevated basal signaling of these mutant receptors was still ligand dependent, they were not studied further. In contrast, all of the mutants that contained substitutions within the TMDs (N84S, S141P, Q149R, I153F, I169K, and L222P) displayed a significant elevation in basal signaling that was α-factor independent (Table 2). SOMMERS *et al.* (2000) previously identified *ste2-N84S* and *ste2-Q149R* mutations; however, they reported relatively weak constitutive signaling from these receptor mutants. In our hands, both of these mutations cause significant increases in basal *FUS1-lacZ* activity when compared to either *STE2* or *ste2*Δ control cells (Table 1 and data not shown). The apparent discrepancy in these results could reflect differences between the assay conditions or in the yeast strains used in the studies. Thus, these results are consistent with previous genetic screens that indicate that CAMs primarily affect the TMDs of the α-factor receptor (KONOPKA *et al.* 1996; STEFAN *et al.* 1998; SOMMERS *et al.* 2000).

New CAMs implicate the cytoplasmic ends of TMDs 2 and 3 in receptor function: Analysis of the positions of the affected residues in the strongest constitutive mutants (N84S, Q149R, and I153F) revealed that they were clustered at the cytoplasmic ends of TMDs 2 and 3, suggesting an important role for this region in regulating receptor activity. Therefore, these mutant receptors were analyzed further by assaying their ability to signal in a ligand-dependent manner. Dose-response assays for *FUS1-lacZ* induction showed that all three mutants displayed a leftward shift in the dose of α-factor required to achieve half-maximal signaling (EC_{50}), indicating increased sensitivity to α-factor (Figure 2B). The *ste2-N84S* mutant displayed about a twofold increase in sensitivity, while the *ste2-Q149R* and *-I153F* mutants were about

sevenfold supersensitive. Thus, these results also implicate the cytoplasmic ends of TMDs 2 and 3 in ligand-dependent receptor signaling.

The *ste2-N84S* and *-I153F* cells were induced to a maximal level that was similar to the wild type (Figure 2B). However, the *ste2-Q149R* mutant was induced to only ~72% of the wild-type maximum, even when exposed to a concentration of α-factor that was >100-fold higher than the saturating dose for a wild-type receptor (Figure 2B and data not shown). The *ste2-Q149R* mutant also showed significant defects in assays for mating and cell division arrest (Figure 2D and data not shown). The impaired signaling activity of the *ste2-Q149R* cells could be due to an intrinsic defect in receptor signaling or to a decrease in the number of cell-surface receptors caused by their mislocalization away from the plasma membrane as has been demonstrated for other CAMs (Dube and Konopka 1998; Stefan *et al.* 1998). Consistent with the latter possibility, microscopic analysis of green fluorescent protein (GFP)-tagged receptors indicated a greatly reduced cell surface fluorescence for *ste2-Q149R-GFP* compared to a wild-type *STE2-GFP* strain (data not shown). In an independent study, Sommers *et al.* (2000) were not able to detect α-factor binding to mutants carrying the *ste2-Q149R* allele in radio-ligand binding assays. Therefore, diminished cell-surface localization of the Q149R substituted receptors apparently contributes to defects in the ability of mutant cells to respond to α-factor.

Two double mutants involving the Q149R substitution and a second substitution in either TMD 2 or TMD 3 were identified in the screen in which the constitutive signaling activity of the Q149R mutant was significantly reduced (Table 2). The I80T substitution at the cytoplasmic end of TMD 2 lowered the basal signaling activity of the *ste2-Q149R/I80T* mutant by ~26% compared to the *ste2-Q149R* mutant alone. The other substitution, V152I, was at the cytoplasmic end of TMD 3 and caused a low basal level of signaling for the *ste2-Q149R/V152I* double mutant that was comparable to the wild type, indicating a complete suppression of the constitutive phenotype. These results raised the possibility that the suppressor mutations may result in a structural change that compensates for the Q149R substitution at the cytoplasmic end of TMD 3. However, suppression of constitutive signaling could also be caused by any type of mutation that uncouples the receptor from G-protein activation. To distinguish between these possibilities, the double mutants were examined for the ability to be induced by α-factor. The *ste2-Q149R/I80T* double mutant was induced to only ~46% of the *ste2-Q149R* mutant alone, indicating that the suppression is caused by diminished receptor signaling (Figure 2, C and D). The *ste2-I80T* single mutant, however, was not defective in responding to α-factor, suggesting that the defects of the double mutant are due to a negative cooperativity of the two single mutations (data not shown). In contrast, the *ste2-Q149R/V152I* mutant responded to α-factor much like the wild type in both short-term assays for *FUS1-lacZ* induction (Figure 2C) and in long-term assays for cell division arrest (Figure 2D). The fact that this double mutant shows improved responses to α-factor relative to the *ste2-Q149R* mutant alone indicates that the suppression of the constitutive signaling is not caused by a defect in receptor function. This suggests that there is a specific genetic interaction between residues at positions 149 and 152 and argues that the V152I substitution may introduce a structural change that com-

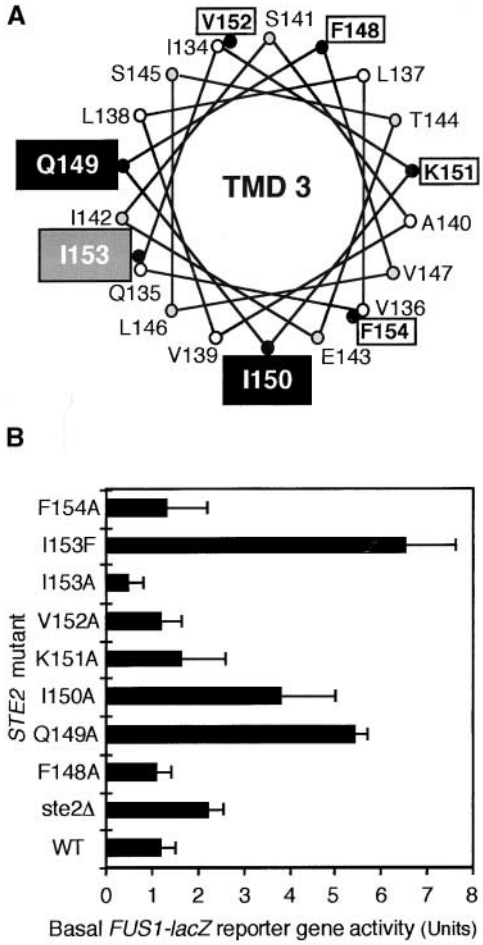

Figure 3.—Alanine-scanning mutagenesis of the base of TMD 3. (A) Residues predicted to comprise TMD 3 (Ile^{134}–Phe^{154}) are plotted on a helical wheel diagram to display their relative orientation in an α-helix as viewed from the cytoplasmic side of the plasma membrane. Residues that were mutated to alanine as part of this study are boxed. Solid boxes identify positions that yield constitutive receptor activity when mutated to alanine. The shaded box indicates that the I153A substitution was not an activating mutation; however, the *ste2-I153F* mutant displayed significant constitutive activity. Open boxes on the opposite side of the helix identify residues that did not cause significant constitutive signaling when mutated to alanine. (B) Basal *Fus1-lacZ* activity of yeast strain JKY131 carrying the indicated *STE2* allele on plasmid pDB02. Results are the average of four independent assays, each done in duplicate (± standard deviation).

TABLE 3

Alanine-scan mutant phenotypes

STE2 allele	Basal *FUS1-lacZ* activity[a]	Relative α-factor-induced *FUS1-lacZ* activity[b]	α-factor-induced zone of growth inhibition (mm)[c]	Relative mating ability[d]
Wild type	1.2 ± 0.3	100%	19	+++
F148A	1.1 ± 0.3	94.2 ± 2.4	18.5	+++
Q149A	5.4 ± 0.3	93.7 ± 3.7	Not detected	++
I150A	3.8 ± 1.2	103.8 ± 7.1	21 (turbid)	+++
K151A	1.6 ± 1	102.4 ± 10.3	18	+++
V152A	1.2 ± 0.4	98.4 ± 5.2	17	+++
I153A	0.5 ± 0.3	100 ± 4.7	15 (turbid)	+++
F154A	1.3 ± 0.9	93.4 ± 4.8	17 (turbid)	+++

[a] Basal *FUS1-lacZ* activity in yeast strain JKY131. Results are the average of four independent assays each conducted in duplicate (±SD).

[b] FUS1-lacZ activity of yeast strain JKY131 in response to 1×10^{-7} M α-factor expressed as percentage of wild type (±SD). Results are the average of four independent assays, each conducted in duplicate.

[c] Halo diameter (mm) of yeast strain yLG123 in response to 0.25 μg α-factor. Results are the average of three independent assays.

[d] Patch mating ability of yLG123 cells transformed with the indicated *STE2* allele on a CEN plasmid. Results are the average of three independent isolates. +++, wild type; ++, partial defect in mating.

pensates for the Q149R mutation. Collectively, the phenotypes displayed by the CAMs and their suppressors identify the cytoplasmic ends of TMDs 2 and 3 as being important in α-factor receptor function.

Constitutively active mutants affect residues on one side of TMD 3: The region surrounding the base of TMD 3 is of interest because it is thought to play a critical role in regulating the activity of many mammalian GPCRs (Oliveira *et al.* 1994; Scheer and Cotecchia 1997; Gether 2000). The cluster of mutations affecting this region of the α-factor receptor, whose primary sequence is very divergent from mammalian receptors, raised the possibility that the cytoplasmic end of TMD 3 may play an important role in regulating signaling in a wider range of receptors than previously recognized. To investigate this domain in more detail, each residue from Phe^{148} through Phe^{154} was mutated individually to alanine. These residues comprise the last two predicted helical turns of TMD 3. Two new mutations that caused constitutive activity were identified, Q149A and I150A. The *ste2-Q149A* mutant affected the same residue as the *ste2-Q149R* mutant that was identified in the genetic screen for CAMs and displayed a fivefold elevation in basal signaling (Figure 3B). The *ste2-I150A* mutant identified a new position and showed about a threefold elevation in basal *FUS1-lacZ* activity. Interestingly, the *ste2-I153A* mutant did not display an elevated basal level of signaling even though a mutation at this position (I153F) was identified in the screen for CAMs. This result suggests that only certain substitutions at position 153 may cause constitutive activity, as will be discussed below. Interestingly, when analyzed on a helical wheel plot, the activating mutations affected residues on one side of TMD 3 (Figure 3A). Control experiments showed that each of the alanine substitution mutants could be induced by α-factor to essentially wild-type levels (Table 3). This indicated that none of these alanine substitutions caused a defect in signaling that would have prevented the detection of constitutive activity. Therefore, the side of TMD 3 containing Gln^{149}, Ile^{150}, and Ile^{153} is predicted to be oriented toward the helix bundle where these residues may play a special role in maintaining the receptor in an inactive state.

Substitution mutants suggest that Gln^{149} plays a direct role in regulating receptor signaling: The alanine-scanning mutagenesis results suggested that there might be a fundamental difference in how substitutions at positions 149 and 153 cause constitutive receptor signaling. As described above, substitution of Gln^{149} with either Ala or Arg caused a significant increase in basal signaling. However, substituting Ile^{153} with Ala did not cause a significant increase in basal signaling, as was seen for the substitution with Phe (I153F). These observations raised the possibility that the polar side chain of Gln^{149} might play an important role in maintaining the receptor in an inactive state, such that many different substitutions at position 149 will result in constitutive signaling. In contrast, the side chain of Ile^{153} may play a different role, perhaps in the proper helix packing arrangement, and thus only residues with certain characteristics may affect the receptor in a manner that causes constitutive activity. Therefore, to examine the effects of different residues at these positions, site-directed mutagenesis was used to introduce a variety of substitution mutations at positions 149 and 153.

Of the 11 different substitution mutants identified at position 149, 8 were constitutively active, displaying a more than threefold elevation in basal *FUS1-lacZ* activity

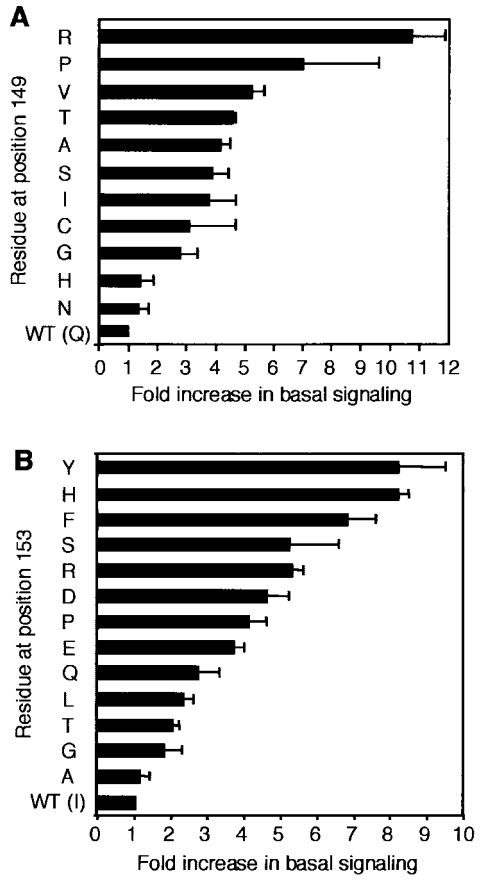

FIGURE 4.—Basal signaling activity of *STE2* mutants with substitutions at positions 149 and 153. Basal *FUS1-lacZ* activity of substitution mutants at position 149 (A) and at position 153 (B) is shown. Yeast strain JKY131 carrying the indicated wild-type or mutant version of the *STE2* plasmid pDB02 was assayed for α-factor-independent β-galactosidase activity to measure the basal levels of *FUS1-lacZ* reporter gene expression. The different mutants are arranged in order of increasing activity. Results are expressed as the fold elevation in basal activity over that of the wild type and represent the average of three independent assays, each done in duplicate (± standard deviations).

(Figure 4A). All of the constitutively active mutants identified at position 149 displayed partial defects in responding to α-factor (Table 4). Nonetheless, it is noteworthy that the strongest constitutive activity was observed in mutants with diverse amino acid substitutions including Arg, Pro, and Val. The substitution mutants that did not display a significant elevation in basal signaling activity (*STE2-Q149N*, *-Q149H*, and *-Q149G*) were interesting in that they showed a slightly increased sensitivity to α-factor in halo assays (Table 4). Thus, even the most conservative substitutions at position 149 (Asn and His) convey a signaling phenotype. Therefore, the Gln^{149} side chain appears to play a key role in maintaining the basal state of the α-factor receptor.

Analysis of 13 substitution mutants at position 153 showed that 8 were constitutively active, displaying a more than threefold elevated basal *FUS1-lacZ* activity. The amino acid substitutions that caused the highest levels of constitutive activity were those with bulky side chains (*e.g.*, His, Phe, and Tyr). The substitutions that caused intermediate levels of activity included a mixture of polar residues (*e.g.*, Gln, Glu, Asp, Arg, and Ser) and proline. It is interesting to note that these residues have the ability to influence transmembrane helix packing arrangements (EILERS *et al.* 2000). In contrast, those that did not display a significant elevation in basal signaling involved substitutions with small aliphatic residues (*e.g.*, Ala and Gly). The majority of the mutants with substitutions at position 153 showed essentially wild-type responses to α-factor, except for those substituted with charged residues (Asp, Glu, and Arg) and proline, which were partially defective in assays for mating and cell division arrest (Table 5). Altogether, the pattern of mutant phenotypes at this position suggests that the Ile^{153} side chain is not specifically required for maintaining the inactive receptor state.

Identification of residues that may interact with Gln^{149}: The results described above suggest that the Gln^{149} side chain is oriented toward the interior of the helix bundle where it is likely to interact with residues in the other TMDs in a manner that could influence receptor structure and function. To identify residues that may interact with Gln^{149}, we first reasoned that the interacting residue would be capable of participating in hydrogen-bond interactions. Second, since Gln^{149} was fully conserved in the homologous α-factor receptors from *S. kluyveri*, *Schizosaccharomyces pombe*, and a putative receptor identified as an open reading frame in *Candida albicans* (Figure 5), the interacting residue should be equally conserved. Finally, since this interaction is predicted to be important for maintaining the receptor in the inactive state, substitutions affecting the interacting residue should also cause constitutive receptor activation.

Two-dimensional structural models of the α-factor receptor were constructed to narrow the search to the most likely candidates. The models were restricted to residues that, like Gln^{149}, reside near the cytoplasmic ends of their respective TMDs. The corresponding residues were ordered into α-helical conformation, and the seven helices of the α-factor receptor were arranged according to the crystal structure of rhodopsin, which is thought to be characteristic of the GPCR family (BALDWIN *et al.* 1997). The TMDs were then oriented on the basis of previous data and also to optimize the shielding of polar residues from the nonpolar lipid environment. The results of this modeling suggested that the most likely interacting partner for Gln^{149} would be at the cytoplasmic end of TMD 2 (Figure 6). Two polar residues at the base of TMD 2 were identified (Asn^{84} and Gln^{85}), which could potentially form hydrogen-bond interactions with Gln^{149} (Figure 7B).

Interestingly, Asn^{84} appears to be a good candidate for interacting with Gln^{149}. It too is conserved in all four

TABLE 4

Gln^{149} substitution mutant phenotypes

Substitution at position 149	Basal *FUS1-lacZ* activity[a]	Relative α-factor-induced *FUS1-lacZ* activity[b]	α-Factor-induced zone of growth inhibition (mm)[c]	Relative mating ability[d]
Wild type (Q)	1.2 ± 0.3	100%	15	+++
N	1.8 ± 0.3	96.6 ± 1.1	18	+++
H	1.8 ± 0.1	102.4 ± 8.3	18	+++
G	2.8 ± 0.6	95.5 ± 11.1	19	++
C	3.1 ± 1.6	57.3 ± 3.1	Not detected	+
S	3.8 ± 0.9	98.9 ± 3.7	13	++
I	3.9 ± 0.5	98.9 ± 1.1	7 (very turbid)	++
A	4.2 ± 0.3	88.2 ± 3.2	Not detected	++
T	4.6 ± 0.1	96.1 ± 3.1	15	++
V	5.3 ± 0.4	58.8 ± 1.9	Not detected	+
P	7 ± 2.6	38.1 ± 4	Not detected	±
R	10.8 ± 1.1	71.7 ± 6.5	Not detected	+

[a] Basal *FUS1-lacZ* activity in yeast strain JKY131. Results are the average of three independent assays, each performed in duplicate (±SD).

[b] *FUS1-lacZ* activity of yeast strain JKY131 in response to 1×10^{-7} M α-factor expressed as percentage of wild type (±SD). Results are the average of three independent assays, each performed in duplicate.

[c] Halo diameter (mm) of yeast strain yLG123 in response to 0.25 μg α-factor. Results are the average of three independent assays.

[d] Patch mating ability of yLG123 cells carrying the indicated *STE2* allele on a CEN plasmid. Results are the average of three independent isolates. +++, wild type; ++, partial mating defect; +, severe mating defect; ±, essentially sterile.

of the α-factor receptors (Figure 5). In addition, an N84S substitution was identified in our genetic screen as a strong constitutively activating mutation. In contrast, the other candidate residue, Gln^{85}, is not found in all of the pheromone receptors, and genetic screening approaches did not identify any constitutively activating mutations at this position in the α-factor receptor. To gain more experimental evidence, Asn^{84} and Gln^{85} were mutated to code for alanine to test their roles for receptor function. The N84A substitution caused a 3.5-fold increase in basal *FUS1-lacZ* activity and resulted in a 2-fold increase in sensitivity to α-factor in halo assays (Figure 7A and data not shown). In contrast, the *ste2-Q85A* mutant was indistinguishable from the wild type (Figure 7A and data not shown). Thus, these results implicated Asn^{84} as the most likely interacting partner for Gln^{149}.

The potential interaction between Asn^{84} and Gln^{149} was analyzed further by a genetic approach in which the residues at positions 84 and 149 were swapped (*ste2-N84Q/Q149N*; SWAP mutant). The rationale for this was that if Asn^{84} and Gln^{149} were involved in a direct interaction, this SWAP mutant might restore a receptor with wild-type signaling properties. The *ste2-N84Q* mutant by itself displayed a 3.5-fold increase in basal *FUS1-lacZ* activity. Interestingly, neither the *ste2-Q149N* mutant nor the SWAP mutant displayed a significant elevation in basal signaling (Figure 8A). This indicated that the Q149N substitution suppressed the constitutive activity caused by the N84Q substitution in the SWAP mutant. Furthermore, although the *ste2-N84Q* and *-Q149N* mutants displayed an ~2-fold supersensitivity to α-factor in halo assays, the SWAP mutant showed essentially wild-type sensitivity (Figure 8B). These results indicate that the phenotypes of each individual mutant were mutually suppressed in the SWAP mutant. In a parallel set of studies we analyzed a double mutant containing the strongest activating mutations at each position (N84S/Q149R). Interestingly, consistent with each of these substitutions activating the receptor in a similar manner, the *ste2-N84S/Q149R* mutant did not display an additive effect on basal signaling compared to the corresponding single mutants (data not shown). Altogether, these genetic analyses suggest that Asn^{84} is the most likely residue to interact with Gln^{149}.

Molecular modeling suggests that Asn^{84} and Gln^{149} form a direct contact: To examine whether a direct contact between Asn^{84} and Gln^{149} could be accommodated structurally, we developed a computer-generated three-dimensional molecular model of the transmembrane region of the α-factor receptor. The model was generated by mapping the residues corresponding to the predicted transmembrane helices of the α-factor receptor onto an α-carbon template of the transmembrane region of the visual pigment rhodopsin (see MATERIALS AND METHODS). Previously characterized helix-helix interactions between TMDs 5 and 6 (DUBE *et al.* 2000) and between TMDs 6 and 7 (DUBE and KONOPKA 1998) were used to set constraints on the resulting molecular model. Energy minimization was then con-

TABLE 5

Ile[153] substitution mutant phenotypes

Substitution at position 153	Basal *FUS1-lacZ* activity[a]	Relative α-factor-induced *FUS1-lacZ* activity[b]	α-Factor-induced zone of growth inhibition (mm)[c]	Relative mating ability[d]
Wild type (I)	0.9 ± 0.3	100%	15	+++
A	1.0 ± 0.3	84.5 ± 2.8	14	+++
G	1.6 ± 0.5	82.4 ± 2.9	12 (Very turbid)	++
T	1.8 ± 0.2	95.1 ± 2.2	14	+++
L	2.1 ± 0.3	99.2 ± 3.1	16	+++
Q	2.4 ± 0.6	87.1 ± 3	13 (turbid)	+++
E	3.3 ± 0.3	87.9 ± 4.4	Filled	+
P	3.7 ± 0.5	69.2 ± 2.7	Not detected	+
D	4.1 ± 0.6	82 ± 2.9	Filled	+
R	4.8 ± 0.3	55 ± 1.6	Not detected	±
S	4.7 ± 1.4	98 ± 3.3	15 (turbid)	++
F	6.1 ± 0.8	105.5 ± 4.1	12 (very turbid)	++
H	7.4 ± 0.3	114.8 ± 4.4	13 (turbid)	++
Y	7.4 ± 1.2	106.9 ± 5.2	16	+++

[a] Basal *FUS1-lacZ* activity in yeast strain JKY131. Results are the average of three independent assays, each performed in duplicate (±SD).

[b] FUS1-lacZ activity of yeast strain JKY131 in response to 1×10^{-7} M α-factor expressed as percentage of wild type (±SD). Results are the average of three independent assays, each performed in duplicate.

[c] Halo diameter (mm) of yeast strain yLG123 in response to 0.25 μg α-factor. Results are the average of three independent assays.

[d] Patch mating ability of yLG123 cells carrying the indicated *Ste2* allele on a CEN plasmid. Results are the average of three independent isolates. +++, wild type; ++, partial mating defect; +, severe mating defect; ±, essentially sterile.

ducted to predict the basal structure of the helix bundle (see MATERIALS AND METHODS). Interestingly, as shown in Figure 9, the resulting structural model predicted a direct interaction between Asn[84] and Gln[149]. The fact that both Asn and Gln have longer than average side chains could help this interaction to form between TMD 2 and TMD 3 without clashing with neighboring residues. These data indicate that a direct contact between these highly conserved polar residues would be sterically permitted in the context of the other residues of the α-factor receptor.

DISCUSSION

A genetic screen for constitutively active mutants that signal in the absence of α-factor was carried out to identify residues in the N-terminal half of the α-factor receptor that are important for function. Twelve unique CAMs were identified in this study, of which 8 were entirely α-factor independent. The majority of the α-factor-independent CAMs contained substitutions in the TMDs. Of particular interest were the strongest CAMs identified in this study (*ste2-N84S* and *ste2-Q149R*), which both displayed >10-fold elevation in basal signaling. These CAMs affected residues at the cytoplasmic ends of TMDs 2 and 3, respectively, which are highly conserved in the family of yeast α-factor receptors. Interestingly, evolutionarily conserved polar residues at the cytoplasmic end of TMD 3 are thought to play a special role in regulating the activity of a large number of mammalian GPCRs (BALDWIN *et al.* 1997; SCHEER and COTECCHIA 1997; WESS 1997; GETHER 2000). In view of this, we investigated the role of this region, and in particular the role of Gln[149], in regulating the activity of the α-factor receptor.

Several lines of evidence suggest that Gln[149] is involved in promoting the inactive receptor conformation by interacting with other TMDs. First, structural analysis of polytopic membrane proteins indicates that polar side chains in transmembrane helices, like that of Gln[149]

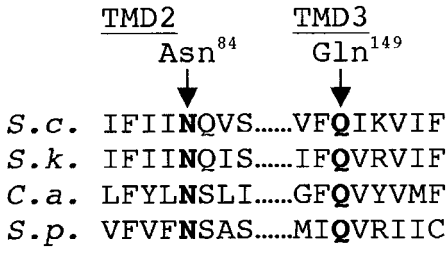

FIGURE 5.—α-Factor receptor multiple sequence alignment. Amino acids corresponding to the last two predicted helical turns of TMDs 2 and 3 on the cytoplasmic side of the α-factor receptors from *S. cerevisiae*, *S. kluyveri*, *C. albicans*, and *S. pombe* were aligned. The positions corresponding to Asn[84] and Gln[149] are highlighted in boldface type.

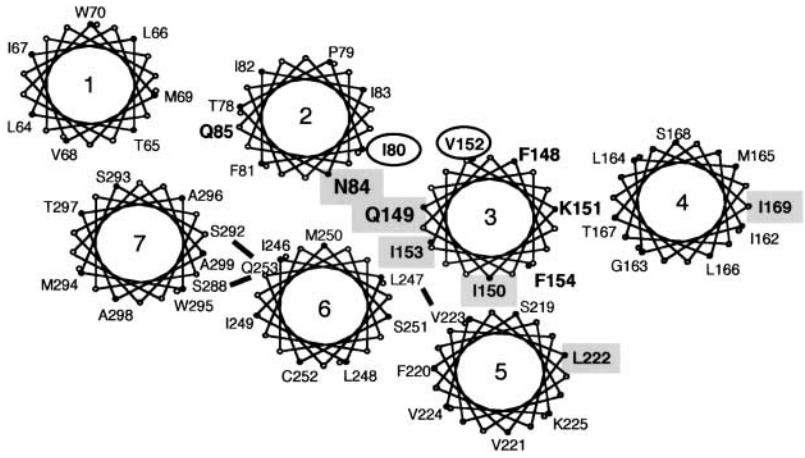

FIGURE 6.—Two-dimensional structural model of the transmembrane region of the α-factor receptor. The residues predicted to form the final two turns of the cytoplasmic end of each TMD are represented as a helical wheel diagram as viewed from the intracellular side of the plasma membrane. The seven helices are arranged according to the structure of rhodopsin. Solid lines between Val^{223} and Leu^{247} and among Gln^{253}, Ser^{288}, and Ser^{292} indicate previously characterized intramolecular interactions that were used to set the respective orientations of TMDs 5, 6, and 7. Shaded boxes indicate positions where constitutively activating mutations were found in this study. The residues affected by the suppressors of *ste2-Q149R* are circled. Other residues that were mutated as part of this study, which did not alter the basal signaling properties of the receptor, are highlighted in boldface type.

in the α-factor receptor, are not likely to be oriented toward the nonpolar lipid environment. Instead, they usually face the core of the protein where they can mediate interactions between adjacent helices (ZHANG and WEINSTEIN 1994; EILERS *et al.* 2000). Second, the Ala-scanning mutagenesis experiments described above predicted that Gln^{149} is on the face of TMD 3 that interacts with the helix bundle, since all of the mutations that caused constitutive signaling affected residues that reside on the same side of TMD 3. In addition, other CAMs identified in an independent study (S145L and I142T; SOMMERS *et al.* 2000) and residues identified by dominant-negative mutations that alter the ligand-binding properties of the receptor (Asn^{132} and Gln^{135}; DOSIL *et al.* 1998) also reside on the same side of TMD 3 as the constitutive mutants described above. Finally, the observation that diverse substitutions at position 149 cause constitutive activity argues that the endogenous Gln residue serves an important function that is lost upon mutation. However, Gln^{149} does not appear to be directly involved in G-protein activation since all of the substitution mutants identified at this position are capable of responding to α-factor. Instead, these results suggest that Gln^{149} has an indirect role in G-protein activation, probably by mediating an intramolecular contact with a residue on another helix that helps restrain the receptor into the inactive conformation.

Molecular modeling studies predicted that Gln^{149} may be oriented toward Asn^{84} on TMD 2. Several lines of additional evidence also implicated Asn^{84} on TMD 2 as the most likely candidate for an interaction with Gln^{149}. First, mutation of Asn^{84} caused strong constitutive activity as expected for a mutation that disrupted an interaction with Gln^{149}. Second, the chemical nature of the Asn side chain would permit interaction between Asn^{84} and Gln^{149} to be stabilized by hydrogen bonding (CREIGHTON 1993). Third, Asn^{84} and Gln^{149} are fully conserved in other members of the α-factor receptor family, as is expected for residues that mediate an important intramolecular contact. Further evidence comes from the residue SWAP experiment in which the phenotypes of each individual substitution mutant (*ste2-N84Q* and *ste2-*

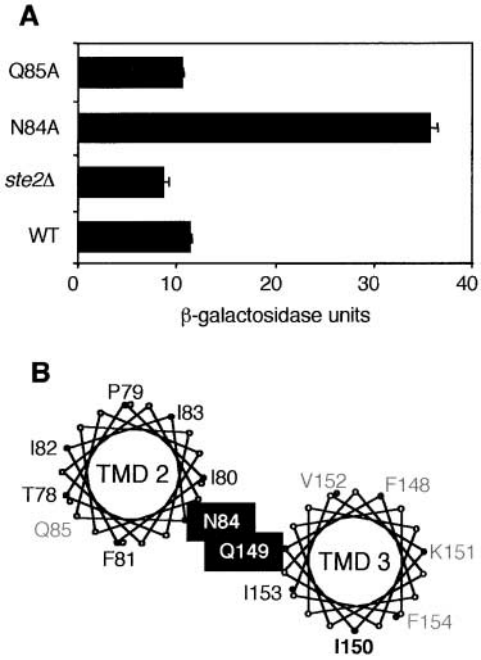

FIGURE 7.—Mutational analysis of polar residues on TMD 2 that may interact with Gln^{149}. (A) Yeast strain JKY127-36-1 carrying the wild-type, *ste2-N84A*, or *ste2-Q85A* substituted versions of plasmid pDB02 was assayed for basal levels of *FUS1-lacZ* reporter gene expression. Results are the average of three independent assays, each done in duplicate (± the standard deviation). (B) The residues predicted to reside in the last two helical turns at the cytoplasmic ends of TMDs 2 and 3 are projected as helical wheel plots as viewed from the cytoplasmic side of the plasma membrane. Gray type indicates positions where substitution with Ala does not significantly affect basal signaling activity. Boldface type identifies positions where Ala substitution mutants cause constitutive signaling. These results suggest that Asn^{84} may interact with Gln^{149} (solid boxes).

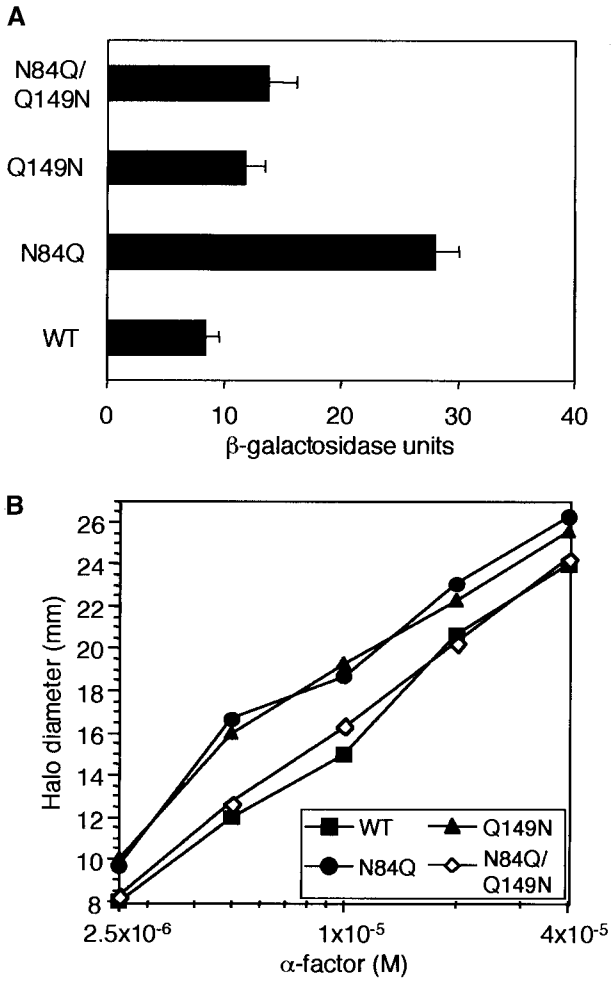

FIGURE 8.—Double-mutant analysis between Asn^{84} and Gln^{149}. (A) Yeast strain JKY127-36-1 carrying the wild-type, *ste2-N84Q*, *ste2-Q149N*, or *ste2-N84Q/Q149N* (SWAP) version of plasmid pDB02 was assayed for basal levels of *FUS1-lacZ* reporter gene expression. Results are the average of three independent assays, each done in duplicate (± the standard deviation). (B) α-Factor-induced cell division arrest (halo) assays were carried out on yLG123 cells transformed with the indicated wild-type or mutant version of *STE2* plasmid pDB02. Results are the average of four independent assays. The standard deviation was <1.5 mm for each data point.

Q149N) were suppressed in the double mutant (*ste2-N84Q/Q149N*). A similar approach was used to test the interaction between a pair of conserved Asp and Asn residues on TMDs 2 and 7 of the serotonin 5HT-2A receptor (SEALFON *et al.* 1995). As part of the SWAP experiment, it was interesting that the *ste2-N84Q* mutant displayed greater constitutive activity than the *ste2-Q149N* mutant. Since Gln has a longer side chain than Asn, the constitutive activity of the *ste2-N84Q* mutant correlates with a potential increase in the distance between the cytoplasmic ends of TMDs 2 and 3. In contrast, the Q149N substitution, which is not expected to increase this distance, did not cause significant constitutive activity. Interestingly, this substitution did result in increased sensitivity to ligand. Thus, all of the genetic evidence implicates Asn^{84} as an interacting partner for Gln^{149} and suggests that this interaction may function to restrain the α-factor receptor in the off state.

Other residues at the cytoplasmic ends of TMDs 2 and 3 are also likely to influence receptor function. For example, molecular modeling predicted that the residues affected by the I80T and V152I suppressor mutations of the *ste2-Q149R* mutant are in close proximity. Ile^{80} is predicted to reside one helical turn below Asn^{84}, and Val^{152} is predicted to reside almost one full helical turn below Gln^{149}. In addition, other constitutive mutants that affected the Ile^{150} and Ile^{153} residues that are nearby in TMD 3 were identified in this study. Thus, these mutants underscore the sensitivity of this region of the α-factor receptor to perturbation and further indicate that contacts between the cytoplasmic ends of TMDs 2 and 3 may be important for α-factor receptor function.

A triad of polar residues (Glu/Asp-Arg-Tyr), termed the E/DRY motif, is found at the cytoplasmic end of TMD 3 in most members of the medically important rhodopsin/adrenergic family of GPCRs (BALDWIN 1993; BALDWIN *et al.* 1997). This sequence is thought to act as a conformationally sensitive switch that regulates the entry of these receptors into the activated conformation (OLIVEIRA *et al.* 1994; SCHEER *et al.* 1996, 2000; WESS 1997; BALLESTEROS *et al.* 1998; GETHER 2000; OKADA *et al.* 2001). As has been seen most clearly in the crystal structure of rhodopsin, these residues appear to form a polar pocket in conjunction with conserved polar residues on other TMDs (BALDWIN *et al.* 1997; PALCZEWSKI *et al.* 2000). In addition, residues on TMDs 1, 2, and 7 are thought to form a hydrogen-bond network that may include the E/DRY motif on TMD 3 that has been implicated in maintaining the inactive receptor conformation (SCHEER *et al.* 1996, 2000; PERLMAN *et al.* 1997; BALLESTEROS *et al.* 1998; GETHER 2000; OKADA *et al.* 2001). Protonation of the Asp/Glu residue of this motif is thought to trigger the disruption of hydrogen-bond interactions between the residues that comprise the polar pocket, allowing the receptors to isomerize to the activated state (ARNIS *et al.* 1994; SCHEER and COTECCHIA 1997; SCHEER *et al.* 1997; OKADA *et al.* 2001). However, many GPCRs, including both the **a**- and α-factor receptors in yeast, lack the E/DRY motif. One possibility is that these receptors employ an alternative mechanism for regulating receptor activity. Alternatively, diverse GPCRs may be activated by a similar mechanism that is not recognizable at the primary sequence level. For example, interaction between Asn^{84} and Gln^{149} in the α-factor receptor may function in a manner analogous to the E/DRY motif in other GPCRs to regulate receptor activation.

Several lines of evidence indicate that disruption of the interaction between TMD 3 and the other TMDs allows for motion between TMDs 3 and 6 that is critical

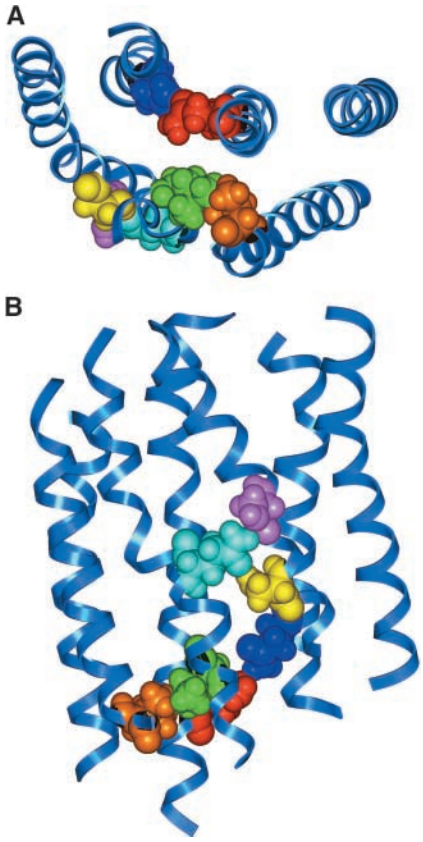

Figure 9.—Three-dimensional molecular model of the α-factor receptor. (A) Intracellular view of the α-factor receptor transmembrane region showing the previously characterized interactions between TMDs 5 and 6 (Val^{223}, orange; Leu^{247}, green) and between TMDs 6 and 7 (Gln^{253}, teal; Ser^{288}, lavender; Ser^{292}, yellow). In the context of these constraints, a direct contact between Asn^{84} and Gln^{149} (blue and red, respectively) is predicted by the model. (B) Same structure as above, but rotated ~90° so that the view is from within the plane of the membrane. TMDs 5, 6, and 7 are in the foreground and the cytoplasmic side is toward the bottom.

for receptor activation. For example, spin-labeling experiments performed with rhodopsin, and studies carried out with the β_2 adrenergic receptor using fluorescent probes, detected movement of TMDs 3 and 6 upon ligand binding (Farrens *et al.* 1996; Gether *et al.* 1997). Engineered disulfide bonds or metal binding sites between the cytoplasmic ends of TMDs 3 and 6 that restrained these TMDs into close proximity were found to impair the signaling activity of the mutant receptors, indicating that the relative movement between these helices is important for receptor signaling (Farrens *et al.* 1996; Sheikh *et al.* 1996, 1999). Interestingly, similar results were obtained for the parathyroid hormone receptor, a GPCR that lacks the E/DRY motif (Sheikh *et al.* 1999). These results suggest that these divergent GPCRs share a conserved mechanism of activation.

Genetic evidence suggests that movement between TMDs 3 and 6 also underlies the activation of the α-factor receptor. For example, substitution of Ser^{254} on TMD 6 with large aromatic residues caused constitutive activity, suggesting that a bulky residue at this position may interfere with the packing of the TMDs in a manner that promotes the activated receptor conformation (Konopka *et al.* 1996; Dube and Konopka 1998). Interestingly, the molecular models described in this study indicate that Ser^{254} may be oriented toward TMD 3. Similarly, substitution of Ile^{153} on TMD 3, which is oriented toward TMD 6 in our models, with a large aromatic residue also caused strong constitutive signaling. Altogether, these results suggest that TMDs 3 and 6 of the α-factor receptor move with respect to each other upon receptor activation.

The relative motion of TMDs 3 and 6 may be important to allow TMD 6 to propagate a signal to the third intracellular loop. TMD 6 is directly connected to the third intracellular loop, which is known to play a key role in G-protein activation in the α-factor receptor as well as in many mammalian GPCRs (Clark *et al.* 1994; Stefan and Blumer 1994; Burstein *et al.* 1998; Gether 2000). Consistent with this, TMD 6 has been identified as a hotspot for constitutive mutations in many different receptors (Shenker *et al.* 1993; Kosugi *et al.* 1994; Scheer and Cotecchia 1997; Dube and Konopka 1998; Spalding *et al.* 1998). In the case of the α-factor receptor, it is interesting that substitutions affecting Pro^{258} in TMD 6 cause strong constitutive receptor signaling (Konopka *et al.* 1996; Stefan *et al.* 1998). Since Pro is expected to cause a bend in a transmembrane helix, substitution with other amino acids is expected to straighten the helix and consequently shift the relative position of TMD 6 as has been observed for peptides modeled after TMD 6 (Arshava *et al.* 1998). Analysis of other constitutive α-factor receptor mutants has also implicated interactions between hydrophobic residues on TMDs 5 and 6 (Dube *et al.* 2000) and an interaction between polar residues on TMDs 6 and 7 (Gln^{253} on TMD 6 and Ser^{288} and Ser^{292} on TMD7) that appear to be important for restraining TMD 6 in the inactive conformation (Dube and Konopka 1998).

Taken together, the analysis of the α-factor receptor suggests that there is a common mechanism of GPCR activation shared by divergent family members, which involves relaxing constraints between transmembrane helices. A similar conformational change irrespective of the primary amino acid sequence could help to explain how receptors like the α-factor receptor and the **a**-factor receptor of *S. cerevisiae*, which do not share significant sequence similarity, can activate the same G protein (Nakayama *et al.* 1987; Sprague and Thorner 1992; Leberer *et al.* 1997). In addition, many mammalian GPCRs can also functionally couple to the yeast G protein when heterologously expressed in the yeast (Price *et al.* 1995, 1996; Pausch 1997; Erickson *et al.* 1998). The ability of these mammalian receptors to activate a yeast G protein further indicates that the structural changes involved in the mechanism of GPCR activation

are conserved in evolution. Finally, the results of this study also underscore the importance of evolutionarily conserved residues that reside in TMDs for maintaining the inactive conformation of GPCRs. Therefore, special consideration should be given to polar residues that reside in the TMDs in studies concerning the structure and function of the large number of newly identified orphan receptors, for which no ligands or signaling pathways are known.

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