Sequences in the Intracellular Loops of the Yeast Pheromone Receptor Ste2p Required for G Protein Activation†

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ABSTRACT: The α-factor receptor of the yeast *Saccharomyces cerevisiae* encoded by the *STE2* gene is a member of the large family of G protein-coupled receptors (GPCRs) that mediate multiple signal transduction pathways. The third intracellular loop of GPCRs has been identified as a likely site of interaction with G proteins. To determine the extent of allowed substitutions within this loop, we subjected a stretch of 21 amino acids (Leu228−Leu248) to intensive random mutagenesis and screened multiply substituted alleles for receptor function. The 91 partially functional mutant alleles that were recovered contained 96 unique amino acid substitutions. Every position in this region can be replaced with at least two other types of amino acids without a significant effect on function. The tolerance for nonconservative substitutions indicates that activation of the G protein by ligand-bound receptors involves multiple intramolecular interactions that do not strongly depend on particular sequence elements. Many of the functional mutant alleles exhibit greater than normal levels of signaling, consistent with an inhibitory role for the third intracellular loop. Removal of increasing numbers of positively charged residues from the loop by site-directed mutagenesis causes a progressive loss of signaling function, indicating that the overall net charge of the loop is important for receptor function. Introduction of negatively charged residues also leads to a reduced level of signaling. The defects in signaling caused by substitution of charged amino acids are not caused by changes in the abundance of receptors at the cell surface.

G protein-coupled receptors (GPCRs)[1] comprise a superfamily of integral, transmembrane proteins that allow cells to communicate with their surroundings. Hundreds of different GPCRs mediate a wide range of physiological signaling responses ranging from sensory transduction to hormone responses and neurotransmission. Once a specific ligand has bound, GPCRs activate an intracellular heterotrimeric G protein, consisting of α, *β*, and *γ* subunits. In the inactive state of the G protein, a GDP molecule is tightly bound to Gα. Interaction between the GPCR and the G protein triggers the exchange of GDP for GTP on the Gα subunit, leading to the dissociation of the G protein from the receptor and of the G protein α subunit from the *βγ* complex. The released Gα-GTP and the *βγ* complex then activate downstream effectors. The Gα subunit eventually hydrolyzes the bound GTP, allowing rebinding to the *β* and *γ* subunits, restoring the unactivated state (for reviews, see refs *1*−*3*).

Despite the availability of three-dimensional structures for trimeric G proteins (*4*, *5*) and one GPCR, rhodopsin (*6*), the molecular mechanisms leading to activation of G proteins upon binding of a ligand to GPCRs remain obscure. All GPCRs share a common topology consisting of seven transmembrane segments connected by hydrophilic loops. Several biochemical and biophysical experiments suggest that binding of ligand to GPCRs changes helix−helix interactions between transmembrane segments 3 and 6 which, in turn, affects the conformation of intracellular regions of receptors, uncovering a site that interacts with the G protein (*1*, *7*). However, the particular surfaces involved in receptor−G protein interactions have not been identified, and the mechanism by which these interactions lead to enhanced nucleotide exchange by the G protein is not understood.

The mating pathway of the yeast *Saccharomyces cerevisiae* has been a valuable tool for studying GPCR signal transduction mechanisms because of the ease of mutagenizing components followed by screening or selecting for desired phenotypes (*8*, *9*). Haploid yeast cells exist as either of two distinct mating types, *MAT***a** or *MAT*α, that can mate to form diploid zygotes. Each cell type secretes a pheromone that binds to receptors on the surface of cells of the opposite mating type, causing the target cells to arrest cell division and to elongate toward the cell of the opposite mating type. *MAT***a** cells secrete **a**-factor, which binds to the **a**-factor receptor encoded by the yeast *STE3* gene. *MAT*α cells secrete

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[1] Abbreviations: GPCR, G protein-coupled receptor; IC, intracellular loop; X-Gal, 5-bromo-4-chloro-3-indyl-*β*-D-galactoside; FDG, fluorescein D-(*β*-D-galactopyranoside).

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α-factor, which binds to the α-factor receptor encoded by the *STE2* gene.

While pheromone receptors are not at all similar in sequence to mammalian receptors, they are members of the GPCR superfamily. They contain seven predicted transmembrane segments and initiate the pheromone response by activating a cytoplasmic trimeric G protein encoded by the *GPA1*, *STE4*, and *STE18* genes with extensive similarity to sequences encoding mammalian Gα, G*β*, and G*γ* subunits, respectively. Furthermore, several mammalian GPCRs expressed in yeast are capable of activating the yeast pheromone pathway upon binding of an appropriate ligand. In some cases, activation of the pheromone response by heterologous receptors requires formation of a chimeric Gα subunit containing mammalian sequences (*10*−*17*). However, despite the extreme sequence divergence between yeast and mammalian receptors, some heterologously expressed mammalian receptors are capable of directly activating the normal yeast G protein (*15*, *16*). Furthermore, the normal yeast α-factor receptor is capable of activating at least one mammalian G protein (*18*).

In all GPCR-mediated signaling events, the cytoplasmic location of the G protein dictates that the interaction with the receptor must involve the intracellular hydrophilic loops, associated regions of transmembrane segments, or the intracellular cytoplasmic carboxyl-terminal tail. The second and third intracellular loops (IC2 and IC3, respectively) and the carboxyl-terminal tail in mammalian receptors are implicated as the most likely sites of interaction with G proteins based on numerous experiments involving cross-linking of loops to G protein subunits, the peptide competition of receptor−G protein interactions, mutagenesis of loops, and creation of chimeric receptors in which loops from one type of receptor are inserted in place of the corresponding regions of a different type of receptor (see refs *1*, *19*, and *20*). However, searches for conserved sequences that might be sites of G protein interaction with GPCRs have uncovered only a conserved "DRY" sequence motif in IC2 at the intracellular end of the third transmembrane segment and a loosely conserved pattern of basic residues in the third intracellular loop.

The carboxyl-terminal tail of the yeast α-factor receptor is dispensable for signaling (*21*, *22*). Furthermore, this receptor contains no DRY motif. The sequence that is most similar to DRY at the intracellular end of the third transmembrane segment of the α-factor receptor consists of a DNF sequence. However, this sequence, as well as most of the second intracellular loop of the α-factor receptor, can be deleted with minimal effects on signaling (*23*). Thus, the most likely site of interaction of the α-factor receptor with the G protein is the third intracellular loop.

The lack of a conserved sequence responsible for interaction of intracellular portions of GPCRs with G proteins suggests that activation is mediated by some structural rearrangement that depends only weakly on the presence of specific amino acids at particular positions. This idea is reinforced by the ability of GPCRs with essentially no sequence similarity, such as mammalian and yeast receptors, to act interchangeably in activating heterologous signaling pathways. The two endogenous yeast pheromone receptors, Ste2p and Ste3p, exhibit no sequence similarity, aside from the general enrichment of basic residues in the third intracellular loop, even though they are known to activate the same cytoplasmic G protein in response to pheromone binding. Furthermore, the third intracellular loop of Ste2p is relatively insensitive to inactivation by point mutations (*24*−*26*).

To establish the range of allowed amino acid substitutions at each position in IC3 of Ste2p, we conducted intensive random mutagenesis of the region, using an approach that has previously been used to define the range of allowed sequences in transmembrane segments of GPCRs (*11*, *27*, *28*). The yeast system has several unique features that make it well-suited for mutagenic analysis of IC3. (1) Large libraries of mutations can be readily introduced into yeast cells. (2) A sensitive screen is available for detection of small populations of cells with functional signaling pathways. (3) Signaling by yeast α-factor receptors is not strongly affected by changes in levels of receptors; thus, compared with other G protein-coupled systems, mutations in pheromone receptors are more likely to affect receptor structure and function rather than receptor biosynthesis, subcellular targeting, or degradation (*26*, *29*, *30*). (4) The α-factor receptor has a relatively short IC3 loop, simplifying the identification of sequences involved in G protein activation.

We report here that IC3 of Ste2p displays an extreme tolerance for amino acid substitution. In addition, we have used site-directed mutagenesis to further characterize patterns arising from the random mutational analysis, particularly the roles of positively charged residues in signaling.

EXPERIMENTAL PROCEDURES

Plasmid Library Construction. The library of plasmids encoding Ste2p with a randomly mutagenized third intracellular loop was constructed as previously described (*27*) in a plasmid derived from the *LEU2 CEN* plasmid pMD357, encoding the *STE2* gene with a COOH-terminal c-myc tag (*31*). Since we sought to screen for rare functional receptors, it was important to eliminate unmutagenized wild-type receptors from the library. This was accomplished using a starting allele in which a termination codon had been inserted in place of R234 in the region targeted for mutagenesis. Thus, any plasmids resulting from direct replication of single-stranded template DNA unmodified by mutagenic oligonucleotide appear as nonfunctional background colonies in the screen for functional receptors. The stop codon was introduced by site-directed mutagenesis (*32*) of plasmid pMD357 using oligonucleotide ON388 with a single base change (AGA → UGA) to create the plasmid pMD794. (Sequences of mutagenic oligonucleotides are available on request.) Plasmid pMD794 was then used as a template to create a library of randomly generated mutations in IC3 of Ste2p by oligonucleotide-directed mutagenesis.

Random mutagenesis was conducted essentially as described previously (*27*). The 61-base mutagenic oligonucleotide ON379 corresponding to the region from Leu228 to Leu248 of the *STE2* gene was synthesized at the University of Rochester Nucleic Acid Core Facility as a mixture containing 80% of the correct base and a total of 20% of the three remaining incorrect bases for every position. To prevent preferential binding of oligonucleotides containing few base pair mismatches to the DNA template, we used a 1:3 molar ratio of oligonucleotide to DNA. Annealing of

the mutagenic oligonucleotide to the template was conducted by heating to 90 °C and cooling to room temperature over a period of 3 h. Extension reaction mixtures with T7 DNA polymerase were incubated on ice for 30 min and heated gradually in steps to 18 °C for 30 min, 25 °C for 30 min, and 37 °C for 2 h to enhance the stability of weakly hybridizing oligonucleotides and reduce library bias. The resulting plasmids were transformed into XL1-Blue *Escherichia coli* cells, and resulting colonies were pooled for plasmid library preparation using the Wizard Miniprep System (Promega Corp.). This library of randomly mutagenized *STE2*-containing plasmids was then transformed into the *S. cerevisae* host strain A575 (MAT**a** *ste2*-Δ *far1*-Δ *bar1*$^{-}$ *cry1*^{R} *ade2-1 his4-580 lys2*$_{oc}$ *trp1*$_{am}$ *tyr1*$_{oc}$ *SUP4-3*^{ts} *leu2 ura3 FUS1::p[FUS1-lacZ TRP1]*) (*31*).

Screening the Mutational Library. Activation of the mating pathway was detected on the basis of the activity of a *FUS1*-lacZ reporter as described previously (*27*, *31*, *33*, *34*). In cells with a functional mating pathway, transcription of the *FUS1* gene is strongly induced after incubation with pheromone. Yeast transformants were replica-plated onto medium containing α-factor and the β-galactosidase substrate X-Gal (5-bromo-4-chloro-3-indyl-β-D-galactoside). To ensure that the observed phenotype was the result of the plasmid-borne *STE2* allele, and to minimize the possibility that the observed phenotype is affected by multiple plasmids in the same yeast strain, plasmids from colonies exhibiting *FUS1-lacZ* induction were recovered from cells, amplified in *E. coli*, and transformed back into the A575 yeast host strain (*27*). If the retransformed strains exhibited pheromone-dependent *FUS1-lacZ* induction, the isolated plasmids were sequenced.

Site-Directed Mutation of STE2. Site-directed changes introduced into *STE2* are listed in Tables 1 and 2. Most of these changes were accomplished by oligonucleotide-directed mutagenesis (*32*) using single-stranded DNA from plasmid pMD357 as a template. Plasmids pMD972−978 and pMD1062−1066 were created using plasmid pMD947 as a template. *STE2* alleles were transferred into multicopy plasmids by excising *Sac*I−*Sph*I fragments from the relevant single-copy plasmids (listed in Table 1) and ligating into *Sac*I−*Sph*I-digested pMD228 a multicopy plasmid which lacks the *STE2* gene (*29*) to create the plasmids listed in Table 2.

Quantitative β-Galactosidase Assay. Recovered *STE2* alleles were quantitatively assayed for the ability to induce *FUS1-lacZ* expression using the fluorescent assay previously developed by the laboratory of H. Dohlman with modifications (*27*). For each allele, we isolated three single colonies which were grown in minimal medium to an A_{600} of ∼1.0, diluted, and regrown overnight to an A_{600} of 0.4. One hundred fifty microliters of each of these cultures was added to a 96-well plate with α-factor at final concentrations of 0, 10, and 500 nM, respectively, in separate wells. Cells were incubated with pheromone at 30 °C for 105 min and optical densities measured at 600 nm using an absorbance plate reader (SpectraMax Plus, Molecular Devices Corp.). A fluorescent substrate solution was added to every well to produce the following final concentrations: 0.8% Triton X-100, 23 mM PIPES, and 117 nM fluorescein D-(β-D-galactopyranoside) (FDG). The plates were incubated at 37 °C for 30 min; then the reactions were stopped by the addition of 32 μL of 1 M $NaCO_3$, and the fluorescence emission was measured at 530 nm with excitation at 485 nm using a fluorescence plate reader (SpectraMax Gemini, Molecular Devices Corp.). The activity of each strain was then compared with a standard calibration curve of the activity of purified β-galactosidase and normalized for the number of assayed cells in each well by dividing by the optical density at 600 nm. Because of considerable day-to-day variation among assays, apparently due in part to the variation in stocks of FDG, levels of *FUS1-lacZ* induction of mutant strains are presented as a percentage of the β-galactosidase activity of a normal control strain measured in the same experiment. Standard deviations for *FUS1-lacZ* expression levels of triplicate samples from different yeast transformants were less than 25% for randomly mutated alleles. Standard deviations for levels of *FUS1-lacZ* induction for site-directed mutants are indicated by error bars in the figures.

Halo Assay. The A575 host strain used for library screening contains a *far1* deletion preventing cell cycle arrest in response to pheromone. To use assays of cell cycle arrest to determine the abilities of mutant receptors to respond to pheromone, it was necessary to transform plasmids containing various *STE2* alleles into the *FAR1*$^{+}$ yeast strain A232 (*MAT***a** *ste2*-Δ *bar1*$^{-}$ *cry1*^{R} *ade2-1 his4-580 lys2*$_{oc}$ *tyr1*$_{oc}$ *SUP4-3*^{ts} *leu2 ura3 FUS1*::p*[FUS1-lacZ TRP1]*). Halo assays were performed as described previously (*23*, *33*, *35*).

Immunoblotting. Extracts were prepared from cells cultured to an OD_{600} of 0.75−1.2 and subjected to electrophoresis and immunodetection using an anti-c-myc antibody (Roche) at a 1:500 dilution essentially as described previously (*23*, *29*).

Radioactive Ligand Binding Assay. Ligand binding was assessed essentially as described previously (*36*). Briefly, cells were grown to an OD_{600} of 0.5 and harvested by centrifugation at 5000*g* at 4 °C. Pelleted cells were washed two times with ice-cold YM-1 medium (*37*) and resuspended to a cell density of 4×10^7 cells/mL. A total of 140 μL of [^{3}H]-α-factor at various concentrations was added to 560 μL of cell suspension such that the final concentration of the radioactive peptide ranged from 7.8×10^{-10} to 10×10^{-8} M. After a 30 min incubation, 200 μL samples were filtered and washed over glass filtermats using a Standard Cell Harvester (Skatron Instruments, Sterling, VA) and placed in scintillation vials for counting. Measurements were taken in triplicate for each strain. The level of nonspecific binding was determined by conducting the assay under identical conditions with strain A1783 that lacks the α-factor receptor. The level of nonspecific binding as a function of the amount of added α-factor was fit to a linear regression, and the nonspecific binding contribution was subtracted from the level of total binding at each α-factor concentration. The remaining specific binding component was fit to the quadratic expression for single-site binding as a function of total added ligand (*38*) via the nonlinear least-squares protocol of the program Sigmaplot.

RESULTS

Intensive Random Mutagenesis of the IC3 Loop. To examine the range of amino acid substitutions compatible with receptor signaling, we used a random mutational approach initially applied to the transmembrane segments

Table 1: *CEN* Plasmids and Yeast Strains

STE2 allele	oligo number	plasmid	yeast strain *ste2*-Δ *far1*-Δ[a]	yeast strain *ste2*-Δ *FAR1*$^{+}$ [b]
STE2^{+}		pMD357	A2149	A451
vector		pMD284	A1738	A608
R231S	ON438	pMD934	A2314	A2368
R233S	ON439	pMD935	A2315	A2369
R234S	ON440	pMD936	A2316	A2370
K239S	ON441	pMD937	A2317	A2371
R231S/R233S	ON442	pMD938	A2318	A2372
R231S/R234S	ON443	pMD939	A2319	A2373
R231S/K239S	ON444	pMD940	A2320	A2374
R233S/R234S	ON445	pMD941	A2321	A2375
R233S/K239S	ON446	pMD942	A2322	A2376
R234S/K239S	ON447	pMD943	A2323	A2377
R231S/R233S/R234S	ON448	pMD944	A2324	A2378
R231S/R233S/K239S	ON449	pMD945	A2325	A2379
R233S/R234S/K239S	ON450	pMD946	A2326	A2380
R231S/R233S/R234S/K239S	ON451	pMD947	A2327	A2381
L236A	ON452	pMD948	A2328	A2382
L236F	ON453	pMD949	A2329	A2383
L236W	ON454	pMD950	A2330	A2384
L236H	ON455	pMD951	A2331	A2385
R231S/R234S/K239S	ON476	pMD971	A2401	A2412
R231S/R233S/R234S/K239S/S243R	ON477	pMD972	A2402	A2413
R231S/R233S/R234S/K239S/G237R	ON478	pMD973	A2403	A2414
R231S/R233S/R234S/K239S/S232R	ON479	pMD974	A2404	A2415
R231S/R233S/R234S/K239S/S243R/G237R	ON480	pMD975	A2405	A2416
R231S/R233S/R234S/K239S/S243R/S232R	ON481	pMD976	A2406	A2417
R231S/R233S/R234S/K239S/G237D/S232R	ON482	pMD977	A2407	A2418
R231S/R233S/R234S/K239S/S243R/G237D/S232R	ON483	pMD978	A2408	A2419
R231S/R233S/G237D	ON484	pMD979	A2409	A2420
R231S/R233S/L238D	ON485	pMD980	A2410	A2421
R231S/R233S/Q240E	ON486	pMD981	A2411	A2422
G237D	ON549	pMD1016	A2467	
L238D	ON550	pMD1017	A2468	
Q240E	ON551	pMD1018	A2469	
R231S/R233S/R234S/D242S	ON553	pMD1020	A2470	
R231S/R233S/K239S/D242S	ON554	pMD1021	A2471	
R231S/R234S/K239S/D242S	ON555	pMD1022	A2472	
R233S/R234S/K239S/D242S	ON556	pMD1023	A2473	
R231S/R233S/R234S/K239S/P258L	ON99	pMD1062	A2535	
R231S/R233S/R234S/P258L	ON448[c]	pMD1063	A2536	
R231S/R233S/K239S/P258L	ON449[c]	pMD1064	A2537	
R231S/R234S/K239S/P258L	ON476[c]	pMD1065	A2538	
R233S/R234S/K239S/P258L	ON450[c]	pMD1066	A2539	
R74S	ON568	pMD1067	A2540	
R76S	ON569	pMD1068	A2541	
K77S	ON570	pMD1069	A2542	
K151S	ON571	pMD1070	A2543	
D157S	ON572	pMD1071	A2544	
K160S	ON573	pMD1072	A2545	
R161S	ON574	pMD1073	A2546	
P258L	ON99	pMD1081	A2558	
L236F/D242S	ON612	pMD1115	A2716	
D242S	ON613	pMD1116	A2717	
R231S/R233S/R234S/K239S/S232P	ON614	pMD1118	A2718	
R231S/R233S/R234S/K239S/S232L	ON615	pMD1119	A2719	

[a] The yeast host strain was A575 *MAT**a** ste2*-ΔΔ *far1*-ΔΔ *bar1*$^{-}$ *cry1*^{R} *ade2-1 his4-580 lys2*$_{oc}$ *trp1*$_{am}$ *tyr1*$_{oc}$ *SUP4-3*^{ts} *leu2 ura3 FUS1*::p*[FUS1-lacZ TRP1]*. [b] The yeast host strain was A232 *MAT**a** ste2*-ΔΔ *bar1*$^{-}$ *cry1*^{R} *ade2-1 his4-580 lys2*$_{oc}$ *tyr1*$_{oc}$ *SUP4-3*^{ts} *leu2 ura3 FUS1*::p*[FUS1-lacZ TRP1]*. [c] These mutations were constructed by site-directed mutagenesis using the indicated oligonucleotide in addition to oligonucleotide ON99 specifying the P258L substitution.

of the C5a receptor by Baranski, Bourne, and co-workers (*11*, *28*) and subsequently applied to transmembrane segments of the α-factor receptor (*27*). The approach relies on the generation of a library of mutated receptors that have been randomly mutagenized so as to contain multiple base substitutions per allele. Mutated functional alleles are then identified by screening for signaling function. Screening is conducted using a yeast host strain that contains a *FUS1-lacZ* reporter gene that is transcriptionally induced by the pheromone response pathway such that colonies with functional receptors can be identified on plates containing the chromogenic *β*-galactosidase substrate X-Gal.

A 21-amino acid region of Ste2p from Leu228 to Leu248 (Figure 1) was targeted for random mutagenesis using partially degenerate oligonucleotides designed to contain 20% substituted bases at each position. Mutated plasmids from approximately 20 000 *E. coli* colonies were transformed into a yeast host strain containing a chromosomal deletion of the *STE2* gene. Out of 64 000 yeast colony total transformants, we recovered 91 strains with detectable pheromone-depend-

Table 2: Multicopy Plasmids and Yeast Strains

	plasmid	yeast strain *ste2*-Δ *far1*-Δ[a]	yeast strain *STE2*$^+$ *FAR1*$^+$ [b]
STE2$^+$	pMD1098	A2683	A2698
R231S/R233S/R234S	pMD1099	A2684	A2699
R231S/R233S/K239S	pMD1100	A2685	A2700
R231S/R234S/K239S	pMD1101	A2686	A2701
R233S/R234S/K239S	pMD1102	A2687	A2702
R231S/R233S/R234S/K239S	pMD1103	A2688	A2703
R231S/R233S/R234S/K239S/S232R	pMD1104	A2689	A2704
R76S	pMD1105	A2690	
K77S	pMD1106	A2691	
vector	pMD559		A2705

[a] The yeast host strain was A575 *MAT***a** *ste2*-ΔΔ *far1*-ΔΔ *bar1*$^-$ *cry1*^{R} *ade2-1 his4-580 lys2*$_{oc}$ *trp1*$_{am}$ *tyr1*$_{oc}$ *SUP4-3*^{ts} *leu2 ura3 FUS1*::p*[FUS1-lacZ TRP1]*. [b] The yeast host strain was A230 *MAT***a** *STE2*$^+$ *bar1*$^-$ *cry1*^{R} *ade2-1 his4-580 lys2*$_{oc}$ *tyr1*$_{oc}$ *SUP4-3*^{ts} *leu2 ura3 FUS1*::p*[FUS1-lacZ TRP1]*.

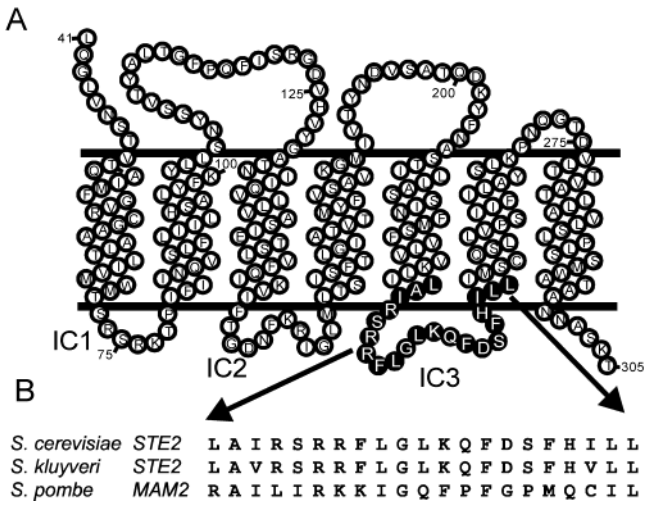

FIGURE 1: (A) Schematic depiction of the predicted topological orientation of the seven transmembrane regions of the Ste2p receptor. The amino acid positions of the third intracellular loop subjected to random saturation mutagenesis are shown as filled circles. (B) Sequence comparison of the third intracellular loop of pheromone receptors from *S. cerevisae*, *Saccharomyces kluyveri*, and *Schizosaccharomyces pombe* (*24*).

ent *FUS1-lacZ* induction following isolation of the plasmid from yeast and retransformation to confirm that the observed phenotype was plasmid-dependent. These are listed in Figure 2. None of the recovered mutant receptors exhibited significant pheromone-independent signaling after retransformation, although we conducted an independent screen for this phenotype (results not shown). This result is consistent with a previous failure to recover constitutive mutations in IC3 of Ste2p (*31*), although constitutive mutations in IC3 of other GPCRs appear to be common (*39*−*41*).

The 91 partially functional receptor alleles contain a total of 350 base substitutions, including 90 substitutions that are translationally silent and 96 unique amino acid substitutions. A total of 78 of the unique amino acid substitutions arose from single base changes, comprising 70% of all possible amino acid substitutions that could have arisen from single base changes.

Once the retransformed plasmids recovered from signaling competent yeast colonies had been retested, 83 alleles containing 201 total and 89 unique amino acid changes exhibited *FUS1-lacZ* induction at a level that was greater than 60% of the wild-type level in the presence of 500 nM α-factor, a concentration of pheromone that would result in maximal activation of normal receptors (Figure 2). This level of induction of the reporter was also used as a cutoff for defining functional receptors in a previous intensive mutagenic analysis (*27*) and was chosen to allow inclusion of receptors that have undergone modest changes in ligand affinity or expression levels within the class of functional alleles. A total of 72 of the recovered functional amino acid substitutions arose from single base substitutions, representing 65% of all possible amino acid substitutions that can arise from single base changes. The requirement for functionality in the screen imposed a significant restriction on the number of recovered single base substitutions, since purely random sampling of the pool of 111 possible single base substitutions resulting in amino acid changes would have been expected to yield 80% of all possible substitutions among the 180 functional single base substitutions that resulted in amino acid changes. This was calculated as $[1 - (1 - 1/111)]^{180}$ (*27*). Furthermore, if sampling of the 111 possible single base changes was random, the odds of recovering only 72 different substitutions in 180 observed changes would be 10^{-34} [calculated as $(72/111)^{180}$]. Given the total of 180 functional substitution events, and the recovery of 72 unique functional single base substitutions, numerical solution of the equation $[1 - (1 - 1/X)^{180} = 72/X]$ was used to determine that the most likely number of different functional amino acid substitutions resulting from single base changes in IC3 is 80. This implies that only 72% of single base substitutions are functional and that we recovered 90% of the functional substitutions, in agreement with an independent calculation presented below.

An average of 2.5 translationally silent mutations was recovered for each possible silent single base substitution that can occur within the targeted sequence. This number can be used to estimate, as described previously (*27*), that the rate of base substitutions in the unselected *STE2* alleles was ∼9%, corresponding to an average of five base substitutions per unselected allele. If certain codons are preferred for *STE2* expression, the calculated mutation rate would actually be higher. There are an average of 3.8 base substitutions for each of the selected partially functional alleles.

The IC3 loop of Ste2p tolerated a wide variety of mutations without diminishing signaling (Figure 2 and Table 3). Aside from positions at the ends of the targeted region, which are mutagenized inefficiently by the procedure we used (*27*), each amino acid position in the loop tolerated at least two different substitutions, eight tolerated at least five substitutions, and one position, R234, tolerated all amino acids that could be introduced via single base substitutions.

Most of the functional alleles recovered from the randomly mutated library contained multiple amino acid substitutions. To conclude that a particular substitution recovered in one of these alleles actually does not affect receptor function, it is necessary to make the assumption that the effects of multiple substitutions are additive and not due to intragenic interactions between multiple mutations in the allele. Additivity of mutational effects is, in most cases, confirmed by recovery of a given substitution in different mutational contexts in different alleles. Furthermore, intragenic sup-

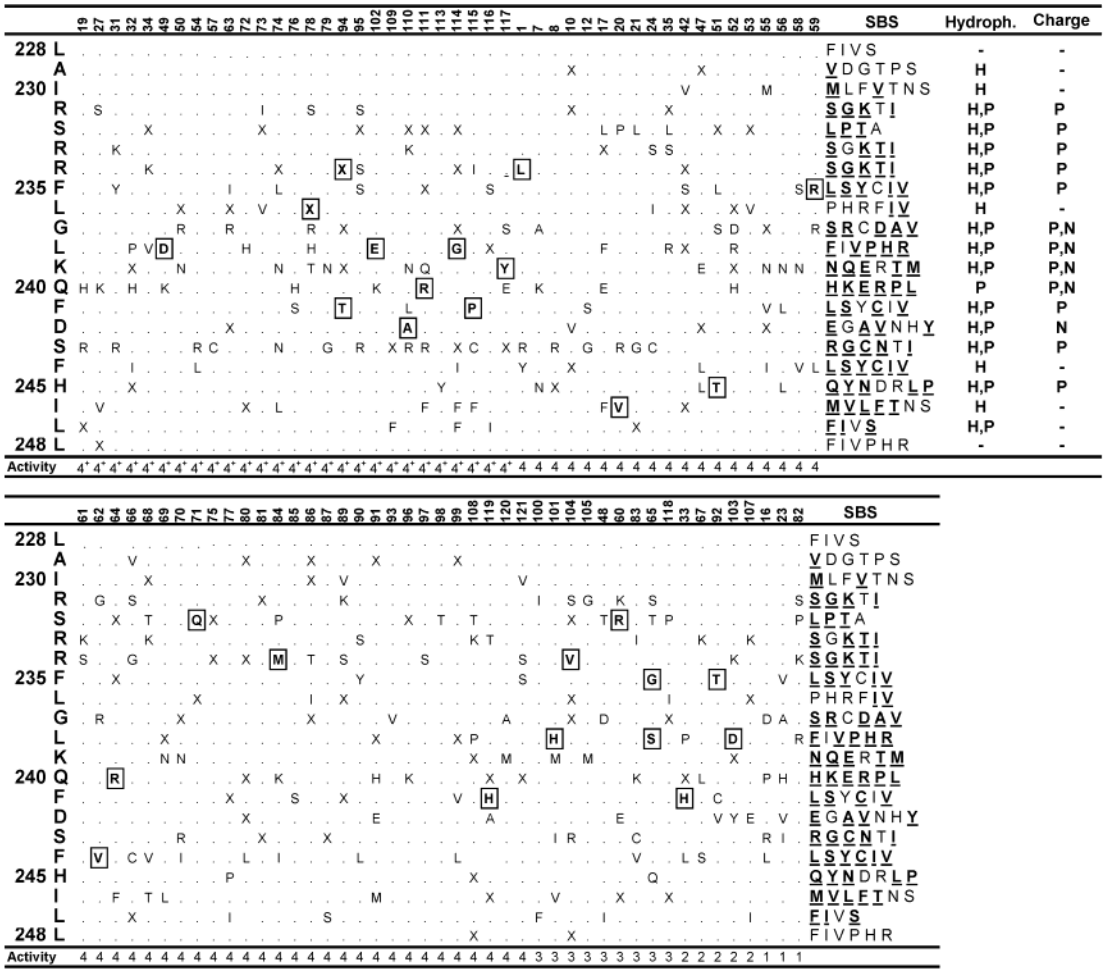

FIGURE 2: Library of recovered alleles in IC3 of Ste2p. Allele numbers are displayed across the top row of the table, listed in approximate order of activity. The wild-type sequence is displayed along the left-hand side. Amino acid substitutions for each allele are displayed using the single-letter notation in the columns under the allele numbers. The letter X denotes sites of translationally silent substitutions. Amino acid substitutions resulting from multiple base changes are shown in boxes. The column labeled SBS indicates the amino acid substitutions that can be introduced through single base substitutions at each position in the sequence. In this column, all the substitutions that were actually recovered, including both functional (at least 60% *FUS1-lacZ* induction) and partially functional (less than 60% *FUS1-lacZ* induction), are indicated in bold and underlined. The column labeled Hydroph. indicates with the letter H positions where only hydrophobic residues were recovered among functional alleles and with the letter P positions where only polar residues were recovered in functional alleles (see the text for details). Dashes in this column indicate positions at the ends of the mutagenized regions where there were insufficient data for assigning a category. The column labeled Charge indicates whether a given position tolerates introduction of potentially charged amino acids into functional receptors. P indicates positions where positively charged residues are tolerated. N indicates positions where negatively charged residues are tolerated. The numbers 0−4 in the row labeled Activity at the bottom indicate the relative level of signaling by the mutant receptors. The recovered alleles were assigned values of 0−4 on the basis of their levels of *FUS1-lacZ* induction in response to 500 nM α-factor following retransformation. A value of 0 indicates 0−20% of the wild-type activity. A value of 1 indicates 20−40% activity. A value of 2 indicates 40−60% activity. A value of 3 indicates 60−80% activity. A value of 4 indicates 80−110% activity. Alleles with greater than 110% of the wild-type activity were designated 4^{+}.

pression events have previously been found to be rare in regions of the *STE2* gene corresponding to transmembrane segments (*34*).

In a random mutational study such as we have conducted, it is difficult to completely saturate all possible amino acid or base substitutions. Thus, failure to recover a particular substitution does not constitute proof that a given change is incompatible with function. In several cases (see below), we have verified that substitutions of certain residues are not compatible with pheromone signaling function by directly testing changes introduced by site-directed mutagenesis. Otherwise, the significance of the failure to recover an allele must be compared with a statistical calculation of the likelihood of failing to recover the allele due to incomplete sampling of the library. For the mutational library we created, containing an average of 2.5 examples of each possible base substitution (based on recovery of translationally silent mutations), the probability of failing to recover any particular single base change based due to incomplete sampling is 8%, calculated as described previously (*27*). This agrees well with the above estimate of 90% efficiency of recovery of functional mutations derived from statistical considerations without reference to the silent mutations.

Following previous intensive mutagenic studies of receptors (*11*, *27*, *28*), we have applied a hydrophobicity scale for amino acids based on their free energies of transfer from water to octanol (*42*). In this scheme, amino acids with free energies of transfer from water to octanol of greater than 0.1 kcal/mol (Ala, Ile, Leu, Met, Phe, Pro, Thr, Trp, Tyr, and Val) are considered hydrophobic. Residues Arg, Asn, Asp, Gln, Glu, Lys, and Ser are classified as polar. Cys, Gly, and His are considered either polar or hydrophobic.

Table 3: Distributions of Recovery of Amino Acids

amino acid	no. of positions[a]	percentage of positions[b]	percentage of positions accessible by single base change[c]
alanine	3	16	67
arginine	9	48	43
asparagine	3	16	50
aspartic acid	3	16	33
cysteine	2	10	40
glutamine	4	21	100
glutamic acid	4	21	100
glycine	6	32	50
histidine	4	21	50
isoleucine	10	53	80
leucine	10	53	75
lysine	5	26	100
methionine	4	21	100
phenylalanine	6	32	60
proline	4	21	50
serine	10	53	54
threonine	6	32	56
tyrosine	4	21	60
valine	10	53	82

[a] This column represents the number of positions at which each amino acid was recovered (excluding the ends) in functional alleles. [b] This column represents the percentage of all 19 mutagenized non-end positions at which the indicated amino acid was recovered in functional alleles. [c] The percentage of positions accessible by single base change was calculated by dividing the number of positions at which the amino acid was introduced with a single base change by the total number of positions where that amino acid could have been introduced with a single base change in 19 non-end positions.

Both hydrophobic and polar amino acids were recovered among the functional alleles at 13 of the 18 non-end positions in the targeted region. Hydrophobic residues were tolerated in the functional alleles at all but one position (Q240). The hydrophobic substitutions Q240L and Q240P at this position were recovered only among alleles with impaired signaling, reinforcing the conclusion that it is important to maintain a polar residue at this site. Given the above estimate of an 8% chance of failure to recover any particular single base substitution, the likelihood of failing to recover either Q240L or Q240P is 0.6%. At five positions in IC3, we recovered only hydrophobic amino acids in the functional mutants, although polar substitutions could have been introduced by single base substitutions at each of these positions (Figure 2). Thus, the IC3 loop of the receptor, which is presumed to be exposed to the cytoplasm, seemed to be more tolerant of hydrophobic than of polar substitutions.

The ranges of radii of gyration of the amino acid side chains recovered among the functional IC3 substitutions, defined according to Levitt (*43*), are shown in Figure 3. Many positions could tolerate both glycine and arginine, corresponding, respectively, to large decreases and increases in the sizes of the side chains compatible with function. One exception was L236. The only amino acids we observed at this position were isoleucine and valine, which have radii similar to that of leucine, although substitution of proline, histidine, arginine, or phenylalanine could also have arisen by single base substitutions in the codon for L236. The probability of failing to recover any of these four possible single base changes at position 236 is 4×10^{-5}. To test the importance of side chain size at this position, we created four additional mutants (L236A, L236F, L236W, and L236H) by site-directed mutagenesis and tested these for

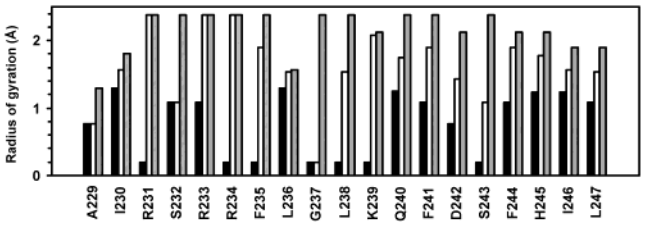

FIGURE 3: Changes in radii of gyration of residues in IC3 of Ste2p observed in functional alleles. Side chain radii were obtained from ref *43*. We arbitrarily assigned a radius of gyration of 0.2 Å to the side of glycine for visualization purposes only. For every non-end mutagenized position, the black bar indicates the smallest side chain radius of gyration recovered in a functional allele, the white bar indicates the native residue radius of gyration, and the gray bar indicates the radius of gyration of the largest side chain recovered in a functional allele.

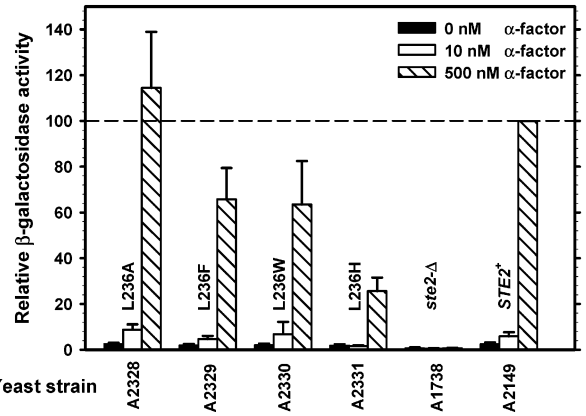

FIGURE 4: Effects of L236 mutations on *FUS1-lacZ* induction. Liquid β-galactosidase assays were conducted in the absence of α-factor and in the presence of 10 and 500 nM α-factor as described in Experimental Procedures.

function (Figure 4). The allele containing L236A has approximately the same maximal response (to 500 nM α-factor) as the normal receptor, while the three other substitutions exhibit less than 65% of this response. Thus, large hydrophilic or hydrophobic substitutions at this position appear to be detrimental for receptor biosynthesis or function, consistent with the failure to recover phenylalanine and histidine at this position among the random mutations. However, a small hydrophobic substitution (L236A) can be tolerated with no loss of function.

A total of 27 of the 80 functional alleles exhibited maximal levels of pheromone response that were more than 30% greater than those seen with the wild-type receptors. This result is consistent with previous findings (*24*−*26*), suggesting that Ste2p contains significant negative regulatory regions whose removal enhances the response to ligand.

All but one of the mutagenized amino acid residues (excluding end positions) could undergo nonconservative amino acid substitutions without suffering the loss of function, where nonconservative substitutions are defined as those with a log odds score of 1.0 or greater in the PAM 250 evolutionary scoring matrix of amino acid substitutions (*11*, *27*, *44*). The one exception was I230. At this position, valine and methionine were the only substitutions recovered in functional alleles. Leucine, phenylalanine, and threonine could have been introduced at position 230 via single base substitutions, maintaining the hydrophobic character of the position; however, these mutations were not recovered. This

indicates that steric interactions of this residue may be critical for receptor folding or function. On the basis of random mutagenesis, L236 also had initially appeared to be a conserved position, capable of being replaced only with isoleucine and valine. However, as described above, the site-directed substitution L236A, which is nonconservative according to the PAM 250 matrix, exhibited near-normal function.

Role of Positively Charged Residues in Intracellular Loops of Ste2p. There are several indications from results of random mutagenesis that the overall positive charge of IC3 may play an important role in pheromone signaling by the α-factor receptor.

(1) Amino acid residues with charged side chains were recovered at a large fraction of all mutagenized positions; however, no single charged group appeared to be essential for signaling. Charged residues were recovered in functional alleles via single or multiple base substitutions (or were pre-existing) at 13 of the 19 non-end mutagenized positions in IC3 (see the right-hand column of Figure 2, top). Residues with charged side chains could have been introduced by single base mutations at 12 positions. Excluding the codons at the ends of IC3, we failed to recover only two possible single base changes encoding amino acids with side chains that can adopt a negative charge (A229D and H245D) and only five single base changes encoding amino acids with side chains that can adopt a positive charge (L236H, L236R, K239R, D242H, and H245R). At two of these positions, A229 and L236, we failed to recover any polar substitutions, indicating that the positions are generally intolerant of polar, as opposed to charged, residues. Two of the substitutions we failed to recover (H245D and D242H) could have introduced changes of two units in charge, depending on the state of ionization of histidine. The remaining two missing substitutions, K239R and H245R, may be cases where substitution of one positively charged group for another is not tolerated because of packing constraints. Introduction of lysine residues seemed particularly well-tolerated, as lysine was recovered at all four positions where it could be introduced by a single base substitution (Figure 2). Arginine substitutions were less uniformly tolerated in functional receptors, showing up at 9 of the 19 non-end positions, including four of the seven positions where arginine could arise by single base mutations.

(2) Approximately half of all alleles that contained substitutions that altered the charge of particular positions in IC3 also contained a charge-compensating additional mutation elsewhere in IC3.

To test the importance of positively charged residues on the intracellular face of the α-factor receptor, we used site-directed mutagenesis to replace each lysine and arginine residue in the predicted intracellular loop regions with serine. This substitution was employed to maintain the polar character of the positions without introducing bulky groups that could be difficult to fit into the site of the removed charged side chain. Substitution of serine for R233, R234, or K239 in IC3 resulted in 20−30% decreases in the level of *FUS1-lacZ* induction in response to 500 nM α-factor (Figure 5). This modest level of impairment is consistent with recovery of functional random mutants in which these charges had been altered (Figure 2). However, the R231S substitution caused an approximately 50% decrease defect

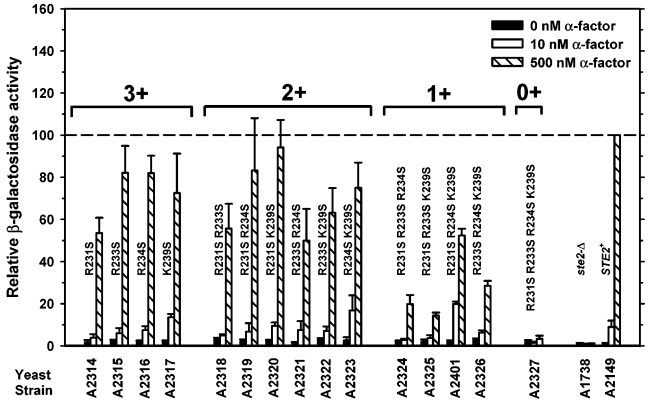

FIGURE 5: Effects of substitutions of serine for positively charged residues in IC3 on *FUS1-lacZ* induction (*CEN* plasmids). Liquid *β*-galactosidase assays were conducted in the absence of α-factor and in the presence of 10 and 500 nM α-factor as described in Experimental Procedures. Results were normalized by setting the wild-type receptor strain A2149 *β*-galactosidase activity to 100% at 500 nM α-factor.

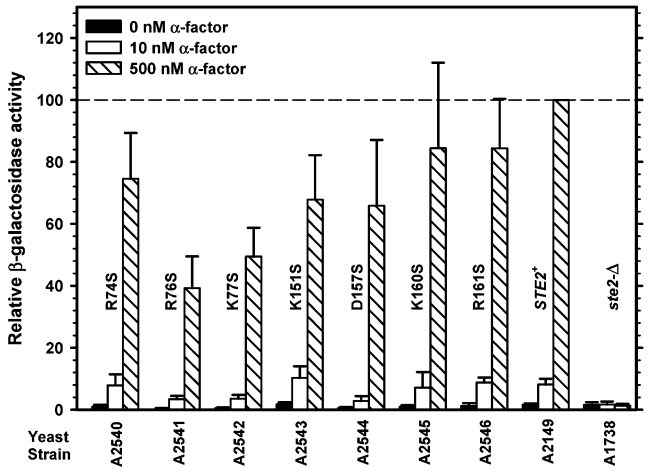

FIGURE 6: Effects of substitutions of serine for positively charged residues in IC1 and IC2 on *FUS1-lacZ* induction (*CEN* plasmids). Liquid *β*-galactosidase assays were conducted in the absence of α-factor and in the presence of 10 and 500 nM α-factor as described in Experimental Procedures.

in signaling competence. While R231S was recovered six times among the functional alleles in the random library, five of these six substitutions were found in combination with additional mutations that introduce an additional positive charge (alleles 27, 78, 95, and 104) or that remove a second positive charge at position R234 or K239 (alleles 66, 95, and 104). As shown in Figure 5, site-directed double substitutions combining R231S with either R234S or K239S exhibit better signaling than R231S alone. The single R231S-containing allele in Figure 2 that did not contain an additional mutation affecting charge also did not exhibit a full pheromone response (allele 65, activity of 3).

As was observed for most residues in IC3, substitution of serine for individual positively and negatively charged amino acids in IC2 caused decreases of less than ∼30% in maximal *FUS1-lacZ* responses to α-factor (Figure 6). This was expected on the basis of the observation of normal signaling by receptors containing deletions of IC2 (*23*). However, substitution of serine for positively charged residues R76 and K77 in IC1 caused a decrease of more than 50% in the maximal response to α-factor in receptors expressed from

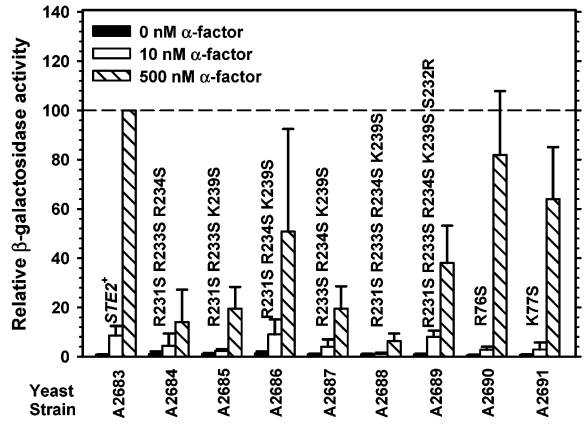

FIGURE 7: Effects of substitutions of serine for positively charged residues in IC1 and IC3 on *FUS1-lacZ* induction (multicopy plasmids). Liquid β-galactosidase assays were conducted in the absence of α-factor and in the presence of 10 and 500 nM α-factor as described in Experimental Procedures.

CEN plasmids (Figure 6). This is most likely caused by a decrease in the number of receptors on the cell surface, because *FUS1-lacZ* induction could be restored to near-normal levels by introducing the mutant alleles into multicopy plasmids (Figure 7). No comparable restoration of signaling was seen upon multicopy expression of mutants with charge substitutions in IC3 (Figure 7 and see below). The decrease in the level of cell surface expression of the IC1 mutants must reflect deficiencies in targeting to the plasma membrane or an increased rate of turnover of cell surface receptors, because overall cellular levels of the mutant receptors expressed from *CEN* (results not shown) or multicopy (Figure 8, lane 10) plasmids are essentially the same as for normal alleles.

Removal of increasing numbers of positive charges from IC3 progressively decreased the maximal response to α-factor as measured by levels of *FUS1-lacZ* induction, regardless of which positive charges were removed (Figure 5). With the exception of single and double substitutions involving R231 (see above), alleles lacking one and two charges exhibited modest impairment of signaling, whereas alleles lacking three of the positive charges exhibit severely compromised signaling, and an allele lacking all four positive charges exhibits no detectable signaling activity. (Because of uncertainty over whether the histidine residues such as H245 would be ionized in cells, we did not include this position in this analysis of charged residues.) Halo assays of α-factor-induced cell cycle arrest in strains expressing receptors with the serine substitutions were consistent with the general progressive loss of function observed in assays of *FUS1-lacZ* induction (results not shown). Strains expressing receptors with any three positive charges remaining in IC3 exhibited nearly normal halos; strains expressing receptors with two positive charges removed from the loop had very turbid halos especially in the presence of small amounts of α-factor, and strains expressing receptors lacking three or all four of the positive charges failed to exhibit any growth arrest even in the presence of 300 ng of pheromone.

The dependence of signaling efficacy on the number of positively charged residues in IC3 suggests that the overall positive charge of this region of the receptor for the pheromone response is important, but that there is redundancy, so that no particular positively charged residue is

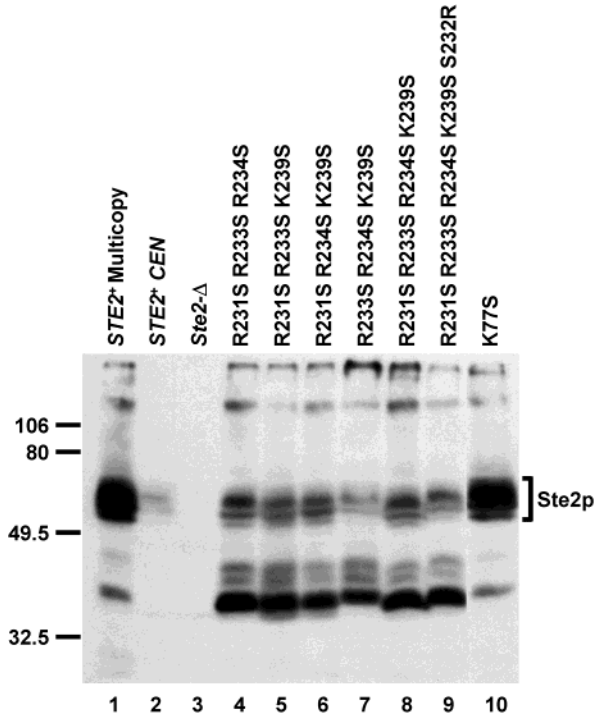

FIGURE 8: Immunoblot analysis of total cellular levels of wild-type and mutant Ste2p receptors. Extracts derived from 3×10^6 cells expressing the indicated *STE2* wild-type and mutant alleles were loaded on each lane of a 12% SDS−polyacrylamide gel. The mutant alleles were encoded on multicopy plasmids in strains listed in Table 2. Blots were developed using anti-c-myc antibodies as described in Experimental Procedures. The migration of standard proteins with the indicated molecular weights is shown on the left-hand side of the figure. The migration of the family of species corresponding to full-length differentially glycosylated Ste2p (*45*) is shown at the right.

required. To test this idea, we asked whether introduction of positively charged residues at new, non-native, positions could restore signaling to receptors lacking all four of the lysine and arginine residues in IC3. Thus, alleles containing the combined R231S, R233S, R234S, and K239S substitutions were further altered by introducing all possible combinations of S232R, G237R, and S243R substitutions. These three sites were chosen for introduction of positive charges because functional alleles with arginine at these positions had previously been recovered from the random mutational libraries (Figure 2). One of these introduced charges, the S232R substitution, restored *FUS1-lacZ* induction at 500 nM α-factor to more than 40% of the wild-type level (Figure 9). This effect was specific for introduction of charge at this position, as substitution of proline or leucine for S232 did not lead to any restoration of signaling in this background (results not shown). However, introduction of positive charges at positions 237 and 243 did not restore signaling to receptors lacking the four normal IC3 positive charges, and combination of the G237R and S243R substitutions with S232R diminished the level of signaling conferred by the S232R substitution.

To further test the idea that there is a generalized requirement for positive charges within the amino-terminal region of IC3, we examined the effects of adding negatively charged residues in this region using the G237D, L238D, and Q240E substitutions, which had been previously isolated among the randomly mutated alleles. When these mutations

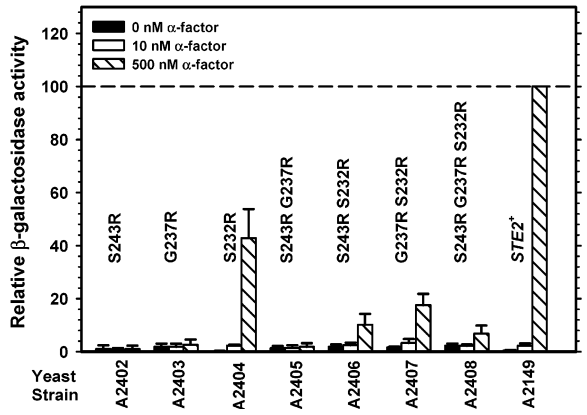

FIGURE 9: Effects of introduction of arginine substitutions into non-native positions in an IC3 loop lacking the all normal positively charged residues. All possible combinations of S243R, G237R, and S232R were introduced into mutant alleles that also lack all four native positive charges in IC3. Thus, in addition to the substitutions indicated in the figure, all alleles except for the *STE2*$^{+}$ control also contained R231S, R233S, R234S, and K239S substitutions. Liquid *β*-galactosidase assays were conducted in the absence of α-factor and in the presence of 10 and 500 nM α-factor as described in Experimental Procedures.

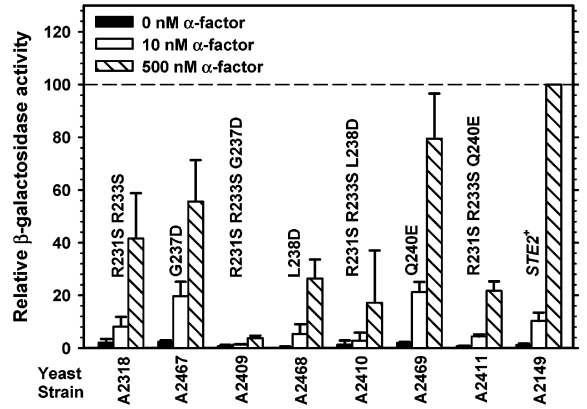

FIGURE 10: Effects of introduction of negative charges in IC3 on *FUS1-lacZ* induction. The negatively charged substitutions G237D, L238D, and Q240E were introduced into both the wild-type *STE2* and a mutant Ste2p receptor containing R231S and R233S removing two of the positively charged residues normally present in IC3. Liquid *β*-galactosidase assays were conducted in the absence of α-factor and in the presence of 10 and 500 nM α-factor as described in Experimental Procedures.

were introduced into wild-type *STE2* alleles, they decreased the maximal level of *FUS1-lacZ* induction by 45, 75, and 20%, respectively (Figure 10). The small decrease caused by Q240E is consistent with recovery of multiple fully functional alleles containing this substitution from the random libraries (Figure 2). The larger decrease caused by G237D is consistent with the assignment of an activity of 3 for signaling exhibited by allele 48 (Figure 2), which contains no other charge alterations. On the other hand, allele 52, with an activity of 4, also contains the G237D mutation, but in this case, it is accompanied by the L238R and Q240H substitutions, which could be compensating for the increased negative charge. The significant decrease in the level of signaling resulting from the site-directed substitution L238D also provides an interesting comparison with the random mutational results. Negatively charged substitutions at this position were recovered in two functional alleles (49 and 102 in Figure 2.), but each of these alleles also contains the same compensatory mutation, Q240K, suggesting the existence of a direct interaction between residues 238 and 240.

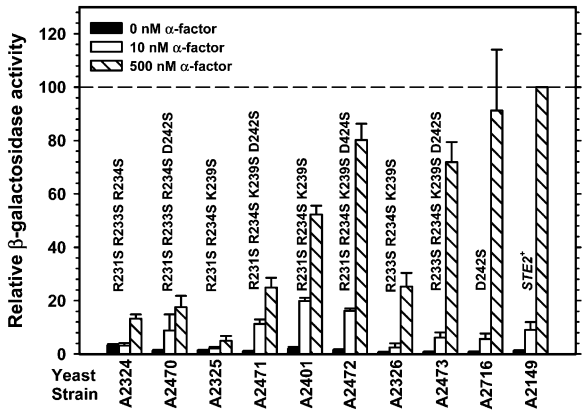

FIGURE 11: Restoration of *FUS1-lacZ* induction in defective alleles of *STE2* by removal of a negatively charged group in IC3. Liquid *β*-galactosidase assays were conducted in the absence of α-factor and in the presence of 10 and 500 nM α-factor as described in Experimental Procedures.

The effects of introducing negative charges in IC3 were also tested in the absence of the normal redundant positive charges. The site-directed substitutions G237D, L238D, and Q240E were introduced into receptors that also contained R231S and R233S substitutions. Compared with signaling by the receptor containing just the R231S and R233S substitutions, introduction of the G237D, L238D, and Q240E substitutions resulted in even greater reductions (95, 85, and 80% of the wild-type level, respectively; see Figure 10) in the level of α-factor-dependent *FUS1-lacZ* induction.

We also tested the role of charge in IC3 of Ste2p by removing the only negatively charged residue in the loop through the site-directed substitution D242S. This particular mutation was not recovered from the random mutational libraries, as it was not accessible via a single base mutation, although alleles containing D242A were fully functional (Figure 2) and site-directed introduction of D242S into an otherwise normal receptor resulted in nearly full signaling function (Figure 11). Introduction of D242S into receptor alleles lacking three of the four normal positive charges in IC3 improved the maximal *FUS1-lacZ* responses to pheromone by factors ranging from 1.3 to 5.1, depending on which positive charges were present (Figure 11). The most likely explanation of these improvements is that removal of the negative charge represents a general increase in the net charge of IC3. An alternative explanation could have been that particular positive charges in IC3 are normally involved in intramolecular electrostatic interactions with D242. Removal of positive charges would leave the unpaired negative charge that would interfere with receptor structure or function. Removal of the remaining unpaired negative charge would then be expected to restore signaling. However, if this were the case, we would expect that in a receptor containing all four normal positive charges, the D242S substitution would leave unpaired positive charges with a detrimental effect on signaling, which is not seen in Figure 11.

Mechanism of the Dependence of Signaling on IC3 Charged Groups. Alteration of pheromone responses by mutations in receptors could take place by altering various processes affecting receptor abundance or function. These include ligand binding, activation of receptors, interaction

with G protein, desensitization, and receptor synthesis, subcellular targeting, or degradation. The following evidence indicates that the charge-dependent effects of the IC3 mutations are mediated primarily by alterations in the efficiency of receptor activation, interaction with G protein, or desensitization, rather than by a decrease in the levels of receptors in cells, even though a decrease is observed. This is in contrast to the signaling defect caused by the R76S and K77S mutations in IC1, where the mutants are expressed at reduced levels but overexpression of mutant alleles provided a restoration of signaling (see Figures 6−8).

To compare levels of expression of normal and mutant receptors, we performed immunoblots of total cell extracts using an antibody directed against the myc epitope tag at the COOH terminus of each of the *STE2* alleles. When the signaling-compromised mutant receptors with charged groups removed from IC3 were expressed from single-copy plasmids, cellular levels were lower than those seen for wild-type *STE2* expressed from similar plasmids (results not shown), suggesting the possibility that a diminished level of signaling was the result of cells expressing fewer receptors. However, this appears not to be the case for the following reasons.

(1) Expression of the mutant receptors from multicopy plasmids increased cellular levels of the mutant receptors to the point where they were present in greater abundance than wild-type alleles expressed from single-copy plasmids (Figure 8); however, the overexpressed mutant receptors exhibit signaling defects similar to those seen when they are expressed in a single copy (compare Figures 5 and 7). Expression of the mutant receptors from multicopy plasmids leads to the appearance of fast-migrating bands on immunoblots that presumably represent proteolytic products resulting from removal of the amino terminus of the receptor (Figure 8). However, the signaling defects are not correlated with this proteolysis because increased levels of the products are not generally seen when these same mutant alleles are expressed from *CEN* plasmids (results not shown).

(2) Pheromone responses mediated by α-factor receptors are remarkably insensitive to changes in levels of receptor expression, including significant decreases in receptor levels (*30*).

(3) The defects in signaling exhibited by the overexpressed receptors are not due to detrimental effects of overexpression of receptors, as high-level expression of these alleles does not lead to any dominant negative effects on signaling by a coexpressed normal receptor. Figure 12 shows that *FUS1-lacZ* induction by a yeast host strain with a normal chromosomal *STE2* locus is approximately the same in the presence of multicopy plasmids encoding receptors with IC3 substitutions as it is in the presence of the vector alone. The lack of dominant negative effects of the overexpressed receptors rules out the possibility that proteolytic products resulting from overexpression of mutant receptors (described above; see Figure 8) are interfering with signaling by intact receptors.

(4) On the basis of the binding of [^{3}H]-α-factor to intact cells, mutant receptors lacking three positive charges in IC3, when expressed from multicopy plasmids, are capable of binding ligand and are present at the cell surface at abundances that are at least as great as the abundance of normal receptors expressed from *CEN* plasmids (Table 4).

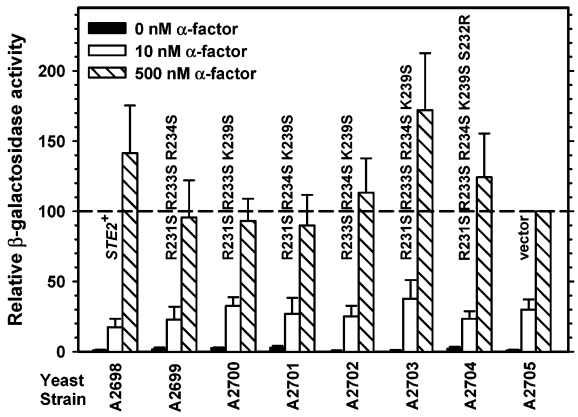

FIGURE 12: Lack of dominant negative effects of IC3 substitutions on *FUS1-lacZ* induction. Multicopy plasmids encoding receptors with serine substituted for positive charges in IC3 as indicated in the figure were transformed into the yeast host strain A232, which contains a chromosomal copy of *STE2*. Liquid β-galactosidase assays were conducted in the absence of α-factor and in the presence of 10 and 500 nM α-factor as described in Experimental Procedures.

Table 4: Ligand Binding to Receptors with Mutations in IC3

strain	IC3	B_{max}[a] (no. of receptors/cell)	K_d[a] (nM)
A2149	*STE2*$^+$ *CEN*	11 000 ± 800	16 ± 3
A2683	*STE2*$^+$ multicopy	189 000 ± 7000	76 ± 4
A2684	R231S/R233S/R234S multicopy	15 000 ± 4000	10 ± 7
A2685	R231S/R233S/K239S multicopy	26 000 ± 5000	79 ± 24
A2686	R231S/R234S/K239S multicopy	11 000 ± 1000	14 ± 4
A2687	R233S/R234S/K239S multicopy	74 000 ± 6000	65 ± 8
A2688	R231S/R233S/R234S/K239S multicopy	ND[b]	ND[b]

[a] The indicated values are the weighted averages of two determinations, each performed in triplicate. In averaging, the applied weighting was the inverse of the standard deviation of the fit of each experiment to a theoretical binding curve. [b] The number of bound counts for these samples was too low to allow reliable fitting to the binding equation.

However, the mutant allele lacking four positively charged residues binds ligand poorly and may be present at the cell surface in levels that are insufficient for efficient signaling. The receptors expressed at the highest abundances exhibit dissociation constants for ligand that are higher than those observed for receptors expressed at normal levels. This may reflect lower-affinity binding by excess receptors that fail to associate with the substoichiometric levels of G protein.

(5) When the charge substitutions causing reduced levels of α-factor signaling were introduced into an *STE2* allele exhibiting constitutive activation of the pheromone response, they significantly reduced the basal level of signaling seen in the absence of pheromone (Figure 13). The substitution P258L is the most strongly constitutively activating single amino acid substitution in *STE2* isolated to date (*31*, *46*, *47*). Since constitutive signaling does not depend on ligand binding, the fact that the charge substitutions inhibit constitutive signaling in alleles containing P258L suggests that they affect a step in signal transduction that is downstream from binding of the ligand to the receptor. However, it is also possible that the IC3 mutations interfere with constitutive signaling by reducing the level of cell surface expression of receptors below the already-low levels seen for alleles containing the P258 substitution alone (*31*, *46*, *47*). The level of pheromone-dependent signaling by receptors containing

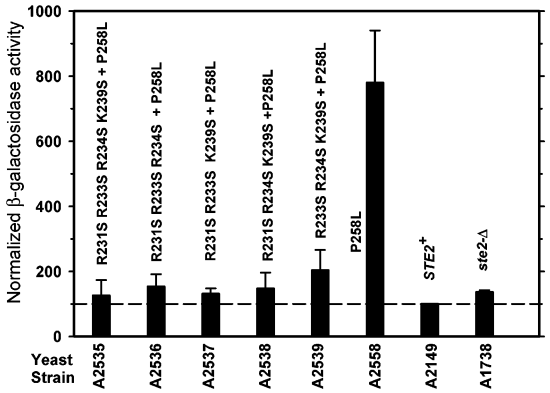

FIGURE 13: Effects of substitutions in IC3 on basal *FUS1-lacZ* induction by a constitutively activating mutation in *STE2*. IC3 mutations were combined in the same allele as the constitutively activating P258L mutations, as described in Experimental Procedures. Liquid *β*-galactosidase assays were conducted in the absence of α-factor as described in Experimental Procedures. The level of ligand-independent signaling by normal *STE2*$^+$ receptors was defined as 100%.

the P258L substitution was also decreased by removal of positively charged residues (results not shown).

DISCUSSION

The results of both the random and site-directed mutational studies reported here support the idea that the IC3 loop of the α-factor receptor plays an important role in signal transduction by the pheromone-bound receptor. Analysis of the random mutants demonstrates the existence of many mutations in the region that interfere with receptor biosynthesis, stability, or function. Although the IC3 loop is presumed to be exposed at the membrane surface, more positions were intolerant of polar substitutions than was the case when a similar analysis was applied to some transmembrane segments (*27*). A previous mutagenic study found an important role for the hydrophobicity of IC3 of *β*-adrenergic receptors in G protein activation (*48*).

Site-directed amino acid substitutions in IC3 that reduce the overall positive charge cause defects in the response to α-factor even when the relevant alleles are expressed from multicopy plasmids and are present at the cell surface and able to bind ligand. However, there is a high degree of functional redundancy in the amino acid sequence of IC3. Removal of only one to two positive charges leads to modest decreases in the pheromone response, whereas removal of three to four charges causes a nearly complete loss of function. Every individual amino acid in this region can be changed to at least two other types of residues, and in no case does the spectrum of allowed substitutions seem to be restricted to highly related amino acid types.

These observations extend previous analyses of IC3 of Ste2p. Clark et al. performed alanine scanning mutagenesis on a region of IC3 and found that introduction of a single alanine at any of 13 positions in the loop had little effect on receptor function (*26*). Furthermore, almost all the double alanine substitutions they tested retained significant signaling function. In the random mutational analysis of IC3 presented here, we recovered alanine in functional alleles at two positions (G237 and D242) where this residue could be introduced by single base substitution, and failed to recover alanine at one additional position (S232) where it could have arisen by single base substitution and where several other hydrophobic residues were recovered. Clark et al. also randomized codons 232−234 of Ste2p and found that most of a group of randomly chosen alleles were functional. Among the functional alleles, they observed a preference, but not an absolute requirement, for a positively charged residue at position 234. In agreement with the current findings, in most cases, Clark et al. found that competence for signaling did not seem to correlate with the level of expression at the cell surface. Most of the defective receptors they identified were expressed at the cell surface in moderately reduced amounts and bound ligand with normal or greater affinity. However, many alleles that signaled normally were also present in reduced amounts at the cell surface. A large fraction of the alleles they isolated exhibited hypersensitivity to pheromone.

Weiner et al. previously screened random mutations in IC3 of Ste2p for alleles that exhibited a reduced level of signaling while maintaining a high number of pheromone binding sites on the cell surface (*24*). They recovered the L236R and L236H single mutations and the R233S/R234G double mutation. As expected, the L236R and L236H substitutions were not recovered among the functional alleles in the current study, and the L236H substitution, when re-created by site-directed mutagenesis, exhibited a significant loss of function. Both individual components of the R233S/R234G double mutation of Weiner et al. were recovered among the functional alleles in the study presented here, consistent with the previous report that both substitutions had to occur simultaneously for a loss of function. None of the functional alleles we recovered from the random mutagenesis contained simultaneous substitution of uncharged residues at positions 233 and 234. Substitution of serine for both these arginines by site-directed mutagenesis resulted in a 50% decrease in the level of *FUS1-lacZ* induction in response to 500 nM α-factor. Another previous study screened for mutations that render the α-factor receptor hypersensitive to pheromone from a pool of receptors that were randomly mutagenized in the IC3 loop and recovered three point mutations conferring this phenotype: G237R, G237A, and K239N (*25*). All of these substitutions were among the functional substitutions recovered in our study from among the randomly mutagenized library.

IC3 regions of various mammalian receptors have been implicated in G protein coupling (see refs *1* and *49*). Clusters of basic residues in IC3s have been shown to be important for determining G protein coupling specificity (*50*) or for actual G protein activation (*51*). The α_{1b}-adrenergic receptor contains 23 basic residues in intracellular loop regions. When Greasley et al. substituted glutamic acid at each of these sites, they found only two positions, R254 and K258, where this conversion from positive to negative charge resulted in a significant loss of signaling function. Introduction of alanine at these two positions caused less severe defects than the introduction of glutamic acid; however, signaling by the double alanine substitution was still very impaired. As we observe with the α-factor receptor, removal of these two positive charges in the α_{1b}-adrenergic receptor caused a modest reduction in the level of cell surface expression of receptors; however, this was insufficient to explain the signaling defect, as other mutant receptors with lower levels of expression were not impaired for signaling (*51*). As

indicated in Figure 1, the clustering of positively charged groups in IC3 is maintained in the *MAM2* receptor from the yeast *Schizosaccharomyces pombe*, even though the exact location of the charged residues is altered.

Cytoplasmic regions of receptors are responsible both for determining the specificity of the G protein interaction and for actually activating the G proteins. The roles of sequences in intracellular regions of GPCRs in determining the specificity of receptor−G protein coupling have previously been examined in a number of cases by interchanging intracellular loops of different receptors that normally interact with different G proteins. However, these two functions could be mediated by different sequences in receptors (*52*). Thus, identification of mutations affecting G protein activation, as performed in the current study, may serve to identify sequences responsible for activation, as distinguished from regions determining coupling specificity.

Redundancy in mechanisms of responding to pheromone may confer an evolutionary advantage for a rapidly reproducing species such as *S. cerevisiae*, allowing it to avoid mutations that cripple the ability of cells to detect mating partners. However, the lack of a rigorous sequence requirement in IC3 must also reflect a generalized mechanism of G protein activation that depends on conserved structural properties rather than a linear sequence, given the interchangeability of unrelated receptors in their abilities to activate G proteins. The IC3 loop of any given GPCRs may be capable of adopting a variety of conformations that allow it to interact with a variety of G protein partners (*1*). This is consistent with the observation that the IC3 loop was disordered in the recent crystal structure of rhodopsin (*6*). Conservation of structural patterns may have been exploited during evolution to allow the two receptors for the mating-type specific yeast pheromones, which exhibit almost no sequence similarity, to activate the same G protein. Direct involvement in the mating process, coupled with the high reproductive rate of yeast, may have allowed rapid co-evolution of divergent receptor−pheromone pairs, while maintaining the crucial interaction with the G protein. Attempts to create a functional chimeric receptor containing the IC3 region of Ste3p inserted into an otherwise normal Ste2p have, so far, been unsuccessful (S. M. Connelly, J. Yuan, F. X. Ding, F. Naider, J. M. Becker, and M. E. Dumont, manuscript submitted for publication), suggesting that IC3 cannot act as a completely autonomous unit, for either protein folding or G protein activation.

The results presented here provide the following evidence for the importance of the net charge of IC3 for receptor function. (1) Competence for signaling decreases progressively as increasing numbers of positive charges are removed from the loop. (2) Introduction of additional negative charges in IC3 has a detrimental effect on signaling. (3) Removal of the normal negatively charged residue D242 from IC3 improves signaling by partially defective receptors. (4) Introduction of a positive charge at a particular non-native position in IC3 enhances signaling by receptors that lack all the normal positive charges. Introduction of positive charges at two other non-native positions failed to enhance signaling by receptors lacking the positive charges, presumably because these positions could not be correctly placed to participate in the relevant electrostatic interactions.

There could be several explanations for the importance of positively charged groups in pheromone signaling. (1) Positively charged groups in the IC3 loop could interact with negatively charged groups on the surface of the G protein that faces the membrane. The regions of trimeric G proteins that have been most directly implicated in interacting with receptors are the carboxyl and amino termini of Gα subunits (*4*, *53*−*59*). In the case of the yeast Gα, Gpa1p, the carboxyl-terminal region contains negatively charged groups at residues D446 and D459 and the carboxyl terminus itself. However, replacement of D446 or D459 with alanine does not appear to affect signaling (*60*). The amino-terminal region contains negatively charged groups at residues D12, D15, D25, E28, E34, D38, E41, and E51. The importance of these charges for signaling has not yet been determined. (2) Positively charged groups in IC3 could be involved in interactions with negatively charged groups elsewhere on the cytoplasmic face of the receptor; however, previous mutagenic studies make this possibility unlikely. The current results demonstrate that the sole negatively charged group in IC3, D242, can be removed with little effect on signaling. The only negatively charged group in IC2, D157, is also in a region that can be removed with little effect on signaling (*23*). Furthermore, the only negatively charged group buried in the transmembrane region, E143, is not required for signaling (*34*). The carboxyl-terminal tail of the receptor, which contains numerous negatively groups, appears to exert only negative effects on pheromone signaling (*21*, *22*). (3) Positively charged groups in IC3 could interact with negatively charged lipid headgroups at the membrane surface. However, it seems unlikely that interaction with these relatively mobile groups could provide the active change in conformation needed to specifically activate the G protein. (4) Positive charges on the receptor could mediate repulsive interactions with the numerous other positively charged regions of the receptor or G protein. However, the studies presented here indicate that no single positive charge in the receptor plays a critical role in signaling.

Many of the alleles that we identified as functional actually exhibited greater than normal levels of *FUS1-lacZ* induction in the presence of high pheromone concentrations, and several site-directed mutants exhibited hypersensitive signaling at 10 nM α-factor (Figures 10 and 11), suggesting a role for IC3 in the negative regulation of some aspect of pheromone signaling as proposed previously (*24*−*26*). This may serve as a safety measure ensuring that yeast cells do not prematurely activate the mating pathway and initiate cell cycle arrest at very low concentrations of pheromone, as might arise from mating-type switching of a few cells or leaky repression transcription of precursors to self-activating pheromones.

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REFERENCES

1. Bourne, H. R. (1997) *Curr. Opin. Cell Biol. 9*, 134−142.
2. Gether, U., and Kobilka, B. K. (1998) *J. Biol. Chem. 273*, 17979−17982.

3. Hamm, H. E. (1998) *J. Biol. Chem. 273*, 669−672.
4. Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996) *Nature 379*, 311−319.
5. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) *Cell 83*, 1047−1058.
6. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) *Science 289*, 739−745.
7. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) *Science 274*, 768−770.
8. Bardwell, L., Cook, J. G., Inouye, C. J., and Thorner, J. (1994) *Dev. Biol. 166*, 363−379.
9. Dohlman, H. G. (2002) *Annu. Rev. Physiol. 64*, 129−152.
10. Erickson, J. R., Wu, J. J., Goddard, J. G., Tigyi, G., Kawanishi, K., Tomei, L. D., and Kiefer, M. C. (1998) *J. Biol. Chem. 273*, 1506−1510.
11. 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) *J. Biol. Chem. 274*, 15757−15765.
12. King, K., Dohlman, H. G., Thorner, J., Caron, M. G., and Lefkowitz, R. J. (1990) *Science 250*, 121−123.
13. Kajkowski, E. M., Price, L. A., Pausch, M. H., Young, K. H., and Ozenberger, B. A. (1997) *J. Recept. Signal Transduction Res. 17*, 293−303.
14. Erlenbach, I., Kostenis, E., Schmidt, C., Serradeil-Le Gal, C., Raufaste, D., Dumont, M. E., Pausch, M. H., and Wess, J. (2001) *J. Biol. Chem. 276*, 29382−29392.
15. Price, L. A., Kajkowski, E. M., Hadcock, J. R., Ozenberger, B. A., and Pausch, M. H. (1995) *Mol. Cell Biol. 15*, 6188−6195.
16. Price, L. A., Strnad, J., Pausch, M. H., and Hadcock, J. R. (1996) *Mol. Pharmacol. 50*, 829−837.
17. Brown, A. J., Dyos, S. L., Whiteway, M. S., White, J. H., Watson, M. A., Marzioch, M., Clare, J. J., Cousens, D. J., Paddon, C., Plumpton, C., Romanos, M. A., and Dowell, S. J. (2000) *Yeast 16*, 11−22.
18. Crowe, M. L., Perry, B. N., and Connerton, I. F. (2000) *J. Recept. Signal Transduction Res. 20*, 61−73.
19. Meng, E. C., and Bourne, H. R. (2001) *Trends Pharmacol. Sci. 22*, 587−593.
20. Wess, J. (1997) *FASEB J. 11*, 346−354.
21. Konopka, J. B., Jenness, D. D., and Hartwell, L. H. (1988) *Cell 54*, 609−620.
22. Reneke, J. E., Blumer, K. J., Courchesne, W. E., and Thorner, J. (1988) *Cell 55*, 221−234.
23. Martin, N. P., Leavitt, L. M., Sommers, C. M., and Dumont, M. E. (1999) *Biochemistry 38*, 682−695.
24. Weiner, J. L., Guttierez-Steil, C., and Blumer, K. J. (1993) *J. Biol. Chem. 268*, 8070−8077.
25. Stefan, C. J., and Blumer, K. J. (1994) *Mol. Cell Biol. 14*, 3339−3349.
26. Clark, C. D., Palzkill, T., and Botstein, D. (1994) *J. Biol. Chem. 269*, 8831−8841.
27. Martin, N. P., Celic, A., and Dumont, M. E. (2002) *J. Mol. Biol. 317*, 765−788.
28. Geva, A., Lassere, T. B., Lichtarge, O., Pollitt, S. K., and Baranski, T. J. (2000) *J. Biol. Chem. 275*, 35393−35401.
29. Leavitt, L. M., Macaluso, C. R., Kim, K. S., Martin, N. P., and Dumont, M. E. (1999) *Mol. Gen. Genet. 261*, 917−932.
30. Shah, A., and Marsh, L. (1996) *Biochem. Biophys. Res. Commun. 226*, 242−246.
31. Sommers, C. M., Martin, N. P., Akal-Strader, A., Becker, J. M., Naider, F., and Dumont, M. E. (2000) *Biochemistry 39*, 6898−6909.
32. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.
33. Sommers, C. M., and Dumont, M. E. (1999) in *Structure−Function Analysis of G Protein-Coupled Receptors* (Wess, J., Ed.) pp 141−166, Wiley-Liss, New York.
34. Sommers, C. M., and Dumont, M. E. (1997) *J. Mol. Biol. 266*, 559−575.
35. Sprague, G. F., Jr. (1991) *Methods Enzymol. 194*, 77−93.
36. Lee, B. K., Khare, S., Naider, F., and Becker, J. M. (2001) *J. Biol. Chem. 276*, 37950−37961.
37. Abel, M. G., Zhang, Y. L., Lu, H. F., Naider, F., and Becker, J. M. (1998) *J. Pept. Res. 52*, 95−106.
38. Dumont, M. E., Corin, A. F., and Campbell, G. A. (1994) *Biochemistry 33*, 7368−7378.
39. Rao, V. R., and Oprian, D. D. (1996) *Annu. Rev. Biophys. Biomol. Struct. 25*, 287−314.
40. Lefkowitz, R. J., Cotecchia, S., Samama, P., and Costa, T. (1993) *Trends Pharmacol. Sci. 14*, 303−307.
41. Kjelsberg, M. A., Cotecchia, S., Ostrowski, J., Caron, M. G., and Lefkowitz, R. J. (1992) *J. Biol. Chem. 267*, 1430−1433.
42. Fauchere, J.-L., and Pliska, V. (1983) *Eur. J. Med. Chem. 18*, 369−375.
43. Levitt, M. (1976) *J. Mol. Biol. 104*, 59−107.
44. Dayhoff, M. O., Schwartz, R. M., and Orcutt, B. C. (1978) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., Ed.) pp 345−352, National Biomedical Research Foundation, Washington, DC.
45. Mentesana, P. E., and Konopka, J. B. (2001) *Biochemistry 40*, 9685−9694.
46. Stefan, C. J., Overton, M. C., and Blumer, K. J. (1998) *Mol. Biol. Cell 9*, 885−899.
47. Konopka, J. B., Margarit, S. M., and Dube, P. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 6764−6769.
48. Cheung, A. H., Huang, R. R., and Strader, C. D. (1992) *Mol. Pharmacol. 41*, 1061−1065.
49. Wess, J., Liu, J., Blin, N., Yun, J., Lerche, C., and Kostenis, E. (1997) *Life Sci. 60*, 1007−1014.
50. Wade, S. M., Lim, W. K., Lan, K. L., Chung, D. A., Nanamori, M., and Neubig, R. R. (1999) *Mol. Pharmacol. 56*, 1005−1013.
51. Greasley, P. J., Fanelli, F., Scheer, A., Abuin, L., Nenniger-Tosato, M., DeBenedetti, P. G., and Cotecchia, S. (2001) *J. Biol. Chem. 276*, 46485−46494.
52. Medici, R., Bianchi, E., Di Segni, G., and Tocchini-Valentini, G. P. (1997) *EMBO J. 16*, 7241−7249.
53. Van Dop, C., Yamanaka, G., Steinberg, F., Sekura, R. D., Manclark, C. R., Stryer, L., and Bourne, H. R. (1984) *J. Biol. Chem. 259*, 23−26.
54. Cerione, R. A., Kroll, S., Rajaram, R., Unson, C., Goldsmith, P., and Spiegel, A. M. (1988) *J. Biol. Chem. 263*, 9345−9352.
55. Hamm, H. E., Deretic, D., Arendt, A., Hargrave, P. A., Koenig, B., and Hofmann, K. P. (1988) *Science 241*, 832−835.
56. Garcia, P. D., Onrust, R., Bell, S. M., Sakmar, T. P., and Bourne, H. R. (1995) *EMBO J. 14*, 4460−4469.
57. Osawa, S., and Weiss, E. R. (1995) *J. Biol. Chem. 270*, 31052−31058.
58. Itoh, Y., Cai, K., and Khorana, H. G. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 4883−4887.
59. Song, J., and Dohlman, H. G. (1996) *Biochemistry 35*, 14806−14817.
60. Kallal, L., and Kurjan, J. (1997) *Mol. Cell Biol. 17*, 2897−2907.

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