THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 12, Issue of March 25, pp. 8831–8841, 1994
Printed in U.S.A.

Systematic Mutagenesis of the Yeast Mating Pheromone Receptor Third Intracellular Loop*

(Received for publication, September 17, 1993, and in revised form, December 22, 1993)

Chris D. Clark‡, Timothy Palzkill§, and David Botstein‡¶

From the ‡Department of Genetics, Stanford University School of Medicine, Stanford, California 94305 and §Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030

Signal transduction in the mating pathway of the yeast *Saccharomyces cerevisiae* is initiated by binding of a peptide pheromone to a G protein-coupled receptor (Ste2). We systematically have mutated the third intracellular loop of the Ste2 receptor to investigate its functional significance. We substituted each of the 13 amino acids in the loop with alanine individually or together with one other residue. In addition, we used a site-directed random replacement mutagenesis technique to replace a region encoding three amino acids in the loop with random sequence. Over 80 such Ste2 mutants have been analyzed by several functional and biochemical criteria in a yeast strain that carries a genomic deletion of the *STE2* gene. The mutant phenotypes range from fully functional to severely compromised in signaling. The observation that amino acid substitutions in the third intracellular loop of the Ste2 receptor can affect activation of the yeast mating response implicates the loop in this signal transduction pathway. The types of mutations that compromise the function of the receptor may provide clues to the physical interaction between the receptor and the G protein.

G protein[1]-mediated signaling pathways modulate a wide array of physiological events. A large family of G protein-coupled receptors has been found that shares a common structural motif of seven putative transmembrane domains. Mutagenesis and domain-swapping studies have suggested that the intracellular loops of these receptors mediate the coupling to G protein (reviewed in Refs. 1 and 2).

In the yeast *Saccharomyces cerevisiae*, mating of the two haploid cell types is initiated by peptide pheromones that bind to seven transmembrane domain receptors on the cell surface. The pheromone α-factor, produced by mating type α cells, binds to the Ste2 receptor on the surface of mating type **a** cells. Ste2p is coupled to a heterotrimeric G protein, which is responsible for triggering a variety of cellular responses in preparation for mating, including cell cycle arrest. In yeast, the β and γ subunits of the G protein activate a downstream effector. The α subunit of the G protein has a negative role; it inhibits βγ activity by forming the inactive heterotrimer. The interaction between the receptor and the G protein is not well understood (the yeast mating pathway is reviewed in Refs. 3–6).

Hydropathy analysis of the Ste2 coding region suggests a seven-transmembrane domain structure containing four putative cytoplasmic regions available for potential contact with the G protein: the first intracellular loop (7 amino acids), the second loop (4 amino acids), the third loop (13 amino acids), and the C-terminal tail (134 amino acids) (7, 8) (Fig. 1). The deduced topology of Ste2p is consistent with studies of post-translational modification of the receptor (reviewed in Ref. 3) and with *in vivo* topological analysis using gene fusions (9). The C-terminal sequences of the receptor regulate receptor number and adaptation to pheromone, but are not essential for pheromone binding or signaling (10, 11).

We chose to focus our studies on the third intracellular loop region of Ste2p because the third loop regions of various similar mammalian receptors have been implicated in G protein contact (reviewed in Refs. 1 and 2). To probe the structure/function relationships of the Ste2p third cytoplasmic loop, we have mutated this region systematically and analyzed the functional and biochemical consequences.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs Inc. The DNA sequence was determined by the dideoxy chain termination method using the Sequenase kit from U. S. Biochemical Corp.

Plasmids—Ste2 alleles were subcloned into autonomous, yeast centromere-containing plasmids pRS315 (marked with *LEU2*) and pRS316 (marked with *URA3*) or into yeast integrating plasmids pRS305 (*LEU2*) and pRS306 (*URA3*) (12).

Yeast Strains and Genetic Methods—The *S. cerevisiae* strains constructed for these studies are derived from strain S288C (Table I). All CAY strains were *MAT***a**, *sst1*Δ, *his3Δ200*, and *lys2-801*. Media were made as described elsewhere (13). Yeast cells were transformed by the lithium acetate method (14). The Ste2 deletion allele (*ste2*Δ) was constructed using PCR to replace 96% of the *STE2* gene (6 amino acids at the N terminus and 12 amino acids at the C terminus remain out of the 431 amino acids total) with the *HIS3* gene. One-step gene disruption (15) was used to replace *STE2* with the *ste2*Δ allele in the yeast genome. To facilitate pheromone response assays, we deleted the *SST1* gene (16, 17), which encodes a specific protease that degrades α-factor (18–20). The *sst1* deletion was made by gene disruption using the plasmid pJGsst1 (21). The *FUS1-lacZ* reporter gene marked by *URA3* was integrated via homologous recombination (22) into the genomic *ura3-52* locus using plasmid pGA1716, which contains the *FUS1* promoter upstream activating sequence (nucleotide positions 3036–3074) fused to the *lacZ* gene (23). The *FUS1-lacZ* reporter marked by *LEU2* was constructed by inserting the *LEU2* gene into the *Apa*I site within the *URA3* gene on plasmid pGA1716, creating plasmid pCD187, which was integrated into the *leu2-3,112* locus. Autonomous centromere-containing plasmids expressing wild type or mutant alleles of Ste2 were introduced into strains CAY164 or CAY200. Some of the Ste2 mutant alleles were integrated into the yeast genome for further analysis. For yeast strains CAY217–CAY224, the Ste2 mutant alleles were cloned into pRS306, which carries the *URA3* gene. The resulting plasmids were recombined into the *STE2* locus of strain CAY199 selecting for Ura$^+$ transformants. Ura$^-$ derivatives of the transformants were obtained on 5-fluoroorotic acid media (24) and then screened for the Ste2 mutant phenotype to identify strains that had replaced the wild type *STE2* gene

* This work was supported in part by a Beckman Center Director's Research Grant funded by SmithKline Beecham and in part by National Institutes of Health Grants GM46888 and GM46406. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom reprint requests should be addressed. Tel.: 415-723-3488; Fax: 415-723-7016.

[1] The abbreviations used are: G protein, guanyl nucleotide-binding regulatory protein; PCR, polymerase chain reaction; bp base pair(s).

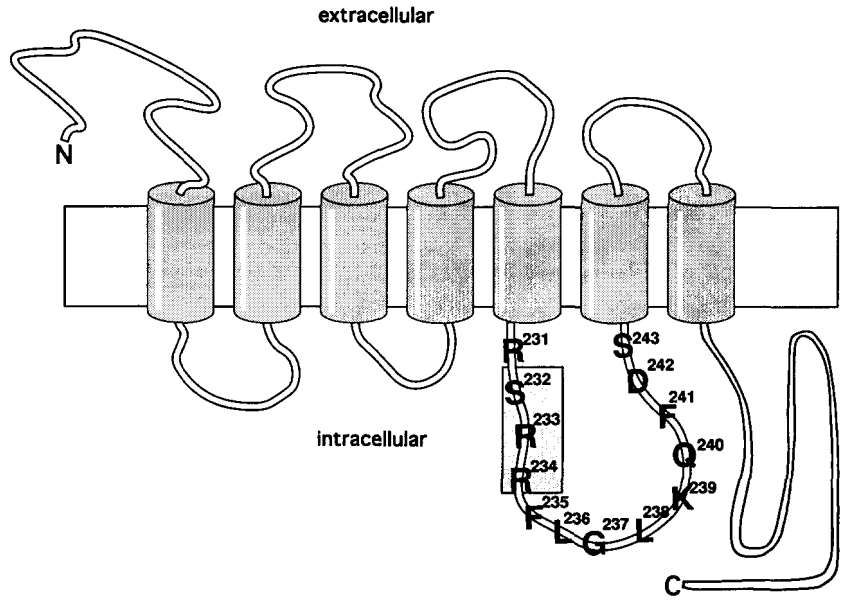

FIG. 1. **Putative structure of the *STE2* receptor.** The amino acid sequence of the entire third intracellular loop is shown. Each of the 13 residues was replaced with alanine individually or in certain pair combinations. Also, the *boxed region* was subjected to random replacement mutagenesis.

TABLE I
Yeast strains

Strain	Genotype
CAY164	*ste2ΔHIS3 ura3–52::FUS1-lacZ::URA3 leu2–3,112*
CAY199	*leu2–3,112::FUS1-lacZ::LEU2 ura3–52*
CAY200	*ste2ΔHIS3 leu2–3,112::FUS1-lacZ::LEU2 ura3–52*
CAY217	*ste2-S232L-R233F-R234Q leu2–3,112::FUS1-lacZ::LEU2 ura3–52*
CAY218	*ste2-S232S-R233P-R234G leu2–3,112::FUS1-lacZ::LEU2 ura3–52*
CAY219	*ste2-S232F-R233L-R234T leu2–3,112::FUS1-lacZ::LEU2 ura3–52*
CAY220	*ste2-S232V-R233D-R234K leu2–3,112::FUS1-lacZ::LEU2 ura3–52*
CAY221	*ste2-S232S-R233T-R234G leu2–3,112::FUS1-lacZ::LEU2 ura3–52*
CAY222	*ste2-S232W-R233S-R234Q leu2–3,112::FUS1-lacZ::LEU2 ura3–52*
CAY223	*ste2-S232S-R233E-R234E leu2–3,112::FUS1-lacZ::LEU2 ura3–52*
CAY224	*ste2-S232I-R233Q-R234R leu2–3,112::FUS1-lacZ::LEU2 ura3–52*
CAY241	*ste2ΔHIS3 leu2–3,112::ste2-D242A::LEU2 ura3–52::FUS1-lacZ::URA3*
CAY242	*ste2ΔHIS3 leu2–3,112::ste2-L236A::LEU2 ura3–52::FUS1-lacZ::URA3*
CAY243	*ste2ΔHIS3 leu2–3,112::ste2-K239A::LEU2 ura3–52::FUS1-lacZ::URA3*
CAY244	*ste2ΔHIS3 leu2–3,112::ste2-G237A::LEU2 ura3–52::FUS1-lacZ::URA3*
CAY245	*ste2ΔHIS3 leu2–3,112::ste2-L238A::LEU2 ura3–52::FUS1-lacZ::URA3*
CAY246	*ste2ΔHIS3 leu2–3,112::ste2-Q240A::LEU2 ura3–52::FUS1-lacZ::URA3*
CAY247	*ste2ΔHIS3 leu2–3,112::ste2-F241A::LEU2 ura3–52::FUS1-lacZ::URA3*
CAY248	*ste2ΔHIS3 leu2–3,112::ste2-R233A-F241A::LEU2 ura3–52::FUS1-lacZ::URA3*
CAY249	*ste2ΔHIS3 leu2–3,112::ste2-R231A-F241A::LEU2 ura3–52::FUS1-lacZ::URA3*
CAY250	*ste2ΔHIS3 leu2–3,112::ste2-R233A-K239A:LEU2 ura3–52::FUS1-lacZ::URA3*
CAY251	*ste2ΔHIS3 leu2–3,112::ste2-R234A-D242A::LEU2 ura3–52::FUS1-lacZ::URA3*
CAY252	*ste2ΔHIS3 leu2–3,112::ste2-F235A-D242A::LEU2 ura3–52::FUS1-lacZ::URA3*
CAY253	*ste2ΔHIS3 leu2–3,112::ste2-S232A-Q240A::LEU2 ura3–52::FUS1-lacZ::URA3*
CAY254	*ste2ΔHIS3 leu2–3,112::ste2-R234A-Q240A::LEU2 ura3–52::FUS1-lacZ::URA3*
CAY255	*ste2ΔHIS3 leu2–3,112::STE2⁺::LEU2 ura3–52::FUS1-lacZ::URA3*
FW786	*MAT***a** *ade8* (from F. Winston)
FW787	*MATα ade8* (from F. Winston)
FY70	*MATα leu2Δ1* (from F. Winston)

with the Ste2 mutants. For yeast strains CAY241–CAY255, the Ste2 mutants were cloned into the integrating plasmid pRS305. The resulting plasmids were recombined into the *leu2-3,112* locus of strain CAY164, selecting for Leu⁺ transformants.

Alanine Substitution Mutagenesis—To replace individually each of the 13 amino acids in the Ste2 third cytoplasmic loop with alanine, we devised a PCR-based mutagenesis method that uses a single oligonucleotide encoding the alanine substitution change for each mutant and two oligonucleotides flanking the region for all of the mutants. The starting plasmid, pCD189, contains the *STE2* gene cloned into plasmid pRS316. In the first PCR, oligonucleotide cdc17 (5′CGCGGATTCA-AAAATGTCTGATGCGGC3′), located at the first codon, and 1 of the 13 alanine mutation oligonucleotides (38-bp primers located at codon positions ranging from 231 to 243) were used to amplify an approximately 700-bp fragment of *STE2* from pCD189. In the second PCR, the first PCR product was used to prime synthesis from a 715-bp *Eco*RV-*Sal*I restriction fragment within *STE2* which had been treated with T4 DNA polymerase in the presence of dideoxynucleotides (to enrich for amplification of the mutant strand, rather than the wild type strand). Two other oligonucleotides in this second PCR flank the mutation: cdc17 (above) and cdc55 (5′GACATCTGTTCCCTGG3′) located at codon 274. These two flanking mutations amplify an 820-bp fragment, which was cut with *Eco*RV and *Cla*I and cloned into the *Eco*RV and *Cla*I sites of pCD189. Each of the alanine mutations and the integrity of the surrounding region that had undergone PCR amplification were confirmed by dideoxy sequencing.

Construction of Double Alanine Substitution Mutants—A *Sty*I restriction site located at codon 236 allowed the construction of 30 double alanine substitution mutants by combining restriction fragments from plasmids containing Ste2 alanine replacement mutations at positions 231, 232, 233, 234, and 235 sequentially with positions 238, 239, 240, 241, 242, and 243. These double mutants were cloned into plasmid pCD189 and the presence of both alanine substitutions was confirmed by dideoxy sequencing.

Random Replacement Mutagenesis—We used a recently described mutagenesis procedure (25) to randomize three codons of the third cytoplasmic loop (Fig. 2). In brief, the technique involved inserting an oligonucleotide containing BspM1 recognition sites at the region of mu-

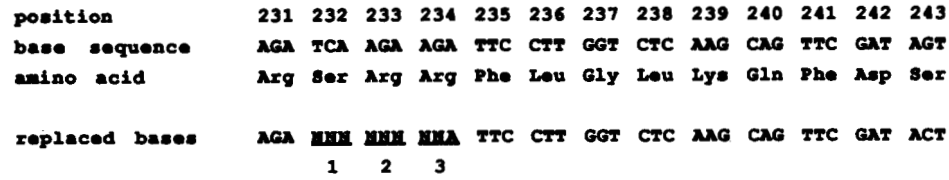

FIG. 2. **Random replacement mutagenesis strategy.** A plasmid library of *ste2* mutants was constructed by replacing 8 bp covering codons 232, 233, and 234 with 8 bp of random sequence. For residue 234, since only the first 2 bp of the codon are randomized, only 16 codons are allowed as replacements, encoding the following ratios of amino acids: 1/8 each of Arg, Leu, and translation termination codons; and 1/16 each of Ala, Gln, Glu, Gly, Ile, Lys, Pro, Ser, Thr, and Val. We expect 22% of the clones in the library to contain translation termination codons.

tagenesis, followed by release of the inserted oligonucleotide by digestion with BspM1, which created an 8-bp deletion. To replace the deleted nucleotides with random sequence, a second linker was inserted that contains 4 bp of random sequence at each end along with embedded BspM1 sites. A library of independent linker insertions was constructed in *Escherichia coli*, and the plasmid DNA was extracted and purified. The DNA was digested with BspM1 again and religated, leaving behind an insertion of eight random nucleotides. This library of independent Ste2 random substitution mutants was transformed into yeast strain CAY164 and transformants were screened for Ste2 function. Plasmids were recovered from yeast (26), transformed into *E. coli*, and prepared for dideoxy sequencing. After determining the sequence of the mutations, individual plasmids were transformed back into yeast for further phenotypic analysis. Several of the mutants were recombined into the genome for further analysis. The inserts in the random replacement library appeared random ($p = 0.5$; $\chi^2 = 3$; degrees of freedom = 3).

Pheromone-induced Responses—Mating tests were performed on cells carrying the plasmid-borne Ste2 mutations (in either CAY164 or CAY200) by spotting a uniform suspension of cells onto the surface of a YEPD plate containing a lawn of tester strain FW786 or FW787. After incubating overnight at 30 °C, cells were replica-plated to SD minimal medium, incubated overnight at 30 °C, then scored for growth of prototrophic diploids. Strains with Ste2 mutations were tested for their ability to induce *FUS1-lacZ* (23) by formation of blue colonies on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (27) in the absence or presence of 0.8 μg/ml synthetic α-factor (Sigma). Uniform suspensions of cells were spotted on the 5-bromo-4-chloro-3-indolyl-β-D-galactoside indicator plates, incubated at 30 °C then scored for blue color over a period of several days. β-Galactosidase assays were performed in duplicate on liquid cultures of Ste2 mutants by a permeabilized cell assay (27). To induce expression of the *FUS1-LacZ* reporter gene, cells were grown to log phase (approximately 2×10^7 cells/ml) and then incubated for 2 h at 30 °C in the presence of 0.4 μg/ml synthetic α-factor. The A_{600} of each strain was used to normalize number of cells before assay. Zone of inhibition assays were performed by plating ~30,000 cells in an overlay of 1% agar YEPD medium onto an agar plate then adding three amounts of α-factor to sterile filter disks (Difco). For the alanine replacement mutants, 50, 250, and 2500 ng of pheromone were added to the disks. For the random replacement mutants, 100, 500, and 5000 ng were added to the disks. After 48 h of incubation at 30 °C, halo diameters were measured, plotted on a semilog scale, and the amount of pheromone required to produce a 2-cm diameter halo was estimated by interpolation (28). Plates were incubated further to observe the ability of Ste2 mutants to recover from arrest.

Preparation of ^{35}S-Labeled α-Factor and Pheromone Binding Assays—Strain FY70 (7×10^7 cells) carrying plasmid pDA6300 (a high copy number plasmid encoding the genes for α-factor) (29) was metabolically labeled with 12.5 mCi carrier-free [^{35}S]H_2SO_4 (ICN Radiochemicals). ^{35}S-Labeled α-factor was purified essentially as previously described (30) with modifications kindly provided by Kim Schandel and Duane Jenness.[2] The concentration of the purified ^{35}S-labeled α-factor was determined by zone of inhibition assays using synthetic α-factor (Peninsula Laboratories Inc., Belmont CA) as a standard. The specific activity of the ^{35}S-labeled α-factor was 251 Ci/mmol and was adjusted as necessary by addition of unlabeled synthetic α-factor. For pheromone binding assays, cells were grown to log phase, centrifuged, and resuspended in binding medium (YEPD with 10 mM each sodium azide and potassium fluoride) at 1×10^8 cells/ml. 9×10^6 cells were used per binding reaction in the presence of various amounts of ^{35}S-labeled α-factor. After 30 min of incubation at room temperature, the 100-μl reaction was diluted with 0.5 ml of binding medium and collected on a premoistened 2.5-cm GF/C filter (Whatman). The cells were rinsed three times with 2 ml of ice-cold binding medium, and the filters were counted by liquid scintillation. For each set of Ste2 mutant binding studies, both wild type Ste2 and *ste2Δ* strains were included as controls. Nonspecific binding was found to be equivalent whether it was measured in the presence of 1000-fold molar excess of unlabeled synthetic α-factor or measured using an isogenic *ste2Δ* strain. The binding data were analyzed by non-linear least squares analysis using the Scatplot program (R. Vandelen, Genentech Inc., South San Francisco, CA).

[2] K. Schandel and D. Jenness, personal communication.

RESULTS AND DISCUSSION

Alanine Replacement Mutagenesis—As a first step toward identifying Ste2 amino acids important for signaling, we replaced each of the 13 amino acids in the third loop with alanine. Since alanine substitution removes the side chains beyond the β-carbon, without changing main chain conformation or imposing severe electrostatic or steric effects (31), substitution with alanine may identify contact sites for proteins that interact with Ste2p (*e.g.* the G protein and other putative proteins involved in recovery).

Plasmids bearing the Ste2 alanine substitution mutants were introduced into a ste2Δ strain (CAY199). As an initial qualitative test for signaling, we assessed the ability of each mutant to mate with a strain of the opposite mating type. All 13 mutants appeared to mate as well as the wild type Ste2 control (Table II), indicating that no single amino acid side chain in the third loop absolutely is required for mating. To address whether there is redundancy of function among these 13 residues, we constructed Ste2 mutants containing two alanine substitutions in the third loop region. One of the double mutants, R233A/F241A, showed reduced mating ability in the qualitative mating test (Table II), suggesting that residues Arg^{233} and Phe^{241} may contribute to signaling by Ste2p.

Next, we tested whether any of these mutations affect an earlier response to pheromone: growth arrest. Wild type Ste2 cells treated with pheromone arrest cell division in the G_1 phase of the cell cycle and thus fail to grow on an agar plate containing α-factor. In contrast, a *ste2Δ* strain is insensitive to the presence of pheromone; it fails to arrest and thus grows normally. We tested all of the alanine replacement mutants for growth arrest in the presence of α-factor (Table II). Growth for many of the mutants, like wild type Ste2, was arrested. However, several of the mutants were able to grow in the presence of pheromone, although not as well as the *ste2Δ* strain. The ability of these mutants to escape the G_1 arrest indicates an alteration in Ste2p signal transduction. The alanine replacements could be affecting various aspects of Ste2p function, including interaction with G protein, ligand binding, desensitization/recovery, and receptor number. Notably, the mating-deficient mutant R233A/F241A also failed to arrest, further implicating residues Arg^{233} and Phe^{234} in Ste2p signaling. Additional evidence for the importance of Arg^{233} comes from a recent study (32) describing a double mutant (R233S/R234G) that perturbs normal receptor-G protein interaction (although this mutant does not inhibit mating). There are 2 serine residues in the Ste2p third loop which provide potential sites for phosphorylation. Since the individual alanine mutants

TABLE II

Alanine replacement mutants: functional and biochemical analyses

For all tests, we included two controls, a wild type (wt) Ste2 strain, and a *ste2Δ* strain that lacks receptor. Initial tests for Ste2 function, mating and growth on pheromone, were performed on plasmid-borne alanine replacement mutants in a *ste2Δ* strain. Further quantitative analyses were performed on mutants that had been integrated into the genome of a *ste2Δ* strain. Pheromone binding data are shown relative to the wild type values for each particular binding assay K_a = affinity constant ($1/K_d$). B_{max} = maximal binding capacity K_a and B_{max} values are indicated ± standard errors.

Allele	Sequence 231–243	Plasmid		Genomic			
		Mating	Growth arrest on pheromone	Constitutive blue color	Pheromone 2-cm halo	K_a (mutant/wt)	B_{max} (mutant/wt)
					ng		
STE2	R S R R F L G L K Q F D S	+	+	–	280	1.0	1.0
STE2Δ	– – – – – – – – – – – – –	–	–	–			
R231A	**A** S R R F L G L K Q F D S	+	+				
S232A	R **A** R R F L G L K Q F D S	+	+				
R233A	R S **A** R F L G L K Q F D S	+	+				
R234A	R S R **A** F L G L K Q F D S	+	+				
F235A	R S R R **A** L G L K Q F D S	+	+				
L236A	R S R R F **A** G L K Q F D S	+	±	–	600	0.9 ± 0.2	0.3 ± 0.2
G237A	R S R R F L **A** L K Q F D S	+	±	+	65	1.3 ± 0.3	0.4 ± 0.2
L238A	R S R R F L G **A** K Q F D S	+	±	–	300	2.0 ± 0.2	0.4 ± 0.1
K239A	R S R R F L G L **A** Q F D S	+	±	–	130	1.5 ± 0.8	0.2 ± 0.6
Q240A	R S R R F L G L K **A** F D S	+	±	+	200	1.8 ± 0.3	0.5 ± 0.3
F241A	R S R R F L G L K Q **A** D S	+	±	+	200	2.1 ± 0.2	1.1 ± 0.1
D242A	R S R R F L G L K Q F **A** S	+	∓	–	600	2.9 ± 0.9	0.1 ± 0.7
S243A	R S R R F L G L K Q F D **A**	+	+				
R231A/L238A	**A** S R R F L G **A** K Q F D S	+	+				
R231A/K239A	**A** S R R F L G L **A** Q F D S	+	+				
R231A/Q240A	**A** S R R F L G L K **A** F D S	+	+				
R231A/F241A	**A** S R R F L G L K Q **A** D S	+	±	–	180	12.0 ± 0.8	0.02 ± 0.4
R231A/D242A	**A** S R R F L G L K Q F **A** S	+	+				
R231A/S243A	**A** S R R F L G L K Q F D **A**	+	+				
S232A/L238A	R **A** R R F L G **A** K Q F D S	+	+				
S232A/K239A	R **A** R R F L G L **A** Q F D S	+	+				
S232A/Q240A	R **A** R R F L G L K **A** F D S	+	+	+	83	3.8 ± 0.4	0.2 ± 0.2
R232A/F241A	R **A** R R F L G L K Q **A** D S	+	+				
S232A/D242A	R **A** R R F L G L K Q F **A** S	+	+				
S232A/S243A	R **A** R R F L G L K Q F D **A**	+	+				
R233A/L238A	R S **A** R F L G **A** K Q F D S	+	+				
R233A/K239A	R S **A** R F L G L **A** Q F D S	+	±	–	300	1.0 ± 0.5	0.3 ± 0.4
R233A/Q240A	R S **A** R F L G L K **A** F D S	+	+				
R233A/F241A	R S **A** R F L G L K Q **A** D S	±	∓	–	ND[a]	12.0 ± 0.8	0.03 ± 0.3
R233A/D242A	R S **A** R F L G L K Q F **A** S	+	+				
R233A/S243A	R S **A** R F L G L K Q F D **A**	+	+				
R234A/L238A	R S R **A** F L G **A** K Q F D S	+	+				
R234A/K239A	R S R **A** F L G L **A** Q F D S	+	+				
R234A/Q240A	R S R **A** F L G L K **A** F D S	+	+	+	80	2.4 ± 0.4	0.3 ± 0.3
R234A/F241A	R S R **A** F L G L K Q **A** D S	+	+				
R234A/D242A	R S R **A** F L G L K Q F **A** S	+	±	–	180	1.4 ± 0.4	0.4 ± 0.3
R234A/S243A	R S R **A** F L G L K Q F D **A**	+	+				
F235A/L238A	R S R R **A** L G **A** K Q F D S	+	+				
F235A/K239A	R S R R **A** L G L **A** Q F D S	+	+				
F235A/Q240A	R S R R **A** L G L K **A** F D S	+	+				
F235A/F241A	R S R R **A** L G L K Q **A** D S	+	+				
F235A/D242A	R S R R **A** L G L K Q F **A** S	+	±	–	270	0.6 ± 0.2	0.7 ± 0.2
F235A/S243A	R S R R **A** L G L K Q F D **A**	+	+				

[a] ND, not determined.

S232A and S243A, as well as the double mutant S232A/S243A, behaved like wild type Ste2, phosphorylation of the loop is not required for Ste2p function (also reported by Weiner *et al.* (32)).

Analysis of Genomic Alanine Replacement Mutants—Fourteen of the alanine replacement mutants were integrated into the yeast genome (see "Experimental Procedures"), to assure greater stability than plasmid-borne mutants and thus constant levels of receptor expression. The resulting yeast strains (CAY241–CAY255) were subjected to additional phenotypic analysis.

The first test we performed on these strains employed the use of a reporter gene, P_{FUS1}::*lacZ* (23), to detect activation of the mating pathway. The *FUS1* gene product is involved in the fusion of cells during conjugation. Transcription of *FUS1* is induced strongly by incubation of cells with pheromone (33). Therefore, a fusion between the *FUS1* promoter and the *lacZ* gene, encoding the enzyme β-galactosidase, allows detection of mating pathway activation by assessing β-galactosidase activity (as measured by formation of blue product). We tested strains CAY241–CAY255 for their ability to form blue colonies on indicator plates in the presence or absence of α-factor. The wild type Ste2 control strain forms blue colonies (as discussed above, the presence of pheromone causes growth arrest for a wild type Ste2 strain; however, since the reporter gene is activated before cell cycle arrest occurs, the strain grows enough to give blue product on the indicator plates). The *ste2Δ* strain, which lacks the receptor entirely, forms white colonies. All of the alanine replacement strains formed blue colonies in the presence of pheromone. We also tested these strains on indicator plates in the absence of pheromone. As expected, the wild

type Ste2 strain remained white. Surprisingly however, several of the mutants (G237A, Q240A, F241A, S232A/Q240A, and R234A/Q240A) formed very pale blue colonies in the absence of pheromone after incubating 5–7 days at 25 °C (Table II). This observation suggests that these mutations cause a weak constitutive activation of the mating pathway. Constitutive activation could be due to mutant receptor conformations that mimic the state of the ligand-bound wild type receptor independent of ligand. Similar constitutively activating mutations have been described for a mammalian G protein-coupled receptor, the α_{1B}-adrenergic receptor (34). Also, a mutation (L194Q) in the third intracellular loop of the other yeast pheromone binding receptor, STE3, has been shown to confer constitutive and hypersensitive phenotypes (35). An additional hypothesis for the apparent constitutivity of these yeast mutants takes into account the following observation. Trace amounts of endogenous α-factor can be generated by either low level "leakage" of expression of the genes encoding α-factor, or by spontaneous mating type switching (36). Perhaps the mutants, by virtue of higher affinity for α-factor, are able to respond to such low pheromone levels.

To determine the dosage-response characteristics of the mutants, we performed the zone of growth inhibition assay, which monitors growth arrest in response to pheromone. Various amounts of α-factor are spotted onto disks placed on a lawn of cells (from strains CAY241–CAY255). The pheromone diffuses into the agar around the disk and interacts with the lawn of cells. After incubation, a pheromone-responsive strain arrests in G_1 and fails to grow, thus forming a clear halo around the disk. To quantify this growth arrest phenotype for each mutant, we interpolated from a dose-response curve the amount of α-factor required to produce a 2-cm zone of inhibition (Table II). The mutants fall into three general categories: (*a*) those that require more pheromone than wild type Ste2 (L236A, D242A, and R233A/K239A); (*b*) those that require less pheromone than wild type (hypersensitive) (G237A, K239A, Q240A, F241A, R231A/F241A, S232A/Q240A, R234A/Q240A, and R234A/D242A; and (*c*) those that require a similar amount as wild type (L238A and F235A/D242A). The pheromone response of mutant R233A/F241A was not quantitated because the halos could not be measured accurately; however, it appeared initially to have larger than wild type halos (*i.e.* hypersensitive). Representative examples of halos for the three classes are shown in Fig. 3.

Surprisingly, some of the mutants that appeared hypersensitive in the halo assay (*i.e.* responded more efficiently than wild type Ste2), had previously exhibited a defect in growth arrest on pheromone plates (*i.e.* responded less efficiently than wild type Ste2). A potential explanation became apparent upon further observation of the halos. After several days, some colonies grew within the otherwise clear halo of these mutants, forming a turbid halo. Turbid halos indicate either incomplete receptor activation of the mating pathway (*i.e.* some cells in the population fail to respond to pheromone) or specific activation of a recovery pathway (11, 28). These mutants had variable amounts of turbid growth, from a few colonies (Fig. 3, G237A) to a completely turbid halo (Fig. 3, R233A/F241A).

A Ste2 mutant with phenotypes similar to our hypersensitive alanine mutants (hypersensitivity and facilitated recovery from growth arrest) previously was reported (28). This Ste2 allele had two mutations (S145L/S219L) in membrane-spanning regions three and five, which may be involved in pheromone binding (28). In addition, a mutation in Gpa1, the α-subunit of the yeast G protein, also can confer initial hypersensitivity and facilitated recovery (11, 37). Thus, it appears that mutations in the putative pheromone binding regions, or in the third cytoplasmic loop of Ste2p, as well as mutations in the downstream G protein α-subunit, can confer

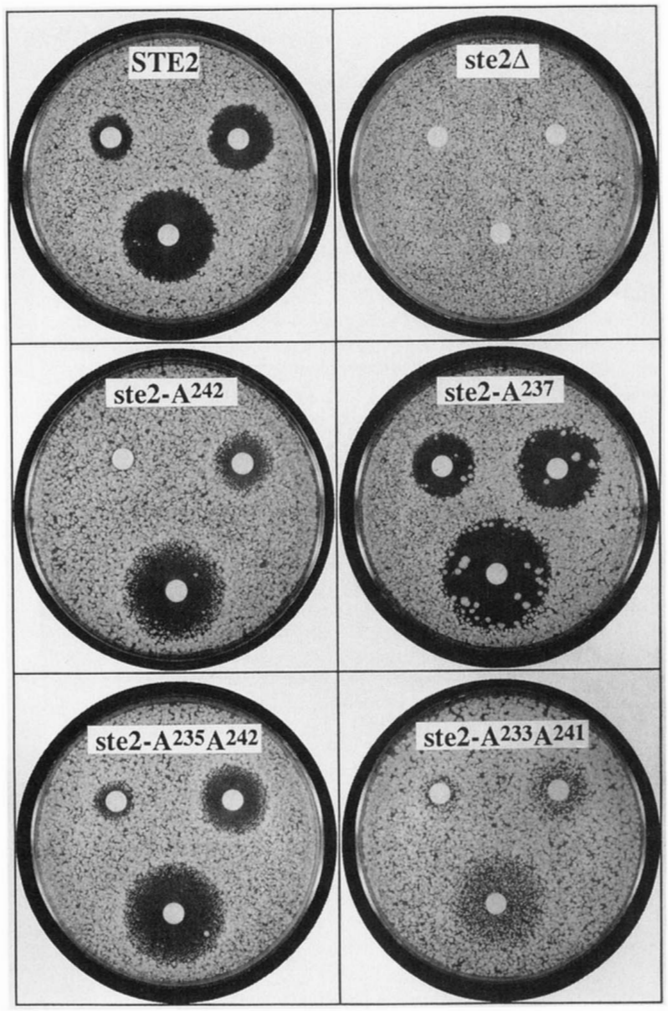

FIG. 3. **Zone of inhibition response for representative alanine replacement mutants.** Synthetic α-factor was spotted on disks (50, 250, and 2500 ng, clockwise from *top left*) on a lawn of cells. Halo diameters were measured after 2 days of incubation. Plates were photographed after 4 days to document turbidity of halos. Controls in the *top row* are wild type *STE2* and *ste2Δ* strains. Mutant A^{242} has smaller than wild type halos (thus requires more pheromone for a similar response); mutant A^{237} has larger than wild type halos (thus requires less pheromone for a similar response); mutant $A^{235}A^{242}$ has halo diameters similar to wild type; and mutant $A^{233}A^{241}$ halos are too turbid to measure accurately.

initial hypersensitivity and facilitated recovery from growth arrest.

Pheromone Binding Properties of Alanine Replacement Mutants—To assess the ligand binding affinity and cell-surface expression of the genomic alanine mutants, we performed equilibrium binding studies with intact cells. The assays were done in the presence of metabolic inhibitors to measure the steady-state levels of surface receptors. Scatchard plots of representative alleles, including apparent equilibrium dissociation constants (K_d)2 and steady-state levels of cell surface receptors (B_{max}) are shown in Fig. 4. The K_d measured for wild type Ste2 ranged in different assays from 0.8 to 3.7 nM, similar to previously reported values of 2 nM (32) and 5–7 nM (38). The B_{max} value measured for wild type Ste2 varied between assays from 1100 to 3100 receptors/cell, slightly less than the previously reported values of 4900/cell (32) and 8000/cell (38). Binding data for all of the integrated alanine replacement mutants are shown in Table II. Most of the mutants (12/14) had expression levels between 20 and 100% of wild type (Table II). The affinity (K_a) and B_{max} values of the mutants help elucidate some of the mutant phenotypes.

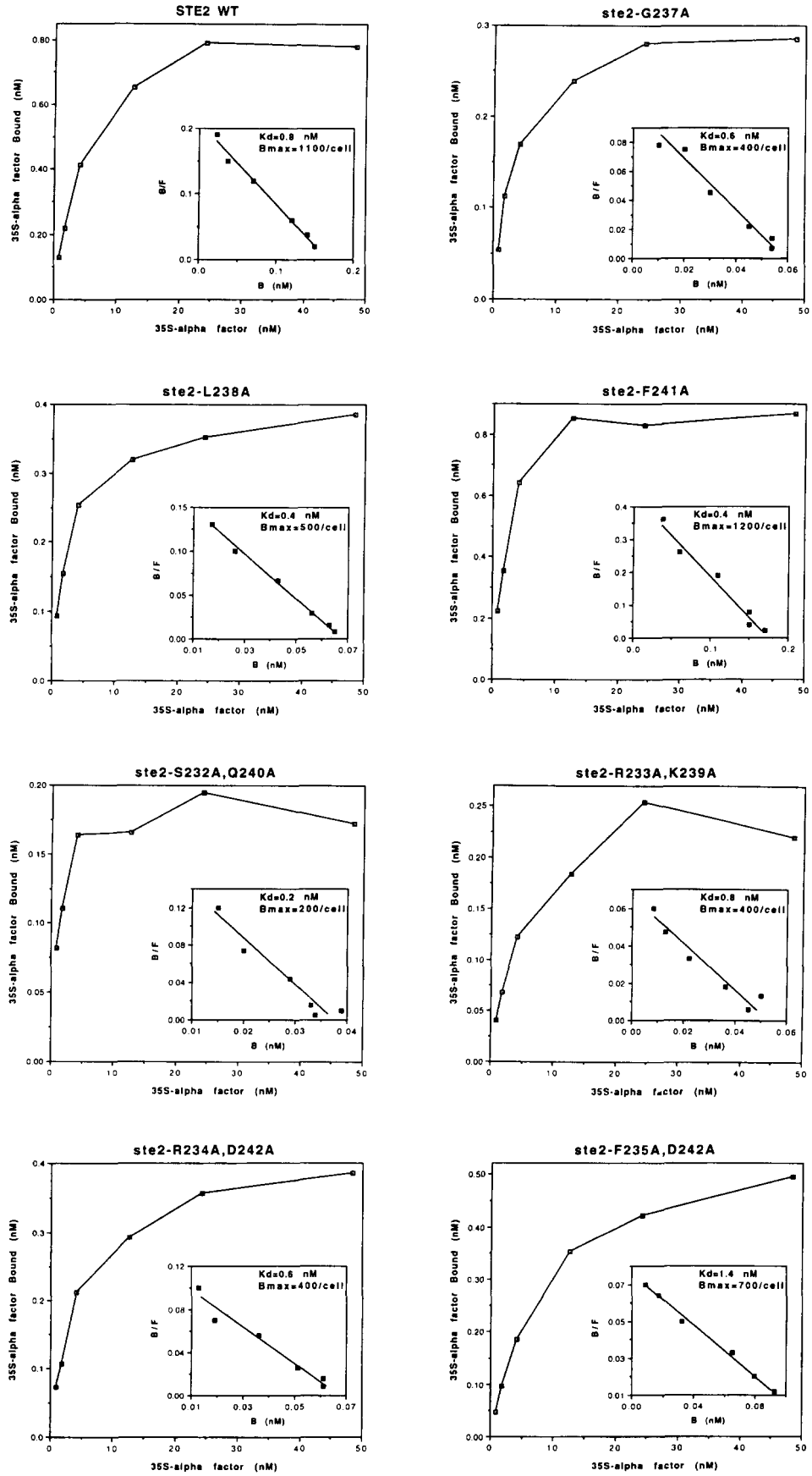

FIG. 4. **Equilibrium binding of labeled α-factor to representative alanine replacement mutants.** The Ste2 allele is indicated *above* each graph. Data were analyzed by the Scatplot program (R. Vandelen, Genentech Inc.) using non-linear fit least-square analysis. The *inset* graphs show the Scatchard analysis. B/F, bound/free; B, bound. Data points are the means of duplicate determinations.

The constitutive phenotype observed in five of the alanine mutants (G237A, Q240A, F241A, S232A/Q240A, and R234A/Q240A) can be explained by one or both of the following reasons. 1) The mutant receptor has a semi-activated conformation that can activate G protein in the absence of pheromone and 2) the receptor has an increased pheromone binding affinity that allows response to trace levels of endogenous pheromone. These explanations are consistent with the observation that all five of the constitutive mutants exhibit hypersensitivity in the dosage-response halo assay (Table II). All five of these mutants also have a 1.3–4-fold higher pheromone binding affinity than wild type. Our Scatchard analysis could not find evidence of a fit for two affinities, probably because, in whole cells, the receptors associate with G protein upon ligand binding (39). The G237A mutant, which has a modest 1.3-fold increase in affinity, shows a marked hypersensitivity; it requires 4-fold less pheromone than wild type Ste2 to form equivalent sized halos. Perhaps these constitutive Ste2 third loop mutations increase affinity by creating a semi-activated receptor structure in the absence of pheromone that allows constitutive coupling to G protein. The constitutive mutants may be analogous to mammalian third loop constitutive mutants in the α_{1B}-adrenergic receptor (40) and the β_2-adrenergic receptor (34) that have increased affinity and appear spontaneously to undergo a critical conformational change that normally requires the binding of agonist. Three of our constitutive mutants have the Q240A replacement, implicating residue Gln^{240} in signaling. Perhaps Gln^{240} serves to constrain the third cytoplasmic loop into a structure unable to interact with G protein until the receptor has bound ligand. Replacing Gln^{240} with alanine may relax this constraint. However, not all mutants with Q240A exhibit a mutant phenotype. Mutants combining Q240A with either R231A, R233A, or F235A behave like wild type Ste2. Thus, mutations at these second positions may provide an alternative constraint to the one provided by Gln^{240}.

The hypersensitive phenotype we observed for eight of the alanine mutants could be caused either by increased binding affinity or by a defect in the recovery pathway. Five of these hypersensitive mutants are also constitutive, and as discussed above, have increased affinity. The remaining three hypersensitive mutants are not constitutive, but do have elevated affinities: mutant K239A by 1.5-fold, R234A/D242A1 by 4-fold, and R231A/F241A by 12-fold (although this mutant is expressed at only 2% of wild type levels, and thus the apparent K_a may be inaccurate). The higher affinity of these three mutants may account for their hypersensitivity, especially since none of them appears to have a defect in recovery; in fact, they all have turbid halos, indicating activation of a recovery pathway. The fact that these three hypersensitive mutants with increased affinity are not constitutive indicates that the constitutive phenotype observed for the other hypersensitive mutants is not due solely to increased affinity allowing sensitivity to trace levels of endogenous pheromone. These findings suggest that a higher affinity conferred by mutations in the third loop is not necessarily the result of locking the receptor into a semi-activated structural state.

All of the hypersensitive mutants exhibited increased pheromone binding affinity; however, not all mutants with increased affinity were hypersensitive. Mutant L238A has a 2-fold higher K_a, yet similarly sized halos as wild type Ste2. Since this mutant is expressed at near wild type levels (41%), it is likely to have impaired coupling to G protein and/or activation of a recovery pathway. Mutant D242A has 3-fold higher K_a, yet significantly smaller than wild type halos. The expression level of D242A is 13% of wild type, which could be contributing to its reduced of signaling ability, in addition to decreased G protein coupling and/or activated recovery. Mutant R233A/F241A has a 14-fold higher affinity than wild type, although because of its low expression level (3% of wild type), the apparent K_a may not be accurate. The mating defect and extremely turbid halos exhibited by R233A/F241A could be due in part to reduced expression. But reduced expression level alone does not explain the severe loss of Ste2 function for R233A/F241A; another mutant with 2% of wild type expression level, R231A/R241A, has larger than wild type halos and no mating defect. Thus, R233A/F241A likely also has reduced G protein coupling and/or activated recovery. It is noteworthy that mutant F241A alone has 100% wild type expression levels, but combining it with either R231A or R233A dramatically reduces expression to 2–3% of wild type.

Mutants L236A and R233A/K239A both have wild type ligand binding characteristics and reasonable expression levels (>20% of wild type). Their mutant phenotypes (reduction in pheromone response in the halo assay and enhanced recovery) are likely due to decreased coupling with G protein and/or an altered recovery response. Weiner *et al.* (32) described two Ste2 mutations at residue 236 (L236H and L236R) that also exhibited wild type binding characteristics and had expression levels >20% of wild type. Those mutants concomitantly blunt G protein coupling and promote the function of an *SST2*-dependent adaptation pathway. The *SST2* gene promotes adaptation to pheromone independent of the C-terminal tail of Ste2 (10, 21), but its function remains elusive (41). It would be interesting to test whether the enhanced recovery phenotype of our Ste2 mutants is *SST2*-dependent. It is intriguing that mutant K239A is hypersensitive with increased affinity, whereas the double mutant R233A/K239A is not hypersensitive and has wild type affinity.

Only one mutant, F235A/D242A, appeared to have reduced ligand binding affinity (2-fold less than wild type). This mutant formed wild type size halos, but they exhibited turbid growth (Fig. 3), probably due to reduced G protein coupling and/or activated recovery. The Asp^{242} residue is unusual in that its substitution by alanine causes very different phenotypes, depending on what it is combined with. The D242A mutation alone had 3-fold increased affinity and small halos, whereas R234A/D242A exhibited 1.4-fold increased affinity and large halos, in contrast to F235A/D242 which has 2-fold reduced affinity and wild type halos.

Random Replacement Mutagenesis—There are two observations suggesting that the function of the Ste2p third cytoplasmic loop is mediated by some general structural feature of the loop rather than by specific residue side chains. First, the alanine scan of the third loop identified residues important for signaling, but did not implicate individual residues as critically important for mating. Also, Weiner *et al.* (32) reported three Ste2 third loop mutations that affected signaling and adaptation, but did not affect mating. Second, Ste3, the other *S. cerevisiae* pheromone receptor which presumably interacts with the same G protein as Ste2 (42), shares no amino acid homology with Ste2 in the third cytoplasmic loop (overall, these two receptors are quite dissimilar (43)). However, it has been suggested that the Ste2 and Ste3 receptors may share a structural motif in that their third cytoplasmic loops have the potential to form an amphipathic helix (3).

To analyze further the structural requirements for transducing the mating signal, we used an additional mutagenesis approach to probe the Ste2p third cytoplasmic loop. The random replacement mutagenesis method replaces stretches of codons with random sequence (25). We chose to mutagenize the three codons Ser^{232}, Arg^{233}, and Arg^{234} (all three residues were implicated in signaling by double alanine replacement mutants). A plasmid library was constructed by replacing 8 bp covering these codons with 8 bp of random sequence (Fig. 2). The result-

TABLE III
Random replacement mutants: functional analyses

Tests for Ste2 function were performed on plasmid-borne random replacement mutants in a *ste2Δ* strain. For the β-galactosidase assays, which were performed in triplicate, the standard errors are ≤25%. The high standard errors are likely due to the inherent instability of autonomous plasmids causing heterogeneity of the cell population. The high values (>150 units) are likely overestimates since they were not within the linear range of the assay. For all tests, we analyzed two independent transformants containing the mutant receptors and included two controls, a wild type Ste2 strain, and a strain that lacks receptor, *ste2Δ*.

Group	Allele	Sequence 232,233,234	Color on X-Gal[a]	β-Gal[b]	Mating	Peptide 2.5-cm halo	Turbidity of halo
				units		*ng*	
	STE2	Ser Arg Arg	Blue	145.0	+	280	None
	Ste2Δ	- - -	White	0.1	-	ND[c]	(No halo)
	65	Val Gln Gln	Blue	148.0	+	160	None
	150	Ser Gln Arg	Blue	227.0	+	350	None
	31	Ser Gln Lys	Blue	273.0	+	350	None
1	179	Phe Arg Thr	Blue	199.0	+	460	None
	67	Leu Gln Lys	Blue	189.0	+	460	None
	50	Leu Lys Arg	Blue	171.0	+	460	None
	9	Leu Gln Arg	Blue	258.0	+	410	None
	2	Leu Arg Arg	Blue	237.0	+	460	None
	7	Met Ala Arg	Blue	242.0	+	460	None
	48	Phe Gln Arg	Blue	168.0	+	460	Slight
	215	Val Ser Lys	Blue	202.0	+	610	Slight
2	11	Leu Ser Lys	Blue	156.0	+	460	Slight
	24	Leu Ser Lys	Blue	171.0	+	460	Slight
	27	Gly Ser Lys	Blue	168.0	+	460	Slight
	64	Ser Thr Lys	Blue	116.0	+	920	Moderate
	175	Gly Leu Lys	Blue	83.0	+	920	Moderate
	69	Gly Val Arg	Blue	78.0	+	530	Moderate
3	15	Leu Tyr Arg	Blue	65.0	+	1490	Moderate
	13	Val Asp Arg	Blue	89.0	+	1490	Moderate
	4	Ser Leu Thr	Blue	55.0	+	1140	Moderate
	206	Ala Ser Glu	Blue	22.0	+	3330	Moderate
	44	Val Asp Lys	Pale blue	32.0	±	ND	Severe
	23	Ser Thr Gly	Pale blue	17.0	±	ND	Severe
4	222	Trp Ser Gln	Pale blue	14.0	±	ND	Severe
	72	Ser Glu Glu	Pale blue	21.0	±	ND	Severe
	168	Ile Gln Arg	Pale blue	3.0	±	ND	Severe
	221	Arg Pro Ala	Pale blue	18.0	±	ND	Severe
	60	Ser Pro Gly	Faint blue	2.5	-	ND	(No halo)
5	28	Pro Leu Gly	Faint blue	1.0	-	ND	(No halo)
	55	Phe Leu Thr	Faint blue	0.5	-	ND	(No halo)
	21	Leu Phe Gln	White	0.3	-	ND	(No halo)
	26	Leu Glu Pro	White	0.5	-	ND	(No halo)
	75	Asp Pro Arg	White	0.5	-	ND	(No halo)
	8	stop codon	White	0.3	-	ND	(No halo)
	22	stop codon	White	0.1	-	ND	(No halo)
6	45	stop codon	White	0.1	-	ND	(No halo)
	47	stop codon	White	0.2	-	ND	(No halo)
	56	stop codon	White	0.3	-	ND	(No halo)
	57	stop codon	White	0.3	-	ND	(No halo)
	61	stop codon	White	0.2	-	ND	(No halo)
	242	stop codon	White	0.3	-	ND	(No halo)

[a] X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.
[b] β-Gal, β-galactosidase.
[c] ND, not determined.

ing library of mutagenized Ste2 clones was transformed into yeast strain CAY164, which carries ste2Δ and the reporter gene P_{FUS1}::*lacZ*.

Phenotypic Analysis of Random Replacement Mutants—Plasmids containing the Ste2 mutants were recovered from yeast, amplified in *E. coli*, and transformed back into CAY164 to ensure that the phenotype was conferred by the plasmid rather than by any spontaneous genomic mutations. Forty individual mutants chosen at random were sequenced, and analysis of the replaced nucleic acid sequences indicated that the mutagenesis was random, as expected. Individual transformants were analyzed for Ste2 function initially by checking their color on indicator plates plus pheromone (Table III). A range of response was observed, from dark blue colonies to white colonies. None of the mutants turned blue on indicator plates minus pheromone (*i.e.* no "constitutive" mutants were found). To quantitate the induction of the reporter gene P_{FUS1}::*lacZ*, we performed β-galactosidase assays on the mutants. In the presence of pheromone, a range of β-galactosidase activity was observed in the different mutants, from fully wild type or greater activity (≥145 units) to none, like the *ste2Δ* strain (0.1 unit) (Table III). No β-galactosidase activity was

found in the absence of pheromone (data not shown). The next test for function was the ability to confer mating with yeast of the opposite mating type (Table III). Some of the mutants mated like wild type Ste2, others exhibited defects in mating, and still others failed to mate at all. Finally, we determined the amount of growth arrest in a zone of inhibition assay and estimated the amount of pheromone required to produce a 2-cm halo diameter (Table III). We also observed the growth of colonies within the halo (turbidity) over a period of several days (Table III). On the basis of these five different but related phenotypic analyses, we classified the mutants into six functional groups, ranging from wild type levels (group 1) to nonfunctional (group 6) (Table III).

Group 1 members are indistinguishable from wild type Ste2 by all criteria except halo size. Most of these mutants showed slightly smaller than wild type halo size (*i.e.* the mutants required more pheromone to attain a 2-cm halo diameter). The single exception to this, which had larger than wild type halos, is mutant S232V/R233Q/R234Q. All of the group 1 mutants had clear halos, like wild type, indicating normal recovery pathways (however, the supersensitivity of S232V/R233Q/R234Q could be due to defective recovery).

Group 2 members closely resemble the group 1 members except that their halos, which were clear on day 2, began to develop turbid growth after 3–4 days. These mutants may have enhanced recovery.

Group 3 members show reduced expression of β-galactosidase from the reporter gene P_{FUS1}::*lacZ*. Also, their halos are turbid on day 2 and they appear to have significantly smaller halo diameters (although this result should be interpreted with caution, since turbid halos are difficult to measure accurately.)

Group 4 members exhibit decreased expression of β-galactosidase. In addition, the qualitative mating assay indicates reduced ability to mate. Their halos were barely detectable, and were too turbid to measure.

Group 5 members show markedly decreased expression of β-galactosidase, no detectable mating, and no halos.

Group 6 members completely lack receptor function and are indistinguishable from *ste2*Δ. In fact, most of these mutants were found to contain stop codons, indicating that truncations in the Ste2p third loop region are not functional.

Analysis of Amino Acid Replacements—To summarize the above phenotypic analyses of 41 random replacement mutants, we observed that eight of the clones in the random replacement library had stop codons (close to the theoretical expectation of 9/41). Thirty of the remaining 33 clones exhibited at least detectable levels of receptor function. Mutant receptors that possess even minimal activity must retain the basic fold of the receptor, although the structure might be altered to some degree. This indicates that most of the amino acid substitutions at positions 232, 233, and 234 are relatively neutral with respect to folding and/or stability of the receptor. However, the substitutions do have varying degrees of functional consequences, that could be due to reduced coupling to G protein, stability, or pheromone binding affinity of the mutant Ste2 receptors.

By correlating the amino acid substitutions of various mutants with the mating pathway phenotypes observed, one can obtain clues to the structural requirements at these positions. The most striking observation is the preponderance in the mutants that have a high level of function (groups 1, 2, and 3) of positively charged residues at position 234. If there were no functional preferences at this position, we would expect to see four Arg or Lys occurrences by random chance for this group of 21 mutants (note that His is not allowed at this position due to the random replacement technique). In contrast, we observe a much greater occurrence of Arg and Lys: 17/21. The probability that this distribution of codons encoding basic side chains would occur by random chance is $p < 0.005$ ($\chi^2 = 44$; 1 degree of freedom). Therefore, a basic side chain apparently is preferred at position 234 for high levels of function (although not absolutely required for function; see mutants S232V/R233Q/R234Q, S232S/R233L/R234T, and S232A/R233S/R234E). A double mutant described previously (R233S/R234G) that lacks a basic residue at position 234 exhibited defective coupling to G protein and activation of an *SST2*-dependent adaptation pathway (32). Mutants in groups 4, 5, and 6 have a random distribution of basic amino acids at 234.

TABLE IV

Genomic random replacement mutants:biochemical analyses

B_{max} indicates maximal binding to ^{35}S-α-factor expressed as a percentage of maximal wild type binding. Each set of assays included wild type (wt) as a control; the B_{max} values for wild type varied from 1100 to 3100 sites/cell. IC_{50} values indicate the concentration of unlabeled pheromone required to inhibit binding by 50%, as determined from competitive displacement plots (Fig. 5).

Strain	Sequence 232,233,234	Function group	Pheromone binding	
			B_{max}	IC_{50}
			% of wt	*nM*
CAY199 (wt)	Ser Arg Arg	1	100	19 ± 1
CAY200 (Δ)	- - -	6	0	ND[a]
CAY220	Val Asp Lys	4	34	1 ± 1
CAY221	Ser Thr Gly	4	44	13 ± 4
CAY222	Trp Ser Gln	4	37	7 ± 1
CAY223	Ser Glu Glu	4	14	4 ± 1
CAY224	Ile Gln Arg	4	24	7 ± 1
CAY218	Ser Pro Gly	5	41	4 ± 2
CAY219	Phe Leu Thr	5	0	ND
CAY217	Leu Phe Gln	6	0	ND

[a] ND, not determined.

At position 232, there appears to be a functional preference for uncharged side chains. Groups 1, 2, and 3 have more uncharged side chains than expected by random chance ($p < 0.025$; $\chi^2 = 6$; 1 degree of freedom). Groups 4, 5, and 6, which have reduced function, show a random distribution of charged *versus* uncharged amino acids.

It may also be significant that no prolines (at any position) are seen in groups 1, 2, and 3 ($p < 0.05$; $\chi^2 = 4$; 1 degree of freedom). There may be more proline residues than expected in groups 4, 5, and 6 ($p < 0.1$; $\chi^2 = 3$; 1 degree of freedom). Since proline interrupts α-helical structure, perhaps its presence reduces signaling by perturbing some secondary structure of the loop. In this context, it is noteworthy that modeling of all of the Ste2 random replacement mutants into α-helical structures did not indicate a functional requirement for helix amphipathicity.

Analysis of Genomic Random Replacement Mutants—Eight mutants from the functionally compromised groups 4, 5, and 6 were cloned into yeast integrating vectors and recombined into the genome, creating strains CAY217–CAY224 (Table I). Dominance tests indicated that the mutations in strains CAY217–CAY224 are recessive to wild type in a qualitative mating assay.

To assess the cell-surface expression of the genomic mutants, we performed equilibrium binding studies on intact cells using an excess of ^{35}S-labeled α-factor (70 nM). Specific binding measured under these conditions is indicative of receptor numbers (assuming that the affinities of the mutants are not reduced; see below). We compared the mutant strains CAY217–CAY224 to the wild type strain CAY199 (Table IV). Six of the mutants showed somewhat reduced receptor numbers, from 14 to 44% of wild type. It is unlikely that these reductions account for the severe loss of function observed in these mutants, since some of the alanine replacement mutants with similar levels of expression were able to function at nearly wild type levels. Two of the random replacement mutants (S232F/R233L/R234T and S232L/R233F/R234Q) fail to bind α-factor at all, suggesting

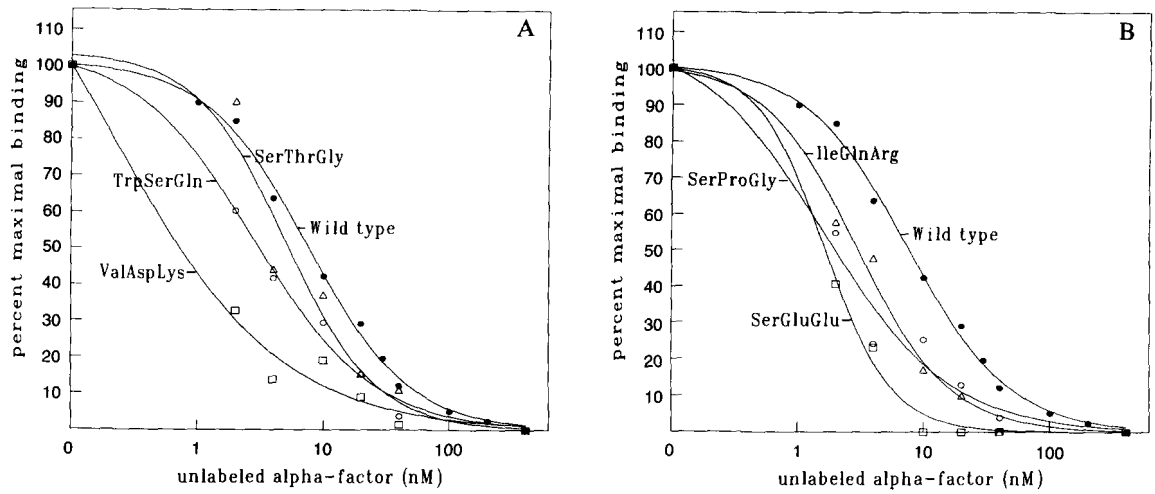

FIG. 5. **Competition binding of labeled α-factor to random replacement mutants.** Bound ^{35}S-labeled α-factor was displaced with increasing concentrations of unlabeled synthetic α-factor. For the Ste2 mutant alleles, the residues named are at positions 232, 232, and 234. The wild type *STE2* control is plotted on both graphs. Data points are the means of triplicate determinations.

that these mutants may be affecting folding and/or stability of the receptor.

To compare the pheromone binding affinity of the mutants to that of wild type, we did competition binding assays in the presence of ^{35}S-labeled and unlabeled α-factor (Fig. 5). We did not include the two mutants that fail to bind any α-factor. All of the mutants tested have IC_{50} values lower than wild type Ste2 (Table IV). Therefore, the loss of function phenotypes caused by these random replacement mutants are not due to reduced affinity for binding pheromone. Thus, since the group 4 mutants formed extremely turbid halos, these mutants may have incomplete activation of the G protein as well as activation of a recovery pathway. In addition, the group 5 and 6 mutants fail to form halos, which may indicate that these mutants are totally deficient in G protein activation.

In summary, our mutational scan of the Ste2 receptor third intracellular loop yielded a wide variety of mutant phenotypes and biochemical consequences. In principle, Ste2 receptor mutations could alter activation of the mating pathway via several potentially interrelated mechanisms. First, a mutant receptor's altered conformation could cause increased or decreased ligand binding affinity. Second, a mutation could affect the receptor's ability to associate with G protein. Third, the receptor's ability to activate the G protein could be affected by mutation. Fourth, a mutation could cause altered desensitization, through changes in receptor internalization and/or receptor uncoupling. For example, desensitization of the adrenergic receptors is regulated by kinases and arrestin proteins (reviewed in Refs. 44 and 45). Fifth, a mutation could affect the number of receptors expressed on the cell surface by altering stability, folding, or transport. Many of the Ste2 third intracellular loop mutants that we have studied exhibit phenotypes consistent with one or more of the above possible mechanisms.

The observation that no single amino acid side chain is absolutely required for mating suggests that the interaction between the third intracellular loop and the G protein (or other putative interacting protein) is not highly specific at the amino acid side chain level, but rather may be mediated by more general structural features. However, the loop is not insensitive to amino acid substitutions; even though the single and double alanine substitution mutants did not obliterate mating, several of them did affect the strength of activation of the mating response. In addition, all of the random replacement triple substitution mutants altered signal transduction in the pathway, ranging from subtle effects to complete loss of Ste2 function. These observations directly implicate the third intracellular loop in the mating signal transduction pathway in yeast. This study is a first step toward elucidating the structural requirements for function of the Ste2p third intracellular loop, with the eventual goal of understanding the physical interaction between the receptor and the G protein.

Acknowledgments—We thank Dr. Avi Ashkenazi for valuable advice and comments on the manuscript and Karen Levy and Stephen E. Bickel for technical assistance.

REFERENCES

1. Dohlman, H. G., Thorner, J., Caron, M. G., and Lefkowitz, R. J. (1991) *Annu. Rev. Biochem.* **60,** 653–688
2. Savarese, T. M., and Fraser, C. M. (1992) *Biochem. J.* **283,** 1–19
3. Blumer, K. J., and Thorner, J. (1991) *Annu. Rev. Physiol.* **53,** 37–57
4. Konopka, J. B., and Jenness, D. D. (1991) *Cell Regul.* **2,** 439–452
5. Kurjan, J. (1992) *Annu. Rev. Biochem.* **61,** 1097–1129
6. Marsh, L., Neiman, A. M., and Herskowitz, I. (1991) *Annu. Rev. Cell Biol.* **7,** 699–728
7. Burkholder, A. C., and Hartwell, L. H. (1985) *Nucleic Acids Res.* **13,** 8463–8475
8. Nakayama, N., Miyajima, A., and Arai, K. (1985) *EMBO* **4,** 2643–2648
9. Cartwright, C. P., and Tipper, D. J. (1991) *Mol. Cell. Biol.* **11,** 2620–2628
10. Konopka, J. B., Jenness, D. D., and Hartwell, L. H. (1988) *Cell* **54,** 609–620
11. Kurjan, J., Hirsch, J. P., and Dietzel, C. (1991) *Genes & Dev.* **5,** 11–22
12. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122,** 19–27
13. Sherman, F., Fink, G., and Lawrence, C. (1974) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153,** 163–168
15. Rothstein, R. J. (1983) *Methods Enzymol.* **101,** 202–211
16. Chan, R. K., and Ott, C. A. (1982) *Mol. Cell. Biol.* **2,** 11–20
17. Hicks, J. B. (1976) *Nature* **260,** 246–248
18. Ciejek, E., and Thorner, J. (1979) *Cell* **18,** 623–635
19. MacKay, V. L., Welch, S. K., Insley, M. Y., Manney, T. R., Holly, J., Saari, G. C., and Parker, M. L. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 55–59
20. Sprague, G. F., Jr., and Herskowitz, I. (1981) *J. Mol. Biol.* **153,** 305–321
21. Reneke, J. E., Blumer, K. J., Courchesne, W. E., and Thorner, J. (1988) *Cell* **55,** 221–234
22. Orr-Weaver, T., Szostak, J., and Rothstein, R. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 6354–6358
23. Rhodes, N., Connell, L., and Errede, B. (1990) *Genes & Dev.* **4,** 1862–1874
24. Boeke, J. D., Trueheart, J., Natsoulis, G., and Fink, G. R. (1987) *Methods Enzymol.* **154,** 164–175
25. Palzkill, T., and Botstein, D. (1992) *Proteins* **14,** 29–44
26. Hoffman, C. S., and Winston, F. (1987) *Gene* **57,** 267–272
27. Rose, M. D., Winston, F., and Hieter, P. (1990) *Methods in Yeast Genetics: A Laboratory Manual*, pp. 155–159, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Marsh, L. (1992) *Mol. Cell. Biol* **12,** 3959–3966
29. Barnes, D. A. (1985) *Isolation and Characterization of the LYS2 Gene and Its Use for Genetic Manipulation of the Yeast Saccharomyces cerevisiae.* Ph.D. dissertation, University of California, Berkeley, CA
30. Blumer, K. J., Reneke, J. E., and Thorner, J. (1988) *J. Biol. Chem.* **263,** 10836–10842
31. Cunningham, B. C., and Wells, J. A. (1989) *Science* **244,** 1081–1085
32. Weiner, J. L., Guttierez-Steil, C., and Blumer, K. J. (1993) *J. Biol. Chem.* **268,** 8070–8077
33. Trueheart, J., Boeke, J. D., and Fink, G. R. (1987) *Mol. Cell. Biol.* **7,** 2316–2328

34. Kjelsberg, M. A., Cotecchia, S., Ostrowski, J., Caron, M., and Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267,** 1430–1433
35. Boone, C., Davis, N. G., and Sprague, G. F. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9921–9925
36. Blumer, K. J., Reneke, J. E., Courchesne, W. E., and Thorner, J. (1988) *Cold Spring Harbor Symp. Quant. Biol.* **53,** 591–603
37. Miyajima, I., Arai, K., and Matsumoto, K. (1989) *Mol. Cell. Biol.* **9,** 2289–2297
38. Jenness, D. D., Burkholder, A. C., and Hartwell, L. H. (1986) *Mol. Cell. Biol.* **6,** 318–320
39. Blumer, K. J., and Thorner, J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4363–4367
40. Samama, P., Cotecchia, S., Costa, T., and Lefkowitz, R. J. (1993) *J. Biol. Chem.* **268,** 4625–4636
41. Dietzel, C., and Kurjan, J. (1987) *Mol. Cell. Biol.* **7,** 4169–4177
42. Bender, A., and Sprague, G. F., Jr. (1986) *Cell* **47,** 929–937
43. Hagen, D. C., McCaffrey, G., and Sprague, G. F. J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83,** 1418–1422
44. Lefkowitz, R. J., Inglese, J., Koch, W. J., Pitcher, J., Attramadal, H., and Caron, M. G. (1992) *Cold Spring Harbor Symp. Quant. Biol.* **57,** 127–133
45. Lefkowitz, R. J., Cotecchia, S., Kjelsberg, M. A., Pitcher, J., Koch, W. J., Inglese, J., and Caron, M. G. (1993) *Adv. Second Messenger Phosphoprotein Res.* **28,** 1–9