Residues in the First Extracellular Loop of a G Protein-coupled Receptor Play a Role in Signal Transduction*

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The Saccharomyces cerevisiae pheromone, α-factor (WHWQLKPGPQPMY), and Ste2p, its G protein-coupled receptor, were used as a model system to study ligand-receptor interaction. Cys-scanning mutagenesis on each residue of EL1, the first extracellular loop of Ste2p, was used to generate a library of 36 mutants with a single Cys residue substitution. Mutation of most residues of EL1 had only negligible effects on ligand affinity and biological activity of the mutant receptors. However, five mutants were identified that were either partially (L102C and T114C) or severely (N105C, S108C, and Y111C) compromised in signaling but retained binding affinities similar to those of wild-type receptor. Three-dimensional modeling, secondary structure predictions, and subsequent circular dichroism studies on a synthetic peptide with amino acid sequence corresponding to EL1 suggested the presence of a helix corresponding to EL1 residues 106 to 114 followed by two short β-strands (residues 126 to 135). The distinctive periodicity of the five residues with a signal-deficient phenotype combined with biophysical studies suggested a functional involvement in receptor activation of a face on a 3₁₀ helix in this region of EL1. These studies indicate that EL1 plays an important role in the conformational switch that activates the Ste2p receptor to initiate the mating pheromone signal transduction pathway.

G protein-coupled receptors (GPCRs) represent a widely distributed family of membrane proteins, ranging in size from 400 to 1000 amino acid residues in a heptahelical topological arrangement that mediates cellular responses to a variety of extracellular signals, such as light, ions, hormones, neurotransmitters, growth factors, and odors. GPCRs are believed to exist in several interchangeable conformations including resting and active states (5). The most widely accepted model to account for hormone action involves stabilization of the active (activated) conformation of the receptor as an outcome of ligand binding (6, 7). The transition into the active conformation results in changes in the physical state of the associated heterotrimeric G protein thereby activating diverse pathways such as protein kinases, adenylate cyclases, phospholipases, and ion channels and culminating in differential gene transcription and characteristic phenotypic changes in cellular physiology (8–11). In S. cerevisiae, binding of the α-factor to Ste2p, its cognate GPCR, initiates a protein kinase-mediated signal transduction cascade that leads to a change in cell morphology, growth arrest, and activation of a number of pheromone-responsive genes in preparation for cellular mating (12–16).

Given the overall common structural features among GPCRs, a detailed structural analysis using x-ray crystallography should bring insights into determinants of ligand binding and mechanisms of receptor activation. However, because of inherent difficulties in solubilizing and obtaining well diffracting crystals of the large, membrane-bound, lipid-associated GPCRs, such studies have been severely impeded. A noted exception is rhodopsin for which a high-resolution crystallographic structure has been determined (17). A crystal structure of the extracellular ligand-binding domain of the metabotropic glutamate receptor with and without the ligand has also been reported (18). An alternative to detailed crystallographic information on GPCRs has been the analysis of mutant receptors for structure–function studies. One area of intense study has been to determine the ligand-binding site as a means to understanding different states of receptors and how the occupation by ligand may initiate signal transduction.

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Detailed analysis of GPCRs has suggested that for larger ligands, such as glycoproteins and peptide hormones, extracellular domains contribute binding determinants (19–22), whereas for smaller ligands, such as catecholamines, the ligand binding pocket is formed by transmembrane domains (19, 23).

More than one extracellular region was found to participate in ligand binding to secretin, human P2Y₄, protease-activated thrombin receptor 1, human prosthaglandin E-prostanoid 2, and cholecystokinin-A receptors (24–29). An extracellular loop was proposed to provide determinants for ligand binding and/or ligand selectivity to prosthaglandin E-prostanoid 2, lutropin lutinizing hormone/chioriogondotropin, human follicle-stimulating hormone, human prosthaglandin E-prostanoid 2 and prostaglandin E-prostanoid 4, monocyte chemotractant protein-1 receptor 2, opioid receptor-like 1, and aminergic receptors (30–36). Involvement of extracellular loops in ligand binding has been demonstrated directly by labeling of loops with photoaffinity peptide probes (37–39). The NH₂ terminus of secretin receptor and corticotropin-releasing factor receptor 1 were found to play an important role in interaction with ligand (40–42). In addition, extracellular loops have been the focus for structural studies and modeling in several GPCRs (29, 43–46).

Finally, GPCRs have been identified in which large chimeric replacements of extracellular regions have caused dissociation of high affinity binding from receptor activation (24). A number of experiments have attempted to identify Ste2p residues or regions involved in ligand binding. Chimeric receptors between the closely related S. cerevisiae and Saccharomyces kluyveri α-factor receptors implicated the involvement of portions of EL1 (extracellular loop one), EL3, and the NH₂-terminal extracellular region of TM1 (transmembrane 1) in the specificity of ligand recognition (47, 48). Studies with random mutagenesis of Ste2p and screening for receptors responding to antagonists proposed an important role for F55 in TM1 in both ligand binding and signal transduction (49). Using site-directed mutagenesis of Ste2p and different α-factor analogues, Lee et al. (50) suggested that the tenth residue of α-factor was in close proximity to Ser⁴⁷ and Thr⁴⁸ in TM1 of Ste2p. Measurement of biophysical properties of fluorescent α-factors bound to Ste2p indicated that the binding pocket was formed by both hydrophobic and hydrophilic regions, suggesting contributions to ligand binding from both extracellular and transmembrane regions of the receptor (51). Mutational analysis of the putative glycosylation sites in the NH₂ terminus and on EL1 indicated that glycosylation was not required for pheromone binding and receptor activation (52). Finally, a recent photoaffinity labeling study identified a region of Ste2p spanning portions of TM6, EL3, and TM7 as the side of cross-linking to the side chain of position 1 of α-factor (53). Despite all of these studies, no previous analysis has discriminated specific residues in the extracellular loops involved in binding and/or receptor activation.

Here we report our studies on residues of EL1, the first extracellular loop of Ste2p. Cysteine-scanning mutagenesis of these residues revealed a region (residues 102–114) of the receptor that appears to be involved in signaling but that is not critical for ligand binding. Moreover, the key residues (102, 105, 108, 111, and 114) required for receptor activation exhibit a periodicity consistent with their spatial presentation on one face of a 3₁₀ helix.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids—**LM102 and LM23–3az yeast strains described by Sen and Marsh (47) were used in these studies. The genotype for the LM102 strain is: MATα, bar1, his4, leu2, trp1, met1, ura3, FUS1-lacZ:URA3, ste3-dl (deleted for the region coding for the α-factor receptor). LM23–3az has the same genotype as LM102 except that it contains an intact chromosomal STE2 gene coding for the α-factor.

**Subcloning of the FLAG™ Epitope and His6 Tag—**The first step in generation of the site-directed mutant Ste2 was the creation of a pGAS14.WT plasmid coding a Ste2p gene product tagged with the FLAG™ epitope and His₆ affinity sequence at the 3’ end of the STE2 gene. For this, the plasmid pNED described by David et al. (54) was used as the template for Cys-scanned site-directed mutagenesis of the receptor’s first extracellular loop, EL1.

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| Oligo Name | Sequence (5’ -3’)* |
|------------|--------------------|
| C59     | TGCTGTCATGACCTCTGCACTC |
| C55     | CGATCGATGACCTCTGCACTC |
| L008    | GGATCGATGACCTCTGCACTC |
| Y101    | GCTCTATCTCTCTGCACTC |
| L102    | GCTCTATCTCTCTGCACTC |
| L040    | CTTCTATCTCTCTGCACTC |
| N058    | CTTCTATCTCTCTGCACTC |
| V101    | CTCTGTCATGACCTCTGCACTC |
| S018    | CTCTGTCATGACCTCTGCACTC |
| L028    | CTCTGTCATGACCTCTGCACTC |
| N082    | CTCTGTCATGACCTCTGCACTC |
| H011    | CTCTGTCATGACCTCTGCACTC |
| L013    | CTCTGTCATGACCTCTGCACTC |
| L014    | CTCTGTCATGACCTCTGCACTC |
| Q014    | CTCTGTCATGACCTCTGCACTC |
| F014    | CTCTGTCATGACCTCTGCACTC |
| P114    | CTCTGTCATGACCTCTGCACTC |
| R114    | CTCTGTCATGACCTCTGCACTC |
| D120    | CGGATCGATGACCTCTGCACTC |
| S121    | CGGATCGATGACCTCTGCACTC |
| H127    | CGGATCGATGACCTCTGCACTC |
| G123    | CGGATCGATGACCTCTGCACTC |
| D125    | CGGATCGATGACCTCTGCACTC |
| H129    | CGGATCGATGACCTCTGCACTC |
| A130    | CGGATCGATGACCTCTGCACTC |
| T131    | CGGATCGATGACCTCTGCACTC |
| Q135    | CGGATCGATGACCTCTGCACTC |

*Used to generate STE2.FT.HT that is devoid of its native cysteine residues. This construct was referred to as the Cys-less STE2.FT.HT, which served as the template for Cys-scanned-site-directed mutagenesis of the receptor’s first extracellular loop, EL1.

**Codons for the mutated amino acid residues are shown in bold face and the mismatched base to incorporate the intended mutation is underlined.**

**Results**

Detection of the pheromone-induced growth arrest (halo assay), pheromone-induced gene expression (FUS1-lacZ assay), and determination of pheromone binding were done in the LM102 host strain. Both LM102 and LM23–3az strains carried the bar1 mutant allele, which inactivated the BAR1 protease responsible for degradation of α-factor, and a FUS1-lacZ gene which serves as a pheromone-inducible reporter. The WT STE2 gene with a native promoter was cloned into a yeast/bacterial shuttle vector pGA314.WT (49) and was used as a template strain for the subcloning of the FLAG™ epitope and His₆ tag and further site-directed mutagenesis of the α-factor receptor gene. This plasmid carried the TRP1 gene as a selectable marker and is a low copy CEN-based plasmid.

**Subcloning of the FLAG™ Epitope and His₆ Tag—**The first step in generation of the site-directed mutant STE2 was the creation of a pGAS14.WT plasmid coding a Ste2p gene product tagged with the FLAG™ epitope and His₆ affinity sequence at the 3’ end of the STE2 gene. For this, the plasmid pNED described by David et al. (54) was used as the source of the COOH-terminal epitope tags. STE2 in pNED contains an 81-bp region at its 3’ end that codes for the 24-bp FLAG™ epitope (DYKDDDDK), the 30-bp Gly-rich flexible tethers (TVGPRSGGS), the 18-bp His₆ affinity tag (HHHHHHH), and the 9-bp unrelated sequences (SSG) followed by a stop codon. The desired region of STE2 containing the 3’-tagged sequences was removed by digestion with SalI and the generated 121-bp fragment was gel purified using the MERmaid kit (Bio 101, Carlsbad, CA). The pGAS14.WT plasmid was digested with SalI, which produced a 6,272-bp fragment coding for the STE2 gene that lacked its 3’ end sequences. This fragment was dephosphorylated with shrimp alkaline phosphatase (U. S. Biochemical Corp.) and gel purified using Geneclean III kit (Bio 101). The 121-bp fragment purified from the pNED vector was ligated into the 6,272-bp fragment purified from the pGAS14.WT vector using T4 DNA ligase (Promega, Madison, WI). Correct orientation of the insert was con-
firmed by DNA sequencing. The 6.393-kb plasmid thus generated coded for the Ste2 gene with 3′ FLAGtag (epitope) and Hisa tag (HT) sequences, and allowed expression of Ste2 under its native promoter. This plasmid was referred to as pGA314.STE2.FT.HT and it served as the template for further site-directed mutagenesis.

**Construction of Cys-less Ste2p.FT.HT**—Single-stranded phagemid DNA of pGA314.STE2.FT.HT was prepared by witnessing an E. coli strain CJ236 (dut ung) carrying pGA314.STE2.FT.HT with the helper phage M13K07 (55). Oligonucleotide-directed mutagenesis of single-stranded phagemid DNA of pGA314.STE2.FT.HT was constructed as described previously (56, 57). The product of the mutagenesis reaction mixture was transformed into DH5α (Invitrogen) E. coli strain, and transformants were selected on ampicillin-containing plates. Plasmids were then isolated from transformants using the Wizard Miniprep kit (Promega). After sequencing of the isolated plasmids to confirm correct incorporation of the intended mutations (C59S and ard Miniprep kit (Promega). After sequencing of the isolated plasmids to confirm correct incorporation of the intended mutations (C59S and C252S), constructs were transformed into yeast strain LM102 (ste2 deletion strain), and transformants were selected by their growth on medium lacking tryptophan. This plasmid, referred to as pGA314.Cysless.STE2.FT.HT, was used as the template on which Cys-scanning mutagenesis of EL1 of Ste2p was performed.

**Cys-scanning Mutagenesis of EL1, the First Extracellular Loop of Ste2p—Preparation of single-stranded phagemid DNA of pGA314.Cysless.STE2.FT.HT and oligonucleotide-directed mutagenesis of single-stranded phagemid DNA of pGA314.Cys-less.STE2.FT.HT DNA were done as described in the previous section for pGA314.STE2.FT.HT. The product of the mutagenesis reaction mixture was transformed into E. coli strain DH5α (Invitrogen), and transformants were selected on ampicillin-containing plates. Plasmids were then isolated from transformants using the Wizard Miniprep kit (Promega). Each construct was subjected to sequencing to confirm correct incorporation of the intended mutations (single Cys substitution on each residue of EL1) prior to transformation into the ste2 deletion yeast strain LM102, and subsequent selection of transformants by their growth on the medium lacking tryptophan. In the case of partially active and nonfunctional cysteine-substituted mutant receptors (as determined by growth arrest assay, see “Results”), plasmids from three independent isolates were transformed into yeast to confirm that the observed phenotypes were because of the intended single cysteine mutation but not because of the presence of spurious mutagenesis.

**Measuring of Protein Expression Levels by Immunoblot**—S. cerevisiae strains LM102 (strain with the ste2 deletion) and LM102 (pGA314.STE2 or pGA314.mutantSTE2) (the strain containing a plasmid encoding Ste2p or mutant Ste2p) were grown, and membranes containing or lacking Ste2p and mutant Ste2p receptors were prepared as described previously (54). All manipulations with membranes and all protein preparation and analysis steps were carried out as described (28). Membranes were solubilized in 1% Triton X-100 (28), and solubilized membrane fractions were separated using 10% SDS-PAGE (50). The proteins were transferred to Hybond-P (Amersham Corp., Arlington Heights, IL). Western and chemiluminescence was used to detect Ste2p and mutant Ste2p, respectively. The Kᵢ for competition binding assays were calculated by using the equation of Cheng and Prusoff (64), where Kᵢ = IC₅₀/(1 + [ligand]/Kᵢ).

**PROSPECT**—PROSPECT is a computer package for finding an optimal alignment between a protein sequence and a protein structural fold (65). PROSPECT finds the globally optimal sequence-structure alignment and does so in an efficient manner, when considering both alignment gap penalty and pairwise potential between residues that are structurally conserved. The scoring function used in the fold recognition was given by PROSPECT (66). The atomic structures were generated using MODELLER (67) based on the alignments obtained from threading. PROSPECT has been applied successfully to many proteins with known experimental structures.

**Three-dimensional Modeling**—The first extracellular loop of Ste2p including a few residues on TM2 and TM3 was modeled in the range of residues 100–137 through the consensus of several tools for transmembrane domain prediction, including SOSUI (68) and MEMSAT (69). Secondary structure predictions for EL1 were carried out using two well known secondary structure prediction tools, i.e. PSIRED (70) and PHD (71), both of which gave similar results.
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RESULTS

Construction of a Functional, Tagged Ste2p Devoid of Cysteine Residues—COOH-terminal tagged Ste2p (FLAGTM epitope and His6 tag) has been constructed with wild-type behavior for both pheromone binding and activation of the signal transduction pathway (54, 74, 75). These tags were engineered to allow detection of receptors on Western blots and to serve for their selective purification using affinity chromatography. To create a template for Cys-scan mutagenesis, we engineered to allow detection of receptors on Western blots and signal transduction pathway (54, 74, 75). These tags were expressed as mean residue ellipticities (degree cm2/decimol).

Circular Dichroism (CD) Analysis—The CD studies of EL1 (103–132) were done from 260 to 190 nm under nitrogen purge using a 0.01-cm path length cylindrical cuvette. The spectra were recorded on an Aviv spectrophotometer (AVIV Associates, Lakewood, NJ), which was interfaced to a computer used for all mathematical calculations. The instrument was calibrated with D-(-)-10-camphorsulfonic acid and all measurements were made at peptide concentrations of 1 × 10^{-4} to 3 × 10^{-4} M. The concentration of the solution was determined spectrophotometrically using tyrosine as standard or by amino acid analysis. The extinction coefficient for Tyr at 280 nm was taken as 1340 (73). The CD intensities were expressed as mean residue ellipticities (degree cm2/decimol).

FIG. 1. Model of Ste2p showing the region targeted in this study. The three transmembrane domains are labeled 1–7. The three extracellular and intracellular loops are labeled EL1–EL3 and IL1–IL3, respectively. Also shown are the COOH-terminal FLAG and His6 tags, locations of mutated C598 in TM1 and C252S in TM6 (dark circles in the respective TM domains), and the region where Cys-scanning mutagenesis was performed over the first extracellular loop of Ste2p (bold face in EL1).

Dose-response curves from growth arrest assays. Paper discs were spotted with various amounts (μg) of α-factor, as indicated on the right panel and halo diameters were measured from plates, a representative of which is shown on the left panel. Amounts of α-factor (μg) spotted on the disc were: 1, 0.0625; 2, 0.125; 3, 0.25; 4, 0.5; and 5, 1. Halo for 2 μg of α-factor is not shown on the plate. Results represent the average from at least three independent experiments with a S.E. of ± 0.2 mm.

TABLE II

| Receptor        | Kᵢ (nM) | No. receptors/cell |
|-----------------|---------|--------------------|
| WT Ste2p        |         |                    |
| Ste2p,FT,HT     | 4.0 ± 1.5 | 10,341 ± 897 |
| C598-Ste2p,FT,HT | 4.7 ± 1.2 | 9,240 ± 592 |
| C252S-Ste2p,FT,HT | 7.5 ± 2.2 | 9,555 ± 698 |
| Cys-less Ste2p,FT,HT | 5.7 ± 1.2 | 9,363 ± 632 |

α Obtained from competition binding assays. Binding of α-factor as determined by saturation binding assays (Ki values) gave almost identical constants compared to the Ki values.

β Obtained from saturation binding assays.

γ WT Ste2p binding constants and cell surface receptor levels were taken from previously published results (12, 54, 58, 76–79).

<sub>nm</sub>(Table II). The complete tolerance of Ste2p to replacement of Cys598 and Cys252 with alanine (74) or isoleucine and alanine, respectively (75), has been reported previously. The substitution of either or both of the Cys residues with Ser residues did...
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Table III

Growth arrest assay results on Ste2p EL1 mutants (Lys100-Gln135)

| Receptor | mm Halo (0.5 μg -factor) | mm Halo (1.2 μg -factor) |
|----------|--------------------------|--------------------------|
| WT Ste2p | 18.5                     | 22.5                     |
| Ste2p FT.HT | 19.5                  | 23.0                     |
| K100C    | 19.0                     | 22.5                     |
| Y101C    | 22.0                     | 26.0                     |
| L102C    | 12.5                     | 14.5                     |
| L103C    | 18.5                     | 22.5                     |
| S104C    | 19.5                     | 22.5                     |
| N105C    | No halo                  | No halo                  |
| Y106C    | 18.0                     | 23.0                     |
| S107C    | 19.0                     | 23.5                     |
| S108C    | No halo                  | No halo                  |
| V109C    | 20.0                     | 24.0                     |
| T110C    | 19.5                     | 23.0                     |
| Y111C    | No halo                  | No Halo                  |
| A112C    | 19.0                     | 23.0                     |
| L113C    | 19.5                     | 23.5                     |
| T114C    | 10.5                     | 12.5                     |
| Q115C    | 20.5                     | 24.0                     |
| F116C    | 18.5                     | 22.5                     |
| P117C    | 20.5                     | 24.0                     |
| Q118C    | 19.9                     | 23.5                     |
| F119C    | 19.5                     | 24.0                     |
| I120C    | 18.5                     | 22.5                     |
| S121C    | 21.0                     | 24.0                     |
| R122C    | 21.0                     | 23.5                     |
| G123C    | 19.5                     | 23.5                     |
| D124C    | 21.0                     | 24.0                     |
| V125C    | 21.5                     | 23.5                     |
| H126C    | 21.5                     | 24.0                     |
| V127C    | 20.5                     | 23.5                     |
| V128C    | 20.0                     | 24.0                     |
| G129C    | 20.0                     | 24.0                     |
| A130C    | 19.0                     | 23.5                     |
| T131C    | 19.5                     | 23.5                     |
| N132C    | 20.0                     | 24.0                     |
| I133C    | 19.5                     | 23.0                     |
| I134C    | 20.5                     | 24.0                     |
| Q135C    | 20.5                     | 24.0                     |

* Halo diameters were also measured at 5 μg of -factor, but no halos were observed for any of these receptor mutants (data not shown).

Gene Induction Response of Cys-scanned Mutants—Signal transduction in response to binding of -factor to Ste2p also results in transcriptional activation of genes involved in mating. One of the early genes to be activated during the latter pathway was FUS1, which was involved in fusion of cells during conjugation. As a measure of response to activation of the mating signal transduction pathway, we tested the ability of 22 of our Cys substitution mutants to activate a FUS1-lacZ reporter gene construct (Fig. 3). Based on the percent maximal induction, E_max (activation obtained from -factor at 1 × 10^-6 M), most mutant receptors were able to activate the FUS1-lacZ reporter gene similarly to, if not better than, the Cys-less receptor. On the other hand, the previously identified five mutant receptors, described as partially active or inactive based on growth arrest assays, induced the reporter gene at much diminished levels. Among these, the two partially active mutant receptors, L102C and T114C, displayed relatively higher induction levels (about 20% of the WT Ste2p) than the three inactive mutant receptors, N105C, S108C, and Y111C, which were severely compromised in their ability to signal (about 10% activity in comparison to WT Ste2p). The dramatic reduction in maximum level of induction was an observation limited to the five mutant receptors. Cys substitutions at residue positions neighboring these five positions did not dramatically affect the function of the mutant receptors because they were able to signal at near normal levels. Interestingly, all mutant receptors tested with FUS1-lacZ assays, including the partially active and inactive mutant receptors, had potencies similar to the Cys-less receptor as determined by EC50 values (Table IV), which reflected the concentration of -factor required to give half-maximal induction.

Effect of Extracellular Loop Cys Replacements on Binding of -factor to Ste2p—We tested nine of the mutant receptors, including the five mutants compromised in signaling, for their ability to bind [3H]- -factor (Fig. 4). The number of cell surface receptors for each receptor tested was determined from saturation binding studies using whole cells, and the K values were determined from competition binding studies (Table V and Fig. 4 showing representative binding curves). These results show that all of these mutant receptors had indistinguishable binding affinities as compared with Cys-less Ste2p. The majority of the mutant receptors, however, were reduced in their expression at the cell surface as determined from saturation binding.

not result in any dramatic change in the number of receptors at the cell surface (Table II). Cys-less Ste2p, Ste2p with two cysteines, or Ste2p with one cysteine replaced by serine, all expressed about 9,000 receptors/cell as measured by saturation binding assays. These expression levels were in agreement with previously published results (12, 54, 58, 76-79). Likewise, Western blot analysis on membrane preparations using antibody showed that Cys-less receptor was expressed at levels similar to Ste2p FT.HT (data not shown).

Growth Arrest Response of Cys-scanned Mutants—Upon treatment with pheromone, cells containing WT Ste2p arrest cell division in the G1 phase of the cell cycle and therefore fail to grow on plates containing agar medium spotted with -factor. We assessed the ability of all our 36 Cys substitution mutants to arrest growth upon treatment with two different amounts of -factor (0.6 and 1.2 μg, Table III). Most mutations did not affect the ability of the mutant receptor to arrest growth. However, we identified five mutant receptors (shown in bold face in Table III) that displayed reduced or complete loss of biological activity.

Interestingly, starting with position 102 and ending at position 114, Cys substitution at every third residue resulted in a dramatic defect in signaling by the mutant receptors. Of these five mutants, L102C and T114C showed only partial activity, whereas the other three mutants, N105C, S108C, and Y111C, completely lost their ability to respond to pheromone growth arrest even at a high amount of -factor (5 μg; data not shown).

FIG. 3. Maximal FUS1-lacZ induction (%) of several Ste2p EL1 mutant receptors. Maximal induction (%) of each mutant was calculated with respect to Cys-less receptors when induced with 1 μM -factor. The labeling of the bars as to whether a mutant is active (hatched), partially active (gray), or inactive (black) was done based on the results from growth arrest assays. Data represent average from two to three independent experiments performed in duplicate with error bars representing mean ± S.E.

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Table IV

| Receptor  | $EC_{50}$ (nM) | % Maximal induction |
|-----------|----------------|---------------------|
| Cys-less  | 31 ± 4         | 100.0               |
| K100C     | 39 ± 8         | 117.0               |
| Y101C     | 25 ± 11        | 97.5                |
| L102C     | 28 ± 11        | 22.8                |
| L103C     | 25 ± 10        | 83.7                |
| S104C     | 33 ± 5         | 71.4                |
| N105C     | 30 ± 7         | 10.6                |
| Y106C     | 25 ± 12        | 98.4                |
| S107C     | 30 ± 2         | 77.4                |
| S108C     | 46 ± 13        | 8.2                 |
| V109C     | 25 ± 9         | 99.3                |
| T110C     | 25 ± 11        | 105.8               |
| Y111C     | 34 ± 2         | 11.2                |
| A112C     | 27 ± 10        | 115.6               |
| L113C     | 60 ± 21        | 78.5                |
| T114C     | 22 ± 13        | 17.6                |
| G115C     | 24 ± 11        | 87.2                |
| Q118C     | 37 ± 6         | 128.4               |
| R122C     | 35 ± 2         | 131.3               |
| V125C     | 31 ± 3         | 116.1               |
| V128C     | 42 ± 7         | 130.4               |
| T131C     | 40 ± 9         | 109.4               |
| Q135C     | 26 ± 12        | 95.8                |

* Cys-less receptor was generated in the Ste2p.FT-HT background. All other receptors with Cys substitutions were in the Cys-less Ste2p.FT-HT background.

Determined from FUS1-lacZ assays. Percent maximal induction (efficacy) of each mutant was calculated with respect to Cys-less receptor when induced with the highest concentration of $\alpha$-factor (1 nM). $EC_{50}$ (potency) reflects the concentration of $\alpha$-factor required to cause half-maximal activation.

assays (Table V) and as corroborated by Western blots (data not shown). For example, Cys-less Ste2p was expressed at about 9,000 receptors/cell, whereas mutants L102C, S108C, and Y111C were expressed at about 4,000 receptors/cell, and N105C and T114C were expressed at about 2,000 receptors/cell. Importantly, Q118C, a fully active receptor, was expressed at levels similar to L102C, S108C, and Y111C, receptors that were impaired in their signaling, and T114C, a partially active receptor, showed the greatest reduction in expression. Nevertheless, we were able to detect excellent ligand binding by all of these mutant receptors regardless of their expression levels. Moreover, we were able to detect excellent ligand binding by all of the mutant receptors, only L102C and T114C were able to cause a slight reduction (about 30%) in signaling by WT Ste2p. The other three receptors, N105C, S108C, and Y111C, showed no dominant negative effect indicating that the WT Ste2p was expressed and fully coupled to the signal transduction pathway. Saturation binding assays on whole cells of each co-expressed strain showed an increase of 1½–2-fold in the total cell surface receptor number when compared with WT Ste2p expression levels alone, indicating that both receptors were expressed at similar levels (data not shown).

Structure of EL1, the First Extracellular Loop of Ste2p—Biological activity and FUS1-lacZ reporter gene induction assays allowed us to identify five mutant receptors with diminished capacity in signaling despite full ability to bind $\alpha$-factor. A striking observation was the periodicity in the positioning of these five mutations (at every third residue in a portion of EL1) that resulted in a signaling deficient phenotype. To explore any structural tendency in EL1, predictions and three-dimensional modeling were done on a peptide sequence corresponding to residues Lys$^{106}$–Leu$^{137}$ of Ste2p. Because EL1 did not have significant sequence similarity to any proteins with known structures, PROSPECT (65) was used to predict its structure based on fold recognition. PROSPECT selected Protein Data Bank 1grj (84) as the best template with the sequence identity of 26.3%. The range in the template 1grj was between residues 117 and 156. An atomic model was built based on the alignment (Fig. 6). Although the confidence level of the prediction was not very high (60% confidence), modeling suggested that the NH$_2$-terminal half of EL1 may contain a $\beta$-sheet followed by a short $\beta$-sheet on the COOH-terminal half of the loop. Interestingly, positions of mutations that resulted in defective signaling in the five mutant receptors coincided very closely with the predicted helical region of the loop.

To obtain some biophysical evidence on the structure of EL1, we synthesized a 30-amino acid sequence corresponding to residues 103–132 of the loop region. The peptide contained a non-natural Gly residue at the carboxyl terminus to facilitate the solid phase synthesis. This peptide was found to have very low solubility in physiological buffers and aggregated strongly in water. We determined that solubility was pH-dependent and increased at higher pH values. Accordingly, we carried out CD analysis in both mixed organic aqueous medium and water, and buffer at pH above 8.0.

The CD spectrum of the EL1-(103–132) peptide in trifluoroethanol:water (8:2 [v/v]) was characterized by minima at 222 and 206 nm and a maximum at 190 nm (Fig. 7). The general shape of the spectrum was similar to those reported for $\alpha$-helical polypeptides. However, the mean residue ellipticities for EL1-(103–132) were approximately $-9700$ degree cm$^2$ dmol$^{-1}$ at 206 nm and $-6900$ degree cm$^2$ dmol$^{-1}$ near 222 nm. These are much lower than the mean residue helicities reported for $\alpha$-helices, and would be indicative of a low percentage of helical structure. When CD studies were carried out in water at higher pH values (adjusted to pH 9.2 by using 1% NH$_4$OH), the spectrum changed significantly with the distinct minima mentioned above changing in both position and relative intensity. For example, the higher wavelength minimum moved below 220 nm. The shape of the spectrum was not typical for any regular polypeptide and would be more consistent with a mixture of helices, $\beta$-sheet, and disordered structures. In 50 mM Tris buffer, pH 8.6, the peptide exhibited a CD spectrum manifesting a very broad minima from 200 to 220 nm that was also indicative of a mixture of secondary structures (Fig. 7). Overall, the CD spectra obtained in both mixed organic media and in water at pH greater than 8 indicated that the synthetic peptide assumed a variety of structures among which were likely helical and $\beta$-sheet elements.
DISCUSSION

An analysis of GPCRs from different families has suggested that peptide ligands interact with extracellular domains of receptors (19–22) unlike small GPCR ligands that bind exclusively in a pocket formed by transmembrane domains (19, 23). The role of extracellular domains in binding of peptides encouraged us to more closely investigate the contribution of Ste2p extracellular loops to ligand binding and receptor activation. The rationale behind focusing on EL1 came from studies by Sen and Marsh (47) and Sen et al. (48), who showed that EL1 contained determinants for ligand selectivity. We therefore decided to follow a systematic, site-directed mutagenesis analysis of EL1 over the entire loop.

For the systematic mutagenesis of EL1, we decided to use the Cys-scanning mutagenesis method, because Cys is average in bulk, highly amenable for subsequent modification using sulfhydryl specific reagents, and generally well tolerated as a replacement for all amino acids in GPCRs. When used in conjunction with biochemical and biophysical techniques, Cys-scanning methodology can provide valuable information on accessibility of residues to the aqueous and lipidic environments, on spatial proximity between domains, and on residues near or at binding crevices that are important for ligand interactions. These important properties have allowed successful use of the Cys-scanning mutagenesis method for several membrane proteins (85–87), including Ste2p (75).

In this study, we generated a library of 36 Ste2p mutants wherein each residue of the loop was replaced with Cys one residue at a time. Because the template used in this study was devoid of Cys, each resulting Ste2p mutant receptor contained only one Cys residue. Halo assays showed that the majority of the Ste2p receptors with mutations of EL1 residues displayed growth arrest indistinguishable from that of WT Ste2p (Table III). On the other hand, we identified five mutations (L102C, N105C, S108C, Y111C, T114C).

TABLE V.

Binding data on several Ste2p EL1 mutant receptors

The data are presented as mean ± S.E. of at least three independent experiments performed in triplicate for competition binding assays and in quadruplicate for saturation binding assays. Data for mutant receptors that are defective in signaling are shown in bold face.

| Receptor       | Biological activity | $K_i$  | No. receptors/cell |
|----------------|---------------------|--------|--------------------|
| C59S/C252S (Cys-less) | Fully active       | 5.7 ± 1.2 | 9,363 ± 632 |
| K100C         | Fully active        | 5.4 ± 1.4 | 6,122 ± 212 |
| L102C         | Partially active    | 5.9 ± 1.1 | 3,917 ± 301 |
| N105C         | Inactive            | 3.4 ± 1.8 | 2,720 ± 252 |
| S108C         | Inactive            | 3.3 ± 1.2 | 3,968 ± 291 |
| Y111C         | Inactive            | 4.7 ± 1.3 | 4,257 ± 308 |
| T114C         | Partially active    | 4.4 ± 0.7 | 1,365 ± 210 |
| Q118C         | Fully active        | 3.2 ± 1.3 | 3,855 ± 328 |
| Y128C         | Fully active        | 6.5 ± 1.3 | Not determined |
| Q135C         | Fully active        | 2.6 ± 0.9 | 7,407 ± 317 |

* C59S/C252S (Cys-less) receptor was generated in the Ste2p.FT.HT background. All other receptors with Cys substitutions were in the Cys-less Ste2p.FT.HT background.

* Determined by comparing the relative halo size of mutants to that of Ste2p.FT.HT at 1.2 μg of α-factor.

* Determined from competition binding assays. Binding of α-factor as determined by saturation binding assays ($K_i$ values) gave almost identical constants compared to the $K_i$ values.

* Determined from saturation binding assays.
our EL1 mutant receptors as to proficiency to activate a FUS1-lacZ reporter gene construct (Fig. 3), only the previously identified five mutant receptors exhibited severely diminished capacity to transduce signal. Mutations in neighboring receptor positions resulted in WT-like FUS1-lacZ induction levels. These results suggested that residues 102–114 of EL1 of Ste2p play an important role in the function of the receptor.

The compromised ability to signal observed for the five mutant receptors could result from improper folding, defective trafficking, reduced level of cell surface expression, and/or diminished or lost binding properties of these receptors. To delineate the specific reasons behind the observed signaling-deficient phenotype of these five mutant receptors, we performed a number of experiments on whole cells including saturation binding assays to quantify the number of cell surface receptors and their binding properties. Collectively, these studies showed that all of these five mutant receptors had WT-like binding affinities for α-factor despite some diminished cell surface expression. On the other hand, the apparent reduction in the cell surface expression of these mutant receptors did not correlate with the observed signaling-deficient phenotype. Our saturation binding studies showed that T114C, a partially active receptor, had the most dramatic reduction in cell surface expression (about 15% of Cys-less Ste2p levels), whereas N105C, S108C, and Y111C were expressed at higher levels (about 40% of Cys-less Ste2p levels) but were completely inactive for signal transduction. Also, the fully active Q118C was expressed at levels similar to the three inactive receptors. Furthermore, it was previously shown that unlike mammalian GPCRs whose function is regulated by cell surface expression levels, yeast cells that express anywhere from as low as 5% to as much as 20-fold excess of the normal level of receptors can transduce signal at near normal levels (80, 81). Taken together, these data indicate that the profound signaling defect of the five mutant receptors was not connected to their level of expression.

The five mutant receptors were able to bind α-factor with near WT affinities, but there was a problem for these receptors to initiate signal transduction. Therefore, we concluded that these five mutations on EL1 prevented Ste2p from assuming its activated state. To investigate this hypothesis, we tested effects of the five signaling-deficient mutant receptors on the function of WT Ste2p when co-expressed in cells. Of these five mutants, only the partially active receptors L102C and T114C were able to moderately interfere with the function of WT Ste2p, whereas the inactive receptors N105C, S108C, and Y111C had no effect even though they were expressed at the cell surface. Studies with previously identified dominant negative mutants of Ste2p concluded that the observed effects were because of the titration of G proteins from WT Ste2p (82, 83). In light of these observations, our studies with the five mutant

FIG. 5. FUS1-lacZ assays of WT Ste2p in the presence of partially active and inactive mutant receptors. Left panel, untransformed strain with intact chromosomal STE2 is referred to as WT STE2 (●). The strain transformed with the mutant receptor is represented by ◼/followed by the name of the mutant receptor. Symbols for the mutant receptors transformed into the WT STE2 strain are: □ Cys-less; ▲ N105C; △ S108C; ■ Y111C; □, L102C; and ◼, T114C. FUS1-LacZ induction (%) of each mutant was calculated with respect to the untransformed WT STE2 strain. Right panel, maximal induction (%) is determined from the data on the left panel with the highest concentration of α-factor (1 μM). Results represent average from two independent experiments performed in duplicate.

N105C, S108C, Y111C, and T114C) in the NH₂-terminal portion of EL1 that caused either partial or severe loss of biological activity of the mutant receptors. Of these, L102C and T114C displayed partial activity, whereas N105C, S108C, and Y111C completely lost their biological activities. When we tested 22 of
receptors suggested that L102C and T114C were able to interact weakly with G protein thereby reducing its interaction with WT Ste2p, whereas N105C, S108C, and Y111C were unable to interact with G protein. The regions of Ste2p known to interact with G protein are the third intracellular loop (IL3) and the distal part of the COOH-terminal tail (77, 88). Therefore, it is possible that the five mutations on EL1 may affect the ability of Ste2p to attain an activated state required for coupling to G protein to initiate signal transduction.

Interestingly, the five mutants manifesting a signaling-defective phenotype were observed at every third position of the loop starting from residue 102 and ending at residue 114. The observation of a distinctive periodicity in the signaling-deficient phenotype at every third position of EL1 from position 102 to 114 was provocative. We reasoned that this region of the loop might contain a $\beta_10$ helix, a secondary structure with 3 residues/tturn rather than the typical 3.6 residues/tturn of a standard $\alpha$-helix. Using PROSPECT, the region of EL1 between residues 105 and 115 was predicted to contain a $\beta_10$ helix, followed by a short $\beta$-sheet region between residues 125 and 136 of EL1. CD studies on EL1 (103–132) suggested that this peptide displayed different structural elements depending on the solvent and pH of the solution used and were thus inconclusive as to the presence of a $\beta_10$ helix at the NH2-terminal half of EL1. Further studies are needed to investigate this possibility.

Previously Schwartz (9) noted that despite an apparent absence of sequence homology between GPCRs, the lengths of extracellular and intracellular loops may be conserved, at least in the rhodopsin superfamily of GPCRs. When we compared extracellular loops of several GPCRs, we noticed that in general GPCRs contain two small extracellular loops and a third larger extracellular loop with no apparent conservation in the loop size relative to its position in the receptor. With this observation, we also noted that the size of rhodopsin EL2 was very similar to that of Ste2p EL1: 29 versus 30 residues, respectively. A crystal structure of rhodopsin in the ground state (17) shows that the extracellular loops fold around two twisted $\beta$-hairpins forming a compact domain, whereas the NH2-terminal domain contributes the second pair of $\beta$-strands. The two highly conserved Cys residues on EL1 and in the middle of EL2 of rhodopsin bring TM3 and TM5 together through a disulfide bond. In this compact structure, EL2 dips into the membrane and caps the retinal-binding site. Importantly, the stability of the activated state of rhodopsin relies strongly on retaining its retinal at the active site by $\beta$-sheet capping via the conserved disulfide bond between EL1 and EL2. Although EL2 is important for receptor structure and folding, it is not involved in ligand binding. Interestingly, our studies show that analogous to EL2 of rhodopsin, EL1 of Ste2p does not participate in binding of $\alpha$-factor but plays a very crucial role in the receptor activation. Our studies also provide the first genetic evidence that indicates that EL1 of Ste2p may have a unique structural fold wherein its first half forms a $\beta_10$ helix and its second half forms a short $\beta$-sheet structure. Obviously, the true three-dimensional structure of EL1 will require either direct biophysical studies on the intact receptor or high-resolution studies on more constrained peptide fragments of this receptor domain.

Structural analysis of extracellular loops of a few other GPCRs has been done using NMR solution studies and a variety of other methods. For example, in the cholecystokinin A receptor, structural studies using portions of TM6, TM7, and the EL3 that connects them showed that this loop has an $\alpha$-helical structure and that the ligand, cholecystokinin-8, interacts with this loop and TM6 (29). Solution structure of the homocysteine disulfide bond-constrained EL2 of human thromboxane $A_2$ receptor (TP) showed that its EL2 contains two $\beta$-turns in the middle of the loop (44). This loop was previously proposed to be involved in ligand binding. In another study, the structure of EL1 of angiotensin II AT1 receptor was determined using CD and fluorescence (45). These studies showed that EL1 of this receptor formed a $\beta$-turn in the middle of the loop and this loop played an important role in ligand binding. Finally, using a combination of CD, fluorescence, NMR, and molecular dynamic modeling on EL2 of the $\kappa$-opioid receptor in dodecylphosphocholine micelles, Zhang et al. (46) showed that this loop, previously proposed to be the ligand-binding site for dynorphin, is highly amphiphilic and contains a well defined helical structure and a $\beta$-turn in the middle of the loop. Consistent with the observations presented for EL1 of Ste2p in the present report, the studies on these other peptide-biding GPCRs clearly demonstrate that their extracellular loops may have distinct structural elements that are important for either ligand binding or overall structural stabilization and proper folding of the receptor.

Activation of GPCRs requires switching of the interhelical constraints that stabilize the inactive state to a new set of contacts in the activated state. The free energy for this activation process comes from binding of the ligand, which in turn results in activation of the G protein. Given that a signaling-deficient phenotype is observed at five periodically located positions of the NH2-terminal region of EL1, this loop of Ste2p may be important in forming part of the network of interhelical constraints that defines the off-state of a general transmembrane switch.

In conclusion, our studies with EL1 of Ste2p show that residues 102–114 of this loop are important in initiation of the pheromone signal transduction pathway in yeast but not in pheromone binding itself. To our knowledge, this is the first example of a GPCR, wherein specific residues in an extracellular loop contribute to the ability of the receptor to initiate signaling, leading us to surmise that specific residues of EL1 are involved in the attainment of the activated state of the receptor. Further studies with this loop and the other extracellular loops of Ste2p should bring additional insights into how this and other GPCRs are activated.

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Residues in the First Extracellular Loop of a G Protein-coupled Receptor Play a Role in Signal Transduction
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