The First Extracellular Loop of the Saccharomyces cerevisiae G Protein-coupled Receptor Ste2p Undergoes a Conformational Change upon Ligand Binding*

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In this study of the Saccharomyces cerevisiae G protein-coupled receptor Ste2p, we present data indicating that the first extracellular loop (EL1) of the α-factor receptor has tertiary structure that limits solvent accessibility and that its conformational changes in a ligand-dependent manner. The substituted cysteine accessibility method was used to probe the solvent exposure of single cysteine residues engineered to replace residues Tyr101 through Gln135 of EL1 in the presence and absence of the tridecapeptide α-factor and a receptor antagonist. Surprisingly, many residues, especially those at the N-terminal region, were not solvent-accessible, including residues of the binding-competent yet signal transduction-deficient mutants L102C, N105C, S108C, Y111C, and T114C. In striking contrast, two N-terminal residues, Y101C and Y106C, were readily solvent-accessible, but upon incubation with α-factor labeling was reduced, suggesting a pheromone-dependent conformational change limiting solvent accessibility had occurred. Labeling in the presence of the antagonist, which binds Ste2p but does not initiate signal transduction, did not significantly alter reactivity with the Y101C and Y106C receptors, suggesting that the α-factor-dependent decrease in solvent accessibility was not because of steric hindrance that prevented the labeling reagent access to these residues. Based on these and previous observations, we propose a model in which the N terminus of EL1 is structured such that parts of the loop are buried in a solvent-inaccessible environment interacting with the extracellular part of the transmembrane domain bundle. This study highlights the essential role of an extracellular loop in activation of a G protein-coupled receptor upon ligand binding.

G protein-coupled receptors (GPCRs) are ubiquitous in eukaryotes and have been found in a diversity of organisms ranging from yeast to humans. In most eukaryotes, GPCRs comprise 1–2% of the total genes in the genome (1) and participate in virtually all aspects of cellular physiology, including hormonal responses, neuronal transmission, and mediation of taste, smell, and vision (2). Modulation of GPCR function is a major pharmaceutical target, currently accounting for >30% of all drugs prescribed (3–5). Thus a thorough understanding of the nature of the receptor-ligand interaction and subsequent signal transduction is essential for the development of more effective and safer therapeutic agents.

The structural hallmarks of this diverse collection of receptors are seven membrane-spanning domains linked by extracellular and intracellular loops, oriented such that the N terminus is external to the cell and the C terminus is internal. Although the structure-function relationships in the membrane-spanning domains and the intracellular loop regions have been well examined in many GPCRs (6–11), few studies have focused on the importance of the extracellular loop structures and their role in receptor activation. In those studies where the extracellular loops were studied in detail, their role in binding ligand was the focus of the investigations (12).

GPCRs sense signals in the extracellular environment, transduce that information across the cell membrane, and trigger specific changes in intracellular physiology. Signal transduction is initiated when a specific ligand binds to its cognate GPCR. Ligand binding can occur within the bundle of transmembrane domains such as for the β-adrenergic receptor and/or with the extracellular domains as occurs for some peptide or protein ligands (10). Ligand binding promotes a conformational change in the receptor, resulting in the exchange of GTP for GDP on the α-subunit of the coupled heterotrimeric G protein. Because signaling depends on interactions between the intracellular G proteins and the receptor, molecular events occurring on the cytoplasmic side of the receptor have been a focus of attention (10, 13, 14). However, concomitant changes are undoubtedly occurring on the extracellular surface of the receptor, including the extracellular loops, and these events have not been systematically investigated.

With the elucidation of the crystal structure of the ground state of bovine rhodopsin (15), the role of the extracellular loops as integral to receptor structure and function has begun to...
EL1 in Ste2p

In yeast, the receptor for the tridecapeptide mating pheromone, a-factor, is the GPCR Ste2p. Our previous work has suggested that the first extracellular loop of Ste2p plays a critical role in receptor activation. Although mutation to cysteine of most residues in the Tyr101 through Gln135 EL1 domain had no effect on binding and signal transduction, mutation of residues Leu102, Asn105, Ser108, Tyr111, and Thr114 to cysteine resulted in receptors that could effectively bind pheromone, but they were partially (Leu102 and Gln135) or severely (Asn105, Ser108, and Tyr111) compromised with respect to signal transduction. The periodicity of every third residue leading to a compromised receptor suggested that this region had structural organization. Subsequent modeling and biophysical studies on a synthetic EL1 peptide indicated that residues 106–114 may form a 3_10 helix, whereas the extreme C terminus of the loop (residues 126–135) may contain _β_-strands (19). Thus this loop appeared to have a defined secondary structure, and we hypothesized that disrupting this structure resulted in a signaling-deficient receptor.

Here we further expand upon the functional role of EL1 in Ste2p by determining the solvent accessibility of the residues in this loop using the substituted cysteine accessibility method (SCAM) (20, 21). To apply this methodology, each residue in the first extracellular loop of a Cys-less Ste2p was mutated to Cys to generate a collection of 35 single Cys receptors. The accessibilities of EL1 single Cys receptors to MTSEA-biotin were compared in the ligand-bound and -unbound state. Our results indicate that many residues are not solvent-accessible, suggesting that EL1 is structured in a way that results in part of the loop being buried in a hydrophobic environment. Furthermore, the presence of pheromone influences the accessibility of specific residues in EL1, suggesting that this loop changes conformation upon binding of a-factor to Ste2p.

**EXPERIMENTAL PROCEDURES**

Strains, Media, and Plasmids—The *Saccharomyces cerevisiae* strain BJ521 was used in all assays (22). The relevant genotype is *MATα, prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 ste2::Kan^R*. For high level, constitutive expression of Ste2p, plasmid pBE1C was constructed from p424GPD, a 2-μm based shuttle vector with a GPD promoter, *CYC1* terminator, and TRP marker for selection in yeast (23). *STE2* containing C-terminal FLAG and His epitope tags was PCR-amplified from plasmid pNED (24) using primers that introduce a BamHI and EcoRI restriction site. The resulting PCR product was subcloned into the complementary sites of p424GPD to yield plasmid pBE1C. For use as a negative control in SCAM analysis, a Cys-less version of pBE1C was generated by *in vivo* ligation (25) of a Cys-less *STE2* PCR product amplified from pGA314.Cys-less.STE2.FT.HT (19) resulting in the generation of the plasmid pBE2C. For SCAM analysis, 35 single Cys mutants ranging from Tyr101 through Gln135 of EL were generated in the pBE2C background by *in vivo* ligation of PCR products from single Cys receptors already described (19). In addition, mutants T199C, Y203C, and Y266C were also constructed for use as controls. The sequences of all *STE2* inserts were verified by DNA sequence analysis completed by the Molecular Biology Resource Facility located on the campus of the University of Tennessee. Primers were purchased from Sigma Genosys or IDT (Coralville, IA). After sequence confirmation, constructs were transformed by the method of Geitz et al. (26) into yeast strain BJ521 (ste2-deletion strain), and transformants bearing the pBE2 constructs were selected by their growth in the absence of tryptophan on MLT medium (24). All media components were obtained from Difco and were of the highest quality available.

Whole-cell MTSEA Labeling, Membrane Preparation, and Immunoblots—Labeling with MTSEA-biotin (Biotium, Hayward, CA) was based on the method originally described by Lin et al. (27), with some modifications. Experiments were completed at least three times as described below. Cells expressing the various pBE2 Cys-scanned Ste2p constructs were grown in MLT overnight at 30 °C. Cells (200 ml) were harvested at mid-log phase, washed, and resuspended in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 3 mM KCl, 150 mM NaCl, pH 7.4). The absorbance (A_600) was determined, and 20–30 absorbance units were resuspended in a final volume of 1 ml of PBS. Cells were incubated on ice for 30 min in the presence or absence of a-factor (WHWLQLKGQPYB (28), where B indicates norleucine) or the des–Trp^3–des–His^2 antagonist (28) at a final concentration of 225 nM. Pheromones were synthesized and characterized as described previously (28, 29). For the Y101C and I120C receptors, an additional agonist ([Ala^3]–a-factor, 225 nM final concentration) and antagonist ([D-Ala^3]–a-factor, 225 nM final concentration) (30) were also tested along with the coumermycin antibiotic novobiocin (Sigma) at a final concentration of 2 μM. The cells were warmed to room temperature and then supplemented with MTSEA-biotin at a final concentration of 0.1 mM. The MTSEA-biotin was prepared immediately before use as a 20 mM stock in dimethyl sulfoxide. The reaction was stopped after 2 min by the addition of a freshly prepared aqueous cysteine solution to a final concentration of 10 mM, which brought the reaction mixture to pH 4, and was incubated on ice for an additional 5 min.

All subsequent steps were performed at 4 °C unless otherwise indicated. MTSEA-biotin-treated cells were pelleted, resuspended in PBS, and lysed by vortexing with glass beads. Following a low speed spin (700 _x_ 5, min) to remove cell wall debris,
unbroken cells, and glass beads, the resulting supernatant was centrifuged at high speed (15,000 × g, 30 min) to pellet membranes. The pellet was resuspended in PBS, and protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad). Membranes were solubilized in RIPA buffer (0.1% SDS, 1% Triton X-100, 0.5% deoxycholic acid, 1 mM EDTA in 1× PBS, pH 7.4) for 1 h at room temperature with end-over-end mixing. The solubilized, biotinylated proteins were collected on UltraLink Immobilized Strepavidin Plus beads (Pierce) by incubation overnight at 4 °C with end-over-end mixing. The beads were washed four times with ice-cold RIPA buffer, once with 2% SDS in PBS (room temperature), followed by a final wash with ice-cold RIPA buffer. During the washes the beads were resuspended and then allowed to settle by gravity for 20 min prior to removal of the supernatant. Bound proteins were extracted from the beads using SDS sample buffer (10% glycerol, 5% 2-mercaptoethanol, 1% SDS, 0.03% bromphenol blue, 62.5 mM Tris, pH 6.8, 55 °C, 5 min) and used for immunoblot analysis. Solubilized proteins were resolved by SDS-PAGE (10% acrylamide) along with pre-stained Precision Plus protein standards (Bio-Rad) and transferred to ImmobilonTM-P membrane (Millipore Corp., Bedford, MA). The blot was probed with anti-FLAG M2 antibody (Eastman Kodak Co.), and bands were visualized with West Pico chemiluminescent detection system (Pierce). Blots were imaged, and the total density of all Ste2p bands in each lane was determined using a ChemiDoc XRS photodocumentation system with Quantity One one-dimensional analysis software (Bio-Rad). To verify Ste2p expression levels, an aliquot of total membrane was solubilized in SDS sample buffer, fractionated by SDS-PAGE (2–20 μg/lane), and immunoblotted in parallel with the biotinylated proteins extracted from the beads.

Deglycosylation of Membrane Proteins—Total membrane proteins (5–25 μg) prepared as described above were resuspended in sodium phosphate (50 mM, pH 7.5), supplemented with 500 units of glycerol-free PNGase F (New England Biolabs, Inc., Beverly, MA), and incubated at 37 °C for 2 h. A negative control was run in parallel in which no enzyme was added prior to incubation at 37 °C. Upon termination of the incubation interval, the membranes were pelleted by centrifugation (15,000 × g, 10 min), and the resulting pellet was dissolved in SDS sample buffer. The samples were used for FLAG immunoblot analysis as described above.

Saturation Binding Analysis—Tritiated α-factor (10.2 Ci/mmol, 12 μl), prepared as described previously (29), was used in saturation binding assays on whole cells. BJS21 cells were grown to mid-log phase in MLT, harvested, washed twice with ice-cold YM1 medium (30), and resuspended at a final concentration of 4 × 10^7 cells/ml in YM1i (YM1 plus potassium fluoride (1 mg/ml), p-toluene-sulfonyl-l-arginine methyl ester (3.8 mg/ml), NaN_3 (0.6 mg/ml), bovine serum albumin (5 mg/ml) and tosyl-lysine chloromethyl ketone (1 mg/ml)). Cells (600 μl) were combined with 150 μl of ice-cold 5× binding medium (YM1i supplemented with 3 H-labeled α-factor) and incubated on ice for 30 min. The final 1× concentrations of pheromone ranged from 0.6 to 50 nM. Upon completion of the incubation interval, 200-μl aliquots of the cell/pheromone mixture were collected in triplicate and washed over glass fiber filter mats using the Standard Cell Harvester (Skatron Instruments, Sterling, VA). Retained radioactivity on the filter was counted by liquid scintillation spectroscopy. BJS21 cells lacking Ste2p, expressing only the empty vector (p424GPD), were used as a nonspecific binding control for the assays. Specific binding for each mutant receptor was calculated by subtracting the nonspecific values from those obtained for total binding. Specific binding data were analyzed by nonlinear regression analysis for single-site binding using Prism software (GraphPad Software, San Diego, CA) to determine the B_max value (receptors/cell) for each mutant receptor.

RESULTS

Expression Levels of Single Cys and Cys-less Receptors—To determine the accessibility of residues in extracellular loop 1 of Ste2p (Fig. 1), mutants with single cysteine residues engineered into positions 101–135 were subcloned into a yeast expression vector under the control of a constitutive GPD promoter. To verify that the mutant receptors were expressed, total membranes were isolated from yeast expressing each of the 35 single Cys mutant receptors and the Cys-less control, immunoblotted, and probed with FLAG antibody (Fig. 2). For most EL1 mutants, 2 μg of total cell protein was loaded per lane. Expression of Ste2p for these mutants (Fig. 2) was determined by quantitation of band density to range from 30 to 110% of the Cys-less control level. Mutants N105C, S108C, Y111C, and T114C were expressed at significantly reduced levels (7–21% compared with the Cys-less wild-type receptor as determined by quantitation of band density); thus 20 μg of total cell protein was loaded per lane to improve visualization and quantitation of these receptors. If only 2 μg of total membrane protein of these poorly expressed mutants had been loaded, the bands corresponding to monomeric Ste2p would have been barely visible on the blot.

In addition to reduced levels of expression in the total membrane fraction, the mutants N105C, S108C, Y111C, and T114C exhibited a different banding pattern as compared with that of the other mutant receptors. For the Cys-less control as well as the other well expressed mutants, Ste2p appeared as a set of three main bands (plus small amounts of some higher molecular weight oligomers), centered roughly about 55 kDa. Based on the primary amino acid sequence of the FLAG- and His-tagged construct used in these studies, the predicted molecular mass for Cys-less Ste2p is 50.8 kDa. Glycosylation of the protein accounts for the observed higher molecular weights of the Ste2p monomer (31). For the poorly expressed mutants, the Ste2p signal on the immunoblot is more diffuse, with signal for the monomer ranging from 52 to 70 kDa. Interestingly, these same mutants had been reported earlier to be compromised with respect to signal transduction, although they were expressed on the cell surface as evidenced by their ability to bind pheromone with affinities equal to that of the wild-type receptor (19).

Although the immunoblot analysis can verify that the EL1 mutants were expressed in the total membrane fraction, it does not provide information regarding their expression on the cell surface. Our previous studies indicated that the EL1 Cys-scanned mutants were expressed at the cell surface and...
bound pheromone with near wild-type affinity, indicating that the receptors were properly folded (19). In this study, to determine the cell surface expression of select EL1 Cys mutants under the control of a constitutive promoter in the BJ521 strain background, saturation binding experiments using whole cells were conducted (Table 1). All mutants assayed were expressed on the cell surface, the majority at wild-type levels. All mutants except S108C, T110C, Y111C, and T114C were expressed at greater than 50% of the Y101C mutant level. Many mutants that did not label with the MTSEA-biotin reagent (L102C, L103C, S104C, N105C, L113C, N132C, and I134C) were expressed at near wild-type levels, suggesting that the absence of labeling with the MTSEA-biotin was because of inaccessibility of the cysteine residue, rather than lack of cell surface expression. Nonlabeled receptors expressed at the cell surface at reduced levels (S108C, T110C, Y111C, and T114C) were still present at sufficient quantity to be detected if they had been biotinylated.

Deglycosylation of Signaling Compromised Mutants—Wild-type Ste2p is glycosylated at residues Asn25 and Asn32 (32). Although residues Asn46, Asn105, and Asn205 are all potential glycosylation sites, analysis by site-directed mutagenesis of these sites indicates that they were not utilized (32). When analyzed by SDS-PAGE, Ste2p migrates as a group of three bands, consisting of the two major glycosylated forms of Ste2p along with the unglycosylated form (31). To determine whether the banding pattern exhibited by the N105C, S108C, Y111C, and T114C mutants was because of differential glycosylation, membranes prepared from cells expressing these mutants were treated with PNGase F, which cleaves N-glycan chains from glycopeptides or glycoproteins. Membranes were incubated in the presence and absence of PNGase F for 2 h at 37 °C. Treat-
ment with the glycosidase resulted in a shift of the multiple high molecular weight bands to a single lower molecular weight species, which corresponds to that of deglycosylated Cys-less-Ste2p.

**TABLE 1**

Surface expression and MTSEA labeling of cysteine-scanned mutants of Ste2p

| Mutant       | % surface expression | % labeling |
|--------------|----------------------|------------|
| Cys-less     | 90 ± 12              | 0'         |
| Y101C        | 100 ± 6              | 100 ± 13   |
| L102C<sup>a</sup> | 119 ± 22             | 0          |
| L103C        | 95 ± 5               | 0          |
| S104C        | 89 ± 11              | 0          |
| N105C        | 100 ± 10             | 0          |
| Y106C        | 156 ± 19             | 90 ± 26    |
| S107C        | –                    | 3 ± 1      |
| S108C        | 9 ± 2                | 0          |
| V109C        | –                    | 6 ± 3      |
| T110C        | 20 ± 3               | 0          |
| Y111C        | 23 ± 4               | 0          |
| A112C        | –                    | 110 ± 19   |
| L113C        | 158 ± 16             | 0          |
| T114C        | 35 ± 3               | 0          |
| G115C        | –                    | 63 ± 15    |
| F116C        | –                    | 31 ± 10    |
| P117C        | –                    | 63 ± 9     |
| Q118C        | –                    | 25 ± 6     |
| F119C        | 80 ± 6               | 11 ± 4     |
| H120C        | 79 ± 6               | 2 ± 1      |
| S121C        | –                    | 8 ± 3      |
| R122C        | –                    | 54 ± 12    |
| G123C        | –                    | 78 ± 9     |
| D124C        | –                    | 18 ± 7     |
| V125C        | 62 ± 17              | 9 ± 3      |
| H126C        | 46 ± 7               | 9 ± 3      |
| V127C        | –                    | 15 ± 4     |
| Y128C        | 109 ± 13             | 121 ± 18   |
| G129C        | –                    | 5 ± 3      |
| A130C        | –                    | 6 ± 2      |
| T131C        | –                    | 0          |
| N132C        | 72 ± 9               | 0          |
| H133C        | –                    | 0          |
| I134C        | 81 ± 8               | 0          |
| Q135C        | –                    | 0          |

<sup>a</sup>Ste2p expressed on the cell surface was determined by saturation binding expressed as a percentage of receptor number normalized to the Y101C receptor (72,400 ± 5,000 receptors/cell).

<sup>b</sup>Ste2p recovered from streptavidin beads was expressed as a percentage of Y101C levels.

<sup>c</sup>0 indicates no detectable labeling.

<sup>d</sup>Boldface indicates signaling-compromised receptors.

<sup>e</sup> indicates that these measurements were not made in this study. Surface expression was verified for all receptors represented in this table by Akal-Strader et al. (19).

**FIGURE 3. Deglycosylation of signaling-deficient Cys-scanned receptors.** Total membrane proteins derived from cells expressing wild-type or signaling-deficient EL1 mutant receptors indicated were treated (+) with PNGase F as described under “Experimental Procedures” or incubated in parallel in the absence of enzyme (−) to control for degradation which might occur as a result of exposure to elevated temperature. The proteins were then analyzed by immunoblot analysis using the anti-FLAG antibody (5 μg for those indicated by boldface with asterisk and 25 μg for the remainder). In control lanes (C) membrane proteins were solubilized directly into denaturing sample buffer immediately prior to SDS-PAGE and subsequent immunoblot analysis. Molecular mass markers (kDa) are indicated on the left-hand side of each panel. The prominent bands visible at ~50 kDa corresponds to the monomeric forms of glycosylated Ste2p, whereas deglycosylated forms appear as more intense bands of reduced molecular weight.

FT-HT Ste2p (Fig. 3). Membranes that were not exposed to PNGase F during the incubation interval (Fig. 3, lanes labeled −) retained the banding pattern observed in membranes solubilized directly in sample buffer (labeled C). This indicates that the shift in the observed banding pattern to a lower molecular weight band associated with PNGase F-incubated receptors N105C, S108C, Y111C and T114C was because of enzyme-dependent removal of N-linked oligosaccharides, rather than a degradation artifact of prolonged incubation at elevated temperature. Although incubation at 37 °C in the absence of the glycosylase did not significantly change the banding pattern of the glycosylated Ste2p monomers, the intensity of the signal was reduced because of aggregation of Ste2p, as evidenced by the presence of high molecular weight bands.

**Differential Accessibility of Residues in the Presence and Absence of Pheromone Determined by SCAM Analysis—**Whole cells were treated with MTSEA-biotin to determine whether the cysteine residues engineered into EL1 were solvent-accessible, although presumably, in the absence of any tertiary structure, all extracellular loop residues should be readily accessible to this reagent. We chose to use whole cells for the SCAM analysis rather than isolated membranes to ensure that the topology of the receptor remained as close as possible to the intact native state. Accessibility was determined in both the presence and absence of α-factor or the des-Trp<sup>d</sup> des-His<sup<d>2</sup> antagonist to determine whether their presence altered the accessibility of the single Cys residue. The des-Trp<sup>d</sup> des-His<sup<d>2</sup> antagonist binds to WT Ste2p, but it does not initiate signal transduction (28). To prevent pheromone degradation and receptor internalization, the cells were maintained on ice during the 30-min pheromone incubation interval. Following exposure to MTSEA-biotin, membranes were prepared, solubilized, and incubated with streptavidin beads. Bound proteins were eluted in reducing sample buffer and immunoblotted. The immunoblots were probed with FLAG antibody, and the extent of labeling for each mutant in the presence or absence of pheromone was assessed. To verify that our method yielded results similar to those previously reported for SCAM as applied to isolated membranes containing Ste2p, we first determined the accessibility of the EL2 mutants T199C and Y203C, which had been described previously to be fully solvent-accessible, and the TM6 mutant Y266C which was not solvent-accessible (27, 33). Compared with the Cys-less receptor, the T199C and Y203C receptors were readily labeled following a 2-min exposure to MTSEA-biotin (Fig. 4), thus demonstrating that as in isolated membranes, these residues are fully accessible in whole yeast cells. In contrast, the Y266C receptor did not label under these same conditions, thus verifying the solvent-inaccessible nature of this residue (Fig. 4). Increasing the MTSEA incubation interval from 2 to 10 min
did not significantly change the extent of labeling or the accessibility of any of the control residues in our whole-cell model (data not shown). In our experiments we used an unbuffered cysteine solution to quench the MTSEA labeling reaction. As recently described by Choi and Konopka (34), the addition of cysteine as a source of excess thiol groups to rapidly quench the MTSEA labeling reaction must be carefully considered. For example, in one report using a mammalian system, addition of cysteine buffered to pH 7.4 caused disulfide exchange between cysteine and the sulfhydryl reagent resulting in loss of labeling (35). In contrast, using yeast cells, addition of unbuffered cysteine resulted in a reduction of the pH of the reaction mixture to 4, well below the 8.3 pKₐ value of the cysteine side chain, and thus cysteine exchange did not occur in this system (34).

Having verified that the whole-cell method yields results similar to those reported for SCAM analysis using isolated membranes, analysis of the entire EL1 loop (Tyr₁₀¹–Gln₁₃₅) was completed. Representative examples of immunoblots from a variety of mutants, exhibiting different degrees of labeling, are shown in Fig. 5. Intensities for all Ste2p bands in a lane for each mutant were quantitated. Because the Y₁₀₁C receptor was readily labeled in the absence of α-factor, this was assigned as the control (100% labeling) and the labeling of all other mutants was expressed as a percentage of this control (Table 1).

Examination of Table 1 reveals that many residues in EL1 are not solvent-exposed as evidenced by the lack of reaction with MTSEA-biotin. At the extreme C terminus, residues Ala¹³₀–Gly¹₃₅ were predicted to be part of TM3 (27); thus it is not unexpected that these residues were not solvent-accessible. However, in the remainder of the loop region, which would be expected to be solvent-accessible, 9 of 29 residues did not react with the MTSEA-biotin reagent, whereas an additional 6 of 29 residues labeled at less than 10% of the control level. Specifically, residues 102–105 at the extreme N terminus of the loop do not react with the sulfhydryl reagent, even though residues 104 and 105 are predicted to be in the extracellular space (Fig. 1). Interestingly, residue Y₁₀₁C, which has been reported to be within TM2, is readily labeled. Starting with Y₁₀₆C most positions of the EL1 up to A₁₃₀C react with MTSEA-biotin with the noticeable exception of S₁₀₈C, T₁₁₀C, Y₁₁₁C, L₁₁₃C, and T₁₁₄C. Three of these mutations, S₁₀₈C, Y₁₁₁C, and T₁₁₄C, along L₁₀₂C confer similar phenotypes, namely efficient ligand binding, but no signal transduction.

In the presence of pheromone, labeling of Y₁₀₁C is significantly reduced (Fig. 5, Fig. 6, and Table 2). The decrease in labeling was also observed if the receptor was incubated with the Ala⁵ variant of α-factor, an agonist with biological activity and affinity similar to that for wild-type pheromone (30). Interestingly, incubation with the antibiotic novobiocin, which has been reported to activate the signal transduction pathway via the Ste2p
EL1 in Ste2p

We now report that the cysteine accessibility of specific residues in EL1 was reduced in the presence of α-factor (60 ± 14% of control levels), although to a lesser extent than observed for Y101C and Y106C mutants, but labeling was not influenced by the antagonist (94 ± 20% control levels). Mutants D124C and V125C were similar to F119C with respect to degree of labeling in the presence of α-factor versus antagonist (Table 2). For the A112C mutant, the same reduction in labeling was observed in both the presence of α-factor and the antagonist. Decreased labeling in the presence of α-factor suggests that a pheromone-dependent change had occurred, rendering these residues less solvent-accessible.

In contrast to the decreased labeling in the presence of α-factor observed with the three receptors mentioned above, two receptors I120C (Fig. 5 and Table 2) and S121C (Table 2) labeled very poorly under control conditions yet produced noticeable labeling in the presence of α-factor. Treatment of the I120C mutant with the Alaα agonist yielded similar results, which can be clearly seen upon increasing the exposure time of the immunoblot (Fig. 6). The labeling of I120C and S121C was not affected by the des-Trp1 des-His2 antagonist and in the case of I120C by the D-Alaα antagonists (Fig. 6), again suggesting an α-factor-specific change in solvent accessibility. Similar to the observation made for the Y101C receptor, novobiocin did not influence labeling of the I120C receptor (Fig. 6).

**DISCUSSION**

Previously we reported that Cys-scanning mutagenesis of the residues in EL1 indicated that this extracellular loop is involved in signal transduction (19). Specifically, mutations of residues 102, 105, 108, 111, and 114 to cysteine results in a pheromone-specific change in solvent accessibility. Similar to the observation made for the Y101C receptor, novobiocin did not influence labeling of the I120C receptor (Fig. 6).

### Table 2

| Mutant    | Percent MTSEA-biotin labeling |
|-----------|-------------------------------|
|           | With α-factor                 | With antagonist               |
| Y101C     | 18 ± 5                        | 82 ± 15                       |
| Y106C     | 11 ± 3                        | 100 ± 16                      |
| S107C     | 94 ± 18                       | 87 ± 19                       |
| V109C     | 98 ± 22                       | 101 ± 24                      |
| A112C     | 62 ± 12                       | 62 ± 10                       |
| G115C     | 135 ± 32                      | 170 ± 35                      |
| F116C     | 135 ± 37                      | 137 ± 36                      |
| P117C     | 129 ± 31                      | 95 ± 17                       |
| Q118C     | 105 ± 27                      | 98 ± 21                       |
| F119C     | 60 ± 14                       | 94 ± 20                       |
| H120C     | 500 ± 100                     | 120 ± 30                      |
| S121C     | 463 ± 93                      | 97 ± 22                       |
| R122C     | 87 ± 17                       | 102 ± 18                      |
| G123C     | 88 ± 12                       | 121 ± 28                      |
| D124C     | 68 ± 18                       | 84 ± 15                       |
| V125C     | 52 ± 14                       | 75 ± 13                       |
| H126C     | 128 ± 33                      | 164 ± 41                      |
| V127C     | 85 ± 21                       | 93 ± 18                       |
| Y128C     | 26 ± 6                        | 65 ± 10                       |
| G129C     | 108 ± 19                      | 118 ± 30                      |
| A130C     | 132 ± 30                      | 186 ± 43                      |

α Labeling was expressed as a percentage of that determined in the absence of pheromone for each individual mutant. Mutants that did not react with MTSEA-biotin in the absence of pheromone were not included in the table. Percent labeling was calculated by quantitating total immunoreactivity of all bands on the FLAG immunoblot.

a Cells were incubated with α-factor prior to MTSEA-biotin exposure.

b Cells were incubated with des-Trp1 des-His2 α-factor antagonist prior to MTSEA-biotin exposure.

receptor in a pheromone super-sensitive strain background (36), had no effect on the accessibility of cysteine at position 101 (Fig. 6) suggesting that this nonpeptide agonist does not interact with the receptor in the same manner as do peptide agonists. To determine whether the decrease in accessibility, which was observed in the presence of the wild-type and Alaα agonists, was because of steric effects of the pheromone physically preventing labeling by MTSEA-biotin or to an α-factor-dependent change in conformation of the receptor, labeling was also completed in the presence of the des-Trp1 des-His2 antagonist, which binds to the receptor, but does not initiate signal transduction. In the presence of the des-Trp1 des-His2 antagonist, labeling of Y101C was not reduced, remaining close to the level observed in the absence of pheromone (Fig. 5 and Table 2). Incubation with another antagonist, [d-Ala3]α-factor (30), in which the N terminus of the peptide is intact, yielded results similar to those for the des-Trp1 des-His2 antagonist (Fig. 6). The effects of agonist and antagonist on MTSEA-biotin labeling were similar for the Y106C mutant. Other mutants exhibited a trend in labeling similar to, but less striking than, that observed for Y101C and Y106C, namely that labeling was reduced in the presence of α-factor but higher in the presence of antagonist. For example, the labeling of the Y128C mutant was greatly reduced in the presence of α-factor (26 ± 6% of control level) and although labeling was higher in the presence of antagonist (65 ± 10% of control level), it did not return to control levels. Labeling of the F119C mutant was also reduced in the presence of α-factor (60 ± 14% of control levels), although to a lesser extent than observed for Y101C and Y106C mutants, but labeling was not influenced by the antagonist (94 ± 20% control levels). Mutants D124C and V125C were similar to F119C with respect to degree of labeling in the presence of α-factor versus antagonist (Table 2). For the A112C mutant, the same reduction in labeling was observed in both the presence of α-factor and the antagonist. Decreased labeling in the presence of α-factor suggests that a pheromone-dependent change had occurred, rendering these residues less solvent-accessible.
ingly, residues L102C, N105C, S108C, Y111C, and T114C, previously shown to confer a unique phenotype to the receptor (19), did not label, even though they were adequately expressed at the cell surface (Table 1). This is despite the fact that other residues close to these positions of EL1 were labeled by MTSEA-biotin. Specifically, mutants Y101C, Y106C, A112C, and G115C, which are adjacent to the Leu105, Asn107, Tyr111, and Thr114 residues, respectively, were readily labeled. In addition, mutants S107C and V109C, which flank residue Ser108, exhibited very low but detectable labeling. Discounting residues Thr131–Gln135, which are predicted to be within TM3 and therefore should not be solvent-accessible, cysteine accessibility at the N-terminal region of EL1 was, in general, less than that for the C-terminal end of the loop. The differential labeling of EL1 residues is surprising as it is expected that all extracellular loop residues should be readily solvent-accessible. An alternative explanation for the lack of labeling of some mutant receptors is that the formation of Cys-Cys cross-bridges might form between EL1 regions of adjacent receptors, thus preventing MTSEA-biotin labeling. However, we feel that this possibility is remote, because at the N-terminal region of EL1, which is in general poorly labeled, there are specific residues that label very well (i.e. Y101C, Y106C, and A112C). If this lack of labeling of the other residues of this region (L102C, S104C, etc.) were due to the formation of disulfide bonds between neighboring EL1 regions, Y101C, Y106C, and A112C should behave in a similar manner.

One observation made during the course of this study was the unusual protein banding pattern observed for the N105C, S108C, Y111C, and T114C signaling-deficient mutants that we attribute to differences in receptor glycosylation (Fig. 3). In our experiments the yeast cell background used for expression of the mutant constructs was deficient in the proteases Pep4p, Prc1p, and Prb1p. A banding pattern similar to that observed for the four receptor mutants was also documented previously for the mutant A52T when expressed in a pep4 mutant background, but not in the wild-type background (31). In our prior experiments in which protease competent strains of S. cerevisiae were used, the multiple glycosylation forms were not detected (37). Pep4p encodes a vacuolar protease essential for proper maturation of many vacuolar enzymes (38) and thus is critical for the down-regulation and degradation of Ste2p in the vacuolar compartment (39). In the Pep4p mutant, the normal degradation pathway is not functioning. Because proteins are not being degraded properly, the result is that membrane proteins, including inappropriately glycosylated receptors, accumulate in intracellular compartments. How mutations in the specific residues of the EL1 domain results in differential glycosylation is unclear, but perhaps changing residues in EL1 influences the conformation of the N terminus of Ste2p and thus alters the glycosylation of the protein. We are currently engaged in experiments to address this issue.

The differential solvent accessibility of the residues in EL1 offers evidence that this loop has tertiary structure (19). There is evidence for structured loop regions in other GPCRs. In bovine rhodopsin, the prototypical GPCR, the crystal structure indicates that the large EL2 region is a tight anti-parallel β-hairpin that penetrates down into the transmembrane helix bundle to contact the 11-cis-retinal, forming a lid over the ligand (15). SCAM studies of the second extracellular loop in the dopamine D2 receptor suggest that this loop penetrates into the binding site in the TM helix bundle (40). The role that the extracellular loops play may extend beyond interactions with the TMs to include participation in the regulation of receptor function. Random saturation mutagenesis of the second extracellular loop of the complement factor 5a (C5a) receptor expressed in S. cerevisiae or mammalian cells suggests that this loop functions to stabilize the receptor in the inactive conformation, because most of the mutations yielded constitutively active receptors (18). Although all of the examples for GPCR loop structure cited above were described for EL2, in Ste2p EL1 is the longest of the extracellular loops and in that respect may function similarly to EL2 of other GPCRs.

Residues Tyr101 through Ser107 were examined in a prior SCAM analysis designed to define the extracellular boundary of TM2 (27). Interestingly, in that study residues Y101C and Y106C were only 35 and 50% accessible, respectively, when normalized relative to the T199C control, a residue in EL2; furthermore, residues that were completely nonreactive in our studies (L102C, L103C, S104C, and N105C) were determined to be 20–60% solvent-accessible. It is possible that the differences in the assay conditions exert an influence on the solvent accessibility of the residues. In our assay MTSEA-biotin labeling was performed on intact cells, whereas in the previous study isolated membranes were used. In fact, in our own pilot experiments performed on isolated membranes,4 MTSEA-biotin labeling appeared to vary among membrane preparations and did not result in a clear differentiation between residues that were either labeled or unlabeled in comparison to whole cells where distinctions were clear. The disruption of the membrane architecture, which must occur when membranes are prepared, could subtly alter the position of the transmembrane domains and the extracellular loops, making fine mapping of the extracellular-membrane boundaries difficult. Underlining the importance of examining receptors in their native environment is the recent finding that formation of disulfide bonds between transmembrane domains in the M3 muscarinic acetylcholine receptor was much more restricted when examined in its native lipid environment rather than as solubilized reconstituted receptors (41). Based on our observations with cysteine accessibility of residues at the N-terminal end of EL1, we propose that residue Tyr101 is positioned at the extracellular boundary of TM2, rather than deeper in the transmembrane bundle as has been proposed (27). At the C-terminal end of EL1, our results are in close agreement with those published previously (27).

An important observation made in this study is that the labeling of specific residues (Y101C, Y106C, F119C, V125C, and Y128C) was diminished, whereas others (I120C and S121C) was greatly enhanced by the presence of α-factor (Table 2). In the MTSEA-biotin labeling experiments, cells expressing the same receptor were treated in parallel with either agonist or antagonist, and the extent of labeling under these two conditions compared with each other and to the labeling observed in the

4 M. Hauser, S. Kauffman, B.-K. Lee, F. Naider, and J. M. Becker, unpublished results.
absence of pheromone. Thus, even though the cell surface expression of the various mutant receptors might differ from one another, making comparison of relative cysteine accessibility difficult within a single experiment, changes in cysteine accessibility for a particular mutant in the presence and absence of pheromone can be determined. If that particular mutant reacts with MTSEA-biotin differently in the presence of agonist and/or antagonist, then it is the ligand that is modulating accessibility, and in this case somehow influencing the structure of EL1.

The accessibility of residues 101 and 106 were greatly reduced in the presence of α-factor. The Ala53α-factor agonist, which binds the receptor with high affinity ($K_i = 60 \text{ nM}$) and can fully activate the signal transduction pathway (30), likewise resulted in reduced accessibility when incubated with the Y101C receptor. This raised the possibilities that either the presence of pheromone physically prevented MTSEA access to the cysteine residue or that pheromone binding induced a conformational change in the receptor that influenced cysteine accessibility. To distinguish between these two possibilities, the receptors were treated with the des-Trp3des-His2 antagonist known to bind to the receptor and compete with the binding of α-factor (28). If decreased cysteine reactivity were because of direct occlusion of these residues by the steric bulk of the α-factor tridecapeptide, then the presence of the antagonist would likely also restrict cysteine accessibility. Because the antagonist did not alter accessibility for residues Y101C and Y106C, we propose that the change in accessibility is because of a conformational change that occurs in the EL1 region of the receptor upon ligand binding. In earlier work, we have demonstrated that TM1 contacts with the C terminus of α-factor whereas TM6 interacts with the N terminus of the pheromone (22, 42–45). Because the des-Trp3des-His2 α-factor antagonist lacks only the first two N-terminal residues and because we (46, 47) and others (33) have indicated that the residues at the N terminus of α-factor interact strongly with Asn205 and Tyr266, which are in TM5 and TM6, respectively, we feel it is highly unlikely that residues on the N terminus of α-factor could interact with EL1 residues near the extracellular face of TM2. An additional experiment was completed using the Y101C receptor using the pheromone antagonist [d-Ala5]α-factor. Unlike the des-Trp3des-His2 antagonist, which is truncated at the N terminus, this antagonist is full-length with a d-isomer of alanine substituted for the native glutamine at position 3. This antagonist binds to the receptor but does promote signal transduction. The results using this antagonist were the same as that obtained with the truncated antagonist. Further evidence that a conformational change in the EL1 region occurs upon binding is the observation that labeling of I120C and S121C receptors as determined by cysteine accessibility. To distinguish between these two possibilities, the receptors were treated with the des-Trp1des-His2 antagonist which did not occur upon binding of antagonist (48). Biophysical analysis of a synthetic peptide of the second extracellular loop of the human thromboxane A2 receptor determined its structure and indicates that a change in loop conformation occurs upon interaction with the ligand for the receptor (49). The possibility also exists that the ligand-induced changes in cysteine accessibility described for the EL1 mutants in Ste2p is the result of conformational changes in other domains of the receptor that somehow indirectly alter the accessibility of cysteine residues in this extracellular loop. To investigate such an intricate model would require knowledge of Ste2p structure in both the active and resting states, which is not available for any GPCR at this time. However, our data indicate that, whatever the mechanism, interaction with pheromone alters the conformation of EL1, and those changes are reflected by differential cysteine accessibility.

Novobiocin, a member of the coumermycin family of antibiotics, is an inhibitor of DNA gyrase (50). It was reported that this nonpeptide molecule could activate the mating pheromone pathway in an Ste2p-dependent manner in a strain that is hypersensitive (sst2Δ) to pheromone (36). Novobiocin also initiated signaling across the F204C and Y266C Ste2p mutant receptors, which normally do not propagate the signal transduction response, when expressed in a sst2Δ background (33). In our experiments, we demonstrated that the presence of novobiocin did not influence the labeling of the target cysteine residues in the Y101C or I120C receptors, expressed in the SST2 background, giving the same response observed in the absence of pheromone (Fig. 6). An elevated concentration of novobiocin was used (22 μM; 100× higher than pheromone) because this compound is a very weak agonist (33, 36). Thus we propose the interaction of novobiocin with the signal transduction system does not change the conformation of EL1 in Y101C and I120C receptors as determined by cysteine accessibility.

Based on our observations of cysteine accessibility in the presence and absence of pheromone, combined with our earlier biophysical studies, we have generated a model for the first extracellular loop of Ste2p (Fig. 7). Our original investigations into the role of EL1 in receptor signaling indicated that residues 106–114 of EL1 form a 310 helix followed by two short β-strands (residues 126–135) (19). In this study, we demonstrate that in the inactive state of the receptor residues 107–114, with the exceptions of residue Ala112, are either not or minimally (<10% of Y101C, see Table 1) solvent-exposed (Fig. 7A). Upon ligand binding, a shift in conformation occurs that results in decreased solvent accessibility of Tyr101, Tyr106, and Ala112. Based on our current data we propose that residues 106–114 comprising the proposed 310 helix are parallel to the plane of the membrane (Fig. 7B) with residues 101, 106, and 112 positioned at the interface with the extracellular surface of the TMs, thus providing ready access to the MTSEA-biotin reagent (Fig. 7B). Although we cannot exclude the possibility that the N-terminal portion of EL1 (Fig. 7B, depicted as a cylinder) is embedded in the lipid bilayer, in our model we hypothesize that most residues are buried within the TM bundle, positioned such that Thr114 is in proximity to Tyr266 and Asn205. For this to occur, the 310 helix must lay across the top of the TM bundle. Recently a model describing interactions between EL2 and TM6 for the thyroid-stimulating hormone

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**EL1 in Ste2p**

April 6, 2007 • Volume 282 • Number 14
We have positioned residue Tyr$^{111}$ such that it is grouped with residues Tyr$^{266}$ and Asn$^{205}$. Mutations of Tyr$^{266}$ and Asn$^{205}$ still allow for pheromone binding, but do not signal, and are extremely intolerant to amino acid substitutions at these positions, and we have demonstrated that these residues physically interact (46, 47). Previous data with the signaling-compromised 102, 105, 108, 111, and 114 cysteine mutants indicate that alanine substitution is tolerated and results in at least partially active receptors at all but position 111, indicating the Tyr$^{111}$ residue is essential for protein function (37). Because the phenotype resulting from mutations at Tyr$^{266}$ and Asn$^{205}$ are similar to that for Y111C, we postulate that these three residues are part of a hydrophobic pocket that has been described to be involved in ligand activation in other GPCRs (52, 53). Because many of the well characterized dominant-negative mutations in Ste2p have been mapped to the junctions between the extracellular loops and the TMs (27), our data provide some insight into this observation. Based on our model, it can be postulated that the conformational change in the receptor upon ligand binding may generate coordinated interactions between the solvent-inaccessible domains of EL1 and those residues in which mutations influence the receptor activation process.

The results presented in this study add to the growing awareness that the functions of GPCR extracellular loop regions are not solely restricted to the binding of ligands. We have shown that residues of EL1 of Ste2p are involved in propagating the signal subsequent to ligand binding and that they may be involved in forming an activation pocket or domain of the receptor required for conformational change necessary for receptor signaling.

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