Interacting Residues in an Activated State of a G Protein-coupled Receptor

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The tridecapeptide α-factor pheromone (1WHWLQLKPGQPMY13) of Saccharomyces cerevisiae and Ste2p, its cognate G protein-coupled receptor (GPCR),3 have been used extensively as models for peptide ligand-GPCR structure and function (1). A major goal of GPCR studies is to ascertain the interactions between ligand and receptor to aid in the identification of analogs used for modulation of receptor activity and to understand how the receptor activates its signal transduction pathway. GPCRs are extremely important for medicine as they represent the target for the majority of prescribed drugs (2).

To identify Ste2p residues or regions involved in α-factor binding, a number of experiments have been performed. Chimeric receptors between the closely related S. cerevisiae and Saccharomyces kluyveri α-factor receptors implicated the involvement of portions of EL1 (extracellular loop 1), EL3, and the N-terminal extracellular region of transmembrane 1 (TM1) in the specificity of ligand recognition (3, 4). Our group using site-directed mutagenesis of Ste2p and binding assays with different α-factor analogs suggested that portions of TM1 and TM6 were important for ligand interaction (5) and that the tenth residue of α-factor is in close proximity to Ser47 and Thr48 in TM1 of Ste2p (6). We found that Tyr266 in TM6 plays an important role in the recognition of the N terminus of α-factor as well as switching of Ste2p into an activated state upon agonist binding (7), and using photo-affinity labeling we suggested that the N terminus of α-factor interacts with a region of the receptor spanning the upper portions of TM5 and TM6 and the connecting EL3 (8). Studies with α-factor analogs and fluorescently modified α-factor indicated that the binding environments of the position one (9) and position three side chains were hydrophobic, whereas the seventh residue was exposed to a partially hydrophilic environment suggesting that the middle portion of the pheromone was not buried in the transmembrane domains (10, 11).

Experiments to identify Ste2p residues and regions involved in receptor activation and signaling have been extensive. An interaction between TM5 and TM6 has been identified by a Cys-cross-linking experiment (12), and other interactions between and among transmembrane domains and between specific residues in transmembrane domains have been demonstrated by genetic experiments (13–15). In addition, extensive searches for dominant-negative mutations in the receptor revealed that most mutants exhibiting this phenotype are mapped to the extracellular ends of the transmembrane domains, especially TM5 and TM6 (16–18). A detailed and elegant analysis concluded that Phe204 in the EL2 loop was important for ligand binding and that Tyr266 at the extracellular end of TM6 was involved in signal transduction (19).

Based on these previous Ste2p findings, we initiated studies described in this communication to map residues interacting with Tyr266 using alanine-scanning mutagenesis of the EL2-TM5 interface (residues Gln200 to Ile209). All mutant receptors were subjected to detailed functional and ligand binding assays. Our results revealed that Asn205 is critical for signal transduction, and based on side-directed mutagenesis and disulfide cross-linking studies we deduced that Asn205 interacts with Tyr266 in a receptor that is activated by mutation to the constitutively activated state.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—LM102 and LM23–3az yeast strains described by Sen and Marsh (3) were used in these studies. The geno-
Interacting Residues of Ste2p

type for the LM102 strain is MATa, bar1, his4, leu2, trp1, met1, urs3, FUS1-lacZ::URA3, ste2-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 receptor. LM23−3az was used only to study dominant negative effects of certain mutant receptors on the function of wild-type (WT) Ste2p (see “Results”). The LM102 strain was used as the recipient for the transformation with WT and the site-directed STE2 mutants. Measurement 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. The WT STE2 gene with a native promoter was cloned into a yeast/bacterial shuttle vector pGA314.WT (5) and was used as a template for the site-directed mutagenesis of the α-factor receptor gene. The plasmid pGA314-T7 was constructed for the disulfide-cross-linking experiment by homologous recombination between pGA314 and pPD225−t7, which was used previously for the identification of interaction between TMS5 and TM6 of Ste2p (12). Ste2p encoded by pPD225−t7 contains no cysteine residues and is identical to wild-type Ste2p in its biological activity. In this report we refer to the Cys-less, T7-tagged Ste2p as WT. The plasmid pMD82 (16), which overexpresses G-αβγ, was a kind gift from Dr. James Konopka, State University of New York, Stony Brook.

Site-directed Mutagenesis—Single-stranded phagemid DNA of pGA314.WT was prepared by infecting Escherichia coli strain CJ236 (ung-, dut-) carrying pGA314.WT with the helper phage M13KO7 (20). Oligonucleotide-directed mutagenesis of single-stranded phagemid DNA was conducted as described by Kunkel et al. (21). After sequence confirmation, constructs were transformed into yeast strain LM102 (ste2-deletion strain), and transformants were selected by their growth in the medium lacking tryptophan. All primers were purchased from Sigma/Genosys (Woodlands, TX) and Integrated DNA Technologies Inc. (Coralville, IA). DNA sequencing was carried out in the DNA sequencing facility located on the campus of the University of Tennessee.

Growth Arrest (Halo) Assay—Yeast nitrogen base medium (Difco) without amino acids (SD medium) supplemented with histidine (20 µg/ml), leucine (30 µg/ml), and methionine (20 µg/ml) was overlaid with 4 ml of cell suspension (2.5 × 10⁶ cells/ml of Nobel agar). Filter disks (sterile blanks from Difco), 7 mm in diameter, were impregnated with 10-µl portions of peptide solutions at various concentrations (adjusted by molar extinction coefficient) and placed onto the overlay (22). The plates were incubated at 30 °C for 24−36 h and then observed for clear zones (halos) around the disks. The halo measured included the diameter of the disk. All assays were carried out at least three times with no more than a 2-mm variation in halo size at a particular amount applied for each peptide. The data were plotted as halo size versus the amount of peptide and linearized by regression analysis using Prism™ software (GraphPad, San Diego, CA).

FUS1-lacZ Gene Induction Assay—S. cerevisiae LM102 contains a FUS1-lacZ gene that is inducible by mating pheromone. Cells were grown overnight in SD medium supplemented with the required amino acids at 30 °C to 5 × 10⁶ cells/ml, washed by centrifugation, and grown for one doubling (hemocytometer count) at 30 °C. Induction was performed by adding 0.1 ml of α-factor at various concentrations (as shown in Fig. 5 or at 10⁻⁶ M for the results reported in Fig. 6A) to 1 ml of concentrated cells (1 × 10⁷ cells/ml). The mixtures were vortexed and, after incubation at 30 °C with shaking for 2 h, cells were harvested by centrifugation, and each pellet was resuspended and assayed for β-galactosidase activity (expressed as Miller units) in duplicate by modification (23) of a standard protocol (24) using o-nitrophenyl-β-D-galactopyranoside (Sigma) as the substrate. The percent activity at various α-factor concentrations was determined by comparing β-galactosidase activity to the maximal activity at 1 µM α-factor for the wild-type receptor. Each experiment was carried out at least two times with similar results in each assay.

Binding Assays—Binding assays were performed using [3H]-α-factor synthesized as described previously (7, 22). The competition binding assay was started by the addition of [3H]-α-factor and various concentrations of non-labeled α-factor or α-factor analogs (140 µM) to a 560-µl cell suspension such that the final concentration of radioactive peptide was 6 × 10⁻⁷ M (20 Ci/mmol). After a 30-min incubation, triplicate samples of 200 µl were filtered and washed over glass fiber filter mats using the Standard Cell Harvester (Skatron Instruments, Sterling, VA) and placed in scintillation vials for counting. Each experiment was carried out at least three times with similar results. Data curves for competition binding assays were fitted from at least eight triplicate data points using Prism™ software (GraphPad) with non-linear regression one site competition. The Ki, values for competition binding assays were calculated by using the equation of Cheng and Prusoff (25), where Ki = IC₅₀/(1 + [ligand]/Kₒ).

Determination of Disulfide Cross-links in α-Factor Receptors—Immunoblot analysis of Ste2p was carried out essentially as described previously (12). Cells were grown to log phase, and then 1 × 10⁶ cells were harvested by centrifugation and lysed by agitation with glass beads in a lysis buffer containing 50 mM Hepes, pH 7.5, 1 mM EDTA, 10 µg/ml phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, and 2 µg/ml pepstatin. The lysate was cleared by centrifugation at 2,000 × g for 5 min, and then membranes were harvested by centrifugation at 15,000 × g for 45 min. The membrane pellet was washed and then resuspended in 100 µl of a buffer containing 100 mM Tris, pH 8.5, 0.1% SDS. The protein concentration was determined by the Lowry assay (Pierce), and then aliquots containing 50 or 100 µg of membrane protein were then digested with 1-1-1tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington Biochemicals Inc.) at 37 °C for 1 h or longer. No oxidizing reagent was used to promote the disulfide bond formation. The gel samples were incubated at room temperature, heated at 37 °C for 15 min, and then separated on NuPAGE 12% Bis-Tris SDS-polyacrylamide gel (Invitrogen) with either non-reducing or reducing conditions and electrophoretically transferred to 0.2-µm nitrocellulose. The blots were probed with anti-T7 antibodies at 1:10,000 dilutions (Novagen Inc.), and then the immunoreactive bands were detected using a Super Signal Ultra ECL kit (Pierce).

Molecular Modeling—A molecular model of Ste2p was generated by homology with the crystal structure of rhodopsin (26) using ExPaSy molecular biology server (27). After selecting rhodopsin as the modeling template, the amino acid sequence of each TMD of Ste2p was used to construct the model. Computer programs used to predict the boundaries of the TMDs of Ste2p were obtained from the same server (ca.expasy.org). The coordinates of each helix were generated by the protein modeling tool ProMod, and final energy minimization was performed by Gromos96 for 200 cycles to improve the stereochemistry of the model and to remove unfavorable clashes. This model is referred to as representing the resting state of Ste2p, because the template used during modeling was the rhodopsin resting state. A similar model of Ste2p (28) resulted in coordinates for transmembrane domains that were almost identical to the ones generated herein. The activated state of Ste2p was generated using the same model by introducing torsion on
FIGURE 1. Targeted residues (box) for site-directed mutation in the region spanning portions of EL 2 and TM5 of α-factor receptor. The predicted two-dimensional topology of the receptor is shown, with the extracellular domains at the top.

the backbone omega, phi and psi angles around Pro^{208} and Ser^{209}. (These residues were mutated to Leu to generate a constitutively active receptor.) The torsion applied was just enough to bring Asn^{205} and Tyr^{206} into close proximity without any unfavorable clashes by using Swiss-PdbViewer (version 3.7) torsion tool. The mutations generated during this study were introduced into the model by using the mutation tool in Swiss-Pdb Viewer, and the distances between the side-chain atoms were measured with the distance tool in the same program.

RESULTS

Biological Activity and Binding of Ste2p Variants after Mutagenesis of Residues 200–209—Ten amino acids spanning a portion of Ste2p at the junction between TM5 and extracellular loop 2 (residues 200–209) were mutated by site-directed mutagenesis (Fig. 1). All residues were mutated to alanine except residue 206, which is alanine in the native protein; in this case the residue was mutated to glycine. The biological activity of each mutant receptor was measured by the growth arrest assay, as described under “Experimental Procedures.” The response of cells expressing each of the receptor constructs, except F204A and N205A, increased linearly throughout the range of α-factor tested (Fig. 2A). Excluding F204A and N205A, which responded only minimally to a high amount of α-factor (8 μg), the biological activity of all receptors measured as the amount of α-factor required to produce a 15-mm zone of inhibition, a halo size in the middle of the dose-response curve, was within 10% of WT Ste2p (Table 1). Measurement of α-factor binding to the receptors indicated that the Kᵢ for WT Ste2p (5.2 nM) was in the range previously reported for this receptor (29–31). Except for the F204A mutant receptor, cells expressing each of the receptor constructs showed affinities for α-factor from 2-fold higher (S207A and I209A) to ∼3-fold lower (Y203A and N205A) when compared with WT receptor (Fig. 2B and Table 1). The F204A mutant showed no measurable binding of α-factor at a concentration up to 1 μM of added pheromone, which agreed with previously reported results for F204S (16). A saturation binding assay using whole cells indicated a level of ∼40,000 receptors/cell for both the wild-type and the N205A receptor. In addition, an immunoblot of membranes demonstrated that the N205A mutant receptor was expressed at a level similar to that of the WT receptor (Fig. 3). The N205A mutant represented a unique phenotype among the ten mutants studied in that α-factor bound well but the receptor was not able to initiate signal transduction.

FIGURE 2. A, dose-response analysis of growth arrest assay, and B, competition binding assay for wild-type and ten mutant receptors. A, the halo zone of growth inhibition of strains carrying the indicated receptors was measured at various concentrations of α-factor, and the data were plotted by regression analysis. The results are the means ± S.E. of two separate experiments. A comparison of the biological activities of the various mutant receptors is shown in Table 1. B, yeast cells expressing wild-type or a mutant Ste2p were incubated with 6 nM [3H]α-factor in the presence of increasing concentrations of α-factor. The results are the average ± S.E. of a representative experiment out of three independent experiments. Derived Kᵢ values are given in Table 1.

| Receptor  | Affinity of α-factor for receptor | Biological activity |
|-----------|----------------------------------|---------------------|
| WT        | 5.2 ± 1.2                        | (100 ± 11)          |
| Q200A     | 3.0 ± 1.3                        | 92 ± 8              |
| D201A     | 4.1 ± 1.4                        | 95 ± 10             |
| K202A     | 5.2 ± 1.3                        | 95 ± 7              |
| Y203A     | 13.3 ± 3.4                       | 98 ± 9              |
| F204A     | >100                             | <5                  |
| N205A     | 14.5 ± 4.3                       | <5                  |
| A206G     | 10.3 ± 2.5                       | 114 ± 13            |
| S207A     | 2.9 ± 1.2                        | 108 ± 9             |
| T208A     | 10.6 ± 3.1                       | 105 ± 9             |
| I209A     | 2.8 ± 1.7                        | 104 ± 9             |

Mutant Receptor N205A Has a Dominant-negative Phenotype Rescued by Overproduction of G Protein—in S. cerevisiae, dominant negative Ste2p receptors have been shown to be expressed and bind G protein effectively thereby down-regulating signaling from an active,
Characteristics of N205A and Y266A Mutant Receptors Are Very Similar—Similarly to Y266A (7), the N205A mutant receptor showed a very good binding affinity ($K_i = 15 \text{ nM}$ as compared with the WT, $K_i = 5 \text{ nM}$), yet had $\leq 5\%$ of WT biological activity as measured by the growth arrest assay (Table 1). Both N205A and Y266A also showed very similar characteristics in the Fus1-lacZ induction assay (Fig. 5). To further compare the N205A and Y266A receptors, we carried out competition binding assays using single Ala-substituted $\alpha$-factor analogs. In both N205A and Y266A receptors the binding affinities of [Ala]$^1$-$\alpha$-factor through [Ala]$^{13}$-$\alpha$-factor increased 2- to 4-fold compared with WT receptor (Table 2). In contrast, Ala substitution at residue 5 and at residues 7 through 13 of $\alpha$-factor applied to N205A and Y266A receptors the binding affinities of [Ala]$^1$-$\alpha$-factor for N205A and the Y266A mutant Ste2ps were similar to wild-type, yet had a 2-fold decrease in the Y266A receptor. None of the Ala-scanned analogs of $\alpha$-factor induced lacZ in either mutant. Overall the affinities and activities of Ala-scanned $\alpha$-factor analogs for wild-type, N205A, and Y266A receptors $K_i$ values for $\alpha$-factor and 13 Ala-scanned analogs were determined in competition binding assays by displacement of [3H]$\alpha$-factor.

**TABLE 2**

| Peptide | WT Binding ($K_i$) | N205A | Y266A$^a$ |
|---------|--------------------|-------|-----------|
| [Ala]$^1$ | 6.81 ± 1.3 | 14.5 ± 4.3 | -2.13 | -6.0 |
| [Ala]$^2$ | 114.1 ± 13 | 45.4 ± 8 | +2.51 | +2.3 |
| [Ala]$^3$ | 388.5 ± 24 | 115.5 ± 15 | +3.36 | +3.8 |
| [Ala]$^4$ | 185.4 ± 19 | 79.1 ± 7 | +2.34 | +4.0 |
| [Ala]$^5$ | 284.0 ± 34 | 58.9 ± 9 | +4.82 | +3.3 |
| [Ala]$^6$ | 7.5 ± 12 | 79.5 ± 8 | -10.6 | -7.2 |
| [Ala]$^7$ | 1303.3 ± 143 | 571.5 ± 9 | +2.28 | +2.2 |
| [Ala]$^8$ | 37.7 ± 7.5 | 186 ± 13 | +4.93 | +10.4 |
| [Ala]$^9$ | 5.6 ± 1.2 | 133 ± 2.5 | +2.38 | +6.9 |
| [Ala]$^{10}$ | 683 ± 58 | 1614 ± 86 | -2.36 | -2.6 |
| [Ala]$^{11}$ | 791 ± 55 | 1354 ± 79 | -1.71 | -5.0 |
| [Ala]$^{12}$ | 151.2 ± 14 | 451.1 ± 36 | -2.98 | -8.0 |
| [Ala]$^{13}$ | 765.7 ± 68 | 4223 ± 186 | -5.51 | -10.6 |
| [Ala]$^{14}$ | >2000 | 893.3 ± 66 | ND$^b$ | -3.4 |

$^a$Data are from Lee et al. (7). All values are the mean ± S.E. from three separate experiments.

$^b$The ratio could not be determined from the binding data obtained.
be pH-dependent because of hydrogen bonding or electrostatic interactions between the two imidazole groups. Therefore, we carried out Fus1-lacZ induction assays in different buffers (pH 4.0, 5.0, 6.0, 7.0, 7.5, and 8.0) so that the pH remained relatively constant under the assay conditions. Starting at pH 5.0, 6.0, 7.0, and 7.5 with MES buffer, the pH of the reaction mixture actually was lowered by one-half pH unit during the course of the 2-h incubation. The pH values for the assays are reported as the final pH value (Fig. 6A). The single N205H mutant receptor was non-functional at all pH values tested (Fig. 6A), whereas the Y266H mutant receptor was partially functional at all pH values between 4 and 7 (50–65% of wild-type signaling). All receptors, including the WT receptor, were not functional at pH 8 as measured by a Fus1-lacZ induction assay. Compared with the WT receptor the N205H/Y266H double mutant receptor recovered partial function at pH 4 and 4.5, was almost as active as the WT receptor at pH 5.5, and was equally active to WT receptor at pH 6.5 and 7.0. A growth arrest assay with the N205H mutant showed this mutant receptor was non-functional at pH 6, whereas the N205H/Y266H mutant receptor was almost as active as the WT receptor at this pH in response to pheromone (Fig. 6B). Although others have used zinc to activate site-directed, histidine-substituted GPCRs (32, 33), the N205H/Y266H mutant was not activated by zinc under the experimental conditions of the gene induction assay (data not shown). In addition, N205A/Y266A, N205Y/Y266N, N205D/Y266K, and N205K/Y266C double mutants were all non-functional in the Fus1-lacZ induction assay (data not shown). These results are consistent with our previous report that an aromatic residue in the 266th position of Ste2p is important for receptor activation in wild-type receptors (7). Whereas the five-membered His residue was able to partially complement the function of the six-membered Tyr residue, substitution with other residues, such as Asn, Asp, and Lys, did not result in functional receptors. The high activity of the N205H/Y266H mutant led us to hypothesize that the interaction between Asn205 and Tyr266 in the resting and/or activated state of the receptor.

WT receptor occurs during ligand-induced receptor activation. This hypothesis guided the next series of experiments, which investigated whether residues at the 205th and 266th positions could interact in the resting and/or activated state of the receptor.

Asn205 Interacts with Tyr266 Only in an Activated State of Ste2p—We could not test whether an interaction between Asn205 and Tyr266 occurred in the receptor activated by ligand addition to WT receptor, because Tyr in position 266 is essential for receptor activation and substitution of Tyr at 266 by any other residue renders the receptor unresponsive to α-factor (7). Therefore to carry out genetic analyses to determine if these positions interacted in an active state of the receptor, we constructed the strong constitutively active mutant receptor of Ste2p (P258L/S259L), which has been used previously to mimic the Ste2p-activated state (34). To test for interactions we introduced cysteine, aspartic acid, and/or lysine in the 205th and 266th positions in the P258L/S259L background. These residues were utilized to determine 1) if a covalent bond would be formed in the activated receptor between N205C and Y266C and 2) if electrostatic interactions between N205D and Y266K/D enhanced or interfered with receptor activity.

The basal signaling activity of the P258L/S259L constitutively active receptor increased ~10-fold compared with wild-type (Fig. 7), as noted previously (34). The introduction of N205K or Y266D mutations into the constitutively active mutant receptor suppressed the constitutive activity ~50 and 65%, respectively. However, the introduction a double mutation N205K/Y266D into the P258L/S259L background restored high constitutive activity (Fig. 7). The reciprocal mutations (N205D, Y266K, or N205D/Y266K) in the constitutively active mutant receptor all led to receptors with 45–55% lowered constitutive activity (Fig. 7). As stated above, neither the N205K/Y266D nor N205D/Y266K was active in the wild-type background.

We used a biochemical analysis to test whether an interaction occurred between N205C/Y266C in the constitutively activated state. Disulfide cross-linking has been previously used to study topology and proximity of residues in GPCRs, including rhodopsin (35, 36) and the M2 muscarinic receptor (37, 38). In addition, a method for determining the interaction between TM5 and TM6 of Ste2p using disulfide cross-linking was established previously using T7 epitope tagging and trypsin digestion (12). Our results demonstrated that the single N205C mutant was partially active, and the Y266C and N205C/Y266C mutants were not active in the WT background (data not shown and Ref. 19). How-
Interacting Residues of Ste2p

FIGURE 8. Disulfide bond formation in the N205C/Y266C mutant receptors. A, diagram of Ste2p with the position of the T7 tag indicated. Sites of trypsin digestion are indicated with a filled diamond. The trypptic fragment containing TM6 is ~2.3 kDa, and the trypptic fragment corresponding to TM6 and TM7 is ~9 kDa. The N- and C-terminal portions of Ste2p are not shown. B, membranes were harvested from yeast LM102 carrying N205C, F204C/Y266C, N205C/Y266C, and N205C/A265C receptors in the WT background (lanes 1–4) and N205C, F204C/Y266C, N205C/Y266C, and N205C/A265C in the P258L/S259L constitutively active background (lanes 5–8). Both receptors contained a T7 epitope tag in TM7. Membranes were digested with trypsin, and samples were run under non-reducing conditions (a), or under reducing conditions (b) by addition of dithiothreitol (200 μM). Membrane protein digests were transferred to nitrocellulose and probed with anti-T7, HRP-conjugated antibody.

However, the quadruple mutant N205C/Y266C/P258L/S259L was as constitutively active as the P258L/S259L mutant (data not shown). After trypsin digestion the N205C/Y266C/P258L/S259L mutant receptor gave only a 11.5-kDa band (Tyr205 to Lys225 of TM5 and Glu240 to Lys264 of TM6-TM7 epitope T7) with a disulfide bond between 205C and 266C in the non-reducing PAGE (Fig. 8B, panel a). In contrast, under reducing conditions (Fig. 8B, panel b) this mutant receptor gave predominantly the 9-kDa band suggesting that a disulfide bond formed in the constitutively active mutant. Under both reducing and non-reducing conditions, N205C/Y266C receptor in the WT background gave a major 9-kDa band (Gln200 to Lys225 comprising TM6 and TM7 with the T7 epitope). To test whether the disulfide cross-link between N205C and Y266C was specific in the constitutively active mutant, we generated N205C/A265C and F204C/Y266C receptors both in the WT and constitutively active background. Similarly to N205C/Y266C, these mutant receptors were not active in the WT background and were constitutively active in the P258L/S259L background (data not shown). After trypsin digestion, N205C/A265C and F204C/Y266C receptors gave a major 9-kDa band upon Western blotting in both non-reducing and reducing gels (Fig. 8B). A minor 11.5-kDa background band appeared in all receptors in both reducing and non-reducing conditions. These results strongly suggest that an interaction between residues at the 205th and 266th positions in Ste2p occurs only, and specifically, in an activated state of this GPCR.

DISCUSSION

Previous investigations on GPCRs have been hampered by the lack of high resolution structures of these important signal-transducing proteins. Indeed, except for the crystal structure of the bovine rhodopsin ground-state, no other x-ray or NMR structure for this class of molecules appears in the literature. The absence of high resolution structures has prevented investigators from following interactions between key receptor residues that appear only in the inactive but not the activated receptor, and vice versa. Thus, important mechanistic information relating to GPCR activation pathways remains obscure.

In this report we used bioassays, receptor mutagenesis, and chemical cross-linking to reveal an interaction between two residues of Ste2p that occurs only in the activated receptor. Our data and those previously reported (16, 17, 19) indicate that Asn240 located in the second extracellular loop is involved in Ste2p signaling. A role for the second extracellular loop in GPCR activation was also reported recently for the C5a receptor (39). In the C5a receptor, EL2 acted as a negative regulator for GPCR activation, whereas in Ste2p, we propose that Asn240 in EL2 is required for an activated state. Of the 9 residues (Gln200–Ile209) that surround Asn240 only Phe204 was important for signaling as judged by Ala-scanning mutagenesis (Table 1). Some of the phenotypes of the Ala-scanned Ste2p were similar to those reported in Cys scanning and random mutagenesis of this receptor. For example N205C resulted in decreased halo size in growth arrest assays and ~80% of LacZ induction when compared with WT. The α-factor binding activity of N205C was measurable, with a Kd of ~30 nM (19, 40). Although there are differences between N205A and N205C mutant receptors regarding binding affinity and LacZ induction, for example our results showed that the N205C mutant is more active than the N205A mutant in the gene induction as well as growth arrest assay (data not shown), in general, these mutants are qualitatively similar in that both mutants are able to bind α-factor with reduced affinity and produce halos of reduced size, relative to WT. Specific differences in the sterio and electronic characteristics of side chains of Ala and Cys residues might account for the quantitative differences observed in various assays. Additionally, the mutants in the present study were expressed in a background strain (LM102) different from that used in their studies. It has been proposed that differences in strain background might affect the quantitative responses in bioassays (41). In contrast, unlike N205A, which exhibited 15 nM affinity for α-factor, F204A did not bind pheromone. Thus the phenotype of Asn240 is that of a receptor residue critical for signaling but not essential for high affinity ligand binding. In this respect Asn240 resembled the phenotype of Tyr246 previously studied by us (7). Indeed as found for the Y266A mutant receptor, N205A appeared to interact primarily with residues near the N terminus of α-factor using pheromone Ala-scanning analysis (Table 2). Moreover, both of these receptors exhibited dominant negative phenotypes that reflect sequestration of G proteins and support the fact that these proteins are expressed intact and localize to the cell membrane.

The phenotypic similarities observed between N205A and Y266A mutants led us to hypothesize that these residues might be near each other in the three-dimensional structure of Ste2p. Indeed, hydrophathy and modeling analyses placed Asn240 and Tyr246 near the extracellular ends of TM5 and TM6 of Ste2p, respectively. It was tempting, therefore, to conclude that these key residues interacted. However, previous studies had shown that in the absence of α-factor N205C, but not Y266C, was accessible to a hydrophilic thiol suggesting distinct environments and a lack of proximity (19). To explore further the putative interactions of Asn240 with Tyr246 we constructed Ste2p mutants containing His at one or both of these positions. N205H did not respond to α-factor from
pH 4 to 7 and Y266H exhibited 50–65% receptor activation as judged using a gene induction assay over the same pH range (Fig. 6A). Intriguingly, the N205H/Y266H mutant receptor gave full activation at pH 6.5 and 7.0 and 30% to 90% signaling at pH 4 to pH 5.5. This suppression of the N205H phenotype in the double mutant can be indicative of an interaction between residues 205 and 266 or between these residues and a side chain of the ligand in the activated receptor. The pH profile suggested that optimum interaction might require one protonated and one unprotonated His (pK_a ~ 6.5). The decrease or lack of activity at pH 4 and 8, respectively, was not due to poor binding of α-factor to the N205H/Y266H receptor, because the α-factor binding affinity to this mutant receptor was not changed significantly when tested at pH values of 4, 6, and 8 (data not shown). Good binding of α-factor at pH 8 has been shown previously for WT Ste2p (42).

To probe the putative proximity of Asn205 and Tyr266 we used a Ste2p background that gave high basal signaling in the absence of α-factor (34). Presumably the P258L/S259L receptor reflects an active conformation of Ste2p and interactions observed in this background indicate groups that are near to each other in the signaling state of this GPCR. In this background, the N205K/Y266D double mutation showed high basal signaling activity similar to that of the P258L/S259L constitutively active receptor, although both the N205K and Y266D single mutations suppressed constitutive activation (Fig. 7). A plausible interpretation of these experimental results is that, in an active state, but not in the inactive state, Asn205 and Tyr266 interact with each other. The reciprocal mutation N205D/Y266K in the constitutively active mutant receptor suppressed constitutive activity to the same level as the single N205D or Y266K mutations in the P258L/S259L receptor (Fig. 7). Nevertheless, the effect of the single mutations was not additive in the double mutant, again suggesting a favorable interaction between side chains of residues at position 205 and 266 in the activated receptor. Perhaps a particular orientation of side chains of both residues and/or the neighboring residue context is required for optimal interaction between Asn205 and Tyr266. It has been reported that a predicted salt-bridge interaction between Arg232 and Glu218 in a GPCR was not interexchangeable as judged in studies on the receptor containing the reciprocal mutation (43). In addition, although the interaction between Arg232 and Glu218 was identified in the crystal structure of crystallin, the R302E/E330R reciprocal mutation showed 31% of WT activity, which is equivalent to the activity of E330R mutant (44). These results suggest that a salt-bridge interaction is not always interchangeable and may account for the suppression of constitutive activity by the introduction of N205D/Y266K but not N205K/Y266D into the P258L/S259L constitutively active mutant.

Another interpretation for the different levels of basal activities of various mutant receptors (Fig. 7) might be due to the difference in the number of receptor molecules expressed at the cell surface. However, it has been proposed that as few as 2% of the wild-type level of receptors can still induce maximal response in a short-term FLIS1-lacZ assay (15, 45). In addition, although the number of cell-surface receptors of the P258Y mutant receptor was 80-fold less than that of the P258L mutant receptor, the P258Y mutant receptor showed 3-fold higher activity than the P258L mutant receptor (41). The different level of basal signaling in our constitutively active Ste2p mutants is not likely due to a different number of cell-surface receptors.

To test further the interaction between Asn205 and Tyr266 in an active state of the receptor we used a biochemical analysis using disulfide-cross-linking, as previously applied to establish an interaction between TM5 and TM6 of Ste2p (12). In that study, formation of a covalent bond between residues of the 5th and 6th TMs was detected by Western blotting in which a larger peptide was apparent in non-reducing gels using an antibody to an epitope appended to TM7 as a result of formation of a disulfide bond between TM5 and TM6. We introduced the N205C/Y266C double mutation into WT and the P258L/S259L constitutively active receptor. We found that a major disulfide-cross-linked fragment of the expected size (~11.5 kDa) formed in this activated receptor with no 9-kDa band observable (Fig. 8B, panel a, lane 7). This 11.5-kDa band was greatly diminished in a reducing gel (Fig. 8B, panel b, lane 7). It has been reported that Ste2p oligomerization promoted receptor biogenesis and signaling (46). Therefore, we were concerned that the 11.5-kDa fragment represented an intermolecular cross-link between N205C-N205C or Y266C-Y266C. However, the N205C-N205C cross-link would not be detected by the antibody to T7, and a Y266C-Y266C cross-link would result in a 18-kDa band. In addition to a major 9-kDa band the N205C, N205C/A266C, and F204C/Y266C WT and constitutively active Ste2ps all showed a minor band, which migrated near the 11.5-kDa band in the non-reducing gel (Fig. 8B, panel a). This latter background band was still apparent in 5 out of 8 lanes in the reducing gel (Fig. 8B, panel b). Although this band may be due to incomplete digestion, we cannot fully explain this background band at this time. Together with the mutagenesis data, these cross-linking results strongly suggest that Asn205 interacts with Tyr266 directly only in an activated state of the receptor.

Based on the results generated in this communication, we have built a model to help interpret the findings. The residues composing the transmembrane domains of Ste2p were superimposed upon the rhodopsin model using the Swiss-Prot data base generating a model for Ste2p (see “Experimental Procedures”). Although there is no significant sequence homology between rhodopsin and Ste2p, rhodopsin has been used for the modeling of Ste2p to explain site-directed mutations that result in functional receptors (19, 40) and to show that the general mechanism of receptor activation between rhodopsin and Ste2p is very similar even though their sequences are very divergent (28). We show Ste2p transmembrane domains in which residues Asn205 and Tyr266 are highlighted (Fig. 9A). The assumption is that this model is in the resting state, because it was generated from rhodopsin in its resting state. A higher resolution depiction of Asn205 and Tyr266 is shown in Fig. 9B. The model placed these residues at a distance of ~6 Å (corresponding to the side-chain O of Asn205 to the C1 of the benzoyl ring of Tyr266), which may not be close enough for productive interaction. Modeling by another group came to a similar conclusion that these residues were separated by a distance of 8 Å.4 These atoms come closer together (~2 Å apart) if we take TM6 and introduce the P258L/S259L mutation (the constitutively active mutant), which causes a conformational change in TM6 bringing Tyr266 closer to N205. Presumably, a similar closing of the distance between these residues occurs upon ligand binding in the native Ste2p. When Cys replaces residues Asn205 and Tyr266 (Fig. 9D), the residues are still separated by ~6 Å (between the S atoms in 205 and 266), whereas in an active state represented by the constitutive P258L/S259L mutant receptor the residues are close enough to form a disulfide bridge, which we detected by a biochemical experiment (Fig. 8). Finally, when histidine substitutes for Asn205 and Tyr266 (Fig. 9, F and G) the residues are brought into close proximity in an active receptor (Fig. 9G) allowing for a productive interaction that may promote signal transduction.

Although numerous intramolecular interactions have been identified for inactive state conformations of GPCRs, much less is known about the intramolecular interactions stabilizing active state conformations.

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4 S.O. Smith, personal communication.
Two reports (32, 33) were interpreted by Kristiansen (47) as demonstrating specific interactions in the active state of a GPCR by protein engineering and biochemical analysis. In these experiments, zinc was used as an artificial ligand to induce activation of the D113H/N312C double mutant of the human \(\beta_2\) adrenergic receptor and the P112H/M291C human NK\(_1\) neurokinin receptor. Although the addition of zinc was able to induce \(\beta_2\)-adrenergic receptor activation, the authors concluded that no conformational change was required to convert the resting receptor to its activated state based on molecular modeling. In addition, interpretation of these experiments is confounded by the observations that allosteric binding sites for zinc have been identified in the \(\beta_2\)-adrenergic receptor (48). Therefore, it is not possible to conclude that the residues 113 and 312 interact only in the active state. In the case of the P112H/M291C NK\(_1\) mutant receptor, the metal ion acted as a partial agonist as well as a pure antagonist when used with Substance P, the natural agonist for this receptor making it unreasonable to conclude,
without further experimentation that residues 112 and 291 interact only in the active state.

We investigated whether the N205H/Y266H mutant receptor could be activated in the presence of zinc. Similarly, we tested whether the N205C/Y266C mutant receptor could be activated in the presence of Cu-phenanthroline. We did not observe an elevated level of reporter gene induction under these conditions (data not shown). Perhaps, in Ste2p, the residues Asn\(^{205}\) and Tyr\(^{266}\) are not in the correct geometry in the resting state for zinc or oxidizing reagent to form a cross bridge between them to activate the receptor. It is also possible given the proposed buried nature of Tyr\(^{266}\) (19) in the inactive receptor that these reagents do not reach the cellular site to produce a cross bridge. Other studies have reported agonist-dependent disulfide bond formation between specific residues in the rat M3 muscarinic receptor (37, 38). However, it is not clear if the disulfide bond formation was indeed specific for the receptor active state because disulfide bonds were formed in the presence of antagonist and in the absence of agonist. These conditions are expected to stabilize non-activated states of the GPCR. In addition, disulfide bond formation necessarily required oxidizing reagents such as Cu-phenanthroline or molecular iodine that may have forced non-native interactions.

Understanding how G protein-coupled receptors are activated by ligand binding is an important goal of workers in the GPCR field. Although many kinetic models have been developed to describe the process of agonist activation, the most widely accepted model is the extended ternary complex model (49, 50). According to the model, the receptor exists in an equilibrium between an inactive conformation (R) and an active conformation (R\(^*\)). However, there is increasing evidence that there are successive conformational changes during the binding of agonist to non-peptide hormone receptors such as a\(_2\)-adrenoreceptor (51) as well as peptide hormone receptors such as the AT\(_1\) (52). Thus, the interpretation of our results based on a resting and active state receptor model are highly simplified representations of what may actually occur during receptor activation.

In this report we have proposed that interaction of residues Asn\(^{205}\) and Tyr\(^{266}\) occurs in the constitutively activated receptor state. We speculate that similar interaction occurs in the agonist-induced active state of Ste2p. However, we were not able to show this interaction in the \(\alpha\)-factor-induced activated receptor, because the N205C/Y266C mutant in the WT background does not respond to \(\alpha\)-factor. For such interaction to occur, it is likely that movement of helices is required because, in the absence of \(\alpha\)-factor, Asn\(^{205}\) is accessible to thiol reagents, whereas Tyr\(^{266}\) is not (19). It has been suggested that, subsequent to ligand binding there are relative movements of transmembrane helices of other GPCRs during receptor activation (53–55). Spin-labeling studies on cysteine-substituted mutants of rhodopsin showed a rigid body movement of TM6 relative to TM3 accompanied by anti-clockwise rotation after light-induced isomerization of retinal (55). Additional evidence for a relative movement between TM3 and TM6 in other GPCRs was provided by fluorescence labeling of the beta\(_2\)-adrenergic receptor or by monitoring the accessibility of Cys residues to a hydrophilic sulfhydryl-specific reagent during receptor activation (56).

As this report neared completion an elegant study of in\(_{\text{site}}\) disulfide cross-linking in the M3 muscarinic acetylcholine receptor provided direct evidence for an agonist-activated conformational change involving residues in TM3 and TM7 of this class A GPCR (57). The M3 muscarinic receptor study provides strong evidence that binding of a diffusible ligand can change the spatial relationships of residues in the inactive and activated state of a GPCR and complements the findings reported herein. The conformational change observed in the muscarinic receptor involved residues that were relatively close to the binding site of the carbachol agonist. Previously, we proposed that Tyr\(^{266}\) of Ste2p plays a role in the recognition of the N terminus of \(\alpha\)-factor (7) suggesting that this residue was close to the ligand binding pocket. The change in the spatial proximity of residues 205 and 266 of Ste2p, which we detected in the constitutively activated mutant, also involves at least one residue (Tyr\(^{266}\) involved in ligand binding. If there is conserved structural homology among all classes of GPCRs as concluded recently (28), it is possible that our results may apply to other members of GPCRs leading to the conclusion that there is an interaction between TM5 and TM6 residues that are close to the extracellular surface.

In conclusion, we report that residue 205 interacts with residue 266 in a constitutively active receptor. Additional experiments will be necessary to determine whether the conformation of the constitutively active mutant receptor is the similar or different from that of the conformation of \(\alpha\)-factor-induced activated receptor. More biophysical and biochemical investigations focusing on interaction between these and other residues in the resting and active state of Ste2p will lead to a better understanding of the activation pathway of this model GPCR.

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Interacting Residues of Ste2p

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2272 JOURNAL OF BIOLOGICAL CHEMISTRY