Interaction between Transmembrane Domains Five and Six of the α-Factor Receptor*

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The α-factor pheromone receptor (STE2) activates a G protein signal pathway that induces conjugation of the yeast Saccharomyces cerevisiae. Previous studies implicated the third intracellular loop of this receptor in G protein activation. Therefore, the roles of transmembrane domains five and six (TMD5 and -6) that bracket the third intracellular loop were analyzed by scanning mutagenesis in which each residue was substituted with cysteine. Out of 42 mutants examined, four constitutive mutants and two strong loss-of-function mutants were identified. Double mutants combining Cys substitutions in TMD5 and TMD6 gave a broader range of phenotypes. Interestingly, a V223C mutation in TMD5 caused constitutive activity when combined with the S251C mutations in TMD6. Also, the L226C mutation in TMD5 caused constitutive activity when combined with either the M250C or S251C mutations in TMD6. The residues affected by these mutations are predicted to fall on one side of their respective helices, suggesting that they may interact. In support of this, cysteines substituted at position 223 in TMD5 and position 247 in TMD6 formed a disulfide bond, providing the first direct evidence of an interaction between these transmembrane domains in the α-factor receptor. Altogether, these results identify an important region of interaction between conserved hydrophobic regions at the base of TMD5 and TMD6 that is required for the proper regulation of receptor signaling.

The α-factor receptor (STE2) binds a peptide-mating pheromone ligand and transduces a signal that promotes the conjugation of the yeast Saccharomyces cerevisiae (1). Like other members of the G protein-coupled receptor (GPCR) family, the α-factor receptor functions by stimulating the α subunit of a heterotrimeric GTPase G-protein. The activated G protein activates the heterotrimeric G protein to bind GTP (2). The binding of GTP to the Gα subunit promotes its release from the Gβγ subunits. Either the α or the βγ subunits then go on to stimulate the next step in the signal pathway. During pheromone signaling, the βγ subunits activate a mitogen-activated protein kinase cascade that leads to transcriptional activation of pheromone-responsive genes and to cell division arrest in G1 (3–5). The βγ subunits also lead to activation of Cdc42p, which promotes polarized growth to form the conjugation bridge that connects the mating cells (6, 7).

The organization of the functional domains in the α-factor receptor are similar to that of many other GPCRs. Receptors in this family are composed of seven transmembrane domains (TMDs) that are connected by intracellular and extracellular loops. The core region of the receptor containing the seven TMDs has been found to be generally responsible for ligand binding and G protein activation (8–10). Mutational analysis of the α-factor receptor indicates that residues near the extracellular ends of the TMDs are involved in ligand binding (11). These residues also appear to play an important role in promoting the activated receptor conformation upon ligand binding. Interestingly, mutations affecting this domain confer a dominant-negative phenotype, apparently because the mutant receptors sequester G proteins and thereby interfere with the ability of wild-type receptors to signal (11, 12). Analysis of the intracellular domains of the α-factor receptor has demonstrated that the third intracellular loop plays a key role in G protein activation (13–16). Consistent with this, the third loop becomes hypersensitive to proteolytic digestion with trypsin in response to ligand binding, indicating that this region undergoes a conformational change that is likely to be important for G protein activation (17). The cytoplasmic C-terminal tail is not required for signaling, but instead a target for phosphorylation that promotes receptor desensitization (18) and down-regulation of receptors by endocytosis (14, 19).

The TMDs are thought to play an important role in GPCR activation by propagating a conformational change from the ligand-binding domain to the intracellular domains that promote G protein activation. In view of the important role of the third intracellular loop in this process, it seems likely that TMD5 and TMD6 will also play an important role in signaling, because they bracket the third intracellular loop. Interaction between TMD5 and TMD6 may play an important role in signaling, because biophysical studies (20) indicate that TMD5 and TMD6 are in close proximity to one another in rhodopsin and modeling studies predict that they are adjacent in other members of the GPCR family (21). TMD6 of the α-factor receptor has been implicated as playing a special role in signaling, because mutations affecting Gln253, Ser254, and Pro258 in this domain cause constitutive receptor activity, indicating that these residues function to restrain the receptor in the inactive conformation (22, 23). Therefore, the role of the residues in TMD5 and TMD6 was investigated in this study by a scanning mutagenesis approach in which each residue was substituted...
with Cys. The advantage of substituting with Cys is that it provides opportunities to take advantage of the chemical reactivity of the sulfur in the Cys side chain to carry out chemical cross-linking studies with the mutant proteins (24, 25). The results of this study identify a site of interaction between conserved hydrophobic regions at the base of TMD5 and TMD6 that is important for the proper regulation of α-factor receptor signaling.

**EXPERIMENTAL PROCEDURES**

**Strains and Media—** Yeast strain yLG123 (MATa ade2-1 his4 :4-580 lys2 -1 trp1 -1 tyr1 -1 leu2 ura3 SUP4-3 bar1-1 mfa2 : FUS1-lacZ ste2 : LEU2) and PMY1 (MATa bar1:HisG far1 stt1-1 ste2-3 malA1 : LEU2 mfa2 : his5-1 lys2 : FUS1-LacZ ade2 his3 leu2 ura3) were used for analysis of receptor mutants. Strain lys1 (MATa lys1) was used for mating tests. Cells were grown in media as described (26). Yeast transformations were performed using the lithium acetate method (27). Plasmid-containing cells were grown in synthetic medium containing adenine and amino acid additives but lacking uracil to select for plasmid maintenance.

**Cysteine Scanning Mutagenesis—** Plasmid pPD215 (YPEp-URA3-STE2-2XHA) was constructed to facilitate cysteine scanning mutagenesis of TMDs 5 and 6. The plasmid is based on pLG59 (11), which carries a modified STE2 gene in which a triple hemagglutinin epitope tag was introduced at the C-terminal end of the receptor coding sequences. Site-directed mutagenesis was carried out by PCR amplification using mutagenic oligonucleotides and either the QUICK CHANGE mutagenesis kit (Stratagene) or Pfu DNA polymerase (Promega). To prevent interference from the two endogenous cysteine codons, Cys29 was changed to Ile, the corresponding amino acid in the homologous receptor from S. kloetreri (28) and Cys227 was changed to Ala. These substitutions did not affect the function of the receptor. Silent mutations were introduced by PCR to add unique restriction sites for BgiII, NheI, and KpnI to facilitate subcloning of mutagenized DNA fragments. Relative to the ATG initiation codon, the NheI site was introduced at position 616, the KpnI site was added at position 814. The resulting STE2 gene was fully functional as judged by its ability to complement a ste2Δ mutation in the functional assays described below and to produce the expected protein product by Western blot analysis. The modified STE2 gene in pPD215 was then used as a starting vector to create a set of Cys substitution mutants in which the codons for each amino acid in TMDs 5 and 6 was changed to TGT to encode Cys. The various TMD5/TMD6 double mutants were created by using a subcloning the 78-base pair NheI-BglII fragment encoding one of the TMD5 Cys mutations into the NheI-BglII site of a plasmid carrying the appropriate Cys substitution in TMD6. Plasmid pPD225-T7 and its derivatives were constructed by using PCR to add the coding sequence for the T7 epitope (MASTMGGQGQMG) in-frame just prior to the trypsin site in the starting vector. All mutagenesis experiments for this study were confirmed by dyeoxy sequencing of the double-stranded DNA using a Sequenase kit from United States Biochemical.

**α-Factor Receptor Analysis—** Immunoblot analysis of Ste2p was carried out essentially as described previously (8). 2.5 × 10^6 mid-logarithmic phase cells were harvested and lysed by agitation with glass beads in 250 μl of lysis buffer (2% SDS, 100 mM Tris, pH 7.5, 8 μl urea, 100 μg of protein extract, as determined by using the BCA Protein Assay kit (Pierce), was separated by electrophoresis on a 10% SDS-polyacrylamide gel, electrophoretically transferred to nitrocellulose, and then probed with rabbit anti-Ste2p antibodies (8), anti-hemagglutinin antibody 12CA5 (Roche Molecular Biochemicals), or anti-T7 antibodies (Novagen, Inc.) as indicated. Immunoactive proteins were detected by enhanced chemiluminescence using an ECL kit (Amersham Pharmacia Biotech). To assay disulfide bond formation, cells were grown to log phase and then 1 × 10^6 cells were harvested by centrifugation and lysed by agitation with glass beads in a lysis buffer containing 10% glycerol, 100 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, 10 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 100 μg/ml TPCK, and 50 μg/ml benzamidine. The lysate was cleared by centrifugation at 300,000 g for 15 min, and then membranes were harvested by centrifugation at 100,000 g for 45 min. The membrane pellet was washed and then resuspended in 100 μl of a buffer containing 100 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, 100 μg/ml of TPCK. The protein concentration was determined by the Bradford assay (Bio-Rad), and then aliquots containing either 50 or 100 μg of membrane protein were then digested with TPCK-treated trypsin (Worthington Biochemicals Inc.) at 37 °C for 1 h. The TPCK-treated trypsin was freshly dissolved in sterile water at a concentration of 10 mg/ml prior to each experiment and then added to membranes at the indicated final concentration. Reactions were stopped by addition of N-ethylmaleimide to 10 mM and 2 × SDS-gel sample buffer. Where indicated, samples were reduced by addition of dithiothreitol to a final concentration of 200 mM. The gel was run at room temperature, heated at 37 °C for 15 min, and then separated on 15% SDS-polyacrylamide gel and electrophoretically transferred to 0.5-μm nitrocellulose. The blots were probed with anti-T7 antibodies at 200 ng/ml (Novagen Inc.), and then the immunoreactive bands were detected using a Super Signal Ultra ECL kit (Pierce).

**α-Factor-induced Responses—** Mating assays were carried out with yeast strain yLG123 (ste2Δ) containing Cys substitution alleles of STE2 on plasmid pPD225. Cells were replica plated to YPD plates containing a lawn of MATa cells (lys1a), incubated at 30 °C for 12 h, replica plated to minimal plates, and then incubated at 30 °C for 48 h to select for the growth of diploids. Halo assays for cell division arrest, summarized in Tables I and II, were performed by spreading on solid medium plates 1.5 × 10^5 yLG123 yeast cells carrying either a wild-type or the indicated mutant receptor gene on a plasmid, and then placing sterile filter disks containing 1500, 750, 375, 187.5, or 93.8 pmol of α-factor were placed on the lawn of cells. Cells were grown at 30 °C for 72 h, and then the diameters of the zones of α-factor-induced cell division arrest were measured. Relative sensitivity to α-factor-induced cell division arrest was determined by plotting the average halo size from two independent experiments versus the log of the amount of α-factor added, which gives a linear relationship. Basal levels of the pheromone-responsive reporter gene FUS1-lacZ were assayed on synthetic medium plates containing 20 μg/ml X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside) and buffered to pH 7. To assay α-factor induction of FUS1-lacZ, cultures were grown overnight to logarithmic phase in selective medium, diluted to 4 × 10^6 cells/ml, and incubated with the indicated concentrations of synthetic α-factor (Bachem) for 2 h. The cells were permeabilized with 0.05% SDS and CHCl3, and then β-galactosidase assays were performed using the colorimetric substrate O-nitrophenyl-β-D-galactopyranoside (29). The results represent the average of two to three independent experiments, each done in duplicate. The standard deviation was usually less than 10% and was always less than 15%.

**RESULTS**

**Cysteine Scanning Mutagenesis of TMD5 and TMD6—** The role of each residue in TMD5 and TMD6 of the α-factor receptor (STE2) was analyzed by mutating the corresponding codons to encode Cys. The advantage of substituting with Cys is that the chemical reactivity of the sulfhydryl group in the Cys side chain may later be exploited in cross-linking studies. A potential drawback of Cys is that deleterious effects can occur due to formation of improper disulfide bonds with endogenous cysteines elsewhere in the protein. To circumvent this potential problem, the two endogenous Cys codons at positions 59 and 252 were changed to encode Ile and Ala, respectively, in the STE2 gene on plasmid pPD225 (see “Experimental Procedures”). Mutation of the endogenous Cys residues did not affect receptor activity, as observed previously (30). This version of the receptor lacking Cys residues will be designated as the wild-type for this study. To identify the residues to target for mutagenesis, hydropathy analysis was used to predict that TMD5 includes residues 209–229 and TMD6 includes residues 246–266 (Fig. 1) (31, 32). The 42 different Cys substitution phenotypes of TMD5/TMD6 were constructed as derivatives of plasmid pPD225, and then the series of plasmids was introduced into a ste2Δ strain that lacks the chromosomal STE2 gene for analysis. Western immunoblot analysis demonstrated that all of the Cys substitution mutant strains producing receptor protein (Ste2p) at a level that was equivalent to the wild-type level and appeared to be properly glycosylated (data not shown).

**Phenotypes of TMD5 Mutants—** Mating assays were performed to screen the Cys substitution mutants to determine whether they produced functional cell surface receptors (Table I). All but one of the TMD5 receptor mutants were able to mate, indicating that most of the mutants retained at least partial
FIG. 1. Residues targeted for mutagenesis in TMD5 and TMD6 of the α-factor receptor. A partial sequence of the α-factor receptor highlighting the residues that were mutated in this study is shown. Residues enclosed in boxes were initially predicted by hydropathy analysis to correspond to TMD5 and TMD6 and were substituted with Cys residues by site-directed mutagenesis.

### Table I

| STE2 allele | Mating ability | Cell division arrest | Elevated basal FUS1-lacZ | 
|-------------|----------------|----------------------|---------------------------|
| Wild-type   | +              | +                    | -                         |
| I209C       | +              | +                    | -                         |
| L210C       | +              | +                    | -                         |
| L211C       | +              | +                    | -                         |
| A212C       | +              | +                    | -                         |
| S213C       | +              | +                    | 2-fold                    |
| S214C       | +              | +                    | -                         |
| S215C       | +              | +                    | -                         |
| S219C       | +              | +                    | -                         |
| F220C       | +              | +                    | -                         |
| V221C       | +              | +                    | -                         |
| I222C       | +              | +                    | -                         |
| V223C       | +              | +                    | -                         |
| V224C       | +              | +                    | -                         |
| K225C       | Sterile        | Defective            | -                         |
| L226C       | +              | +                    | -                         |
| I227C       | +              | +                    | -                         |
| L228C       | +              | +                    | -                         |
| A229C       | +              | +                    | 3-fold increased          |

* Yeast strain yLG123 carrying the indicated STE2 allele on plasmid pPD225.
* Ability to mate with lys1a cells in a patch mating assay.
* Relative α-factor sensitivity was determined by halo assays for cell division arrest. + indicates essentially wild-type response. Defective indicates no detectable halo after 2 days. Partial response indicates a turbid zone of cell division arrest.
* Basal levels of the FUS1-lacZ reporter gene activity were determined by comparing the relative intensity of blue color for colonies on X-gal plates of strain yLG123 carrying the indicated receptor plasmid. To quantify the -fold increase in basal signaling, receptor plasmids were introduced into strain PMY1 and assayed as described under "Experimental Procedures."

receptor activity. In contrast, the substitution of Lys225 with Cys (K225C) caused a strong defect and appeared to be completely defective in mating (Fig. 2A). The TMD5 mutants were next examined for ability to promote α-factor-induced cell division arrest, which is a more stringent test of receptor function that can be used to compare the relative sensitivity to α-factor. The cell division arrest response was quantitated by measuring the zone of growth inhibition (halo) surrounding filter disks containing α-factor that are added to a lawn of cells on solid medium. This assay also monitors the ability of the mutant strains to maintain the pheromone response for a longer time period (2 days). Most of the Cys substitution mutants produced halos that were equivalent to that of the wild-type (Table I). The only TMD5 mutant that failed to respond in this assay carried the K225C substitution (Fig. 2B). The K225C substitution also caused a nearly complete defect in activation of the pheromone-responsive FUS1-lacZ reporter gene, a very sensitive assay for pheromone response, indicating that the ste2-K225C mutant is strongly defective in signal transduction (Fig. 2C). In contrast, the A229C mutant showed about 3-fold increased sensitivity to α-factor (Table I and Fig. 2). The close proximity of Lys225 and Ala229 to each other at the junction between TMD5 and the third cytoplasmic loop suggests that this region plays an important role in signaling.

The TMD5 mutants were next examined for the ability to activate signaling in a ligand-independent manner using methods similar to those used previously to characterize constitutively active α-factor receptors mutated in TMD6 (22, 23). For this analysis, yeast carrying a Cys substitution mutant receptor plasmid were replica-plated to solid medium containing X-gal, a chromogenic substrate for β-galactosidase, that was used to compare the basal activity of the FUS1-lacZ reporter gene. Plates were monitored for the appearance of cells displaying a blue color, indicating elevated basal expression of FUS1-lacZ. From the set of TMD5 mutants, only cells carrying the S214C substitution consistently displayed a darker blue color in the X-gal plates. Quantitative analysis demonstrated that the basal FUS1-lacZ reporter gene activity of the ste2-S214C mutant was increased 2-fold over the wild-type. Altogether, these data demonstrate that, with the exception of K225C, Cys...
substitutions were very well tolerated in TMD5.

Phenotypes of TMD6 Mutants—All of the receptor mutants carrying Cys substitutions in TMD6 (Table II) were able to promote a high degree of mating with the exception of the Y266C substitution affecting Tyr266. STE2-Y266C was identified previously as a dominant-negative mutant of the α-factor receptor (11). The Y266C protein was shown to be produced at levels equivalent to the wild-type receptor and to be stable at the cell surface but defective for signaling. The STE2-Y266C mutant was also the only TMD6 mutant that was completely defective for responding to pheromone in the halo assay (Fig. 2B). Four TMD6 mutants (Q253C, S254C, P258C, and S259C) showed significant growth in the zone of division arrest, indicating that these mutants were impaired in maintaining the cell division arrest response (Fig. 2B and Table II). The A265C mutant displayed a lightly turbid zone of growth arrest indicating that cells formed a partial zone of growth inhibition in response to α-factor but were not able to maintain the zone of inhibition. Sterile indicates that cells did not mate detectably in a patch mating assay. All single mutants show wild-type function except for the ste2-K225C mutant (Fig. 2). Double mutant combinations were also constructed with the S254C mutation but are not shown because the single ste2-S254C mutant displays strong phenotypes on its own (Table I).

Table II

| STE2 allele | Mating ability | Cell division arrest | Elevated basal FUS1-lacZ |
|-------------|---------------|----------------------|--------------------------|
| Wild-type   | +             | +                    | −                        |
| I246C       | +             | +                    | −                        |
| L247C       | +             | +                    | −                        |
| L248C       | +             | +                    | −                        |
| I249C       | +             | +                    | −                        |
| M250C       | +             | +                    | −                        |
| S251C       | +             | +                    | −                        |
| C252        | +             | +                    | −                        |
| Q253C       | Partial response | 4.5-fold             | −                        |
| S254C       | Partial response | 2-fold               | −                        |
| L255C       | +             | +                    | −                        |
| L256C       | +             | +                    | −                        |
| V257C       | +             | +                    | −                        |
| P258C       | Partial response | 47-fold             | −                        |
| S259C       | Partial response | 2-fold               | −                        |
| I260C       | +             | +                    | −                        |
| I261C       | +             | +                    | −                        |
| F262C       | +             | +                    | −                        |
| I263C       | +             | +                    | −                        |
| L264C       | +             | +                    | −                        |
| A265C       | +             | Partial response     | −                        |
| Y266C       | Sterile       | Defective            | −                        |

STE2-Y266C was identified previously as a dominant-negative mutant of the α-factor receptor (11). The Y266C protein was shown to be produced at levels equivalent to the wild-type receptor and to be stable at the cell surface but defective for signaling. The STE2-Y266C mutant was also the only TMD6 mutant that was completely defective for responding to pheromone in the halo assay (Fig. 2B). Four TMD6 mutants (Q253C, S254C, P258C, and S259C) showed significant growth in the zone of division arrest, indicating that these mutants were impaired in maintaining the cell division arrest response (Fig. 2B and Table II). The A265C mutant displayed a lightly turbid zone of growth arrest indicating that cells formed a partial zone of growth inhibition in response to α-factor but were not able to maintain the zone of inhibition. Sterile indicates that cells did not mate detectably in a patch mating assay. All single mutants show wild-type function except for the ste2-K225C mutant (Fig. 2). Double mutant combinations were also constructed with the S254C mutation but are not shown because the single ste2-S254C mutant displays strong phenotypes on its own (Table I).

Constitutive sensitivity was determined by halo assays for cell division arrest. + indicates essentially wild-type response. Defective indicates no detectable halo after 2 days. Partial response indicates a turbid zone of cell division arrest.

Relative α-factor sensitivity was determined by halo assays for cell division arrest. + indicates essentially wild-type response. Defective indicates no detectable halo after 2 days. Partial response indicates a turbid zone of cell division arrest.

Constitutive sensitivity was determined by halo assays for cell division arrest. + indicates essentially wild-type response. Defective indicates no detectable halo after 2 days. Partial response indicates a turbid zone of cell division arrest.

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tollerated individually did not alter receptor function. In contrast, the 
ste2-V223C-M250C mutant showed no detectable mating and the ste2-L226C-L247C mutant was partially impaired in its ability to mate (Fig. 3B and Table III). These mutants also failed to form clear halos, indicating strong defects in the cell division arrest response. A few of the double mutant combinations that could mate well also showed defects in the halo assay even though the corresponding single mutants were not significantly impaired (L222C/L248C, L222C/I249C, L222C/M250C, and V224C/L248C). Thus, certain double mutant combinations caused defects in receptor function that were not observed for either single substitution.

The ligand-independent signaling activity of the double mutant strains was examined next to identify those displaying elevated basal levels of signaling as compared with the corresponding single Cys mutants. Two sets of double mutants were identified by this criterion (Fig. 3). Double mutant combinations involving V223C with L247C, L248C, or S251C and the combinations involving L226C with M250C or S251C displayed constitutive activity in the plate assay. Quantitative assays showed that the mutants displayed 1.9- to 3.7-fold increased basal activity (Table III). The constitutive phenotype was not due to specific effects of the cysteine residues, such as disulfide bond formation, because double mutations in which either Val223 or Leu226 were changed to Ser also caused similar levels of ligand-independent signaling (not shown).

| TMD5/TMD6 substitutions | Mating ability | Cell division arrest | Elevated basal FUS1-lacZ |
|--------------------------|----------------|---------------------|-------------------------|
| Wild-type                | +              | +                   | -                       |
| L222C/L248C             | +              | Partial response    | -                       |
| L222C/L249C             | +              | Partial response    | -                       |
| L222C/M250C             | +              | Partial response    | -                       |
| V223C/L247C             | +              | +                   | 1.9-fold                |
| V223C/L248C             | +              | +                   | 2.2-fold                |
| V223C/M250C Sterile     | +              | Sterile             | -                       |
| V223C/S251C             | +              | +                   | 2.5-fold                |
| V224C/L248C             | +              | Partial response    | -                       |
| V224C/A252C             | +              | Partial response    | -                       |
| K225C/stere2-L226C-L247C| Sterile        | Sterile             | -                       |
| L226C/L247C             | +              | +                   | 3.7-fold                |
| L226C/S251C             | +              | +                   | 3.4-fold                |

* Yeast strain yLG123 carrying the indicated STE2 allele on plasmid pPD225.
  
  + Ability to mate with lys1α cells in a patch mating assay.

  Relative α-factor sensitivity was determined by halo assays for cell division arrest. + indicates essentially wild-type response. Defective indicates no detectable halo after 2 days. Partial response indicates a turbid zone of cell division arrest.

  Basal levels of the FUS1-lacZ reporter gene activity were determined by comparing the relative intensity of blue color for colonies on X-gal plates of strain yLG123 carrying the indicated receptor plasmid. To quantify the + + + increase in basal signaling, receptor plasmids were introduced into strain PMY1 and assayed as described under “Experimental Procedures.”

  * All double mutant combinations tested involving K225C were defective.

The constitutive signaling phenotype associated with certain double mutants could be indicative of an important physical interaction between TMD5 and TMD6, because other residues that cause constitutive activity when mutated have been implicated in inter-helix interactions in the α-factor receptor (22). To examine this possibility, helical wheel analysis was used to predict the orientation of the residues in their respective α-helices. Interestingly, Val223 and Leu226 are expected to fall on one side of TMD5, and Leu247, Leu248, Met250, and Ser251 are predicted to cluster on one side of TMD6 (Fig. 4). Furthermore, all of the residues implicated by the constitutive double mutants are expected to be within a little more than one helical turn from each other at the cytoplasmic ends of TMD5 and TMD6. Thus, it is possible that these residues may interact.

**Intramolecular Cross-linking**—To determine whether the residues identified by genetic analysis may interact physically, the spatial relationship of the substituted cysteine was examined by testing their ability to form an intramolecular disulfide bond. The initial experiments were patterned after similar studies with other membrane proteins such as the bacterial chemotaxis receptors, lac permease, and rhodopsin in which samples were oxidized by treatment with I2 or Cu(II)-1,10-phenanthroline to promote disulfide bond formation (24, 25, 34). However, treatment of yeast membranes with these strong oxidants led to a high degree of nonspecific aggregation of the α-factor receptor. Total yeast membrane proteins were also aggregated as judged by Coomassie Blue staining of proteins separated by gel electrophoresis. The aggregation was due, at least in part, to nonspecific effects rather than to disulfide bond formation, because a receptor protein lacking Cys residues also aggregated under these conditions. In contrast, experiments carried out in the absence of strong oxidants showed that incubation with ambient oxygen was sufficient to promote disulfide bond formation in some mutant receptors without causing aggregation (see below). Interestingly, in the course of these studies, Oprian et al. (35) reported that disulfide bonds that formed after exposure of rhodopsin to ambient oxygen were more specific and were more likely to indicate a close spatial relationship of the residues involved than were disulfide bonds formed after treatment with strong oxidants. Therefore, all further experiments were carried out with ambient oxygen treatment.

Disulfide bond formation between TMD5 and TMD6 was initially assayed by comparing the partial trypsin digestion products of wild-type and mutant receptors on Western blots probed with anti-Ste2p antibodies. This approach attempted to take advantage of the ability of trypsin to cleave preferentially the third loop between TMD5 and -6 (17), but the complex pattern of the partial trypsin digestion made the results difficult to interpret. Therefore, to specifically detect disulfide bonds involving TMD6 after complete digestions with trypsin, a T7 epitope tag was introduced between residues 303 and 304 at the C-terminal end of the trypptic fragment that includes TMD6 and TMD7 (Fig. 5A). The T7 tag was selected for these
The T7 tag thus permitted a strategy where complete digestion with trypsin could be used to assay disulfide bond formation. As diagrammed in Fig. 5A, the T7-tagged tryptic peptide should be approximately 10 kDa, whereas cross-linking between TMDs 5 and 6 should generate a larger 13-kDa peptide. 

![Diagram](image)

**FIG. 5. Properties of T7-epitope-tagged receptor.** The T7 epitope tag was introduced just prior to the trypsin site at Lys304 in STE2 to create pPD225-T7. A, diagram of Ste2 protein with the position of the T7 tag indicated by an arrow and the sequence of the T7 epitope shown in the box. Sites of trypsin digestion are indicated with an asterisk. The tryptic fragment containing TMD5 is shown with diagonal hatching, and the tryptic fragment corresponding to TMD6 is shaded gray. B, halo assay for α-factor-induced cell division arrest. yLG123 cells carrying plasmids encoding the untagged (pPD225) or the T7 epitope-tagged receptor (pPD225-T7) were spread on solid medium, and then filter disks containing 187.5, 375, or 750 pmol of α-factor were applied to induce the observed zones of cell division arrest. C, Western blot analysis of Ste2-T7 proteins produced by ste2Δ cells (yLG123) carrying the indicated STE2 allele on a plasmid. The blot was probed with anti-T7 monoclonal antibody.

Trypsin digestion of the Ste2-T7 protein devoid of cysteine resulted in the detection of the expected 10-kDa peptide containing TMD6 and TMD7 on a Western blot probed with anti-T7 antibody (Fig. 6A). Interestingly, analysis of the T7-tagged versions of the double mutants involving V223C showed that one of the mutant receptors (V223C/L247C) consistently yielded a 13-kDa tryptic peptide as expected for cross-linking (Fig. 6B). This 13-kDa band did not shift down in mobility even when 33-fold more trypsin was used than was required to detect the lower 10-kDa band. These data provide, to our knowledge, the first direct demonstration of the physical proximity between any of the TMDs in the α-factor receptor and indicate that Val223 and Leu247 are likely to interact in the wild-type α-factor receptor.

**FIG. 6. Disulfide bond formation in the V223C/L247C mutant receptors.** Membranes were harvested from yeast yLG123 carrying (A) wild-type, (B) V223C/L247C, or the (C) V223S/L247C derivatives of a T7 epitope-tagged allele of STE2 on plasmid vector pPD225-T7. The membranes were oxidized with ambient oxygen and digested with the indicated concentration of trypsin for 1 h at 37 °C. Where indicated, samples were reduced by addition of dithiothreitol to 200 mM final concentration. Samples were fractionated on a 15% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with an anti-T7 antibody (Novagen Inc.). Immunoreactive bands were detected with ECL (Super-Signal Ultra, Pierce). Positions of protein markers are noted to the left.
FIG. 7. Predicted membrane topology of the α-factor receptor.

The central amino acids of the 431-residue long α-factor receptor are indicated by the one-letter code. The black line between TMD5 and TMD6 highlights the positions of Val223 and Leu247 that are predicted by the results of this study to be in close proximity. A set of lines between Gln253 in TMD6 and Ser288 and Ser292 in TMD7 mark the predicted intramolecular contact that was identified by analysis of constitutive mutants (22). Black circles indicate the residues affected by dominant-negative mutations (11, 12). The open circle indicates the position of Lys225, and the triangle indicates the position of Ala229; these are sites of Cys substitutions that caused loss of function and supersensitivity, respectively.

The Cys scanning mutagenesis did, however, identify two mutants that indicate that the junction between TMD5 and the third intracellular loop is important for α-factor receptor signaling. The R225C mutation caused loss of function, and A229C caused 3-fold hypersensitivity to pheromone. Interestingly, double mutant analysis carried out by combining Cys substitutions in TMD5 and TMD6 identified a neighboring cluster of residues that are also important for receptor function. This cluster of residues was identified, because certain double mutant combinations caused synergistic defects that were not seen in the corresponding single mutants. For example, the V223C/M250C double mutant was strongly defective in signaling and other double mutants that combined V223C in TMD5 with L247C, I248C, or S251C substitutions in TMD6 caused constitutive activity. Constitutive phenotypes were also identified when L226C in TMD5 was combined with the M250C or S251C substitutions in TMD6. The residues mutated in all of these double mutant combinations show an interesting relationship in that they are predicted to fall on one face of their respective helices (Fig. 4). Thus, the genetic interactions displayed by these mutants suggest that the residues involved are important for receptor function and may define regions of TMD5 and TMD6 that interact.

Interaction between TMD5 and TMD6—The spatial relationship of the residues identified in the double mutants analysis was examined by determining if the residues were close enough to form a disulfide bond. Screening the mutant proteins identified a cross-link between Cys residues substituted for Val223 and Leu247. Interestingly, detection of this disulfide bond did not require the addition of strong oxidizing agents that have been used in most previous Cys cross-linking studies. This is significant because studies on rhodopsin indicated that disulfide bonds promoted by ambient oxygen resulted in cross-linking that was more specific and was indicative of a close spatial relationship between the Cys residues (35). Another difference in our approach relative to previous cross-linking studies was that we assayed disulfide bond formation by analyzing the gel mobility of an epitope-tagged tryptic fragment. The α-factor receptor could not be analyzed by the previously used protease digestion methods (35, 40, 41), because it lacks a unique protease site between the Cys residues that can be used to assess whether they are disulfide-linked. The α-factor receptor also did not tolerate the introduction of a novel protease site (Factor X) in the third loop to use for this analysis. Thus, introduction of an epitope tag should be applicable to other receptors that, like the α-factor receptor, cannot be analyzed by previously used protease digestion approaches. In addition, the epitope tag strategy may also be useful for confirming the results of cross-linking studies that detect disulfide bonds formed between coexpressed halves of split receptors (34, 35), because the split receptors may not always efficiently associate to form a native receptor structure.

The detection of a cross-link between Val223 and Leu247 provides an important point of reference for the orientation of the residues in TMD5 and TMD6 (Fig. 4). For example, it is interesting that Ser254 in TMD6 is predicted to face in the general direction of TMD5, because substitution of Ser254 with residues of large van der Waals radius (i.e. S254F) caused higher constitutive activity than did substitution with smaller residues, suggesting that the constitutive activity is a consequence of altered helix packing (22). This orientation also predicts that Ser219 in TMD5 faces toward TMD6, consistent with the interpretation that Ser219 substitution mutants respond to a broader range of agonists because of altered helix packing (42, 43). In addition, this model indicates that TMD5 interacts with the side of TMD6 that is opposite from Gln253, a residue that is predicted to interact with TMD7 (Fig. 4). Gln253 is thought to interact with Ser288 and Ser292 in TMD7 based on the analysis of constitutively active receptor mutants (22). The overall arrangement of TMD5, -6, and -7 predicted by these studies is significant, because it is consistent with the organization of the helix bundle predicted for members of the rhodopsin family of GPCRs (20, 44). Thus, although the α-factor receptor does not share significant sequence homology with the large rhodopsin family of GPCRs, there is underlying structural and functional similarity.

The identification of a cross-link between Cys residues substituted for Val223 and Leu247 also has interesting implications for models of the membrane topology of TMD5. Based on hydrophathy, TMD5 was predicted to encompass residues 209–229 (31, 32). However, for residues 223 and 247 to be in close juxtaposition, the core of TMD5 is now predicted to span residues 203–225 (Fig. 7). This new positioning of TMD5 has two interesting consequences. One is that this alignment places Lys225 at the cytoplasmic interface, instead of being buried in the membrane. As there is no obvious counterion for Lys225 in the other TMDs, positioning this residue nearer the polar lipid head groups and cytoplasm is more energetically favorable. The other interesting aspect of this topology is that it places the TMD5 residues affected by dominant-negative mutations near the extracellular end of the transmembrane segment (Fig. 7). The four dominant-negative mutations in TMD5 are now in better juxtaposition with the 10 other sites of dominant-negative mutations found in TMD2, -3, -4, -6, and -7 that are all predicted to map near the extracellular ends of membrane-spanning regions (11, 12). Thus, shifting the predicted topology of TMD5 also helps to better define the domain affected by the

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dominant-negative mutations that plays an important role in ligand binding and receptor activation. Altogether, these results significantly improve the two-dimensional models of receptor structure and provide an important frame of reference for the development of a three-dimensional model of the \( \alpha \)-factor receptor.

Implications for Signal Transduction—The identification of an interaction between a cluster of nonpolar residues at the base of TMD5 and -6 in the \( \alpha \)-factor receptor has interesting implications for the function of other GPCRs. This may be a conserved feature of GPCRs, because disruption of intramolecular contacts between the base of TMD5 and TMD6 is thought to constitutively activate the Lutropin receptor and cause precocious puberty (45). Similarly, the interaction of a hydrophobic cluster of amino acids at the base of TMD5 and TMD6 appears to be important for restraining the thyrotropin-releasing hormone receptor in the inactive state (46). Although, the particular amino acids identified in these studies may function in an analogous way. In this regard, it is interesting that the corresponding regions of TMD5 and TMD6 of rhodopsin are closely packed (20). Collectively, these data indicate that, despite a lack of sequence similarity among the GPCR family, there is a remarkable similarity in the structure and function of these receptors.

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Interaction between Transmembrane Domains Five and Six of the α-Factor Receptor

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