

Agonist-Specific Conformational Changes in the Yeast α -Factor Pheromone Receptor

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The yeast α -factor pheromone receptor is a member of the G-protein-coupled receptor family. Limited trypsin digestion of yeast membranes was used to investigate ligand-induced conformational changes in this receptor. The agonist, α -factor, accelerated cleavage in the third intracellular loop, whereas the antagonist, desTrp1,Ala3- α -factor, reduced the cleavage rate. Thus, the enhanced accessibility of the third intracellular loop is specific to the agonist. α -Factor inhibited cleavage weakly at a second site near the cytoplasmic terminus of the seventh transmembrane helix, whereas the antagonist showed a stronger inhibition of cleavage at this site and at another site in the C-terminal domain of the receptor. The α -factor-induced conformational changes appeared to be inherent properties of the receptor, as they were retained in G-protein-deficient mutants. Moreover, a mutant receptor (*ste2-L236H*) that affects the third loop and is defective for G-protein coupling retained the ability to undergo the agonist-induced conformational changes. These results are consistent with a model in which G-protein activation is limited by the availability of specific contacts between the G protein and the third intracellular loop of the receptor. The antagonist appears to promote a distinct conformational state that differs from either the unoccupied or the agonist-occupied state.

Receptors coupled to heterotrimeric GTP-binding proteins (G proteins) contain seven transmembrane helices and control cellular responses to a variety of stimuli, including hormones, neurotransmitters, light, and odorants (2, 13, 46). It is widely believed that the conformational changes induced by agonist binding permit these receptors to associate productively with G proteins, thus resulting in the exchange of GDP for GTP in the G_{α} subunit. Three lines of evidence suggest that the third intracellular loop of the receptor provides at least some of the intermolecular contacts that promote guanine nucleotide exchange: (i) work with chimeric receptors indicates that the third intracellular loop influences the specificity of G-protein interactions (13); (ii) mutations in the third intracellular loop (2, 11, 46, 48) block coupling of receptors to their respective G proteins; and (iii) synthetic peptides that comprise the third loop bind G proteins *in vitro*, thereby stimulating nucleotide exchange and blocking the association of the G protein with the receptor (47). It has been proposed that structural constraints in receptors prevent the third intracellular loop from contacting the G protein when agonists are absent and that these structural constraints are relieved when the receptor assumes an agonist-activated conformation (36). However, there is as yet no direct evidence which demonstrates that increased exposure of the third intracellular loop is a specific consequence of agonist binding.

The ability of ligands to promote changes in protein conformation has been largely pioneered by studying hemoglobin and allosteric enzymes (1, 37, 41). In general, allosteric proteins are thought to assume two conformational states that are in dynamic equilibrium. Ligands that activate the protein shift the equilibrium toward the more active state, since they bind the active conformation preferentially, whereas inhibitors shift the

equilibrium toward the inactive state by binding preferentially to the inactive conformation (29). The allosteric models that have been proposed for G-protein-coupled receptors predict that agonists and certain antagonists (termed inverse agonists) should have opposing effects on receptor structure (7, 36). Relatively little structural information that pertains to the allosteric transitions in G-protein-coupled receptors is available. Biophysical and biochemical investigations of rhodopsin suggest that photoactivation leads to conformational changes at several sites in this protein, including changes in the extracellular loops; however, the relationship of the conformational changes at these sites to G-protein activation remains unclear (15).

This study concerns ligand-induced conformational changes in the α -factor pheromone receptor from the yeast *Saccharomyces cerevisiae*. The agonist, α -factor, is synthesized by haploid cells of the α mating type, and it binds to receptors located on the surface of haploid cells of the a mating type. Both partial agonists and antagonists of this receptor have been described (27, 30, 43). The receptor (encoded by the *STE2* gene) requires homologs of the mammalian G_{α} , G_{β} , and G_{γ} protein subunits (encoded by the *GPA1*, *STE4*, and *STE18* genes, respectively) for their ability to cause G_1 arrest of cell division and to induce the expression of genes controlling the conjugation of the two cell types (3, 28, 40). The receptor appears to form a direct physical association with these G-protein subunits, since α -factor binds *ste4* mutant cells more weakly (20) and since it dissociates more rapidly from membranes assayed in the presence of GTP analogs or when the membranes are prepared from *gpa1*, *STE4^{Hpl}*, or *ste18* mutants (6). Certain mutations affecting the third intracellular loop of the receptor severely block its ability to couple with the G protein and to generate an intracellular signal (48). Other α -factor responses include receptor endocytosis (10, 21, 38) and phosphorylation (9, 34), mating-partner selection (19, 42), and the promotion of changes in cellular morphology (23). In this report, we show that α -factor-induced conformational changes in the receptor result in enhanced accessibility of the

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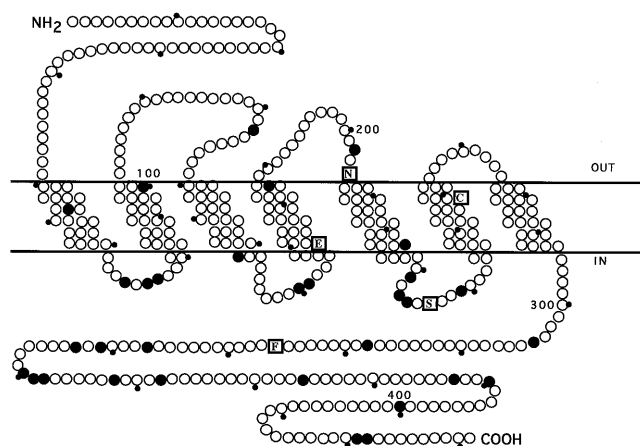


FIG. 1. Primary structure of the α -factor receptor protein. Filled circles indicate potential trypsin cleavage sites. Residues labeled E, N, S, and C indicate C termini predicted for the truncated receptors that were synthesized in vitro. Mutation *ste2-T326* results in translational frameshift after residue 326 (labeled F) and a protein product containing 334 residues (24).

third intracellular loop whereas the antagonist, desTrp1,Ala3- α -factor, reduces accessibility.

MATERIALS AND METHODS

Yeast strains and culture conditions. All strains used are congenic with strain 381G (18). The genotype of strain DJ211-5-3 (deposited in the American Type Culture Collection; ATCC 96515) is *MATa cryl bar1-1 ade2-1 his4-580 leu2 lys2 trp1 tyr1 ura3 SUP4-3*. Strain DJ903-A-1 (38) is identical to strain DJ211-5-3 except that it contains mutation *ste2-T326* (24). Strain DJ803-2-1 (provided by Jodi Hirschman) is identical to strain DJ211-5-3 except that it contains mutation *ste5-3* (18) and mutation *scg1::lacZ(LEU2)*, which disrupts the *GPA1* gene (12). Cells were cultured in YM-1 medium (17) at 30°C unless indicated otherwise.

Peptide ligands. α -Factor (WHWLQLKPGQPMY) was obtained from Sigma Chemical Co. (St. Louis, Mo.). The antagonist desTrp1,Ala3- α -factor (HALQLKPGQMY) was synthesized and analyzed by Quality Controlled Biochemicals (Hopkinton, Mass.); the sequence was confirmed by amino acid analysis and mass spectrometry.

Analysis of partial trypsin digestion products. A cleared cellular lysate of strain DJ211-5-3 was prepared as described previously (38). Membranes were collected by centrifugation (45 min, 140,000 \times g), washed once with 1 mM magnesium acetate–0.1 mM dithiothreitol–0.1 mM EDTA–7.6% glycerol–10 mM morpholinopropanesulfonic acid (MOPS; pH 7.0), and stored at –70°C. Digestion of membranes (0.4 mg of protein per ml) was initiated by adding tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma Chemical Co.) to 30 μ g/ml at 30°C and terminated by adding HCl to 0.016 N. In parallel reactions, 2 μ M synthetic α -factor was added prior to the addition of trypsin. Membranes were collected by centrifugation in a Beckman Airfuge, suspended in a solution of 1% sodium dodecyl sulfate (SDS) and 1% β -mercaptoethanol, incubated for 20 min at 40°C, and diluted eightfold with 50 mM sodium phosphate buffer (pH 7). Asparagine-linked carbohydrate was removed with endoglycosidase H (2 h, 37°C). Proteins were denatured with SDS sample buffer (10 min, 37°C), resolved electrophoretically on an SDS–12% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (Millipore Corp, Bedford, Mass.), probed with affinity-purified antireceptor antiserum directed against the N-terminal extracellular domain (24), and visualized with the ECL chemiluminescence detection system (Amersham Life Science).

In vitro protein synthesis. Plasmids pJBK019 and pJBK020 (provided by James Konopka, State University of New York, Stony Brook) contain the coding region of the *STE2* gene cloned into the pGEM1 and pGEM2 vectors, respectively. Plasmids were cleaved with *HindIII*, *ClaI*, *SpyI*, *NsiI*, or *EcoRV* and transcribed with either SP6 or T7 RNA polymerase. The RNA product was purified with sequential precipitations with 5 M LiCl and 70% ethanol. In vitro translation reactions containing [³⁵S]methionine used either the wheat germ (full-length control and truncated proteins S, N, and E [Fig. 1]) or the rabbit reticulocyte lysate (truncated protein C) system as recommended by the supplier (Promega Biotec). The translation products were resolved on a single SDS-polyacrylamide gel together with trypsin-treated wild-type membranes (strain DJ211-5-3) and with *ste2-T326* mutant membranes (strain DJ903-A-1), transferred to a polyvinylidene difluoride membrane, and probed with antireceptor antibody as described above. The radioactive translation products were detected by autoradiography.

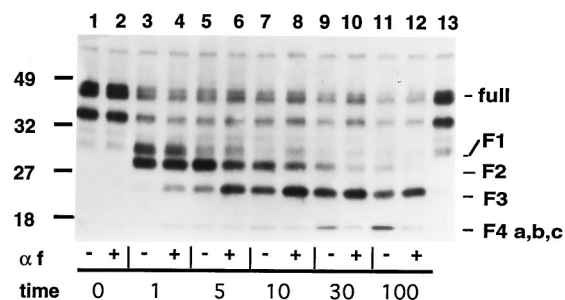


FIG. 2. Influence of α -factor on the receptor cleavage pattern. Membranes were incubated with trypsin for the number of minutes indicated in the presence or absence of α -factor (α f). The resulting cleavage products were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting methods. The antiserum was against the N terminus of the receptor (24). The control reaction was incubated for 100 min in the absence of trypsin and α -factor (lane 13). The major cleavage products (F1, F2, F3, and F4 a,b,c) are indicated at the right. Molecular weights of the marker proteins are indicated in thousands at the left.

RESULTS

Effect of α -factor on the receptor cleavage sites. Limited trypsin digestion was used to monitor ligand-induced conformational changes in the α -factor receptor. The receptor contains 32 potential trypsin sites (Fig. 1), whereas no sites are present in α -factor. Initial attempts to cleave receptors on whole cells were unsuccessful, presumably because the two trypsin sites that are predicted to face the extracellular surface are shielded either by the receptor conformation or by other cell surface components. In contrast, receptors in crude membrane preparations were cleaved readily. Figure 2 depicts the electrophoretic analysis of the cleavage products that resulted from various periods of trypsin digestion. Since antiserum specific for an N-terminal segment of the receptor (24) was used to detect these tryptic fragments, their electrophoretic mobilities reflect the locations of the various cleavage sites relative to the N terminus (the N-terminal domain of the receptor contains no trypsin sites). The undigested sample contained two electrophoretic species corresponding to the full-length receptor (approximately 43 kDa) and to a smaller fragment (35 kDa) that apparently resulted from cleavage during membrane preparation (Fig. 2, lanes 1 and 2). In the absence of α -factor, the receptor was cleaved within 1 min to yield the fragments designated F1 and F2 (lane 3). By 5 min, F2 was the predominant form (lane 5). The cleavage events giving rise to fragment F3 were considerably slower (lane 7). Cleavages closer to the N terminus of the receptor eventually gave rise to a collection of fragments designated F4 a, b, and c (lanes 9 and 11) which were resolved on SDS–15% polyacrylamide gels (not shown).

The binding of α -factor influenced the rates at which certain trypsin sites were cleaved. Fragment F1 persisted slightly longer in the presence of α -factor (Fig. 2; compare lanes 7 and 8), suggesting that the cleavage site giving rise to fragment F2 is less accessible in the agonist-occupied conformation. Once F2 was formed, however, it was converted to F3 more rapidly in the presence of α -factor (lanes 5 through 8), suggesting that the site giving rise to F3 is more accessible to trypsin in the agonist-occupied conformation. By 30 min, the formation of F3 was essentially complete in both the presence and the absence of α -factor (lanes 9 and 10). When α -factor was present, F3 was relatively resistant to further cleavages (compare lanes 11 and 12), thus indicating that the trypsin cleavage sites giving rise to the F4 fragments are apparently protected by α -factor binding. Although the overall rate of receptor digestion varied over a 10-fold range for different membrane and trypsin prep-

arations, α -factor consistently inhibited the formation of F2 and accelerated the formation of F3 among all five of the independent experiments.

Protease digestion offers a powerful phenomenological tool for detecting changes in protein conformation. However, its usefulness for defining specific aspects of protein conformation is subject to some limitations. First, the accumulation of a specific intermediate is a function of the cleavage event that forms it as well as the cleavages that destroy it. The increased levels of fragment F3 that occur in the presence of α -factor are apparently due to an increased rate of formation rather than decreased rates of destruction, since differential accumulation of F3 is apparent after only 1 and 5 min of digestion; at these time points, the F4 fragments have not yet begun to accumulate. The second limitation that complicates the kinetic analysis is the potential influence of one cleavage event on the cleavage rate at a second site. Since F1 and F2 form before F3, it could be possible that α -factor influences the formation of F3 only as an indirect consequence of its action on the formation of F1 or F2. This explanation appears to be inconsistent with our findings; α -factor does not influence the formation of fragment F1 and has only a modest inhibition on the formation of F2, yet it dramatically stimulates formation of F3. Moreover, as discussed below, the binding of an α -factor antagonist changes the relationship of these two cleavage events. Third, the steric restrictions that influence the interaction of trypsin with a site on the receptor may be different from the restrictions that govern interactions with the physiologically relevant molecules. Finally, it is not possible to detect slow cleavage events that occur at sites distal to rapid cleavage events.

Assignment of trypsin cleavage sites. The trypsin cleavage sites were assigned to positions in the primary structure of the receptor by comparing the electrophoretic mobilities of tryptic fragments with receptor fragments of known length. Figure 1 shows the potential trypsin cleavage sites and the positions of the C-terminal residues of the various truncated receptors (E, N, S, C, and F). Full-length receptors that had been synthesized in vitro (Fig. 3, lane 4) migrated in the same position as those synthesized in vivo (lane 3). Fragment F3 (lanes 1, 2, and 9) was larger than synthetic receptors truncated at position 204 (truncation N, lane 7) yet smaller than synthetic molecules truncated at position 236 (truncation S, lane 6). Thus, the cleavages that occur at or near the third intracellular loop (position 225, 231, 233, or 234) are stimulated by α -factor binding. Fragment F2 (lanes 1, 2, 9, and 10) was larger than synthetic receptors truncated at position 259 (truncation C, lane 5) and smaller than the *ste2-T326* mutant receptors (truncation F, lane 11); thus, α -factor inhibits cleavages at the beginning of the C-terminal cytoplasmic domain (position 304 or 318). Fragment F1 was larger than the *ste2-T326* mutant receptors, indicating that the most rapidly cleaved sites are at or beyond position 337.

Analysis of mutant cells. The possible influence of the G protein on the conformational change was evaluated by examining the digestion pattern of membranes that had been prepared from mutant cells lacking G-protein subunits. Specifically, we were concerned that the increased rate of third-loop cleavage upon α -factor binding (Fig. 2) may have been a consequence of dissociation of the G protein from the receptor rather than a direct consequence of a receptor conformational change. When membranes were assayed from *gpa1* mutant cells that lack the G_α subunit (Fig. 4A), α -factor both slowed the formation of fragment F2 (lanes 5 and 6) and accelerated the formation of fragment F3 (lanes 5 through 10), as was observed for the wild-type strain (Fig. 2). The overall digestion rate was slower than that shown in Fig. 2; however, it was

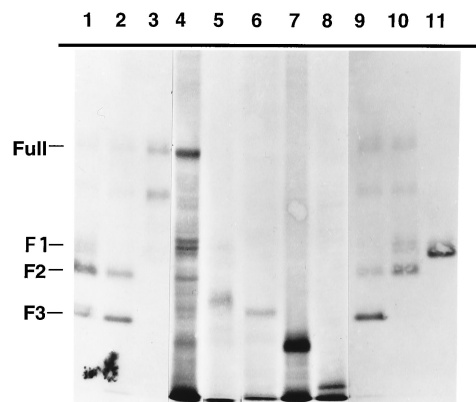


FIG. 3. Positions of the major receptor cleavage sites. Full-length receptors (lane 4) and the truncated receptors depicted in Fig. 1 (lanes 5 to 8) were synthesized in vitro and resolved by SDS-polyacrylamide gel electrophoresis. Translation is predicted to terminate at residue 431 for full-length receptors, at residue 259 for truncation C (lane 5), at residue 236 for truncation S (lane 6), at residue 204 for truncation N (lane 7), and at residue 158 for truncation E (lane 8). Wild-type membranes (lanes 1 to 3, 9, and 10) and *ste2-T326* mutant membranes (lane 11) were prepared and analyzed as for Fig. 2. The membrane preparations were undigested (lanes 3 and 11) or digested for 1 min (lanes 1 and 10) or 10 min (lanes 2 and 9) with trypsin in the absence (lanes 2, 10, and 11) or presence (lanes 1 and 9) of α -factor. All of the samples were visualized on a single SDS-polyacrylamide gel that had been transferred to a polyvinylidene difluoride filter. The figure was generated by superimposing the autoradiographic (lanes 4 to 10) and the chemiluminescent (lanes 1 to 3 and 9 to 11) images. Positions of the major cleavage products are indicated on the left.

within the range that we observed for wild-type membrane preparations. The additional digestion intermediate that is larger than F1 (Fig. 4A, lanes 3 and 4) was consistently observed with both wild-type and mutant membranes when the overall digestion rate was slow. The effects of α -factor were similar for the *ste4* and *ste18* mutants lacking the G_β and G_γ subunits, respectively (not shown). We conclude that the α -factor-induced changes in the tryptic digestion pattern are not a simple consequence of G-protein dissociation and that the α -factor sensitivity of the digestion pattern is likely to reflect conformational changes in the receptor itself. We cannot determine whether the G-protein deficiencies lead to small differences in the overall digestion rate, since the limitations of our assay permit us to compare only the effects of α -factor on the digestion of a given membrane preparation.

The *ste2-L236H* mutation affects the third intracellular loop of the α -factor receptor and results in a partial block in signal transduction activity (38, 48). Previous studies (48) showed that the affinity of this mutant receptor for α -factor was relatively unaffected by GTP analogs, suggesting that the coupling between the mutant receptor and the G protein was impaired. Any one of three changes in receptor structure could conceivably result in this coupling defect. (i) Alterations in the α -factor binding site may obviate the energetic contribution of agonist binding to the allosteric transition; (ii) structural constraints in the mutant receptor may disfavor the conformational change; or (iii) the receptor in its active conformation may be unable to associate productively with the G protein. We found that ability of α -factor to promote the conformational transition was retained in *ste2-L236H* mutant receptors (Fig. 4B). Thus, the signal transduction defect of *ste2-L236H* mutant receptors apparently reflects a failure of the receptor to form a productive complex with G protein in that we were unable to detect a defect in the ligand-induced conformational change. However, we cannot rule out the possibility that other conformational deficiencies that were not detected in our assay

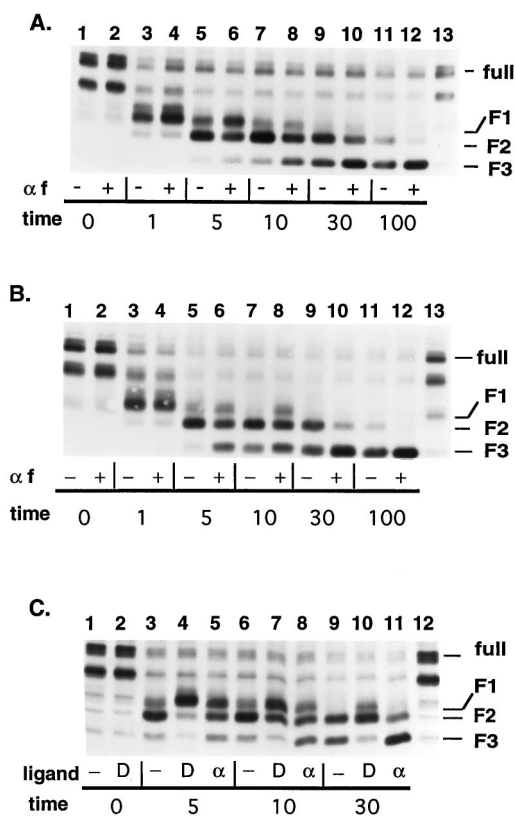


FIG. 4. Effects of mutations and antagonist on the cleavage pattern. (A) Membranes from the *gpa1* mutant were prepared and analyzed as described for Fig. 2 except that the strain contained the temperature-sensitive mutation *ste5-3* (18) and was cultured at 34°C. These conditions avoided the haploid-lethal phenotype exhibited by *gpa1* mutants (4). α f, α -factor. (B) Membranes from the *ste2-L236H* mutant (38) were prepared and analyzed as described for Fig. 2. (C) Wild-type membranes (strain 211-5-3) were treated with trypsin for the period indicated in the absence of ligand (–), in the presence of 2 μ M α -factor (α), or in the presence of 2 μ M desTrp1,Ala3- α -factor (D). Membranes were also incubated for 30 min in the absence of trypsin and α -factor (lane 12).

led to the signal transduction phenotype of the *ste2-L236H* mutant.

Antagonist-induced conformations. To identify the structural features of the ligand-occupied receptor that pertain to G-protein activation, we examined effects of antagonist desTrp1,Ala3- α -factor (43) on receptor cleavage rates. Although this peptide binds receptors, it is unable to trigger cell division arrest or to stimulate induction of α -factor-responsive genes (32). Figure 4C shows that desTrp1,Ala3- α -factor differed from α -factor in its ability to influence receptor cleavage, in that the formation of all proteolytic fragments was slowed dramatically when the antagonist was present. First, although both α -factor and desTrp1,Ala3- α -factor inhibited appearance of fragment F2, the antagonist showed a significantly greater effect (lanes 3 to 5). Second, antagonist decreased the rate at which fragment F3 was formed (lanes 9 and 10), whereas α -factor accelerated the formation of F3 (lanes 6, 8, 9, and 11). Finally, at the 5-min time point (lanes 3 to 5), a weak band that migrates more slowly than fragment F1 is apparent only in the reaction containing antagonist (lane 4); thus, the cleavage that gives rise to F1 is also apparently inhibited by antagonist. In control reactions, the antagonist did not perturb the proteolytic activity of trypsin per se, as judged by cleavage of the chromogenic substrate *N* α -benzoyl-L-arginine ethyl ester (BAEE)

(not shown). Our results are consistent with the third intracellular loop playing an important role in G-protein activation, since this region is exposed to the cytosolic environment only upon agonist binding. In contrast, as both the agonist and the antagonist inhibited the formation of fragment F2, we were unable to define a specific role for the conformational changes that influence the C-terminal domain, except that the reduced accessibility afforded by the antagonist does not in itself provide a signal that is sufficient to activate the G protein. The distinct proteolytic pattern induced by the antagonist implies that it promotes a conformational state of the receptor that differs from either the agonist-occupied or the unoccupied state.

It could be argued that antagonist reduces the rate of third-loop cleavage as an indirect consequence of its inhibitory action at the distal cleavage sites. This does not appear to be the case, since the 5-min digestion of the α -factor-bound receptor (Fig. 4C, lane 5) and the 10-min digestion of the antagonist-bound receptor (lane 7) are degraded equally to fragments F1 and F2; yet after an additional 5 min of digestion, the α -factor-bound receptor (lane 8) shows a greater accumulation of fragment F3 than the antagonist-bound receptor shows after an additional 20 min of digestion (lane 10). Hence, the reduction in rate of third-loop cleavage that is caused by the antagonist is apparently not an indirect consequence of slower cleavages at distal sites in the receptor.

DISCUSSION

In this study, we used limited trypsin digestion of the α -factor receptor to identify the conformational changes that are induced by the binding of α -factor and its antagonist, desTrp1,Ala3- α -factor. α -Factor caused the third intracellular loop of the receptor to become more accessible to trypsin, and it caused a second site in the C-terminal domain near the seventh transmembrane helix to become less accessible. Both of these α -factor-induced changes were also observed with G-protein-deficient membranes; thus, the relative rates of cleavage appear to reflect conformational states of the receptor rather than the association of the receptor with the G protein. Previous workers have found similar results for mammalian rhodopsin in that photoactivation accelerates proteolytic cleavages in the third extradiscal loop (31) and inhibits cleavages in the C-terminal domain (25). We show for the first time that the changes in the third intracellular loop are specific for the agonist, since α -factor and its antagonist had opposing effects on the accessibility of trypsin to this region. The importance of the third intracellular loop in the α -factor receptor is also apparent from the mutants that block G-protein coupling (48). The fact that the *ste2-L236H* mutant retains the ability to undergo the agonist-induced conformational change (Fig. 4B) suggests that the amino acid substitution in this loop blocks its ability to associate with the G protein.

A two-state allosteric model has been used to describe the initial action of agonists and antagonists on the conformation of G-protein-coupled receptors (7, 36). In this model, agonists shift the dynamic equilibrium of receptor toward the activated R* state, whereas certain antagonists, termed inverse agonists, stabilize the inactive R state. Only the R* state is thought to form a productive complex with G protein. The model makes two predictions. First, agonists and inverse agonists should have opposing influences on the aspects of receptor conformation that are relevant to signal transduction. Second, these conformational changes should be an inherent property of the receptor (i.e., they should be G protein independent). The desTrp1,Ala3- α -factor peptide appears to have inverse agonist

activity in that a related antagonist (desTrp1,Ala3,Nle12- α -factor) has a negative influence on G-protein coupling (6); i.e., GTP analogs increase the affinity of the receptor for antagonist under conditions that decrease the affinity for agonist. With regard to the exposure of the third intracellular loop, our results satisfy both predictions of the two-state allosteric model, since α -factor and desTrp1,Ala3- α -factor have opposing influences on the rate of third-loop cleavage (Fig. 4C), even in the absence of G protein (Fig. 4A). Hence, one essential attribute of the R* state of the α -factor receptor may include the exposure of the third intracellular loop. However, the receptor can clearly exist in more than two conformational states, as the antagonist also retards the cleavage rates at two sites in the C-terminal domain; the physiological significance of the additional state(s) is not yet clear. Fluorescent modifications of the β_2 -adrenergic receptor have also been used to address the relative influences of agonists and inverse agonists on receptor conformation (16); the fluorescence emission from one or more of the modified cysteine residues (located only in the second and third extracellular loops) is decreased by agonists, whereas the fluorescence emission is increased by inverse agonists.

For many G-protein-coupled receptors, genetic evidence suggests that the third intracellular loop plays a central role in signal transduction. Constitutive mutants affecting mammalian α_2 - and β_2 -adrenergic receptors exhibit higher affinities for agonists (but not antagonists) even in the absence of G-protein function (33, 36). Hence, the association of the third intracellular loop with the body of the receptor may provide an energetic contribution to the relative stability of the R state. Certain yeast mutants affecting the third intracellular loop in both the α -factor receptor (11, 44) and the related α -factor receptor (8) exhibit either a partially constitutive signal or enhanced pheromone sensitivity. Interestingly, many of the partially constitutive *ste2* mutants also show increased affinity for α -factor (11, 44), consistent with the hypothesis that they reduce the energetic requirement for the allosteric transition. Other α -factor receptor mutants fail to undergo the allosteric transition (8a) or permit desTrp1,Ala3- α -factor to function as an agonist (27, 44). It should now be possible to test directly whether specific amino acid substitutions affect the dynamic properties of the α -factor receptor conformation.

In addition to the third intracellular loop, other portions of the G-protein-coupled receptor structure are likely to play a role in G-protein activation. The binding of receptors to their cognate G proteins can be competed for with antibodies directed against the second intracellular loop or the C-terminal domain (49). Similarly, binding of the G protein to the receptor is competed for by the presence of synthetic peptides that comprise a portion of the second loop or the C-terminal domain of rhodopsin (22, 39). Many α -factor antagonists (14), including desTrp1,Ala3- α -factor (our unpublished results), appear to elicit partial activation of the G protein, in that they show agonist activity when the target cells are supersensitive to α -factor as a result of the presence of the *sst2* mutation. Since desTrp1,Ala3- α -factor does not increase the exposure of the third intracellular loop, conformational changes at other sites in the α -factor receptor may contribute to signal transduction. In the presence of the antagonist, activation may result either from the reduced exposure of the C-terminal domain or from other structural changes that were not detected by our trypsin digestion assay. However, the C-terminal domain of the α -factor receptor is unlikely to play an essential role in G-protein activation, since truncated receptors lacking this region remain responsive to agonist (24, 34).

The binding of α -factor also stimulates endocytosis of the

α -factor receptor in addition to G-protein activation (38). The conformational change necessary for endocytosis does not appear to require G-protein function (5, 21, 50). Endocytosis requires the C-terminal domain of the receptor (35, 38) and is not blocked by mutations in the third intracellular loop (38, 48). It is possible that the agonist-induced conformational changes that promote receptor endocytosis are reflected by the reduced rates of trypsin cleavage in the C-terminal domain. It is of interest that these cleavages (amino acid residue 304 or 318) occur close to the DAKSS sequence (residues 335 to 339) that is believed to mediate the regulation of endocytosis (35). In addition to endocytosis, other α -factor responses, including adaptation to the stimulus (24, 34), receptor phosphorylation (34), and changes in cellular morphology (23, 24), are mediated by the C-terminal domain; it is conceivable that the conformational change that we detect in the C-terminal domain may also play a role in these α -factor responses as well.

No high-resolution structures for G-protein-coupled receptors are yet available. However, for many of these receptors, models for the packing of the seven helices have been proposed (2, 45). Upon ligand binding, an individual helix may become altered in its relationship to its neighbors by undergoing a translational (piston model) or a rotational motion along its helical axis, by altering the angle at which it traverses the membrane, or by undergoing a lateral translational motion. One possible model for the agonist-induced conformational change in the α -factor receptor would be that movements in helix 6 and helix 7 shield the cytoplasmic region adjacent to helix 7 (resulting in slower formation of fragment F2) and expose the third intracellular loop, adjacent to helix 6 (resulting in accelerated formation of fragment F3). Interestingly, a mutation affecting the short extracellular loop that connects helices 6 and 7 prevents exposure of the third intracellular loop upon agonist binding (8a). Consistent with this interpretation, computer-generated simulations of the 5-hydroxytryptamine receptor (26, 51) suggest that agonist binding causes a rotation of helix 7 that is coupled with rotation of helix 6, with lateral translation of helix 4, and ultimately with exposure of the third intracellular loop.

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