University of Massachusetts Medical School

eScholarship@UMMS

1998-01-28

Genetic and Biochemical Analysis of the Activation Mechanism of the Saccharomyces Cerevisiae Pheromone Receptor

Gul H. Bukusoglu
University of Massachusetts Medical School

Let us know how access to this document benefits you.

GENETIC AND BIOCHEMICAL ANALYSIS OF THE ACTIVATION MECHANISM OF THE *SACCHAROMYCES CEREVISIAE* PHEROMONE RECEPTOR

A Dissertation Presented

By

Gül H. Büküsoglu

Submitted to the faculty of the

University of Massachusetts Graduate School of Biomedical Sciences,

Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN

MOLECULAR GENETICS AND MICROBIOLOGY

January 1998

Copy Rights

Chapter I was published as:

Büküsoglu, G., and D. D. Jenness. (1996) Agonist-specific conformational changes in the yeast α-factor pheromone receptor. Molecular and Cellular Biology, 16: 4818-4823.

GENETIC AND BIOCHEMICAL ANALYSIS OF THE ACTIVATION MECHANISM OF THE *SACCHAROMYCES CEREVISIAE* PHEROMONE RECEPTOR

A Dissertation Presented

By

Gül H. Büküsoglu

Approved as to style and content by:

Dr. Donald Tipper, Chair of Committee

Dr. Peter Pryciak, Member of Committee

Dr. Reid Gilmore, Member of Committee

Dr. William Royer, Member of Committee

Dr. James Konopka, Member of Committee

Dr. Duane D. Jenness, Thesis Advisor

Dr. Thomas B. Miller, Jr., Dean of the

Graduate School of Biomedical Sciences

Department of Molecular Genetics and Microbiology

University of Massachusetts Medical Center

January 28, 1998

Acknowledgments

Now that graduate school is over, I can admit that it was a very pleasant learning experience. These years at UMass have passed easily in large part due to my adviser and our lab members. Duane Jenness has been a wonderful adviser. He is caring and patient as he is knowledgeable; I will not forget when a personal problem before the birth of my son caused more distress to him than to me. He expected first rate work without interfering (too much). I would like to thank him here for the excellent training I received. Thanks also to my lab-mates Kim, Jodi, Yu and Ayce. Kim Schandel and Jodi Hirschman provided me with advice and perspective on science, life and after-life; I have bent their ears on many occasions and have found relief. The graduate students in the lab, Yu Li and Ayce Yesilaltay, have given me unwavering support and friendship. Thanks to all my friends at UMass who enhanced this experience and especially to Christine Clemson with whom we shared major and minor problems of life. Thanks to all MGM members for contributing to the friendly atmosphere.

I would also like to express my appreciation to the committee members for being as thorough as they have been, and for their insight and advice.

Last but not least, I am grateful to my husband for his encouragement through these years and for going through graduate school all over again.

As a final note, I would like to advise graduate students to enjoy their training instead of agonizing over when it will end. I suspect Duane was right (yet again) when he told me that these were the best years of my scientific career.

Respective Contributions

Kim A. Schandel sequenced the subcloned *ste2-T274P* allele, constructed the strain 902-A-1, and also performed the endocytosis assay with this mutant.

Ayce Yesilaltay constructed and performed the halo assays with the *ste2-T274P* on a multicopy plasmid, as well as the *sst2-1 bar1-1* strain (6360-1-2).

Ming Cui carried out the initial competition binding experiment with the *ste2-T274P* allele with agonist and antagonist.

ABSTRACT

Activation mechanism of the α-factor pheromone receptor of *Saccharomyces cerevisiae* was analyzed using biochemical and genetic techniques. An *in vitro* partial proteolysis assay was developed to determine the conformational change of the receptor that occurs upon binding of agonist. The activation specific cleavages were established by comparing cleavage products with antagonist versus agonist occupied receptor. Of the changes in peptide pattern that were revealed by trypsinization, the fragment resulting from the exposure of the third loop to the protease was found to be agonist specific and to be G-protein independent. A low-affinity binding receptor mutant was isolated which failed to undergo this agonist induced conformational change. Four intra-allelic suppressors of this receptor mutant were isolated and all were mapped to the ends of transmembrane helices 4, 5, 6 and 7; all were found to be replacements of non-polar residues by polar ones. The role of the suppressor mutations in conformational change was analyzed.

TABLE OF CONTENTS

INTRODUCTION

The superfamily of G-protein coupled receptors (GPCR) are responsible for transducing signals in response to a number of diverse stimuli such as light, odorants, neurotransmitters, peptide hormones, biogenic amines, opiods and ions. Sequence of these receptors suggests seven hydrophobic domains of sufficient length to span the lipid bilayer (20-28 amino acids), hydrophilic intervening sequences, an extracellular N-terminal domain and an intracellular C-terminal tail, Figure 1A. The presence of the seven transmembrane-spanning segments is seen clearly by using high-resolution electron cryo-microscopy with the retinal-linked bacteriorhodopsin (Henderson et al. 1990). Bacteriorhodopsin is a light activated proton-pump that is not coupled to G-proteins and occurs naturally as two-dimensional crystals within the purple membranes of *Halobacterium halobium*. Recently, using membrane-mimetic matrices, highly ordered 3D crystals of bacteriorhodopsin were obtained which allowed the determination of the X-ray structure of the molecule at 2.5 Angstroms and confirmed the predicted transmembrane topology (Pebay-Peyroula et al. 1997). Two-dimensional electron crystallography of bovine rhodopsin which is a member of the GPCR superfamily has confirmed this overall structure (Schertler 1993), although the packing of the helices is apparently different. It is assumed that these receptors undergo a conformational change upon binding of the agonist and that the conformational change is then propagated to the G-protein. The altered G-protein structure results in the exchange of the bound GDP for GTP in the $G\alpha$ subunit, which causes the dissociation of $G\alpha$ from the $G\beta\gamma$ subunits. The free $G\alpha$ and $G\beta\gamma$ can interact and regulate effector functions such as adenyl cyclase, phospholipase, cyclic GMP phosphodiesterase and ion-channels that regulate K^+ or Ca^{++} (Hedin et al. 1993). The fact that GPCRs couple to a wide variety of ligands and yet share the same structure has led to

the idea that these receptors have a common activation mechanism. The initial step of activation has been difficult to delineate since crystallization of membrane proteins is problematic. Therefore, the work on the mechanism of conformational change of these receptors has relied on other techniques.

Evidence for Ligand-induced Conformational Change

In order to convert extracellular stimuli into intracellular response, it is accepted that receptors undergo a conformational change upon binding of ligand. This conformational change "turns on" the effectors which in turn trigger a signal. Most investigations of GPCRs were pioneered by studying rhodopsin which is abundant in the rod cells of the retina. Apart from its abundance, rhodopsin forms a spectrally distinct form upon activation by light; this property allows dissection of its mechanism of activation.

Although x-ray crystallographic structures of GPCRs with and without ligand are not currently available, other observations provide evidence for ligand-induced conformational change. Limited proteolysis used to detect light-sensitive changes in rhodopsin revealed that photobleached rhodopsin is more susceptible to digestion compared to the unbleached pigment, suggesting that a conformational change results from the phototransition (Kühn et al. 1982; Pellicone et al. 1985b). Whereas photoexcitation was found to inhibit the cleavage of C-terminal tail of rhodopsin (Pellicone et al. 1985b; Kühn et al. 1982), residues in transmembranes 4 and 6 (TM4 and TM6 respectively) become more susceptible to cleavage by CNBr (Pellicone et al. 1985a). Synthetic peptides derived from rhodopsin inhibit its interaction with transducin. These results implicate the intracellular regions of the receptor as playing an essential role in the interaction with the G-protein and lead to the general ideas that the unliganded receptor is in a more compact form and that the binding of agonist releases the intracellular regions to interact with the G-

2

protein (Bourne 1997). Similarly, time resolved electron spin resonance (ESR) spectroscopy of the cysteine residues in rhodopsin suggests that photoactivation induces a conformational transition at the junction of TM3 and second intracellular loop; this transition coincides with the spectral appearance of metarhodopsin (Farahbakhsh et al. 1993). Combination of site-directed mutagenesis and electron paramagnetic resonance (EPR) spectroscopy were used to deduce the distances between pairs of nitroxide-tagged cysteine residues. By determining the distance between a fixed residue at the junction of TM3 and the second intracellular loop (i2) and residues on TM6 before and after photobleaching, Farrens et al. (1996) concluded that TM6 moves with respect to TM3. The importance of this rigid body movement was demonstrated by comparing the properties of cysteine-containing mutants in the reduced and the oxidized states. The mutant rhodopsin was found to activate transducin (G_T) when the cysteine residues were reduced and were free to rotate; however, when the cysteine residues were oxidized, the mutant rhodopsin was unable to stimulate G_T (Farrens et al. 1996). Presumably, the formation of the disulfide bond blocks the conformational transition.

Labeling of cysteine residues of the β2-adrenergic receptors was informative as well. In this case, cysteine-reactive fluorescent tags which are highly sensitive to the polarity of the environment were used to show dose-dependent decreases in fluorescence upon agonist binding. The decrease in fluorescence was found to correlate with the efficacy of the agonist used. Antagonists on the other hand produced small but significant increases in fluorescence (Gether et al. 1995). This implies that the labeled cysteine residues are exposed to a more polar environment upon agonist binding and that this is due to receptor activation and G-protein coupling since these changes are specific for the agonist. Systematic labeling of the cysteine residues of β2-adrenergic receptor localized residues responsible for conformational change to TM 3 and 6 (Gether et al. 1997).

3

Chimeras of $\alpha2$ and $\beta2$-adrenergic receptors which couple to Gi and Gs respectively were used to identify the determinants of agonist and antagonist binding and G-protein coupling (Kobilka et al. 1988).

The structural changes associated with receptor activation have also been explored by using a combination of molecular dynamic simulations and site-directed mutagenesis. These studies investigate the role that the conserved Asp/Glu-Arg-Tyr sequence (junction of TM3 and the second intracellular loop) plays in maintaining the equilibrium between the inactive (R) form and the active (R*) form of the receptor. The deprotonated aspartic acid in this motif was predicted to stabilize the R state of the α_{1B}-adrenergic receptor; consistent with this prediction, mutagenesis of D142 to alanine was found to result in high constitutivity (Acharya and Karnik 1996). Further dissection of the role of this residue in rhodopsin suggested a more complex scenario, since although D134A, D134Q and D134I were constitutive, D134F was not. These results imply that a combination of charge and side chain-packing at this junction influence the inactive state of rhodopsin (Acharya and Karnik 1996). These same researchers also found that R135 in this motif was not responsible for binding of rhodopsin to transducin (G_T) but rather for triggering GDP release from transducin. Using the same combination of techniques, (Scheer et al. 1996) found that R143 is tucked into a polar pocket formed by the residues on TM1, 2 and 7; disruption of these interactions resulted in constitutivity. Spontaneous mutations that affect the GPCRs and lead to disease have also been useful in understanding the role of these residues as well as other residues in TM2, TM3, TM6 and TM7. These spontaneous mutations have also drawn attention to unexpected regions of the receptor such as the extracellular loops that apparently affect receptor activation (Birnbaumer 1995).

Receptor-G-protein complexes have been immunoprecipitated with antibodies against both G-proteins and the receptor itself, and the effect of agonists or antagonists on co-immunoprecipitation has been evaluated. Results from these studies provide the basis for the hypothesis that the receptor isomerizes to the active form even in the absence of agonist and that the binding of agonists and antagonists result in the stabilization of the active and the inactive states, respectively (Matesic and Luthin 1991). The ability of the unliganded receptor that is in the inactive form (i.e., uncoupled from G-proteins) to isomerize to the activated form (i.e., able to couple with G-proteins) is consistent with the basal levels of signalling (so-called "noise") observed for G-protein coupled receptor systems. The level of noise varies among the receptor systems; for example rhodopsin produces very low noise levels (which allows a large number of receptors per cell), whereas TSH shows much higher noise levels (Parma et al. 1995). The addition of agonist is thought to stabilize the activated form and lead to the formation of a ternary complex (HRG) containing agonist, receptor and G-protein. The ratio of signal (HRG) to noise (RG) determines the strength of the signal. Thus, the activation of the receptor requires three processes: binding of agonist, conformational change and coupling to G-protein. Information regarding the receptor will be reviewed in terms of the domains that are responsible for these three functions.

Ligand Binding Domains

The receptors that belong to the superfamily of GPCRs can be subdivided into receptors that bind either small molecules (such as biogenic amines and retinal), peptides or large glycoproteins. Among the GPCRs that bind amines, $\beta 2$-adrenergic receptor is one of the most extensively studied. The initial scanning deletion experiments with this receptor showed the hydrophilic loop regions as unimportant for ligand binding (Dixon et al. 1987) and therefore implicate the transmembrane regions. As a general strategy, conserved

residues of receptors that bound different ligands were ruled out as being important for ligand binding. To further narrow in on the specific residues involved in ligand binding, polar residues in the transmembrane regions were selected for mutagenesis. Since all the agonists and the antagonists of the adrenergic receptors are basic amines, it was suspected that there would be an acidic residue on the receptor that would act as the counter-ion for the amine group. The mutagenesis of the two aspartic acid residues in TM2 and TM3 of the β-adrenergic receptors was found to affect K_d and EC_{50} but to varying degrees; whereas the Asp79Ala mutation increased both parameters by 10-fold, mutagenesis of Asp113Glu resulted in an increase of 40,000 and 1,500-fold respectively. In contrast to the wide conservation of the Asp79 residue, Asp113 is conserved only among the receptors that bind amine ligands suggesting that both residues are involved in ligand binding through different mechanisms; while the Asp113 provides the counterion that interacts with ligand, the Asp79 is likely to be necessary in maintaining the conformation of the agonist-bound receptor (Strader et al. 1988). Apart from these residues, the disulfide bond between the extracellular cysteines on most GPCRs is believed to be important for stabilization of receptor structure and the ligand binding pocket (Noda et al. 1994).

Rhodopsin, the first GPCR to be cloned, and the thrombin receptors are unlike the rest of the superfamily in that they are both pre-coupled with their ligands. Rhodopsin has a covalently bound ligand (retinal) that is buried in the TM domain. Retinal forms a Schiff base with a lysine residue (296) in TM7, and glutamic acid 113 in TM3 provides the counterion that interacts with the protonated retinal (Probst et al. 1992). The covalent binding of ligand results in increased occupancy of the receptor, which will ensure efficient detection of an incoming photon and decrease noise that arises from opsin. The activation of the thrombin receptor by thrombin is achieved through a different mechanism. The first 41 amino acids of the thrombin receptor is proteolyzed by thrombin; the new N-terminus

6

interacts with the binding pocket and activates the receptor (Birnbaumer 1995).
Generalization regarding the position of ligand binding sites in GPCRs have been derived
from the analysis of mutant receptors as shown in Figure 1B (Coughlin 1994). Receptors
with relatively small ligands such as seratonin, histamine, catecholamines bind to the
hydrophobic pocket. Larger ligands such as peptide hormones, endothelin, IL-8 and
neurokinin bind also to the extracellular portions of TM regions. Glycoprotein hormones
(TSH, FSH, LH/CG) and thyrotropin use the extracellular loops and the N-terminal tail
along with the transmembrane pocket. The ligands that bind the N-terminus are Glutamate
and Ca^{+2}, as well as thrombin. The overall conclusion of work in this area is that the site
to which the agonist binds is not important; the activating function of the ligand is still
exerted at the level of the transmembrane domain of receptors. Current thought is that
agonists function by stabilizing an active conformation of the receptor that is present as
only a minor species in the absence of ligand (Schwartz and Rosenkilde 1996).
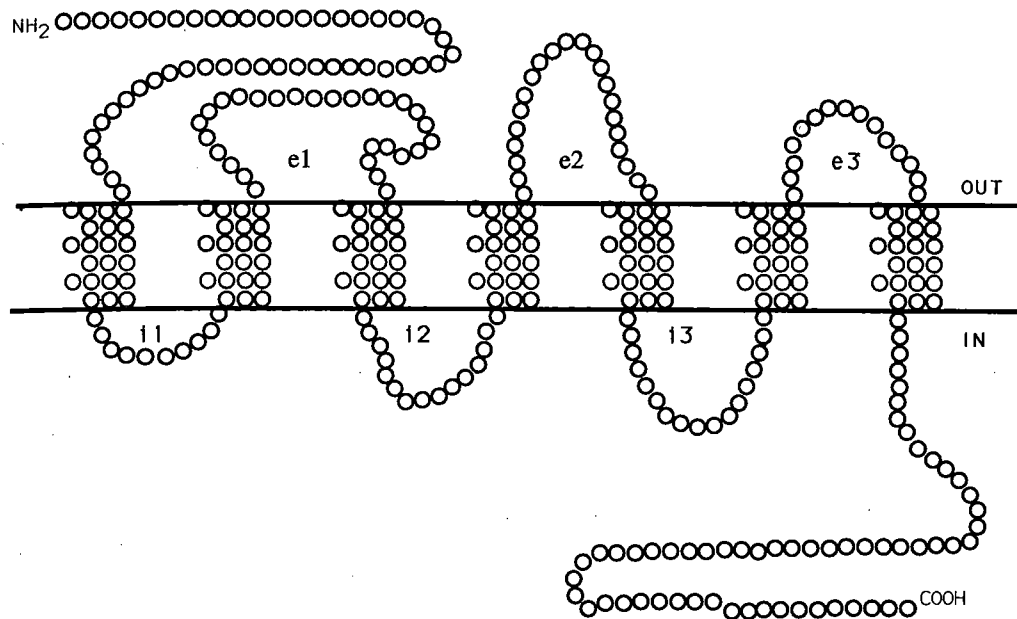
Figure 1A. The general organization of the GPCRs. The amino-terminus and the 3 extracellular loops (e1-e3) face the extracellular side while the carboxy-terminus and the 3 intracellular loops (i1-i3) face the intracellular side of the cell.
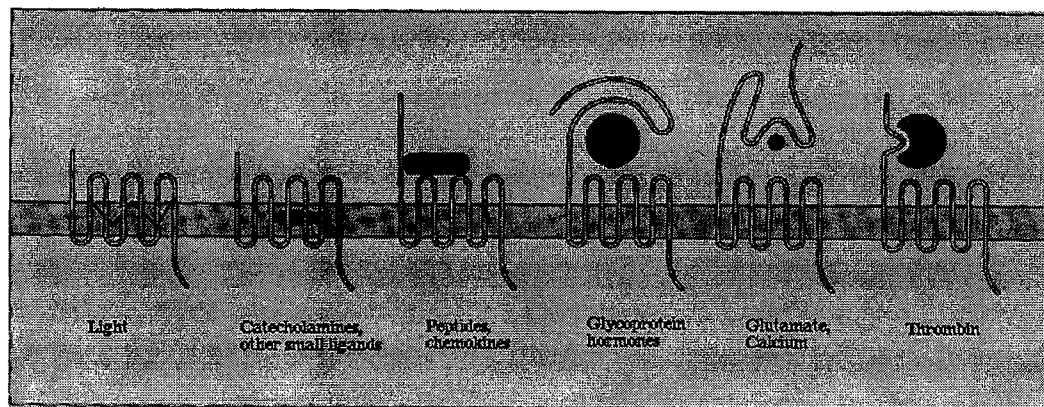
Figure 1B. Diverse strategies for ligand detection by GPCRs. From S. Coughlin, 1994.

Activation Domains

GPCRs consist of hundreds of amino acids which can assume multiple molecular structures which will be altered upon binding of ligand. Although ligand binding might result in many changes in the overall structure of the receptor, the changes associated with receptor activation are those that lead to productive interaction with G-proteins. Some of the interactions important for activation have been deduced for rhodopsin. It has been concluded that the inactive R state of rhodopsin which is not capable of activating transducin is bonded covalently to 11-*cis*-retinal through Lys296 and this association with the R state is stabilized by the salt bridge with Glu113 in TM3. The R* state which activates transducin lacks the salt bridge and binds preferentially to all-*trans*-retinal. Likewise, the angiotensin AT1A receptor is constrained through specific residues on TM3 (Asn111) and TM7 (Asn295) which are believed to be in close spatial proximity as determined by molecular modeling. Disruption of this interaction by mutant receptors affecting either residue resulted in no detectable binding with antagonist and only high-affinity binding with agonists, high-affinity binding was G-protein independent. The mutant receptors had increased basal levels of signalling as compared to wild-type receptor. This suggests that the interaction between these TM helices are important in maintaining the receptor in the inactive state and that disruption of this interaction leads to activation. These residues in the AT1A receptor are comparable to the Glu113 in TM3 and to Lys296 in TM7 of the rhodopsin, that is, mutation of either residue causes constitutivity. These residues in rhodopsin are two and one helical turn above the Asn111 and Asn295 respectively. A residue that has an equivalent position to that of Asn111 in the AT1A receptor is Cys128 in TM 3 of the α1B-adrenergic receptor. Substitution of this residue to phenylalanine was found to cause constitutive activation of the phospholipase C pathway (Balmforth et al. 1997). These results reinforce the idea that the unliganded receptor is in a more constrained conformation and that binding of ligand relaxes the receptor by disruption of the specific

intramolecular interactions. These findings provide an explanation as to why GPCRs are susceptible to constitutive activation by mutations that are not localized to the same region; any mutation that results in a "looser" conformation can lead to constitutivity (Bourne 1997).

G-protein Coupling Domains

It has been shown that one-third of the unliganded α2-adrenergic receptors in the cell are found physically associated with the G-protein, and the remaining population of receptors becomes coupled to the G-protein only after they have bound agonist (Neubig 1994). These findings are consistent with the notion that unliganded receptors isomerize to the R* state capable of interacting with G-proteins and that binding of agonist stabilizes this state and unmasks G-protein-binding sites. Work with chimeric receptors that couple to two different G-proteins, Gs and Gi, implicate the third intracellular loop of the receptor as being one of the specific G-protein contacts. However, high efficiency coupling requires additional structural features; some of these features are specified by the second intracellular loop (Hedin et al. 1993). Recently, it has been shown that the second intracellular loop is important for coupling to both Gi and Gs, and the third intracellular loop contains two potent coupling domains: one of these domains is sufficient for binding to Gi, whereas both domains are required for binding to Gs (Eason and Liggett 1996). The aspartate/glutamate-arginine-tyrosine "D/ERY" motif in the C-terminal portion of the second intracellular loop is important in coupling a subclass of receptors to G-proteins. A naturally occuring variant contains a histidine instead of the arginine in this motif in the VP2-vasopressin receptors. It was found to cause congenital nephrogenic diabetes insipidus as a consequence of uncoupling the receptor and Gs (Rosenthal et al. 1993). Substitution of the equivalent arginine in rhodopsin results in a mutant receptor that can bind to, but not activate G_T; these rhodopsin mutants presumably cannot induce the release

of the GDP from the heterotrimeric G-protein (Acharya and Karnik 1996). In addition to these points of contact, the G-protein interaction of biogenic amine receptors, angiotensin receptor and rhodopsin was found to also require the carboxy-terminal tail (Strader et al. 1994). Apart from intracellular regions of the receptor, mutations affecting the extracellular loops have been found to cause constitutivity (Nanevicz et al. 1996) and (Perez et al. 1996). Apparently, changes in distant parts of the receptor can result in release of G-protein from the coupling domain of the receptor.

Although GPCRs that bind similar ligands possess common sequence motifs in their ligand binding and the activation domains, there are no common sequence motifs among receptors that bind to the same G-proteins. For the receptor-G-protein interaction, importance of the overall tertiary structure of the intracellular loops, as opposed to sequence identity, has been implicated from coexpression of mammalian receptors in yeast. Price et al. (1996) have shown that the rat A_{2a} adenosine receptor could be functionally coupled to the pheromone pathway when expressed in *Saccharomyces cerevisiae*. This is surprising since mammalian Gs and the yeast Gα homolog, *GPA1*, share little sequence homology; notably homology is lacking in the C-terminal region which is thought to interact with the receptor. Moreover, the fact that these receptors share little sequence homology underscores the importance of the higher order structural features, rather than primary sequence, in determining the coupling site (Price et al. 1996).

The Yeast α-Factor Pheromone Receptor

The pheromone receptor from *Saccharomyces cerevisiae* contains seven membrane-spanning domains (Cartwright, 1991) and is coupled to G-proteins (Blumer and Thorner 1990). The presence of a GPCR in yeast provides an experimental system where genetics

can be used to complement molecular and biochemical approaches. Yeast cells can exist either as diploids or as haploids; haploid cells exist in one of two mating types, α and **a**. The peptide pheromones α-factor (synthesized by α cells) and **a**-factor (synthesized by **a** cells) bind to receptors located on the surface of haploid cells of the opposite mating type. The receptors are cell-type specific proteins of the mating pathway and are encoded by the *STE2* and *STE3* genes. Both receptors contain seven transmembrane segments and require homologs of the mammalian $G\alpha$, $G\beta$ and $G\gamma$ 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 (Marsh et al. 1991). The receptor appears to form a direct physical association with these G-protein subunits since α-factor binds *ste4* mutant cells more weakly (Jenness et al. 1987) and since it dissociates more rapidly from membranes assayed in the presence of GTP analogs or when the membranes are prepared from *gpa1*, *STE4Hpl* or *ste18* mutants (Blumer and Thorner 1990). Other α-factor responses include receptor endocytosis (Schandel and Jenness 1994), mating-partner selection (Jackson et al. 1991) and the promotion of changes in cellular morphology (Konopka 1993). Neither **a**-factor nor its receptor show homology to their counterparts in the opposite cell type; unlike α-factor, **a**-factor is farnesylated at its C-terminus. Although the pheromone response pathways for **a** cells and α cells apparently use the same post-receptor gene products, the receptors show no obvious sequence homology.

Comparison of STE2p with other GPCRs

Although a wealth of information has been gathered from the mammalian G-protein coupled receptors using biochemical and biophysical approaches, yeast offers the advantage of an *in vivo* system where genetic analysis can complement the biochemical findings.

The yeast pheromone response pathway consists of a G-protein- coupled pheromone receptor and a mating specific MAP kinase cascade (Leberer et al. 1997). Although neither of the pheromone receptors show sequence homology with the mammalian receptors, they share the common structural organization in that they consist of seven transmembrane spanning regions, as well as an extracellular N-terminus and a cytoplasmic C-terminal tail (Cartwright and Tipper 1991). Although the Ste2p is not stabilized through disulfide bonds, and does not contain a palmitoyl modification at its carboxy tail, as do the mammalian counterparts, it is glycosylated at its N-terminus and is phosphorylated at its C-terminus (Blumer et al. 1988). The C-terminal tail is not required for binding or for signalling (Konopka et al. 1988) but mediates desensitization through phosphorylation of certain residues (Reneke et al. 1988) and (Chen and Konopka 1996). As in mammalian receptors, the C-terminal tail is also involved in desensitization and endocytosis which requires the DAKSS sequence (Rohrer et al. 1993; Zanolari et al. 1992).

As is the case with the mammalian receptors, the intracellular third loop has been implicated for interaction with G-proteins in yeast as well (Clark et al. 1994; Sprague and Thorner 1992; Stefan and Blumer 1994). Binding of the agonist to the receptor results in a conformational change which leads to the dissociation of G-proteins (Bukusoglu and Jenness 1996); in yeast, it is the free Gβγ subunits that interact with the downstream elements of the yeast pheromone pathway (Dietzel and Kurjan 1987; Whiteway et al. 1989). As mentioned above, pheromone receptors possess no obvious sequence homology to mammalian receptors; conserved residues in other GPCRs responsible for ligand interaction (such as an Asp or Glu in TM3) or the activation motifs (such as the DRY in the N-terminal portion of the second intracellular loop) are either not essential (Sommers

13

and Dumont 1997) or absent in the α-factor receptor. However, structural motifs for coupling to G-proteins seem to be conserved in pheromone receptors since heterologous expression of some mammalian receptors induce the pheromone response pathway in a manner that is dependent on the mammalian receptor agonist (Price et al. 1996).

Whereas the mammalian systems are complicated by the presence of multiple types of G-proteins, yeast has only one known Gβγ (Whiteway et al. 1989) and two Gα subunits; the *GPA1* gene product is the subunit that is involved in the pheromone response (Dietzel and Kurjan 1987; Miyajima et al. 1987). Unlike the other G-protein subunits, the *GPA2* gene product is not haploid-specific and has been implicated in the activation of adenylyl cyclase and invasion in yeast (Nakafuku et al. 1988). This suggests the possibility that there may be another Gβγ subunit that Gpa2p interacts with. Personal communication from J. Hirsch's lab suggests that another GPCR in yeast interacts with Gpa2p. Nevertheless, the mating pathway is not affected by the lack of the Gpa2p (Nakafuku et al. 1988). Another apparent difference from the mammalian system regarding the G-proteins is that they are apparently limiting relative to the receptor since the overexpression of Ste2 does not increase pheromone sensitivity (Konopka et al. 1988).

STE2p Ligand binding domains.

Through the employment of mutagenesis and chimeric receptors, it has been shown that the transmembrane domains of the yeast pheromone receptor are involved in ligand binding. Insertional mutagenesis of the second and fourth transmembrane helices of the pheromone receptor show reduced binding to α-factor and are signalling defective (Konopka and Jenness 1991). Analysis of chimeric receptors of *S. cerevisiae* and *S. kluyveri* have also leads to the conclusion that the transmembrane segments of receptor are important for binding of α-factor (Sen and Marsh 1995). The two non-contiguous

14

important for binding of α-factor (Sen and Marsh 1995). The two non-contiguous domains of STE2 which include the first and the third extracellular loop and their associated transmembrane helices are sufficient to change the specificity of the *S. kluyveri* receptor to α-factor. Recent work from our laboratory has led to the finding that residues on TM2 (Y98), TM3 (T131), TM4 (S184) and TM7 (G273) are important for binding of agonist. Mutations of these residues lead to at least 10-fold reduced α-factor binding; the levels of protein at the plasma membrane were found to be normal (A. Yang and D.D. Jenness, unpublished observation).

STE2p conformational change

A number of published observations pertain to the existence of a conformational change in the α-factor pheromone receptor.

1. Phosphopeptides generated in the presence and absence of α-factor treatment resulting from the partial digestion of ^{32}P-labelled *STE2* showed that most fragments had increased radioactivity in the presence of α–factor. This was taken to indicate that α-factor causes a conformational change of the carboxy tail of the receptor that alters its accessibility to trypsin (Reneke et al. 1988). However, it is also possible that post-receptor signals may activate a kinase which phosphorylates the Ste2p.

2. Mutagenesis of Ste2p yielded mutant forms of the receptor which were responsive to a partial agonist and an antagonist. Mutations in the third intracellular loop were found that make the receptor responsive to *S.kluyveri* α-factor and to the antagonist DesTrp1, Ala3-α-factor (Stefan and Blumer 1994). Similarly, mutations in Ste2p were found that are deep in the TM region that make the receptor responsive to antagonist and improved response to *S.kluyveri* α-factor (Marsh 1992). This phenotype of responsiveness to partial agonist and antagonist may reflect the disruption of the interactions that maintain the receptor in its

inactive form. As a result, the mutant receptor is thought to be in a partially relaxed state such that binding of weak agonist can trigger activation.

3. Mutation of a proline residue to leucine on transmembrane segment 6 which is conserved between yeast and mammalian GPCRs was found to lead to constitutive activation. Since prolines are likely to introduce kinks in transmembrane helices and since the position of the residue is close to the third intracellular loop, it is thought that this mutation allows the unliganded receptor to assume the activated state (Konopka et al. 1996).

G-protein coupling domains of STE2p

A priori, the intracellular loops and the C-terminal tail are all possible sites for G-protein coupling domains since they are exposed to the cytoplasm. As in mammalian GPCRs, certain mutations affecting the third intracellular loop of the receptor were shown to severely block its ability to couple with the G-protein and to generate an intracellular signal (Schandel and Jenness 1994; Weiner et al. 1993). Unpublished observations from our lab also implicate a role for the carboxy-tail of the receptor in interaction with the G-proteins; a temperature-sensitive destabilizing mutation in the Gα subunit leads to the constitutive arrest phenotype at 38 °C that is observed in *gpa1* null mutants at all growth temperatures. This phenotype is exacerbated in *ste2* deletion mutants and in mutants that produce receptors lacking the carboxy-terminal domain. One interpretation of these results is that the presence of the receptor C-terminal tail leads to stabilization of the G-protein subunits by a direct interaction (Schandel, K.A. and Jenness, D. D., unpublished work).

Endocytosis and Signalling Functions of STE2p

Work in yeast suggest that signals for endocytosis are carried on the third intracellular loop and the carboxy-tail of the receptor. A mutant form of Ste2p has been identified that contains 4 more residues than the wild-type receptor in the C-terminal portion

16

of the intracellular third loop (i3); this mutant receptor is internalized faster than wild-type in a ligand independent manner. Although this mutant has a higher affinity for agonist, it does not result in a constitutive phenotype (Stefan and Blumer 1994). Work from our lab showed that a non-signalling mutant receptor had normal ligand-induced endocytosis implying that the signals for endocytosis and signalling are not necessarily the same (Schandel and Jenness 1994). The carboxy-terminus of the receptor is essential for basal endocytosis (Schandel and Jenness 1994) and (Konopka et al. 1988) and for ligand-induced endocytosis; Rohrer et al. (1993) have localized a sequence in this region (DAKSS) that is a signal for endocytosis. It has been shown that endocytosis in yeast does not require G-protein coupling, (Jenness and Spatrick 1986; Blinder et al. 1989; Schandel and Jenness 1994; Zanolari et al. 1992); in mammalian systems, certain β–adrenergic receptor mutants that couple inefficiently to G-proteins were shown to undergo ligand-mediated endocytosis as well (Cheung et al. 1990).

Ligands of STE2p

In general, receptor ligands can be classified into three groups: Full agonists, partial agonists and antagonists. Full agonists bind to the receptor and elicit maximal biological response; they generally show G-protein- dependent high-affinity binding, whereas partial agonists elicit a submaximal response and usually do not show G-protein dependent binding (Samama et al. 1994). Antagonists are molecules that bind the receptor but do not produce a biological response. There are two types of antagonists. Neutral antagonists elicit no effect alone but compete for activation by agonists; they are thought to bind with similar affinity to R and R* forms. Negative antagonists (also referred to as inverse agonists) reduce the basal activity of receptors and they generally reduce the affinity of the receptor for the G-protein. Negative antagonists are thought to have a greater affinity for the R form of the receptor (Samama et al. 1994).

The only full agonist for the α-factor receptor is α-factor itself. Its sequence is shown in Table 1. It is encoded by the two genes MFα1 and MFα2; these genes contain four and two tandem copies respectively of the information encoding the α-factor peptide. α-factor binds its receptor with an equilibrium dissociation constant (K_d) of 6 nM (Jenness et al. 1986). The peptide is thought to contain a β-bend that may be important for bioactivity (Xue et al. 1996). It is also thought that the first tryptophan of the molecule stabilizes the interaction with the receptor by increasing the hydrophobicity of the molecule. α–factor is synthesized in a similar fashion to the mammalian peptide hormones, as prepropoly-α-factor is processed by proteases before it is secreted. Its sequence is similar to the mammalian gonadotropin-releasing hormone (GnRH) also known as luteinizing hormone releasing hormone, see table below, (Naider and Becker 1986). Using different analogs of α-factor (Eriotou-Bargiota et al. 1992; Manfredi et al. 1996), it has been shown that the N-terminal portion (residues 1 through 4) of the molecule is important for signalling activity and the C-terminal portion is important for binding. Blumer and Thorner (1990) found that the interaction of α–factor with the receptor is pH-dependent (i.e., pH 6 gives tighter binding than pH 8); this suggests either that a residue in α-factor requires protonation or that the conformation of the ligand binding site is affected by pH.

Peptides that differ from α-factor at one or more positions have been shown to serve as partial agonists or antagonists (Raths et al. 1988). In this study, two forms of α-factor, (desTrp1-α-factor and Ala3-α-factor) were found to act as partial agonists. Double substitution of these forms, (desTrp1,Phe3-α-factor and desTrp1,Ala3-α-factor) converts them into antagonists in that they fail to induce normal pheromone responses (i.e., morphogenesis and agglutination); competition binding studies confirm the conclusion that these analogs are inactive and rule out the possibility that they have low activity which

18

would have been manifest at high ligand concentrations. Ayce Yesilaltay and Ming Cui have shown that desTrp1,Ala3-α-factor binds a-cells 125-fold more weakly than α-factor in that the EC_{50} of α-factor is 8 nM, whereas antagonist's is 1000 nM. Another partial agonist for this receptor is the α-factor from *S.kluyveri*; (Table 1). Although *S.kluyveri* and *S.cerevisiae* do not interbreed, high concentrations of α-factor from *S.kluyveri* (10-100 fold over that required for *S.cerevisiae* α-factor) induces agglutinability of *S.cerevisiae MATa* cells (Marsh and Herskowitz 1988). The two partial agonists that were also used in this thesis are AN-37 and AN-44 (table 1). These novel peptides were identified in a genetic screen to study the structure-function relationship of α-factor (Manfredi et al. 1996).

Table 1.

Sequence of α-factor and its analogs

α-factor	WHWLQLKPGQPMY
Gonadotropin-releasing hormone	EHWSYGLRPG
S.kluyveri α-factor	WHWLSFSKGEPMY
AN-37 (α-factor analog)	CRGPQLKPGQPMY
AN-44 (α-factor analog)	WHWLSLGGRQPMY
desTrp1,Ala3-α-factor	HALQLKPGQPMY

Desensitization mechanisms in yeast

A common feature of hormone regulated systems is that the continued presence of agonist results in a waning of the response. This ligand-induced loss of responsiveness is referred to as adaptation or desensitization. In mammalian GPCR systems, desensitization

mechanisms are known that either limit receptor activity or cause inhibition of post-receptor steps in the response pathway. The sequestration and phosphorylation of the receptor facilitates binding of arrestins to the receptor and decrease its ability to activate the post-receptor signal. G$\beta\gamma$ subunits regulate receptor desensitization by potentiating the translocation of β-adrenergic receptor kinase from cytosol to the plasma membrane (Hedin et al. 1993). Pathways that dampen the post-receptor signal act generally on the G-protein subunits. Hydrolysis of the GTP bound to GPA1p is likely to reverse the activation of the Gα subunit. While phosducins act on G$\beta\gamma$, the "RGS" proteins (regulators of G-protein signalling proteins) negatively regulate Gα subunits. The RGS proteins are evolutionarily conserved among *Saccharomyces cerevisiae, Caenorhabditis elegans* and humans; the yeast homolog is the pheromone inducible *SST2* gene product. Mutations in *SST2* cause greater than 100-fold increase in pheromone sensitivity and result in the inability to adapt and recover from pheromone-induced arrest of cell division (Dohlman and Thorner 1997). In yeast, the product of the *SST1* gene is also involved in desensitization of *MATa* cells through a completely different mechanism. This gene (also known as *BAR1*) encodes a protease that specifically degrades α-factor and, as a consequence, results in the dampening of responsiveness in *MATa* cells (Sprague and Herskowitz 1981) and (Chan and Otte 1982).

Overexpression of certain genes in yeast were found to lead to resistance to α-factor induced arrest of cell division. One of these genes is the Gα homologue, Gpa1. The $gpa1^{Val50}$ mutation which is analogous to the constitutively-active allele of *ras*, accelerates the rate of recovery from pheromone. It has been found that this adaptation pathway depends on the *MSG5* gene, which is a protein phosphatase that inactivates FUS3 (a MAP kinase) (Doi et al. 1994). Other genes that inhibit responsiveness when overproduced are *SST2*, *KSS1*, and *AFR1*. The *KSS1* gene product is normally involved

in activation of the pheromone pathway. Unlike the other gene products mentioned thus far, the *AFR1* gene product seems to act through the C-terminal of the pheromone receptor. There is some redundancy in its function since deletion of *AFR1* does not lead to increased sensitivity to α–factor as does the *sst2* deletion. Although it interacts with the carboxy-tail of the receptor, there is no homology to protein kinases and very little homology to arrestin (Konopka et al. 1995).
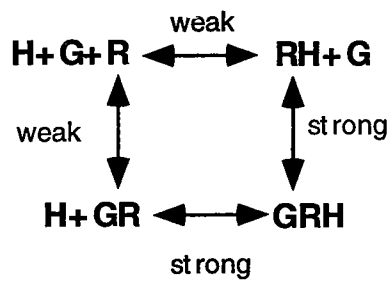
Yet another pathway for desensitization was demonstrated by Hirsch and colleagues (Hirsch and Cross 1993). This study shows that the *STE3* gene (which encodes the **a**-factor receptor normally expressed only in *MATα* cells) when expressed in *MATa* cells reversed the constitutive-signalling phenotype of the *gpa1* null allele. Inhibition of the post-receptor signal is thought to result from the sequestration of the free βγ subunits by the *STE3* gene product. This does not require α–factor, or the products of *STE2, SST2* or *KSS1*; thus it appears to constitute a different desensitization mechanism.

The Ternary Complex Model

The Ternary Complex Model (TCM) depicted in Figure 2A is one of number of models that have been used to explain the activation of G-proteins by GPCR's (Cotecchia et al. 1990; Monod et al. 1965; Onaran and Costa 1997; Samama et al. 1993; Weber 1972). The name ternary complex emphasizes the importance of the trimolecular complex between agonist, receptor, and G-protein in the process of signal transfer. It states that the interaction between the agonist and the receptor results in a form that can interact productively with the G-protein. According to this model, the receptor exists in two

21

dynamic states as reflected by changes in agonist affinity. The high-affinity state of the receptor that is ultimately stabilized by the agonist is designated the GRH. The ability of agonists to promote this state depends on their greater affinity for the GR form than for the R form of the receptor; this property describes the efficacy or the intrinsic activity of the agonist. This model accounts for the ability of GTP to convert the receptors from high affinity state to the low affinity state.

A. Ternary complex model

$$
\begin{array}{ccc}
& \text{weak} & \\
\text{H+G+R} & \longleftrightarrow & \text{RH+G} \\
\text{weak} \updownarrow & & \updownarrow \text{strong} \\
\text{H+GR} & \longleftrightarrow & \text{GRH} \\
& \text{strong} &
\end{array}
$$

B. Extended model

$$
\begin{array}{ccc}
& K & \\
\text{R} & \longleftrightarrow & \text{RH} \\
J \updownarrow & & \updownarrow bJ \\
\text{R}^* & \longleftrightarrow & \text{R}^*\text{H} \\
& bK & \\
M \updownarrow & & \updownarrow aM \\
\text{GR}^* & \longleftrightarrow & \text{GR}^*\text{H} \\
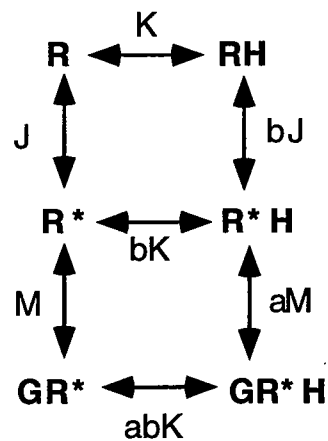& abK &
\end{array}
$$

Figure 2. The ternary complex model and the extended model. H, G and R are hormone, G protein and receptor respectively. a and b are the coupling constants while J, M and K are the isomerization constant, G protein association and agonist association equilibrium constants respectively.

The extended TCM model (Figure 2B) takes into consideration the isomerization step of the receptor from the R to R* form. Only the R* form is capable of binding to the G-protein. Direct evidence for the presence of R* state comes from recent work with nicotinic acetylcholine receptor which is a gated channel which is not a GPCR. This work demonstrates that the channels open spontaneously in the absence of agonist and that the signal is qualitatively and quantitatively not different from agonist-stimulated response. The conductance of the channel was observed even after the disruption of the ligand binding site and suggests that the molecule undergoes allosteric transitions, both in the presence and absence of ligand (Jackson 1994).

Indication for isomerization of GPCRs to the R* state in the absence of ligand comes from several observations:

1-Receptors with high and low affinity for agonists are present in the absence of G nucleotides (Kent et al. 1980).

2-Receptors associate with G-proteins even in the absence of agonist (Okuma and Reisine 1992).

3-Overexpression of β-adrenergic receptors leads to an increase in the basal level of signalling by increasing the number of receptors in the R* state (Samama et al. 1993; Milano et al. 1994).

4-Constitutively activating mutations affecting the receptor result in increased affinity for agonists even in the absence of G-proteins (Samama et al. 1993). Therefore, these mutations result in a high-affinity, G-protein-independent state that cannot be accounted for by the simpler TCM model. According to the extended model, the constitutivity of the mutant receptors is due to the increase in the isomerization constant; in other words, due to their ability to adopt the R* form more easily in the absence of agonist.

In yeast, overexpression of Ste2p has been found to result in a lower-affinity form; whereas the K_d in wild-type cells is 2-6 nM (Blumer, 1988; Jenness,1986), the K_d in cells containing multiple copies of *STE2* ranges from 12 nM (Blumer 1988) to 150 nM (Blumer and Thorner 1990; David et al. 1997). These results suggest that in yeast, the availability of the G-protein may limit the formation of the ternary complex. Since increased expression of receptors affects the binding to α-factor (as indicated by K_d measurements), this could also be interpreted to indicate that G-proteins were not limiting to begin with. This apparent conflict can be settled when the relative ratio of the receptor to G-proteins is determined in yeast. For most physiological systems, the number of G-proteins seems to exceed the number of the receptors (Kenakin 1995; Neubig 1994). However, in *in vitro* systems where the amount of G-protein is insufficient to permit coupling to an excess of receptors, overexpression of β-adrenergic receptor was found to result in a low-affinity state for ligand binding, similar to observations in yeast (Milano et al. 1994).

CHAPTER I

ALLOSTERIC TRANSITION OF THE YEAST PHEROMONE RECEPTOR

An allosteric transition is a change in the binding or catalytic properties of a molecule brought about by the binding of an effector molecule at another site (Jackson 1994). The ability of ligands to promote changes in protein conformation has been largely pioneered by studying hemoglobin and allosteric enzymes (Ackers and Hazzard 1993; Schachman 1988; Schumacher et al. 1995). In general, allosteric proteins are thought to assume at least 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 (Monod et al. 1965). 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 (Bond et al. 1995; Samama et al. 1993). Relatively little structural information is known that pertains to the allosteric transitions in G-protein-coupled receptors. Biophysical and biochemical investigations of rhodopsin suggest that photoactivation leads to conformational changes at several sites in this protein, including changes in the extradiscal loops (corresponding to intracellular loops) ; however, the relationship of the conformational changes at these sites to G-protein activation remains unclear (Fahmy et al. 1995).

This chapter concerns ligand-induced conformational changes in the α-factor pheromone receptor from the yeast *Saccharomyces cerevisiae*. Using partial trypsin

25

digestion, it is shown that α-factor-induced conformational changes in the receptor result in enhanced accessibility of the 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 (Hartwell 1980). The genotype of strain DJ211-5-3 (deposited in the American Type Culture Collection, ATCC 96515) is *MATa cryl barl-1 ade2-1 his4-580 leu2 lys2 trp1 tyr1 ura3 SUP4-3*. Strain DJ903-A-1 (Schandel and Jenness 1994) is identical to strain DJ211-5-3 except that it contains mutation *ste2-T326* (Konopka et al. 1988). Strain DJ803-2-1 (provided by Jodi Hirschman) is identical to strain DJ211-5-3 except that it contains mutation *ste5-3* (Hartwell 1980) and mutation *scg1::lacZ(LEU2)* which disrupts the *GPA1* gene (Dietzel and Kurjan 1987). Cells were cultured in YM-1 medium (Hartwell 1967) 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, MA); the sequence was confirmed by amino acid analysis and mass spectrometry.

Analysis of partial trypsin digestion products. A cleared cellular lysate was prepared as described previously (Schandel and Jenness 1994). Membranes were collected by centrifugation (45 min, 140,000 x g); washed once with 1 mM magnesium acetate, 0.1 mM dithiothreitol, 0.1 mM EDTA, 7.6% glycerol and 10 mM MOPS, pH 7.0; and stored

at -70°C. Digestion of membranes (0.4 mg/ml protein) was initiated by adding TPCK-treated trypsin (Sigma Chemical Co.) to 30 µg/ml at 30° C and terminated by adding HCl to 0.016 N. In parallel reactions, 2 µM synthetic α-factor was added prior to the addition of trypsin. Membranes (150 µl from each time point) were collected by centrifugation in a Beckman Airfuge, suspended in a solution of 1% SDS and 1% β-mercaptoethanol (10 µl); incubated for 20 min at 40°C; and diluted 8-fold with 50 mM sodium phosphate buffer, pH 7. Asparagine-linked carbohydrate was removed with 20 mU of endoglycosidase H (2 hours, 37°C). Proteins were denatured by adding 50 µl of SDS sample buffer (50 mM Tris, pH6.8, 0.1 mM EDTA, 2% SDS, 0.01% bromophenol blue, 10% glycerol; to this, β-mercaptoethanol was added at 33 µl/ml) and incubated for 10 min at 37°C, resolved electrophoretically on a 12% SDS-polyacrylamide gel, transferred to a PVDF membrane (Millipore Corp, Bedford MA), probed with affinity-purified anti-receptor antiserum directed against the N-terminal extracellular domain (Konopka et al. 1988), and visualized with the ECL chemiluminescent detection system (Amersham Life Science).

In vitro protein synthesis. Plasmids pJBK019 and pJBK020 (provided by James Konopka; SUNY, Stony Brook) contain the coding region of the STE2 gene cloned into the pGEM1 and pGEM2 vectors, respectively. Plasmids were cleaved with Hind III, Cla I, Sty I, Nsi I or Eco RV and transcribed with either SP6 or T7 RNA polymerase according to Promega Transcription in vitro Systems technical manual intructions (20 µl of 5x transcription buffer, 10 µl 100 mM DTT, 0.5 mM rNTP, 2 µg DNA template, 50 U RNA polymerase, 100 U RNAsin ribonuclease inhibitor and 0.5 U of yeast inorganic pyrophosphatase in a final volume of 100 µl with DEPC-treated water was incubated at 37°C for 2 hours). The RNA product was purified with sequential precipitations with 5M LiCl and 70% ethanol (Sambrook et al. 1989). In vitro translation reactions containing ^{35}S-methionine utilized either the wheat germ (full-length control and truncated proteins S,

N and E, Fig. 1) or the rabbit reticulocyte lysate system (truncated protein C) as recommended by the supplier (Promega Biotech). 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 PVDF membrane, and probed with anti-receptor antibody as described above. The radioactive translation products were detected by using autoradiography.
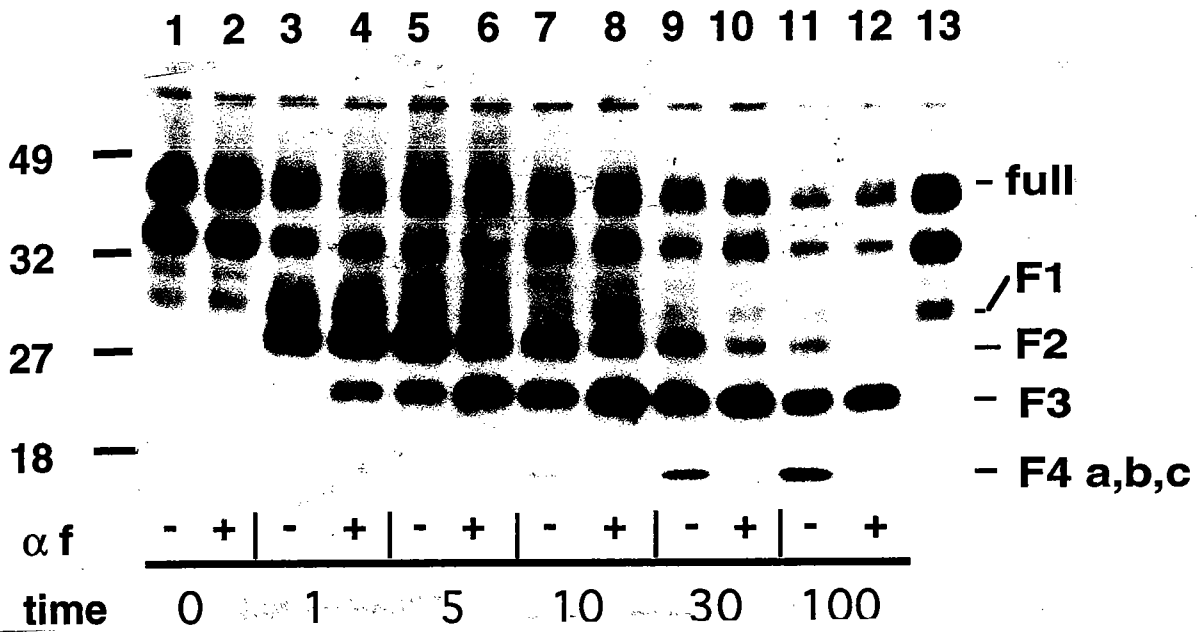
Results

Effect of α-factor on the receptor cleavage sites. Limited trypsin digestion was used to monitor ligand-induced conformational changes at multiple sites in the α-factor receptor. The receptor contains 32 potential trypsin sites (Figure 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 varying periods of trypsin digestion. Since antiserum specific for an amino-terminal segment of the receptor (Konopka et al. 1988) was used to detect these tryptic fragments, their electrophoretic mobility reflects the relative distances of the various cleavage sites from the amino-terminus. The undigested sample contained two electrophoretic species corresponding to the full-length receptor (approximately 43 kD) and to a smaller fragment (35 kD) that apparently resulted from cleavage during the membrane preparation (Figure 2, lanes 1 and 2). In the absence of α-factor, the receptor was cleaved within one minute to yield the fragments designated F1 and F2 (lane 3). By five minutes, F2 was the predominant form (lane 5). The cleavage events giving rise to fragment F3 were considerably slower (lane

28
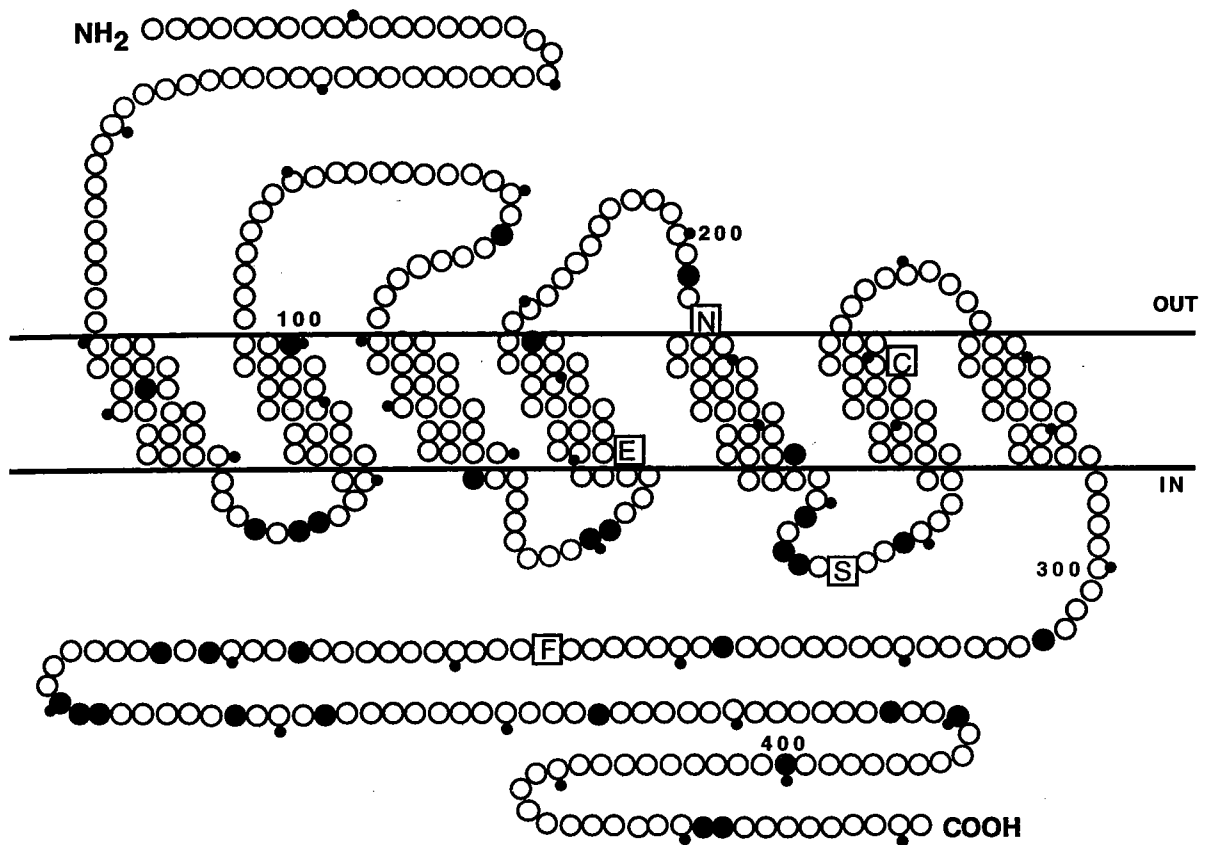
Figure 1. Primary structure of the yeast pheromone receptor. Filled circles indicate potential trypsin cleavage sites. Residues labeled E, N, S, and C indicate C termini predicted for the truncated receptors that were synthesized in vitro. Mutation Ste2-T326 results in translational frameshift after residue 326 (labeled F) and a protein product containing 334 residues (24).

Figure 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. The resulting cleavage products were analyzed by SDS-PAGE and immunoblotting methods. The antiserum was against the N-terminus of the receptor (Konopka, 1988). The control reaction was incubated for 100 minutes in the absence of trypsin and α-factor (lane 13). The major cleavage products (F1, F2, F3 and F4abc) are indicated at the right. Molecular weights of the marker proteins are indicated at the left.

7). Cleavage events close to the amino-terminus of the receptor eventually gave rise to a collection of smaller fragments designated F4a, b, and c (lanes 9 and 11) which were resolved on 15% SDS-polyacrylamide gels with molecular weigths of about 18K, 16K and running at the gel front respectively.

The binding of α-factor influences the rates at which certain trypsin sites are cleaved. Fragment F1 persists slightly longer in the presence of α-factor (Figure 2, compare lanes 7 and 8), suggesting that the cleavage site giving rise to fragment F2 is protected when α-factor is bound. Once F2 is formed, however, it is converted to fragment F3 more rapidly in the presence of α-factor (lanes 5 through 8), indicating that the site giving rise to F3 is more exposed 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 cleavage in the presence of α-factor (compare lanes 11 and 12), 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 preparations, α-factor consistently inhibited the formation of F2 and accelerated the formation of F3 among the five 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 are 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 minutes 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

31

event on the cleavage rate at a second site. Although I have not determined whether the cleavage events in the assay are interdependent, the accelerated formation of fragment F3 is unlikely to be an indirect consequence of prior cleavages, since α-factor produces only a modest inhibition in formation of fragment 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 electrophoretic mobility of the tryptic fragments with receptor fragments of known length. Figure 1 shows the potential trypsin cleavage sites and the positions of the carboxy-terminal residues of various truncated receptors (E, N, S, C and F). Full-length receptors that had been synthesized *in vitro* (Figure 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 206 (truncation N, lane 7) yet smaller than synthetic molecules truncated at position 235 (truncation S, lane 6). Thus, the α-factor-stimulated cleavages that give rise to F3 occur in or near loop i3 (position 225, 231, 233 or 234). Fragment F2 (lanes 1, 2, 9 and 10) was larger than synthetic receptors truncated at position 258 (truncation C, lane 5) and smaller than the *ste2-T326* mutant receptors (truncation F, lane 11), indicating that the α-factor-inhibited cleavage occurs at the beginning of the carboxy-terminal cytoplasmic domain (position 304 or 318). Fragment F1 was larger than the *ste2-T326* mutant receptors, indicating cleavage at or beyond position 337.

32
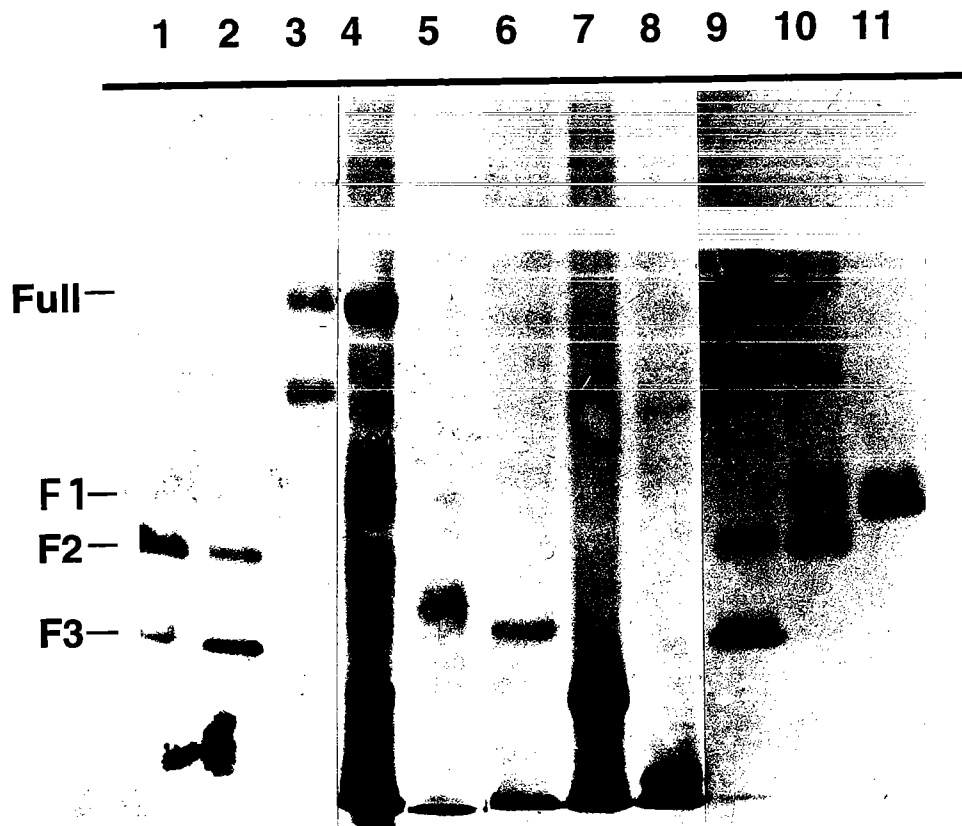
Figure 3. Positions of the major receptor cleavage sites. Full-length receptors (lane 4) and the truncated receptors depicted in Fig. 1 (lanes 5, 6, 7, and 8) were synthesized in vitro and resolved by SDS-PAGE. Translation is predicted to terminate at residue 431 for full-length receptors, at residue 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 1-3, 9 and 10) and *ste2-T326* mutant membranes (lane 11) were prepared and analyzed as in 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 absence (lanes 2, 10 and 11) or presence of α-factor (lanes 1 and 9). All of the samples were visualized on a single SDS-PAGE gel that had been tranferred to a PDVF filter. The figure was generated by superimposing the autoradiographic (lanes 4-10) and the chemiluminescent images (lanes 1-3 and 9-11). Positions of the major cleavage products are indicated on the left.

33

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, I was concerned that the increased rate of third-loop cleavage upon α-factor binding (Figure 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$_\alpha$ subunit (Figure 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 (Figure 2). The overall digestion rate was slower than that shown in Figure 2; however it was within the range that we observed for wild-type membrane preparations. The additional digestion intermediate that is larger than F1 (Figure 4A, lanes 3 and 4) was consistently observed with both wild-type and mutant membrane when the overall digestion rate was slow. The effect of α-factor was similar for the *ste4* and *ste18* mutants lacking the G$_\beta$ and G$_\gamma$ subunits, respectively (Figure 4B and C). I 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. I could not determine whether the G-protein deficiencies lead to small differences in the overall digestion rate since the limitations of the assay permit only the comparison of the effect of α-factor on the digestion of a given membrane preparation.
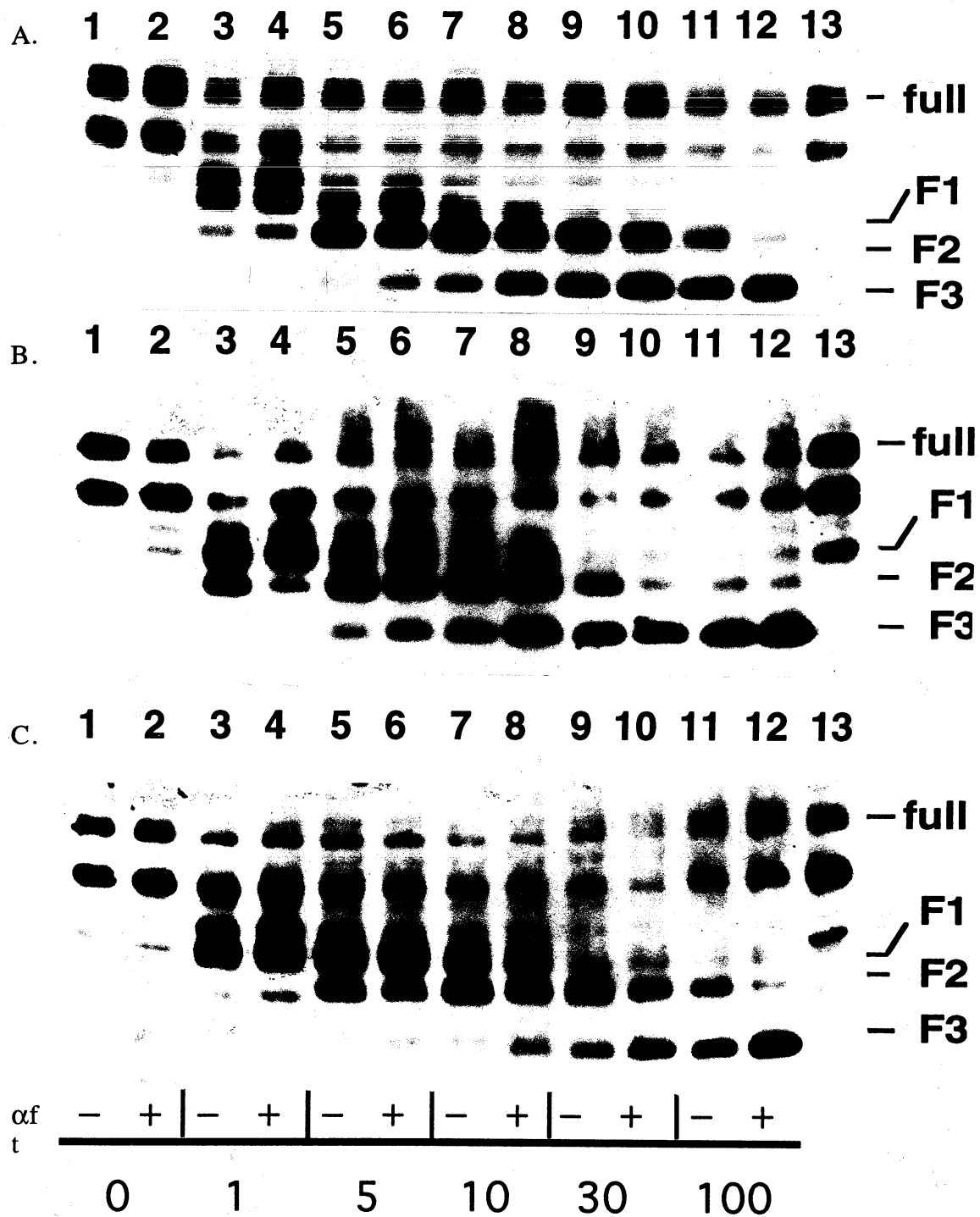
Figure 4. Effects of G protein deletions on the cleavage pattern. (A) Membranes from the *gpa1* mutant were prepared and analyzed as described in Figure 2, except that the strain contained the temperature-sensitive mutation *ste5-3* (Hartwell, 1980) and was cultured at 34°C. These conditions avoided the haploid-lethal phenotype exhibited by *gpa1* mutants (Blinder, 1989). (B)

Membranes from the *ste4* mutation, and (**C**) membranes from the *ste18* mutation were prepared and analyzed as described in Figure 2.

The *ste2-L236H* mutation affects the third intracellular loop of the α-factor receptor and results in a partial block in signal transduction activity (Schandel and Jenness 1994; Weiner et al. 1993). Previous studies (Weiner et al. 1993) 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 (Figure 5A). 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 I was unable to detect a defect in the ligand-induced conformational change. However, I cannot rule out the possibility that other conformational deficiencies that were not detected in this assay lead to the signal transduction phenotype of the *ste2-L236H* mutant.
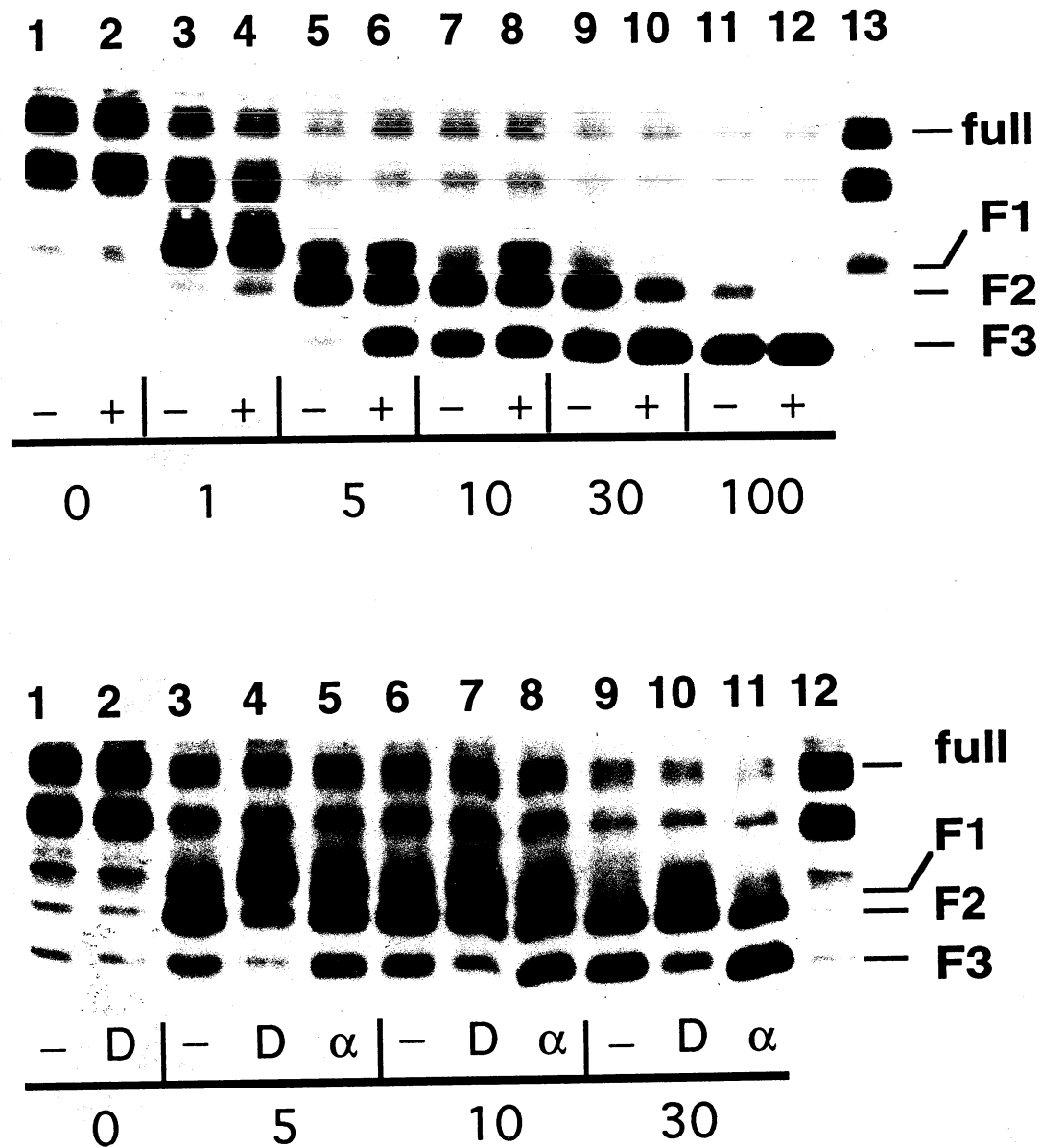
Figure 5. Effects of mutations and antagonist on the cleavage pattern. **(A)** Membranes from the *ste2-L236H* mutant (Schandel, 1994) were prepared and analyzed as described in Figure 2. **(B)** Wild-type membranes (strain 211-5-3) were treated with trypsin for the period indicated in the absence of ligand (-), in the presence of 2 μM α-factor (α), or in the presence of 2 μM desTrp1,Ala3-α-factor (D). Membranes were also incubated for 30 minutes in the absence of trypsin and α-factor (lane 12).

Antagonist-induced conformations. To identify the structural features of the ligand-occupied receptor that pertain to G-protein activation, I examined effects of antagonist desTrp1,Ala3-α-factor (Shenbagamurthi et al. 1983) 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 (Raths et al. 1988). Figure 5B 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, 4 and 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 five minute time point (lanes 3, 4 and 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 BAEE (not shown). These 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, I was 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 the cleavages that form fragment F1 or F2 are required before trypsin can cleave the receptor in its third intracellular loop. If this were true then antagonist could reduce 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 minute digestion of the α-factor-bound receptor (Figure 5B, lane 5) and the 10 minute digestion of the antagonist-bound receptor (lane 7) are degraded equally to fragments F1 and F2; yet after an additional 5 minutes of digestion, the α-factor-bound receptor (lane 8) shows a greater accumulation of fragment F3 than the antagonist-bound receptor shows after 20 additional minutes 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 chapter, using limited trypsin digestion of the α-factor receptor, I identified 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 (Pellicone et al. 1985b) and inhibits cleavages in the C-terminal domain (Kühn et al. 1982). Here, it is shown 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 (Weiner et al. 1993). The fact that the *ste2-L236H* mutant retains the ability to undergo the agonist-induced conformational change (Figure 5B) 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 (Bond et al. 1995; Samama et al. 1993). 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

41

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.*, 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 (Blumer and Thorner 1990) -- *ie.*, 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, these 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 (Figure 5B), even in the absence of G-protein (Figure 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 β2 adrenergic receptor have also been used to address the relative influences of agonists and inverse agonists on receptor conformation (Gether et al. 1995); 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 α2- and β2-adrenergic receptors exhibit higher affinities for agonists (but not antagonists) even in the absence of G-protein function (Ren et al. 1993; Samama et al. 1993). 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 (Clark et al. 1994;

Stefan and Blumer 1994) and the related **a**-factor receptor (Boone et al. 1993) 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 (Clark et al. 1994; Stefan and Blumer 1994), 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 (Chapter III) or permit desTrp1,Ala3-α-factor to function as an agonist (Marsh 1992; Stefan and Blumer 1994). I believe that this assay will make it 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-protein can be competed with antibodies directed against the second intracellular loop or the C-terminal domain (Weiss et al. 1988). Similarly, binding of the G-protein to the receptor is competed by the presence of synthetic peptides that comprise a portion of the second loop or the C-terminal domain of rhodopsin (Konig et al. 1989; Schreiber et al. 1994). Many α-factor antagonists (Eriotou-Bargiota et al. 1992) including desTrp1,Ala3-α-factor (Appendix) appear to elicit partial activation of the G-protein, in that they show agonist activity when the target cells are supersensitive to α-factor due to 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 (Konopka et al. 1988; Reneke et al. 1988).

The binding of α-factor stimulates endocytosis of the α-factor receptor in addition to G-protein activation (Schandel and Jenness 1994). The conformational change necessary for endocytosis does not appear to require G-protein function (Blinder et al. 1989; Jenness and Spatrick 1986; Zanolari et al. 1992). Endocytosis requires the C-terminal domain of the receptor (Rohrer et al. 1993; Schandel and Jenness 1994) and is not blocked by mutations in the third intracellular loop (Schandel and Jenness 1994; Weiner et al. 1993). 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 335-339) that is believed to mediate the regulation of endocytosis (Rohrer et al. 1993). In addition to endocytosis, other α-factor responses are mediated by the C-terminal domain, including adaptation to the stimulus (Konopka et al. 1988; Reneke et al. 1988), receptor phosphorylation (Reneke et al. 1988), and changes in cellular morphology (Konopka 1993; Konopka et al. 1988); 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 (Baldwin 1994; Strader et al. 1995). 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 (i. e., slower formation of fragment F2) and expose the third intracellular loop, adjacent to helix 6

(*i. e.*, 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 (Chapter 2). Consistent with this interpretation, computer-generated simulations of the 5-hydroxy-tryptamine receptor (Luo et al. 1994; Zhang and Tipper 1993) 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.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF A MUTANT RECEPTOR
DEFECTIVE IN THE ALLOSTERIC TRANSITION

Two models account for receptor activation by agonist: conformational induction and conformational selection. The conformational induction model (exemplified by the induced-fit model) states that binding of agonist to the inactive state of the receptor results in a conformational state that would otherwise be inaccessible. The conformational selection model (exemplified by the Monod-Wyman-Changeaux model for allosteric proteins) states that the receptor exists in two or more conformations even in the absence of ligand, and agonist selectively binds and stabilizes the activated form. One view is that neither of these mechanisms are very different and that they are only extremes of the same mechanism (Kenakin 1996).

The conformational changes associated with GPCR's are most often interpreted in terms of the second model. The Ternary Complex Model (TCM) is an example of such a model because agonist binds selectively to the receptor-G-protein complex. Using the β-adrenergic receptors as an example, it was shown that GPCRs exist in high and low affinity states and that agonist, antagonist and guanine-nucleotides influence the relative abundance of the two states. The TCM model was recently modified (Samama et al. 1993) to account for the properties of certain constitutively-activated receptors and is referred to as the extended TCM. This extended TCM model has three basic tenets:

(i) The receptors in the inactive and active state are at an equilibrium even in the absence of ligands. The conformational equilibrium constant is expressed as J; J=R/R*.

(ii) Kd is affected by the transition between R and R* states, and ligand binding makes a free energy contribution to the transition of RT ln(K); K=k_R/k_{R*}.

(iii) The G-protein associates only with the R* conformation.

The molecular changes constituting the switch from the R to R* form have been the topic of much research. Furthermore, determining the residues involved in allosteric transition has been useful in the elucidation of the activation mechanism. To this end, mutations that render the receptor constitutively active have been especially helpful (Scheer and Cotecchia 1997). The constitutively active mutant receptors are thought to mimic the activated (R*) form of the receptor in the absence of the agonist. Analysis of naturally occuring receptor mutations that lead to disease have shown that in some cases the disease was due to a constitutively-activated form of the receptor. Disruption of a salt bridge between Lys296 in TM7 and Glu113 in TM3 in rhodopsin was found to result in retinitis pigmentosa; mutations affecting either residue lead to constitutive activation of rhodopsin (Robinson et al. 1992). Mutations in the TM6 such as the Asp578Gly of the Luteinizing hormone receptor (LRH) have been identified in patients with familial male precocious puberty (Shenker et al. 1993). Mutations affecting Thyroid Stimulating Hormone (TSH) receptor have been identified in patients with thyroid adenomas (Parma et al. 1993); the resulting amino acid changes have been mapped to TM 3, 6 and 7 as well as to the third intracellular loop. In addition to the changes in the TM regions, mutational alterations in the extracellular loops of two different receptors (TSH and thyrotropin receptors) were found to result in constitutive activation (Parma et al. 1995) and (Nanevicz et al. 1996) (Figure 1). Biochemical and computer simulation analysis of such mutants have led to the suggestion that in the R form, the receptors are constrained through specific interactions in
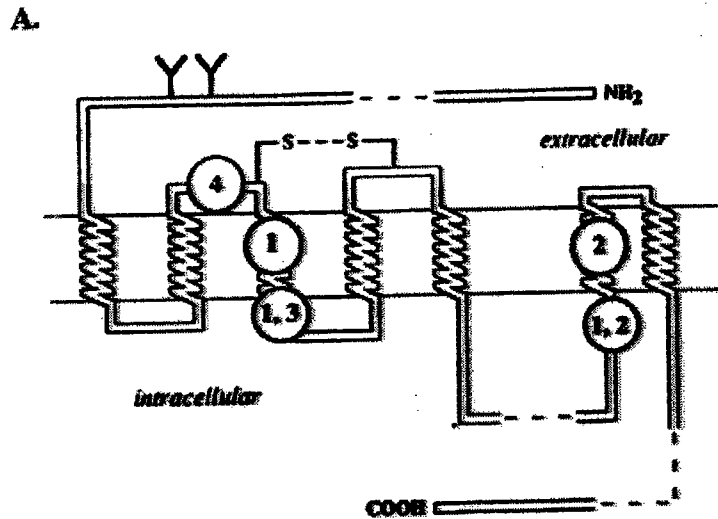
A.

B.
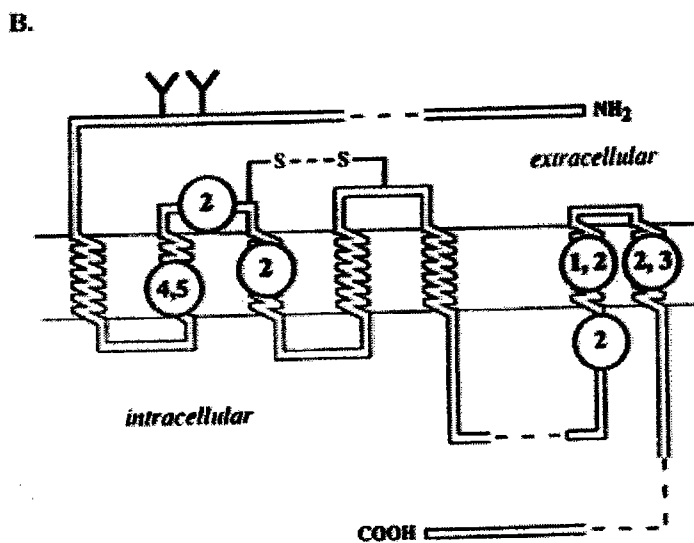
Figure 1. A. Artificial activating mutations:
(1)Adrenergic,(2) Muscarinic, (3) Rhodopsin, (4) Thrombin receptor.
B. Spontaneous activating mutations: (1) LH, (2) TSH,
(3 and 4) Rhodopsin, (5) MSH receptor. From A. Scheer, 1996.

the transmembrane regions and that binding of the agonist leads to disruption of these constraints and to a more relaxed R* form.

Common structural features among GPCRs that are associated with different ligands or with different G-protein specificities suggest the identity of amino acids that are essential structural determinants of receptor function. Highly-conserved residues are therefore targets of site-directed mutagenesis. Mutation of conserved residues of the α1B-adrenergic receptor (C128 in TM3 (Perez et al. 1996) and S204 in TM5, (Strader et al. 1989)) were found to result in increased affinity for agonists as well as higher basal signalling levels than the wild-type control. The mutation of residues in the third intracellular loop have also been found to lead to agonist-independent signalling; the systematic mutation of the Ala293 (located in i3) of the α1B-adrenergic receptor by any of the 19 other amino acids leads to (different levels of) constitutive activation (Samama et al. 1992). Activating mutations in the N- and C-terminal of the third loop of muscarinic acetylcholine Hm1 receptors have been identified as well (Hogger et al. 1995). Moreover, substitutions or deletions of the a-factor receptor in yeast also result in constitutive activation (Boone et al. 1993). These results suggest that certain molecular interactions are essential for constraining the unoccupied receptor and that interruption of these constraints lead to agonist-independent activation.

A reciprocal approach for identifying residues involved in receptor activation is to search for receptor mutants that are trapped in a low-affinity conformation. Mutant proteins that are unable to attain an activated conformation are expected to show a reduced affinity for agonist as well as diminished signalling capacity. The same phenotype would also result from a receptor mutation that affects the ligand binding site. Nevertheless, certain mutants of the NK1 and the β-adrenergic receptors have been proposed to cause the

stabilization of the R form. In the NK1 receptor, alanine substitution of either residue Glu78 (in TM2) or residue Tyr205 (TM5) lead to modest reduction (26- and 38-fold respectively) in the binding affinity for agonists accompanied by a total loss of the ability of agonists to mediate a signal transduction response (Huang et al. 1994). These authors conclude that both residues which are highly conserved, play a role in receptor activation rather than in the initial binding step. Another residue conserved not only among receptors that bind structurally similar ligands but in most GPCRs is Asp79. Therefore this residue is thought to be involved in maintaining the agonist-bound conformation of the receptor. The Asp79Ala substitution in the β-adrenergic receptor leads to a 10-fold increase in K_d for agonist in the presence and absence of G-proteins and a 10-fold increase in EC_{50} for signalling (Strader et al. 1988). In the V2 vasopressin receptor, substitution of residue Arg113 with tryptophan (first extracellular loop) has been found in patients with congenital nephrogenic diabetes insipidus. This polar to non-polar substitution was found to result in a 20-fold increase in K_d and a 60-fold increase in EC_{50} (Birnbaumer et al. 1994).

In this chapter, I describe the identification and characterization of a mutation in the yeast *STE2* gene which leads to a receptor that is apparently locked in a low-affinity allosteric state.

Materials and Methods

Isolation of Signalling Mutant. This was done essentially as described by Schandel& Jenness,1994. The entire *STE2* coding region was mutagenized by hydroxylamine treatment (Rose and Fink 1987) of plasmid pJBK008 (Konopka et al. 1988). The mutagenized plasmid DNA was used to transform strain 213-7-3 (*MATa ste2Δ*) and Ura$^+$ transformants were replica plated to plates containing 4×10^{-8} M α-factor.

The α-factor resistant isolates were chosen for further study. These candidates were tested for their ability to bind ^{35}S-labelled α-factor (5 nM) (Jenness et al. 1986). Only one mutant (pDJ270) retained the ability to bind pheromone; affinity was 35% of the level observed for wild-type cells.

To determine the location of the mutation, I subcloned four restriction fragments (HindIII-DraI, HpaI-AatII, AatII-PstI and ClaI-PstI) from the mutant copy of *STE2* (pDJ270) into an otherwise wild-type copy of *STE2* carried on the integrating plasmid pDJ251 (pPS3-*tet*^{R},*STE2,URA3*). Recombinant plasmids were digested with StuI and used to transform strain 213-7-3. Halo assays were performed to identify the ClaI-PstI fragment as the region of *STE2* conferring the mutant phenotype. This fragment was sequenced by using double-stranded plasmid DNA and the Sequenase kit from United States Biochemicals. A single mutation (*Ste2-T274P*) was found; an A to C transversion at position 819 was predicted to result in a threonine to a proline substitution at residue 274. A two-step gene replacement was used to replace the chromosomal copy of *STE2* in 211-5-3 with *ste2-T274P* (pDJ283) creating strain 902-A-1. Southern blot analysis was used to confirm the overall structure of the *STE2* locus.

Assays of Responsiveness to Pheromone. Halo assays were conducted by placing sterile filter discs, containing 20 µl of various concentrations of α-factor on a lawn of 5×10^6 cells on an agar plate. The size and clarity of the halos were noted after 24 and 48 hr at 30 °C and the diameter of the halo measured after 48 hr.

α-Factor-binding measurements. Cells were cultured overnight at 30°C to a final density of 10^7 cells/ml. NaN3 and KF were added to a final concentration of 10 mM and cells were harvested by centrifugation, rinsed twice in inhibitor medium (YM-1 containing

10 mM NaN$_3$, 10 mM KF, and 10 mM *p*-tosyl-L-arginine methyl ester) and the cell concentration was determined as described previously (Jenness et al. 1983). The binding reaction was initiated by addition of cells to a final concentration of 2×10^8 cells/ml with serial dilutions of ^{35}S-α-factor (5 Ci/mmol) in inhibitor medium in the presence and absence of 20 μM cold α-factor. The amount of ^{35}S-α-factor ranged from 1nM to 100 nM. After 30 min at room temperature, a 0.09 ml of sample was diluted to 2 ml with inhibitor medium, and the cells were collected on a presoaked filter (Whatman, GF/C). The filters were rinsed twice with 2 ml of inhibitor medium for 0.4 to 0.5 min. The filters were then dried and counted in liquid scintillation counter. The amount of nonspecific binding was determined for each ^{35}S-α-factor dilution. Specific binding was calculated as the counts bound to filter. The free α–factor concentration was the concentration of α–factor bound to the cells subtracted from the total α-factor concentration in the binding reaction mixture. Binding data were analyzed by the method of Scatchard to find K$_d$ and Bmax (Scatchard 1948).

Competition binding was done in a similar fashion except that the final cell concentration for wild-type strain was 3×10^8 cells/ml, final ^{35}S-α-factor concentration was 2 nM, the concentration of unlabeled α-factor ranged between 2 nM to 2 μM. For the *ste2-T274P* mutant cells, the final cell concentration used was 5×10^8 cells/ml, final ^{35}S-α-factor was 10 nM and the range of the unlabeled agonist ranged from 5 nM to 2 μM.

The binding of ^{35}S-α-factor to crude membranes was determined as well. The reaction was started by the addition of 400 μg membranes in lysis buffer (1 mM magnesium acetate, 0.1 mM dithiothreitol, 0.1 mM EDTA, 7.6% glycerol and 10 mM MOPS, pH 7.0, plus 1mM EDTA, 100 μg/μl PMSF and 100 μg/μl pepstatin) into tubes containing either 10 nM ^{35}S-α-factor or 10 nM ^{35}S-α-factor plus 2 μM cold α-factor.

After incubation at 30°C for 10 min, membranes were collected on filters that had been dipped in 0.3% PEI, pH=7, washed twice with 2 ml lysis buffer, dried and counted in a liquid scintillation counter. Strains assayed were 211-5-3, 902-A-1 and 213-7-3, each in triplicate.

Quantitative mating experiments. Cells were grown overnight at 30°C to 10^7 cells/ml. 2×10^6 **a** cells to be tested for mating were mixed with equal number of α cells (strain EMS63), collected on a type HA filter (0.45-μm pore size), and rinsed with 10 ml of YM-1 medium and the filter was transferred to a YEPD plate, which was incubated for 6 h. The cells were then suspended in 2 ml of ice-cold minimal medium (without ammonium sulfate or glucose), dispersed by vortexing at high speed for 1 min, diluted and plated out onto unsupplemented minimal medium.

β-galactosidase assays. Assays of β-galactosidase activity in permeabilized cells were performed as described by Hagen and Sprague Jr. (1984). Cultures were grown to exponential phase in -trp+CAA at 34°C, diluted to about 5×10^6 cells/ml and shifted to 30°C for 1.5 h. Cells were treated with α-factor for 30 minutes, and the reaction was stopped by the addition of cycloheximide (final concentration, 0.02 mg/ml). Cells were collected by centrifugation and resuspended in 0.1 volume of Z-buffer (10 mM KCl, 1mM $MgSO_4$, 0.1 M sodium phosphate, pH7). To permeabilize cells, suspensions were adjusted to 60 μl/ml chloroform, 28 mM β-mercaptoethanol and 0.0075 % SDS and vortexed. The reaction was initiated with 0.8 mg/ml of *o*-nitrophenyl-β-D-galactosidase and terminated after 30 min at 28°C by addition of one-third volume of 0.1 M sodium carbonate. The A_{420} was determined after clarification by centrifugation. Units of activity were calculated by using

the formula (1000 X A420 of reaction) divided by (A600 of culture X volume of culture used in ml's X time of reaction in min).

Results

Mutant isolation. In order to identify residues involved in activation of α-factor receptors, I mutagenized a plasmid-borne copy of the *STE2* gene with hydroxylamine and selected mutants that exhibited a loss-of-function phenotype. Random mutagenesis was employed since the *STE2* gene product has no sequence homology with the rest of the GPCRs and since random mutagenesis avoids bias towards any particular residue. Binding of α-factor to wild-type receptors induces cell division arrest, therefore the library of plasmid-borne mutations was screened for mutations that resulted in α-factor resistance. Fifty three colonies from three thousand transformants were identified to be resistant by replica plating to α-factor-containing plates (40 nM of α-factor). This α-factor concentration was sufficient to cause arrest of cells expressing wild-type receptors. When single-colony isolates were retested, three were found to display the α-factor-resistance phenotype. The mutant plasmids were recovered from these isolates and then reintroduced into the original *ste2* deletion strain to confirm the presence of a plasmid-borne mutation. In principle, a mutant could show an α-factor resistance phenotype for one of several reasons:

i) It fails to express receptors on the cell surface.

ii) The α-factor binding site is defective.

iii) α-factor binding site is intact but the receptor is unable to assume the activated conformation.

iv) The mutant receptors are defective for G-protein interaction.

54

Only one of the mutants was found to be expressed on the plasma membranes comparable to wild-type levels and to show 35% wild-type binding at 5 nM α-factor concentration. Hence, this mutant expresses defective receptors on the plasma membrane. The mutation was found to result in a threonine to proline substitution at position 274; this residue is located near the extracellular end of TM 7 (Figure 2). The localization of the mutation rules out the possibility that it interferes with the G-protein association of the receptor.
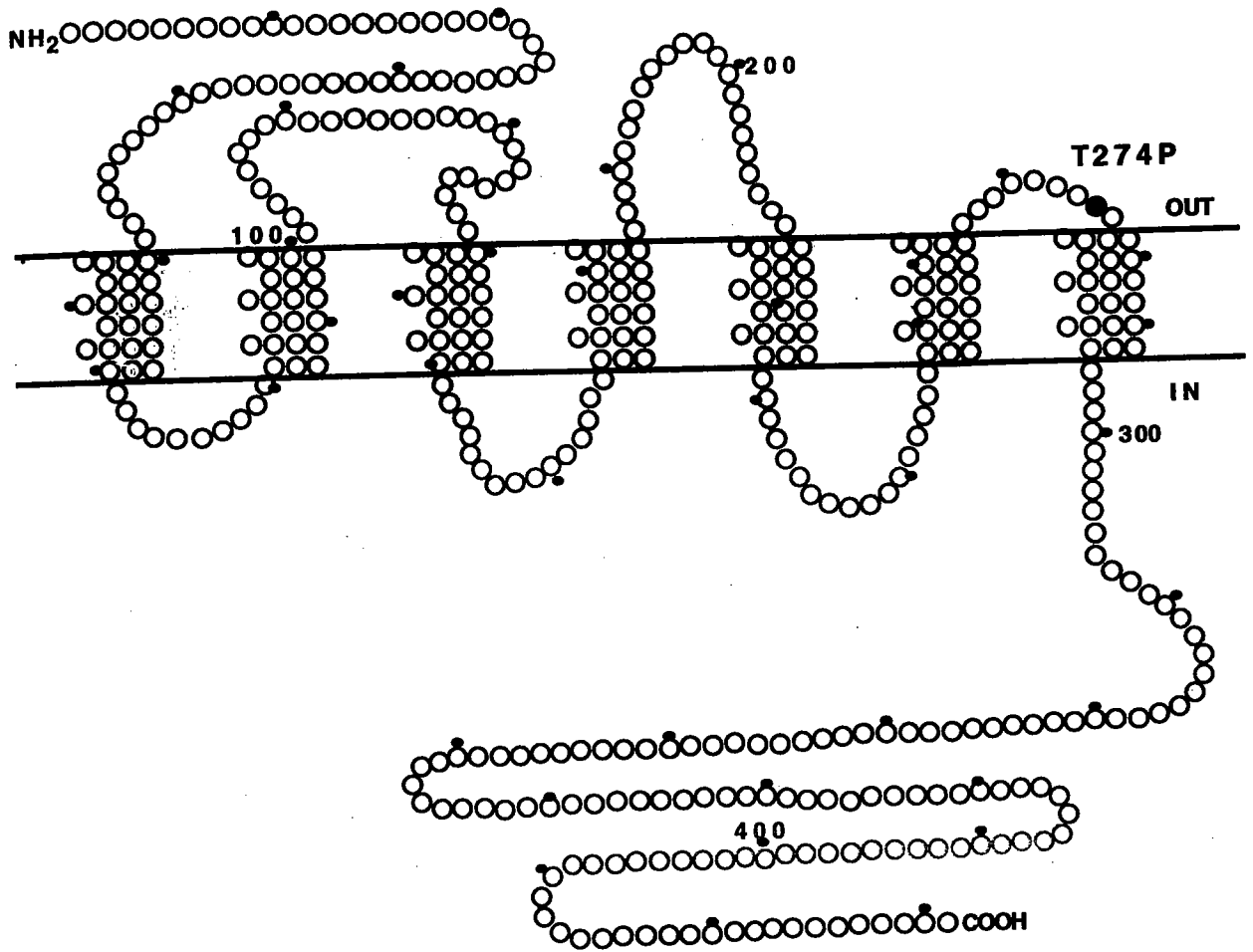
Figure 2. Localization of the *ste2-T274P* mutation.

Characterization of the mutant receptor. A receptor mutant that is defective for conformational change will be locked into the R state and will have a higher K_d value for agonists. To assess the agonist affinity and cell-surface expression of the mutant, I performed equilibrium α-factor binding assays with intact cells. Metabolic inhibitors were added to block receptor internalization. The results of the binding experiment were plotted according to the method of Scatchard (Scatchard 1948) (Figure 3). The K_d value for the wild-type control cells was 3 nM and the number of sites was 4800 receptors/cell similar to previously reported values (Jenness et al. 1986) and (Weiner et al. 1993). The mutation was found to increase the K_d value of the receptor to roughly 60 nM, and the results were consistent with a nearly wild-type number of binding sites (Bmax). The amounts of receptor protein from lysates of wild-type and mutant cells was compared by using immunoblotting methods. This analysis indicated that the level of the mutant receptor protein was roughly half the level found in wild-type cells. Fractionation of the membranes from the mutant cells on Renografin density gradients indicated that essentially all of the mutant receptor protein was associated with the plasma membrane (K.A. Schandel and D.D. Jenness, unpublished data).

Mutant receptors that are locked in the inactive R state are predicted to show a decreased affinity for agonists and an increased affinity for negative antagonists (inverse agonist). The desTrp1,Ala3-α-factor peptide appears to have properties similar to that of a negative antagonist since it induces a hyperconstrained conformation of the receptor as detected by trypsin digestion assay (Bukusoglu and Jenness 1996) and since a related antagonist (desTrp1,Ala3,Nle12-α-factor) was shown to have a negative influence on G-protein coupling (Blumer and Thorner 1990). Ligand binding affinities of the *ste2-T274P* mutant cells were determined by assays that employed radioactive α-factor and various
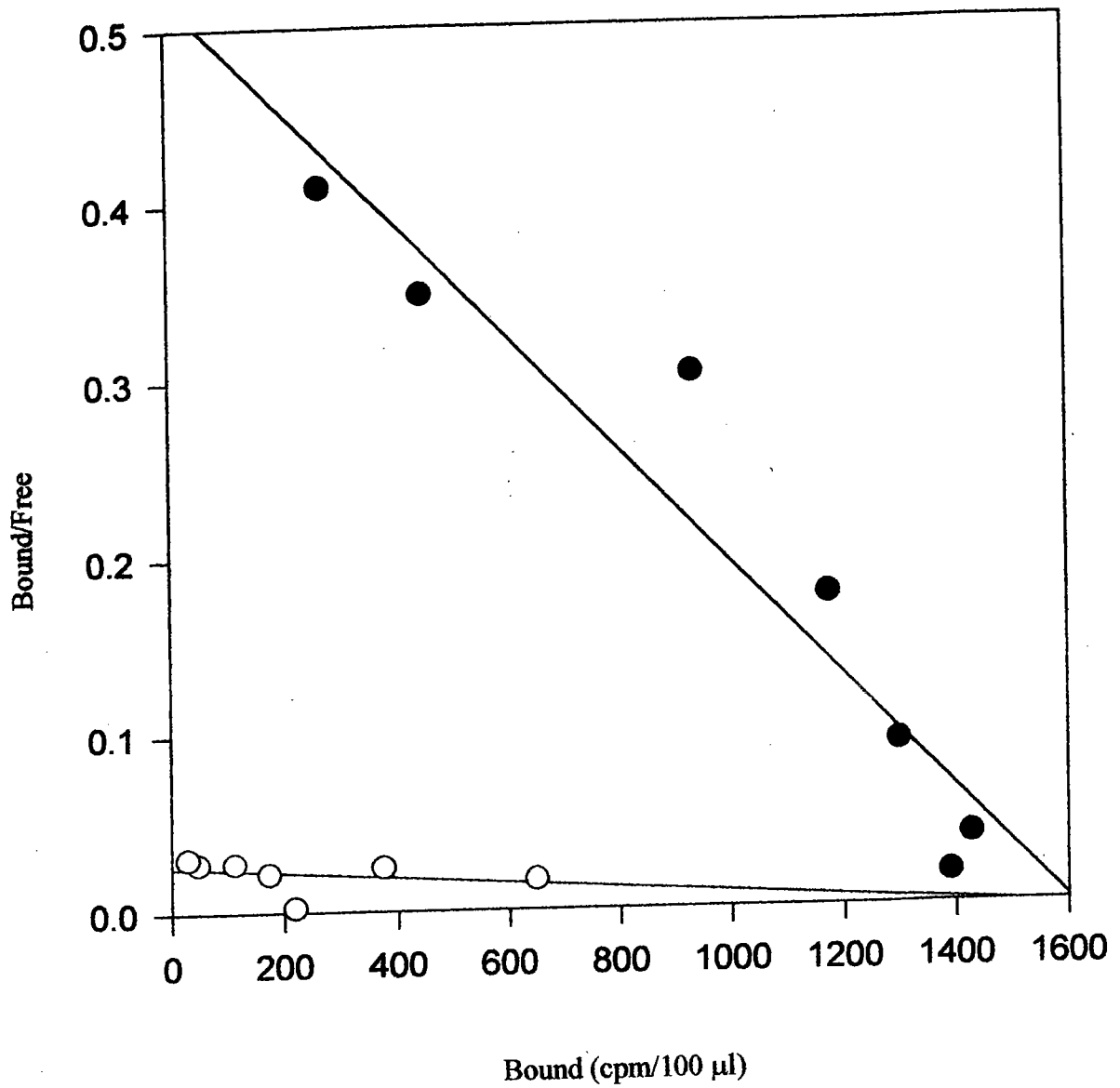
Figure 3. Equilibrium binding of ^{35}S-labeled α-factor to cells expressing wild-type ($\bullet$) or mutant (o) receptors. Data obtained from equilibrium binding experiments are plotted according to the method of Scatchard and were done in triplicate.

concentrations of unlabeled ligand as competitor. This assay permits comparison of the affinities of the mutant and wild-type receptors for agonist and antagonist. If the conditions of this experiment are adjusted so that the concentration of radioactive ligand used and the concentration of receptor sites are small compared with the K_d value, then the 50% inhibitory concentration for radioactive ligand binding (IC_{50}) will correspond to the K_d value. The IC_{50} value obtained with the wild-type cells and unlabeled α-factor was 8 nM, while the IC_{50} value obtained with the antagonist was 1000 nM. The IC_{50} value obtained with the *ste2-T274P* mutant and unlabeled α-factor was 80 nM, while a value of 800 nM was obtained with antagonist (Figure 4). As expected for a receptor that is in a low-affinity form, the mutant receptors show decreased affinity for agonist whereas the affinity for the negative antagonist is increased.
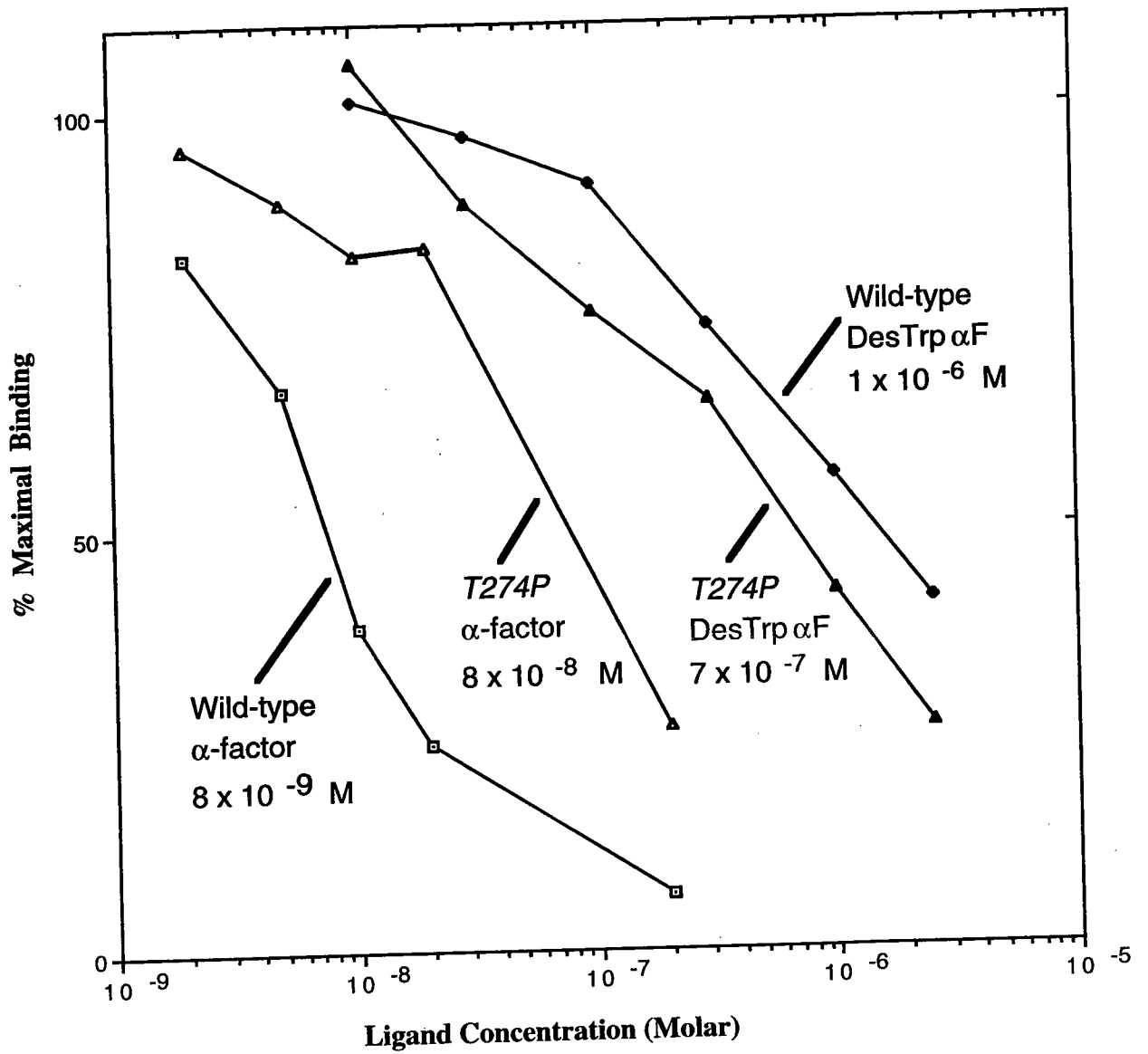
Figure 4. Competition binding curves of cells with the wild-type (STE2p) and the mutant receptor (ste2-T274Pp) with the agonist and antagonist.

The ability of the mutant receptor to undergo the conformational change in response to ligand was determined by using the trypsin digestion assay described in Chapter I. In this *in vitro* assay, membranes containing mutant and wild-type receptors are digested partially with trypsin in the presence of agonist or antagonist, and cleavage events are detected by using immunoblotting methods. The concentration of ligand used in this assay was above K_d (2 μM of both ligand). At this concentration of agonist, over 90% of the receptors are expected to be occupied, whereas for the antagonist, over 65% occupancy is expected. Membranes prepared from cells containing either the wild-type or the *ste2-T274P* mutant receptor were digested for various periods of time. The partial digests were run on gels and probed with an antibody against the N-terminal portion of Ste2p, (Figure 5). As was discussed in the previous chapter, activation of the wild-type receptor by α-factor results in the exposure of the third loop, as a consequence, the rate at which fragment F2 is converted to F3 is slower. Unlike the wild-type Ste2p, the mutant receptor did not show increased exposure of the third intracellular loop upon addition of agonist (compare lanes 4 & 5 and 7 & 8 for the mutant receptor, and lanes 10 & 11 for the wild-type receptor). Since the mutation results in decreased affinity for agonist, it could be argued that at the concentration used, α-factor was not bound to the mutant receptor. This issue was addressed by performing radioactive α-factor binding experiments with the membranes that had been prepared for the trypsin digestion assay. Addition of 2 μM unlabelled α-factor was found to compete for the binding of radioactive α-factor when the membranes had been obtained from either wild-type or *ste2-T274P* mutant cells (Figure 6). These results show that under the conditions of the digestion assay, a significant portion of the mutant receptors bind α-factor.
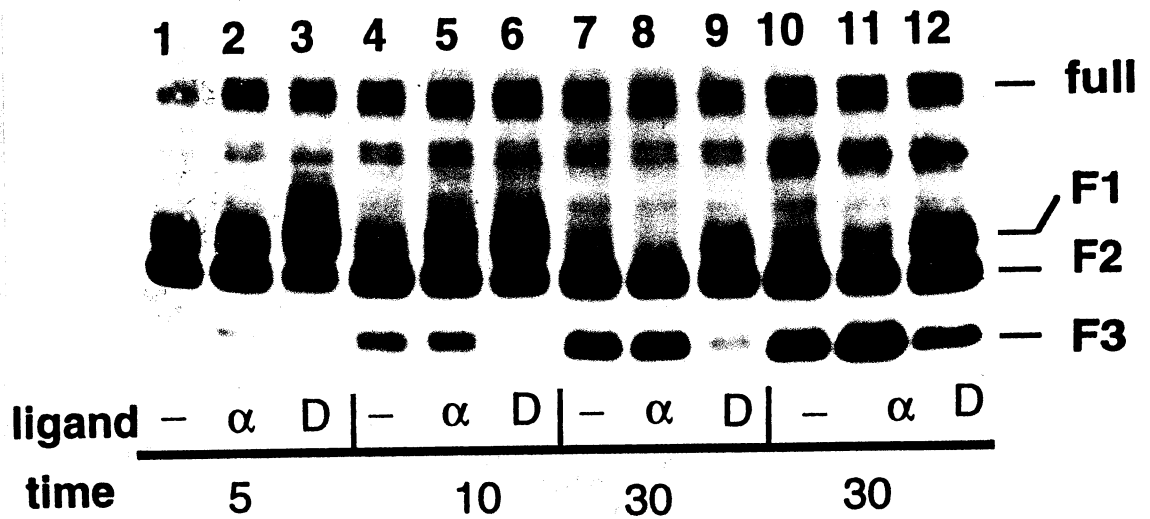
Figure 5. Trypsin digestion assay of membranes prepared from cells containing the T274P mutation in the pheromone receptor. Lanes 1-9 are membrane preparations containing *ste2-T274P* mutation, lanes 10-12 are membrane preparations of cells containing the wild-type STE2 receptor. – is for no ligand, α is for α-factor, D is for antagonist.
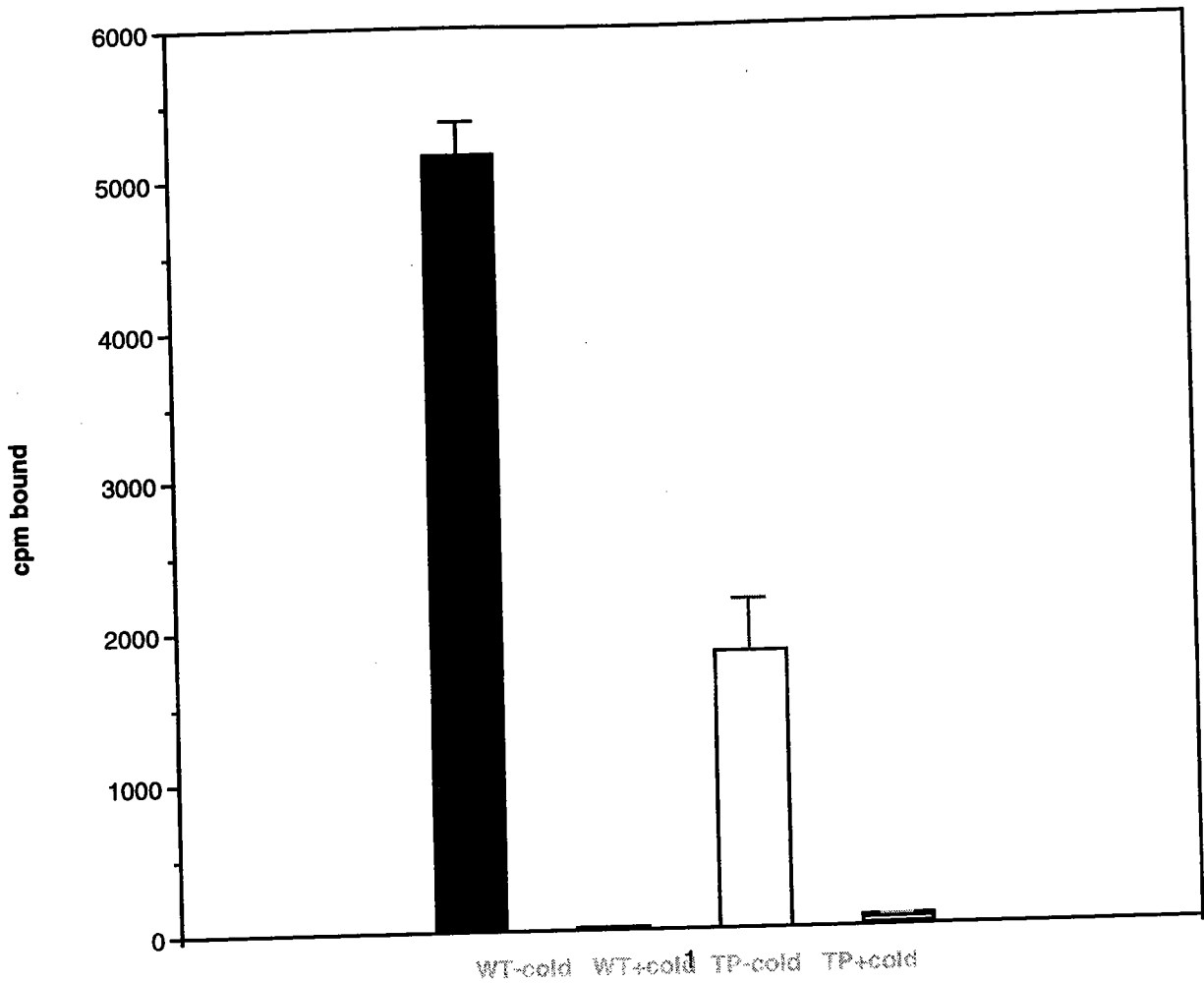
Figure 6. Results of the binding experiments using membranes prepared from cells containing the wild-type receptor or the *ste2-T274P* mutation.

It was shown previously that antagonist binding results in a hyper-constrained form of the wild-type receptor which is less accessible to trypsin than either unoccupied receptor or receptors occupied with agonist (Bukusoglu and Jenness 1996). The effect of the antagonist on the mutant receptor was analyzed by the same method (Figure 5). Whereas the agonist apparently did not influence the conformation of the mutant receptor, the antagonist resulted in the same digestion pattern with the mutant receptor as with wild-type (compare lanes 7 & 9 for the mutant, and lanes 10 & 12 for the wild-type). This observation provides further support for the argument that the *ste2-T274P* mutant receptor can bind α-factor under the conditions of the assay (since the antagonist binds the mutant and wild-type receptors with similar affinity) but cannot assume the activated, "relaxed" conformation whereas it can assume the antagonist-induced, hyper-constrained conformation.

To evaluate the activity of the mutant receptors *in vivo*, I examined the dose-response relationship for the ability of α-factor to induce transcription of a pheromone responsive reporter gene. Genes that respond to α-factor contain one or more copies of the pheromone response element (PRE) upstream of the transcriptional start site (Kronstad et al. 1987). The reporter plasmid that was used in this study is the multicopy plasmid pJD10 that contains a promoter with 8 tandem copies of PRE fused to the coding sequence for the *E.coli* β-galactosidase (Hasson et al. 1994). Since this assay measured short-term responses to α-factor, it avoided the complications resulting from long-term assays (i.e., desensitization). Cells were incubated at 30°C for an hour after addition of various concentrations of α-factor. The *ste2-T274P* mutant receptor was found to signal up to wild-type levels at high α-factor concentrations (Figure 7). The EC_{50} in this assay is the α-factor concentration that gives 50% of maximal signalling. The EC_{50} value for the wild-
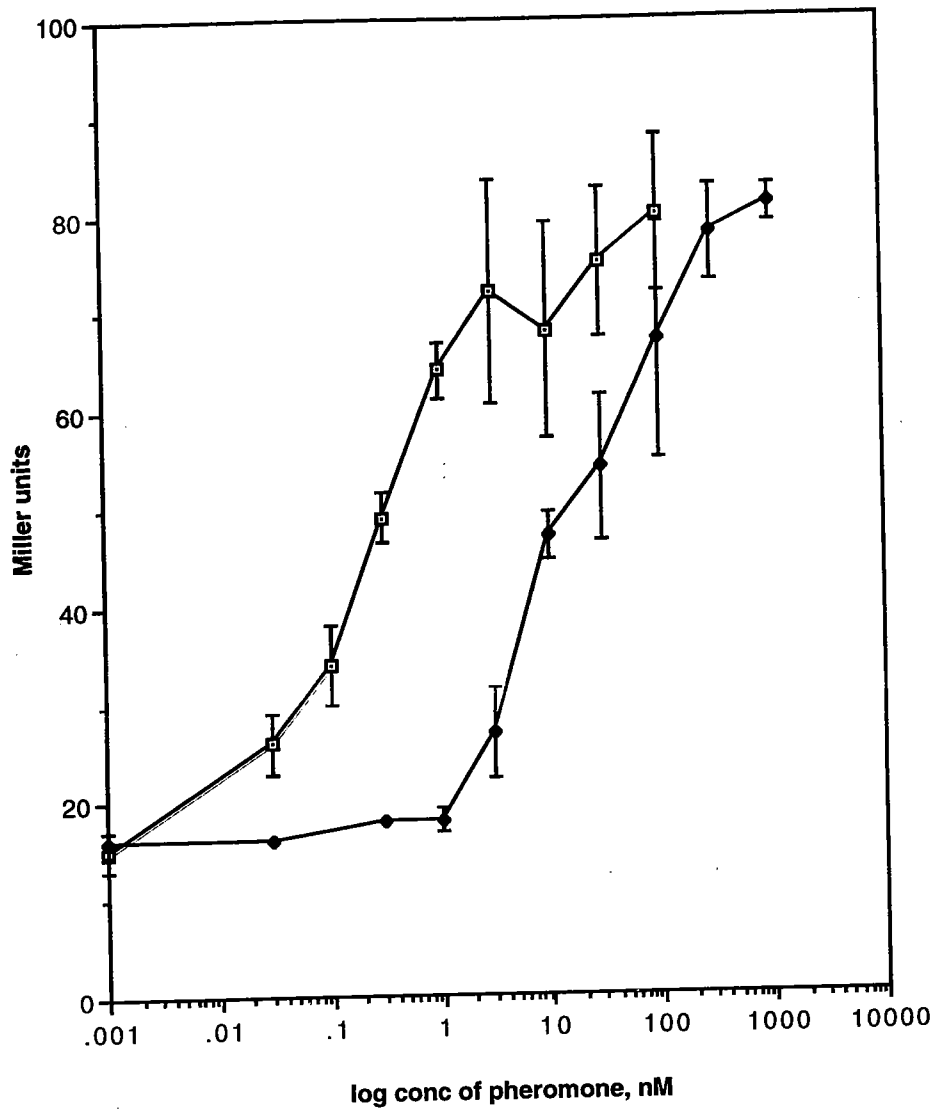
Figure 7. Transcriptional induction assay. Open squares represent wild-type, closed diamonds represent the ste2-T274P mutant receptors. Data shown are the mean values ± SEM.

type control cells was 0.3 nM whereas the EC_{50} for the *ste2-T274P* mutant was 10 nM. This result indicates that whereas the *ste2-T274P* results in a 10-fold decrease in agonist affinity, it causes a 33-fold difference in the dose-response.

As a second measure of mutant receptor activity *in vivo*, K. Schandel assayed the ability of the *ste2-T274P* mutant to undergo ligand-induced endocytosis of cell-surface receptors. The assay was performed as described (Schandel and Jenness 1994) except that 500 nM α-factor was used for the mutant cells (as opposed to 10 nM for the wild-type cells) to account for the K_d defect of the mutant receptor. It was observed that at this concentration of agonist, the mutant receptor was endocytosed almost as rapidly as the wild-type receptors (Figure 8). At 10 nM, endocytosis of mutant receptors was undetectable (not shown). These results are in agreement with the transcriptional induction experiments (Figure 7), in that the mutant receptors are active in both assays but require higher α-factor concentrations to achieve a comparable effect.

In order to determine whether the *ste2-T274P* mutation is dominant or recessive, I performed α-factor halo assays that monitor the ability of the pheromone to arrest the division of cells on an agar surface. In this assay, filter disks containing different quantities of α-factor were placed on a petri plate that had been seeded with cells of the test strain. After 1-2 days, the ability of cells to grow in the area surrounding the disk was evaluated by measuring the diameter of the inhibitory zone ("halo") and by evaluating the clarity of the zone. When a single-copy plasmid encoding the *ste2-T274P* mutant receptor (pDJ283) was used to transform the *ste2* deletion strain, I found that the resulting transformants gave halos that were smaller and more turbid than the clear halos obtained with the wild-type control strain (Figure 9A). Turbidity in the halo assay (which measures
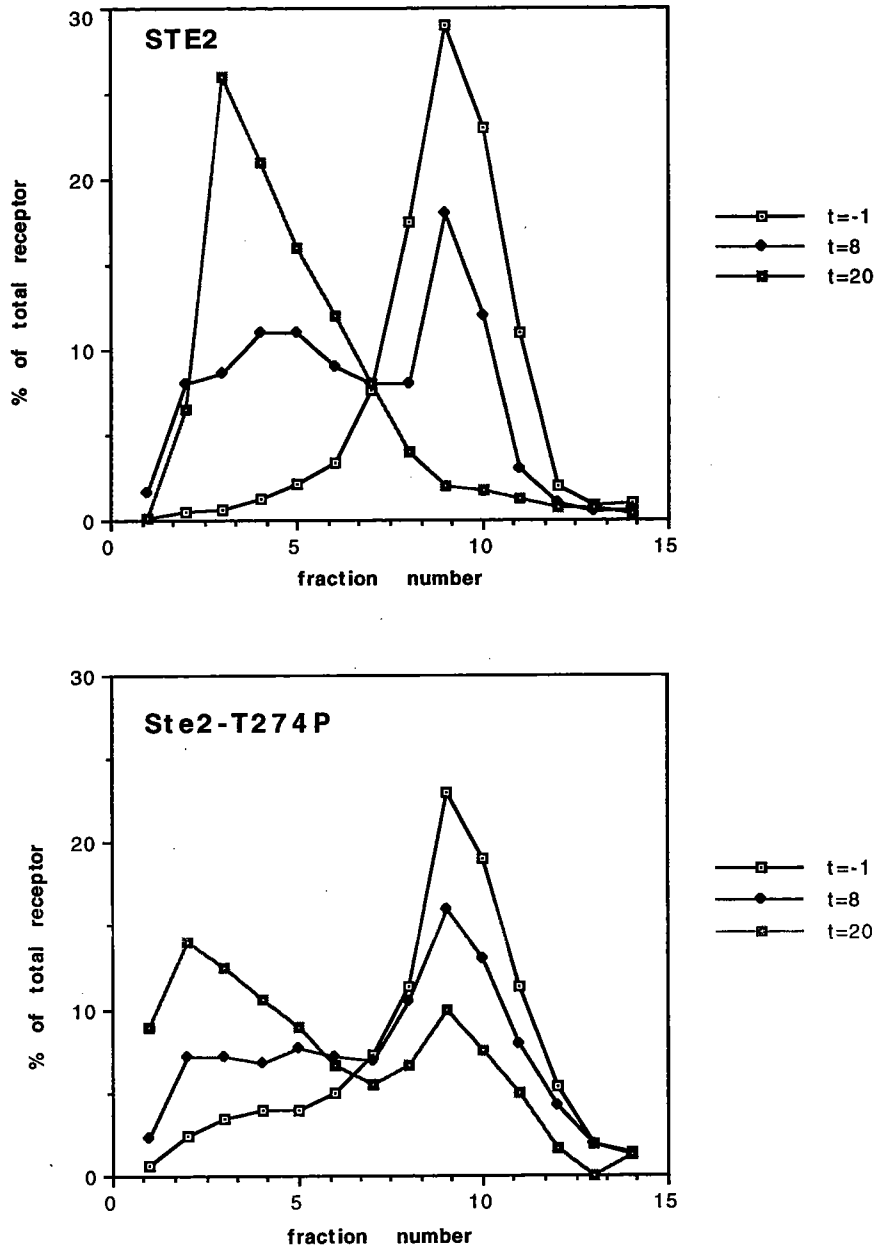
Figure 8. Endocytosis of wild-type STE2 and Ste2-T274P receptor.
Renografin gradients of 1 min before, 8 min and 20 min after addition
of α-factor are plotted. 10 nM and 500 nM of agonist was used respectively.

67

ste2Δ/ YCp STE2   ste2Δ/ YCp Ste2-T274P   ste2Δ/ YCp Ste2-L236H
     (clear)   (t urbid)   (t urbid)
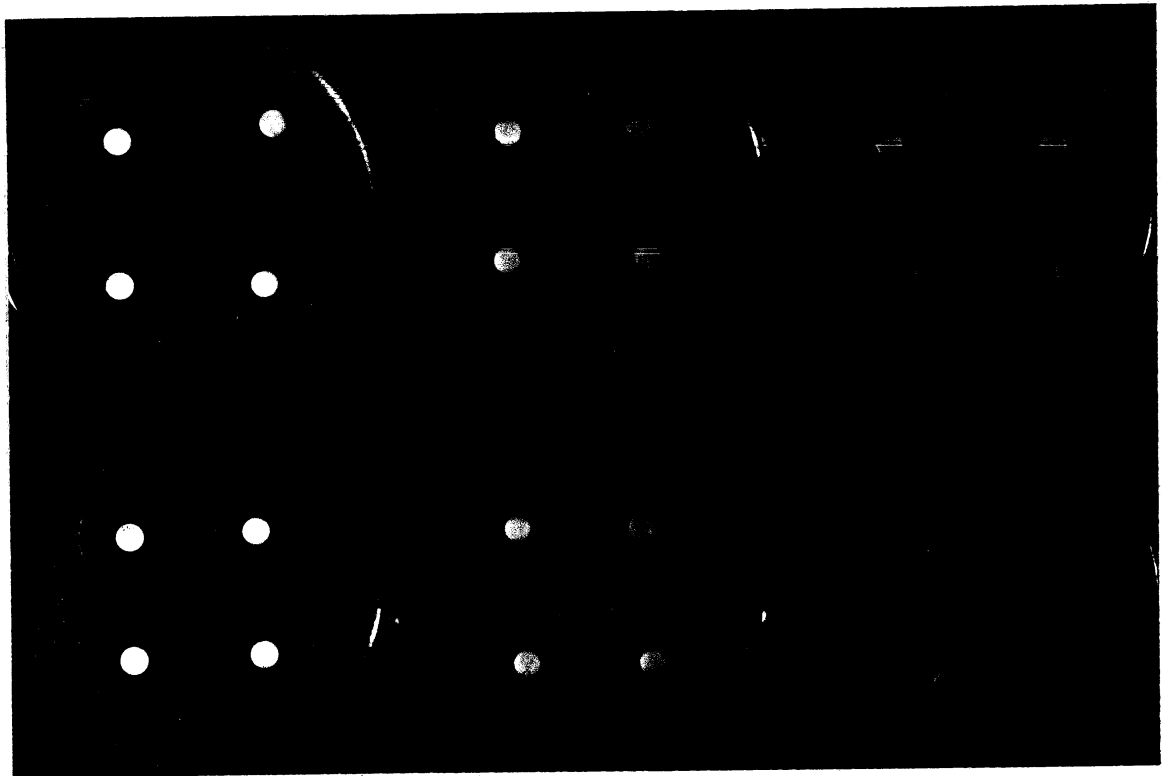
STE2 / YCp STE2   STE2 / YCp Ste2-T274   STE2 / YCp Ste2-L236H
     (clear)   (semi-clear)   (clear)

Figure 9A. Dominance assay. Halo assays were done with cells containing either a chromosomal copy (*STE2*) or a deletion (*ste2Δ*) of the wild-type receptor expressing different *STE2* alleles from a single-copy plasmid. The clarity of the halos are indicated. Strains used are 211-5-3 (*STE2*) and 213-7-3 (ste2Δ) transformed with pJBK008 (*STE2*), pDJ253 (*ste2-L236H*) and pDJ282 (*ste2-T274P*). Filter disks contain 50, 25, 12.5 or 6.25 μM of α-factor.
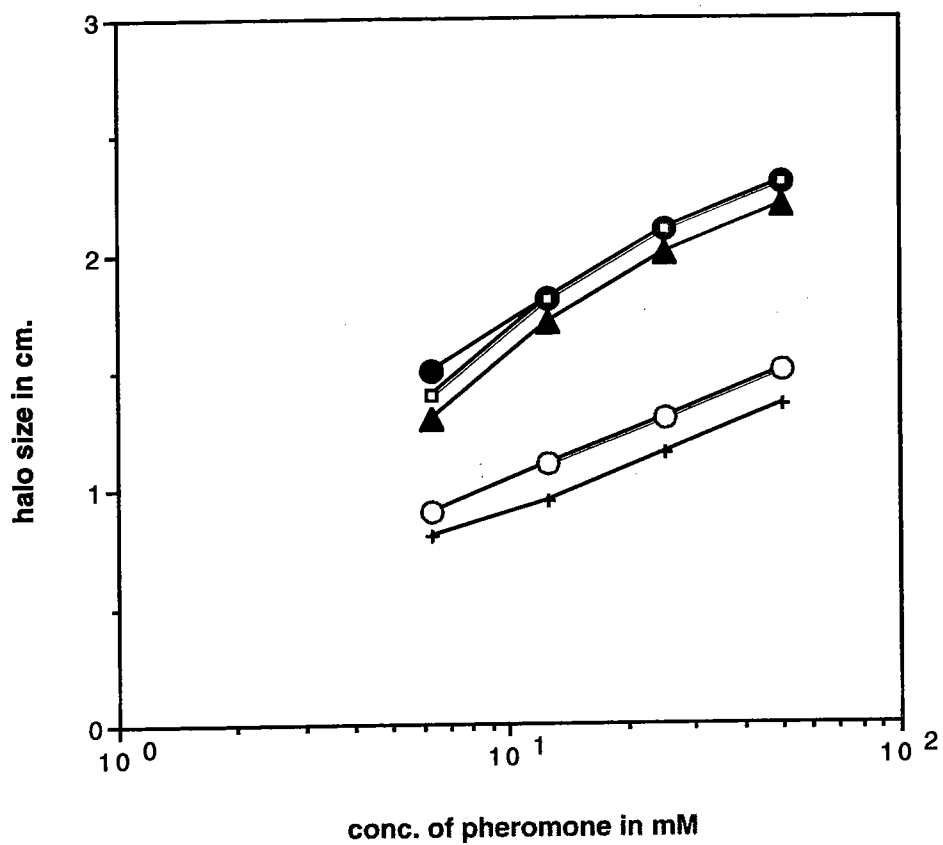
In the plot above, each sign represents the following: closed circles STE$^+$/STE2$^+$, closed squares ste2$^-$/STE2$^+$, open squares STE$^+$/ste2L236H, closed triangles STE$^+$/ste2-T274P, open circles ste2$^-$/ste2-L236H and pluses ste2$^-$/ste2-T274P.

long-term response to pheromone) indicates either reduced signalling ability and/or increased recovery rate of the mutant receptor. The same plasmid containing the *ste2-T274P*, introduced into a *STE2+* strain resulted in turbid halos that were larger than the *ste2-T274P* mutant plasmid in the *ste2* deletion strain but not as large as the the halos produced by wild-type cells. This intermediate phenotype indicates that the *ste2-T274P* is semi-dominant. In comparison, *ste2-L236H* mutation was shown to be recessive since plasmids containing this allele resulted in wild-type halos when introduced into a *STE2+* strain. This phenotype could be due to receptor oligomerization (between wild-type and mutant receptors) or to the sequestration of cellular components such as G-proteins by the weak-signalling *ste2-T274P* mutant receptors.

The halo assays were also repeated with high-copy plasmids containing the mutant and the wild-type receptors by A. Yesilaltay. When the wild-type *STE2+* was contained on a multi-copy plasmid, the halo size was the same as that obtained with a single-copy of *STE2+* (Konopka et al. 1988), (Figure 9b). Interestingly, the multi-copy plasmid containing the *ste2-T274P* resulted in halos that were larger than those observed for the single-copy plasmid that contained the *ste2-T274P* but was still turbid and smaller than wild-type *STE2* on a single-copy plasmid (Figure 9B). Increasing the number of the wild-type receptors did not enhance signalling (presumably the G-proteins are limiting), whereas overexpressing the mutant receptors led to bigger (but still turbid) halos (Figure 9B). This is the expected result of a receptor with low-affinity binding since increase in the receptor numbers of such a receptor would result in a stronger signal (by increasing the number of receptors bound to ligand). It is also possible that the *ste2-T274P* mutation leads to a decrease in the absolute number of receptors such that even in the cells that are overexpressing the *ste2-T274P*, the mutant receptor concentration in the cell is limiting.
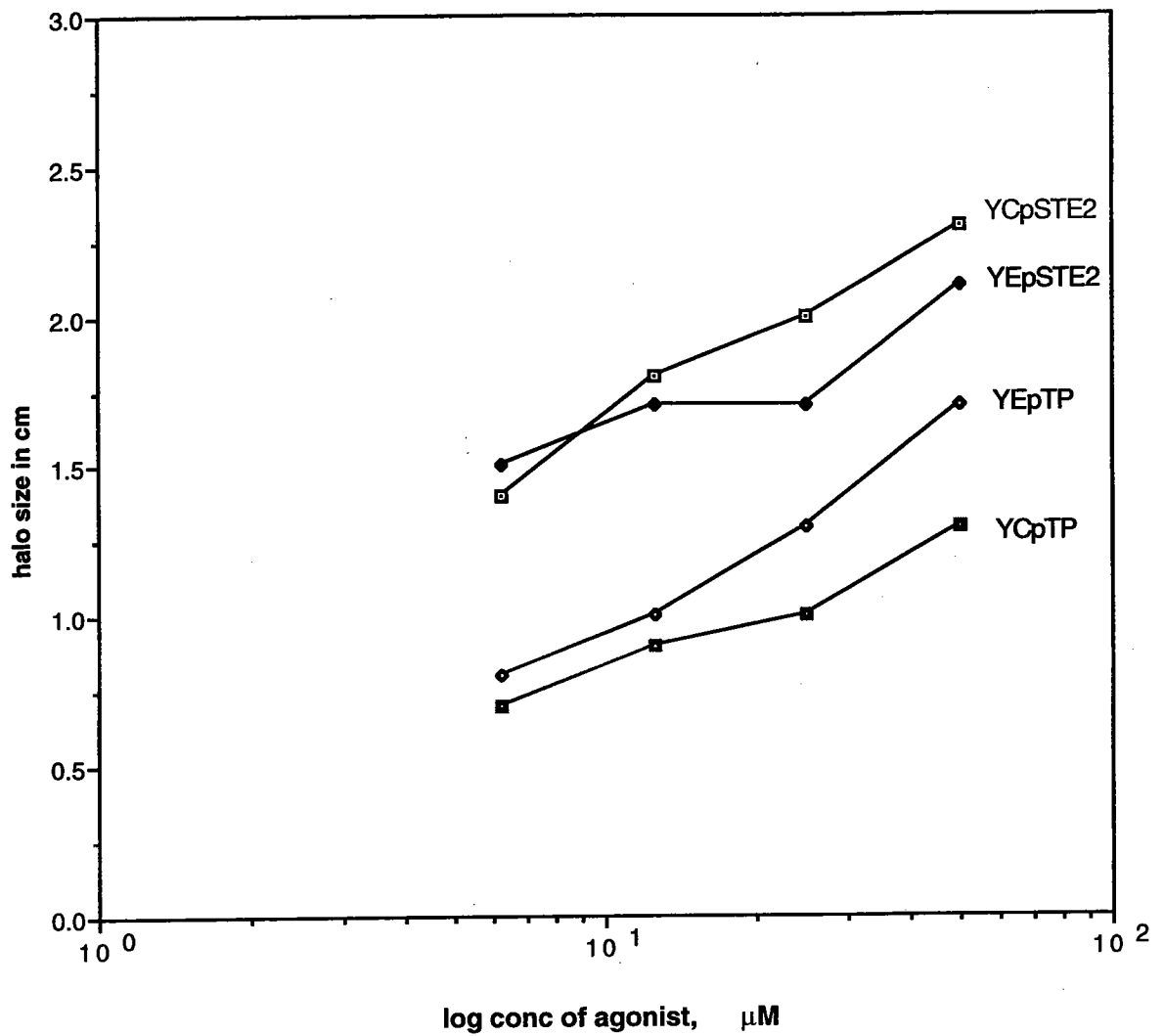
Figure 9B. Halo assay plots of cells lacking chromosomal receptor but expressing the wild-type receptor or the Ste2-T274Pp from either a single copy (YCp) or a multicopy (YEp) plasmid.

Discussion

In order to dissect regions of the receptor that are involved in allosteric transition of the yeast pheromone receptor, I sought a receptor mutant that was defective for this transition. Such a receptor mutant would be expected to have a lower affinity for α-factor and decreased signalling capacity. A single mutation was identified that fulfilled the above criteria; it resulted in the substitution of a Thr to a Pro at residue 274. When a receptor mutant exhibits a decrease in the affinity for the agonist as does the *ste2-T274P*, it is difficult to discern whether this is due to an alteration in the ligand binding site or in allosteric transition (Figure 10). The difficulty in differentiating between these two phenomena is due to the fact that the isomerization constant cannot be measured directly for GPCRs. The issue can be addressed, however, by comparing the apparent binding affinity, and the basal and induced levels of signalling for the wild-type and the mutant receptors. I will discuss the results in terms of those that substantiate the 274th residue's role in either binding the ligand or mediating allosteric change.

The *ste2-T274P* mutation results in a decrease in the affinity of the receptor for α-factor, and an increase in the affinity of the receptor for the antagonist desTrp1,Ala3-α-factor. This is the expected phenotype of a receptor that is locked in a low-affinity form. As discussed in Chapter I, the desTrp1,Ala3-α-factor is thought to be an inverse agonist since a related antagonist (desTrp1,Ala3,Nle12-α-factor) has a negative influence on G-protein-coupling (Blumer and Thorner 1990) and since it induces a hyper-constrained conformation of the receptor (Bukusoglu and Jenness 1996). Inverse agonists show preferential binding to the low-affinity form of receptors (Bond et al. 1995); computer
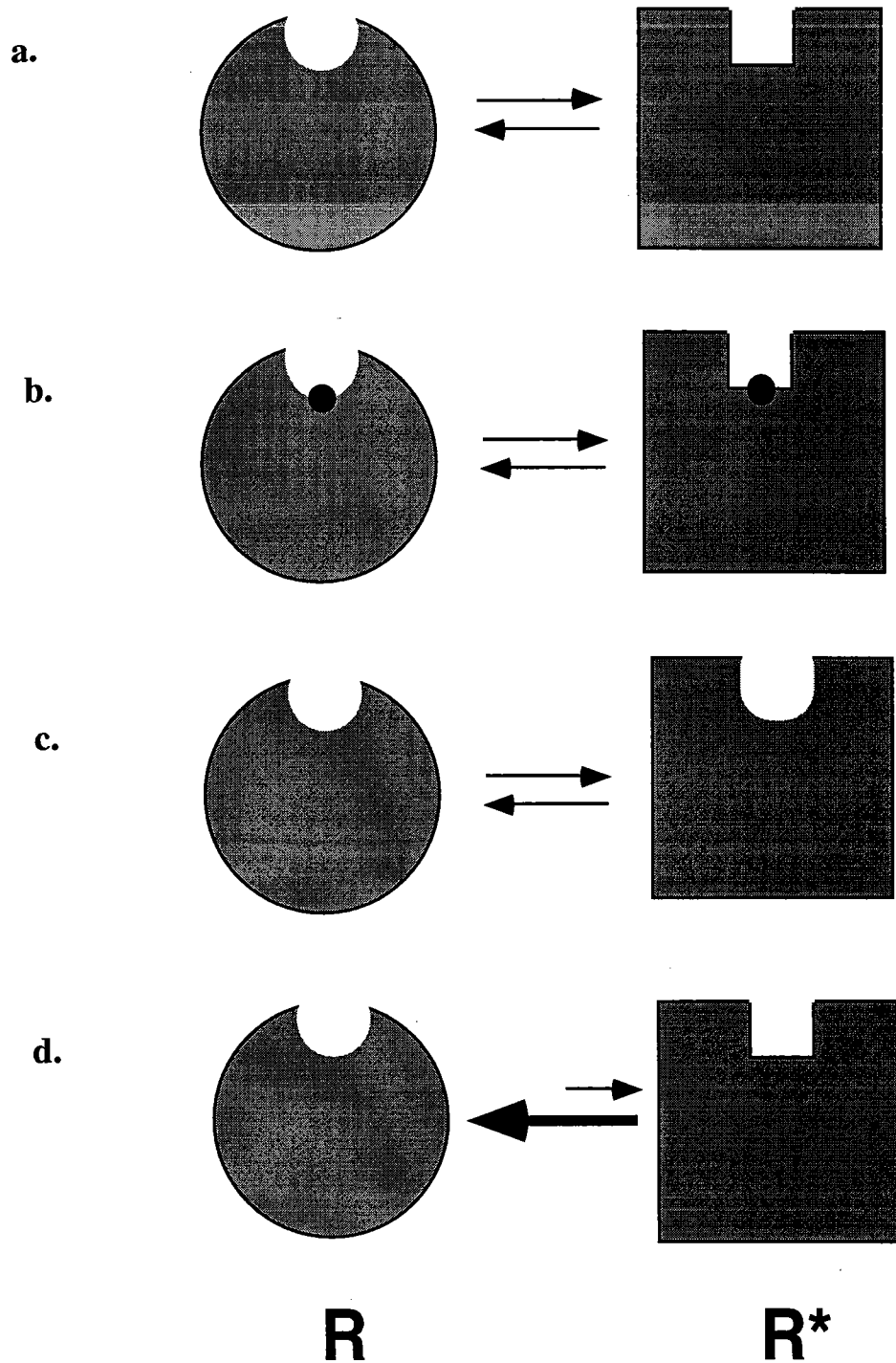
a.

b.

c.

d.

R   R*

Figure10. Allosteric transition model showing: a. equilibrium of the 2
states of the wild-type receptor, b. receptors with a simple-binding site
mutation, c. a mutation affecting the affinity of one state of a receptor ,
d. a mutation altering the equilibrium in favor of the inactive (R) form.

73

aided simulations of the β2-adrenergic receptors predicted that an increase in the isomerization constant (R/R*) would result in an increase in the affinity for negative antagonists and decrease in the affinity for agonists (Samama et al. 1994).

In addition to the K_d defect, the *ste2-T274P* mutation reduces the ability of the receptor to undergo the agonist-induced conformational change. Nevertheless, the mutant receptors are able to undergo the antagonist-induced change. These results are consistent with the idea that Thr274 is involved in the agonist-induced activation of the receptor and that its substitution leads to a receptor that is locked in a low-affinity conformation.

The transcriptional induction assay showed that the mutant receptor can signal up to wild-type levels although its signalling ability was impaired 33-fold. The decrease in the signalling ability is more than could be accounted for by the decrease in the K_d which suggests that the *ste2-T274P* mutation affects allosteric transition. Even if the change in K_d and the change in signalling ability of the mutant receptor were exactly the same, this would still not be inconsistent with the *ste2-T274P* mutation changing the allosteric equilibrium according to the theoretical calculations of the two-state model.

Initially, it seems surprising that a mutant that is defective for allosteric transition can achieve maximal signalling levels when more agonist is added. Upon analysis of the data, it can be seen that although the K_d of the wild-type STE2p is about 3-8 nM, the EC$_{50}$ is determined to be 0.3 nM from the transcriptional induction assay; this suggests that at most 10% of the receptors need to be occupied to result in 50% maximal signalling. Unless the *ste2-T274P* mutation leads to less than 10% receptor occupancy by the agonist, the mutant receptor would be expected to achieve wild-type levels of signalling in the

74

transcriptional induction assay. The halo-assay results also suggest that, even at very high α-factor concentration the mutant receptor responds differently than the wild-type receptor; halos with mutant receptor are smaller and turbid, suggesting that either the signal generated by the mutant receptor is weaker and/or is desensitized quicker. This argues for the idea that the signal generated from the mutant receptor is qualitatively and quantitatively different than wild-type.

Another conclusion of the transcriptional induction assay results is that the mutant receptor must be able to assume the R* conformation to some extent (since it can signal); this is in disagreement with the trypsin assay. One explanation for this discrepancy could be the limitation of the trypsin assay which can detect only the change regarding the third loop related to activation. It is highly likely that there are other changes associated with the R* form, e.g., the exposure of the second intracellular loop and the changes associated with the C-terminal tail, which the *ste2-T274P* might not be impaired for. Another argument could be the difference between the *in vivo* and the *in vitro* assay; it could be that the allosteric equilibrium (R*/R) is smaller *in vitro* than *in vivo*. In other words, the factor that stabilizes the R* form of the receptor *in vivo* is lacking from the membrane preparations used in the trypsin digestion assay. The presence of a putative protein that stabilizes the R* form could be addressed by looking for suppressors of the *ste2-T274P* that would enable it to mate like wild-type.

A direct way of differentiating between the affect of *ste2-T274P* on the binding site versus the conformational change would be to look at the basal levels of signalling with the mutant receptor. If *ste2-T274P* is indeed a simple binding-site mutant, the basal levels would be expected to be the same as wild-type receptor. If, on the other hand, the mutation changes the equilibrium between the two states of the receptors in favor of the inactive

75

form, it should decrease the basal level of signalling. Unfortunately, this expectation cannot be tested directly since, in yeast cells, post-receptor steps are responsible for majority of the basal signal or "noise" (Hasson et al. 1994). Isolating suppressors of the *ste2-T274P* mutation would help resolve this issue; the effect of the *ste2-T274P* on basal levels could then be determined indirectly by comparing the basal levels of the suppressed and the unsuppressed *ste2-T274P* mutant.

CHAPTER III

SUPPRESSORS OF *ste2-T274P*

It appears that the substitution of proline for threonine at position 274 of the pheromone receptor affects the allosteric transition. In principle, the allosteric transition could be altered by either of two mechanisms: by altering the relative free energy of the R and R* states or by altering the relative affinity of the R and R* states for agonist. Accordingly, the properties of the *ste2-T274P* mutant receptors reflect increased free energy of the R* form or decreased free energy of the R form; alternatively, the ligand binding site may have been altered so as to reduce the relative affinity of agonist for the R* form of the receptor. Any of these outcomes would lead to reduction in the affinity of the receptor for agonist, without reducing its affinity for antagonist.

A suppressor search was initiated to resolve the mechanism by which the *ste2-T274P* blocks conformational change as well as to identify residues on the receptor or other proteins that contribute to the allosteric transition. Suppressors that reverse the trypsin digestion pattern and the K_d phenotype would strengthen the hypothesis that the *ste2-T274P* is locked in the R state. The possibility that other proteins affect conformational change of Ste2p is suggested by the following:

1. It is known that *ste4* deletion alters the K_d of the receptor for α-factor which suggests that at least Gβ affects the conformation of the receptor (Jenness et al. 1987).

2. My preliminary results suggest that SST2p affects the conformation of the receptor (Appendix).

3. Most significantly, in the *in vitro* proteolysis assay, the *ste2-T274P* does not undergo the conformational change but it signals *in vivo* as determined by the β-galactosidase assay. This suggests that a factor present in whole cells stabilizes the *ste2-T274P* receptors in the activated state and that this factor is absent in the membrane preparations used for the trypsin assays.

Materials and Methods

Isolation and characterization of suppressor mutants. Haploid cells of strain 910-1-4 (*MATa 381G ste2 ::LEU2 ade2 ADE6 leu2 ura3 TYR1*) containing the *ste2-T274P* allele on a plasmid (pDJ270; *CEN, URA3*) were mutagenized by ethylmethane sulfonate (Prakash and Sherman, 1973) to about 70% survival. Mutagenized cells from each of the twelve independent pools were cultured overnight to a density of 4×10^6 cells per ml in minimal media lacking uracil (with casamino acids). 10^7 mutagenized **a** cells were challenged to mate with 10^7 α-cells (strain 913-1-4; *MATα 381G ste2 ::LEU2 ADE2 ade6 cyh2 ^{r} leu2 ura3 TYR1*) and collected on a type HA filter, the filter was transferred to a YEPD plate. After a 6 hr incubation at 30 °C, the filters were transferred to selective liquid medium (minimal medium supplemented with lysine, tryptophan, histidine and casamino acids) and allowed to grow overnight at the same temperature. The resulting diploid cells were subcultured in selective liquid medium to enrich further for diploid cells. The population enriched for diploids was induced to sporulate and random spores were spread on YEPD plates with 10 μg/ml cycloheximide plates (with no additional adenine). Mating type of the red, cycloheximide resistant haploid cells (*ade2 ADE6 cyh2 ^{r}*) was determined by using the cross-stamp mating test; fertile spores of the **a** mating type were selected which showed the presence of a suppressor mutation. Forty-three *MATa* isolates were first tested for the presence and for the specificity of extragenic suppressors. Isolates

78

that had been cured of the plasmid (leaving chromosomal *ste2Δ* as the only *STE2* allele) were retested for mating to rule out by-pass suppression. The allele-specificity of each suppressor was tested by introducing the vector (pJBK007), the *STE2* plasmid (pJBK008), the *ste2-L236H* mutant plasmid (pDJ253), the *ste2-T274P* plasmid (pDJ270) and then repeating the cross-stamp mating test. The *ste2-L236H* mutant receptors are proficient for the agonist-induced conformational change (see Chapter I). Of the five extragenic suppressors that reproduced the fertility phenotype, none were found to be specific for the *ste2-T274P*; they allowed both receptor mutants as well as the wild-type *STE2* to signal better as determined by halo assays. Therefore, they were not considered to be relevant to the conformational change of the receptor.

Linkage of the suppressor mutations to the plasmid (i.e., intragenic suppressors) was determined by extracting the plasmid from yeast cells, propagating it in *E.coli* and retransforming the original *MATa ste2Δ* strain (910-1-4). For the forty-two isolates that were found to be fertile, five of the suppressor mutations were found to be linked to the EMS mutagenized plasmid for fertility, indicating that the suppressing mutation was in the receptor itself. These intragenic suppressor mutations were sequenced and then recreated either by subcloning or site-directed mutagenesis both as single mutations and in combination with the initial *ste2-T274P* mutation and were integrated into the chromosome for further analysis.

Cross-stamp mating test. Strains to be tested were patched onto YEPD master plate and cross-stamped with an inoculum of tester strains PT1 (*MATa hom3 ilv1 can1*) and PT2 (*MATα hom3 ilv1 can1*) perpendicular to the patches. The plates were incubated overnight at 30°C and then replica plated onto a minimal plate to select for diploids. Fertile *MATa* strains produced prototrophic diploids when crossed with PT1, fertile *MATα* strains

79

produced prototrophic diploids when crossed with PT2, and sterile strains failed to produce prototrophic diploids with PT1 or PT2.

Integrating the suppressor mutations onto the chromosome. To determine the location of the suppressor mutations in the *STE2* gene, the mutagenized plasmids (pDJ349-352) were purified, propagated in *E.coli* and sequenced by using the double-stranded sequencing method (Sequenase kit from United States Biochemicals). A single mutation was found in each mutant: G to A transition at positions 448, 684, 791 and 884 which lead to glycine to aspartic acid substitution at position 163 for the first and alanine to threonine substitution at positions 229, 265 and 295 for the rest. The first two mutations which were carried on restriction fragments AatII-DraI (for G163D) and AatII-ClaI (for A229T), were subcloned into an integrating vector carrying either a wild-type copy of *STE2* (pDJ251; *URA3*) or the *ste2-T274P* (pDJ283; *URA3*). The other two mutations (A265T and A295T) could not be separated from the T274P mutation due to their proximity to the original mutation; therefore the ClaI-PstI fragment containing both the suppressor mutation and the original T274P mutation were subcloned into pDJ283; in order to create them individually, site-directed mutagenesis was employed. The subcloned plasmids were linearized with PstI in order to direct integration at the *STE2* locus and were tranformed into strain 1378-A-1 (*MATa 381G leu2 ura3 bar1 ste2-T100*); strain 1378-A-1 was created by transforming 211-5-3 with the PstI linearized pDJ276 (pPS3, *tetR, ste2-SspΔ, URA3*, K. Schandel; unpublished work) and selecting FOA^R colonies that were sterile. This strain contains a deletion of the *STE2* coding sequence; therefore it is a non-mater; therefore integrants were selected by picking FOA resistant isolates (indicates a ura⁻ phenotype) that were fertile.

Site-directed mutagenesis. The suppressor mutations *ste2-A265T* and *ste2-A295T* could not be separated from the original *ste2-T274P* mutation by subcloning because of the lack of unique restriction enzyme sites in this region. Therefore, they were recreated in the *STE2* gene by using site-directed mutagenesis; Muta-gene M13 *in vitro* Mutagenesis Kit, version 2, BioRad, Richmond, CA 94804. For this purpose, *STE2* was cloned into the RF form of phage M13mp18 and the resulting recombinant phage was propagated in *E. coli dut, ung* strain (CJ236). Phage particles whose single-stranded DNA contains uracil in thymine positions are produced in this strain. The uracil containing DNA was purified and used as template in the *in vitro* mutagenesis reaction. This was then transformed into a strain with a functional uracil N-glycosylase, thus selecting against the parent strand. Single-stranded DNA from the resulting phage particles were sequenced in the Nucleic Acid Facility. The primers used for the *in vitro* mutagenesis were

5'CGATAATATTCATCCTCACATACAGTTTGAAACC3' and

5'CATCAATGTGGACCACGGCTGCTAATAATG3', respectively (from Genemed Biotechnologies, South San Francisco, CA).

Other assays such as determination of IC_{50}, β-galactosidase assays, trypsin digestion assay and quantitative matings were performed as described in the Materials and Methods section of Chapter III.

Results

In order to identify factors that affect receptor conformation, I sought suppressors of the loss-of-function alleles of *ste2-T274P*. A mutant isolation scheme was designed so as to allow the detection of both extragenic and intragenic suppressors (Figure 1). EMS mutagenesis was employed to randomly mutate the *ste2* deletion strain that carried the

910-1-4 / YCp Ste2-T274P   (mates poorly)

EMS mutagenesis   (improved mating)
mate with 913-1-4
sporulate diploids
plate random spores

perform cross-stamp mating test on haploid segregants

43 fertile MATa isolates

cure strains of plasmid   extract and purify plasmid
transform 910-1-4

42 plasmid-dependent isolates

5 mutants specific for suppression
of ste2-T274P

transform with vector, STE2,
ste2-L236H, ste2-T274

none of the 5 extragenic suppressors
were specific for Ste2-T274P

Figure 1. Isolation scheme for the suppressors of ste2-T274P the mutant receptor.

ste2-T274P allele on a single-copy plasmid. Suppressors that reversed the poor-mating phenotype of the parent strain were selected by challenging separate mutagenized pools of the *MATa* parent cells to mate with *MATα* cells that contained the *ste2Δ*. Diploid cells were selected and then induced to sporulate. Random spores were spread on YEPD plates containing cycloheximide and allowed to germinate. To ensure that the haploids were a result of a cross between the two parent strains, I selected red, cycloheximide resistant colonies (*ade2 ADE6 cyh2ʳ*). These isolates were transferred to plates lacking uracil to select for strains that contained the plasmid. The mating type and fertility of the surviving isolates were determined by using the cross-stamp mating test. Isolates that had been cured of the plasmid were retested for mating to rule out by-pass suppression. The presence and the specificity of the extragenic suppressors was tested first. Of the forty-two fertile isolates, five contained suppressor mutations that were linked to the plasmid, and the rest of the suppressors were chromosomal. None of the extragenic (i.e., chromosomal) suppressor strains were found to be allele-specific as they exhibited enhanced mating (determined by cross-stamp test) and α-factor sensitivity (halo-assays) when transformed with plasmids containing three of the alleles of *STE2* tested (*ste2-T274P, ste2-L236H, STE2*). I conclude that the mutated gene products resulting in suppression must be involved in one or more general aspect of pheromone response such as desensitization or post-receptor events in the pheromone response pathway.

Two criteria were used to confirm the presence of intragenic suppressor mutations. First, no suppression was observed when the mutant was cured of the *ste2-T274P* plasmid and retransformed with a second unmutagenized copy of the same plasmid. Second, when the plasmid was extracted from the mutant and used to transform an unmutagenized *ste2* deletion strain, the resulting transformant exhibited the suppressed phenotype. Suppression was evaluated by testing for the reversal of the *ste2-T274P* mating defect and

for α-factor responsiveness as judged by halo-assays. Five intragenic suppressors were found to fulfill both criteria (pDJ 348 through 352). The position of the suppressor mutations were determined by subcloning and DNA sequencing. Of the five suppressor mutants, two were found to contain the identical mutation, therefore further work was limited to the four suppressor mutations (pDJ 349 through 352). The suppressor mutations were separated from *ste2-T274P* either by subcloning or by using site-directed mutagenesis. Thus, I created plasmids containing each suppressor mutation alone and in combination with the initial *ste2-T274P* mutation. These single and double mutant alleles were introduced into the chromosome at the *STE2* locus in an otherwise normal genetic context for subsequent analysis. These strains are isogenic to strain 211-5-3 except at the *ste2* locus they contain *ste2-G163D-T274P* (1379-A-1), *ste2-G163D* (1380-B-1), *ste2-A229T* (1381-B-1), *ste2-A229T-T274P* (1382-A-1), *ste2-A265T* (1389-B-1), *ste2-A265T-T274P* (1390-A-1), *ste2-A295T* (1391-B-1), *ste2-A295T-T274P* (1392-A-1).

Localization and mapping of suppressors. All four suppressors affected amino acids near the ends of transmembrane helices (Figure 2). They resulted in the substitution of glycine to aspartic acid at residue 163 (*ste2-G163D*), and alanine to threonine substitutions at residues 229 (*ste2-A229T*), 265 (*ste2-A265T*) and 295 (*ste2-A295T*). The *ste2-A265T* mutation is predicted to affect the extracellular end of the sixth transmembrane helix; hence, it potentially affects the binding site. Its functional assignment is therefore subject to the same ambiguity as the *ste2-T274P* with respect to its direct effects on α-factor binding or to its effects on overall conformation. In contrast, the other three mutations affect amino acids at the junction of the transmembrane helices and the intracellular loops; these regions are unlikely to play direct roles in binding of α-factor.

84
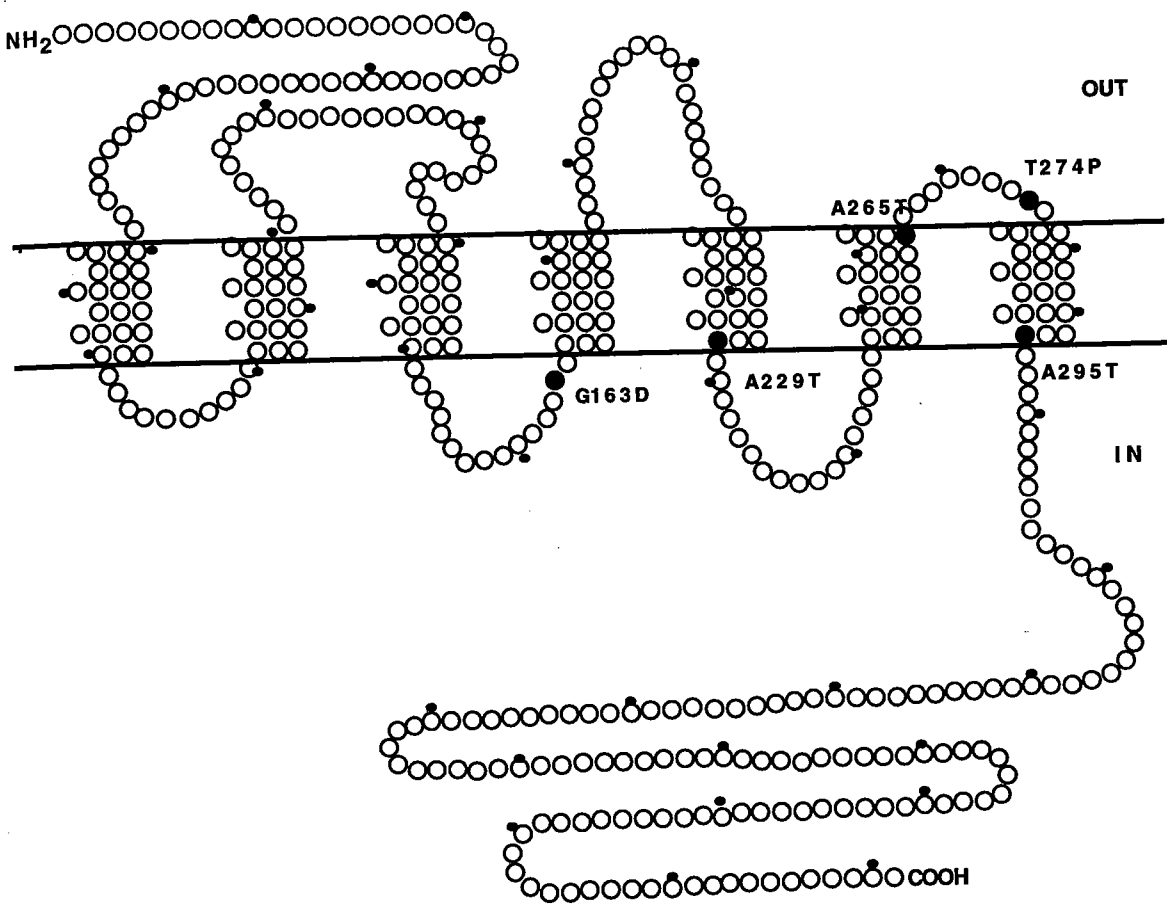
Figure 2. Amino acid substitutions associated with the suppressor mutations in the *ste2* gene.

Quantitative mating assays. The suppressors of *ste2-T274P* were originally identified as causing reversal of the mating defect in a qualitative mating assay (cross-stamp mating assay). To obtain a quantitative assessment of the suppression phenotype, I performed filter mating assays with the suppressor mutants containing *ste2-T274P* and with suppressor mutants in an otherwise *STE2+* genetic background (Table I). Whereas the *ste2-T274P* mutant showed a severe mating defect (5% of the wild-type activity), mating activity was reversed to essentially normal levels by all four suppressor mutations (52-100% of the wild-type activity). Small reductions in mating activity as compared to wild-type were observed upon the introduction of the *ste2-G163D, ste2-A265T* and *ste2-A295T* alleles into the otherwise wild-type background (44-58% of the wild-type activity).

α-factor affinity. The original *ste2-T274P* mutation results in a lower affinity for the agonist compared to the wild-type receptor (IC_{50} of 80 nM as compared to 8nM, Chapter II). In order to determine whether the suppressor mutations correct the α-factor binding defect in the *ste2-T274P* mutant receptors, I examined the α-factor binding properties of the *ste2-T274P* mutant strains containing the various suppressor mutations. To determine whether the suppressor mutations affect the affinity of the *ste2-T274P*, I also examined the binding properties of the suppressor mutations in the absence of the *ste2-T274P* mutation. α-factor affinity was determined by examining the ability of cold α-factor to compete with the radioactive α-factor for binding to the various mutant strains. The IC_{50} value is the concentration of the cold α-factor that inhibits binding of 50% of radioactive α-factor. The assay conditions were such that the receptor concentration and the amount of the radiolabeled-agonist (L) were below K_d. Under these conditions, the IC_{50} approximates the equilibrium dissociation constant K_i, since;

$$K_i = IC_{50} / (1 + L / K_d) \text{ (Siegel et al. 1989)}$$

86

Strain	Quantitative mating (%)	IC50 (nM)
STE2	100	10
Ste2-T274P	5	80
Ste2-A229T	100	15
Ste2-A229T-T274P	100	21
Ste2-A265T	58	15
Ste2-A265T-T274P	54	11
Ste2-A295T	52	15
Ste2-A295T-T274P	150	13
Ste2-G163D	44	15
Ste2-G163D-T274P	66	52

Table I. Results of the quantitative mating and competition binding experiments with cells containing the wild-type receptor, the *ste2-T274P* and the suppressor in combination with the *ste2-T274P* mutation or in an otherwise wild-type genetic background. Quantitative matings are expressed as % of wild-type. Strains used are 211-5-3 (*STE2*), 1379-A-1 (*ste2-G163D,T274P*),1380-B-1 (*ste2-G163D*), 1381-B-1 (*ste2-A229T*), 1382-A-1 (*ste2-A229T,T274P*), 1389-B-1 (*ste2-A265T*), 1390-A-1 (*ste2-A265T,T274P*), 1391-B-1 (*ste2-A295T*), 1392-A-1 (*ste2-A295T,T274P*) and 902-A-1 (*ste2-T274P*).

The results of the binding experiment are tabulated in Table I. If the decreased affinity of the *ste2-T274P* mutant is a consequence of its inability to undergo the allosteric transition, then the suppressor mutations that affect allostery would be predicted to increase the receptor's affinity to agonist. As expected, the three suppressors brought IC$_{50}$ to near wild-type levels, and the *ste2-G163D,T274P* mutant gave an intermediate value of 50 nM.

Levels of receptor protein in the suppressor mutants. An important consideration in the analysis of any mutant protein is the level of protein accumulation. The effect of the suppressor mutations on the expression levels of the receptors was determined by performing Western blotting analysis on crude lysates that had been prepared from the mutant strains. The total Ste2p levels of two of the suppressors (*ste2-A229T* and *ste2-A295T*) were found to be considerably lower than the wild-type levels (Figure 3). This could be due to either increased rates of degradation or decreased rates of synthesis. In contrast, the total Ste2p levels of the *ste2-A265T* mutant and the *ste2-A265T,T274P* double mutant were higher than the *STE2*+ control. This could be due to a feedback mechanism of the mating pathway since pheromone response (and presumably signalling) results in an increased accumulation of *STE2* mRNA (Hartig et al. 1986).

Basal levels of signalling in the suppressor mutants. If the suppressor mutants affect the conformational state of the receptor, they would also be expected to increase the basal level of signalling (i.e., in the absence of agonist). This issue was addressed by determining the expression levels of β-galactosidase in cells containing the *lacZ* gene fused to an α-factor inducible promoter. Strains containing the suppressors, both alone and in combination with *ste2-T274P* were examined. The assay was performed by using cells that had received either no α-factor or 1 μM of α-factor for an hour (Table II). Except for

88

Genotype: STE2   ste2A229T   ste2A229T,TP   ste2G163D   ste2G163D,TP   ste2A265T
μg protein: 5 10   5   10   5   10   5   10   5   10   5   10
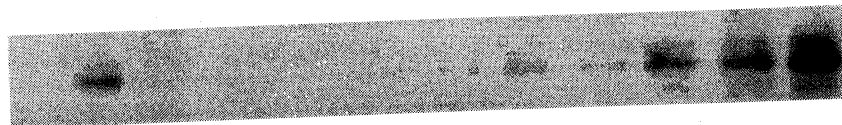
[Ste2p]:   1x   0.2x   0.2x   0.3x   1.3x   3x

Genotype: STE2   ste2A265T,TP   ste2A295T   ste2A295T,TP   ste2T274P
μg protein: 5 10   5   10   5   10   5   10   5   10
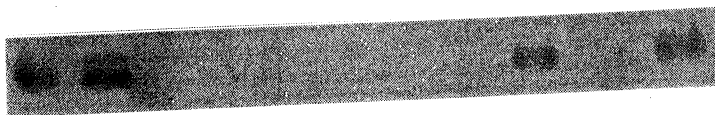
[Ste2p]:   1x   0.25x   0.1x   0.7x   0.75x

Figure 3. Amounts of receptor protein in the *ste2* mutants. Cleared cell lysates were resolved by using anti-C-terminal Ste2p and immunoblotting methods. The relevant genotypes and the total amount analyzed are indicated. The relative concentration of receptor protein in the cell was estimated from densitometric analysis of the immunoblot and normalized to wild-type value. Strains used are 211-5-3 (*STE2*), 1379-A-1 (*ste2-G163D,T274P*),1380-B-1 (*ste2-G163D*), 1381-B-1 (*ste2-A229T*), 1382-A-1 (*ste2-A229T,T274P*), 1389-B-1 (*ste2-A265T*), 1390-A-1 (*ste2-A265T,T274P*), 1391-B-1 (*ste2-A295T*), 1392-A-1 (*ste2-A295T,T274P*) and 902-A-1 (*ste2-T274P*).

strain	no agonist		1 μM α–factor	
	mean±SD	median	mean±SD	median
STE2	31±7	30	246±34	246
Ste2-T274P	25±5	22	194±43	162
Ste2-A229T-T274P	35±4	35	188±10	185
Ste2-A265T-T274P	42±20	34	217±28	217
Ste2-A295T-T274P	52±33	35	253	253
Ste2-G163D-T274P	31±12	29	222±84	182
Ste2-A229T	51±4	50	237±71	286
Ste2-A265T	43±1	44	259±163	243
Ste2-A295T	45±12	41	216±44	238
Ste2-G163D	18	18	176	176

The significance of the differences between the basal levels for the different strains were determined by the paired student t-test:

STE2 and *ste2-A229T*	p=0.015, different at the 0.05 level
STE2 and *ste2-A265T*	p=0.03, different at the 0.05 level
ste2-T274P and *ste2-A229T,T274P*	p=0.018, different at the 0.05 level
ste2-T274P and *ste2-A229T*	p=0.00157, different
ste2-A229T and *ste2-A229T,T274P*	p=0.015

Table II. Results of β-galactosidase assays with and without agonist (basal). Mean±SD for each strain is given. Induced levels of the suppressors in the T274P background were not significantly different from those in the wild-type background except for the Ste2-A229T-T274Pp. Strains used are 211-5-3 (*STE2*), 1379-A-1 (*ste2-G163D,T274P*),1380-B-1 (*ste2-G163D*), 1381-B-1 (*ste2-A229T*), 1382-A-1 (*ste2-A229T,T274P*), 1389-B-1 (*ste2-A265T*), 1390-A-1 (*ste2-A265T,T274P*), 1391-B-1 (*ste2-A295T*), 1392-A-1 (*ste2-A295T,T274P*) and 902-A-1 (*ste2-T274P*).

the *ste2-G163D* mutant, the suppressor mutants had significantly higher basal levels than the wild-type control strain (as determined by the paired student t-test). In the suppressed *ste2-T274P* mutants, the basal levels were not significantly different than the wild-type *STE2*. This is consistent with the idea that the *ste2-T274P* mutation leads to constraints in the receptor that favors the inactive state (low-affinity), and that these suppressor mutations reverse this constraint and result in a receptor that is activated more easily. The *ste2-G163D* mutant had low basal activity both in the absence and the presence of the *ste2-T274P* mutation.

The maximal levels of signalling (i.e., in the presence of α-factor) for the *ste2-A265T* and *-A295T* mutants, both alone and in combination with *ste2-T274P*, were comparable to the values obtained with wild-type cells. In contrast, the *ste2-G163D*, the *ste2-G163D,T274P* and the *ste2-A229T,T274P* mutants showed lower signalling capacity compared to the wild-type control. A decreased signalling capacity could either be due to the direct negative effect of the mutations on signalling or due to the negative effect that these mutations have on the accumulation of receptor protein.

Arrest of cell division. α-factor causes **a** cells to arrest division in G1. Since the suppressor mutations resulted in increased basal transcription of α-factor inducible genes, they may also lead to slower progress of the cell division cycle. To address this issue, I introduced the plasmid containing the strongest suppressor mutation *ste2-A229T* (pDJ355) into a strain which contained a temperature-sensitive allele of *STE5* {strain 751-11-2 (*MATa ste5-3 ste2-10::LEU2*)}. The *STE5* gene product is required for a post-receptor step in the pheromone response pathway; hence it should block cell-cycle arrest when the receptor is constitutively activated (Figure 4). Plates containing the transformants that had been cultured at 34°C were replica-plated; the replicas were incubated at 22°C and at 34°C.

Activated Receptor

↓

Gαβγ

↓

STE20

↓

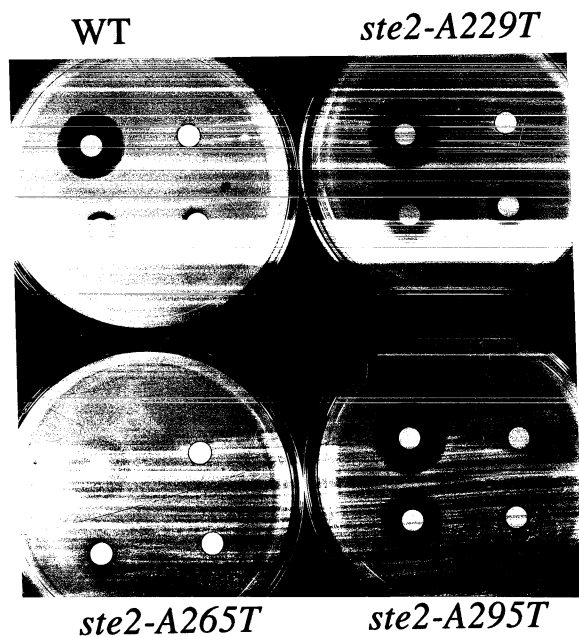| S T E 5 | STE11
STE7
FUS3 KSS1 |

↓
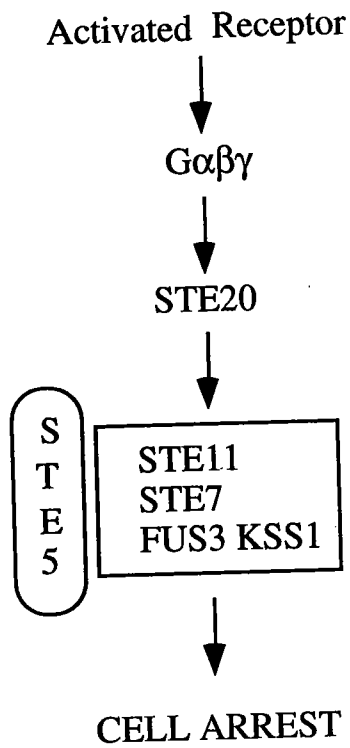
CELL ARREST

Figure 4. The constitutivity assay. Mutations leading to constitutively active receptors will grow at 34°C but not at 22°C; at the restrictive temperature there will not be any stable *STE5* gene product that could signal cell arrest.
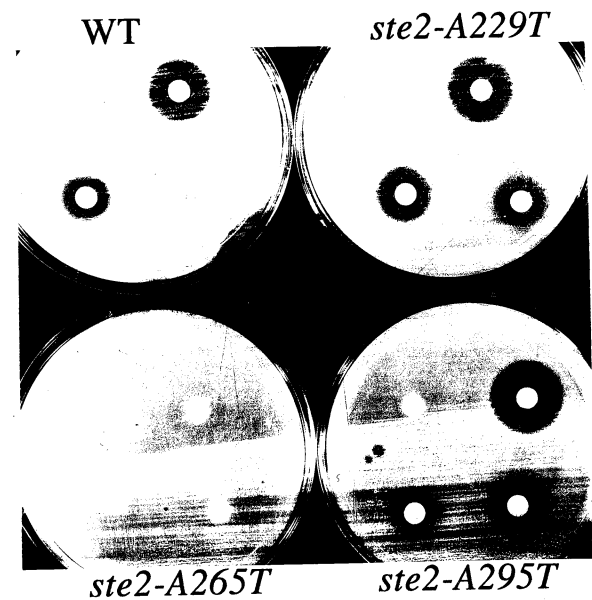
If the mutant receptors influence the cell cycle, then the cells would show growth defects only at the restrictive temperature due to continuous signalling. At both temperatures suppressor mutants grew at comparable rates to the wild-type control, indicating there was no strong influence of the mutant receptor on cell-cycle. These results suggest that the increase in basal activity observed with the suppressor mutations is not strong enough to provide a sustained block for cell-cycle progression.

Responsiveness to partial agonists. Increased basal signalling suggests that the receptors containing the suppressor mutations are in a more readily activatable state than the wild-type receptor. In this case, it is reasonable to expect that they would respond better to partial agonists. This possibility was tested by performing halo assays with the suppressor mutants *ste2-A229T, ste2-A265T* and *ste2-A295T* strains 1381-B-1, 1389-B-1 and 1391-B-1 respectively. The partial agonists tested were the α-factor from the related yeast *S.kluyveri* and the AN-37 and AN-44 analogs of α-factor prepared by Cadus Pharmaceuticals. AN-37 contains multiple amino acid substitutions in the N-terminal one-third of the α-factor molecule whereas AN-44 and *S.kluyveri* α-factor contain substitutions in the middle one-third of the α-factor (Table III). AN-37 and AN-44 bind wild-type receptors with K_d values of $3x10^{-7}$ and $2x10^{-5}$ M respectively (M. Cui and D. D. Jenness, unpublished). Both were diluted in dimethyl formamide (DMF); DMF alone does not result in halos as shown in Figure 5. The concentration used was $5x10^{-7}$M for AN-37, $5x10^{-5}$ M for AN-44, and $2x10^{-4}$ M for *S.kluyveri* α-factor. As seen in Figure 5, both the *ste2-A229T* and *ste2-A295T* mutants produced larger halos with kluyveri α-factor and AN-44 as well as with α-factor than the wild-type receptor; the *ste2-A229T* mutant was also found to produce larger halos with AN-37. The *ste2-A265T* mutant produced turbid halos with every agonist tested after 48 hours of incubation. Since *ste2-A265T* mutant was similar to the *ste2-A295T* mutant as tested by quantitative mating and

93

WT   ste2-A229T

α-factor   DMF

AN-44   AN-37

ste2-A265T   ste2-A295T

WT   ste2-A229T

antagonist   α-factor

ant+α-factor   kα-factor

ste2-A265T   ste2-A295T

Figure 5. Halo assay of the suppressors in the wild-type background to partial agonists. The order of the ligands is indicated on the left side of the culture plates. kα-factor is kluyveri α-factor. AN-37 and AN-44 were diluted in dimethyl formamide (DMF); DMF alone does not result in halos as shown in figure.

agonist	sequence of agonist	halo diameter (in cm)		
		STE2$^+$	ste2-A229T	ste2-A295T
α-factor (1.1x)	WHWLQLKPGQPMY	1.8	2 (1.1x)	2.1
desTrp1Ala3	-HALQLKPGQPMY	the antagonist results in no haloes		
AN-37	CRGPQLKPGQPMY	0.8	1.25 (1.56x)	0.8 (1x)
AN-44 (1.65x)	WHWLSLGGRQPMY	1	1.65 (1.65x)	1.65
α-kluyveri (1.76x)	WHWLSFSKGEPMY	0.85	1.4 (1.64x)	1.5

Table III. Responsiveness of the suppressor mutations to partial agonists was analyzed by halo assays conducted using the agonist, antagonist and the partial agonists of the yeast pheromone receptor. The actual halo sizes are given, the fold induction with the receptor mutations compared to the wild-type receptor are indicated in parenthesis. AN-37 and AN-44 were diluted in dimethyl formamide (DMF); DMF alone does not result in halos.

95

by β-galactosidase assay, this qualitative difference in halo size is likely to be a consequence of an increased role of adaptation for the *ste2-A265T*. As was the case with the wild-type, neither mutant was found to signal with the antagonist DesTrp1,Ala3-α-factor.

Trypsin digestion assay. As shown previously (Chapter II), the *ste2-T274P* mutant receptors fail to undergo detectable agonist-mediated conformational change as determined by the *in vitro* proteolysis assay (also Chapter III, compare panels A and B of Figure 6). The expectation therefore was that the *ste2-T274P* mutation in combination with the suppressor mutation would lead to an α-factor-inducible conformational change similar to that of the wild-type. Since the suppressor mutations result in an increased basal activity as determined by the activity of the α-factor-inducible reporter gene, it was also anticipated that they would influence the allosteric equilibrium. If the suppressor mutations resulted in an increase of the R* state of the receptor, in the absence of agonist, they would be expected to have cleavage patterns that resemble the wild-type in the presence of α-factor. This issue can be addressed by comparing the cleavage patterns of the membrane preparations containing either wild-type receptor, the *ste2-T274P* mutation, the suppressor mutations, and the suppressor mutations in combination with *ste2-T274P*.

The difficulty with comparing digestion patterns from different membrane preparations is that the overall rate of trypsin digestion is influenced by other proteins that compete for the enzyme activity. The quantity and the quality of these competinG-proteins vary among the different membrane preparations. Including an internal control would standardize these variations among the different membrane fractions. Trypsin digestion results in two major cleavages of the receptor; cleavage #1 results in the formation of receptor fragment F2, and cleavage #2 results in the formation of receptor fragment F3.

A.

B.

C.

D.

| agonist | - | + | | - | + | | - | + | | - | + | | - | + | | - | + |
| time | 0 | | 1 | | 5 | | 10 | | 30 | | 100 | |

97
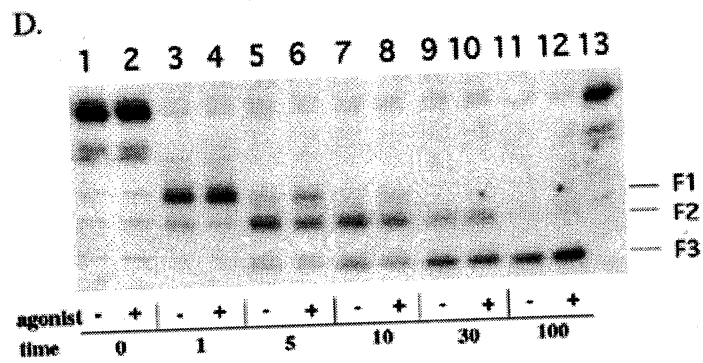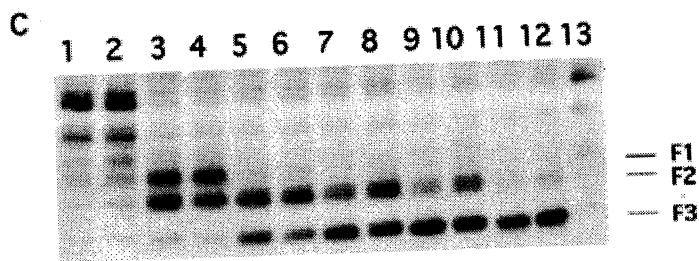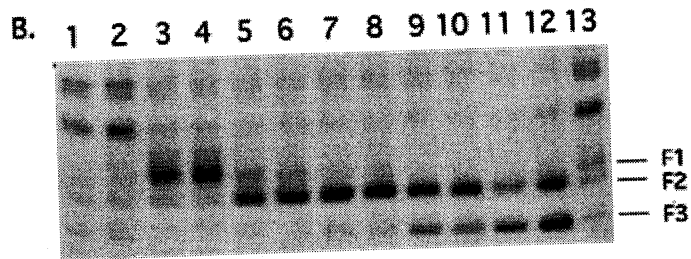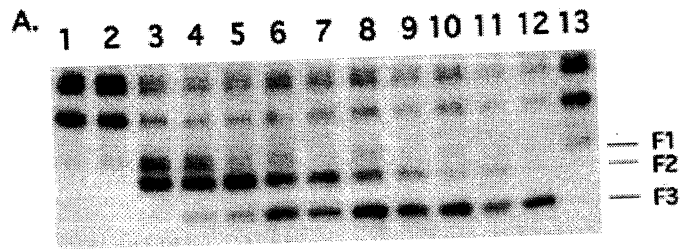
Figure 6. Membranes prepared from cells with the different receptor alleles; A.wild-type (211-5-3), B. ste2-T274P (902-A-1), C. ste2-A229T,T274P (1382-A-1), D. ste2-A229T (1381-B-1), were incubated with trypsin for the indicated number of minutes in the presence or absence of agonist. The resulting cleavage products were analyzed by SDS-PAGE and immunoblotting methods. The antiserum was against the N terminus of the receptor. The control reaction was incubated for 100 minutes in the absence of trypsin and agonist (lane 13). The major cleavage products are indicated on the right.

Ratio of the cleavage rates at position #1 and position #2 provides a diagnostic indicator for the conformational state of the receptor. Although the cleavage pertinent to activation is cleavage #2, including the rate of cleavage #1 provides an internal control for each membrane preparation.

The trypsin assays were performed as described in Chapter I. Crude membranes were prepared from wild-type cells as well as from cells containing the *ste2-T274P* mutation, the suppressor mutations, and the suppressor mutations in combination with *ste2-T274P*. Membranes were incubated with trypsin with and without agonist (even and odd numbered lanes, respectively) and at various time points, cleavage products were detected by using Western blot analysis. The relative rates of cleavage was inferred by estimating the time at which 50% of F1 is converted into F2 (for cleavage #1) and the time at which 50% of F2 is converted into F3 (for cleavage #2); the ratio of these two time points reflects the relative cleavage rates. As summarized in Table IV, cleavage #1 occurs by 1 minute for wild-type receptor both in the absence and the presence of α-factor. Cleavage #2 in the absence of α-factor is by 10 minutes, and addition of α-factor decreases this period to 5 minutes. The ratio of cleavage #2 to cleavage #1 was found to be 10 in the absence and 5 in the presence of agonist for the wild-type, whereas the ratio of the *ste2-T274P* was found to be 30 in the absence and 50 in the presence of α-factor. Hence, as concluded in Chapter II, the defects in the *ste2-T274P* mutant receptor interfere with the ability of agonist to enhance the relative rate of cleavage at position #2. According to this criterion, the ste2-T274P mutant receptor is more in the inactive conformation than the unoccupied wild-type receptors.

Figure 6, panel C shows the cleavage pattern obtained with the suppressed mutant *ste2-A229T,T274P*. With this mutant, 50% of F2 was converted to F3 by 5 minutes (panel C, lane 5) in the absence of α-factor, and this process was slowed to 10 minutes in the presence of agonist (panel C, lane 8). The relative cleavage rates for

strain	Cleavage 1[a]		Cleavage 2[b]		ratio of 2 to 1[c]	
	-α-f	+α-f	-α-f	+α-f	-α-f	+α-f
STE2						
	1	1	10	5	10	5
	3	3	30	7	10	2.5
	3	3	30	7	10	2.5
	10	20	100	30	10	1.5
Ste2-T274P	1	1	30	65	30	65
	1	1	30	65	30	65
	3	3	65	65	22	22
ste2-A229T,T274P	1	1	7	10	7	10
ste2-A265T,T274P	5	5	10	20	2	4
ste2-A295T,T274P	7	7	10	20	1.5	3
ste2-G163D,T274P	7	7	20	30	3	4
ste2-A229T	2	5	20	20	10	4
ste2-A265T	3	3	10	10	3	3
ste2-A295T	5	5	20	20	4	4
ste2-G163D	3	3	10	5	3	1.7

Table IV. Tabulated results of trypsin digestion assays with membranes containing wild-type receptor (STE2), and the receptors containing the ste2-T274P mutation, the suppressor mutations with the ste2-T274P and the same mutations in an otherwise wild-type background. The digestion pattern was analysed by Western blotting methods at 0,1,5,10,30 and 100 minutes, except for the ste2-A229T which was analyzed at 2 min.s in addition to the above.

[a] Cleavage 1 is the time in minutes when amounts of F1 versus F2 were approximately equal.

[b] Cleavage 2 is the time when amounts of F2 versus F3 were approximately equal (1,5,10,30 and 100 min). When equal amounts of the two intermediates occured between two time points, the mean value was assigned (i.e., time interval between 1 to 5 min was 3, between 5 to 10 min was 7, between 10 to 30 min was 20, and 30 to 100 min was assigned a value of 65).

c Last column is the ratios of Cleavage 2 to 1 in the absence and the presence of α-factor respectively.

Strains used are 211-5-3 (*STE2*), 1379-A-1 (*ste2-G163D,T274P*),1380-B-1 (*ste2-G163D*), 1381-B-1 (*ste2-A229T*), 1382-A-1 (*ste2-A229T,T274P*), 1389-B-1 (*ste2-A265T*), 1390-A-1 (*ste2-A265T,T274P*), 1391-B-1 (*ste2-A295T*), 1392-A-1 (*ste2-A295T,T274P*) and 902-A-1 (*ste2-T274P*).

ste2-A229T,T274P mutant both in the absence and presence of agonist differed from what was observed for *ste2-T274P* mutant alone, and more closely resembled the pattern observed for wild-type receptors in the presence of α-factor. The same results were obtained with the rest of the suppressor mutations in combination with the *ste2-T274P* mutation, Table IV. Figure 6, panel D depicts the digestion kinetics of the *ste2-A229T* mutant; whereas the cleavage that gave rise to F2 was slowed by α-factor, as was observed for wild-type receptors, the cleavage that gives rise to F3 was not enhanced by α-factor upon addition of α-factor. The trypsin digestion assays revealed that the cleavage rates of all four suppressor mutations (both in an otherwise wild-type background and in combination with the *ste2-T274P* mutation) resemble the wild-type rather than the *ste2-T274P* mutation. Of the rest of the suppressor mutations, the *ste2-G163D* displayed α-factor induced exposure of the third loop, whereas the *ste2-A265T* and *ste2-A295T* receptor mutants did not. In conclusion, the presence of the suppressor mutation causes the wild-type and the *ste2-T274P* mutant receptors to assume a conformation that resembles the α-factor-activated state. The finding that the third loop shows a high rate of cleavage both in the presence and absence of α-factor in the suppressed receptor mutants none of which are constitutively active is surprising and it suggests either that the balance between the R and R* states in the *in vitro* assay differs from *in vivo* case or that exposure of the third intracellular loop is not sufficient to cause constitutive activation of the post-receptor signal.

Interaction with the G-proteins. Since most of the suppressor mutations are close to the intracellular loops, they could be enhancing mating by altering interactions with G-proteins. This point was addressed by a genetic test developed by Kim Schandel. This test is based on a synthetic lethal phenotype of the *gpa1-A345T* mutation that was identified as a suppressor of the *ste2-L236H* mutant. The *ste2-L236H* mutation leads to an amino acid

102

change in the third intracellular loop and results in decreased responsiveness to α-factor (Schandel and Jenness 1994). The *gpa1-A345T* mutation was found to reverse the signalling defect of this mutant receptor (Schandel, K.A. and Jenness, D.D., unpublished work). In addition to its ability to suppress the receptor mutation, the *gpa1-A345T* mutation leads to the constitutive cell-cycle arrest at $38^{\circ}C$. This phenotype is consistent with instability of Gpa1p at $38^{\circ}C$ and with a consequent failure to bind the Gβγ subunits. This $38^{\circ}C$ phenotype is exacerbated in a ste2 deletion background. This observation provides a convenient way to test whether the mutant receptors retain contact with the G-protein. If the receptors containing the suppressor mutations had lost the stabilizing interactions with the mutant G-protein, the double mutant would be temperature-sensitive for growth. No synthetic lethal phenotype was detected for the double mutants containing *gpa1-A345T* together with *ste2-T274P*, *ste2-G163D*, *ste2-A229T*, *ste2-A265T* and *ste2-A295T*.

Discussion

In an attempt to identify factors involved in the activation of the pheromone receptor, I used a genetic screen to isolate suppressor mutants that allowed the inactive *ste2-T274P* receptor to become more active. No allele-specific extragenic suppressors were found in this screen. Extragenic suppressors would have potentially identified gene products that interact with the receptor and affect the allosteric transition. However, four intragenic suppressors were found to alleviate (at least partially) the receptor defects caused by the *ste2-T274P* mutation. Interestingly, all four mutations mapped to the ends of the transmembrane helices implying that allosteric transition involves cooperative interactions among transmembrane helices IV, V, VI and VII. This provides further evidence that the *ste2-T274P* mutant receptor is defective for allosteric transition; as opposed to a simple

binding-site defect. It is also interesting that all of the suppressors resulted in the substitution of a polar residue in place of the existing non-polar residue. In light of this fact, one possible mode of action of the suppressor mutations could be to "stretch" the helices out of the membrane. In this respect, it is noteworthy that one of the suppressor mutations (*ste2-A229T*) affect the N-terminus of the third intracellular loop which is exposed to the cytoplasm upon α-factor binding. A variety of evidence implicates the exposure of the third loop to the cytoplasm and to G-protein in the activation of both mammalian and yeast receptors (Bourne 1997; Hogger et al. 1995).

Since the reversal of mating defect was the basis of selection, it was expected that all the suppressed mutants would exhibit increased mating as compared to the unsuppressed *ste2-T274P* mutant in the quantitative mating assay. This expectation was borne out. All four suppressor mutations led to increased mating in the *ste2-T274P* background. Determination of IC50 values of the suppressor mutants for α-factor in either the *ste2-T274P* or otherwise wild-type genetic background led to the conclusion that three of the four mutant receptors have close to a wild-type K_d value, whereas the *ste2-T274P* receptors exhibit a value that is significantly greater than the wild-type. The K_d of the *ste2-G163D,T274P* mutant gave an intermediate value, whereas the *ste2-G163D* single mutant had wild-type K_d. In support of the idea that the *ste2-T274P* mutant is not a simple binding-site mutant, the residues affected by three of the suppressors were not readily accessible to the extracellular ligand.

One of the expected results was that the suppressor mutations would lead to a reversal of the conformational defect of the *ste2-T274P* mutant receptor, as detected by the limited-trypsin digestion assay. I suspected that the receptors from the suppressor mutants would be in a more activated state than the wild-type receptors even in the absence of

agonist. According to previous findings (Wess 1997), this activated state is thought to be a less-constrained state, in which the conformation of the intracellular loops would allow productive coupling to the G-proteins. Analysis of my data revealed that the relative cleavage rates in the C-terminal tail and the third intracellular loop were more disparate in the *ste2-T274P* receptor than in the wild-type receptor. In the absence of α-factor, the suppressor mutants exhibited an increase in the cleavage rate of the third intracellular loop compared to the cleavage in the C-terminal tail. However, upon α-factor addition, accumulation of fragment F3 that resulted from this cleavage was not enhanced significantly compared to wild-type receptors. Results of the trypsin assay suggest that the effect of the suppressors is to relax the overall receptor structure, making the third intracellular loop more accessible to trypsin. In the mammalian system Gether et al. (1997) and Samama et al. (1997) both showed that the unoccupied receptors with constitutive activity were more susceptible to proteases as assessed by a loss of binding sites after incubation at 37°C; addition of ligand (agonist or antagonist) increased stability, presumably by constraining the receptor. Although in the absence of agonist, the digestion pattern of the receptors from the suppressor mutants resembles the digestion pattern of wild-type receptors in the presence of agonist, the suppressor mutations do not result in strong constitutivity, in that they do not lead to constitutive cell-cycle arrest. This suggests that the exposure of the third loop which can be detected by the trypsin assay is not the only essential criterion for activation or that receptors in the *in vitro* assay are not as constrained as they are *in vivo*.

The activating effect of the suppressor mutations on the overall receptor structure was corroborated by measuring the level of activity from an α-factor inducible reporter gene. One difficulty in comparing the relative levels of signalling among the different receptor mutants is that the observed levels of signalling (both constitutive and inducible)

may be limited due to decreases in the number of the mutant receptors. Nevertheless, it was observed that the suppressor mutations by themselves lead to a higher basal activity than the double mutants containing the same suppressor mutation together with the *ste2-T274P*. Since the increased basal activity did not in general correlate with increased receptor number, it appeared that *ste2-T274P* constrains the receptor in a less active conformation. The increase in basal activity seen with the receptors with suppressor mutations was used to differentiate between the two possible models underlying the inactivation of the *ste2-T274P* mutant receptor. The *ste2-T274P* mutation could result in a change either in the allosteric equilibrium (the ratio of R to R*) or in the relative affinity of the two states for the agonist. The reversal of inactivation could also be a consequence of either mechanism. Hence there are four possibilities for the inactivation and suppression of the mutant receptors. These four models make different predictions for the basal level of β-galactosidase activity in the *ste2-T274P*, suppressor and the double mutant (Figures 7A and 7B).

Model 1. If the *ste2-T274P* changes the relative affinity of either state for the agonist, the basal levels will not be affected by this mutation since basal signalling does not involve the addition of agonist. If the suppressors reverse this defect, the basal levels of the *ste2-T274P*, suppressor in the *ste2-T274P* background, and suppressor by itself would all be the same (Figure 7A).

Model 2. If the *ste2-T274P* altered the relative affinity, and the suppressors affected the equilibrium of the two states, then the suppressors both alone and with the *ste2-T274P* mutation would have higher basal levels than the *ste2-T274P* (Figure 7A).

pheromone

weak

tight

Wild-type receptors

weak
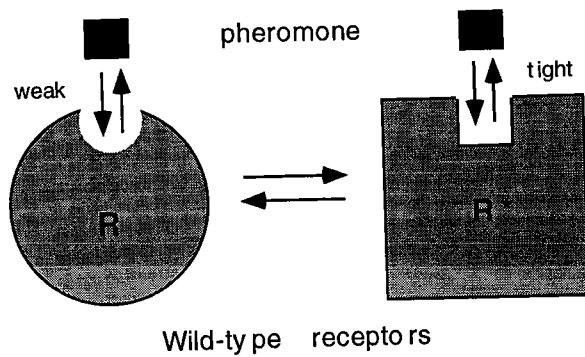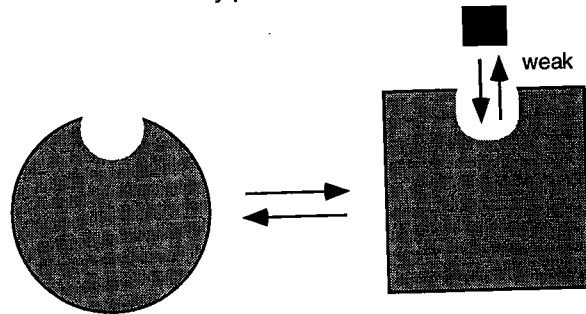
I. If Ste2-T274P affects the binding site of the R* form

tight
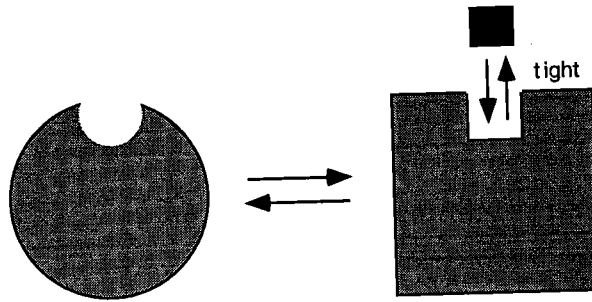
Model 1. Suppressor changes back the binding site

weak
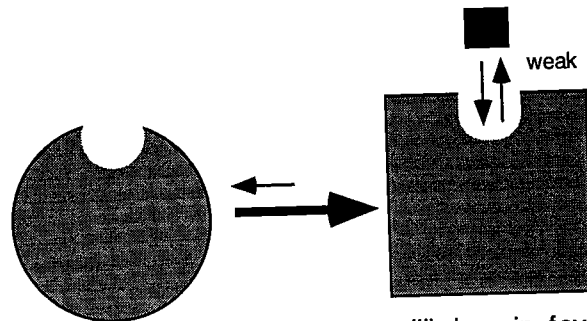
Model 2. Suppressor alters the equilibrium in favor of the R* form

Figure 7A. Affect of mechanism of suppression on basal levels of signalling when the *ste2-T274P* alters the binding site.
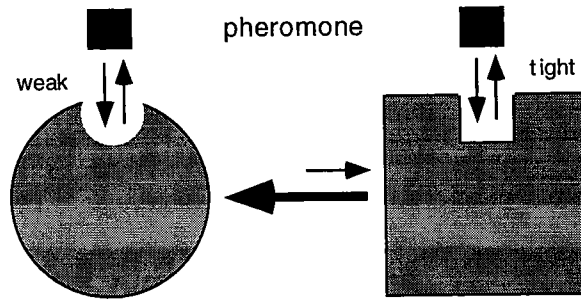
II. If Ste2-T274P alters the equilibrium between R and R*
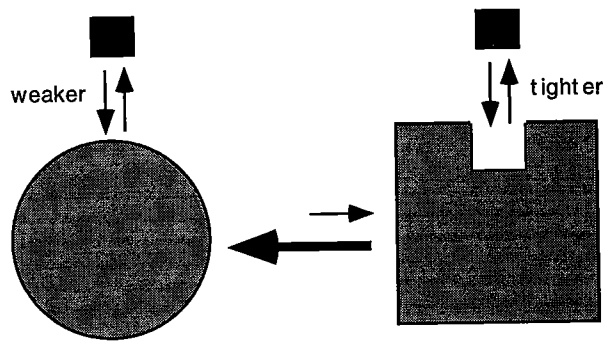
Model 3. Suppressor alters the binding site
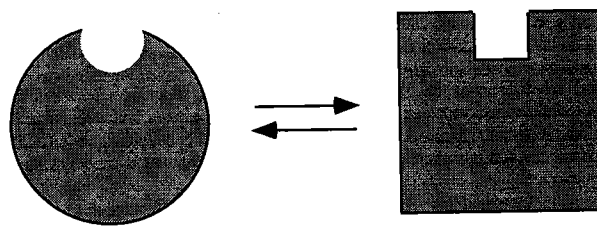
Model 4. Suppressor changes back the equilibrium

Figure 7B. Affect of the mechanism of suppression on basal levels of signalling when the *ste2-T274P* alters the allosteric equilibrium.

Model 3. If the *ste2-T274P* affected the equilibrium, and if the suppressors altered the relative affinity; the basal levels with the *ste2-T274P* would be expected to be lower than wild-type. However, experimentally, the basal level will be essentially unaltered because the basal signal originating from the receptor is small compared to the level of signal that arises from post-receptor signals. In this scenario, the presence or absence of the suppressor mutation is not predicted to have any effect on the basal signalling levels (Figure 7B).

Model 4. If the *ste2-T274P* mutation and the suppressor mutations have opposing influences on the allosteric equilibrium (Figure 7B). In this case, the *ste2-T274P* mutant is predicted to signal less than wild-type, and the suppressor in the *ste2-T274P* background is predicted to show an intermediate level of signalling, and the suppressor mutation is predicted to signal more than wild-type. The basal level of suppressor in the *ste2-T274P* background would depend on the strength of the suppression. If the suppressor mutation reverses the effects of the *ste2-T274P* mutation back to wild-type levels, then the basal level would be the same as *ste2-T274P* by itself. If the suppressor alters the equilibrium such that there was more than wild-type levels of R*, than the basal signalling would be higher. Regardless, the suppressor alone would have an increased level of signalling relative to the suppressor in the *ste2-T274P* background in this model.

The observations of the transcriptional induction assay are the following:

1. Suppressors in an otherwise wild-type background have high basal levels which rules out Models 1 and 3.

2. Suppressors with the *ste2-T274P* mutation have a lower basal level than suppressors alone which rules out Model 2.

109

Therefore the experimental results are consistent only with Model 4. The basal levels of β-galactosidase activity in the suppressed ste2-T274P mutant was significantly greater than either wild-type or the ste2-T274P single mutant. This suggests that the ste2-T274P mutation leads to an alteration of the allosteric equilibrium and the suppressors have the opposite effect. A further increase in the basal levels is expected to occur if the receptors containing the suppressor mutations are over-expressed since the accumulation of receptor protein in the suppressor mutants was lower than that observed for wild-type receptors. Examples of constitutively-active β2-adrenergic receptors have been described in which the receptor protein is unstable and the addition of either agonist or antagonist increases the levels of accumulation (Gether et al. 1997; Samama et al. 1997). Considerably higher basal levels were observed with the constitutively active mutant ste2-P258L when it was over-expressed; this mutant on a single copy plasmid had protein levels comparable to wild-type, but the Bmax was 4 times less than wild-type (Konopka et al. 1996) implying that a minority of the receptors were at the cell surface. Birnbaumer and colleagues have shown that a 6-fold decrease in Type 2 vasopressin receptors causes a 60-fold increase in the EC_{50} as well as a decrease in the maximal stimulation (Birnbaumer et al. 1994). Decreased levels of receptor protein accumulation could also be the reason for the lower level of signalling (compared to wild-type) that was observed when the ste2-A229T,T274P mutant was treated with α-factor. It is also possible that this mutation leads to the induction of a desensitization mechanism, resulting in lower maximal signalling.

I suspect that the suppressor mutations do not have an all-or-none effect on the allosteric transition but instead decrease the difference in the free energy between the R and the R* states since these suppressors do not lead to strong constitutivity. Generally, GPCR activation is not thought to alter the receptor structure in a major way, but is thought

to affect intracellular loops as a result of subtle changes in the transmembrane helices (Wess 1997). Consistent with this idea, the positions of the amino acids affected by the suppressors implicate the involvement of the second and third intracellular loop as well as the carboxy-terminal tail in receptor activation. The two strongest suppressor mutations *ste2-A229T* and *ste2-A295T* affect residues that are conserved in distantly related pheromone receptors (*S.cerevisiae, S.kluyveri* and *S. pombe.*) The *ste2-A229V* mutation which was identified by Marsh and colleagues was found to result in increased responsiveness to antagonist, *S.cerevisiae* α-factor and the *S.kluyveri* α-factor (Marsh 1992). This provides additional evidence for involvement of this residue in conformational change.

DISCUSSION

Saccharomyces cerevisiae was used as a model system for understanding the mechanism by which G-protein-coupled receptors are activated. The yeast pheromone response pathway consists of a G-protein-coupled pheromone receptor and a mating-specific MAP kinase cascade (Leberer et al. 1997). Although yeast containing multiple MAP kinase cascades (Herskowitz 1995), there is a single pheromone receptor and it shares the general molecular architecture with the mammalian receptors. Given the ease with which laboratory manipulations and advanced genetic analysis are performed with yeast cells, the presence of a yeast GPCR provides an ideal system for studying the mechanism of receptor activation. Moreover, yeast provides a "cleaner" system as there is no interference from multiple G-proteins as there is in the mammalian cells.

The structural similarity among the members of this superfamily of receptors that bind a diverse range of ligands suggests that the different receptors share mechanistic features for their activation. Although extensive computer modelling has been performed with the transmembrane regions of several GPCRs, lack of structural information makes it difficult to evaluate the accuracy of these models. Most contributions to the activation mechanism of GPCRs comes from work with rhodopsin that has spectrally-distinct R and R* forms. This property makes it useful for analysis of mutations that activate or inactivate it, as well as for biophysical dissection of the activation process. As mentioned in the introduction, the chromophore 11-*cis*-retinal associates with the apoprotein by forming a Schiff base with residue Lys296 in transmembrane 7, and this association stabilizes the R state of the receptor. The Glu113 in transmembrane 3 provides the counterion for the Schiff base. The activation of the receptor by a photon results in the isomerization of the

11-*cis* to 11-*trans* retinal and the disruption of the salt-bridge between TM3 and TM7 (Rao and D. 1996). Transition to the R* state of rhodopsin also requires the protonation of the D142 at the cytoplasmic end of TM3. This residue is a part of a conserved motif (DRY) found in most GPCRs (DRY however is absent in the STE2p). Computer simulations and site-directed mutagenesis of the α_{1B}-adrenergic receptor, were used to suggest that the interaction among polar residues on the cytoplasmic portions of the receptor contribute to the stabilization of the R state. The disruption of these interactions involving Asn63, Asp91, Asn344 and Tyr348 (TM 1, 1, 2, and 7 respectively), is thought to cause the R143 residue to exit this "polar pocket" and to become more exposed to cytoplasmic environment (Scheer et al. 1996). Apart from the DRY motif, the presence of a hydrophobic residue in the second intracellular loop was found to be important for the coupling of G-proteins to adrenergic and muscarinic receptors (Moro et al. 1993). Time-resolved spin-label studies have indicated the involvement of the second intracellular loop of rhodopsin in activation as well (Farahbakhsh et al. 1995).

The importance of the third intracellular loop of the receptor in G-protein coupling and activation has been underscored by mutations that affect this region of the receptor and result in a constitutively-active phenotype. The third intracellular loop is thought to have an α-helical structure, and this secondary structure as well as the sequence identity were both found to influence G-protein activation (Franke et al. 1992; Burstein et al. 1996). Apart from examining the individual role of each intracellular loop, the cooperative effects of the loops 2, 3 and 4 (formed between TM7 and and the site of palmitoylation in some GPCRs) in the activation of G-proteins have also been investigated. One study pertaining to the role of the intracellular loops employed the use of synthetic peptides corresponding to these regions in competition binding studies with rhodopsin and G_t (Konig et al. 1989). It was found that two peptides showed synergy in competing for binding of G_t with activated

113

rhodopsin; this effect was thought to be due to the stabilizing effects of the peptides on the R* form of rhodopsin.

Analysis of two-dimensional rhodopsin crystals have allowed the calculation of a three-dimensional map for the transmembrane helices, including the tilt angles (Unger et al. 1997). This work concludes that helices 2 and 3 are positioned such that they separate helices 6 and 7 and helix 4. The transmembrane helices are positioned such that the extracellular surface is more open (allowing ligand binding) and the cytoplasmic portion is more closely packed. This model is consistent with the current ideas for receptor activation which suggest that the cytoplasmic pocket opens up to create new sites for contact with the G-protein.

The activation mechanism of the yeast pheromone receptor STE2p is not understood as well as the mammalian GPCRs. Work by Sen and Marsh (1994) indicates that the ligand-binding site is likely to involve contacts in multiple transmembrane domains since mutations within multiple transmembrane segments alter ligand binding specificity. Separate studies from Clark et al. (1994), Schandel and Jenness (1994) and Stefan and Blumer (1994) confirm the importance of the third intracellular loop in signalling for Ste2p as established for mammalian receptors. Systematic alanine substitution of the intracellular third loop (Clark et al. 1994) was used to identify determinants of signalling; it was concluded that the general aspects of the loop structure are more important than specific residues. The importance of the transmembrane domains in receptor activation was shown by two groups. Konopka et al. (1996) showed that the Pro258 on TM6, one of the few conserved residues between the yeast receptor and mammalian systems, is important for maintenance of the inactive state since the *ste2-P258L* mutant shows constitutive activation. Unlike the constitutively activating mutations of the mammalian receptors which are dominant, this mutation was found to be partially dominant. In order to identify sites important for receptor activation, Sommers and Dumont (1997) took the approach of

114

targeting a particular transmembrane region for mutagenesis. This work identified residues in the third transmembrane helix that result in loss-of-function *ste2* alleles; the affected residues were I142N, E143K and T144P. The second site suppressors of these *ste2* alleles affected the first (R58G), fifth (M218T), and the sixth (Y266C) helices suggesting either direct physical interaction between those residues or a more indirect or global stabilization of the receptor. The same work in addition showed that substitution of the only acidic residue on TM3 does not affect function. This is unlike some mammalian receptors where the acidic residue acts as the counter-ion for the ligand. Thus, this finding rules out the argument that the basic residues on α-factor (histidine and lysine) require interaction with this residue.

In order to study the activation mechanism of the yeast α-factor pheromone receptor, I started out by developing an *in vitro* assay that was sensitive to ligand-induced changes of the receptor. Using limited trypsin digestion, I showed that binding of the agonist results in the enhanced cleavage of the third intracellular loop. The same ligand-induced changes in the digestion pattern were also obtained with membrane preparations from strains lacking G-proteins; this suggests that the exposure of the third loop upon binding of agonist is an inherent property of the receptor and not an indirect consequence of G-protein dissociation. 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. This finding correlates with work from mammalian GPCRs which implicate an important role for the third intracellular loop in receptor activation (Hogger et al. 1995; Bourne 1997) as well as work in yeast. The agonist specificity of the third intracellular cleavage was tested by performing trypsin

digestion experiments in the presence of the antagonist, DesTrp1, Ala3-α-factor. The antagonist was found not to accelerate the cleavage of the third loop; in fact, its presence resulted in the deceleration of all cleavages. This suggests that binding of the antagonist promotes a distinct form of the receptor that is different than both the unliganded and agonist-bound form of the receptor.

In Chapter II, I describe the isolation and the characterization of a non-signalling receptor mutant, *ste2-T274P*. I took the approach of randomly mutagenizing the *STE2* gene in order to prevent bias toward a particular residue or a region of receptor. The non-signalling receptor mutant exhibited a low-affinity for α-factor; upon addition of saturating amount of the agonist, it was found to be endocytosed and to signal like wild-type receptor and yet *in vitro*, it failed to undergo the agonist-induced conformational change at saturating levels of α-factor. These results points to an apparent discrepancy between the *in vivo* (endocytosis and signalling) and *in vitro* (conformational change) assays. One possible explanation is that the change detected by the trypsin digestion assay that is pertinent to activation is the only one to be impaired in the *ste2-T274P* and that other changes that occur upon the addition of agonist are sufficient for the endocytosis and weak signalling functions to be observed. Another possible explanation is that a factor that stabilizes the active form of the mutant receptor *in vivo* is lacking under the conditions of the trypsin assay. In other words, the allosteric parameters are less stringent under the conditions of the *in vitro* assay. The fact that the mutation results in reduced affinity for agonist and an increased affinity for the antagonist are consistent with the *ste2-T274P* mutation affecting both the conformational change and the binding site. The observation that the effect of the mutation on dose-response is more extreme than the effect on binding affinity are inconsistent with a simple alteration of the ligand-binding site.

116

Two types of structural defects could account for the failure of a mutant receptor to undergo an allosteric transition: The defect could result in increased free energy for the R* form or decreased energy for the R form, alternatively, it could result in an alteration of the ligand binding site that reduces the relative affinity of agonist for the R* form of the receptor. One way to differentiate between the two possibilities is to examine the Effect of the receptor mutation on the basal levels of signalling. A mutation that changes the allosteric equilibrium in favor of the inactive form of the receptor would be expected to decrease basal activity. This can not be tested directly in yeast because post-receptor signalling leads to the majority of the basal signal; deletion of the *STE2* gene results in even a higher basal level. An indirect way of testing the Effect of the *ste2-T274P* mutation on basal levels was to examine the effect of *ste2-T274P* on a receptor mutant that exhibits higher levels of basal signal.

Suppressors of the *ste2-T274P* were sought in order to identify other residues involved in the allosteric transition as well as to define the specific defect in the *ste2-T274P* mutant receptor. The second site suppressors of *ste2-T274P* as well as the initial *ste2-T274P* mutation itself have a commonality in that they affect residues at the end of transmembrane helices; these regions are thought to be involved in receptor activation (Luo et al. 1994). The second site suppressor mutations result in the substitution of non-polar to polar residues and affect the fourth, fifth, sixth and the seventh transmembrane helices; these TM helices are connected to i2, i3 and the carboxy-terminal of the receptor. Therefore, the positions of the primary defects in these mutants underscores the involvement of the second and third intracellular loops as well as the carboxy-terminal tail in receptor activation. This is consistent with the notion that multiple intracellular receptor domains are involved in receptor activation and G-protein coupling (Shi et al. 1995). My

findings are also consistent with the computer-generated simulations of the tryptamine receptors which suggest that agonist binding causes a rotation of helix 7 that is coupled with the rotation of helix 6, with lateral transition of helix 4 and ultimately with the exposure of the the third intracellular loop (Luo et al. 1994; Zhang and Tipper 1993).

The suppressor mutations were further characterized by determining mating ability and agonist affinity. As expected, all four of the suppressor mutations reversed the mating defect and reduced α-factor affinity of the *ste2-T274P* mutant. The transcriptional induction assay revealed that at least two of the suppressors increased the basal level of signalling. Moreover, the suppressor mutant enhanced responsiveness to certain α-factor analogs as assessed by halo-assays. Both of these findings are consistent with the idea that the suppressor mutations cause the receptor to be poised towards the more activated state (R*). The analysis of the trypsin digestion data are also consistent with this idea since the relative cleavage rate of the third intracellular loop was found to be increased in the unoccupied receptors in the suppressor mutants, resembling the activated state. Activated forms of receptors are thought to assume a more relaxed state which may result in enhanced protease cleavage (Scheer and S. 1997; Gether et al. 1997; Samama et al. 1997). A surprising finding is that the addition of α-factor does not lead to a further enhancement of the accessibility of the third intracellular loop. This is surprising since the addition of α-factor results in increased signalling that is comparable to wild-type. One possibility is the difference between the *in vivo* and *in vitro* conditions; it could be that *in vivo* the third loop is more exposed upon the addition of agonist. Second possibility is that further exposure of the third intracellular loop is not a sufficient criterion for signalling; changes in other parts of the receptor are capable of additional signalling observed in the presence of agonist. Data that provides support for this notion comes from Kim et al. (1997) where electron paramagnetic resonance (EPR) spectroscopy was used to determine the domains of

118

rhodopsin that move upon photoactivation. Comparison of the EPR spectra of the photoactivated wild-type and the constitutively-active mutant in the absence of agonist detected similar motion at TM 3 and 7, whereas only the wild-type rhodopsin showed movement at TM6 upon photoactivation. This suggests that different domains can move independently and argues against concerted changes in receptor. Another point of interest is, although the third loop is exposed in the suppressed receptor mutants even before the addition of α-factor, this alone is not sufficient for strong constitutive signalling although the suppressor mutations result in increased basal levels. Since some of the suppressor mutations decrease the protein levels of the receptor, it could be that if protein levels were identical among the receptor mutants, they would exhibit higher basal activity.

Second site suppressors are often associated with global stabilization of the protein. If this were the case with the suppressor mutants analyzed in this work, they would be expected to alleviate the defects of other receptor mutants that harbor structural defects. But, since the *ste2-T274P* binds the antagonist, it is unlikely to have an unstable structure. Aside from the global stabilization, the second site suppressors identified in this work are thought to stabilize the R* state of the receptor. The effect of the suppressors on the stabilization of the R* form of the receptor can be tested with other loss-of function *ste2* alleles such as those identified by Sommers and Dumont (1997).

According to the Ternary Complex Model which is used to study the activation mechanism of GPCRs, there are two forms of the receptor; the inactive form (R) and the active form (R*). The Ternary Complex Model (TCM) is a derivatization of the Monod, Wyman, Changeux's two-state model that was proposed to explain the characteristics of hemoglobin in the inactive and the active form which have distinct crystal structures. This idea of two forms of hemoglobin is being challenged by the finding of three energy states

for the protein (Ackers and Smith 1987). In the GPCR field, recent findings have also led to the suggestion that multiple forms of receptor exist and that each form displays unique characteristics for binding to the G-protein. A mutation in the α_{1B}-adrenergic receptor which results in higher affinity to agonist and a slight increase in basal levels but does not show induction with agonist suggests the presence of more than one R* form (Scheer et al. 1996). Another mutation in the α_{1B}-adrenergic receptor which couples to two different effectors was shown to constitutively activate only the phospholipase C but not the phospholipase A2 pathway (Perez et al. 1996). The idea of different forms of GPCRs is also suggested by work with negative antagonists. Work from Samama et al. (1994) and Barker et al. (1994) indicates that ligands can serve as inverse agonists for signal transduction even though they elicit a conformational change and cause increased endocytosis of the receptor. My findings provide further evidence for the presence of multiple forms of the receptor in that the antagonist, DesTrp1,Ala3-α-factor promotes a highly constrained conformational state *in vitro* that differs from the unoccupied or agonist-occupied state.

The extended TCM model states that only the R* form interacts with the G-protein. This does not appear to be a general feature of GPCRs since examples have been found where receptor-G-protein complexes can be co-immunoprecipitated in the absence of the agonist (Okuma and Reisine 1992). A rhodopsin mutant that can bind G-protein but not activate it argues against this facet of the model as well (Franke et al. 1990). The stabilization of the temperature-sensitive Gpa1p (*gpa1-A345T*) by the *ste2-T274P* mutant receptors (locked in the R state) suggests that the R form of the Ste2p can also interact with G-proteins (K.A. Schandel and D.D. Jenness, unpublished). Recently, the extended model has been modified further and the modified model has been referred to as the "general model"; this model takes into account the interaction between G-proteins and

120

inactive receptors and the presence of more than two states of the receptor (Figure 1; Fong 1996). Nevertheless, these complications underscore the complexity of the mode of activation of the GPCRs and point to the need for combined approaches in this field.

In summary, although the α-factor pheromone receptor does not share sequence homology and lacks most of the conserved motifs of the mammalian GPCRs, its structure-function relationships seem similar to the mammalian GPCRs. As in mammalian systems, exposure of the third loop was found to be an important, although perhaps not the sole determinant of receptor activation. There will undoubtedly be discrepancies with the mammalian systems since certain evolutionary pressures (such as providing selectivity for the specific G-protein) in higher eukaryotes do not apply in yeast.

A. Ternary complex model

$$H + G + R \longleftrightarrow RH + G$$

$$H + GR \longleftrightarrow GRH$$

B. Extended model

$$R \xleftrightarrow{K} RH$$

$$J \qquad\qquad bJ$$

$$R^* \xleftrightarrow{bK} R^* H$$

$$M \qquad\qquad aM$$

$$GR^* \xleftrightarrow{abK} GR^* H$$

C. General model

Agonist binding

R → Receptor activation

G protein binding

RH ⟷ R* H

R ⟷ R*

GRH ⟷ GR* H

GR ⟷ GR*
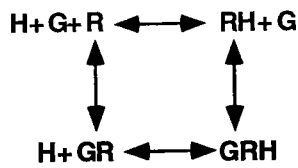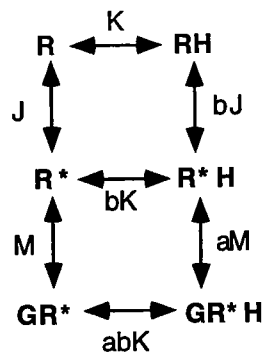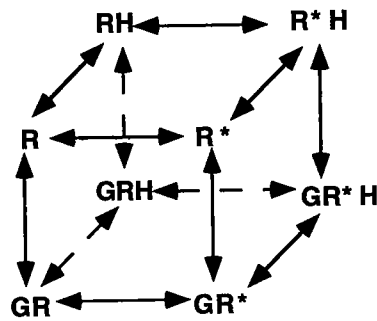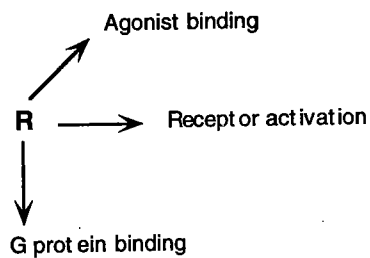
Figure 1. Thermodynamic models of GPCR activation. A and B are the same as Figure 2 in Introduction. C. shows receptor can undergo three processes either separately or simultaneously. This also illustrates that the agonist can bind to the receptor in 4 different states with 4 different affinities. From Cell. Signal. 8, 217-224, 1996, T.M. Fong.

APPENDIX

This section contains a summary of experiments that were started as preliminary work as well as suggestions for future experiments.

I. Experiments with the *SST2Δ* strain

SST2 is a negative regulator of the pheromone response pathway; its deletion leads to enhanced signalling. It is thought not to interact directly with the Ste2p because of the following:

1. *Ste2* deletion still gives an *sst2* deletion phenotype (Konopka and Jenness 1991).

2. Deletion of *SST2* and the *Ste2-T326* mutation have synergistic effects on sensitivity. Synergy suggests that these two gene products affect two different pathways. This result also indicates that the C-terminal domain of the pheromone receptor is not required for interacting with Sst2p (Konopka et al. 1988).

3. Linker scanning mutations of the *STE2* gene have resulted in the finding of an N-terminal mutated Ste2p which signals in an *sst2-1* strain. Cells with the *ste2-I101* mutation produce no halos, but the *ste2-I101 sst2-1* double mutant produces halos. Allele-specific suppression could be indicative of interaction between the two gene products. It could also mean that wild-type *SST2* dampens the signal via another protein and loss of this interaction allows the signal generated from the mutant receptor to be sufficient for halo formation (Konopka and Jenness 1991).

4. Genetic data from our lab and others suggests that *SST2* acts at the level of the G-protein. *gpa1-7* mutation bypasses the α-factor receptor and results in constitutive activation of the signalling pathway. Recovery from division arrest in the *gpa1-7 sst2-1* mutant is slower than in the *sst2-1* mutant alone which suggests a role for *SST2* gene in the recovery of *gpa1* mutant (Blinder, MCB,1989). Both *STE4* and *SST2* are required to stop the signal from a *Ste5Hyp* strain (Hasson et al. 1994). Certain alleles of *GPA1* bypass the requirement for a fully active *SST2* (Kurjan et al. 1991). Moreover, *SST2* was found to be in a complex with *GPA1* and appears to accelerate the rate of GTP hydrolysis (Dohlman et al. 1996).

Effect of sst2 deletion on ligand interaction

1. Does the antagonist DesTrp1,Ala3 α-factor behave like agonist in an *sst2Δ* strain?

Two findings suggests that in the *sst2* deletion strain, the antagonist behaves like an agonist. It was shown that an analog of α-factor lacking one amino acid from its N-terminus was 10-fold less active in an *SST2+* strain and the same molecule was half as active as α-factor in an *sst2⁻* strain (Eriotou-Bargiota et al. 1992). Therefore I asked whether the antagonist used in the trypsin assay would also lead to the same result. If it does, then what is the mechanism by which it signals in this strain? Halo assays were performed with *SST2+bar1⁻*, *sst2⁻Bar1+*, and *sst2⁻bar1⁻* strains. The results showed that the DesTrp1, Ala3 α-factor also leads to larger halos in an *sst2* deletion background than it does in the *SST2* control strain (Figure 1). Comparing the results of *BAR+* and *bar1⁻* strains, I conclude that the protease encoded by *BAR1* can not degrade the antagonist as efficiently as it degrades α-factor, since the halos with the antagonist in an *sst2-1, BAR1* are larger than halos with α-factor and the opposite is true in the *sst2⁻bar1⁻* strain.

124

HALO SIZES OF THE DIFFERENT STRAINS

	$SST2^+$ bar1-1	sst2-1 $BAR1^+$	sst2-1 bar1-1
5×10^{-5} M α-factor	1.8 cm	1.15 cm	
10^{-3} M DesTrp1,Ala3-α-factor	no halo	2.6 cm	
5×10^{-5} M α-factor	2.1 cm		3.8 cm
10^{-3} M DesTrp1,Ala3-α-factor	no halo		2.1 cm

Figure 1. Testing the signalling capacity of the sst2 deletion strain with the antagonist used in our experiments. Strains used were 211-5-3 ($SST2^+$ bar1-1), 3268-8-2 (sst2-1 $BAR1^{+)}$ and 6360-17-2 (sst2-1 bar1-1). The K_d of the antagonist was determined to be 1 mM from competition assays. Unlike the halos from the $SST2^+$ strain which have a well-defined circumference, the edges of the halos wiht the sst2 deletion strain with both the agonist and the antagonist were hazy. The halo assay with the 6360-17-2 strain was performed by Ayce Yesilaltay.

A.

| 5 min | 10 min | 30 min |
| - Des α | - Des α | - Des α |

B.

| 5 min | 10 min | 30 min |
| - Des α | - Des α | - Des α |
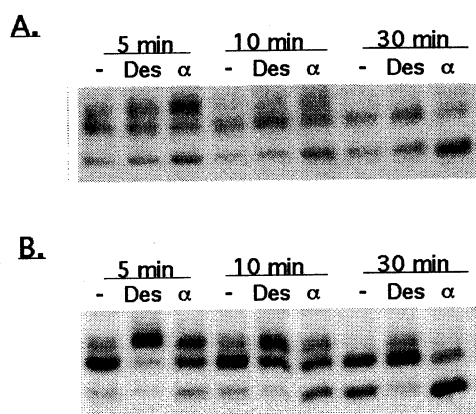
Figure 2. Limited tryptic digestion of the sst2 mutant, **(A)** and the wild-type control **(B)**. Membranes were digested with trypsin for 5, 10, and 30 min. Reactions included no ligand (-), antagonist (Des) or agonist (α).

126

Trypsin digestion assays were performed for the purpose of probing the mechanism by which the antagonist signals in an *sst2⁻* strain (Figure 2). With the *sst2⁻* strain, the antagonist was not found to expose the third loop as does the agonist, but it also did not induce the hyperconstrained state that was seen with $SST2^+$ strain. At all time points tested, the cleavage pattern with the antagonist looked the same as no ligand control. As was observed with the wild-type receptor, the agonist accelerated the cleavage of the third loop (formation of F3) in the *sst2* deletion strain, and it reduced the cleavage rate in the C-terminal domain as judged by the increased accumulation of F1 and the decreased accumulation of F2 at the 5 min time point. This result supports the model summarized in Figure 3. In this model, the hyperconstrained state of the receptor results from a complex of receptor, G-protein and Sst2p, and in an *sst2* deletion strain exposure of the third loop is not a constraint for activation. Since there are data indicating that Sst2p does not directly act on the receptor itself, the effect of Sst2p on the receptor is likely to be more indirect. Since there are genetic data that suggest Sst2p acts through G-proteins, it is possible that Sst2p keeps the $G\alpha\beta\gamma$ subunits together and that the binding energy of the antagonist is not sufficient to dissociate this complex. However, in the *sst2* deletion strain, the heterotrimer is not as strongly associated; consequently, the binding of antagonist is sufficient to dissociate $G\alpha$ from $G\beta\gamma$ and to result in signalling (Figure 3).

2. Does novobiocin potentiate the effects of the antagonist in an *sst2Δ* background?

Novobiocin is a coumarin antibiotic that targets the DNA gyrase in bacteria. Its mechanism of action in higher eucaryotes is not known. In an attempt to define a target for this drug in yeast, Pocklington and Orr identified a yeast mutant that was sensitive to this drug. This phenotype was concluded to be a consequence of a mutation in the *SST2* gene, and it was
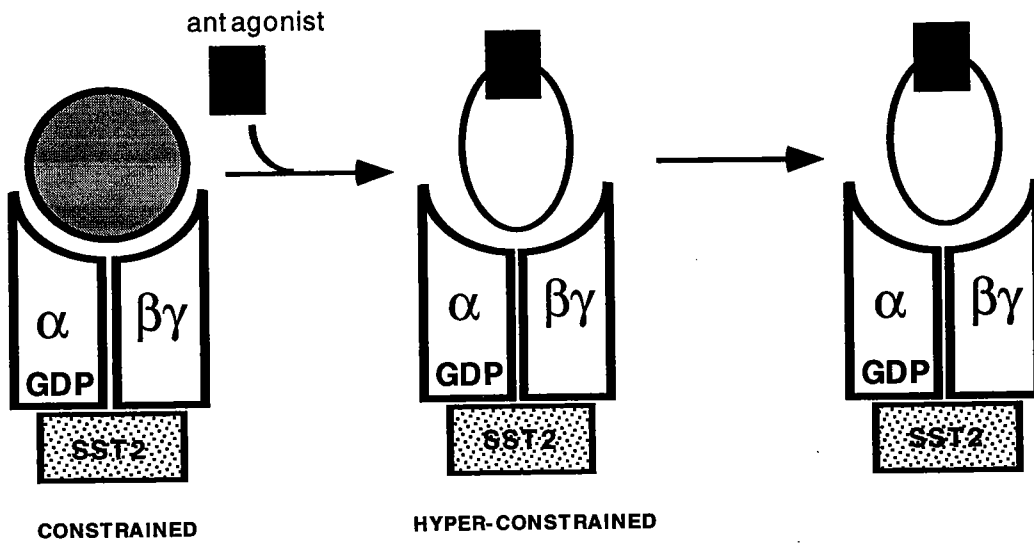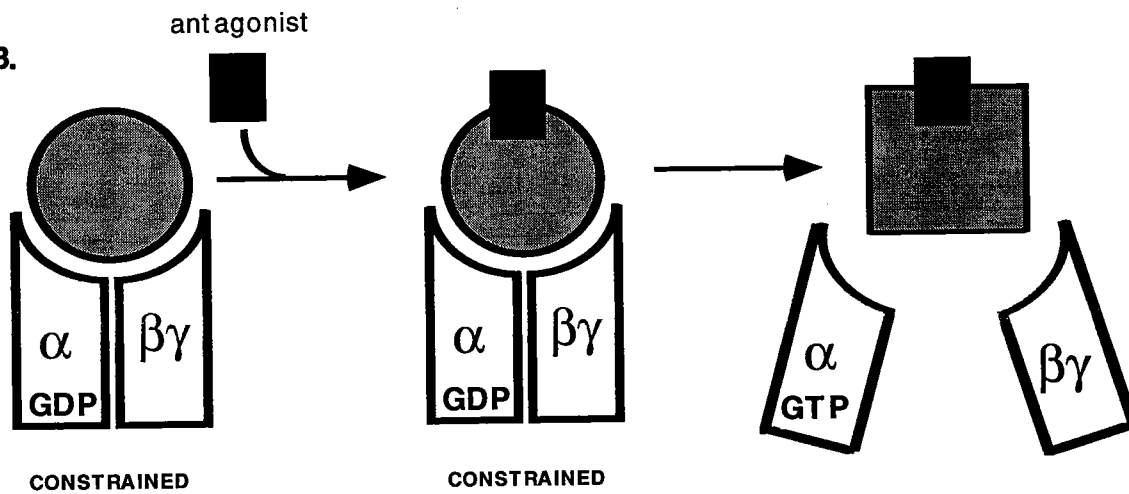
Figure 3. Signalling by the antagonist: A. in an SST2+ strain,
B. in an sst2 deletion strain

shown that, in an *sst2* deletion strain, novobiocin acts as an agonist for a-cells but not for α-cells (Pocklington and Orr 1994). Since the results summarized in the previous section indicated that in the same strain background the antagonist behaved like an agonist, I considered the possibility that novobiocin potentiates the activity of the antagonist. (Eriotou-Bargiota et al. 1992) had shown potentiation of α-factor with a truncated form of α-factor which lacks two amino acids from its C-terminus. This work also points to the N-terminus of α-factor as being important for signalling. Furthermore, the antagonist, DesTrp1,Ala3-α-factor lacks the two tryptophans (aromatic residues) in its N-terminus, and novobiocin has an aromatic structure (Figure 4) therefore it seemed reasonable to ask whether the two agents show synergistic activity. I performed halo assays with $SST2^+$ and *sst2* deletion strains to test whether cell division arrest was caused by antagonist, novobiocin or the two agents together. Although novobiocin produced halos in the *sst2* deletion strain and not in the $SST2^+$ strain, the antagonist alone produced larger halos on its own than in combination with novobiocin. Hence, novobiocin does not complement the defects of the antagonist, DesTrp1,Ala3-α-factor.

II. CHARACTERIZATION OF THE ANTAGONIST

There are two types of antagonists; neutral antagonists and inverse agonists (which are also referred to as negative antagonists as well). Neutral antagonists simply compete for the binding of agonists and they are thought to have no binding preference for the R or the R* state. Inverse agonists are ligands that are thought to reduce the activity of the receptor because they have a higher affinity for the R form of the receptor and stabilize this inactive conformation. The increase in the affinity of the *Ste2-T274P* mutant receptor for the antagonist suggests that this molecule is an inverse agonist. This is also consistent with the trypsin assay results from Chapter I (Bukusoglu and Jenness 1996) which show that binding of the antagonist induces a different conformation of the receptor.
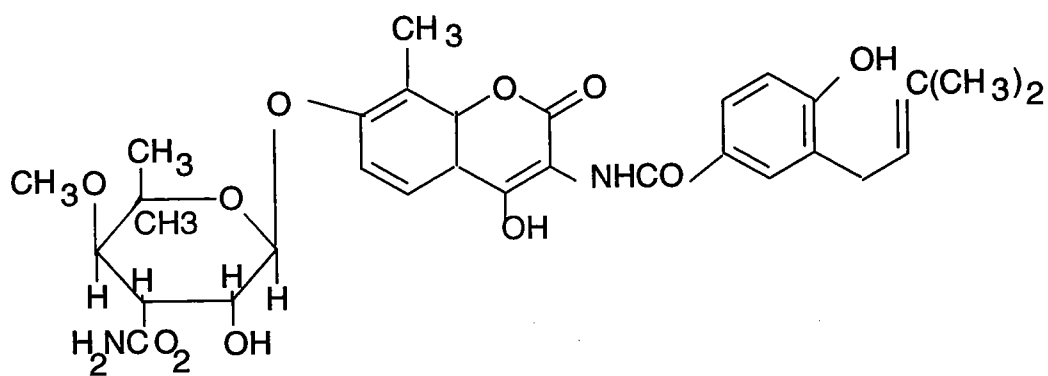
Figure 4.a. The chemical structure of novobiocin
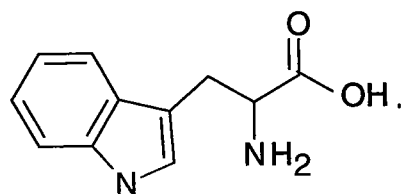
Figure 4.b. The chemical structure of tryptophan

130

1. Is DesTrp1,Ala3-α-factor an inverse agonist of STE2p?

The high level of basal signal afforded by the suppressors of *ste2-T274P* provide a basis for future studies of the antagonist DesTrp1,Ala3-α-factor. One property of inverse agonists is that they inhibit the basal level of signalling. As mentioned before, this is not possible in wild-type yeast due to post-receptor steps generating more noise than with the receptor itself. Since the suppressor mutations of *STE2* result in elevated levels of "noise", it should be possible to turn this off by the addition of inverse agonist but not by neutral antagonist. Future experiments with the suppressors should aid the further characterization of the DesTrp1,Ala3-α-factor.

2. Does the antagonist affect the endocytosis of the *STE2* receptor?

It was shown that chronic treatment with inverse agonist, but not neutral antagonist causes 5-HT$_{2C}$ receptor downregulation (Barker et al. 1994). Consistent with this, the α$_{1B}$-adrenergic receptor internalization was shown to be affected by addition of ligand: agonist addition results in the receptor redistribution into endosomes whereas antagonist prevents this process (Fonseca et al. 1995).

The trypsin digestion data indicate that the DesTrp1,Ala3-α-factor used in the assay could be an inverse antagonist since it induces a conformation that is different than that of the unliganded receptor. Therefore the effect of the antagonist on endocytosis was investigated. Renografin gradient centrifugation was used to assay cells for the movement of the receptor after the cells had been treated with either agonist or antagonist. Results did not indicate an effect of the antagonist on endocytosis.

III. EXTRAGENIC SUPPRESSORS

It was curious that although we had set out for extragenic suppressors of the *ste2-T274P*, none was found in this screen. Upon reflection, the design of the screen might not have been the best for this purpose. The sequence of the intracellular interaction sites are not altered due to the mutation of residue at 274. Therefore suppressor mutations that allow the *ste2-T274P* mutant to mate better because of stabilization of the R* form of the mutant receptors are also likely to stabilize the R* form of the wild-type receptor. Due to this caveat, the extragenic suppressors of the *ste2-T274P* mutant may not have shown allele-specificity. Hence, they would have been eliminated in my suppressor screen (Chapter III).

The work with the extragenic suppressors was carried out as follows:
After the mutants were ruled out for bypass suppression, suppression specificity for the *ste2-T274P* allele was determined. Cross-stamp mating tests indicated that none of the suppressor mutants were fertile in a *ste2* deletion background and all were fertile in the *STE2*+ background. Five mutants were fertile when retransformed with plasmid pDJ270 containing the *ste2-T274P* but not with pDJ253 containing the *ste2-L236H* allele. Halo assays with these five resulted in three different phenotypes:
1. Two suppressors resulted in a slightly larger halo phenotype in the *STE2*+ background and smaller or same-size clear halos in genetic backgrounds containing either of the *ste2* alleles.

2. This suppressor (gb 1-3) resulted in same size halos for all three alleles of *STE2* tested (the wild-type, the *ste2-L236H* and *ste2-T274P*). Both mutant receptor alleles produced semi-turbid halos.

3. Two suppressors (gb 10-13 and 13-13) resulted in very large halos with wild-type *STE2*, larger and clearer halos with *ste2-T274P* compared to *ste2-L236H* but not as big as with wild-type *STE2*$^+$.

Since the response to pheromone can only be studied in *MATa* haploid cells, MAT locus was deleted to facilitate further genetic analysis; cells that are *matΔ* mate as *MATa* cells, while *matΔ* cells that are transformed with a plasmid carrying the *MATα* gene mate as *MATα* cells. The dominance of each suppressor was tested by crossing them to strain 1350-TP (*MATΔ::LEU2 ste2-T274P leu2 ura3 ADE2 ade6 trp1 cyh2^r TYR1$^+$ lys2 his4-580 SUP4-3 arg4 his3*) carrying plasmid pDJ102 (YCp *MATα,TRP1$^+$*). The resulting diploids were cured of the plasmid; hence, they behaved as *MATa* maters in the cross-stamp mating test; five of the diploids tested were fertile. Since the diploids are heterozygous for the suppressor mutation, the fact that they are fertile indicates that the suppressor mutation is dominant. None of the five mutants were specific for *ste2-T274P*; nevertheless, further characterization of the isolated suppressors might lead to interesting results. Since most of these mutations result in an enhanced signalling with all the receptors tested, the suppressor is likely to be in the *SST2* gene. This could be tested by a simple complementation experiment where the mutant strains can be transformed with a plasmid carrying the *SST2* gene.

IV. MORE SIGNALLING MUTANTS

133

The screen used to isolate *ste2-T274P* was utilized once more to yield six more α-factor resistant receptor mutants. With the preliminary binding experiments, of the 118 isolates tested, these six showed between 30-87% binding to α-factor compared to wild-type receptor; the rest had close to no binding.

One of these signalling mutants was found to effect the 258th residue; the proline at this position was replaced by a serine (pGB195). Mutation of the same residue to a leucine was found to lead to constitutive activation (Konopka et al. 1996). It is interesting in this regard that substitution by a hydrohilic amino acid (serine) results in the opposite phenotype. This is reminiscent of the second site suppressors of the *ste2-T274P* which when replaced by a hydrophilic residue affected activation (Chapter III). The difference in the substitution of the two amino acids is apparent also from the preliminary binding experiments; whereas with the constitutively active Pro258Leu receptor, the binding affinity is increased to twice that of the wild-type, the Pro258Ser substitution results in decreased affinity (30% of wild-type). This mutant seems a likely candidate for a receptor that is blocked for allosteric transition like the *ste2-T274P*.

The other two mutant alleles that were sequenced do not seem to express a full-length protein since mutations detected for both result in an opal codon at Gly56 (pGB134) and ochre at E135 (pGB136) although they retain about 50% α-factor binding capacity. The remaining three mutants have binding ability ranging from 40-87% (pGB138, 146 and 218). Further characterization of these receptor mutants is likely to lead to information on the functional domains of the receptor.

V. IMPROVEMENTS IN THE TRYPSIN DIGESTION ASSAY

With the present trypsin-digestion assay, ligand-induced conformational changes can be reliably detected; however, the overall rate of the reaction differs for different membrane preparations making it difficult to compare results directly. The time required for trypsin to complete a given cleavage in the receptor is likely to be limited by the presence of other yeast proteins that compete for the trypsin activity. The quantity and the composition of these competing proteins undoubtedly vary among the different preparations of particulate fractions used in this assay. In order to control these competing reactions, wild-type receptors that have been tagged with six histidinyl residues at the N-terminus will be used as internal control. Before the digestion products are resolved on SDS-PAGE, the tagged standard will be separated from the untagged test protein by using commercially available spin-columns (Ni-NTA). In this way, the rates of cleavage of mutant receptor can be compared directly to the wild-type control. This alteration will allow the comparison of the conformational states of the various single and double mutants in the presence and absence of ligand. Recently, the pheromone receptor was purified by overexpressing the his-tagged Ste2p (David et al. 1997). The purification scheme consisted of solubilizing the cell membranes containing the overexpressed receptor and subjecting it to immobilized metal affinity chromatography.

The pGAL-His-Ste2 construct was cloned into pRS304 vector through a two-step, three way ligation of BamHI-EcoRI Gal promoter fragment, EcoRI-ClaI fragment of the histidine tag, ClaI-KpnI fragment of a truncated STE2. The primers used as the histidine tag were AATTCATAATGTCTCACCACCATCACCACCACGGTTCCAT and CGATGGAACCGTGGTGGTGATGGTGGTGAGACATTATG which were annealed together and ligated to the vector that had a galactose promoter attached. The ligated product was digested with EcoRI in order to remove the multimerized inserts that apparently caused instability in the construct. The insert was sequenced at the U. Mass.

Nucleic Acid Facility using the primers PO80 and PO111 and was integrated into yeast at the STE2 locus by linearizing the plasmid pDJ375 with HpaI, transforming strain 211-5-3 and plating it out on -trp+CAA plates. The resulting duplication contains one complete copy of the STE2 coding sequence fused to the His insert under the control of the GAL1 promoter. The other copy contains truncated STE2 coding sequence under the control of its own promoter. The strain carrying the integration was tested by halo assays on either rich media (lacking galactose) or plates containing galactose; although the construct produced halos only on the plates containing galactose (no halos were evident on the YEPD plates), the halos were smaller compared to the wild-type strain (211-5-3) on galactose containing plates. The strain (1394-A-1) was further tested by using Western analysis of crude extracts; the primary antibody recognizes the histidine tag (His-probe {G-18} from Santa Cruz Biotechnology, Inc.). This antibody failed to produce specific bands with the His-tagged Ste2 construct; antibody directed against the C-terminal tail of receptor resulted in faint bands.

VI. SERIES OF QUESTIONS

a. To provide further evidence for the idea that substitution of the polar residues at the locations of the suppressors is the reason for the suppression, these residues can be replaced with more hydrophobic residues which should reverse the phenotype.

b. Amounts of total receptor was found to be about 10% of the wild-type with the suppressor *ste2-A295T*. A possible explanation for this could be decreased synthesis or increased endocytosis of the mutant receptor. Renografin gradients could be used to differentiate between these possibilities. If it is found that this mutation leads to higher

basal endocytosis, this would identify a unique residue having a function in both basal signalling and basal endocytosis.

c. The suppressor mutations alter the expression levels of the receptor protein, therefore they should be normalized to wild-type by expression through an inducible promoter to determine more precisely the increase in their basal level.

d. It is reasonable to think that the basal endocytosis levels of the *ste2-T274P* receptor would be lower than wild-type if the mutant receptor was locked in the low affinity form. The assumption here is that the endocytosis signals would be effected in the same way as the signalling cues in the *ste2-T274P*. In fact, there is evidence to the contrary; that the 2 processes use different signals from Schandel and Jenness (1994) with the non-signalling receptor mutants where α-factor induced signalling is effected but not endocytosis. Also, Stefan et.al. (1994) identified a mutant receptor which has 4 amino acid insertions at the C-terminal part of its third loop. Although this mutant receptor has higher basal endocytosis compared to wild-type, it did not lead to constitutivity suggesting that signalling and endocytosis signals are different.

e. Is the dominance of the *ste2-T274P* more obvious when it is overexpressed?

f. Binding experiments in a gpa1 deletion strain containing the *ste2-T274P* allele might prove useful. If the *ste2-T274P* is locked in a low-affinity form that does not couple to G-proteins (or does so only to a small degree), then the binding of agonist will not be affected by *gpa1Δ*.

137

g. To deduce whether the suppressors of *ste2-T274P* are global suppressors or not, they could be tested in combination with other ste2-loss-of-function alleles from our lab (our unpublished receptor mutants) as well as Konopka and Dumont's receptor mutants; global suppressors are expected to reverse the phenotype of the otherwise unrelated loss-of-function alleles.

h. Since the suppressors increase basal levels of signalling, it would be interesting to ask if they induce basal level of endocytosis.

BIBLIOGRAPHY

Acharya, S. and Karnik, S. S. (1996). Modulation of GDP release from transducin by the conserved Glu134-Arg135 sequence in rhodopsin. J. Biol. Chem. *271*, 25406-25411.

Ackers, G. K. and Hazzard, J. H. (1993). Transduction of binding energy into hemoglobin cooperativity. Trends Biochem. Sci. *18*, 385-390.

Ackers, G. K. and Smith, F. R. (1987). The hemoglobin tetramer: A three-state molecular switch for control of ligand affinity. Ann. Rev. Biophys. Biophys. Chem. *16*, 583-609.

Baldwin, J. M. (1994). Structure and function of receptors coupled to G-proteins. Curr. Opin. Cell Biol. *6*, 180-190.

Balmforth, A. J., Lee A. J., Warburton P., Donnely D., Ball S. G. (1997). The conformational change responsible for AT1 receptor activation is dependent upon two juxtaposed asparagine residues on transmembrane helices III and VII. J. Biol. Chem. *272*, 4245-4251.

Barker, E. L., Westphal, R. S., Schmidt, D. and Sanders-Bush, E. (1994). Constituitively active 5-hydroxytrytamine$_{2C}$ receptors reveal novel inverse agonist activity of receptor ligands. J. Biol. Chem. *269*, 11687-11690.

Birnbaumer, M. (1995). Mutations and diseases of G-protein coupled receptors. J. Rec. & Sig. Trans. Res. *15*, 131-160.

Birnbaumer, M., Gilbert S., Rosenthal W.. (1994). An extracellular congenital nephrogenic diabetes insipidus mutation of the vasopressin receptor reduces cell surface expression, affinity for ligand, and coupling to the Gs/adenylyl cyclase system. Mol. Endo. *8*, 886-894.

Blinder, D., Bouvier, S. and Jenness, D. D. (1989). Constitutive mutants in the yeast pheromone response: ordered function of the gene products. Cell *56*, 479-486.

Blumer, K. J., Reneke, J. E. and Thorner, J. (1988). The *STE2* gene product is the ligand-binding component of the α-factor receptor of *Saccharomyces cerevisiae*. J. Biol. Chem. *263*, 10836-10842.

Blumer, K. J. and Thorner, J. (1990). β and γ subunits of a yeast guanine nucleotide-binding protein are not essential for membrane association of the α subunit but are required for receptor coupling. Proc. Natl. Acad. Sci. U S A *87*, 4363-4667.

Bond, R. A., Leff, P., Johnson, T. D., Milano, C. A., Rockman, H. A., McMinn, T. R., Apparsundaram, S., Hyek, M. F., Kenakin, T. P., Allen, L. F. and Lefkowitz, R. J. (1995). Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the β_2-adrenoreceptor. Nature *374*, 272-276.

Boone, C., Davis, N. G. and Sprague, G. F., Jr. (1993). Mutations that alter the third cytoplasmic loop of the a-factor receptor lead to a constitutive and hypersensitive phenotype. Proc. Natl. Acad. Sci. USA *90*, 9921-9925.

Bourne, H. R. (1997). How receptors talk to trimeric G-proteins. Curr. Opin. Cell Biol. *9*, 134-142.

Bukusoglu, G. and Jenness, D. D. (1996). Agonist-specific conformational changes in the yeast α-factor pheromone receptor. Mol. Cell. Biol. *16*, 4818-4823.

Burstein, E. S., Spalding, T. A. and Brann, M. R. (1996). Amino acid side chains that define muscarinic receptor/G-protein coupling - Studies of the third intracellular loop. J. Biol. Chem. *271*, 2882-2885.

Cartwright, C. P. and Tipper, D. J. (1991). In vivo topological analysis of Ste2, a yeast plasma membrane protein, by using β-lactamase gene fusions. Mol. Cell. Biol. *11*, 2620-2628.

Chan, R. K. and Otte, C. A. (1982). Isolation and genetic analysis of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by a-factor and α-factor pheromones. Mol. Cell. Biol. *2*, 11-20.

Chen, Q. and Konopka, J. B. (1996). Regulation of the G-protein-coupled α-factor pheromone receptor by phoshorylation. Mol. Cell. Biol. *16*, 247-257.

Cheung, A. H., Dixon, R. A., Hill, W. S., Sigal, I. S. and Strader, C. D. (1990). Separation of the structural requirements for agonist-promoted activation and sequestration of the beta-adrenergic receptor. Mol. Pharmacol. *41*, 775-779.

Clark, C. D., Palzkill, T. and Botstein, D. (1994). Systematic mutagenesis of the yeast mating pheromone receptor third intracellular loop. J. Biol. Chem. *269*, 8831-8841.

Cotecchia, S., Exum, S., Caron, M. C. and Lefkowitz, R. J. (1990). Regions of the α1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. Proc. Natl. Acad. Sci. USA *87*, 2896-2900.

Coughlin, S. R. (1994). Expanding horizons for receptors coupled to G-proteins: diversity and disease. Curr. Op. Cell Biol. *6*, 191-197.

David, N. E., Gee, M., Andersen, B., Naider, F., Thorner, J. and Stevens, R. C. (1997). Expression and purification of the *Saccharomyces cerevisiae* α-factor receptor (Ste2p), a 7-transmembrane-segment G-protein-coupled receptor. Jour. Biol. Chem. *272*, 15553-15561.

Dietzel, C. and Kurjan, J. (1987). The yeast *SCG1* gene: a Gα-like protein implicated in the a- and α-factor response pathway. Cell *50*, 1001-1010.

Dixon, R. A., Sigal I.S., Rands E., Register R.B., Candelore M.R., Blake A.D., Strader C.D. (1987). Ligand binding to the b-adrenergic receptor involves its rhodopsin-like core. Nature *326*, 73-77.

Dohlman, H. G., Song, J. P., Ma, D. R., Courchesne, W. E. and Thorner, J. (1996). Sst2, a negative regulator of pheromone signaling in the yeast *Saccharomyces cerevisiae*: Expression, localization, and genetic interaction and physical association with Gpa1 (the G-protein alpha subunit). Mol. Cell. Biol. *16*, 5194-5209.

Dohlman, H. G. and Thorner, J. (1997). RGS proteins and signalling by heterotrimeric G-proteins. J. Biol. Chem. *272*, 3871-3874.

Doi, K., Gartner, A., Ammerer, G., Errede, B., Shinkawa, H., Sugimoto, K. and Matsumoto, K. (1994). MSG5, a novel protein phosphatase promotes adaptation to pheromone response in S.cerevisiae. EMBO J. *13*, 61-70.

Eason, M. G. and Liggett, S. B. (1996). Chimeric mutagenesis of putative G-protein coupling domains of the α_{2A}-adrenergic receptor. J. Biol. Chem. *271*, 826-832.

Eriotou-Bargiota, E., Xue, C. B., Naider, F. and Becker, J. M. (1992). Antagonistic and synergistic peptide analogues of the tridecapeptide mating pheromone of *Saccharomyces cerevisiae*. Biochemistry *31*, 551-557.

Fahmy, K., Siebert, F. and Sakmar, T. P. (1995). Photoactivated state of rhodopsin and how it can form. Biophys. Chem. *56*, 171-181.

Farahbakhsh, Z. T., Hideg, K. and Hubbell, W. L. (1993). Photoactivated conformational changes in rhodopsin: a time-resolved spin label study. Science *262*, 1416-1419.

Farahbakhsh, Z. T., Ridge, K. D., Khorana, H. G. and Hubbell, W. L. (1995). Mapping light-dependent structural changes in the cytoplasmic loop connecting helices C and D in rhodopsin: a site directed spin labeling study. Biochem. *34*, 8812-8819.

Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L. and Khorana, H. G. (1996). Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin. Science *274*, 768-770.

Fong, T. M. (1996). Mechanistic hypotheses for the activation of G-protein coupled receptors. Cell. Signal. *8*, 217-224.

Fonseca, M. I., Button, D. C. and Brown, R. D. (1995). Agonist regulation of α_{1B}-adrenergic receptor subcellular distribution and function. J. Biol. Chem. *270*, 8902-8909.

Franke, R. R., König, B., Sakmar, T. P., Khorana, H. G. and Hofmann, K. P. (1990). Rhodopsin mutants that bind but fail to activate transducin. Science *250*, 123-125.

Franke, R. R., Sakmar, T. P., Graham, R. M. and Khorana, H. G. (1992). Structure and function in rhodopsin. J. Biol. Chem. *267*, 14767-14774.

Gether, U., Ballesteros J.A., Seifert R., Sanders-Bush, E., Weinstein H., Kobilka B.K. (1997). Structural instability of a constitutively active G-protein-coupled receptor. J. Biol. Chem. *272*, 2587-2590.

Gether, U., Lin, S. and Kobilka, B. K. (1995). Fluorescent labeling of purified adrenergic receptor. Evidence for ligand-specific conformational changes. J. Biol. Chem. *270*, 28268-28275.

Hagen, D. C. and Sprague Jr. G. F. (1984). Induction of the yeast α-spesific STE3 gene by the peptide pheromone a-factor. J. Mol. Biol. *178*, 835-852.

144

Hartig, A., Holly, J., Saari, G. and MacKay, V. L. (1986). Multiple regulation of STE2, a mating-type-specific gene in Saccharomyces cerevisiae. Mol. Cell. Biol. *6*, 2106-2114.

Hartwell, L. H. (1967). Macromolecule synthesis in temperature-sensitive mutants of yeast. J. Bacteriol. *93*, 1662-1670.

Hartwell, L. H. (1980). Mutants of *Saccharomyces cerevisiae* unresponsive to cell division control by polypeptide mating hormone. J. Cell Biol. *85*, 811-822.

Hasson, M. S., Blinder, D., Thorner, J. and Jenness, D. D. (1994). Mutational activation of the *STE5* gene product bypasses the requirement for G-protein β and γ subunits in the yeast pheromone response pathway. Mol. Cell. Biol. *14*, 1054-1065.

Hedin, K. E., Duerson K., Clapham D.E. (1993). Specificity of receptor-G-protein interactions: searching for the structure behind the signal. Cell. Signalling *5*, 508-515.

Henderson, R., Baldwin J.M., Ceska T. A., Zemlin F., Beckmann E., Downing K.H. (1990). Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. J. Mol. Biol. *213*, 899-929.

Herskowitz, I. (1995). MAP kinase pathways in yeast: for mating and more. Cell *80*, 187-197.

Hirsch, J. P. and Cross, F. R. (1993). The pheromone receptors inhibit the pheromone response pathway in Saccharomyces cerevisiae by a process that is independent of their associated Gα protein. Genetics *135*, 943-953.

Hogger, P., Shockley, M. S., Lameh, J. and Sadee, W. (1995). Activating and inactivating mutations in N- and C-terminal i3 loop junctions of muscarinic acetylcholine Hm1 receptors. J. Biol. Chem. *270*, 7405-7410.

Huang, R.-R. C., Yu, H., Strader, C. D. and Fong, T. M. (1994). Interactions of Substance P with the second and seventh TM domains of the neurokinin-1 receptor. Biochem *33*, 3007-3013.

Jackson, C. L., Konopka, J. B. and Hartwell, L. H. (1991). *S. cerevisiae* α pheromone receptors activate a novel signal transduction pathway for mating partner discrimination. Cell *67*, 389-402.

Jackson, M. B. (1994). Single channel currents in the nicotinic acetylcholine receptor: a direct demonstration of allosteric transitions. TIBS *19*, 396-399.

Jenness, D. D., Burkholder, A. C. and Hartwell, L. H. (1983). Binding of α-factor pheromone to yeast **a** cells: Chemical and genetic evidence for an α-factor receptor. Cell *35*, 521-529.

Jenness, D. D., Burkholder, A. C. and Hartwell, L. H. (1986). Binding of α-factor pheromone to Saccharomyces cerevisiae a cells: Dissociation constant and number of Binding Sites. Mol. Cell. Biol. *6*, 318-320.

Jenness, D. D., Goldman, B. S. and Hartwell, L. H. (1987). *Saccharomyces cerevisiae* mutants unresponsive to α-factor pheromone: α-Factor binding and extragenic suppression. Mol. Cell. Biol. *7*, 1311-1319.

Jenness, D. D. and Spatrick, P. (1986). Down regulation of the α-factor pheromone receptor in *S. cerevisiae*. Cell *46*, 345-353.

Kenakin, T. (1995). Agonist-receptor efficacy II: Agonist trafficking of receptor signals. TIPS *16*, 232-238.

Kenakin, T. (1996). Receptor conformational induction versus selection: all part of the same energy landscape. TiPS *17*, 190-191.

Kent, R. S., Lean, A. D. and Lefkowitz, R. J. (1980). A quantitative analysis of beta-adrenergic receptor interactions: Resolution of high and low affinity states of the receptor by computer modelling of ligand binding data. Mol. Pharm. *17*, 14-23.

Kim, J.-M., Altenbach, C., Thurmond, R. L., Khorana, H. G. and Hubbell, W. L. (1997). Structure and function in rhodopsin: Rhodopsin mutants with a neutral amino acid at E134 have a partially activated conformation in the dark state. Proc. Natl. Acad. Sci. USA *94*, 14273-14278.

Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G. and Lefkowitz, R. J. (1988). Chimeric α_2-, β_2-adrenergic receptors: delineation of domains involved in effector coupling and ligand specificity. Science *240*, 1310-1316.

Konig, B., Arendt, A., McDowell, J. H., Kahlert, M., Hargrave, P. A. and Hofmann, K. P. (1989). Three cytoplasmic loops of rhodopsin interact with transducin. Proc. Natl. Acad. Sci. USA *86*, 6878-6882.

Konopka, J. B. (1993). *AFR1* acts in conjunction with the α-factor receptor to promote morphogenesis and adaptation. Mol. Cell. Biol. *13*, 6876-6888.

Konopka, J. B., Demattei, C. and Davis, C. (1995). *AFR1* promotes polarized apical morphogenesis in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *15*, 723-730.

Konopka, J. B. and Jenness, D. D. (1991). Genetic Fine-structural analysis of the *S. cerevisiae* α-pheromone receptor. Cell Reg. *2*, 439-452.

Konopka, J. B., Jenness, D. D. and Hartwell, L. H. (1988). The C-terminus of the *S. cerevisiae* α-pheromone receptor mediates an adaptive response to pheromone. Cell *54*, 609-620.

Konopka, J. B., Margarit S.M., Dube P. (1996). Mutation of Pro-258 in transmembrane domain 6 constitutively activates the G-protein-coupled α-factor receptor. Proc. Natl. Acad. Sci. USA *93*, 6764-6769.

Kronstad, J. W., Holly, J. A. and MacKay, V. L. (1987). A yeast operator overlaps an upstream activation site. Cell *50*, 369-377.

Kühn, H., Mommertz, O. and Hargrave, P. A. (1982). Light-dependent conformational change at rhodopsin's cytoplasmic surface detected by increased susceptibility to proteolysis. Biochim. Biophys. Acta *679*, 95-100.

Kurjan, J., Hirsch, J. P. and Dietzel, C. (1991). Mutations in the guanine nucleotide-binding domains of a yeast Gα protein confer a constitutive or uninducible state to the pheromone response pathway. Genes & Dev. *5*, 475-483.

Leberer, E., Thomas, D. Y. and Whiteway, M. (1997). Pheromone signalling and polarized morphogenesis in yeast. Curr. Opin. Genet. & Dev. *7*, 59-66.

Luo, X., Zhang, D. and Weinstein, H. (1994). Ligand-induced domain motion in the activation mechanism of a G-protein-coupled receptor. Protein Eng. *7*, 1441-1448.

Manfredi, J. P., Klein, C., Herrero, J. J., Byrd, D. R., Trueheart, J., Wiesler, W. T., Fowlkes, D. M. and Broach, J. R. (1996). Yeast alpha mating factor structure-activity relationship derived from genetically selected peptide agonists and antagonists of Ste2p. Mol. Cell. Biol. *16*, 4700-4709.

Marsh, L. (1992). Substitutions in the hydrophobic core of the α-factor receptor of *Saccharomyces cerevisiae* permit response to *Saccharomyces kluyveri* α-factor and to antagonist. Mol. Cell. Biol. *12*, 3959-3966.

Marsh, L. and Herskowitz, I. (1988). STE2 protein of *Saccharomyces Kluyveri* is a member of the rhodopsin/β-adrenergic receptor family and is responsible for recognition of the peptide ligand α factor. Proc. Natl. Acad. Sci. USA *85*, 3855-3859.

Marsh, L., Neiman, A. M. and Herskowitz, I. (1991). Signal transduction during pheromone response in yeast. Annu. Rev. Cell Biol. *7*, 699-728.

Matesic, D. F. and Luthin, G. R. (1991). Atropine dissociates complexes of muscarinic acetylcholine receptor and guanine nucleotide-binding protein in heart membranes. FEBS Lett *284*, 184-186.

Milano, C. A., Allen, L. F., Rockman, H. A., Dolber, P. C., McMinn, T. R., Chien, K. R., Johnson, T. D., Bond, R. A. and Lefkowitz, R. J. (1994). Enhanced myocardial function in transgenic mice overexpressing the β2-adrenergic receptor. Science *264*, 582-586.

Miyajima, I., Nakafuku, M., N.Nakayama, Brenner, C., Miyajima, A., Kaibuchi, K., Arai, K., Kaziro, Y. and Matsumoto, K. (1987). *GPA1,* a haploid-specific essential gene, encodes a yeast homolog of mammalian G-protein which may be involved in mating factor signal transduction. Cell *50,* 1011-1019.

Monod, J., Wyman, J. and Changeux, J.-P. (1965). On the nature of allosteric transitions: a plausible model. J. Mol. Biol. *12,* 88-118.

Moro, O., Lameh, J. and Sadee, P. H. W. (1993). Hydrophobic amino acid in the i2 loop plays a key role in receptor-G-protein coupling. J. Biol. Chem. *268,* 22273-22276.

Naider, F. and Becker, J. M. (1986). Structure-activity relationships of the yeast α-factor. Crit. Rev. Biochem. *21,* 225-248.

Nakafuku, M., Obara, T., Kaibuchi, K., Miyajima, I., Miyajima, A., Itoh, H., Nakamura, S., Arai, K., Matsumoto, K. and Kaziro, Y. (1988). Isolation of a second yeast *Saccharomyces cerevisiae* gene (*GPA2*) coding for guanine nucleotide-binding regulatory protein: studies on its structure and possible functions. Proc. Natl. Acad. Sci. USA *85*, 1374-1378.

Nanevicz, T., L, W., M, C., M, I. and S.R., C. (1996). Thrombin receptor activating mutations. J. Biol. Chem. *271*, 702-706.

Neubig, R. R. (1994). Membrane organization in G-protein mechanisms. FASEB J *8*, 939-946.

Noda, K., Saad, Y., Graham, R. M. and Karnik, S. S. (1994). The high affinity state of the β2-Adrenergic receptor requires unique interaction between conseved and non-conserved extracellular loop cysteines. J. Biol. Chem. *269*, 6743-6752.

Okuma, Y. and Reisine, T. (1992). Immunoprecipitation of α_{2a}-adrenergic receptor-GTP-binding protein complexes using GTP-binding protein selective antisera. J. Biol. Chem. *267*, 14826-14831.

Onaran, H. O. and Costa, T. (1997). Agonist efficacy and allosteric models of receptor action. Ann. N.Y. Acad. Sci. *812*, 98-115.

Parma, J., Van Sande J., Swillens S., Tonacchera M., Dumont J., Vassart G. (1995). Somatic Mutations causing constitutive activity of the Thyrotropin receptor are the major

cause of hyperfunctioning throid adenomas: identification of additional mutations activating both the cyclic adenosine 3',5'-monophosphate and inositol phosphate-Ca cascades. Mol. Endocrinol. *9,* 725-733.

Parma, J., Duprez L., Van Sande J., Cochaux P., Gervy C., Mokel J., Dumont J., Vassart G.(1993). Somatic mutations in the Thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. Nature *365,* 649-651.

Pebay-Peyroula, E., Rummel, G., Rosenbusch, J. P. and Landau, E. M. (1997). X-ray Structure of Bacteriorhodopsin at 2.5 Angstroms from Microcrystals Grown in Lipidic Cubic Phases. Science *277,* 1676-1681.

Pellicone, C., Cook, N. J., Nullans, G. and Virmaux, N. (1985a). Light-induced conformational change in rhodopsin detected by modification of G-protein binding, GTP gamma S binding and cGMP phosphodiesterase activation. FEBS Lett. *181,* 184-188.

Pellicone, C., Nullans, G. and Virmaux, N. (1985b). Localization of light-induced conformational changes in bovine rhodopsin. FEBS Lett. *181,* 179-183.

Perez, D. M., Hwa, J., Gaivin, R., Mathur, M., Brown, F. and Graham, R. M. (1996). Constitutive activation of a single effector pathway: Evidence for multiple activation states of a G-protein-coupled receptor. Mol Pharmacol *49,* 112-122.

Pocklington, M. J. and Orr, E. (1994). Novobiocin activates the mating response in yeast through the alpha-pheromone receptor, Ste2p. Biochimica et Biophysica Acta *1224,* 401-412.

Price, L. A., Strnad J., Pausch M. H., Hadcock J. R. (1996). Pharmacological characterization of the Rat A2a Adenosine receptor functionally coupled to the yeast pheromone response pathway. Mol. Pharm. *50,* 829-837.

Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J. and Sealfon, S. C. (1992). Sequence Alignment of the G-protein Coupled Receptor Superfamiy. DNA & Cell Biol. *11,* 1-20.

Rao, V. R. and Oprian D. D. (1996). Activating mutations of rhodopsin and other G-protein-coupled receptors. Annu. Rev. Biophys. Biomol. Struct. *25,* 287-314.

Raths, S. K., Naider, F. and Becker, J. M. (1988). Peptide analogues compete with the binding of α-factor to its receptor in *Saccharomyces cerevisiae*. J. Biol. Chem. *263,* 17333-17341.

Ren, Q., Kurose, H., Lefkowitz, R. J. and Cotecchia, S. (1993). Constitutively active mutants of the α_2-adrenergic receptor. J. Biol. Chem. *268,* 16483-16487.

Reneke, J. E., Blumer, K. J., Courchesne, W. E. and Thorner, J. (1988). The carboxy-terminal segment of the yeast α-factor receptor is a regulatory domain. Cell *55,* 221-234.

Robinson, P. R., Cohen, G. B., Zhukovsky, E. A. and Oprian, D. D. (1992). Constitutively activating mutants of rhodopsin. Neuron *9,* 719-725.

Rohrer, J., Benedetti, H., Zanolari, B. and Riezman, H. (1993). Identification of a novel sequence mediating regulated endocytosis of the G-protein-coupled α-pheromone receptor in yeast. Mol. Biol. Cell *4*, 511-521.

Rose, M. D. and Fink, G. R. (1987). KAR1, a gene required for function of both intracellular and extracellular microtubules in yeast. Cell *48*, 1047-1060.

Rosenthal, W., Antaramian, A., Gilbert, S. and Birnbaumer, M. (1993). Nephrogenic diabetes insipidus: A V2-vasopressin receptor unable to stimulate adenylyl cyclase. J. Biol. Chem. *268*, 13030-13033.

Samama, P., Cotecchia, S., Costa, T. and Lefkowitz, R. J. (1993). A mutation-induced activated state of the β_2-adrenergic receptor. J. Biol. Chem. *268*, 4625-4636.

Samama, P., Cotecchia, S., Ostrowski, J., Caron, M. G. and Lefkowitz, R. J. (1992). Constitutive activation of the α1B-adrenergic receptor by all amino acid substitution at a single site. J. Biol. Chem. *267*, 1430-1433.

Samama, P., Pei, G., T., C., Cotecchia, S. and Lefkowitz, R. J. (1994). Negative antagonists promote an inactive conformation of the β_2-adrenergic receptor. Mol. Pharm.*45*, 390-394.

Samama, P., Bond R.A., Rockman H.A., Milano C.A., Lefkowitz R.J.(1997). Ligand-induced over-expression of a constitutively active β2-adrenergic receptor: pharmacological creation of a phenotype in transgenic mice. Proc. Natl. Acad. Sci. USA *94*, 137-141.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning, Cold Spring Harbor Laboratory Press.

Scatchard, G. (1948). The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. *51*, 660-672.

Schachman, H. K. (1988). Can a simple model account for the allosteric transition of aspartate transcarbamoylase? J. Biol. Chem. *263*, 18583-18586.

Schandel, K. A. and Jenness, D. D. (1994). Direct evidence for ligand-induced internalization of the yeast α-factor pheromone receptor. Mol. Cell. Biol. *14*, 7245-7255.

Scheer, A., Fanelli F., Costa T., De Benedetti P. G., Cotecchia S. (1996). Constitutively active mutants of the α1B-adrenergic receptor: role of highly conserved polar amino acids in receptor activation. EMBO J. *15*, 3566-3578.

Scheer, A. and S., C. (1997). Minireview: Constitutively active G-protein-coupled receptors: Potential mechanisms of receptor activation. J. Recept. Signal Transduct. Res. *17*, 57-73.

Schertler, G. F. (1993). Projection structure of rhodopsin. Nature *362*, 770-772.

Schreiber, R. E., Prossnitz, E. R., Ye, R. D., Cochrane, C. G. and Bokoch, G. M. (1994). Domains of the human neutrophil N-formyl peptide receptor Involved in G-protein coupling - mapping with receptor-derived peptides. J. Biol. Chem. *269*, 326-331.

Schumacher, M. A., Dixon, M. M., Kluger, R., Jones, R. T. and Brennan, R. G. (1995). Allosteric transition intermediates modelled by crosslinked haemoglobins. Nature *375*, 84-87.

Schwartz, T. W. and Rosenkilde, M. M. (1996). Is there a 'lock' for all 'keys' in 7 TM receptors? TIPS *17*, 213-216.

Sen, M. and Marsh, L. (1994). Noncontiguous domains of the alpha-factor receptor of yeasts confer ligand specificity. J. Biol. Chem. *269*, 968-973.

Sen, M. and Marsh, L. (1995). Novel antagonist to agonist switch in chimeric G-protein-coupled alpha-factor peptide receptors. Biochem. Biophys. Res. Commun. *207*, 559-564.

Shenbagamurthi, P., Baffi, R., Khan, S. A., Lipke, P., Pousman, C., Becker, J. M. and Naider, F. (1983). Structure-activity relationships in the dodecapeptide α factor of *Saccharomyces cerevisiae*. Biochemistry *22*, 1298-1304.

Shenker, A., Laue, L., Kosugl, S., Jr., J. M., Minegishi, T. and Cutler, G. B. (1993). A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. Nature *365*, 652-654.

Shi, W., Osawa, S., Dickerson, C. D. and Weiss, E. R. (1995). Rhodopsin mutants discriminate sites important for the activation of rhodopsin kinase and G_t. J. Biol. Chem. *270*, 2112-2119.

Siegel, G. J., Agranoff B. W., Albers R. W., Molinoff P. B. (1989). Basic
Neurochemistry, Raven Press, Ltd., 1185 Avenue of the Americas, New York, New
York 10036).

Sommers, C. M. and Dumont, M. E. (1997a). Genetic interactions among the
transmembrane segments of the G-protein coupled receptor encoded by the yeast STE2
gene. J. Mol. Biol. *266,* 559-575.

Sprague, G. F., Jr. and Thorner, J. (1992). Pheromone response and signal transduction
during the mating process of *Saccharomyces cerevisiae.* In The Molecular and Cellular
Biology of the Yeast *Saccharomyces,* J. R. Broach, J. R. Pringle and E. W. Jones ed.
(Cold Spring Harbor, NY, Cold Spring Harbor Press), pp. 657-744.

Sprague, J. G. F. and Herskowitz, I. (1981). Control of yeast cell type by the mating type
locus. I. Identification and control of expression of the **a**-specific gene, *BAR1.* J. Mol.
Biol. *153,* 305-321.

Stefan, C. J. and Blumer, K. J. (1994). The third cytoplasmic loop of a yeast G-protein-
coupled receptor controls pathway activation, ligand discrimination, and receptor
internalization. Mol. Cell. Biol. *14,* 3339-3349.

Strader, C. D., Candelore, M. R., Hill, W. S., Sigal, I. S. and Dixon, R. A. F. (1989).
Identification of two serine residues involved in agonist activation of the β-adrenergic
receptor. J. Biol. Chem. *264,* 13572-13578.

Strader, C. D., Fong, T. M., Graziano, M. P. and Tota, M. R. (1995). The family of G-protein-coupled receptors. FASEB J *9*, 745-754.

Strader, C. D., Fong, T. M., Tota, M. R. and Underwood, D. (1994). Structure and function of G-protein-coupled receptors. Annu. Rev. Biochem. *63*, 101-132.

Strader, C. D., Sigal I. S., Candelore M. R., Rands E., Hill W.S., Dixon R. A. F. (1988). Conserved aspartic acid residues 79 and 113 of the β-adrenergic receptor have different roles in receptor function. J. Biol. Chem. *263*, 10267-10271.

Unger, V. M., Hargrave, P. A., Baldwin, J. M. and Schertler, G. F. X. (1997). Arrangement of rhodopsin transmembrane α-helices. Nature *389*, 203-206.

Weber, G. (1972). Ligand binding and internal equilibria in proteins. Biochem. *11*, 864-878.

Weiner, J. L., Guttierez-Steil, C. and Blumer, K. J. (1993). Disruption of receptor-G-protein coupling in Yeast promotes the function of an SST2-dependent adaptation pathway. J. Biol. Chem. *268*, 8070-8077.

Weiss, E. R., Kelleher, D. J. and Johnson, G. L. (1988). Mapping sites of interaction between rhodopsin and transducin using rhodopsin antipeptide antibodies. J. Biol. Chem. *263*, 6150-6154.

Wess, J. (1997). G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. FASEB J. *11*, 346-354.

Whiteway, M. S., L.Hougan, Thomas, D. D. D. Y., Bell, L., Saari, G. C., Grant, F. J., O'Hara, P. and MacKay, V. L. (1989). The *STE4* and *STE18* genes of yeast encode potential β and γ subunits of the mating factor receptor-coupled G-protein. Cell *56*, 467-477.

Xue, C.-B., McKinney, A., Lu, H.-F., Jiang, Y., Becker, J. M. and Naider, F. (1996). Probing the functional conformation of the tridecapeptide mating pheromone of Saccharomyces cerevisiae through study of disulfide-constrained analogs. Int. J. Peptide Protein Res. *47*, 131-141.

Zanolari, B., Raths, S., Singer-Kruger, B. and Riezman, H. (1992). Yeast pheromone receptor endocytosis and hyperphosphorylation are independent of G-protein-mediated signal transduction. Cell *71*, 755-763.

Zhang, M. and Tipper, D. J. (1993). Suppression of a dominant-G-protein beta-subunit mutation in yeast by G-alpha-protein expression. Mol Microbiol *9*, 813-821.