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**Studies on the role of specific residues of the
Saccharomyces cerevisiae α -factor pheromone
receptor (Ste2p) in the inactive and active state**

A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Yong-Hun Lee

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Dedication

I would like to dedicate this dissertation to my parents, Mr. and Mrs. Keun-Sang Lee and to my parents-in-law, Mr. and Mrs. Ju-Won Kim who stood by me and supported me during my education.

I would also like to dedicate this work to my wife, Jeong-Yeon Kim, and my children, Linus Lee and Annette Lee who endured their life as a dependent on a student househead with great patience and love during the course of this research.

Without their love and support, I could never have completed this work.

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Abstract

G protein-coupled receptors (GPCRs) are a class of integral membrane receptor proteins that are characterized by seven-transmembrane (7TM) domains connected by intracellular and extracellular loops, an extracellular N-terminus, and an intracellular C-terminus. To date more than 1000 GPCRs have been identified, and these proteins recognize neurotransmitters, sensory molecules and chemotactic agents. These receptors are involved in the control of many aspects of metabolism and play important roles in diverse processes such as pain perception, growth and blood pressure regulation, and viral pathogenesis. Therefore, these proteins became important target for therapeutic agents and recent reports indicate that nearly 40% of drugs currently prescribed for human ailments target this family of proteins.

The tridecapeptide α -factor pheromone ($W^1H^2W^3L^4Q^5L^6K^7P^8G^9Q^{10}P^{11}M^{12}Y^{13}$) of *Saccharomyces cerevisiae* and Ste2p, its cognate GPCR, have been used extensively as a model for peptide ligand-GPCR structure and function. The power of yeast genetic was used to examine the structure –function relationship of α -factor receptor. Upon the α -factor binding to Ste2p, a signal is transduced via an associated guanine-nucleotide binding protein initiating a cascade of events that leads to the mating of haploid yeast cells. As only two GPCRs and two G proteins are encoded in the *S. cerevisiae* genome, *S. cerevisiae* provides an ideal system to study the relation between a peptide ligand and its GPCR in the absence of interfering biological complexities.

Part I of this dissertation is an overview of the structure, receptor theory and conformational changes in receptor activation with specific emphasis on the peptide pheromone α -factor and its receptor Ste2p.

Part II of this dissertation is a study of TM6 of Ste2p. Site-directed mutagenesis of a targeted portion of Ste2p TM6 was carried out. Among the Alanine substituted residues in the 262-270 region of Ste2p, only the Y266A mutant did not transduce signal yet exhibited only a small decrease in α -factor binding affinity. In comparison to WT Ste2p, the mutant Y266A receptor showed increased binding affinity for N-terminal, alanine-substituted α -factor analogues (residues 1-4). Data from these studies suggest that Y266 is part of the binding pocket that recognizes the N-terminal portion of α -factor and is involved in the transformation of Ste2p into an activated state upon agonist binding.

Part III of this dissertation describes the specific interaction between residues N205 and Y266 of Ste2p in an active state not in resting state. Using a series of biological and biochemical analyses of wild-type and site-directed mutant receptors, we identified N205 as a potential interacting partner with the Y266 residue. To test the interaction between N205 and Y266 residues of Ste2p, a series of biological and biochemical analysis coupled with mutation was carried out. First, a pH-dependent functional activity of N205H/Y266H double mutant suggests that the 205H and 266H residues interact in the activated state of the receptor. Second, the introduction of N205K or Y266D mutations into the P258L/S259L constitutively-active receptor suppressed the constitutive activity; in contrast, the N205K/Y266D/ P258L/S259L quadruple mutant was

fully constitutively active, again indicating an interaction between residues at the 205 and 206 positions in the receptor active state. Finally, we showed a di-sulfide formation only between N205C and Y266C in constitutively-active receptor not in WT receptor. Data from these studies suggest a specific interaction between N205 and Y266 in an active state, but not the resting state, of Ste2p.

The final part of this dissertation reviews the overall conclusions and discussion. This part also contains suggestions for future experiments that could help to understand a conformation change during receptor activation. These studies should contribute to an understanding of the conformational differences between resting and active states of GPCRs.

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PART I

General Introduction

CHAPTER 1

G Protein-coupled Receptors: An overview

G protein-coupled receptors (GPCRs) are transmembrane proteins that mediate most of their intracellular actions through pathways involving activation of G-proteins (Gether, 2000; Gether et al., 2002; Pierce et al., 2002; Schoneberg et al., 2002). GPCRs comprise the largest superfamily of proteins in humans with more than 1000 different GPCRs identified by sequencing of the human genome since the first receptors were cloned more than a decade ago (Fredriksson et al., 2003; Kolakowski, 1994; Lander et al., 2001)

The endogenous ligands of the GPCR superfamily are exceptional diverse. They include peptide and protein hormones (such as angiotensin, bradykinin, endothelin, and melanocortin), biogenic amines (for example, adrenaline, dopamine, histamine, and serotonin), nucleosides and nucleotides (adenosine, adenosine triphosphate, uridine triphosphate), peptide pheromones (i.e. α -factor, **a**-factor of various fungal species), and lipids and eicosanoids (cannabinoids, leukotrienes, prostaglandins, and thromboxanes). Moreover, the sensation of exogenous stimuli, such as light, odors, and taste, is mediated via this class of receptors (Hoon et al., 1999; Kolakowski, 1994). GPCRs are named due to their ability to recruit and regulate the activity of intracellular heterotrimeric G proteins. These G proteins are composed of three subunits (α , $\beta\gamma$ -dimer) and each subunit plays important roles in determining the specificity and temporal characteristics of the cellular responses to signals (Hamm, 1998). Activation of GPCRs upon ligand binding

induces a conformational change in the associated G protein α -subunit leading to release of GDP followed by binding of GTP (Bourne et al., 1991). This change initiates the dissociation of the α -subunit from the receptor and the $\beta\gamma$ -dimer. G-proteins transmit the signal to effector proteins, such as enzymes and ion channels, resulting in rapid changes in the concentration of intracellular signaling molecules, cAMP, cGMP, inositol phosphates, diacylglycerol, arachidonic acid, and cytosolic ions (Cabrera-Vera et al., 2003; Landry and Gies, 2002). However, not all receptors that activate G-proteins are members of the superfamily of GPCRs. Activation of G-proteins has also been implicated in signal transduction mediated by several tyrosine kinase receptors, including the receptors for epidermal growth factor (EGF), insulin, insulin-like growth factor-II, and colony-stimulating factor-1 and insulin-like growth factor-I (Kuemmerle and Murthy, 2001).

Since the GPCR superfamily comprises receptors for many hormones, paracrine (local hormones), neurotransmitters, peptides, amino acids, glycoproteins, prostanoids, phospholipids, fatty acids, nucleosides, nucleotides, Ca^{2+} ions and neuromodulators, it is not surprising that abnormal signaling by these receptors are at the origin of disorders that affect tissues and organs in the human body (Karnik et al., 2003). Their dysfunction causes human diseases (Table 1) therefore, many GPCRs have been targeted for pharmaceuticals (Table 2). It has been predicted that about 80% of known hormones and neurotransmitters activate signal transduction mechanisms by activating GPCRs (Birnbaumer et al., 1990), and it has been predicted that GPCRs represent 50% of all modern prescription drugs and 25% of the top-selling drugs directly

Table 1: Various diseases linked to naturally occurring inherited mutations in different GPCRs

Disease	Receptor	Mode of inheritance	Type/function of mutation
Colour blindness	Red and green opsins	X-linked	X chromosome rearrangement
Stationary night blindness	Rhodopsin	AR	Missense mutation
Retinitis pigmentosa	Rhodopsin	AD	Apoptosis of rod cells
Retinitis pigmentosa	Rhodopsin	AR	Null mutations
Nephrogenic Diabetes Insipidus	V ₂ receptor	X-linked	Loss of function
Isolated glucocorticoid deficiency	ACTH receptor	AR	Loss of function
Hyperfunctioning thyroid adenomas	TSH receptor	Somatic mutation	Missense mutation
Familial precocious puberty	LH receptor	AD male limited	Missense mutation
Familial hypocalciuric hypercalcemia	Ca ²⁺ -sensing receptor	AD	Missense mutation
Neonatal severe hyperparathyroidism	Ca ²⁺ -sensing receptor	AR	Missense mutation

Abbreviations: ACTH, Adrenocorticotrophic hormone receptor; TSH, thyroid stimulating hormone; LH, Luteinizing hormone; AR, autosomal recessive; AD, autosomal dominant. Adapted from (Flower, 1999).

Table 2: Examples of marketed drugs acting at GPCRs drawn from the 500 top selling pharmaceutical products worldwide in 2004*

GPCR target	Drug	Disease
Histamine receptors	Zantac	Ulcers
	Pepcid	Ulcers
	Claritin	Allergies
	Allegra	Allergies
5-HT receptors	Risperdal	Psychosis
	Imitrex	Migraine
	BuSpar	Anxiety
	Zyprexa	Schizophrenia
Angiotensin receptors	Cozaar	Hypertension
Adrenoceptors	Toprol-XL	Hypertension
	Coreg	Congestive heart failure
	Serevent	Asthma
Muscarinic acetylcholine receptors	Atrovent	COPD
GnRH receptors	Zoladex	Cancer
Dopamine receptors	Requip	Parkinson's diseases
Prostaglandin (PGE1) receptors	Cytotec	Ulcers
ADP receptors	Plavix	Stroke

***Source:** Adapted and modified from “http://www.pharmalive.com/special_reports”

Abbreviations: COPD, chronic obstructive pulmonary disease; 5HT, 5-hydroxytryptamine (serotonin); GnRH, Gonadotrophin-releasing-hormone

or indirectly affecting GPCRs (Drews, 2000; George et al., 2002; Hopkins and Groom, 2002; Klabunde and Hessler, 2002; Lundstrom, 2005; Maudsley, 2005). In addition to the successful discovery of drugs functioning at GPCRs in the past, there is sufficient evidence to suggest that GPCRs will continue to be drug targets of the future. Human genome sequencing has revealed hundreds of members of the GPCR superfamily excluding those GPCRs involved in olfaction (Fredriksson et al., 2003; Kolakowski, 1994; Lander et al., 2001). Many of these orphan receptors have not been assigned functions. Even amongst those receptors whose ligands are known, there are still many receptors for which small molecule agonists or antagonists are lacking. Considering that only a very small portion of the known GPCRs represent targets of currently marketed drugs, it is thus obvious that future studies of GPCRs should make a striking impact on understanding and treatment of a variety of diseases.

Classification of GPCRs:

Sequence comparison among the different GPCRs showed the little or no sequence similarities (Kolakowski, 1994). However, members of the superfamily have structural similarities including seven transmembrane (TM)-spanning α -helical segments connected by alternating intracellular and extracellular loops (IL and EL, respectively), with the amino terminus located on the extracellular side of the cytoplasmic membrane and the carboxy terminus on the intracellular side (Strader et al., 1994). While each of seven TMs is generally comprised of 20-27 amino acids, the size of N-terminal domains (7-600 amino acids), loop domains (5-230 amino acids), and C-terminal domains (12-360

amino acids) are various indicating diverse functions of GPCRs to exogeneous stimuli (Hamm, 1998).

The GPCR superfamily has been classified by phylogenetic analysis of their amino acid sequences, by their native ligands, and by analysis of globular domains and motifs in the receptors. The superfamily of GPCRs has previously been divided into 6 different families (Kolakowski, 1994), with each family having >20% amino acid sequence identity within the TMDs (Transmembrane domains). The three major subfamilies include the receptors related to the rhodopsin and the β_2 -adrenergic receptor (family A), the receptors related to the glucagon receptor (family B), and the receptors related to the metabotropic neurotransmitter receptors (family C) (Figure 1).

The rhodopsin-like family A receptor has the largest number of receptors. Therefore, this class is the most studied so far. This class contains receptors for peptides, hormone peptide, odorants, glycoprotein hormones, and small molecules such as the catecholamines and amines (Lee et al., 2002). Among family A receptors, peptide receptors form the largest subgroup and mediate important physiological roles of endogenous peptides that act as neurotransmitters, hormones, and paracrines.

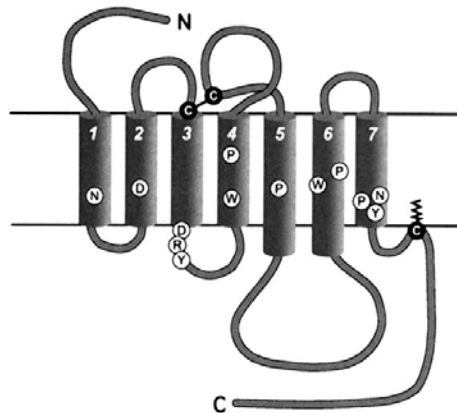
For the majority of peptide receptors studied, peptide agonists have been shown to interact directly with residues in the N terminus and extracellular loops (ECLs) (Gether, 2000). Site-directed mutagenesis experiments have provided convincing data suggesting that residues in the extracellular part of the TM2, TM3, and TM5–7 interact directly with certain neuropeptides in the human Y₁ NPY (Kanno et al., 2001; Sautel et al., 1996), rat AT_{1A} angiotensin II (Noda et al., 1996), rat B₂ bradykinin (Jarnagin et al., 1996), and human CXCR₄ (Brelot et al., 2000) receptors. The glucagon like family B receptors are

Figure 1. The diagrams of the three major subfamilies of GPCRs. Family A

receptors (*upper panel*) can phylogenetically be divided into six subgroups as indicated.

Family A receptors are characterized by a series of highly conserved key residues (*black letter in white circles*). In most family A receptors, a disulfide bridge is connecting the second (ECL2) and third extracellular loop (ECL3) (*white letters in black circles*). In addition, a majority of the receptors have a palmitoylated cysteine in the carboxy-terminal tail causing formation of a putative fourth intracellular loop.

Family B receptors (*middle panel*) are characterized by a long amino terminus containing several cysteines presumably forming a network of disulfide bridges. The B receptors contain, similar to the A receptors, a disulfide bridge connecting ECL2 and 3. However, the palmitoylation site is missing. Moreover, the DRY motif at the bottom of TM 3 is absent. Family C receptors (*lower panel*) are characterized by a very long amino terminus (~600 amino acids). Except for two cysteines forming a putative disulfide bridge, the C receptors do not have any of the key features characterizing A and B receptors. Some highly conserved residues are indicated (*black letter in white circles*). A unique characteristic of the C receptors is a very short and highly conserved third intracellular loop. Adapted from (Gether, 2000).



Family A. Rhodopsin/ β 2 adrenergic receptor-like

Biogenic amine receptors (adrenergic, serotonin, dopamine, muscarinic, histamine)

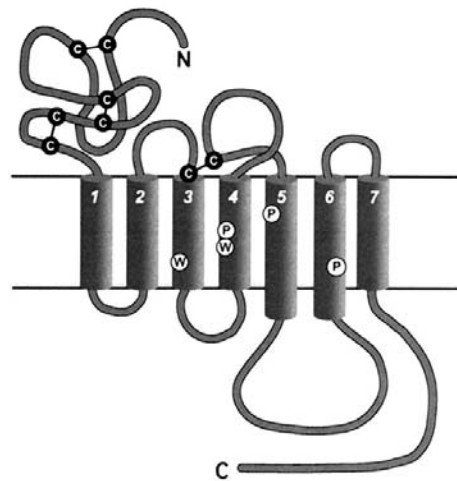
CCK, endothelin, tachykinin, neuropeptide Y, TRH, neurotensin, bombesin, and growth hormone secretagogues receptors plus vertebrate opsins

Invertebrate opsins and bradykinin receptors

Adenosine, cannabinoid, melanocortin, and olfactory receptors.

Chemokine, fMLP, C5A, GnRH, eicosanoid, leukotriene, FSH, LH, TSH, fMLP, galanin, nucleotide, opioid, oxytocin, vasopressin, somatostatin, and protease-activated receptors plus others.

Melatonin receptors and other non-classified



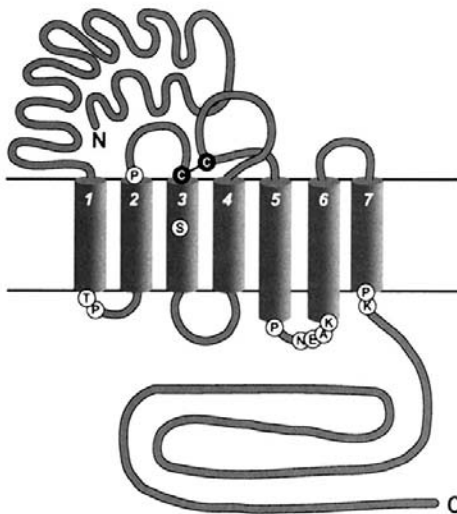
Family B. Glucagon/VIP/Calcitonin receptor-like

Calcitonin, CGRP and CRF receptors

PTH and PTHrP receptors

Glucagon, glucagon-like peptide, GIP, GHRH, PACAP, VIP, and secretin receptors

Latrotoxin



Family C. Metabotropic neurotransmitter/Calcium receptors

Metabotropic glutamate receptors

Metabotropic GABA receptors

Calcium receptors

Vomeronasal pheromone receptors

Taste receptors

presently the next largest family including receptors for a variety of gastrointestinal peptide hormones (secretin, glucagons, vasoactive intestinal peptide, and growth-hormone-releasing hormone), calcitonin, corticotrophin-releasing hormone and parathyroid hormone (Pierce et al., 2002). These receptors have a N-terminal extracellular (~120 residues) hormone binding domain (HBD). Site-directed mutagenesis and cross-linking experiments with family B receptors indicate that the peptide ligand binding site is discontinuous and consists of contact points from the extended N terminus and the 3 ECLs (Unson, 2002). The metabotropic glutamate family C receptors are relatively a smaller group, containing the metabotropic glutamate and GABA (γ -aminobutyric acid) receptors, the calcium sensing receptors, putative mammalian pheromone receptors, as well as some taste receptors (T1R) (Hoon et al., 1999). This family C receptors have a very large extracellular amino terminus (500-600 amino acids) suggesting the N-terminus may play an important role for ligand binding and receptor activation (Conn and Pin, 1997; O'Hara et al., 1993). The family D is the STE2 yeast pheromone receptors, and family E is the STE3 yeast pheromone receptors. The family F receptors are related to slime mold cyclic adenosine monophosphate (cAMP) receptors and archaeobacterial opsins. Since my Ph.D studies focused on the structure and function of Ste2p, more detail on this receptor system is described in Chapter 2 of Part I of this dissertation.

Heterotrimeric G-proteins:

There are two main classes of G-proteins. One is heterotrimeric G-proteins that associate with GPCRs and the other is small cytoplasmic G-proteins. Since we are interested in GPCRs, we described only heterotrimeric G-proteins in this dissertation. The heterotrimeric G-proteins are composed of α , β , and γ subunits, with molecular masses of about 39–45, 35–39, and 6–8 kDa, respectively. To date, at least 28 distinct G-protein α subunits, which are the products of 16 different genes and splice variants have been identified in mammalian cells (Downes and Gautam, 1999). β and γ subunits are tightly associated and can be regarded as one functional unit. Five different β and 12 different γ subunits have been described excluding splice variants in mammalian cells (Downes and Gautam, 1999). This diversity allows formation of many combinations, which may confer increased diversity and specificity of the signaling. Heterotrimeric G-proteins have a principal activation/inactivation cycle by the exchange of guanine nucleotides (GTP: GDP) allowing reversible and specific transmission of signals into cells. Upon binding of ligand, the receptor becomes activated and undergoes a conformational change resulting in increased affinity for the G-protein. This allows rapid release of GDP from its binding site on the α subunit. Under physiological conditions, the GDP is immediately replaced by GTP, the concentration of which exceeds that of GDP by several-fold. The exchange in the guanine nucleotides leads to a reduction in the affinity of the α subunit for the $\beta\gamma$ complex and functional dissociation of the heterotrimer. The active state of $G\alpha$ continues until GTP is hydrolyzed to GDP by the intrinsic GTPase activity of $G\alpha$ subunits (see reviews, (Cabrera-Vera et al., 2003; Pierce

et al., 2002). In addition, G-proteins may be inactivated by GTPase-activating proteins (GAPs). Once GTP is cleaved to GDP, the α -GDP and $\beta\gamma$ subunits reassociate and become inactive. Phosphodiesterase (PDE) and phospholipase C β have been known to effectors act as GTPase-activating proteins (GAPs). Several regulator of G-protein signaling (RGS) proteins have been identified to be effective GAPs for α subunits of G_i proteins (Landry and Gies, 2002) and the first RGS identified was the yeast Sst2p (Dohlman and Thorner, 1997). The details of G proteins and RGS in yeast are described in Chapter 2 of Part I of this dissertation.

Receptor theory:

It was thought that an agonist ligand was a molecule that selected or induced an active conformation of the receptor (De Lean et al., 1980). It was postulated that receptor activation required ternary complex and this ternary complex defines the active form of the receptor as a ternary complex involving the hormone, the receptor, and membrane component X that is identified as a G-protein later (De Lean et al., 1980). Modern theories (extended ternary complex) have shifted to receptor states that can exist even without the effects of an agonist. Extended ternary complex model of receptor theory was based on the important finding that mutations in the third intracellular loop of the β_2 -AR resulted in its constitutive activation. The exchange of a short stretch of amino acids at the intracellular end of TM6 of the human β_2 adrenergic receptor with the homologous sequence from the α_{1B} adrenergic receptor resulted in constitutive activity (Samama et al., 1993). This phenotype resulted in the theory that this mutant receptor might have an

increased tendency to adopt an active conformation in the absence of ligand. To interpret the molecular properties of mutant receptors, an extension of the original model was proposed and designated the extended ternary complex model. This model includes an explicit isomerization step regulating the equilibrium between at least two inter-convertible states, R (inactive) and R* (active). According to this model, constitutive activation was explained as an alteration of the normal equilibrium between the inactive state (R) and the active state (R*), shifting a higher proportion of receptor molecules in the active R* state. In the absence of agonist, inactive R state is dominant, however, the low energy barrier between inactive R state and active R* state allowed a small portion of receptors spontaneously to assume R* state. Agonists were predicted to bind with higher affinity to the active conformation of receptor and increase the portion of active state of receptor by shifting the equilibrium from the inactive state (R) and the active state (R*). Inverse agonists were predicted to stabilize the inactive R state shifting the equilibrium away from R* state. Neutral agonists were defined as molecules that bind with the same affinity to both inactive state (R) and the active state (R*) of receptor.

Another revised ternary complex model was proposed as the cubic ternary complex model (Weiss et al., 1996). This model included all the features of the ternary complex model but allowed G-proteins to bind to inactive receptors. This results in a complete equilibrium picture of the three-way interaction between ligand, receptor, and G-proteins. In the extended ternary complex model, a ligand with high affinity for a receptor conformation coupled to G-protein would result in agonist action. However, the cubic ternary complex model implies the existence of an inactive receptor conformation coupled to G-protein and active receptor conformation coupled to G-protein. This is a

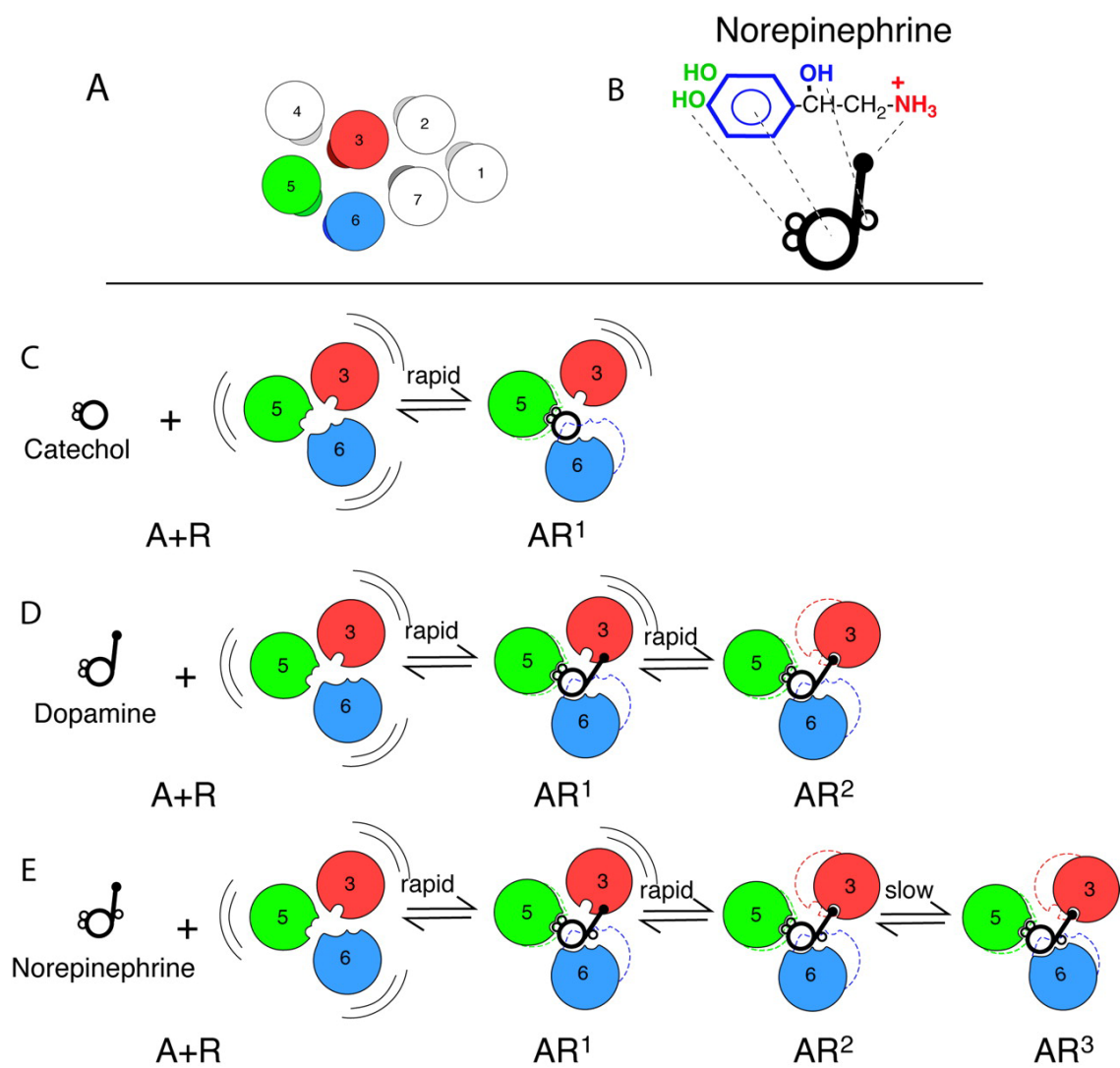
distinctive difference of the cubic model compared with the revised ternary complex model.

Therefore, a multi-state model has been proposed to explain observations that cannot be explained by previous models. For example, different constitutively active mutants of $\alpha 1b$ -receptor are differentially phosphorylated and internalized although they convey a similar agonist-independent activity to the receptor (Mhaouty-Kodja et al., 1999). In addition, fluorescence spectroscopy analysis of the purified $\beta 2$ -adrenergic receptor (AR) showed that most ligands promote alterations in receptor structure consistent with the existence of multiple agonist-specific conformation states (Kobilka et al., 1999). G-protein activation is achieved by changing an inactive receptor conformation to active conformation, which is mediated by ligand binding to receptor. In rhodopsin, the 11-*cis* bond conformation is the "inactive" state and the 11-*trans* is the "active" state. Any amino acid chain that is involved in receptor conformational change should follow two different states, which can be designated inactive or active depending on whether the particular state is associated with the active or the inactive receptor. The multi-state model can explain a generation of multiple discrete conformational states because the receptor activation involves a sequential progression of conformational changes. For example, if 10 amino acids in a GPCR are involved in the conversion of the inactive to active state conformation, each of the 10 amino acids has two distinct states. The number of distinct conformations that may be theoretically possible is 2^{10} .

In addition to the above theoretical assumption of multi-state of receptor, there is lots of evidence supporting multi-state of receptor from kinetic studies as well as pharmacological studies of GPCRs. One of the first studies to support the multiple states

was done using the type-1 pituitary adenylyl cyclase-activating polypeptide (PACAP) receptor with truncated ligand. While the agonists (PACAP-27 and -38) stimulated adenylyl cyclase (AC) with equal potencies, only PACAP-38 could invoke the inositol phosphate response through phospholipase C (PLC) (Spengler et al., 1993). Another evidence for multiple signaling conformations came from the studies of β_2 -adrenergic receptor (AR)(Ghanouni et al., 2001). Spectral changes could be direct evidence reflecting receptor conformational changes in the receptor activation when a fluorescent reporter molecule covalently attached to the receptor. To study the mechanism of how different classes of ligands can modulate receptor function, a fluorescence fluorophore was covalently attached to Cys 265 located in the third intracellular loop at the cytoplasmic end of the TM6. When the labeled receptor was bound to a full agonist, the intracellular loop domain existed in two distinct conformations. In addition, the conformations induced by a full agonist were distinguishable from those induced by partial agonists. These results suggest that each agonist may have its unique conformational state of receptor. In support, it had also been shown earlier in the β_2 -AR that agonists and antagonists can induce distinct conformational states of the receptor (Gether et al., 1995). Recently, this study was further dissected using a panel of chemically related catechol derivatives to show the specific chemical groups on the agonist responsible for the rapid and slow conformational changes in the receptor (Swaminath et al., 2004) (Figure 2). The conformational changes connected with biologic responses in biochemical assays, suggesting that these conformers were physiologically relevant. Dopamine, which induces only a rapid

Figure 2. Sequential binding model. *A*, arrangement of the TM domains of the β_2 AR as viewed from the extracellular surface. The agonist binding domains are shown in *red* (TM3), *green* (TM5), and *blue* (TM6). *B*, schematic representing structural components of norepinephrine. As shown in *C-E*, in the absence of ligand, the receptor (**R**) is conformationally flexible. Conformational state **R**¹ is stabilized by interactions between TMs 5 and 6 and the catechol ring. The transition to state **R**² occurs when Asp-113 in TM3 binds the amine nitrogen. The transitions from **R** to **R**² are rapid. The slow transition from **R**² to **R**³ involves interactions between the chiral β -hydroxyl and Asn-293 on TM6. Adapted from (Swaminath et al., 2004).



conformational change, activates Gs but not receptor internalization. In contrast, norepinephrine and epinephrine, which induce both rapid and slow conformational changes, could activate both Gs and receptor internalization. These studies demonstrate that the endogenous agonist can induce at least two kinetically and functionally distinct conformational states: a rapid state capable of activating Gs and a slow state required for efficient agonist-induced internalization, suggesting that agonist activation follows a series of multiple conformational states with distinct cellular functions (Swaminath et al., 2004). This study indicates that one endogenous agonist can activate multiple conformers that are physiologically active. Taken together, it is likely that a single agonist can induce or stabilize multiple functionally distinct conformational states (conformer). Such an activation mechanism might be common to other GPCRs, particularly those activated by peptides and protein hormones where there are a larger number of sites of interaction between receptor and agonist. A better understanding of this conformational heterogeneity will facilitate the design of more effective and selective pharmaceuticals.

The three-dimensional structures of GPCRs:

The X-ray structure of bovine rhodopsin is the only crystal structures of any GPCR that includes the 7TM domain (Okada et al., 2002; Palczewski et al., 2000; Teller et al., 2001). The crystal structure of rhodopsin revealed a highly organized heptahelical transmembrane bundle with 11-cis-retinal as a key cofactor involved in maintaining rhodopsin in an inactive state. Ten amino acids were missing in this crystal structure; four from the intracellular loop 3 and the other six from the C-terminal tail (Palczewski et al., 2000). While both the extracellular loop 1 and 3 run along the periphery of the molecule,

a part of extracellular loop 2 folds deeply into the center of rhodopsin (Palczewski et al., 2000). The EL2 is connected to TM3 via a disulfide bridge and fits tightly into this helix bundle (Okada et al., 2001). A significant feature, from the crystal structure, is that the transmembrane helices in rhodopsin are not regular α -helices (Filipek et al., 2003b). In general, the transmembrane helices in rhodopsin are bent and contain segments with 3_{10} - or π -helical conformation. The bovine rhodopsin structure revealed several kinks at proline and other residues in TMs, 12° at Pro53(TM1), 30° at Gly89Gly90(TM2), 12° at Gly120Gly121(TM3), 11° at Ser127(TM3), 30° at Pro170Pro171(TM4), 25° at Phe203(TM5), 15° at His211(TM5), 30° at Pro267(TM6), 24° at Pro291(TM7), and 21° at Pro303(TM7) (Palczewski et al., 2000). One of the strongest distortions is in TM6 induced by the presence of Pro 267 which is a highly conserved residue among GPCRs. It has been shown that mutations at this residue can have long range effects such as conformational changes on IL3 (Ernst et al., 2000). The crystal structure also revealed an unexpected short 8th helix, which runs parallel to the cytoplasmic membrane. This helix and the generated fourth cytoplasmic loop cover part of the binding site for the C-terminus of the G_α subunit and plays a role in G_γ binding.

A set of residues including portions of IL3 and the 8th helix likely undergoes a conformational change upon photoactivation of the chromophore that leads to receptor activation and signal transduction (see review (Filipek et al., 2003a)). The arrangements of the seven transmembrane helices in the crystal structure of rhodopsin confirmed a previously proposed arrangement of TMs (Baldwin et al., 1997). In addition, the three-dimensional structures of rhodopsin was in good agreement with the models of various GPCRs derived from low-resolution structures of rhodopsin (Davies et al., 1996; Krebs et

al., 1998; Unger et al., 1997) and showed all major structural features as predicted from years of biochemical and biophysical studies on wild-type and mutated receptors (Altenbach et al., 1999; Elling and Schwartz, 1996; Liu et al., 1995; Turcatti et al., 1996; Zhou et al., 1994). Details of the molecular mechanism involved in the structural changes will possibly be revealed by solving the structure of the photoreaction intermediates of bovine rhodopsin in three-dimensional crystal structure or by solving structures of GPCRs complexed with heterotrimeric G-proteins.

Intramolecular interactions of GPCRs:

It has been proposed that many GPCRs are constrained by specific intramolecular interactions in the absence of agonist binding and that constitutively active mutations may lead to disruption of such constraints. Specific intramolecular interactions in GPCRs have been proposed according to data obtained from protein engineering experiments. The majority of these intramolecular interactions have been identified in the seven TM domains, including the cytoplasmic helical extension of TM6. Analysis of the crystal structure of bovine rhodopsin suggests that the inactive state conformation of rhodopsin is stabilized by multiple interhelical salt bridges and hydrophobic interactions (Filipek et al., 2003a). There is a Schiff base formation between Glu113 in TM3 and Lys296 in TM7 of bovine rhodopsin in the resting state. Single point mutations of Glu113 and Lys296 in bovine rhodopsin have been found to lead to constitutive receptor activity (Hargrave et al., 1993; Khorana, 1992). Site-directed mutagenesis experiments with bovine rhodopsin have demonstrated that a mutation of Gly 90 to Asp 90 in TM2 can substitute for Schiff base counterion Glu113 in TM3 (Rao et al., 1994). This

demonstrated the proximity of Asp90 and Lys296 in the three-dimensional structure of rhodopsin.

In addition to the mutational analysis, biochemical analyses coupled with mutagenesis have been used to probe intramolecular interaction of GPCRs. The introduction of histidine mutations as an artificial Zn^{2+} binding site has been successfully used to probe the intrahelical interaction between TM3 and TM6 in rhodopsin, parathyroid hormone receptor, and β_2 -adrenergic receptor (Sheikh et al., 1999; Sheikh et al., 1996). By substituting histidines for residues at the cytoplasmic ends of TM3 and TM6 in these GPCRs, it was shown that Zn^{2+} prevented the receptor from activating G protein signaling indicating these residues (Val138, Lys141 and Thr251) interact to stabilize the inactive state of receptor. Another method for probing spatial proximity of residues in TMs is to construct artificial disulfide bridges, which has been applied to bovine rhodopsin (Cai et al., 1997; Yang et al., 1996; Yu et al., 1999), M3 muscarinic receptor (Han et al., 2005; Ward et al., 2002), and Ste2p (Dube et al., 2000). For example, a disulfide bridge was built successfully between Val204C in TM5 and Phe276C in TM6 of bovine rhodopsin (Yu et al., 1999), which previously had been probed by metal ion site construction (Elling et al., 1995) and between Val223C in TM5 and Leu247C in TM6 of Ste2p (Dube et al., 2000).

Whereas numerous intramolecular constraints for inactive state conformations of GPCRs have been identified, much less is known about the intramolecular interactions stabilizing the active state conformations. The identification of intrahelical interactions that appear only in the inactive or activated receptor may provide a molecular basis for understanding mechanistic information relating to GPCR activation pathways.

Ligand binding and conformational changes in GPCR activation:

The superfamily of GPCRs binds and is activated by a wide variety of ligands, ranging in size from small molecules to large glycoproteins. Genetic, biochemical, and biophysical approaches have been used to identify the binding domains for these structurally divergent ligands (Strader et al., 1994). The binding sites of endogenous “small-molecule” ligands in family A receptors, such as for the retinal chromophore in rhodopsin and for catecholamines in the adrenergic receptors are the most widely studied and well characterized of these receptor-binding domains (Ji et al., 1998; Kobilka, 1992; Strader et al., 1995). Recent studies on several peptide receptors such as the receptors for gonadotropin-releasing hormone (Lu et al., 2005; Millar et al., 2004), angiotensin (Bondensgaard et al., 2004; Hunyady et al., 2003; Rihakova et al., 2002), parathyroid hormone (Chorev, 2002; Tsomaia et al., 2004), secretin (Dong et al., 2004), bradykinin B2 (Bellucci et al., 2003; Schroeder et al., 2003), opioids (Janecka et al., 2004; Judd et al., 2003), neurokinin (NK) (Sachon et al., 2003; Ulfers et al., 2002), vasopressin/oxytocin (Breton et al., 2001; Politowska et al., 2002; Wesley et al., 2002), cholecystokinin/gastrin (Silvente-Poirot et al., 1998), and neurotensin 1 (Barroso et al., 2000) supplied valuable information on the binding domains for other classes of GPCRs.

Although the specific ligand receptor interactions vary, certain similarities are observed in ligand GPCR interactions. For example, while small molecules bind within the transmembrane region of receptor, large ligands bind to extracellular loops. Peptide ligands exhibit a combined binding mode whereby they bind primarily to the extracellular loops while part of the structure penetrates the transmembrane region and interacts with residues buried in lipids (Fanelli et al., 1999; Fathy et al., 1998; Flanagan et al., 2000).

The binding of α -factor to the Ste2p is detailed in the Chapter 2 of Part I in this dissertation.

Studies suggested that relative movements of the transmembrane domains occurred during agonist-induced GPCR activation. In bovine rhodopsin, site-directed spin-labeling experiments have been performed to examine structural changes in the intracellular extensions of TM 1–7 and helix 8 on photoactivation (Altenbach et al., 1999; Farrens et al., 1996). According to these experiments, photoactivation has been shown to induce movements of TMs 3 and 6, relative to the rest of the 7-helix bundle, a significant rigid body movement of TM6 in a counterclockwise direction when viewed from the extracellular side, and a movement of the cytoplasmic end of TM6 away from TM3(Farrens et al., 1996). Moreover, disulfide cross-linking of these 2 TMs(Farrens et al., 1996) or Zn^{2+} binding to an artificial Zn^{2+} binding site between these TMs (Sheikh et al., 1999) prevents the activation of receptor, further supporting the relevance of their movement in the activation of rhodopsin.

Similar to rhodopsin, the evidence for a relative movement of TM3 and TM6 in the β_2 adrenergic receptor (AR) was provided by direct fluorescence labeling of specific positions in TM3 and TM6(Gether, 2000) and by monitoring the accessibility of cysteine residues to a hydrophilic sulfhydryl-specific reagent during receptor activation(Javitch et al., 1997; Jensen et al., 2001). Recently, agonist activation of the leukotriene B4 BLT_1 receptor in solution has been shown to lead to an increase in accessibility of residues in the intracellular end of TM6 (Baneres and Parello, 2003). These data support the hypothesis that agonist triggering might lead to exposure of a cytoplasmic binding site in between the 7 TMs by outward movement of at least 3 TMs, allowing heterotrimeric G-

proteins to associate with receptors. These results suggested that there are changes of intramolecular interactions allowing the receptor to undergo conversion between its inactive and active state.

CHAPTER 2

α -factor Pheromone and its G Protein-coupled Receptor (Ste2p) in *Saccharomyces cerevisiae*

S. cerevisiae is a budding yeast which is the most intensively studied eukaryotic model organism in molecular and cell biology. The *S. cerevisiae* α -factor receptor system has been used as an ideal system to understand GPCR structure-function as a model for peptide hormones (Dohlman et al., 1991). The power of yeast genetic was used to examine the structure –function relationship of α -factor receptor.

Complete genome sequence of *S. cerevisiae* revealed three GPCRs (Goffeau et al., 1996). Two of these are pheromone receptors (Ste2p and Ste3p) and the other is the Gpr1p, which is a carbohydrate sensor. Though the pheromone and carbohydrate-sensing pathways share some down-stream components, there appears to be no cross-talk between the two systems at the cytoplasmic membrane as the pheromone receptors and the Gpr1p couple to two different G-proteins (Gpa1p and Gpa2p, respectively) (Lorenz et al., 2000a; Lorenz et al., 2000b). Therefore, *S. cerevisiae* provides an ideal system to study the relation between a peptide ligand and its GPCR in the absence of interfering biological complexities.

Mating response in Saccharomyces cerevisiae:

S. cerevisiae may exist as a haploid or diploid cell (Sprague et al., 1983). The haploid cells have two sexual phenotypes characterized by the expression of a set of genes involved in mating that are not expressed in diploids. Mating of the *MATa* and *MATα* haploid cells of *S. cerevisiae* is initiated by the secretion of diffusible peptide pheromones that are recognized by receptors on the opposite cell type (for review see (Dohlman, 2002; Dohlman et al., 1991) and see Figure 3 for schematic representation) . The mating pheromones are essential to trigger the mating cycle, therefore, the cells that cannot produce these molecules or lack their cognate receptors Ste2p or Ste3p become sterile. α -Factor, a tridecapeptide pheromone synthesized constitutively by *MATα* cells and acting on *MATa* cells is encoded by two genes *MFα1* and *MFα2* (Kurjan and Herskowitz, 1982). The tridecapeptide α -factor pheromone WHWLQLKPGQPMY peptide was chemically synthesized and found to exhibit all the properties of the natural pheromone (Ciejek et al., 1977; Masui et al., 1977). The **a**-factor of *S. cerevisiae* is a dodecapeptide pheromone (YIIKGVFWDPAC[Farnesyl]-OCH₃) (Anderegg et al., 1988), and it has been known that post-translational modification with a farnesyl isoprenoid and carboxymethyl group is required for full biological activity (Marcus et al., 1991).

α -factor pheromone binding to Ste2p its GPCR induces signal transduction to regulate the activity of transcription factors required for the expression of components of the mating pathway (Figure 4)(Banuett, 1998; Herskowitz, 1995; Madden and Snyder, 1998). This results in a number of biological responses. The cells synthesize cell surface molecules necessary for fusion with their mating partners, arrest in the G1 phase of the

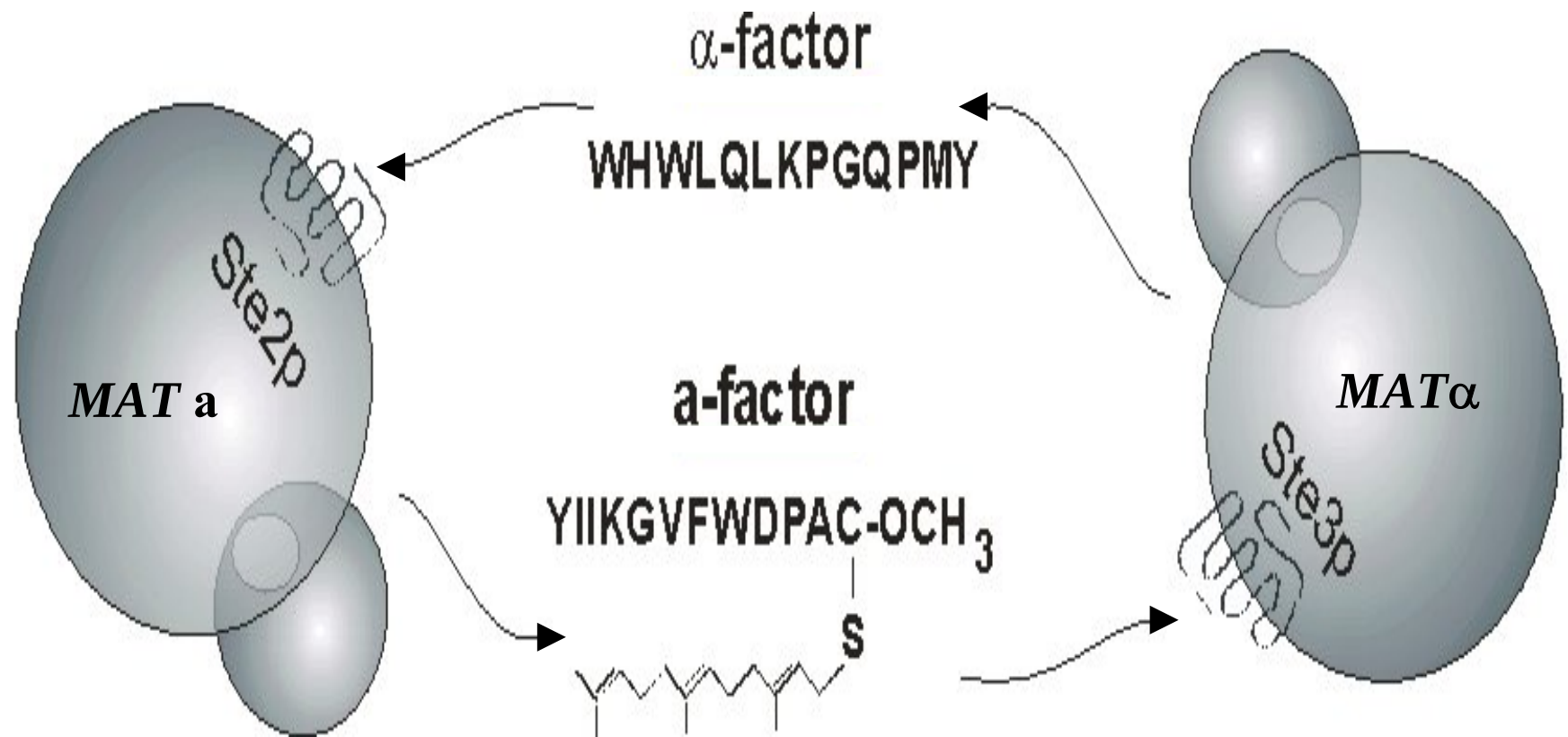
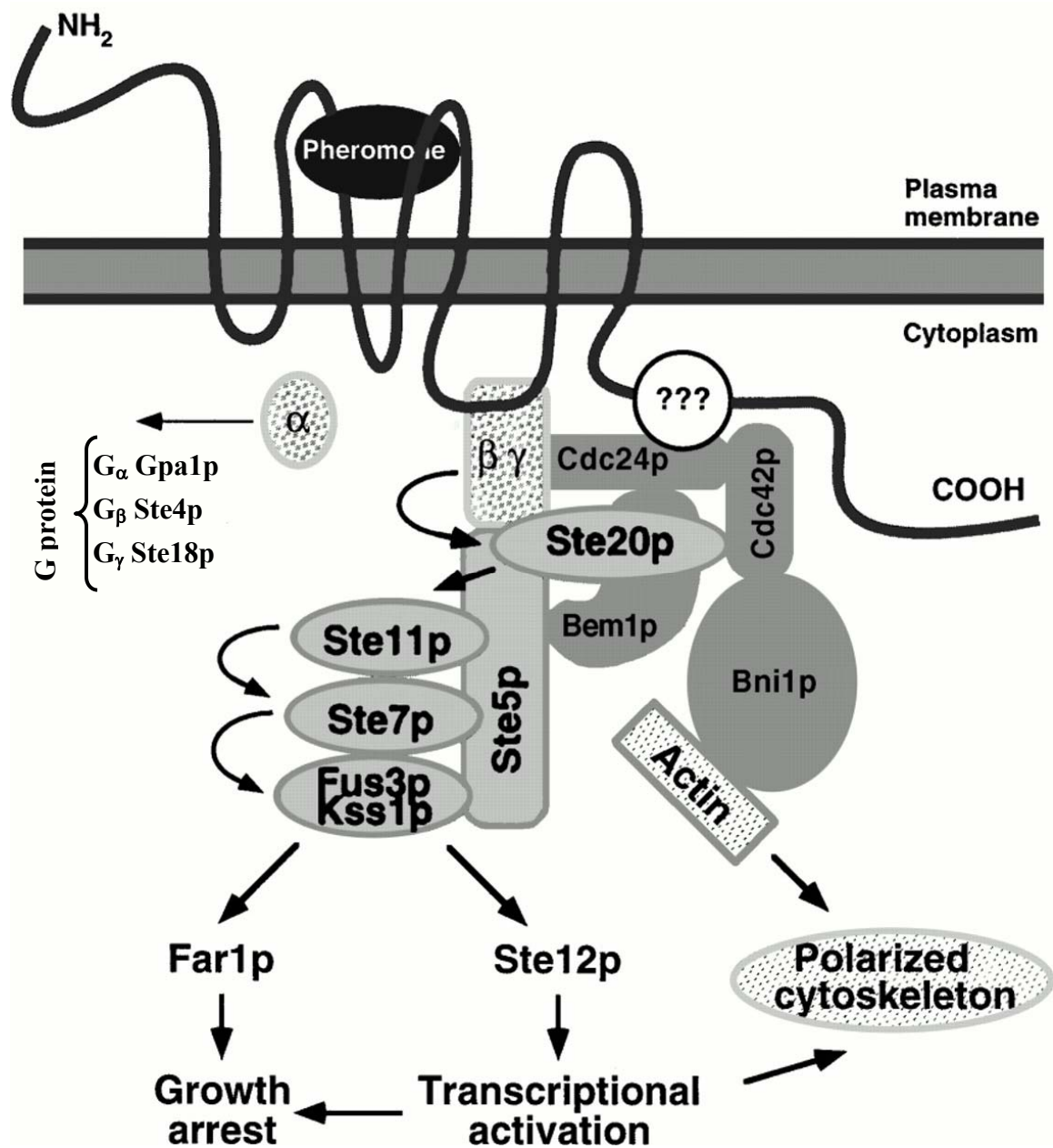


Figure 3: Pheromone mediated mating in *Saccharomyces cerevisiae*. Schematic representation of pheromone/receptor mediated communication between MATa and MATα haploid cells prior to mating.

Figure 4. *Saccharomyces cerevisiae* mating pathway. Binding of the pheromone to the receptor stimulates downstream responses such as transcriptional activation of pheromone induced genes, cell cycle arrest, and polarization of the cytoskeleton. Ste5p is the scaffold protein, Ste20p is mitogen-activated quadruple protein kinase (MKKKK), Ste11p is MKKK, Ste7p is MKK, Fus3p and Kss1p are MAPK. A proposed but unidentified protein is denoted as ??? in a white circle. Adapted from (Madden and Snyder, 1998).

Saccharomyces cerevisiae pheromone receptor (Ste2p or Ste3p)



growth cycle by eliminating the function of G1 cyclin complex and Cdc28 protein kinase to obtain synchrony for mating, form mating projections that are involved in the fusion process, thereby exhibiting a marked change in shape, and activate a number of genes that are necessary for sexual conjugation (Dohlman et al., 1991; Marsh et al., 1991). Many of these cellular responses have been used to measure the activity of this receptor and potency of pheromones and their analogs. Growth arrest (Halo) assay and β -galactosidase activity assay generated by a *FUS1-lacZ* construct are the examples of methods based on the cellular response.

Regulation of GPCRs signaling:

One ubiquitous feature of signaling through G-protein-coupled receptors is the short-term and long-term loss of cellular sensitivity following a stimulus, a phenomenon referred as desensitization (Ferguson, 2001). Typically, activation of a GPCR results in : (1) activation and inhibition of specific signaling pathways in the cell, (2) desensitization mediated by phosphorylation of GPCRs by G-protein-coupled receptor kinases (GRKs) followed by effector protein binding to GPCRs that uncouple the receptor at the plasma membrane from the G-protein, and (3) endocytosis of the receptor followed by postendocytic sorting of the receptor either back (4) to the plasma membrane (receptor recycling) or (5) to lysosomes for degradation. Short-term desensitization may also involve phosphorylation of GPCRs by second messenger-dependent protein kinases, which uncouples GPCRs at the plasma membrane from G-proteins. Long-term desensitization may include one or more of the following processes: (1) down-regulation of receptors and/or downstream components in the signaling pathway (e.g., G-proteins

and effector proteins) by proteolytic protein degradation in lysosomes or at the plasma membrane, (2) decreased synthesis of receptor protein and/or downstream proteins, and (3) enhanced mRNA degradation. Like many other GPCRs, Ste2p is also regulated by general GPCR signaling mechanisms. Among the general regulation mechanisms, three distinct processes contribute to α -factor receptor regulation. These are : (1) rapid phosphorylation and desensitization (Chen and Konopka, 1996), (2) internalization and recycling (Hicke and Riezman, 1996) and (3) down-regulation and degradation (Mulholland et al., 1999; Stefan and Blumer, 1999).

In addition to the regulation of GPCRs signaling at the receptor level, the mating signaling in yeast is further controlled by the expression levels or the activities of the downstream elements in the signaling pathway. Since the pheromone response is transient, yeast cells should reenter the cell cycle in the absence of pheromone. One of the well characterized regulators is Sst2p, a yeast homologue of regulators of G protein signaling (RGS), which effect the G protein signaling by accelerating the GTP hydrolysis and promoting re-association of the heterotrimer (Dohlman and Thorner, 1997) (Dohlman, 2002). Sst2p is regulated by proteolytic cleavage and this process is regulated by the signaling pathway (Hoffman et al., 2000). The phosphatase Msg5p and Mpt5p have been reported that have a role in adaptive mechanism at higher concentrations of pheromone (Zhou et al., 1999). Additionally, expression of some proteases like Bar1p (Sst1p), which specifically cleaves α -factor pheromone, adds further complexity to the regulation of mating (Ballensiefen and Schmitt, 1997).

Structure-function analysis of α -factor pheromone:

The α -factor is a tridecapeptide secreted by *MAT α* cells and has the sequence of Trp¹His²Trp³Leu⁴Gln⁵Leu⁶Lys⁷Pro⁸Gly⁹Gln¹⁰Pro¹¹Met¹²Tyr¹³. The pheromone binds to Ste2p that is encoded by the *STE2* gene and expressed on the cell surface of *MAT α* cells. Extensive structure-function analyses coupled with receptor mutagenesis have provided insights into the structural basis of α -factor activity (Abel et al., 1998; Eriotou-Bargiota et al., 1992; Liu et al., 2000; Xue et al., 1996; Yang et al., 1995). Systematic Ala-substituted α -factor analogs have been used to study the structure-function of the α -factor pheromone (Abel et al., 1998). Among the D-Ala series of α -factor analogs, specifically [D-Ala²] α -factor, [D-Ala³] α -factor and [D-Ala⁴] α -factor revealed no measurable biological activity. On the other hand, these peptides bound relatively strongly to Ste2p and antagonized the biological activity of α -factor in halo and *lac Z* gene induction assays. These results were consistent with previous studies from our laboratory showing that most of the antagonists discovered involved changes in residues near the amine terminus. Based on these findings it seemed clear that residues near the amine terminus play an important role in Ste2p activation or in stabilizing the activated state of this receptor.

The center of α -factor contains a Pro⁸Gly⁹ sequence. Biochemical and biophysical analyses on α -factor and its constrained analogs provided evidence that a β -turn involving the Pro⁸Gly⁹ residues is an important determinant of the biologically active structure of the pheromone (Antohi et al., 1998; Xue et al., 1989; Xue et al., 1996; Yang et al., 1995; Zhang et al., 1998). On the other hand the residues at the carboxyl terminus of α -factor

strongly influence binding. Replacement of the carboxyl terminus of α -factor by either L- or D-Ala caused decreases in affinity of up to 3000-fold (Abel et al., 1998). Removal of the carboxyl terminal residues resulted in pheromones with drastically reduced receptor affinity and, in some cases, also with complete loss of affinity. A genetic study of structure-activity relationships of α -factor also showed functional separation of parts of this ligand (Manfredi et al., 1996). Taken together, three functional domains were assigned in the proposed model of the functional domains of α -factor (Figure 5) (Abel et al., 1998). The separation of functional domains for binding and activation has been documented in other peptide hormones (Carpenter et al., 1998; de Gasparo et al., 2000; Greenberg et al., 2000).

Considering the multi-state model of receptor (discussed in chapter 1: receptor theory), the separation of a binding domain and an activation domain of α -factor suggests that there is a sequential binding of the pheromone to its receptor. One functional group of α -factor might initially interact with the receptor (possibly binding domain). Following the initial binding of binding domain of α -factor, binding of remaining groups (loop domain of α -factor) might occur in a sequential manner as a result of movement of TM domains to positions that permit interaction with functional group (possibly signaling domain of α -factor). The bend region of α -factor may give the structural flexibility to the signaling domain so as to fit to active receptor conformation. In correspond with this hypothesis, using a fluorescent alpha-factor analogue [K(7)(NBD), Nle(12)] coupled with flow cytometry and fluorescence microscopy showed that the α -factor associates with

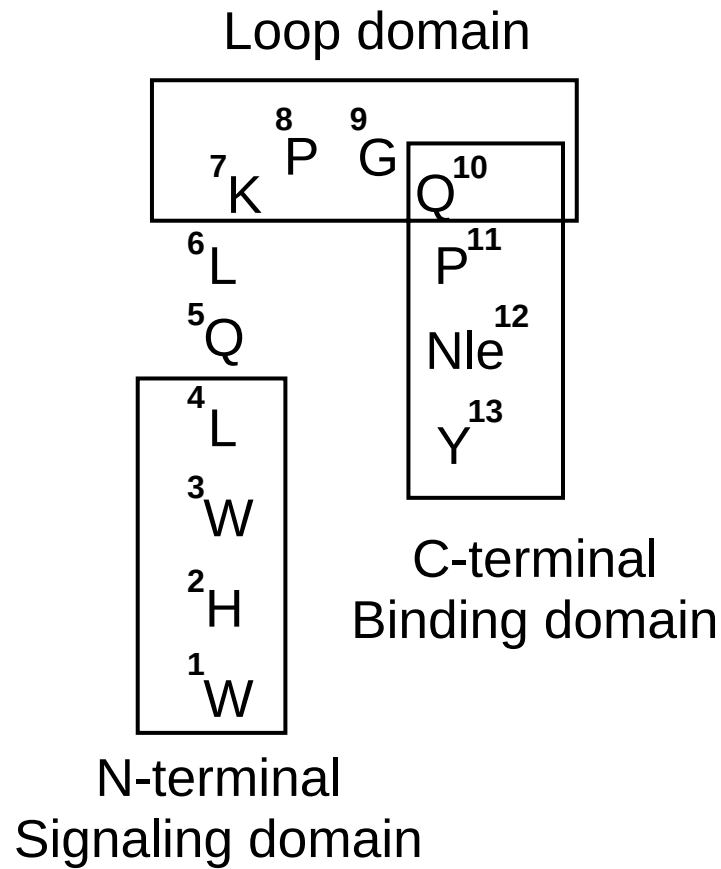


Figure 5. Functional domains of α -factor. The residues at the N-terminus appeared to mainly function in activation of the receptor signaling, the residues at the C-terminus strongly contribute to the binding affinity. The loop domain corresponds to residues of the peptide which are thought to produce a bend in the α -factor structure. Figure adapted from (Abel et al., 1998).

receptor via a two-step process involving an initial interaction that places the fluorophore in a hydrophobic environment, followed by a conversion to a state in which the fluorophore moves to a more polar environment (Bajaj et al., 2004).

Interactions between α -factor pheromone and its GPCR, Ste2p:

One of the important objectives of studies on GPCRs is learning how ligands bind and how this recognition results in information transfer. Since pheromones can be designed with chemically or photochemically active groups that promote crosslinking to the receptor, crosslinking to the receptor was used to identify the pheromone binding site.

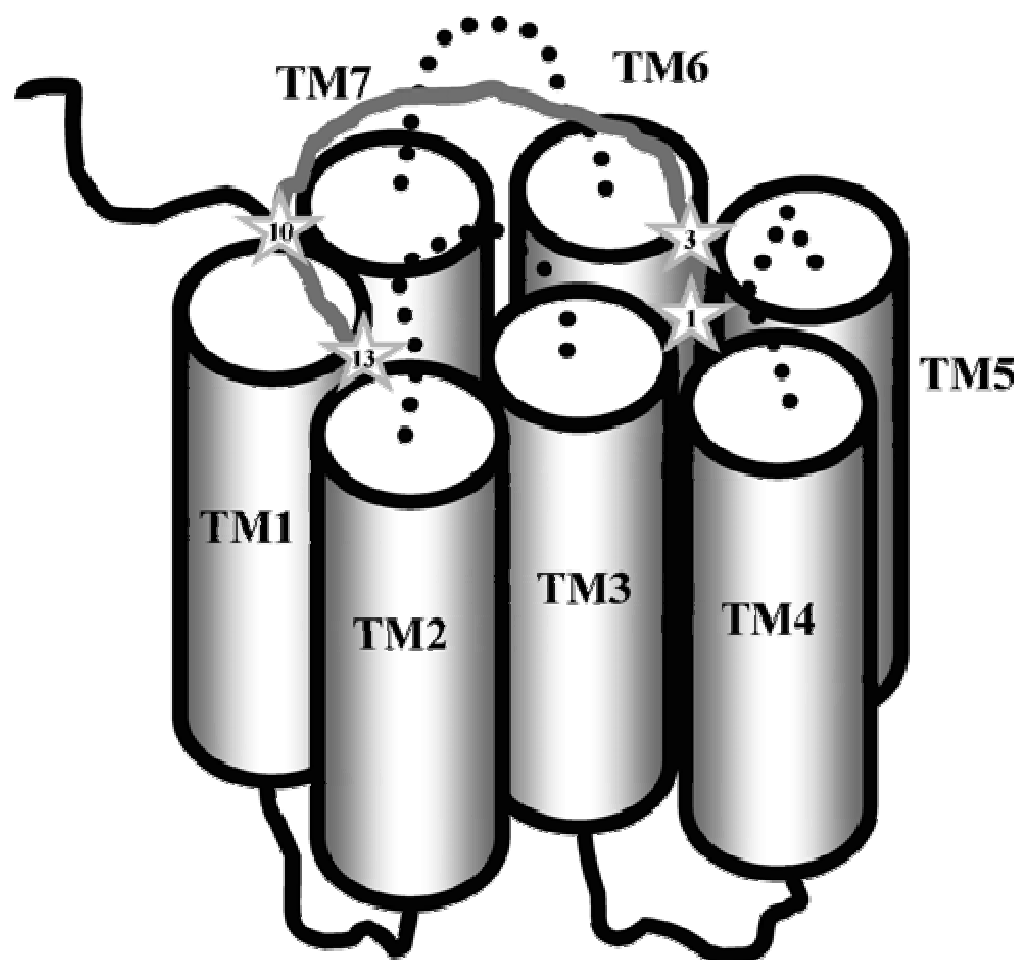
A series of α -factor analogs in which *p*-benzoyl-L-phenylalanine (Bpa), a photoactivatable group, was scanned throughout the backbone were synthesized (Henry et al., 2002). Chemical and enzymatic fragmentation of the receptor/radioprobe complex ([Bpa¹, Tyr³(¹²⁵I), Arg⁷, Phe¹³] α -factor-crosslinked-Ste2p) indicated that cross-linking occurred on a portion of Ste2p spanning residues 251-294 which encompasses a portion of transmembrane domain 6, the extracellular loop between transmembrane domains 6 and 7, and a portion of transmembrane domain 7 (Henry et al., 2002). This was the first determination of a specific contact region between a Class IV GPCR and its ligand. Similarly, analogues of α -factor containing Bpa and biotin as a tag, were synthesized to identify the pheromone binding site to receptor (Son et al., 2004). Digestion of Bpa(1), Bpa(3), and Bpa(13) cross-linked receptors with chemical and enzymatic reagents suggested that the N-terminus of the pheromone interacts with a binding domain consisting of residues from the extracellular ends of TM5-TM7 and portions of EL2 and

EL3 close to these TMs and that there is a direct interaction between the position 13 side chain and a region of Ste2p (F55-R58) at the extracellular end of TM1 (Son et al., 2004).

In addition to crosslinking studies, chimeric receptors between *S. cerevisiae* and *S. kluyveri* α -factor receptors also supplied important information to understand the ligand receptor interaction(s) (Sen and Marsh, 1995; Sen et al., 1997). A series of studies using constructed chimeric receptors indicated that a significant element of the respective α -factor recognition by *S. cerevisiae* and *S. kluyveri* receptors resides in positions 47–49 of the respective GPCRs. Scrutiny of the sequence of these regions indicated that residues Lys-Lys-Ile in the *S. kluyveri* Ste2p receptor are Ser-Thr-Val in the *S. cerevisiae* Ste2p. In position 10, *S. kluyveri* α -factor and the *S. cerevisiae* α -factor have Glu and Gln, respectively, suggested that possible interaction between positions 47–49 of Ste2p and position 10 of α -factor. The detailed and elegant analyses using position 10 α -factor analogs coupled with receptor mutagenesis revealed that the side chain of the tenth residue of α -factor is in close proximity with the side chains of residues 47 and/or 48 of Ste2p when the pheromone is bound to the receptor (Lee et al., 2001).

Taking together crosslinking and chimeric receptor studies, a model for α -factor bound to Ste2p was proposed (Figure 6). The model places α -factor bent around the Pro-Gly center of the peptide with the Lys side chain facing away from the transmembrane domains and interacting with a binding pocket formed by the extracellular loops. The Gln¹⁰ side chain is proximal to residues 47 and 48 near the extracellular side of TM1, whereas the Trp¹ side chain is near a pocket formed by TM6-E3-TM7. It is likely that Trp¹ and Trp³ interact with an aromatic group near the interface of the transmembrane

Figure 6. Working model for fitting of the α -factor pheromone into a ligand binding site on its GPCR Ste2p. This schematic shows a counterclockwise orientation of TMs of the Ste2p. Two amino acid residues (Ser 47 and Thr 48), were proposed that there are in the proximity to Gln¹⁰ of bound α -factor. The Tyr¹³ side chain of bound α -factor interacts with a region of Ste2p (F55-R58) at the extracellular end of TM1. The Trp¹ and Trp³ side chain of bound α -factor interact with the extracellular ends of TM5-TM7. The α -factor and the receptor extracellular loops are represented by the gray curve and dotted lines, respectively. Contact residues are denoted with stars with the residue number of the pheromone. This figure was adapted from (Son et al., 2004).



domains and the outside surface of the membrane. The Tyr¹³ side chain interact with a region of Ste2p (F55-R58) at the extracellular end of TM1.

S. cerevisiae α -factor system is an ideal system for the study of understanding peptide hormone to its GPCRs and receptor activation mechanisms. However, the lack of information about the α -factor binding site to Ste2p make it difficult to understand the mechanism of receptor activation. Mapping the α -factor binding region(s) in Ste2p could supply great insights for understanding the overall mechanism of GPCR activation by peptide ligands. Recently, our group using site-directed mutagenesis of Ste2p and binding assays with different α -factor analogs suggested that portions of TM1 and TM6 were important for ligand interaction (Abel et al., 1998) and that the tenth residue of α -factor is in close proximity to Ser⁴⁷ and Thr⁴⁸ in TM1 of Ste2p (Lee et al., 2001). These results suggested that N-terminus of α -factor, which is known as signaling domain, binds to TM5 or TM6 of Ste2p. Based on this hypothesis, we studied the fifth and sixth transmembrane domain using systematic ala-scan mutagenesis coupled with biological and biochemical assays.

In this dissertation, Part II describes that the Tyr 266 in TM6 play a key regulatory role in selective recognition of the α -factor as well as receptor activation. Part III describes that Asn 205 in TM5 has similar function to that of the residue 266 in TM6 and these residues interacts in an activated state of receptor. Finally, Part IV is an overall evaluation of these studies, contributions to the working model of activation mechanisms of Ste2p and suggested future studies that could be performed to improve the model of mechanism of receptor activation.

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PART II

Tyr266 in the Sixth Transmembrane Domain of the Yeast α -Factor

Receptor Plays Key Roles in Receptor Activation and Ligand

Specificity^s

§Part II was published in its entirety as Byung-Kwon Lee, Yong-Hun Lee Melinda Hauser, Cagdas D. Son, Sanjay Khare, Fred Naider, and Jeffrey M. Becker (2002) *Biochemistry*, 41 (46), pp. 13681 -13689. Dr. Naider's laboratory was responsible for synthesis and purification of the peptides used in the study. Dr. Byung-Kwon Lee was responsible for the mutagenesis of TM6 and characterization of mutant receptors. Yong-Hun Lee was involved in the characterization of mutant receptor using binding assay and carried out Dominant-negative experiment.

CHAPTER 1

Introduction

Receptors in the cell membrane coupled to heterotrimeric GTP-binding proteins (G protein) control cellular responses to a variety of stimuli including hormones, neurotransmitters, light, and odorants (Bockaert et al., 2002; Wess, 1997). Within the human genome, there are hundreds of different G protein-coupled receptors (GPCRs) (Lander et al., 2001; Venter et al., 2001). Although differing in primary amino acid sequence, all GPCRs have the same overall topology: seven transmembrane domains connected by intracellular and extracellular loops, an extracellular N-terminus, and an intracellular C-terminus (Baldwin, 1993; Hall et al., 1999).

Ste2p of the yeast *Saccharomyces cerevisiae* has been used frequently as a model for studying the structure and function of GPCRs (Dohlman et al., 1991; Leberer et al., 1997). This receptor recognizes the tridecapeptide α -factor pheromone (WHWLQLKPGQPMY). Binding of α -factor to Ste2p activates a G protein-mediated signaling cascade that is highly conserved within eukaryotic signaling pathways. In addition to using Ste2p as a model GPCR, α -factor itself has been studied extensively as a representative GPCR peptide ligand. Covalently constrained analogues (Yang et al., 1995), peptidomimetic analogues (Zhang et al., 1998), analogues with residue substitutions (Levin et al., 1993; Liu et al., 2000) including Ala-scanning (Abel et al., 1998b), and antagonists (Eriotou-Bargiota et al., 1992) have been synthesized and studied with regard to their receptor binding, ability to initiate signal transduction, and

conformation. These studies have revealed residues or segments of α -factor with dominant roles in forming a β -turn in the middle of the bound pheromone (residues 7-10), in binding to the receptor (residues 10-13), and in stabilizing the active conformation of the receptor to initiate signal transduction (residues 1-4).

Recently we initiated studies to define specific residue-to-residue interactions between α -factor and Ste2p. We reported that Q10 of α -factor closely interacted with S47 and T48 of Ste2p at the junction between TM1 (transmembrane one) and the N-terminal tail (Lee et al., 2001), and photoaffinity labeling (Henry et al., 2002) showed that the N-terminus of α -factor interacted with a region of Ste2p encompassing TM6-EL3-TM7 (transmembrane domain six-extracellular loop three-transmembrane domain seven). On the basis of these recent results, we proposed a model in which the C-terminus of α -factor is positioned near TM1, while the N-terminus of α -factor binds in a pocket composed of TM3, TM5, and TM6 (Lee et al., 2001).

A number of studies with GPCRs, including Ste2p, have demonstrated the involvement of TM5 and TM6 in receptor activation. In the rhodopsin-like family of G protein-coupled receptors TM6 has been shown to play a major role in ligand binding and in producing the resulting functional response (Gether and Kobilka, 1998). In Ste2p, analysis of constitutively active mutants indicated that residues in the sixth transmembrane domain play a key role in receptor activation (Konopka et al., 1996; Sommers et al., 2000). TM5 and TM6 of Ste2p have been implicated as important for receptor activation because the third intracellular loop, flanked by TM5 and TM6, interacts with the $G\alpha$ protein (Clark et al., 1994; Stefan and Blumer, 1994). Extensive

screens to identify dominant-negative mutations in the receptor revealed that most mutants exhibiting this phenotype were mapped to the extracellular ends of the transmembrane domains, especially in TM5 and TM6 (Dosil et al., 1998; Dosil et al., 2000; Leavitt et al., 1999). Prior work revealed that mutation Y266C in TM6 of Ste2p resulted in a receptor that retained significant binding affinity but was defective in signaling (Dosil et al., 1998). Furthermore, the Y266C receptor exhibited a strong dominant-negative phenotype when coexpressed with WT; this phenotype could be reversed by concomitant overexpression of the G protein subunits. This same dominant-negative receptor had also been identified as a second-site suppressor of the mutant E143K in TM3 of Ste2p (Sommers and Dumont, 1997), suggesting that the presence of Y266C results in alterations in the packing of the transmembrane domains, resulting in suppression.

To more fully test our hypothesis that the extracellular portion of TM6 of Ste2p interacts with the N-terminus of α -factor, we carried out the present study. Part II of this dissertation describes the effects resulting from mutation of nine residues in a portion of TM6 adjoining EL3 adjacent to the membrane extracellular interface. Focusing on the special properties of the Y266A mutation, we carried out detailed binding and biological studies on these mutated receptors using a series of Ala-scanned α -factor analogues previously synthesized in our laboratory. Part II of this dissertation results indicate that Y266 is a strategic residue critical for receptor function and for the recognition of the N-terminus of α -factor.

CHAPTER 2

Materials and Methods

Strains and plasmids:

The strain LM102 (Sen and Marsh, 1994) was used for receptor binding and growth arrest assay. The relevant genotype is *MAT α , bar1 leu2 ura3 FUS1-lacZ::URA3 ste2-dl* (α -factor receptor coding region deletion). LM102 was used as the recipient for plasmids encoding WT and site-directed mutants of *STE2*. Constructs expressed in this strain background were then used to measure pheromone-induced growth arrest (halo assay) and to determine pheromone binding. The strain carried the *bar1* mutant allele inactivating the Bar1p protease that is responsible for degradation of α -factor. The *CEN*-based yeast/bacterial shuttle vector, pGA314.WT (Abel et al., 1998a), containing a *TRP1* selectable marker and WT *STE2* under the control of its native promoter was used as a template for the site-directed mutagenesis of the receptor. This plasmid contains a strong terminator sequence, resulting in high transcription efficiency and overexpression of Ste2p. To test for receptor mutants that exhibit a dominant-negative phenotype, pGA314 plasmids encoding either WT or site-directed mutations in *STE2* were transformed into the strain LM23-3az, a parental strain of LM102, that has a genomic copy of the WT *STE2* gene in its native chromosomal locus (Sen and Marsh, 1994). Additionally, the dominant-negative phenotype was assessed

in the presence of the high-copy (2 μ m) plasmid pMD82, encoding the G protein α , β , and γ subunits (*GPA1*, *STE4*, and *STE18*) under the control of their native promoters (Dosil et al., 1998). Since pMD82 and the pGA314-based plasmids contained different selectable markers (*LEU2* and *TRP1*, respectively) both could be maintained simultaneously in LM23-3az. For the limited trypsin digest experiments, the strain BJS2-1 was generated from the protease-deficient strain BJ2168 (Jones, 1991) by disrupting the chromosomal copy of *STE2* with the kanamycin resistance cassette as described by Guldener et al. (Guldener et al., 1996). The relevant genotype of BJS2-1 is *MATa prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 ste2::kan^r*. Limited trypsin digests were also completed in a diploid host strain, CG990 (*MATa/ α ura3-52 lys2-801 trp1-~~A~~1 ade2-101*). This strain was transformed with the plasmid pNED (David et al., 1997), a high-copy-number plasmid encoding epitope-tagged *STE2* under the control of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. PCR primers were designed to amplify a 400 bp region from pGA314 containing the Y266A mutation. The mutation was engineered into pNED by in vivo ligation (Oldenburg et al., 1997) and transformed into CG990.

Site-directed mutagenesis:

Single-stranded phagemid DNA of pGA314.WT was prepared by infecting *E. coli* strain CJ236 (*ung⁻, dut⁻*) carrying pGA314.WT with the helper phage M13KO7 (Vieira and Messing, 1987). Oligonucleotide-directed mutagenesis of single-stranded phagemid DNA was completed as described by Kunkel et al. (Kunkel et al., 1987). The product of the mutagenesis reaction mixture was transformed into *E. coli* strain DH5 α .

(Invitrogen, Carlsbad, CA), and transformants were selected on medium containing ampicillin. Plasmids were then isolated from transformants using the Wizard Miniprep kit (Promega, Madison, WI). After sequence confirmation, constructs were transformed into yeast strain LM102 (*ste2*-deletion strain), and transformants were selected by their growth in the media lacking tryptophan. All primers were purchased from Sigma/Genosys (The Woodlands, TX). DNA sequencing was carried out in the Molecular Biology Resource Facility located on the campus of the University of Tennessee.

Peptides used in this study:

L-Norleucine, which is isosteric with L-methionine, was incorporated at position 12 to replace L-methionine in all of the analogues. The insertion of Nle in place of methionine improved the synthesis and the stability of the resulting peptide. This replacement to form [Nle¹²] α -factor was previously shown to result in an analogue with activity and binding affinity equal to those of the native pheromone (Raths et al., 1988). Syntheses of α -factor and its analogues used in this study were described previously (Abel et al., 1998b; Eriotou-Bargiota et al., 1992). The structures of all peptides were verified by amino acid analysis and mass spectrometry. For [³H] α -factor, synthetic [Nle¹²] α -factor was labeled by reduction of dehydropyrroline-containing α -factor by the TR3 hydrogenation procedure of Amersham Biosciences (Piscataway, NJ) and purified as described previously (Raths et al., 1988). The photoaffinity labeling analogue Bpa¹Y³(¹²⁵I)R⁷F¹³ α -factor was prepared as described previously (Henry et al., 2002).

Growth arrest (Halo) assay:

Yeast nitrogen base medium with ammonium sulfate without amino acids (Difco, Kansas City, MO) with 2% glucose (SD medium), supplemented with histidine (20 µg/mL), leucine (30 µg/mL), and methionine (20 µg/mL), was overlaid with 4 mL of cell suspension (2.5×10^5 cells/mL, 1.1% Nobel agar). Filter disks (sterile blanks from Difco), 6 mm in diameter, were impregnated with 10 µL portions of peptide solutions at various concentrations and placed onto the overlay. The plates were incubated at 30 °C for 24-36 h and then observed for clear zones (halos) around the disks. The data were expressed as the diameter of the halo including the diameter of the disk. All assays were completed at least three times, with no more than a 2 mm variation in halo size for a particular amount of each peptide. The data were plotted as halo size versus the amount of peptide, and linearized by regression analysis using Prism software (GraphPad, San Diego, CA). To compare the relative activities of α -factor with different receptors, the amount of peptide producing a halo of a particular size was determined from the regression line of dose-response curves.

Binding assays:

Saturation and competition binding assays were performed using tritiated α -factor as previously described (Lee et al., 2001). Each experiment was repeated at least three times, and each data point measured in triplicate. Data curves were fit using single-site competition, nonlinear regression analysis software (GraphPad Prism). K_D and B_{max}

values were determined from saturation binding assays for WT and all receptors mutated at Y266. K_i values with WT and Y266A receptor for a series of α -factor analogues were calculated using the equation of Cheng and Prusoff (Cheng and Prusoff, 1973), where $K_i = EC_{50}/(1 + [\text{ligand}]/K_D)$. For the Ala-scanned series of receptors (F262A, I263A, L264A, A265G, S267A, L268A, K269A, and P270A) where K_D values were not determined, IC_{50} values were reported as determined directly by nonlinear regression of the competition binding data.

Trypsin digest:

Plasmid pGA314 encoding WT or Y266A mutant *STE2* was transformed into strain BJS2-1. In a parallel set of experiments, plasmid pNED encoding WT or Y266A mutant *STE2* was transformed into the diploid strain CG990. Cells were grown in MLT medium without tryptophan to maintain selection for the plasmid. Membranes were prepared from mid-log-phase cells. All steps were performed at 4 °C. The cells were lysed with glass beads in buffer (50 mM HEPES, pH 7.5, 5 mM EDTA) supplemented with protease inhibitors (1.5 µg/mL PMSF, 1 µg/mL leupeptin, 1 µg/mL pepstatin A) using a cell homogenizer (B. Braun Instruments, Allentown, PA). Unbroken cells, cell wall debris, and glass beads were removed by centrifugation (700g, 30 min) and the membranes collected by centrifuging the supernatant (100000g, 60 min). The resulting membrane pellet was resuspended in buffer (50 mM HEPES, pH 7.5, 20% glycerol) plus protease inhibitors and protein concentration determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). The membranes were stored at -80 °C until use.

Membranes (80 µg per sample) were harvested, washed, and resuspended in YM1-i (Abel et al., 1998a) and incubated with either α -factor or the antagonist [desW¹desH²] α -factor at a final concentration of 5 µM for 30 min at room temperature. A control with no pheromone supplement was run in parallel. Tryptic digestion was performed essentially as previously described (Bukusoglu and Jenness, 1996). Upon completion of the incubation interval, membranes were collected by centrifugation (15000g, 10 min, 4 °C) and resuspended in 500 µL of trypsin digest buffer (1 mM magnesium acetate, 0.1 mM EDTA, 7.6% glycerol, 10 mM MOPS, pH 7.0, 0.1 mM dithiothreitol) supplemented with the appropriate pheromone ligand at 5 µM. TPCK-treated trypsin (Sigma Chemical, St. Louis, MO) was added to a final concentration of 30 µg/mL, and samples were incubated for various times at 30 °C. Parallel reactions were run on samples in the absence of trypsin to control for degradation that might occur during incubation. Digestion was terminated by addition of NaOH to a final concentration of 0.015 N. Membranes were collected by centrifugation in an Airfuge ultracentrifuge (Beckman Coulter, Fullerton, CA) and solubilized in SDS sample buffer. Proteins were resolved by SDS-PAGE (15% acrylamide) using prestained Kaleidoscope molecular weight markers (Bio-Rad Laboratories, Hercules, CA) transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA) and probed with affinity-purified antireceptor antiserum directed against the N-terminal domain of the α -factor receptor (Konopka et al., 1988), which were kindly provided by James Konopka, State University of New York, Stony Brook, NY. The bands were visualized with the Western lightning chemiluminescent detection system (Perkin-Elmer Life Sciences, Boston, MA).

CHAPTER 3

Results

Biological activity and binding of Ste2p variants produced by mutagenesis of residues 262-270:

Nine amino acids near the junction between TM6 and extracellular loop 3 (residues 262-270) were mutated by site-directed mutagenesis (Figure 1). All residues were mutated to alanine except residue 265, which is alanine in the native protein; in this case the residue was mutated to glycine. The biological activity of each mutant receptor was measured by the growth arrest assay, as described in the Experimental Procedures. The response of cells expressing each of the receptor constructs, except Y266A, increased linearly throughout the range of pheromone tested (Figure 2A). Excluding Y266A, the amount of α -factor required to produce a 15 mm zone of inhibition, a halo size in the middle of the dose response curve, was similar for all mutant receptors (Table 1, Figure 2A). Cells expressing the Y266A receptor did not respond to α -factor even using a large amount (8 μ g) of pheromone (data not shown). The IC₅₀ value for each mutant receptor was determined from competition binding assays (Figure 2B, Table 1). Ligand binding was reduced 2-4-fold compared to the WT by mutations F262A, A265G, and Y266A. Mutations of the remaining six residues resulted in an increased α -factor binding affinity (up to 5-fold), without any significant change in biological activity as determined by the growth arrest assays.

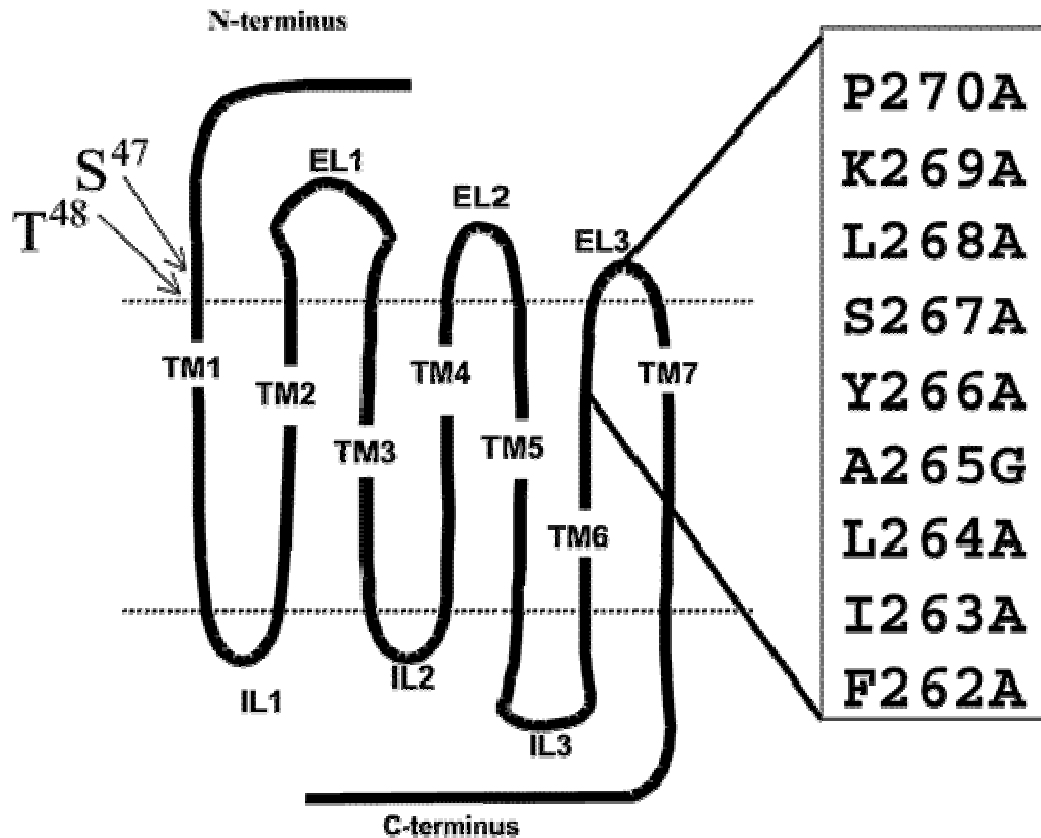


Figure 1: Topology of Ste2p and residues targeted for site-directed mutation. The predicted two-dimensional topology of the receptor is shown with the indicated extracellular domains (EL1, EL2, EL3), intracellular loops (IL1, IL2, and IL3), and transmembrane domains (TM1-TM7). Receptors containing the mutations indicated in the box were the subject of this study. Shown are two amino acid residues (S47 and T48) at the junction between TM1 and the N-terminus identified previously as contacts with the 10th residue (Q) of α -factor.

Figure 2: Biological activity and competition binding of α -factor in the Ste2p wild type and mutants in TM6. (A) The halo (zone of growth inhibition) sizes were measured at various concentrations of α -factor, and the data were plotted by regression analysis. The results are the mean SE of two separate experiments (see Table 1 for comparative activities). Key: WT (■), F262A (▲), I263A (▼), L264A (◆), A265G (·), Y266A (□), S267A (△), L268A (▽), K269A (♣), and P270A (⊙). (B) Yeast cells expressing the wild type or one of the mutant constructs were incubated with 6 nM [^{3} H] α -factor in the presence of increasing concentration of unlabeled α -factor as indicated on the abscissa. The experiments were repeated three times, and the results shown are the average SE of a representative experiment (see Table 1 for IC₅₀ values). Key: WT (■), F262A (▲), I263A (▼), L264A (◆), A265G (·), Y266A (□), S267A (△), L268A (▽), K269A (♣), and P270A (⊙).

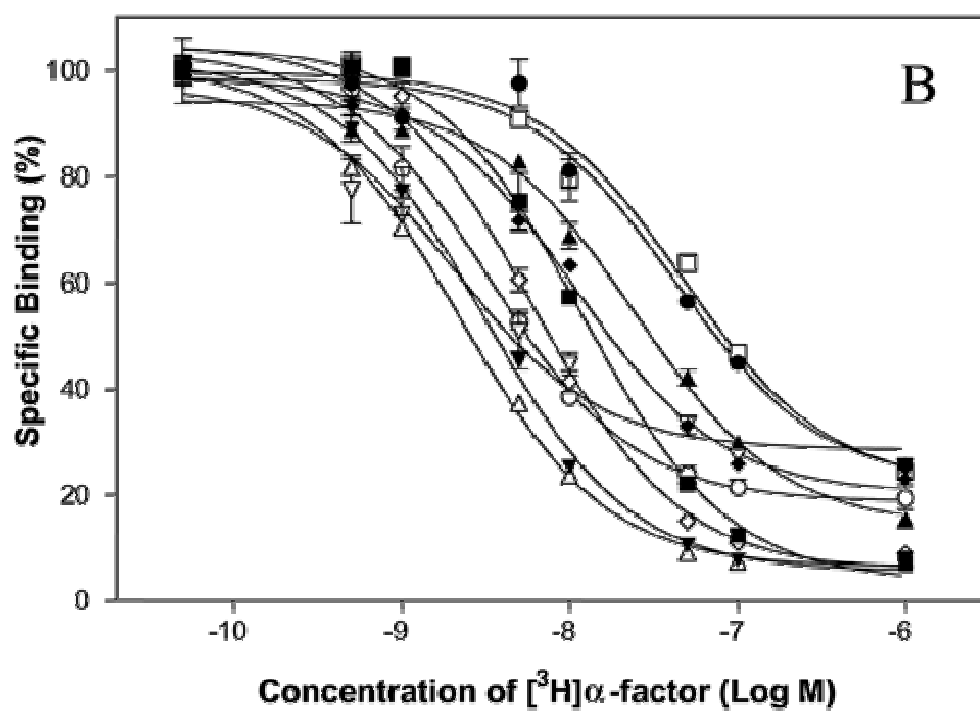
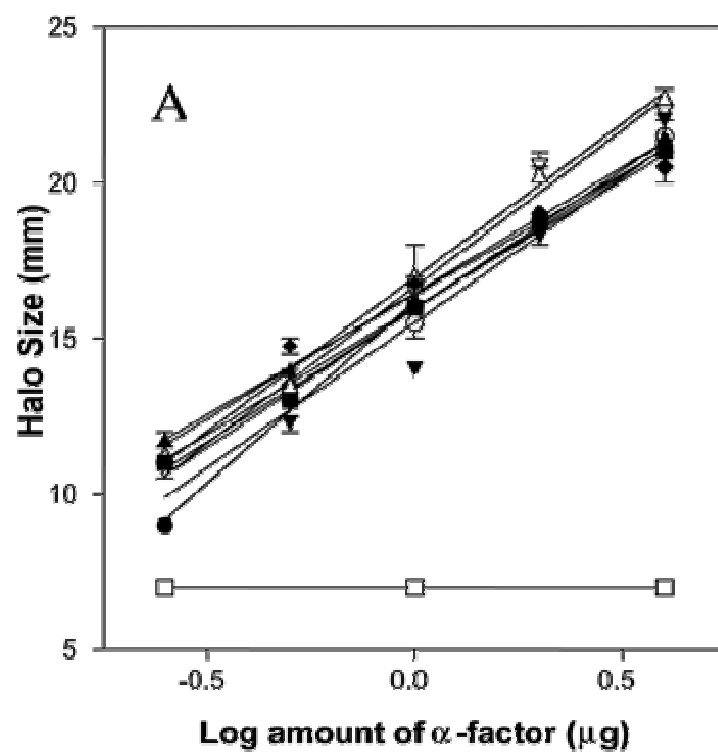


Table 1: Affinities and biological activities of Wild-Type and mutant receptors^{a1}

Receptor	Affinity of α-factor for receptor (IC₅₀, nM)	Biological activity of receptor (% of WT)
WT	11.8 $\pm$ 2.1	100 $\pm$ 11
F262A	24.3 $\pm$ 3.4	120 $\pm$ 11
I293A	3.2 $\pm$ 0.9	89 $\pm$ 6
L264A	3.2 $\pm$ 0.9	115 $\pm$ 13
A265G	38 $\pm$ 4.1	97 $\pm$ 8
Y266A	47 $\pm$ 3.9	<5
S267A	2.3 $\pm$ 0.7	121 $\pm$ 15
L268A	2.1 $\pm$ 1.1	112 $\pm$ 9
K269A	6.2 $\pm$ 1.4	103 $\pm$ 7
P270A	3.4 $\pm$ 1.6	99 $\pm$ 8

^a Affinities of various receptors for α -factor were measured by competition binding with 3[H] α -factor and expressed as IC₅₀ values. Biological activities were calculated from the dose-response curves of a growth arrest assay. Results are expressed as mean $\pm$ SE of two to three separate experiments.^b The amount of α -factor to produce a 15 mm halo was 0.68 μ g for WT receptor.

The Y266A receptor is expressed at the cell surface:

To determine whether the Y266A mutant receptor was expressed at the cell surface, whole cells (strain LM102) expressing the plasmid-encoded Y266A receptor or the WT receptor were incubated with a radiolabeled, photoactivatable ligand [Bpa¹Y³(¹²⁵I)R⁷F¹³ α -factor] and cross-linked by exposure to UV light as described previously (Henry et al., 2002). After the cross-linking, membrane proteins were resolved by SDS-PAGE. A radiolabeled band corresponding in size to the receptor-ligand complex (54 kDa) was detected, verifying the surface expression of the Y266A mutant (Figure 3).

Another experiment to measure the levels of cell surface expressed Y266A was done using a saturation binding assay on whole cells. This experiment demonstrated that the number of binding sites ($B_{\max}$) was reduced in the Y266A receptor (~14000 per cell) as compared to the WT Ste2p (~40000 per cell) (Table 2). Previous studies indicate that 14000 binding sites per cell is sufficient for full signaling with Ste2p (David et al., 1997; Dube et al., 2000; Jenness et al., 1986; Raths et al., 1988). This suggests that Y266A is expressed at levels fully capable of signal transduction if this receptor were indeed signaling-competent.

The aromatic ring structure at residue 266 is important for receptor function:

To study the role of tyrosine at position 266 more thoroughly, five additional amino acid substitutions (Ser, Phe, Trp, Lys, and Leu) were made at this position and characterized by growth arrest and saturation binding assays to directly determine the ligand affinity (K_D) and number of binding sites ($B_{\max}$) (Table 2, Figure 4). Substitution to lysine was not tolerated at this position and resulted in a complete lack of binding and

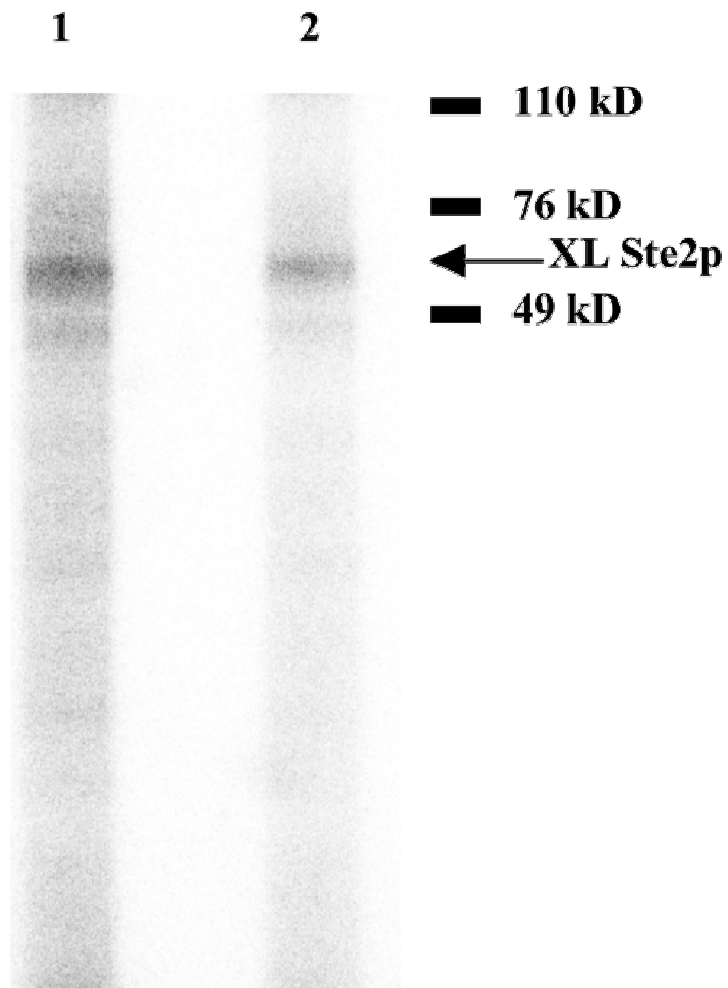


Figure 3: Photoaffinity labeling of Ste2p. Membranes derived from cells expressing wild-type (lane 1) or Y266A (lane 2) receptor were incubated with Bpa¹Y³(¹²⁵I)R⁷F¹³ α -factor in the presence of UV irradiation. Membrane proteins were solubilized and run on SDS-PAGE. Labeled bands were detected by autoradiography.

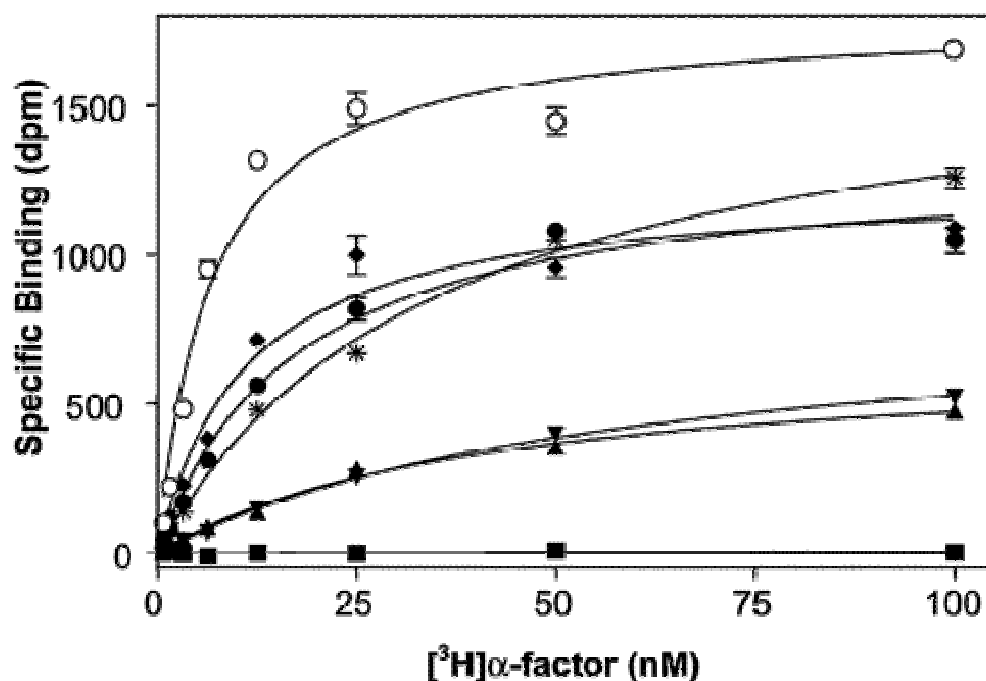


Figure 4: Saturation binding isotherms of [³H] α-factor for wild-type and various mutant receptors at position Y266. Cells harboring wild-type (○) and Y266F (◆), Y266W (○), Y266L (*), Y266S (▼), Y266A (▲), and Y266K (■) mutant receptors were incubated with increasing concentrations of [³H] α-factor and assayed as described in the Experimental Procedures. Specific binding was determined by subtracting counts in the presence of excess cold α-factor. Assays were carried out at least three times, and each concentration point was performed in triplicate. The results shown are from a representative experiment with calculated K_D and B_{max} values presented in Table 2.

Table 2: Affinities and biological activities of Ste2p with substitutions at Y266^a

	Binding		
Receptor	K_D (nM)	B_{max} (no. of binding sites/cell)	biological activity (% of WT)
WT	5.8 ± 1.4	40210 ± 3250	100 ± 9^b
Y266A	40.5 ± 5.7	14150 ± 1070	<5
Y266S	57.6 ± 4.5	16500 ± 1100	<5
Y266F	10.5 ± 2.8	25700 ± 1540	88 ± 7
Y266W	16.5 ± 3.2	27700 ± 1800	92 ± 6
Y266K	ND ^c	NA ^d	<5
Y266L	34.4 ± 2.7	37600 ± 3020	<5

^a Saturation binding assays were performed using whole cells expressing WT or mutant receptors. K_D and B_{max} values, calculated using a one-site binding model, are presented as the mean $\pm$ SE of at least three experiments. Each determination was performed in triplicate. ^b The amount of α -factor to produce a 15 mm halo was 0.65 μ g for WT receptor. ^c ND = not detectable. ^d NA = not applicable. The number of binding sites per cell could not be determined. Cells expressing this receptor had undetectable levels of binding.

biological activity. Although the Y266K receptor was verified by immunoblot analysis to be expressed at levels greater than the WT, the electrophoretic mobility of this mutant suggests that this receptor forms large amounts of aggregate material, and is most likely not correctly targeted to the cell membrane (data not shown). Substitution to serine or leucine produced receptors with properties most similar to those of Y266A. Binding affinity was decreased 3-5-fold ($K_D = 57.6$ and 34.4 nM for Y266S and Y266L, respectively) compared to the WT, and the number of receptors expressed per cell was similar to (16500 for Y266S) or 3-fold greater than (37500 for Y266L) that of the Y266A mutant (14150). In contrast, substitution to aromatic residues resulted in near wild-type binding affinities ($K_D = 10.5$ and 16.5 nM for Y266F and Y266W, respectively). Although there was a decrease in receptor cell surface expression ($B_{max} = 40000$ binding sites/cell for the WT vs 25700 and 27700 binding sites/cell for Y266F and Y266W, respectively), these mutants retained full biological activity as assayed by growth arrest assay (88% and 92% of WT activity).

Mutation Y266A confers a strong dominant-negative phenotype:

A "dominant-negative" mutant masks the phenotype of the corresponding wild-type protein when both are coexpressed. We tested whether Y266A conferred a dominant-negative phenotype when coexpressed with WT receptor. Plasmids encoding the Y266A mutant receptor were expressed in LM23-3AZ, a strain containing a chromosomal copy of WT Ste2p. This same strain was also transformed with a plasmid encoding either the WT Ste2p or the mutant F262A or K269A. Coexpression of WT or F262A or K269A mutant constructs did not significantly influence halo formation. In

contrast, when used in a growth arrest assay (Figure 5), strains coexpressing the WT and the Y266A mutant were much less sensitive to the pheromone, compared to the WT control, requiring more than 10 times the amount of ligand to produce a detectable halo. This dominant-negative phenotype could be suppressed by coexpression of the Y266A mutant construct with pMD82, a plasmid which overexpressed the G protein subunits. In this case, sensitivity to the pheromone was restored, and dose-dependent halos were generated in growth arrest assays using low amounts (0.25-2 μ g) of ligand (Figure 6).

Y266 is essential for conformational change of the receptor upon α -factor binding:

Membranes expressing either the WT or Y266A mutant receptor were subjected to partial tryptic digest in the absence of ligand or in the presence of either α -factor or [desW¹desH²] α -factor, an α -factor antagonist. The cleavage products were resolved by SDS-PAGE using 15% Tris-glycine gels, immunoblotted with an antibody directed against the N-terminal domain of the receptor and visualized by chemiluminescence. In the absence of trypsin, a doublet corresponding in size to Ste2p (approximately 50 kDa) is the major species present, with a less intense band at approximately 35 kDa (Figure 7). The smaller band is likely the result of degradation during the preparation of the membranes as observed in previous studies (Bukusoglu and Jenness, 1996; Henry et al., 2002). Digestion with trypsin for 60 min in the absence of ligand resulted in the generation of four major fragments, labeled as 1-4. No difference in digestion pattern was observed when the WT was compared to the Y266A mutant receptor in the absence of ligand.

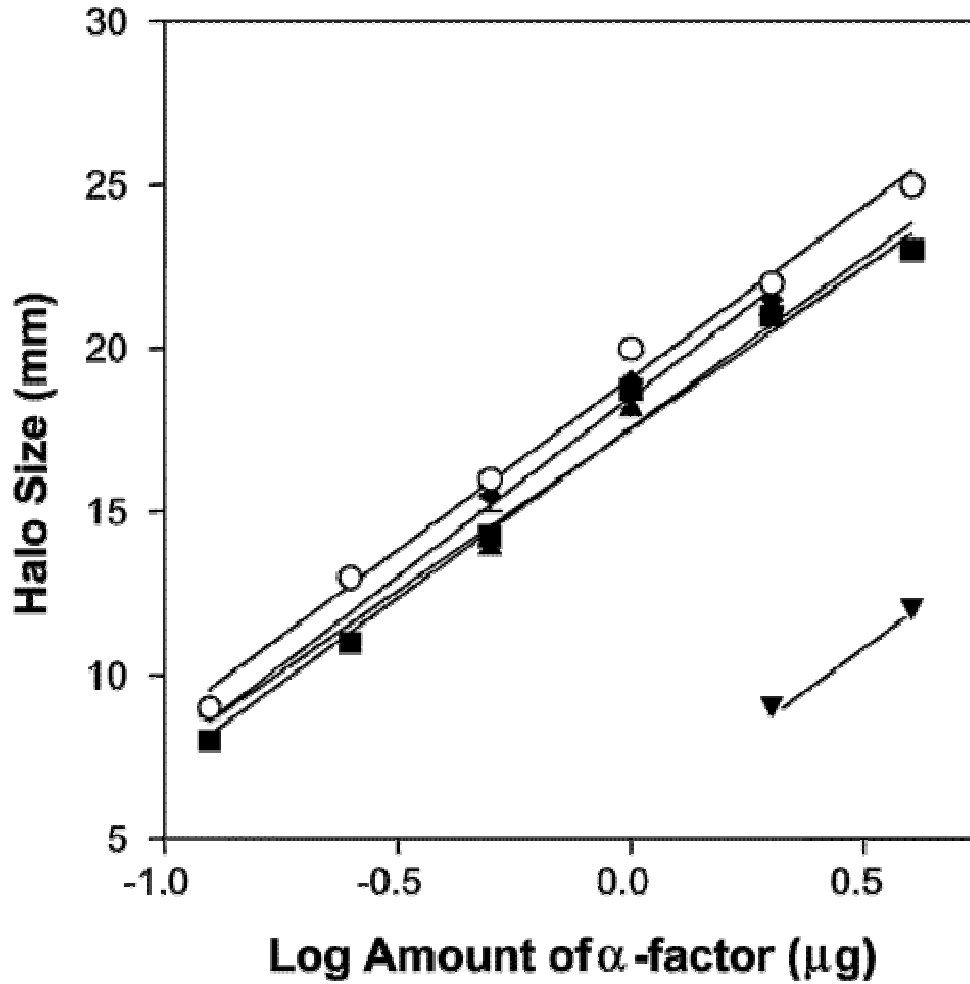
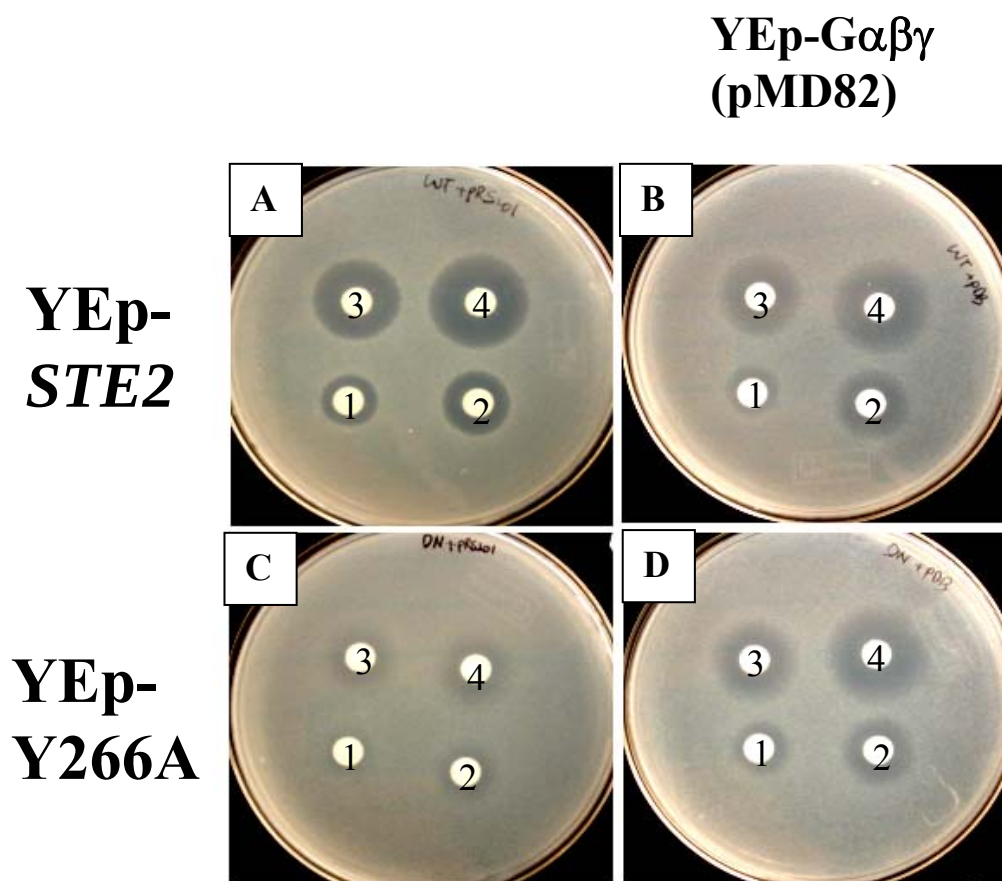


Figure 5: Dominant-negative effects of the Y266A mutant receptor on growth arrest. The indicated mutant or wild-type receptors were expressed in strain LM23-3az that contains a chromosomal copy of *STE2*. The growth arrest assays were conducted as described in the Experimental Procedures. The results are the average $\pm$ SE of two independent experiments. Key: LM23-3az expressing *STE2* from the chromosome (○), *cSTE2*, *cSTE2* coexpressed with plasmid-encoded *STE2* (■), F262A (▲), Y266A (▼), or K269A (◆).

Figure 6: Dominant-negative phenotype of Y266A receptor. Cells expressing wild-type Ste2p (chromosomal) and wild-type or mutant (Y266A) plasmid-encoded Ste2p with or without overexpression of heterotrimeric G proteins [GPA1 ($G\alpha$), STE4 ($G\beta$), and STE18 ($G\gamma$) genes on pMD82] were assayed for growth arrest by addition of α -factor. The amounts of α -factor applied to disks on each plate were 2, 1, 0.5, 0.25 μ g, indicated by numbers 4 to 1, respectively. Panel A: Ste2p co-expressed from chromosome and plasmid; Panel B: chromosomal Ste2p co-expressed with $G_{\alpha\beta\gamma}$ proteins and plasmid-encoded wild-type Ste2p; Panel C: chromosomal Ste2p co-expressed with Y266A mutant; Panel D: chromosomal Ste2p co-expressed with $G_{\alpha\beta\gamma}$ proteins and plasmid-encoded N205A Ste2p.



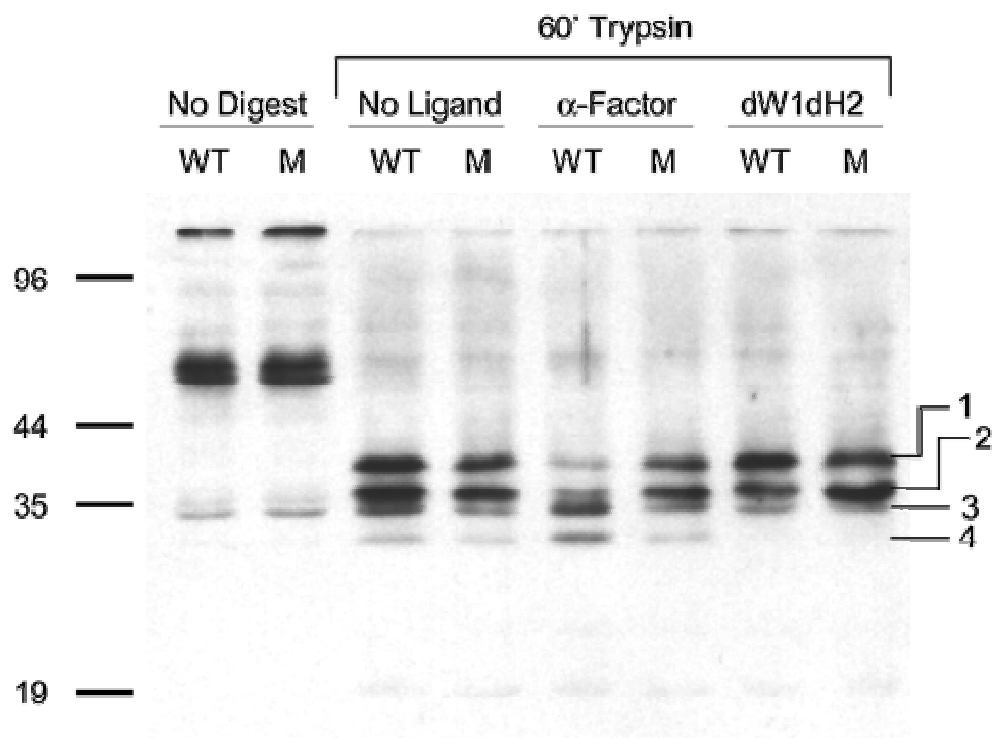


Figure 7: Partial tryptic digest of Wild-Type and Y266A mutant receptors.

Immunoblot of membranes (20 µg/lane) incubated with either native α -factor or [desW¹desH²] α -factor prior to trypsin digestion (60 min, 30 °C). Parallel controls were completed in the absence of ligand or trypsin. Molecular size markers (kDa) are indicated at the left. Four major digest products (1-4) are indicated on the right.

Digestion in the presence of α -factor rendered the WT receptor more susceptible to digestion, in agreement with results previously reported (Bukusoglu and Jenness, 1996). Under these conditions, bands 1 and 2 became less intense, while product accumulated in bands 3 and 4. In the presence of [desW¹desH²] α -factor, the WT receptor was less readily digested; the banding pattern was similar to that observed for the unoccupied receptor; however, band 4 was absent. The results for the Y266A mutant receptor are somewhat different. As noted above, in the absence of ligand, the digestion pattern was very similar to that observed for the WT receptor, suggesting that both have similar topologies in the ligand-unbound, or inactive, state. In the presence of α -factor, the digestion pattern of the Y266A mutant was the same as that of the unoccupied mutant receptor. Upon binding the antagonist, the Y266A receptor digestion pattern changed and was similar to that observed for antagonist bound to the WT receptor.

To determine whether the differential sensitivity of the Y266A receptor was due to an intrinsic difference in receptor structure or was related to the nature of its interaction with the G protein, the tryptic digest was completed in the diploid background CG990. In diploid cells, the mating loci are silent; thus, these cells do not express G proteins. The WT and Y266A mutant constructs were expressed under the control of the constitutive GPD promoter, since the native *STE2* promoter would not be active in a diploid background. As was observed in the haploid background, in the presence of α -factor the tryptic digest pattern of the Y266A receptor did not change; bands 1 and 2 remained resistant to digestion (data not shown). This suggests that the differential trypsin sensitivity of the mutant when compared to the WT receptor was due to a change in receptor structure, rather than in the nature of its interaction with the G protein.

Mutation of Y266A alters binding of N-terminally modified α -factor analogues:

To further probe the uncoupling of ligand binding and signal transduction in the Y266A receptor, a wide variety of α -factor analogues, including antagonists and partial agonists, were tested for biological activity in an effort to determine whether any particular feature of the ligand could compensate for the receptor defect. Thirteen Ala-scanned analogues of α -factor were tested in binding and growth arrest assays (Table 3). These analogues had a wide spectrum of growth arrest activity, when tested using WT receptor, ranging from partial to enhanced agonist. In contrast, none of the 13 analogues were able to induce growth arrest in cells expressing the Y266A mutant receptor. In the competition binding assay, the Ala-scanned α -factor analogues exhibited a range of affinities, reported as K_i values, for binding to the Y266A receptor. Alanine substitution in the first four residues of the ligand ([Ala¹]- through [Ala⁴] α -factor) resulted in pheromone that bound better to the Y266A receptor than to the WT. For example, for the [Ala¹] α -factor, in which the native tryptophan residue was replaced with alanine, the binding affinity (K_i) for the WT receptor was 216 nM, approximately 30-fold less than the binding affinity of native ligand to the WT receptor (6.8 nM). The binding affinity of this same ligand to the Y266A receptor was 96 nM, indicating an increase in binding affinity (216 nM for WT vs 96 nM for Y266A). Similar changes (2-4-fold increases in affinity) were observed for the [Ala²]-through [Ala⁴] α -factor analogues. Although none of the [Ala¹]- through [Ala⁴] α -factors had a better affinity than native ligand for the WT receptor, all of these N-terminally substituted ligands bound better to the mutant receptor than they did to the WT receptor. In contrast, [Ala⁵]- through [Ala¹³] α -factors exhibited

Table 3: Affinities and biological activities of Ala-scanned α -factor analogues for Wild-Type and Y266A mutant receptors^a

Peptide	Binding (K_i , nM)			Biological activity ^b (%)	
	WT	Y266A	Fold change	WT	Y266A
α -factor	6.8 $\pm$ 1.5	41 $\pm$ 4.2	-6.0	100 $\pm$ 11	<5
[Ala ¹]	216 $\pm$ 18	96 $\pm$ 10	+2.3	110 $\pm$ 12 ^c	<5
[Ala ²]	505 $\pm$ 33	132 $\pm$ 12	+3.8	38 $\pm$ 4	<5
[Ala ³]	250 $\pm$ 16	63 $\pm$ 6	+4.0	13 $\pm$ 2	<5
[Ala ⁴]	365 $\pm$ 21	110 $\pm$ 17	+3.3	14 $\pm$ 2	<5
[Ala ⁵]	8 $\pm$ 2	58 $\pm$ 4.5	-7.2	124 $\pm$ 12	<5
[Ala ⁶]	1560 $\pm$ 110	3420 $\pm$ 175	-2.2	43 $\pm$ 5	<5
[Ala ⁷]	27 $\pm$ 2.5	280 $\pm$ 17	-10.4	141 $\pm$ 13	<5
[Ala ⁸]	9 $\pm$ 2	62 $\pm$ 5	-6.9	68 $\pm$ 6	<5
[Ala ⁹]	2150 $\pm$ 160	5590 $\pm$ 410	-2.6	13 $\pm$ 2	<5
[Ala ¹⁰]	570 $\pm$ 30	2840 $\pm$ 135	-5.0	45 $\pm$ 5	<5
[Ala ¹¹]	140 $\pm$ 17	1110 $\pm$ 70	-8.0	70 $\pm$ 6	<5
[Ala ¹²]	1050 $\pm$ 90	11100 $\pm$ 1180	-10.6	53 $\pm$ 5	<5
[Ala ¹³]	4900 $\pm$ 450	16600 $\pm$ 1700	-3.4	23 $\pm$ 11	<5

K_i values for α -factor and 13 Ala-scanned analogues were determined in competition binding assays by displacement of [³H] α -factor. Biological activities were measured by the growth arrest assay. All values are the mean $\pm$ SE from three separate experiments.

^b The amount of α -factor to produce a 15 mm halo was 0.62 μ g for WT receptor.

^c This halo was not clear and disappeared within 36 h.

2-10-fold decreases in their affinities for the mutant receptor in comparison to their affinities for the WT Ste2p. These decreases were similar in magnitude to the decrease in affinity exhibited by α -factor for the Y266A mutant in comparison to WT Ste2p (6-fold).

To further examine the increased binding affinity which resulted upon changes in the N-terminus of the ligand, α -factor analogues modified by substitutions other than alanine, [Y¹]-, [D-Ala⁹]- and [Orn¹⁰] α -factors as well as the antagonists [desW¹,desH²] α -factor and [desW¹Ala³] α -factor were tested (Table 4). Similar to the observation made for the [Ala⁵]- through [Ala¹³] α -factors, the affinity of [D-Ala⁹]- and [Orn¹⁰] α -factors for the Y266A receptor was decreased (8- and 4-fold, respectively) when compared to their affinity for the WT receptor. In contrast, the N-terminal substitution of tyrosine for tryptophan in the [Y¹] α -factor resulted in a ligand with 4-fold increased affinity for the mutant receptor, similar to what was observed for the [Ala¹]- through [Ala⁴] α -factors. The affinities of the antagonists for the Y266A receptor were also enhanced. The [desW¹Ala³] α -factor analogue exhibited a slight increase in affinity (1.4-fold change), while [desW¹,desH²] α -factor showed a 3.5-fold change. Interestingly, the affinity of [desW¹,desH²] α -factor was the greatest of all ligands tested (K_i = 19 nM vs 42 nM for native α -factor). Despite the fact that these various substituted analogues bound to the Y266A receptor, none of these analogues were able to induce growth arrest (data not shown).

Table 4: Binding affinities of antagonists and α -factor analogues^a

Peptide	WT	Y266A	Fold change
α -factor	6.5 ± 2	42 ± 4	-6.5
[desW ¹ ,desH ²] α -factor	67 ± 7	19 ± 3.5	+3.5
[desW ¹ ,A ³] α -factor	84 ± 7	61 ± 5	+1.4
[Y ¹] α -factor	121 ± 13	30 ± 4	+4.0
[D-A ⁹] α -factor	6.0 ± 1.8	48 ± 4.5	-8.0
[Orn ¹⁰] α -factor	205 ± 19	940 ± 83	-4.0

^a K_i values (nM) obtained from competition binding assay using [³H] α -factor were the mean of two separate experiments, each of which was performed in triplicate.

CHAPTER 4

Discussion

Recent studies of Ste2p indicated that residues in the sixth transmembrane domain play important roles in signal transduction to the heterotrimeric G proteins as a consequence of α -factor binding. For example, mutation of P258L in the center of this domain resulted in constitutive activation of Ste2p (Konopka et al., 1996), suggesting that P258 may be involved in the switch from the inactive to the active form of the receptor. Interestingly, mutation of Y266C in TM6 was found to result in an inactivatable form of the receptor (Sommers and Dumont, 1997) despite the fact that mutagenesis of adjacent residues to Cys had no effect on signal transduction (Dube et al., 2000). However, no comprehensive studies regarding ligand interactions with this receptor domain have been performed.

Our group has been exploring binding site interactions between Ste2p and α -factor using molecular biological (Abel et al., 1998b; Lee et al., 2001), spectroscopic (Ding et al., 2001; Ding et al., 2002), and photo-cross-linking (Henry et al., 2002) approaches. Evidence was found that α -factor interacts with residues near the extracellular face of Ste2p. Furthermore, binding sites for most peptide hormones are proposed to involve extracellular loops and the outer ends of transmembrane domains (Marshall, 2001). Therefore, we decided to thoroughly explore the unique phenotype manifested by Y266C. Our strategy was to mutate Y266 and all residues from F262 to

P270 to Ala, to evaluate signaling and pheromone binding by the resulting mutant receptors, and to investigate their interaction with selected α -factor analogues.

Pheromone-induced growth arrest and binding affinity analyses indicated that the majority of Ala substitutions in this critical receptor domain did not interfere with receptor function, with the notable exception of Y266A. Mutations at certain residues resulted in even better α -factor binding and/or increased signaling ability (Table 1). Although Ala mutation at Y266 yielded a receptor inactive for signaling, α -factor still binds to this receptor with relatively high affinity (Tables 1 and 2). The apparent reduction in the cell surface expression of Y266A receptor in comparison to WT Ste2p as shown from saturation binding experiments is not likely the source of the observed signaling-deficient phenotype. Our data showed that there is no clear correlation between expression levels and receptor function. For example, the inactive Y266L receptor has near wild-type expression levels whereas the highly active Y266F and Y266W receptors are reduced in expression by approximately one-third (Table 2). Furthermore, there is also no strict correlation between the K_D values of the receptor and signaling proficiency since Y266L and A265G have virtually identical K_D values despite the fact that the former receptor is inactive whereas the latter receptor exhibited wild-type signaling (Tables 1 and 2). Finally, it was previously shown that, unlike mammalian GPCRs whose function is regulated by cell surface expression levels, yeast cells that express anywhere from as low as 5% to as much as 20-fold excess of the normal level of receptors can transduce signal at near normal levels (Martin et al., 2002; Sommers et al., 2000). Many studies measuring levels of Ste2p expression by saturation binding experiments have reported that between 6000 and 10000 receptors are expressed on the cell (David et al.,

1997; Dube and Konopka, 1998; Jenness et al., 1986; Raths et al., 1988), making the WT Ste2p expression levels seen in this report unusually high. If the Y266A receptor were functional, 14000 receptors/cell should be more than sufficient to initiate signal transduction. When coexpressed with WT receptor, Y266A strongly interfered with the WT signaling activity (Figure 5); this is consistent with previous identification of the Y266C receptor as a dominant-negative mutant (Dosil et al., 1998). On the basis of the work presented by Leavitt et al. (Leavitt et al., 1999), the expression level of chromosomally encoded WT receptor was unaffected even when the Y266C dominant-negative receptor was coexpressed 20-fold on a multicopy plasmid. Since Y266A was expressed using a low-copy-number (CEN) plasmid, under its native promoter, it is expected that the ratio between WT and Y266A would be very similar. Although receptor dimerization could be a possible explanation for the dominant-negative phenotype, the ability to sequester G proteins from wild-type receptor by dominant-negative mutations has been experimentally confirmed in the yeast α -factor receptor system (Dosil et al., 1998; Dosil et al., 2000; Leavitt et al., 1999). Our current finding that overexpression of the G-protein subunits reverses the Y266A dominant negative phenotype lends additional credence to the postulate that G protein sequestration may be the mechanism underlying this phenotype. Additionally, the observation that a dominant-negative mutant in the α -adrenergic receptor, which specifically inhibited signaling via G_q, was rescued by the overexpression of G_q (Chen et al., 2000) further supports this postulate. On the basis of this scenario, we propose that the signaling deficiency of the Y266A receptor is due to its inability to activate G proteins. More specifically it seems likely that Y266A mutation prevents Ste2p from assuming the activated state of Ste2p induced by α -factor binding.

A previous study demonstrated that Ste2p assumed unique topologies in the ligand-free, agonist-bound and antagonist-bound states as judged by trypsin digest patterns found by SDS-PAGE. We, therefore, tested the Y266A mutant receptor using this approach. The relatively high affinity for α -factor binding indicated that, in the resting state, the mutant receptor had a conformation similar to that of WT Ste2p. Supporting this conclusion, the tryptic digest of unoccupied Y266A receptor was nearly identical to that of WT Ste2p (Figure 6). Upon binding α -factor, the WT receptor underwent a shift in conformation, resulting in an increased sensitivity to trypsin, as previously reported (Bukusoglu and Jenness, 1996). Although the active site of Y266A was saturated with pheromone, the digestion pattern was the same as that of the ligand-free state. The digestion pattern was independent of the interaction of the mutant receptor with G proteins; essentially identical results were observed in diploid cells that do not express G proteins. This indicates that mutation of Y266 to Ala prevented Ste2p from undergoing the conformational change necessary for subsequent signal transduction in response to ligand binding. Additionally, binding of the desW¹desH² antagonist to both WT and Y266A mutant receptors resulted in a tryptic digest pattern distinct from either the resting or agonist-bound state. This suggests that antagonist binding resulted in a novel conformational state, one differing from those of both the unoccupied and the agonist-bound receptor. Similar results were also reported for binding of the antagonist [desW¹A³] α -factor to WT receptor (Bukusoglu and Jenness, 1996).

Additional mutations at Y266 revealed that signal transduction required an aromatic ring moiety at this position since substitution of this residue with other aromatic amino acids (Y266F and Y266W) maintained high-affinity binding and receptor

signaling ability. A specific interaction between an aromatic side chain of residue 266 and residues from other TMs may be critical for the induction or stabilization of the activated conformation of the receptor since neither the aliphatic leucine nor cationic lysine substitution could support receptor function (Table 2). In fact, the Y266C mutant receptor was found, in an allele-specific manner, to restore function in the nonsignaling E143K mutation in the third transmembrane domain. This was interpreted as indicating a specific interaction between residues E143 and Y266 in the third and sixth transmembrane domains of Ste2p, respectively (Sommers and Dumont, 1997). Although the critical involvement of hydrophobic residues at TM6 in receptor function was reported for other GPCRs (Gether, 2000), the Y266A mutant receptor differs from those previously characterized in that it appears to affect primarily signaling by the receptor, while having little effect on agonist binding.

To gain more insight into the nature of the Y266A signaling defect, various α -factor analogues, including antagonists, were tested for their binding and growth arrest activity. The Y266A mutation markedly impaired the binding of α -factor analogues with single alanine substitutions in positions 5-13, while the same substitution at positions 1-4 of α -factor showed increased binding affinity for this mutant compared to WT Ste2p (Table 3). Moreover, the antagonist [desW¹,desH²] α -factor had the highest affinity for this mutant receptor (2.5-fold better binding compared to that of native α -factor, Table 4). However, none of these analogues resulted in growth arrest of the yeast cell. It appears that in addition to its involvement in the Ste2p activation pathway Y266 likely contributes to receptor-ligand interactions. Specifically, our data would be consistent with

a direct interaction between Y266 and residues near the N-terminus of α -factor. It is difficult to explain how a single substitution in the receptor can influence binding with four successive residues (residues 1-4) in the ligand. It is possible that, rather than interfering with a direct contact, changing Y266 to alanine results in a significant but localized perturbation of this region of Ste2p at the membrane interface. Others have shown that aromatic residues such as Trp and Tyr influence the insertion of transmembrane helices into bilayers (Braun and von Heijne, 1999) and may play a key role in keeping the transmembrane domains of integral membrane proteins in proper register with respect to the membrane. Alternatively, the recognition of the N-terminal end of α -factor by Ste2p may be mediated by a conformational compatibility mechanism, as shown in the neurokinin receptor system (Huang et al., 1995). In this view, the outcome of mutations in the receptor or substitutions in the ligand would be reflected in a regional conformational change in the receptor binding site or ligand, rather than disruption of specific contacts between receptor and ligand. Y266 would be a key residue involved in the recognition of aspects of the conformation of the N-terminus of α -factor, thus allowing the agonist to bind productively and trigger a transition into an activated state. The important role of an aromatic cluster in receptor activation and ligand binding has been described for peptide hormone receptors (Chauvin et al., 2000; Lin et al., 2000; Renzetti et al., 1999) as well as for the dopamine receptor (Javitch et al., 1998). Since the N-terminus of α -factor is highly aromatic and hydrophobic (W¹HWL⁴), this domain probably binds to hydrophobic residues comprising the receptor pocket. In addition to Y266 two aromatic residues (Y203 and F204) at the EL2-TM5 junction would be

reasonable candidates for interaction with the N-terminus of α -factor. Interestingly, mutation of these residues also resulted in dominant-negative mutants (Dosil et al., 1998).

The Y266A mutant receptor maintained relatively high agonist binding, but was deficient with respect to signaling activity. Interestingly, this same mutant receptor exhibited enhanced antagonist affinity, and to the best of our knowledge, this is the first report of a mutation in a G protein-coupled receptor to possess all of these characteristics. It is striking that the [desW¹desH²] α -factor antagonist binds more strongly than the WT agonist to the Y266A receptor (Table 4). Considering the fact that WT Ste2p binds α -factor 10-fold more avidly than it does the desW¹desH² antagonist, the Y266A mutant appears to prefer this N-truncated antagonist. Furthermore, the Y266A receptor displays increased affinity for other N-terminally modified ligands, including [desW¹A³] α -factor, [Y¹] α -factor, and the position 1-4 Ala-scanned α -factor analogues. This suggests that the structure of the Y266A mutant receptor better accommodates analogues in which the overall hydrophobicity at the N-terminus has been reduced by either substitution or elimination of hydrophobic residues. Interestingly, all antagonists of Ste2p discovered to date fall into this category of α -factor analogue; therefore, Y266A appears to possess a binding site that would be highly favorable to Ste2p antagonists. Currently GPCRs are a major drug target representing nearly 50% of current drugs under development (Gershengorn and Osman, 2001; Gurrath, 2001). Often the pharmaceutical agent acts as an antagonist to prevent undesired effects from excessive receptor signaling. Thus, factors that result in antagonist selectivity may be relevant to drug design, and structural studies of this type of mutant receptor may be highly significant.

In conclusion, our results indicate that the Y266A mutation in TM6 of Ste2p abolished agonist-induced growth arrest, without compromising high binding affinity for α -factor. The loss of signaling is thought to result from the inability of the mutant receptor to achieve the active state required for productive interaction with its cognate G protein. We also report that, upon binding ligand, the conformation of Y266A receptor remained unchanged and did not resemble the tryptic digest pattern observed in agonist-bound WT receptor. Finally, we provide evidence that Y266 likely interacts with the amine terminal residues of α -factor (W¹HWL⁴) possibly via interactions involving aromatic side chains. Biophysical and biochemical investigations focusing on the N-terminus of α -factor and its interactions with other site-directed mutants of the receptor are under way.

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the *Saccharomyces cerevisiae* alpha-factor tridecapeptide. *Biochemistry*, **37**, 12465-12476.

PART III

Interacting Residues in an Active State of a G protein-coupled receptor

CHAPTER 1

Introduction

The tridecapeptide α -factor pheromone ($W^1H^2W^3L^4Q^5L^6K^7P^8G^9Q^{10}P^{11}M^{12}Y^{13}$) of *Saccharomyces cerevisiae* and Ste2p, its cognate G protein-coupled receptor (GPCR), have been used extensively as models for peptide ligand-GPCR structure and function (Dohlman et al., 1991). A major goal of GPCR studies is to ascertain the interactions between ligand and receptor to aid in the identification of analogs used for modulation of receptor activity and to understand how the receptor activates its signal transduction pathway. GPCRs are extremely important for medicine as they represent the target for the majority of prescribed drugs (Klabunde and Hessler, 2002).

To identify Ste2p residues or regions involved in α -factor binding, a number of experiments have been performed. Chimeric receptors between the closely related *S. cerevisiae* and *Saccharomyces kluyveri* α -factor receptors implicated the involvement of portions of EL1 (extracellular loop 1), EL3, and the N-terminal extracellular region of TM1 (transmembrane 1) in the specificity of ligand recognition (Sen and Marsh, 1994; Sen et al., 1997). Our group using site-directed mutagenesis of Ste2p and binding assays with different α -factor analogs suggested that portions of TM1 and TM6 were important for ligand interaction (Abel et al., 1998a) and that the tenth residue of α -factor is in close proximity to Ser⁴⁷ and Thr⁴⁸ in TM1 of Ste2p (Lee et al., 2001). We found that Y266 in TM6 plays an important role in the recognition of the N-terminus of α -factor as well as switching of Ste2p into an activated state upon agonist binding (Lee et al., 2002), and

using photo-affinity labeling we suggested that the N-terminus of α -factor interacts with a region of the receptor spanning the upper portions of TM5 and TM6 and the connecting EL3 (Henry et al., 2002). Studies with α -factor analogs and fluorescently modified α -factor indicated that the binding environments of the position one (Zhang et al., 1997) and position three side-chains were hydrophobic while the seventh residue was exposed to a partially hydrophilic environment suggesting that the middle portion of the pheromone was not buried in the transmembrane domains (Ding et al., 2001; Ding et al., 2002).

Experiments to identify Ste2p residues and regions involved in receptor activation and signaling have been extensive. An interaction between TM5 and TM6 has been identified by a Cys-cross-linking experiment (Dube et al., 2000), and other interactions between and among transmembrane domains and between specific residues in transmembrane domains have been demonstrated by genetic experiments (Dube and Konopka, 1998; Parrish et al., 2002; Sommers and Dumont, 1997). In addition, extensive searches for dominant-negative mutations in the receptor revealed that most mutants exhibiting this phenotype are mapped to the extracellular ends of the transmembrane domains, especially TM5 and TM6 (Dosil et al., 1998; Dosil et al., 2000; Leavitt et al., 1999). A detailed and elegant analysis concluded that F204 in the EL2 loop was important for ligand binding and that Y266 at the extracellular end of TM6 was involved in signal transduction (Lin et al., 2003).

Based on these previous Ste2p findings, we initiated studies described in this part to map residues interacting with Y266 using alanine-scanning mutagenesis of the EL2-TM5 interface (residues Q200 to I209). All mutant receptors were subjected to detailed

functional and ligand binding assays. Our results revealed that N205 is critical for signal transduction and based on site-directed mutagenesis and disulfide cross-linking studies we deduced that N205 interacts with Y266 in a receptor that is activated by mutation to the constitutively-activated state.

CHAPTER 2

Materials and Methods

Strain and plasmids:

LM102 and LM23-3az yeast strains described by Sen and Marsh (Sen and Marsh, 1994) were used in these studies. The genotype for the LM102 strain is: *MATa*, *bar1*, *his4*, *leu2*, *trp1*, *met1*, *ura3*, *FUS1-lacZ::URA3*, *ste2-dl* (deleted for the region coding for the α -factor receptor). LM23-3az has the same genotype as LM102 except that it contains an intact chromosomal *STE2* gene coding for the α -factor receptor. LM23-3az was used only to study dominant negative effects of certain mutant receptors on the function of wild-type (WT) Ste2p (see "Results"). The LM102 strain was used as the recipient for the transformation with WT and the site-directed *STE2* mutants. Measurement of the pheromone-induced growth arrest (halo assay), pheromone-induced gene expression (*FUS1-lacZ* assay), and determination of pheromone binding were done in the LM102 host strain. Both LM102 and LM23-3az strains carried the *bar1* mutant allele, which inactivated the *BARI* protease responsible for degradation of α -factor, and a *FUS1-lacZ* gene. The WT *STE2* gene with a native promoter was cloned into a yeast/bacterial shuttle vector pGA314. WT (Abel et al., 1998a) and was used as a template for the site-directed mutagenesis of the α -factor receptor gene. The plasmid pGA314-T7 was constructed for the disulfide-crosslinking experiment by homologous recombination between pGA314 and pPD225-T7, which was used previously for the identification of interaction between

TM5 and TM6 of Ste2p (Dube et al., 2000). Ste2p encoded by pPD225-T7 contains no cysteine residues and is identical to wild-type Ste2p in its biological activity. In this paper we refer to the Cys-less, T7-tagged Ste2p as WT. The plasmid pMD82(Dosil et al., 1998), which over-express G- $\alpha\beta\gamma$, was a kind gift from Dr. James Konopka, State University of New York, Stony Brook.

Site-directed mutagenesis:

Single-stranded phagemid DNA of pGA314.WT was prepared by infecting *Escherichia coli* strain CJ236 (*ung*⁻,*dut*⁻) carrying pGA314.WT with the helper phage M13KO7 (Vieira and Messing, 1987). Oligonucleotide-directed mutagenesis of single-stranded phagemid DNA was conducted as described by Kunkel *et al.* (Kunkel et al., 1987). After sequence confirmation, constructs were transformed into yeast strain LM102 (*ste2*-deletion strain), and transformants were selected by their growth in the medium lacking tryptophane. All primers were purchased from Sigma/Genosys (Woodlands, TX) and Integrated DNA Technologies Inc (Coralville, IA). DNA sequencing was carried out in the DNA sequencing facility located on the campus of the University of Tennessee.

Growth arrest (Halo) assay:

Yeast nitrogen base medium (Difco) without amino acids (SD medium) supplemented with histidine (20 μ g/ml), leucine (30 μ g/ml), and methionine (20 μ g/ml) was overlaid with 4 ml of cell suspension (2.5×10^6 cells/ml of Nobel agar). Filter disks (sterile blanks from Difco), 7 mm in diameter, were impregnated with 10- μ l portions of peptide solutions at various concentrations (adjusted by molar extinction coefficient) and

placed onto the overlay (Abel et al., 1998b). The plates were incubated at 30 °C for 24-36 h and then observed for clear zones (halos) around the disks. The halo measured included the diameter of the disc. All assays were carried out at least three times with no more than a 2-mm variation in halo size at a particular amount applied for each peptide. The data were plotted as halo size *versus* the amount of peptide, and linearized by regression analysis using PrismTM software (GraphPad, San Diego, CA).

FUS1-lacZ gene induction assay:

S. cerevisiae LM102 contains a *FUS1-lacZ* gene that is inducible by mating pheromone. Cells were grown overnight in SD medium supplemented with the required amino acids at 30 °C to 5×10^6 cells/ml, washed by centrifugation, and grown for one doubling (hemocytometer count) at 30 °C. Induction was performed by adding 0.1 ml of α -factor at various concentrations to 1 ml of concentrated cells (1×10^8 cells/ml). The mixtures were vortexed and after incubation at 30 °C with shaking for 2 h, cells were harvested by centrifugation, and each pellet was resuspended and assayed for β -galactosidase activity (expressed as Miller units) in duplicate by modification (Kippert, 1995) of a standard protocol (Guarente, 1983) using *o*-nitrophenyl- β -D-galactopyranoside (Sigma) as the substrate. The percent activity at various α -factor concentrations was determined by comparing β -galactosidase activity to the maximal activity at 1 μ M α -factor for the wild-type receptor. Each experiment was carried out at least two times with similar results in each assay.

Binding assay:

Competition binding assays were performed using [^3H] α -factor synthesized as described previously (Abel et al., 1998b). The competition binding assay was started by the addition of [^3H] α -factor and various concentrations of non-labeled α -factor or α -factor analogs (140 μl) to a 560- μl cell suspension such that the final concentration of radioactive peptide was 6×10^{-9} M (20 Ci/mmol). After a 30-min incubation, triplicate samples of 200 μl were filtered and washed over glass fiber filtermats using the Standard Cell Harvester (Skatron Instruments, Sterling, VA) and placed in scintillation vials for counting. Each experiment was carried out at least three times with similar results. Data curves for competition binding assays were fitted from at least eight triplicate data points using PrismTM software (GraphPad) with nonlinear regression one site competition. The K_i values for competition binding assays were calculated by using the equation of Cheng and Prusoff (Cheng and Prusoff, 1973), where $K_i = \text{IC}_{50}/(1 + [\text{ligand}]/K_d)$.

 α -Factor receptor analysis:

Immunoblot analysis of Ste2p was carried out essentially as described previously (Dube et al., 2000). Cells were grown to log phase and then 1×10^8 cells were harvested by centrifugation and lysed by agitation with glass beads in a lysis buffer containing 50 mM Hepes, pH 7.5, 1 mM EDTA, 10 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride, 2 $\mu\text{g}/\text{ml}$ leupeptin, and 2 $\mu\text{g}/\text{ml}$ pepstatin. The lysate was cleared by centrifugation at $2000 \times g$ for 5 min, and then membranes were harvested by centrifugation at $15,000 \times g$ for 45 min. The membrane pellet was washed and then resuspended in 100 μl of a buffer containing 100 mM Tris, pH 8.5, 0.1% SDS. The protein concentration was determined by the

Lowry assay (Pierce), and then aliquots containing either 50 or 100 µg of membrane protein were then digested with TPCK-treated trypsin (Worthington Biochemicals Inc.) at 37 °C for 1 h or longer. No oxidizing reagent was used to promote the disulfide bond formation. The gel samples were incubated at room temperature, heated at 37 °C for 15 min, and then separated on NuPAGE 12% Bis-Tris SDS-polyacrylamide gel (Invitrogen) with either non-reducing or reducing conditions and electrophoretically transferred to 0.2-µm nitrocellulose. The blots were probed with anti-T7 antibodies at 1:10,000 dilutions (Novagen Inc), and then the immunoreactive bands were detected using a Super Signal Ultra ECL kit (Pierce).

Molecular modeling:

A molecular model of the Ste2p was generated by homology with the crystal structure of rhodopsin (Palczewski et al., 2000) using ExPASy molecular biology server (Schwede et al., 2003). After selecting rhodopsin as the modeling template, the amino acid sequence of each TMD of Ste2p was used to construct the model. Computer programs used to predict the boundaries of the TMDs of Ste2p were obtained from the same server (<http://ca.expasy.org>). The co-ordinates of each helix were generated by the protein modeling tool ProMod and final energy minimization was performed by Gromos96 for 200 cycles to improve the stereochemistry of the model and to remove unfavorable clashes. This model was referred to as the resting state, as the template used during modeling was the rhodopsin resting state. A similar model of Ste2p (Eilers et al., 2005) resulted in coordinates for transmembrane domains that were almost identical to the ones generated herein. The activated state of Ste2p was generated using the same

model by introducing torsion on the backbone omega, phi and psi angles around Pro258 and Ser 259 (These residues were mutated to Leu in order to generate a constitutively active receptor.). The torsion applied was just enough to bring N205 and Y266 into close proximity without any unfavorable clashes by using Swiss-PdbViewer (version 3.7) torsion tool. The mutations generated during this study were introduced into the model by using the mutation tool in Swiss-Pdb Viewer and the distances between the side chain atoms were measured with the distance tool in the same program.

CHAPTER 3

Results

Biological activity and binding of Ste2p variants produced by mutagenesis of residues 200-209:

Ten amino acids spanning a portion of Ste2p at the junction between TM5 and extracellular loop 2 (residues 200-209) were mutated by site-directed mutagenesis (Figure 1). All residues were mutated to alanine except residue 206, which is alanine in the native protein; in this case the residue was mutated to glycine. The biological activity of each mutant receptor was measured by the growth arrest assay, as described in the Methods. The response of cells expressing each of the receptor constructs, except F204A and N205A, increased linearly throughout the range of α -factor tested (Figure 2A). Excluding F204A and N205A, which responded only minimally to a high amount of α -factor (8 μ g), the amount of pheromone required to produce a 15 mm zone of inhibition, a halo size in the middle of the dose response curve, was similar for all mutant receptors (Table 1). Measurement of α -factor binding to the receptors indicated that the K_i for WT Ste2p (5.2 nM) was in the range previously reported for this receptor (Chen and Konopka, 1996; Jenness et al., 1986; Rath et al., 1988). Except for the F204A mutant receptor, cells expressing each of the receptor constructs showed affinities for α -factor from 2-fold higher [S207A, I 209A] to about 3-fold lower [Y203A, N205A] when compared to WT receptor (Figure 2B, Table 1). The F204A mutant showed no

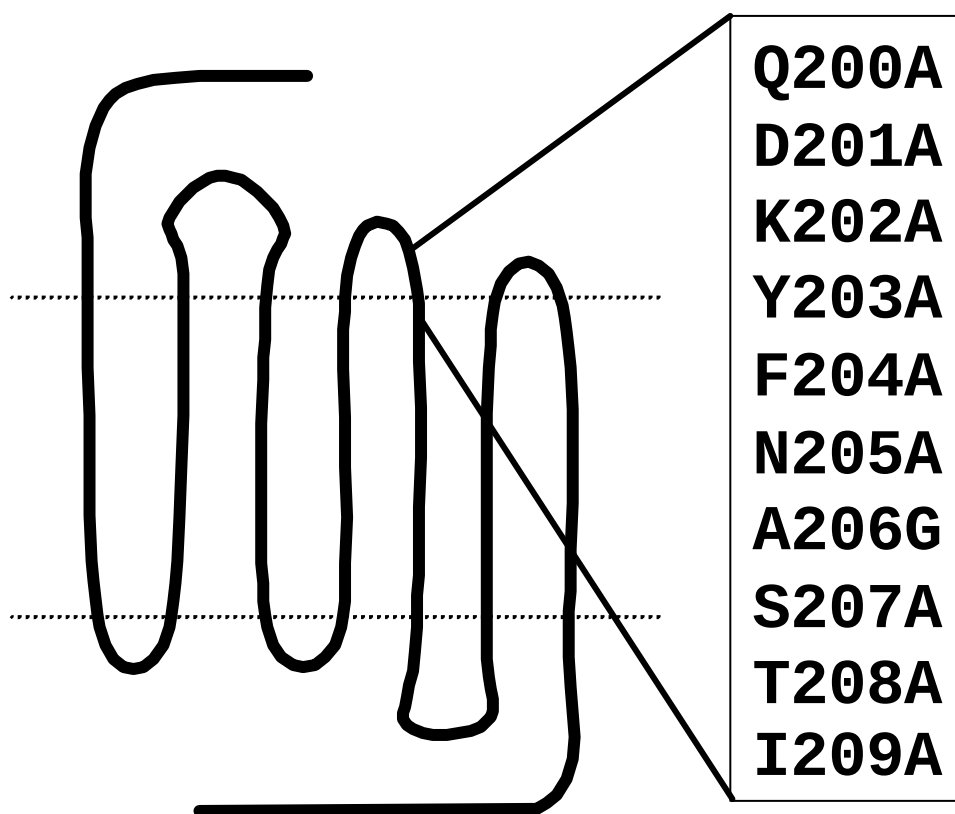
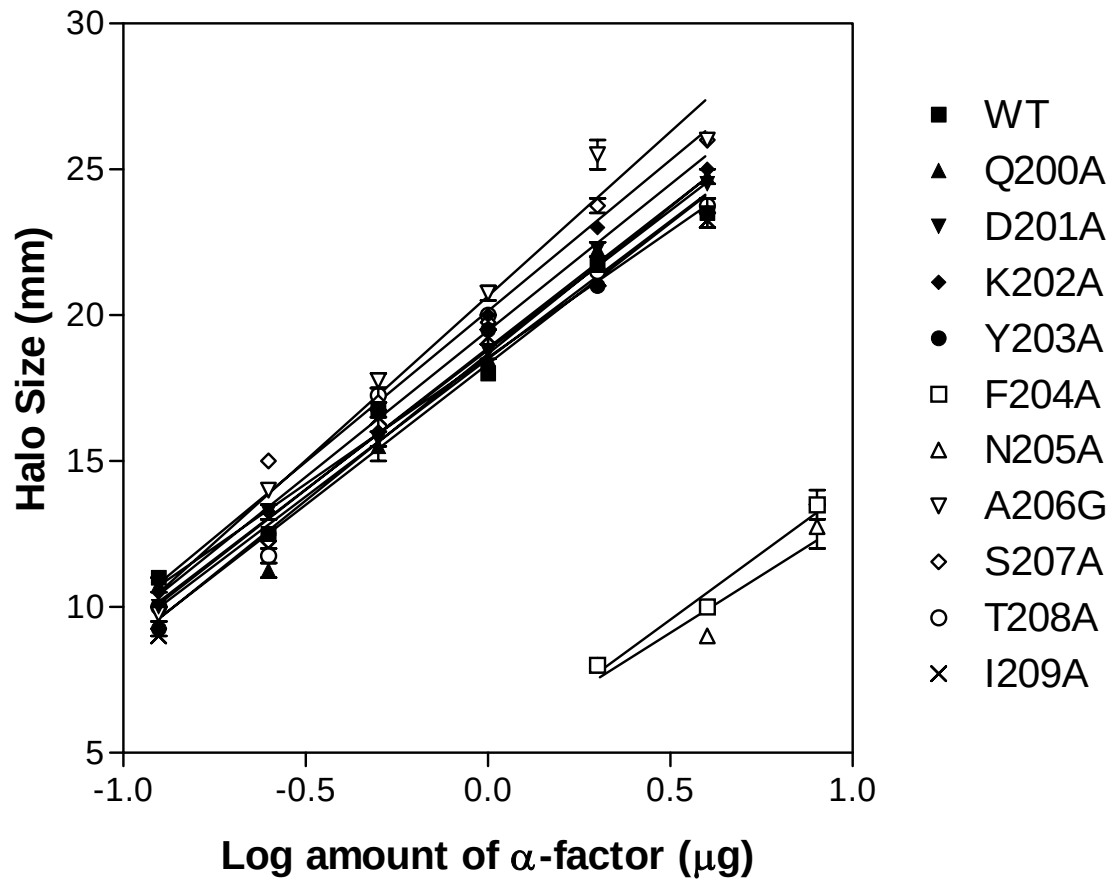


Figure 1. Targeted residues (Box) for site-directed mutation in the region spanning portions of EL 2 and TM5 of α -factor receptor. The predicted two-dimensional topology of the receptor is shown, with the extracellular domains at the top.

Figure 2. (A) Dose-response analysis of growth arrest assay, and (B) competition binding assay for wild type and ten mutant receptors. A. The halo zone of growth inhibition of strains carrying the indicated receptors was measured at various concentrations of α -factor and the data were plotted by regression analysis. The results are the mean $\pm$ standard error (S.E.) of two separate experiments. A comparison of the biological activities of the various mutant receptors is shown in Table I. B. Yeast cells expressing wild type or a mutant Ste2p were incubated with 6 nM of [^{3} H] α -factor in the presence of increasing concentrations of α -factor. The results are the average $\pm$ S.E. of a representative experiment out of three independent experiments. Derived K_i values are given in Table I.

A



B

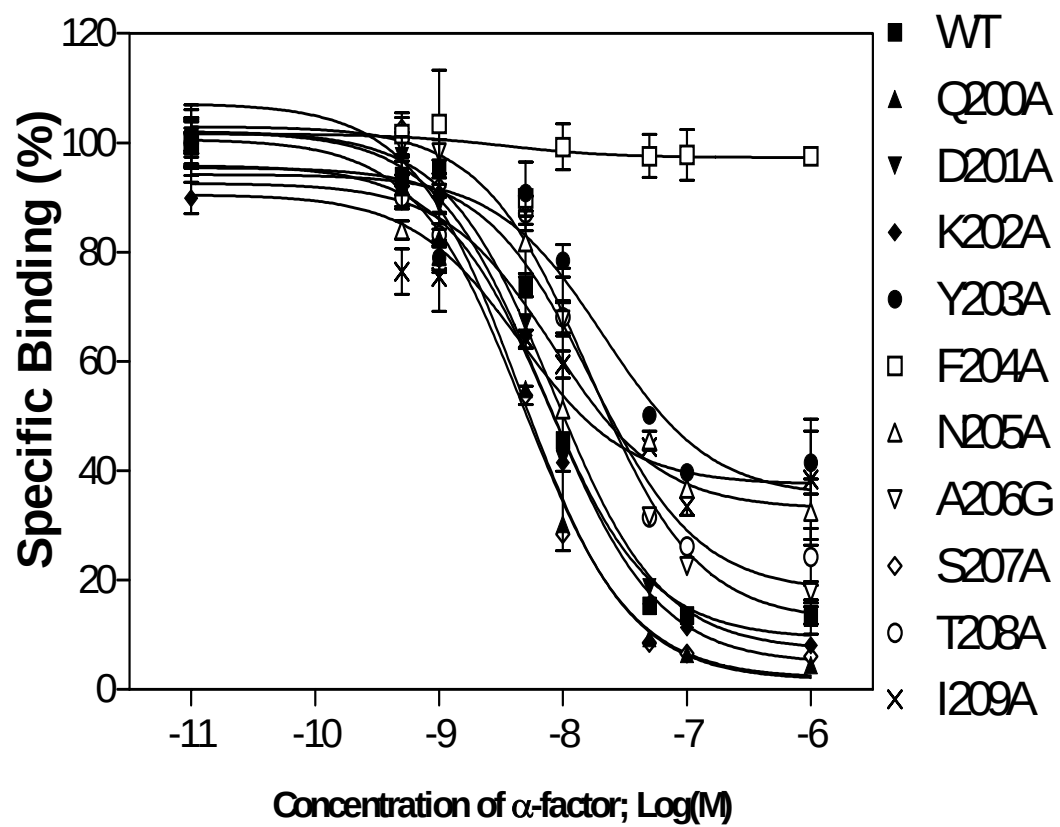


Table 1. Affinities and biological activities of Wild-Type and mutant receptors^a 2

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

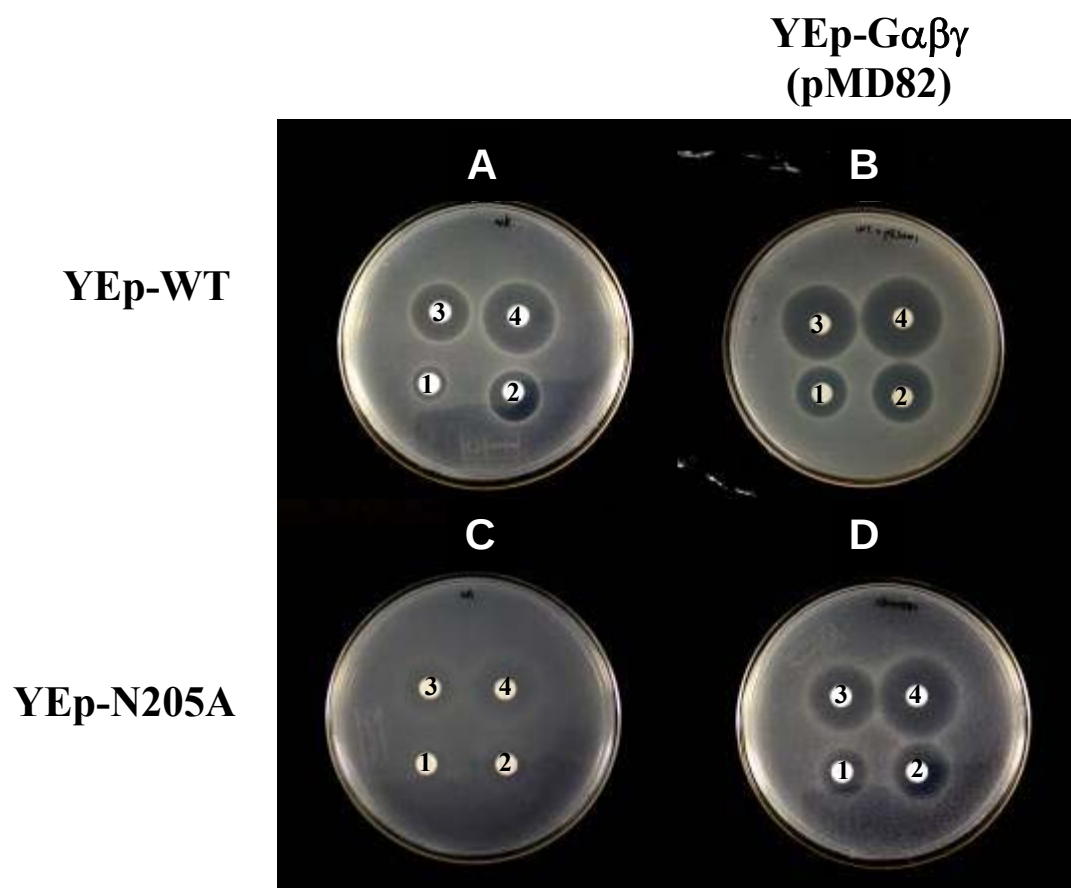
^a Affinities (K_i values in nM) of various receptors for α -factor were measured by competition binding with 3[H] α -factor. Biological activities are the amount of α -factor required to produce a halo of 15 mm from dose-response curves of a growth arrest assay. The percent is compared to the WT receptor as 100% activity.

measurable binding of α -factor at a concentration up to 1 μ M of added pheromone, which agreed with previously reported results for F204S (Dosil et al., 1998). The N205A mutant represented a unique phenotype among the ten mutants studied in that α -factor bound well but the receptor was not able to initiate signal transduction.

Mutant receptor N205A has a dominant-negative phenotype rescued by overproduction of G protein:

In *S. cerevisiae*, dominant negative Ste2p receptors have been shown to be expressed and bind G protein effectively thereby down-regulating signaling from an active, co-expressed receptor (Dosil et al., 1998; Leavitt et al., 1999). Plasmids encoding either wild-type or N205A Ste2p were transformed into LM23-3az, a strain containing a chromosomal copy of WT Ste2p. Co-expression of a WT construct on the plasmid did not effect the strong response to pheromone (Figure 3, Panel A). In contrast, a strain co-expressing the chromosomally-encoded WT and the plasmid-encoded N205A mutant responded weakly to α -factor (Figure 3, Panel C). Additional transformation of these yeast with pMD82, a plasmid expressing the G proteins *GPA1*(G α), *STE4*(G β), and *STE18*(G γ), resulted in a cell with regained sensitivity to α -factor (Figure 3, Panel D) while having little effect on the co-expressed wild-type Ste2p from the chromosome and plasmid (Figure 3, Panel B). These results suggest that the dominant-negative phenotype of the N205A mutant receptor (Fig. 3C) was due to G protein sequestration. Moreover, the results substantiate that N205A is expressed and localized to the cell membrane where it may interact with G protein.

Figure 3. Dominant-negative phenotype of N205A receptor. Cells expressing wild-type Ste2p (chromosomal) and wild-type or mutant (N205A) plasmid-encoded Ste2p with or without overexpression of heterotrimeric G proteins [GPA1 ($G\alpha$), STE4 ($G\beta$), and STE18 ($G\gamma$) genes on pMD82] were assayed for growth arrest by addition of α -factor. The amounts of α -factor applied to disks on each plate were 2, 1, 0.5, 0.25 μ g, indicated by numbers 4 to 1, respectively. Panel A: Ste2p co-expressed from chromosome and plasmid; Panel B: chromosomal Ste2p co-expressed with $G_{\alpha\beta\gamma}$ proteins and plasmid-encoded wild-type Ste2p; Panel C: chromosomal Ste2p co-expressed with N205A mutant; Panel D: chromosomal Ste2p co-expressed with $G_{\alpha\beta\gamma}$ proteins and plasmid-encoded N205A Ste2p.



The characteristics of the N205A and Y266A mutant receptors are very similar:

Similarly to Y266A (Lee et al., 2002), the N205A mutant receptor showed a very good binding affinity ($K_i = 15$ nM as compared to the WT, $K_i = 5$ nM), yet had less than 5% of WT biological activity as measured by the growth arrest assay (Table 1). Both N205A and Y266A also showed very similar characteristics in the *FusI-lacZ* induction assay (Figure 4). To further compare the N205A and Y266A receptors, we carried out competition binding assays using single Ala-substituted α -factor analogs. In both N205A and Y266A receptors the binding affinities of [Ala¹] α -factor through [Ala⁴] α -factor increased about two- to four-fold compared to WT receptor (Table 2). In contrast, Ala substitution at residue 5 and at residues 7 through 13 of α -factor resulted in a decreased affinity of two- to ten-fold for both N205A and Y266A compared to WT Ste2p. Substitution at residue 6 led to a two-fold increase in affinity for the N205A receptor compared to wild-type and a two-fold decrease in the Y266A receptor. None of the Ala-scanned analogs of α -factor induced growth arrest in either mutant. Overall the affinities and activities of Ala-scanned α -factor analogs for N205A and the Y266A mutant Ste2ps were similar (Table 2). Because the N205A and Y266A receptors had a similar recognition pattern to residue 1-4 analogs of α -factor, which are in close proximity to each other, we hypothesized that residues at positions 205 and 266 might occupy a common space to work together to initiate receptor activation.

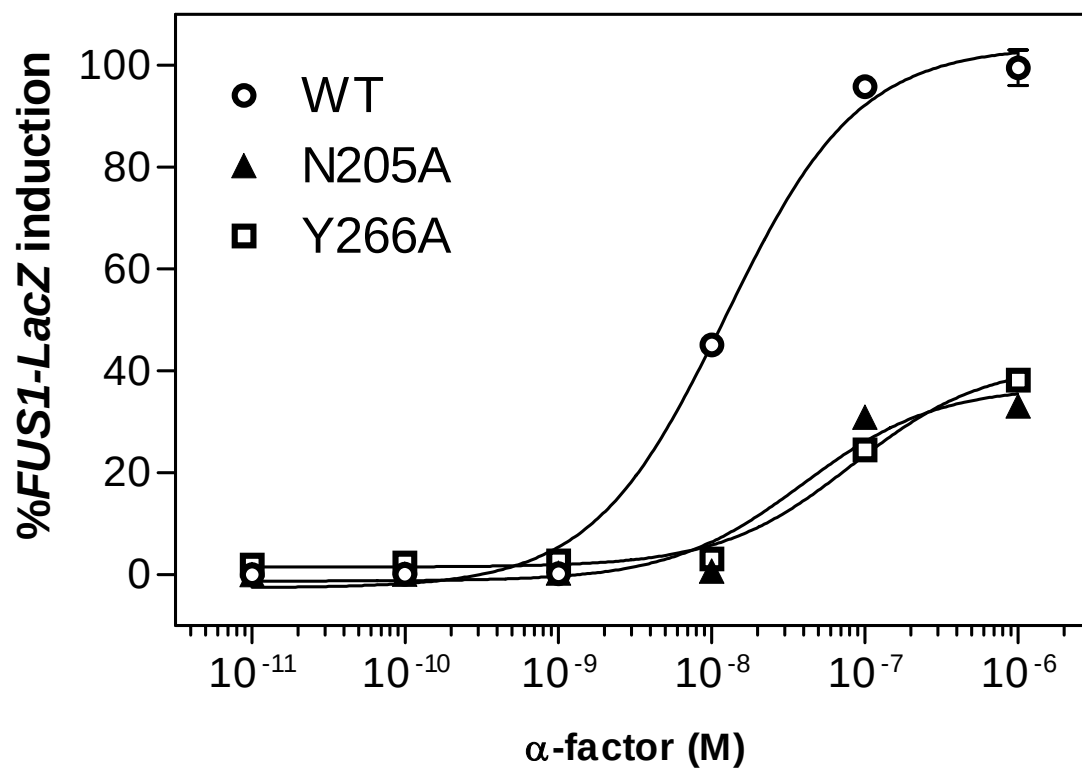


Figure 4. *FUS1-LacZ* assays of N205A mutant receptor and Y266A mutant receptor.

Results represent average from two independent experiments performed in duplicate for Wild-type receptor (o), N205A ($\blacktriangle$), and Y266A ($\square$).

Table 2. Binding affinities of Ala-scanned α -factor analogs for Wild Type, N205A, and Y266A receptors^a

Peptide	Binding(Ki=nM)		Fold Change	
	WT	N205A	N205A	Y266A ^b
α -factor	6.8±1.3	14.5±4.3	-2.13	-6.0
[Ala1]	114±13	45.4±8	+2.51	+2.3
[Ala2]	388±24	115±15	+3.36	+3.8
[Ala3]	185±19	79.1±7	+2.34	+4.0
[Ala4]	284± 34	58.9±9	+4.82	+3.3
[Ala5]	7.5±1.2	79.5±8	-10.6	-7.2
[Ala6]	1300±143	570±9	+2.28	-2.2
[Ala7]	37.7±7.5	186±13	-4.93	-10.4
[Ala8]	5.6±1.2	13.3±2.5	-2.38	-6.9
[Ala9]	680±58	1600±86	-2.36	-2.6
[Ala10]	790±55	1350±79	-1.71	-5.0
[Ala11]	151±14	451±36	-2.98	-8.0
[Ala12]	760±68	4200±186	-5.51	-10.6
[Ala13]	> 2000	890±66	ND ^c	-3.4

^a K_i values for α -factor and 13 Ala-scanned analogs were determined in competition binding assays by displacement of [³H] α -factor. ^b Data from Lee *et al* (Lee et al., 2002). All values are the mean $\pm$ S.E. from three separate experiments. ^cThe ratio could not be determined from the binding data obtained.

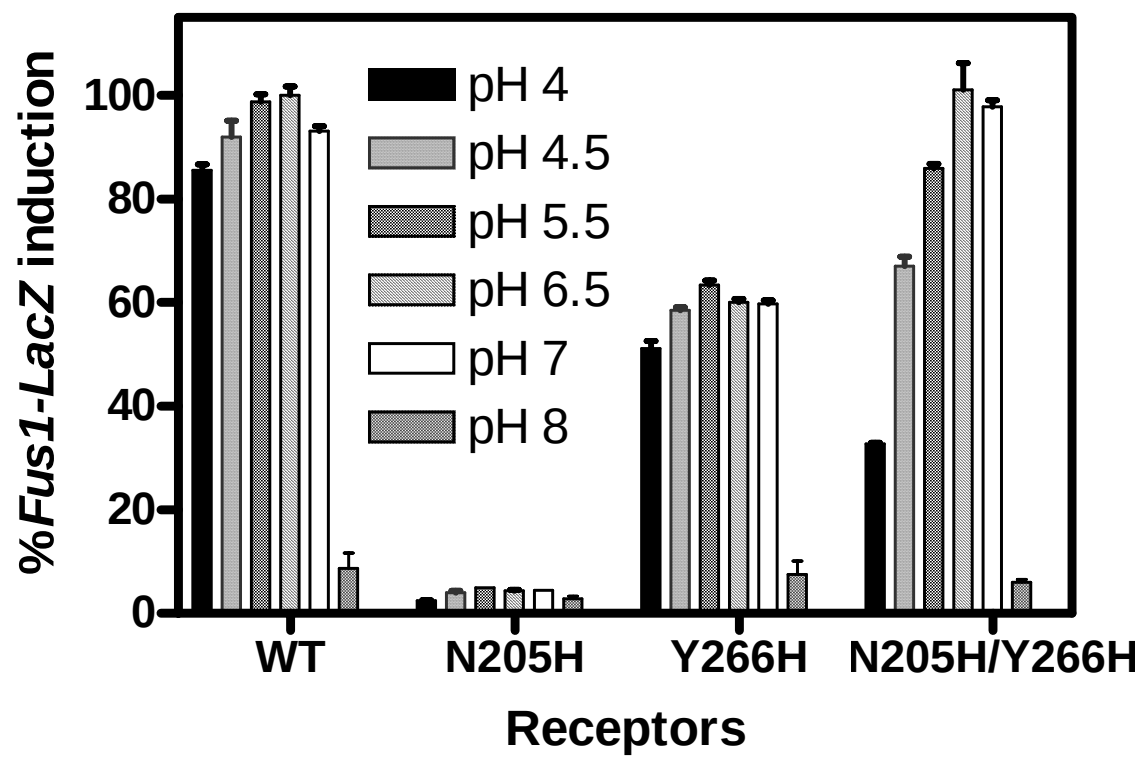
N205H mutant receptor is non-functional; however, the Y266H mutant is partially functional and the double mutant N205H/Y266H is fully functional:

To test for possible interaction between the residues residing at positions 205 and 266, we introduced histidines at these positions. Interactions between these histidine residues are expected to be pH-dependent because of hydrogen bonding or electrostatic interactions between the two imidazole groups. Therefore, we carried out *Fus1-lacZ* induction assays in different buffers (pH 4.0, 5.0, 6.0, 7.0, 7.5, and pH 8.0) so that the pH remained relatively constant under the assay conditions. Starting at pH 5.0, 6.0, 7.0 and 7.5 with MES buffer, the pH of the reaction mixture actually was lowered by one-half pH unit during the course of the 2 hours incubation. The pH values for the assays are reported as the final pH value (Figure 5A). The single N205H mutant receptor was non-functional at all pH values tested (Figure 5A), whereas the Y266H mutant receptor was partially functional at all pH values between 4 and 7 (50% to 65% of wild-type signaling). All receptors, including the WT receptor, were not functional at pH 8 as measured by a *Fus1-lacZ* induction assay. Compared to the N205H single mutant the N205H/Y266H double mutant receptor recovered partial function at pH 4 and 4.5, was almost as active as the WT receptor at pH 5.5, and was equally active to WT receptor at pH 6.5 and 7.0. A growth arrest assay with the N205H mutant showed this mutant receptor was nonfunctional at pH 6 whereas the N205H/Y266H mutant receptor was almost as active as the WT receptor at this pH in response to pheromone (Figure 5B). Although others have used zinc to activate site-directed, histidine-substituted GPCRs (Elling et al., 1999; Holst et al., 2000), the N205H/Y266H mutant was not activated by zinc under the experimental conditions of the gene induction assay (data not shown). In

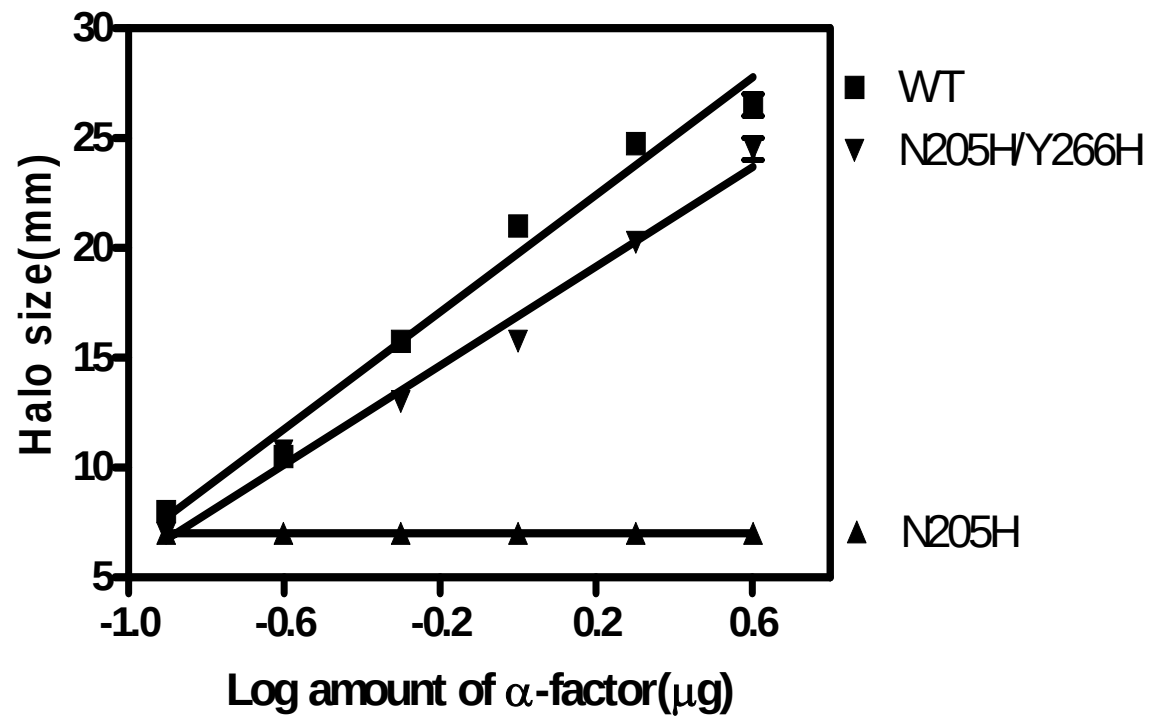
Figure 5. *FUS1-LacZ* assays and growth arrest assay of WT and mutant receptors.

A, pH-dependent *LacZ* activity of WT, N205H, Y266H and N205H/Y266H mutant receptors. Results represent average from two independent experiments performed in duplicate. All data are normalized to the activity of wild-type receptor responding to 1 μ M α -factor at a particular pH value. B, The halo (zone of growth inhibition) sizes measured at various concentrations of α -factor at pH 6. The data were plotted by regression analysis. The results are the mean $\pm$ S.E. of two separate experiments.

A



B



addition, N205A/Y266A, N205Y/Y266N, N205D/Y266K, and N205K/Y266D double mutants were all non-functional in the *Fus1-LacZ* induction assay (data not shown). These results are consistent with our previous report that an aromatic residue in the 266th position of Ste2p is important for receptor activation (Lee et al., 2002). While the five-membered His residue was able to partially complement the function of the six-membered Tyr residue, substitution with other residues, such as Asn, Asp, and Lys did not result in functional receptors. The high activity of the N205H/Y266H mutant led us to hypothesize that the interaction between N205 and Y266 in the WT receptor occurs during ligand-induced receptor activation, or in a constitutively-active state of receptor. This hypothesis guided the next series of experiments which investigated whether residues at the 205th and 266th positions could interact in the resting and/or activated state of the receptor.

N205 interacts with Y266 only in an activated state of Ste2p:

In order to determine if these positions interacted in an active state of the receptor, we constructed the strong constitutively active mutant receptor of Ste2p (P258L/S259L) which has been previously used to mimic the Ste2p activated state (Konopka et al., 1996). Unfortunately, we could not test whether the interaction occurred in the receptor activated by ligand addition, since Tyr in position 266 is essential for receptor activation; substitution of Tyr at 266 by any other residue renders the receptor unresponsive to α -factor (Lee et al., 2002). We also introduced cysteine, aspartic acid, and/or lysine in the 205th and 266th positions in the P258L/S259L background. These residues were utilized to determine (1) if a covalent bond would be formed in the

activated receptor between N205C and Y266C and (2) if electrostatic interactions between N205K/D and Y266K/D enhanced or interfered with receptor activity.

The basal signaling activity of the P258L/S259L constitutively active receptor increased about 10-fold compared to wild-type (Figure 6), as noted previously (Konopka et al., 1996). The introduction of N205K or Y266D mutations into the constitutively active mutant receptor suppressed the constitutive activity about 50% and 65%, respectively. However, the introduction a double mutation N205K/Y266D into the P258L/S259L background restored high constitutive activity (Figure 6). The reciprocal mutations (N205D, Y266K or N205D/Y266K) in the constitutively active mutant receptor all led to receptors with 45-55% lowered constitutive activity (Figure 6). As stated above, neither the N205K/Y266D nor N205D/Y266K was active in the wild-type background.

We used a biochemical analysis to test whether an interaction occurred between N205C/Y266C in the constitutively activated state. Disulfide cross-linking has been previously used to study topology and proximity of residues in GPCRs including rhodopsin (Cai et al., 1997) (Yang et al., 1996) (Yu et al., 1999) and M3 muscarinic receptor (Ward et al., 2002); (Han et al., 2005). In addition, a method for determining the interaction between TM5 and TM6 of Ste2p using disulfide cross-linking was established previously using T7 epitope tagging and trypsin digestion (Dube et al., 2000). Our results demonstrated that the single N205C mutant was partially active and the Y266C and N205C/Y266C mutants were not active in the WT background (data not shown and (Lin et al., 2003)).

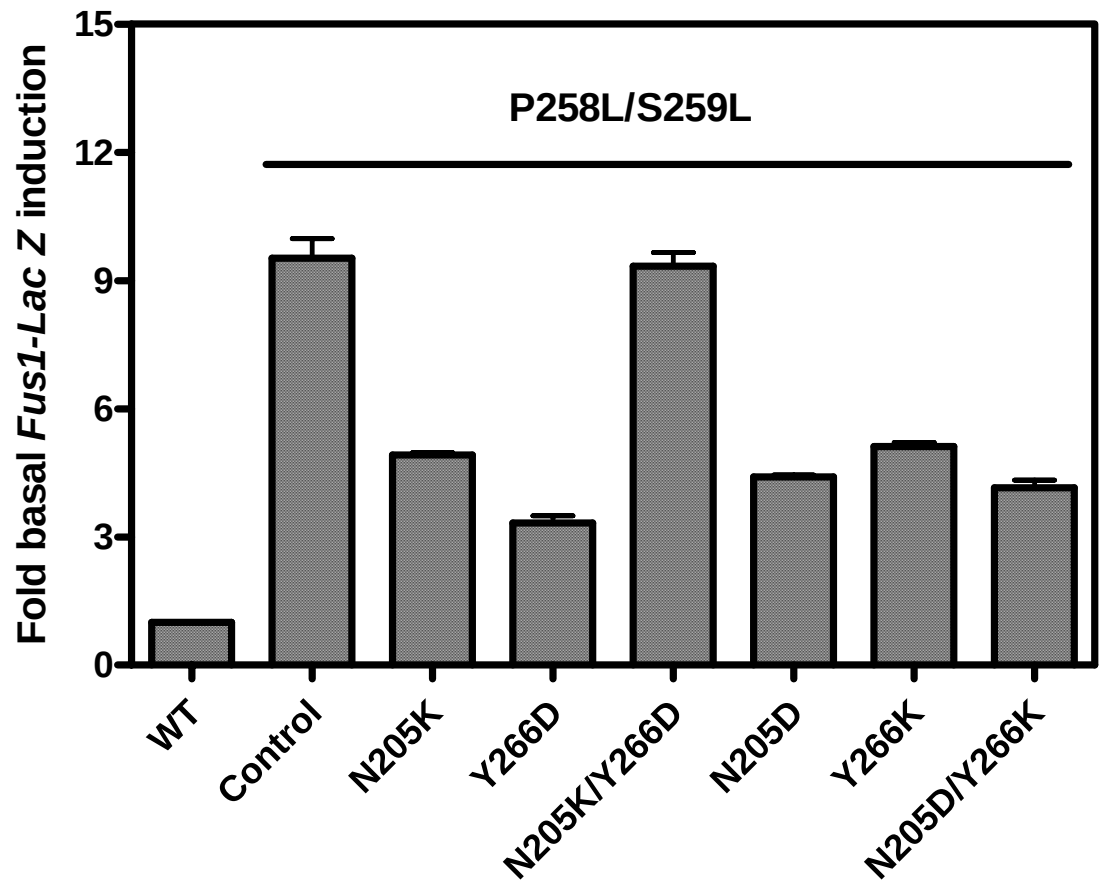
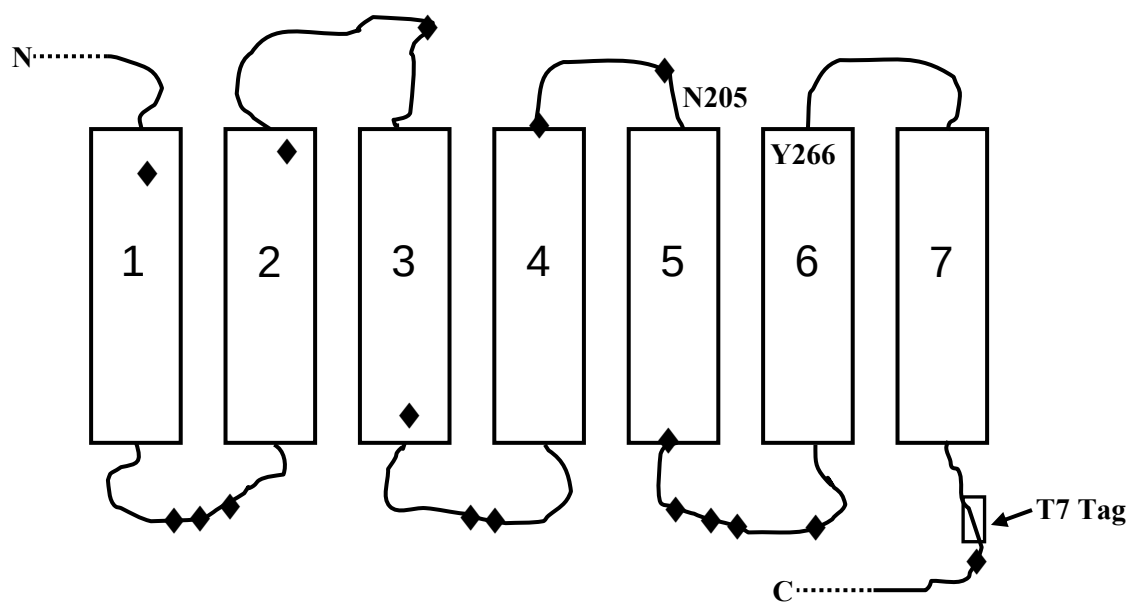


Figure 6. *FUS1-LacZ* assays of WT and various mutant receptors containing P258L/S259L constitutively active mutation. Basal signaling activity of *Fus1-LacZ* induction was measured in the absence α -factor with various P258L/S259L mutant receptors. The fold increase of basal activity of *Fus1-LacZ* induction was determined by relative increase of activity compared to wild-type receptor. Results represent average from two independent experiments performed in duplicate.

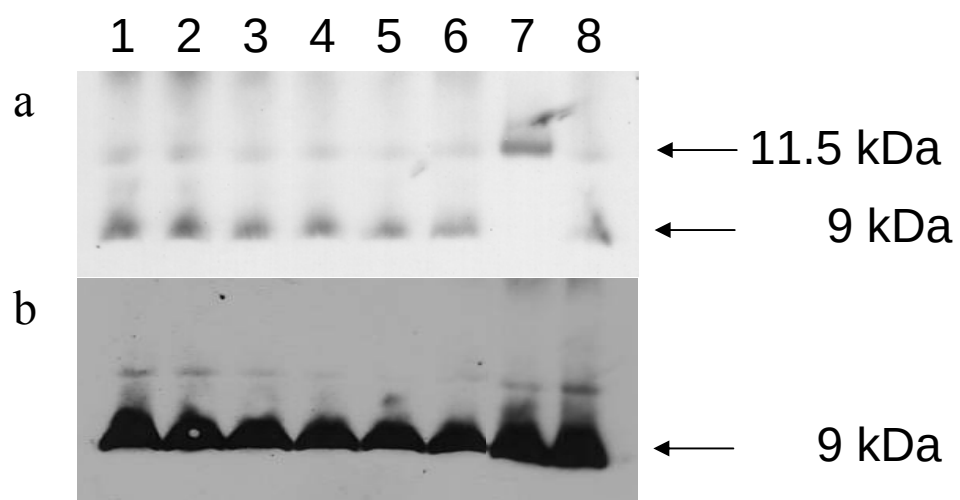
However, the quadruple mutant N205C/Y266C/P258L/S259L was as constitutively active as the P258L/S259L mutant (data not shown). While many studies of disulfide cross-linking have been done in the presence of oxidizing reagent to promote the bond formation (Yu and Oprian, 1999) (Yu et al., 1999) (Ward et al., 2002) (Han et al., 2005), our system did not require an oxidizing reagent such as Cu-phenanthroline to force disulfide formation between N205 and Y266 residues. In addition, the Cu-phenanthroline reagent did not activate N205C/Y266C mutant Ste2p (data not shown). After trypsin digestion, the N205C/Y266C/ P258L/S259L mutant receptor gave only a 11.5 kDa band (Y203 to K225 of TM5 and Q240 to K304 of TM6-TM7-epitope T7 with a disulfide bond between 205C and 266C) in the non-reducing PAGE (Figure 7B-a). In contrast, under reducing conditions (Figure 7B-b) this mutant receptor gave predominantly the 9 kDa band suggesting that a disulfide bond formed in the constitutively active mutant. Under both reducing and non-reducing conditions, N205C/Y266C receptor in the WT background gave a major 9 kDa band (Q240 to K304 comprising TM6 and TM7 with the T7 epitope). To test whether the disulfide crosslink between N205C and Y266C was specific in the constitutively-active mutant, we generated N205C/A265C and F204C/Y266C receptors both in the WT and constitutively-active background. Similarly to N205C/Y266C, these mutant receptors were not active in the WT background and were constitutively active in the P258L/S259L background (data not shown). After trypsin digestion, N205C/A265C and F204C/Y266C receptors gave a major 9 kDa band upon western blotting in both non-reducing and reducing gels (Figure 7B). A minor 11.5 kDa background band appeared in all receptors in both reducing and non-reducing conditions.

Figure 7. Disulfide bond formation in the N205C/Y266C mutant receptors. Panel A. diagram of Ste2p with the position of the T7 tag indicated. Sites of trypsin digestion are indicated with a *filled diamond*. The tryptic fragment containing TMD5 is ~2.5 KDa, and the tryptic fragment corresponding to TMD6 and TMD7 is ~9 KDa. The N- and C-terminal proteins of Ste2p are not shown. Panel B. Membranes were harvested from yeast LM102 carrying N205C, F204C/Y266C, N205C/Y266C, and N205C/A265C receptors in the WT background (Lane 1-4) and N205C, F204C/Y266C, N205C/Y266C, and N205C/A265C in the P258L/S259L constitutively active background (Lane 5-8). Both receptors contained a T7 epitope-tag in TM7. Membranes were digested with the trypsin and samples were run under non-reducing conditions (a), or under reducing conditions (b) by addition of dithiothreitol (200 mM). Membrane protein digests were transferred to nitrocellulose and probed with anti-T7, HRP-conjugated antibody.

A



B



These results strongly suggest that an interaction between residues at the 205th and 266th positions in Ste2p occurs only, and specifically, in an activated state of this GPCR.

CHAPTER 4

Discussion

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

In this dissertation we used bioassays, receptor mutagenesis and chemical crosslinking to reveal an interaction between two residues of Ste2p that occurs only in the activated receptor. Our data and those previously reported (Dosil et al., 1998; Leavitt et al., 1999; Lin et al., 2003) indicate that N205 is involved in Ste2p signaling. Of the 9 residues (Q200—I209) that surround N205 only F204 was important for signaling as judged by Ala-scan mutagenesis (Table I). Some of the phenotypes of the Ala-scanned Ste2p were similar to those reported in Cys scanning and random mutagenesis of this receptor. For example N205C resulted in decreased halo size in growth arrest assays and about 80 % of *Lac Z* induction when compared to WT. The α -factor binding activity of N205C was measureable, with a K_d greater than 30nM (Lin et al., 2003) (Lin et al., 2004). Although there are differences between N205A and N205C mutant receptors

regarding binding affinity and *Lac Z* induction, in general, these mutants are similar in that both mutants are able to bind α -factor with reduced affinity and produce halos of reduced size, relative to WT. The characteristic difference between Ala and Cys substitution might account for this difference. Our result showed that N205C mutant is more active than N205A mutant in the gene induction as well as growth arrest assay (data not shown). Additionally, the mutants in the present study were expressed in a background strain (LM102) different from that used in their studies. It has been proposed that differences in strain background might account for quantitative differences (Stefan et al., 1998). In contrast, unlike N205A which exhibited 15 nM affinity for α -factor, F204A did not bind pheromone. Thus the phenotype of N205 is that of a receptor residue critical for signaling but not essential for high-affinity ligand binding. In this respect N205 resembled the phenotype of Y266 previously studied by us (Lee et al., 2002). Indeed as found for the Y266A mutant receptor, N205A appeared to interact primarily with residues near the N-terminus of α -factor using pheromone Ala-scanning analysis (Table II). Moreover, both of these receptors exhibited dominant negative phenotypes that reflect sequestration of G proteins and support the fact that these proteins are expressed intact and localize to the cell membrane.

The above findings suggested that N205 and Y266 might be near each other in the 3D structure of Ste2p. Indeed hydropathy and modeling analyses placed N205 and Y266 near the extracellular ends of TM5 and TM6 of Ste2p, respectively. It was tempting, therefore, to conclude that these key residues interacted. However, previous studies had shown that in the absence of α -factor N205C, but not Y266C, was accessible to a

hydrophilic thiol suggesting distinct environments and a lack of proximity (Lin et al., 2003). To explore further the putative interactions of N205 with Y266 we constructed Ste2p mutants containing His at one or both of these positions. N205H did not respond to α -factor from pH4 to 7.0 and Y266H exhibited 50-65% receptor activation as judged using a gene induction assay over the same pH range (Figure 5A). Intriguingly, the N205H/Y266H mutant receptor gave full activation at pH6.5 and 7.0 and 30% to 90% signaling at pH 4 to pH 5.5. This suppression of the N205H phenotype in the double mutant can be indicative of an interaction between residues 205 and 266 or between these residues and a side chain of the ligand in the activated receptor. The pH profile suggested that optimum interaction might require one protonated and one unprotonated His ($pK_a \sim 6.5$). The decrease or lack of activity at pH 4 and 8, respectively, was not due to poor binding of α -factor to the N205H/Y266H receptor, as the α -factor binding affinity to this mutant receptor was not changed significantly when tested at pH values of 4, 6, and 8 (data not shown). Good binding of α -factor at pH 8 has been shown previously for WT Ste2p (Blumer and Thorner, 1990).

To probe the putative proximity of N205 and Y266 we used a Ste2p background that gave high basal signaling in the absence of α -factor (Konopka et al., 1996). Presumably the P258L/S259L receptor reflects an active conformation of Ste2p and interactions observed in this background indicate groups that are near to each other in the signaling state of this GPCR. In this background, the N205K/Y266D double mutation showed high basal signaling activity similar to that of the P258L/S259L constitutively active receptor, although both the N205K and Y266D single mutations suppressed

constitutive activation (Figure 6). A plausible interpretation of these experimental results is that in an active state, but not in the inactive state, N205 and Y266 interact with each other. The reciprocal mutation N205D/Y266K in the constitutively active mutant receptor suppressed constitutive activity to the same level as the single N205D or Y266K mutations in the P258L/S259L receptor (Figure 6). Nevertheless, the effect of the single mutations was not additive in the double mutant, again suggesting a favorable interaction between side chains of residues at position 205 and 266 in the activated receptor. Perhaps a particular orientation of side chains of both residues and/or the neighboring residue context is required for optimal interaction between N205 and Y266. It has been reported that a predicted salt bridge interaction between Arg 173 and Glu 318 in a GPCR was not interchangeable when reciprocal mutation was done (Shapiro et al., 2002). In addition, although the interaction between R302 and E330 was identified in the crystal structure of crystallin, the R302E/E330R reciprocal mutation showed 31% of WT activity which is equivalent to the activity of E330R mutant (Yu et al., 2004). These results suggest that a salt bridge interaction is not always interchangeable and may account for the suppression of constitutive activity by the introduction of N205D/Y266K but not N025K/Y266D into the P258L/S259L constitutively active mutant.

Another interpretation for the different level of basal activities of various mutant receptors (Figure 6) might be due to the difference in the number receptor molecules expressed at the cell surface. However, it has been proposed that as little as 2% of the wild-type level of receptors can still induce maximal response in a short-term *FUS1-lacZ* assay (Dube and Konopka, 1998) (Shah and Marsh, 1996). In addition, although the number of cell surface receptor of the P258Y mutant receptor was 80-fold less than that

of P258L mutant receptor, the P258Y showed 3-fold higher activity than P258L mutant receptor (Stefan et al., 1998). These results suggest that the different level of basal signaling is not be due to a different number of cell surface receptors.

To test further the interaction between N205 and Y266 in an active state of receptor, in addition to the genetic analysis of the interaction, we used a biochemical analysis using disulfide-crosslinking, as previously applied to establish an interaction between TM5 and TM6 of Ste2p (Dube et al., 2000). In that study, formation of a covalent bond between residues of the 5th and 6th TMs was detected by western blotting in which a larger peptide was apparent in non-reducing gels using an antibody to an epitope appended to TM7 as a result of formation of a disulfide bond between TM5 and TM6. We introduced the N205C/Y266C double mutation into WT and the P258L/S259L constitutively active receptors. We found that a major disulfide-crosslinked fragment of the expected size (~11.5 kDa) formed in this activated receptor with no 9 kDa band observable (Figure 7B-a, Lane 7). This 11.5 kDa band was greatly diminished in a reducing gel (Fig. 7B-b, lane 7). It has been reported that Ste2p oligomerization promoted receptor biogenesis and signaling (Overton et al., 2003). Therefore, we were concerned that the 11.5 kDa fragment represented an intermolecular crosslink between N205C-N205C or Y266C-Y266C. However, the N205C-N205C crosslink would not be detected by the antibody to T7 and a Y266C-Y266C crosslink would result in a 18 kDa band.

While N205C/Y266C in constitutively active mutant receptor gave a 11.5 kDa band after trypsin digestion in non-reducing gel and 9 kDa band in reducing gel, the N205C, N205C/A265C, and F204C/Y266C in WT as well as constitutively active mutant background gave a major 9 kDa band both in non-reducing and reducing gel.

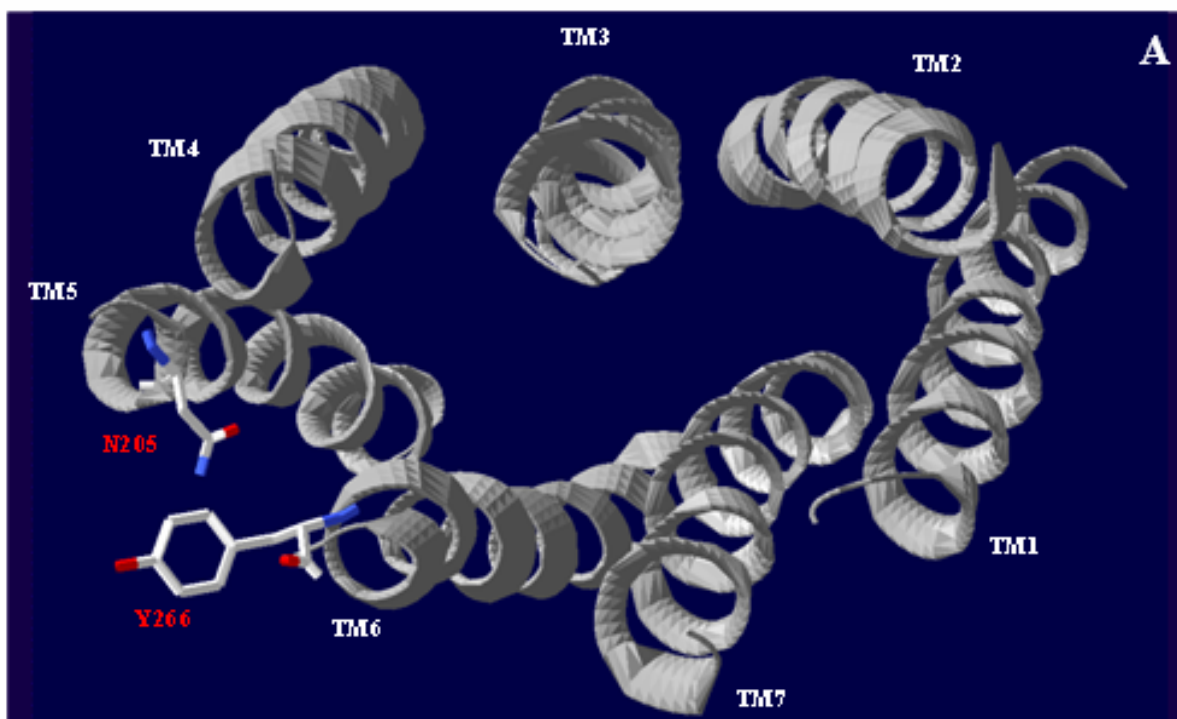
These data suggest that the interaction is not a non-specific in an activated state and not an intermolecular crosslink between N205C-N205C or Y266C-Y266C. Thus, the 11.5 kDa band in Fig 7B-a represents a fragment with a disulfide bond between N205C and Y266C in an activated state of receptor. While the N205C/Y266C in constitutively active background gave a 11.5 kDa band only, the other mutants gave a minor 11.5 kDa band in addition to a major 9 kDa band in non-reducing gel (Figure 7B-a). To test this minor 11.5 kDa band is due to the disulfide crosslinking product, we ran the samples in reducing gel (Figure 7B-b). The minor 11.5 kDa band was not reduced by reducing agent and correlated with the intensity of 9 kDa band suggesting this band may be a partially digested product. This partially digested 11.5 kDa might suggest the less accessibility of trypsin into this region due to the interaction between G proteins and intracellular loop 3 (Figure 7A, (Madden and Snyder, 1998)) Together with the mutagenesis data, these cross-linking results strongly suggest that N205 interacts with Y266 directly only in an activated state of the receptor.

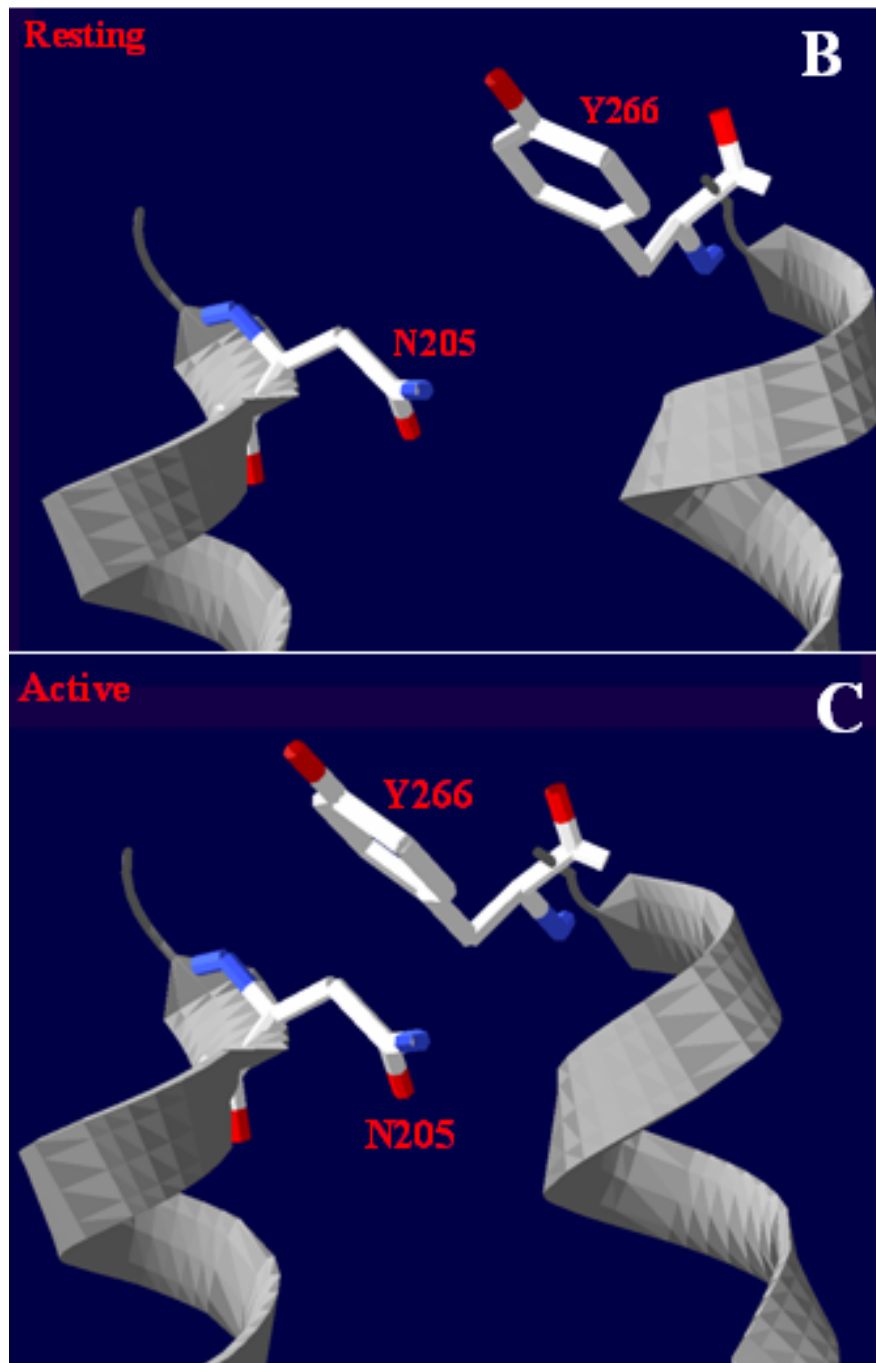
Based on the results generated in this communication, we have built a model to help interpret the findings. The residues composing the transmembrane domains of Ste2p were superimposed upon the rhodopsin model using the Swiss-Prot database generating a model for Ste2p (see Methods). Though there is no significant sequence homology between rhodopsin and Ste2p, rhodopsin has been used for the modeling of Ste2p to explain site-directed mutations that result in functional receptors (Lin et al., 2003) (Lin et al., 2004) and to show that general mechanism of receptor activation between rhodopsin and Ste2p are very similar even though their sequences are very divergent (Eilers et al., 2005). We show Ste2p transmembrane domains in which residues N205 and Y266 are

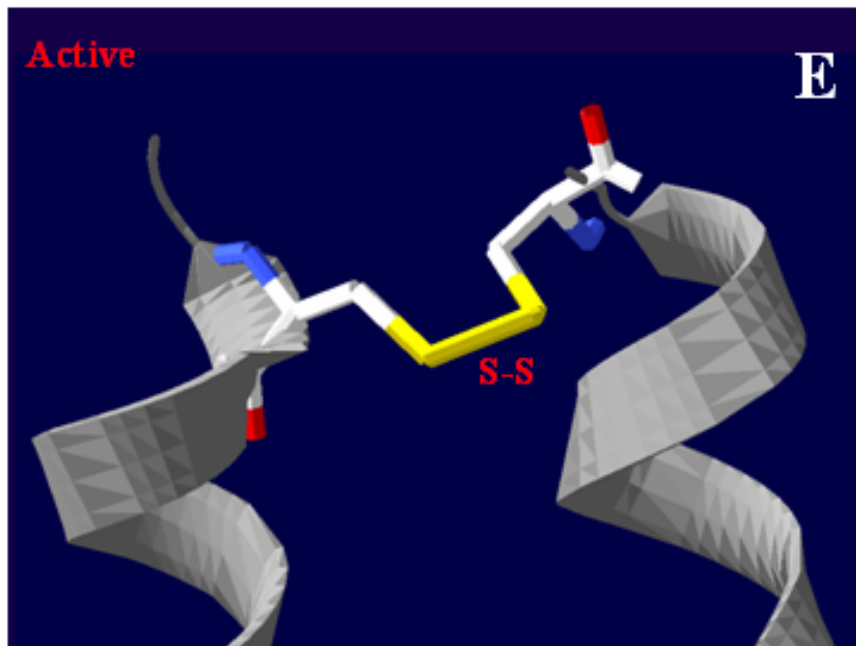
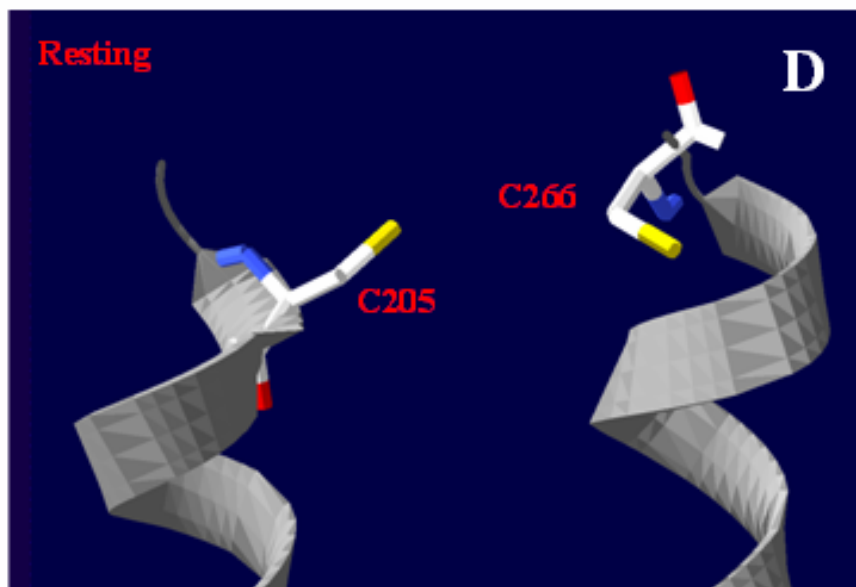
highlighted (Figure 8A). The assumption is that this model is in the resting state, because it was generated from rhodopsin in its resting state. A higher resolution depiction of N205 and Y266 is shown in Figure 8B. The model placed these residues at a distance of ~ 6 Å (corresponding the side-chain O of Asn205 to the C1 of the benzoyl ring of Tyr266) which may not be close enough for productive interaction. Modeling by another group came to a similar conclusion that these residues were separated by a distance of 8 Å (S. O. Smith, personal communication). These atoms come closer together (to ~ 2 Å) if we take TM6 and introduce the P258L/S259L mutation (the constitutively active mutant) which causes a conformational change in TM6 bringing Y266 closer to N205. Presumably, a similar closing of the distance between these residues occurs upon ligand binding in the native Ste2p. When Cys replaces residues N205 and Y266 (Figure 8D), the residues are still separated by ~ 6 Å (between the S atoms in 205 and 266), whereas in an active state the residues are close enough to form a disulfide bridge which we detected by a biochemical experiment (Figure 7). Finally, when histidine substitutes for N205 and Y266 (Figure 8F and G) the residues are brought into close proximity in an active receptor (Figure 8G) allowing for a productive interaction that may promote signal transduction.

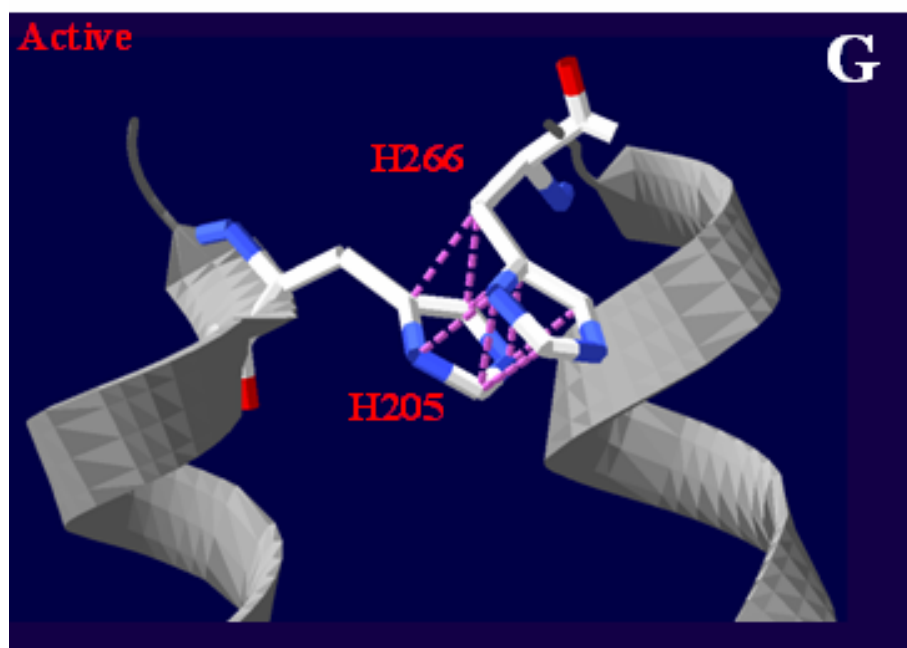
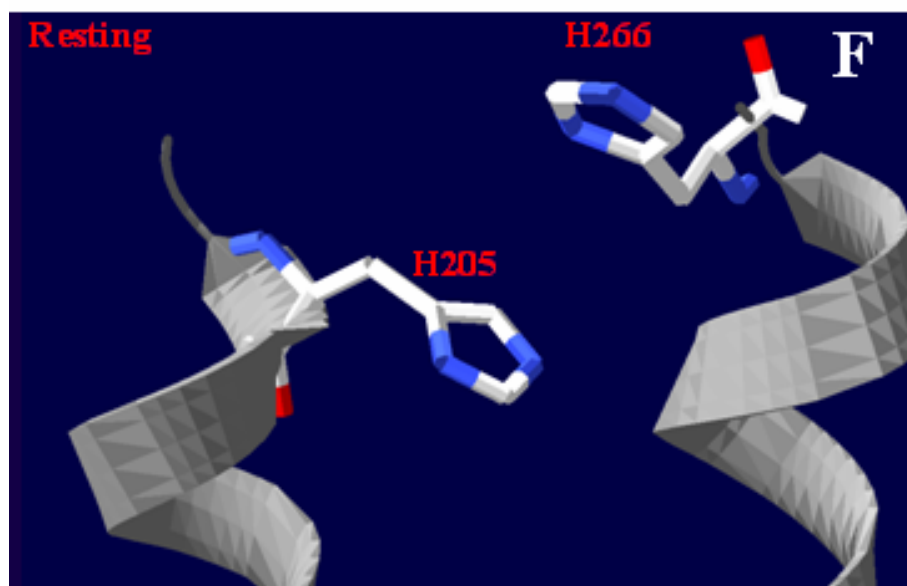
While numerous intramolecular interactions have been identified for inactive state conformations of GPCRs, much less is known about the intramolecular interactions stabilizing active state conformations (Kristiansen, 2004). Two reports (Elling et al., 1999; Holst et al., 2000) were interpreted by Kristiansen (Kristiansen, 2004) as demonstrating specific interactions in the active state of a GPCR by protein engineering and biochemical analysis.

Figure 8. Model of Ste2p. The TMs of the receptor are represented with residues N205 and Y266 shown in detail in the presumed resting state as drawn by superimposing Ste2p residues onto the rhodopsin model (Panel A). TM 5 and 6 and various residues in the 205th and 266 positions are represented in Panels B-G. N205 and Y266 are represented in the resting (Panel B) and active state (Panel C), the double cysteine receptor is shown in the resting (Panel D) and active state (Panel E), and the double histidine receptor is represented in the resting (Panel F) and active state (Panel G).









In these experiments, zinc was used as an artificial ligand to induce activation of the D113H/N312C double mutant of the human β_2 adrenergic receptor and the P112H/M291C human NK₁ neurokinin receptor. Although the addition of zinc was able to induce β_2 adrenergic receptor activation, the authors concluded that no conformational change was required to convert the resting receptor to its activated state based on molecular modeling. In addition, interpretation of these experiments is confounded by the observations that allosteric binding sites for zinc have been identified in the β_2 -adrenergic receptor (Swaminath et al., 2003). Therefore, it is not possible to conclude that the residues 113 and 312 interact only in the active state. In the case of the P112H/M291C NK₁ mutant receptor, the metal ion acted as a partial agonist as well as a pure antagonist when used with Substance P, the natural agonist for this receptor making it unreasonable to conclude that residues 112 and 291 interact only in the active state.

We investigated whether the N205H/Y266H mutant receptor could be activated in the presence of zinc. Similarly, we tested whether the N205C/Y266C mutant receptor could be activated in the presence of Cu-phenanthroline. We did not observe an elevated level of reporter gene induction under these conditions (data not shown). Perhaps, in Ste2p, the residues N205 and Y266 are not in close enough proximity in the resting state for zinc or oxidizing reagent to form a cross bridge between them to activate the receptor, or these reagents did not reach the cellular site to produce a cross bridge. Other studies have reported agonist-dependent disulfide bond formation between specific residues rat M3 muscarinic receptor (Han et al., 2005) (Ward et al., 2002). However, it is not clear if the disulfide bond formation was indeed specific for the receptor active state because disulfide bond were formed in the presence of antagonist and in the absence of agonist.

These conditions are expected to stabilize non-activated states of the GPCR. In addition, disulfide bond formation necessarily required oxidizing reagents such as Cu-phenanthroline or molecular iodine that may have forced non-native interactions.

Understanding how G protein-coupled receptors are activated by ligand binding is an important goal of workers in the GPCR field. Though many kinetic models have been developed to describe the process of agonist activation, the most widely accepted model is the extended ternary complex model (Leff, 1995; Lefkowitz et al., 1993). According to the model, the receptor exists in an equilibrium between an inactive conformation (R) and an active conformation (R*). However, there is increasing evidence that there are successive conformational changes during the binding of agonist to non-peptide hormone receptors such as β_2 -adrenoreceptor (Swaminath et al., 2004) as well as peptide hormone receptors such as the AT₁ (Hunyady et al., 2003). Thus, the interpretation of our results based on a resting and active state receptor model are highly simplified representations of what may actually occur during receptor activation.

In this correspondence we have proposed that interaction of residues N205 and Y266 occurs in the constitutively-activated receptor state. We speculate that similar interaction occurs in the agonist-induced active state of Ste2p. However, we were not able to show this interaction in the α -factor-induced activated receptor, because the N205C/Y266C mutant in the WT background does not respond to α -factor. For such interaction to occur, it is likely that movement of helices is required because, in the absence of α -factor, N205 is accessible to thiol reagents whereas Y266 is not (Lin et al., 2003). It has been suggested that subsequent to ligand binding there are relative

movements of transmembrane helices of other GPCRs during receptor activation (Farrens et al., 1996; Karnik et al., 2003; Stefan and Blumer, 1994). Spin-labeling studies on cysteine-substituted mutants of rhodopsin showed a rigid body movement of TM6 relative to TM3 accompanied by anti-clockwise rotation after light-induced isomerization of retinal (Farrens et al., 1996). Additional evidence for a relative movement between TM3 and TM6 in other GPCRs was provided by fluorescence labeling of the β_2 -adrenergic receptor or by monitoring the accessibility of Cys residues to a hydrophilic sulfhydryl-specific reagent during receptor activation (Gether et al., 1997).

As this paper neared completion an elegant study of *in situ* disulfide crosslinking in the M3 muscarinic acetylcholine receptor provided direct evidence for an agonist activated conformational change involving residues in TM3 and TM7 of this class A GPCR (Han et al., 2005). The M3 muscarinic receptor study provides strong evidence that binding of a diffusible ligand can change the spatial relationships of residues in the inactive and activated state of a GPCR and complements the findings reported herein. The conformational change observed in the muscarinic receptor involved residues that were relatively close to the binding site of the carbachol agonist. Previously, we proposed that Y266 of Ste2p plays a role in the recognition of the N-terminus of α -factor (Lee et al., 2002) suggesting that this residue was close to the ligand binding pocket. The change in the spatial proximity of residues 205 and 266 of Ste2p, that we detected in the constitutively activated mutant, also involves at least one residue (Y266) involved in ligand binding. If there is conserved structural homology among all classes of GPCRs as concluded recently (Eilers et al., 2005), it is possible that our results may apply to other members of GPCRs leading to the conclusion that there is an interaction between

TM5 and TM6 residues that are close to the extracellular surface.

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

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PART IV

General Conclusions and Future Studies

CHAPTER 1

General Conclusions and Discussion

This dissertation describes how the residues of N205 and Y266 of Ste2p contribute to α -factor binding and receptor activation. Systematic ala-scan of TM5 and TM6 of Ste2p coupled with biochemical and biological analysis have been used to gain insights into structure-function relationships between α -factor, the *Saccharomyces cerevisiae* peptide pheromone, and Ste2p, its G protein-coupled receptor. The major findings of the studies are that the N205 and Y266 residues in TM5 and TM6 respectively play an important role in the recognition of the N-terminal portion of α -factor and are involved in the transformation of Ste2p into an activated state upon agonist binding. Furthermore, N205 interacts with the Y266 residue of Ste2p in an activated state not in an inactive state. These findings contribute to the current knowledge of how peptide ligands interact with their GPCRs, and possible future experiments would provide further details on the mechanism(s) of receptor activation upon ligand binding.

The functional role of Y266 in the TM6 of Ste2p:

Previously, we reported that Q10 of α -factor closely interacted with S47 and T48 of Ste2p at the junction between TM1 and the N-terminal tail (Lee et al., 2001), and photoaffinity labeling (Henry et al., 2002) showed that the N-terminus of α -factor interacted with a region of Ste2p encompassing TM6-EL3-TM7. Based on these results, we proposed a model in which the C-terminus of α -factor is positioned near TM1, while

the N-terminus of α -factor binds in a pocket composed of TM3, TM5, and TM6 (Lee et al., 2001). To identify interactions between Ste2p and α -factor, a variety of α -factor analogues were used in conjunction with site-directed mutagenesis of a targeted portion of Ste2p transmembrane domain six (covered in Part II). Among the Alanine substituted residues in the 262-270 region of Ste2p, only the Y266A mutant did not transduce signal yet exhibited only a small decrease in α -factor binding affinity. Substitutions with Ser, Leu, or Lys at Y266 also generated signaling-defective receptors. In contrast, Phe or Trp substitution at Y266 retained receptor function, suggesting that aromaticity at this position was critical. When coexpressed with WT receptor, the Y266A receptor exhibited a strong dominant-negative phenotype, indicating that this mutant bound G protein. A partial tryptic digest revealed that, in the presence of agonist, a different digestion profile for Y266A receptor was generated in comparison to that for WT receptor. The difference in trypsin-sensitive sites and their negative dominance indicated that the Y266A receptor was not able to switch into an "activated" conformation upon ligand binding. In comparison to WT Ste2p, the mutant Y266A receptor showed increased binding affinity for N-terminal, alanine-substituted α -factor analogues (residues 1-4). A substantial decrease in affinity was observed for α -factor analogues with Ala substitutions from residues 5-13. The results suggest that Y266 is part of the binding pocket that recognizes the N-terminal portion of α -factor and is involved in the transformation of Ste2p into an activated state upon agonist binding.

The interaction between the residues N205 and Y266 of Ste2p in an active state not in resting state:

Ste2p, the G protein-coupled receptor (GPCR) for the tridecapeptide pheromone α -factor of *Saccharomyces cerevisiae*, was used as a model GPCR to investigate the role of specific residues in the resting and activated state of the receptor. Using a series of biological and biochemical analyses of wild-type and site-directed mutant receptors, we identified N205 as a potential interacting partner with the Y266 residue (covered in Part III). To test the possible interaction between N205 and Y266 residues of Ste2p, a series of biological and biochemical analysis coupled with mutation was carried out. First, an N205H/Y266H double mutant showed a pH-dependent functional activity, whereas the N205H receptor was non-functional and the Y266H receptor was partially active indicating that the 205H and 266H residues interact in the activated state of the receptor. Second, the introduction of N205K or Y266D mutations into the P258L/S259L constitutively-active receptor suppressed the constitutive activity; in contrast, the N205K/Y266D/ P258L/S259L quadruple mutant was fully constitutively active, again indicating an interaction between residues at the 205 and 206 positions in the receptor active state. Finally, to further test this interaction, we introduced the N205C/Y266C, F204C/Y266C, and N205C/A265C double mutation into wild-type and P258L/S259L constitutively-active receptors. After trypsin digestion, we found that a disulfide-crosslinked product formed only in the constitutively-activated receptor at the molecular weight expected for a receptor fragment with a cross-link between N205C and Y266C. This study represents the first experimental demonstration of an interaction between specific residues in an active state, but not the resting state, of Ste2p. The information

gained from this study should contribute to an understanding of the conformational differences between resting and active states of GPCRs.

Conformational changes during the activation of Ste2p:

There are numerous references to experimental observations that reveal important movements in GPCRs (Karnik et al., 2003; Perez and Karnik, 2005) (Reviewed in Chapter 1 of Part I). Upon ligand binding, it has been proposed that spatial relationship between TMs become altered by movement of TMs. There is emerging experimental evidence supporting the conformational changes of Ste2p during the ligand induced receptor activation. A study of Ste2p indicated that agonist binding led to an increased trypsin-accessibility of the IL3 (intracellular loop 3), whereas an antagonist led to a reduction of proteolytic cleavage within this loop (Bukusoglu and Jenness, 1996). A different trypsin digestion profile for Y266A mutant Ste2p receptor compared to WT Ste2p in the presence of agonist also suggested changes in conformation during receptor activation (Lee et al., 2001). In the part III of this dissertation, we proposed the interaction between N205 and Y266 in an active state not in resting state. This result would explain conformational changes between resting state and an active state because previous studies had shown that N205C, but not Y266C, was accessible to a hydrophilic thiol reagent in the resting state suggesting they are in distinct environments (Lin et al., 2003). A model hypothesized from these data was that TM5 and/or TM6 are displaced via a piston-like movement and/or tilting during receptor activation (Figure 1). This conformational change might induce movement of IL3 (intracellular loop 3) so that IL3 interacts with G protein in an active state complex.

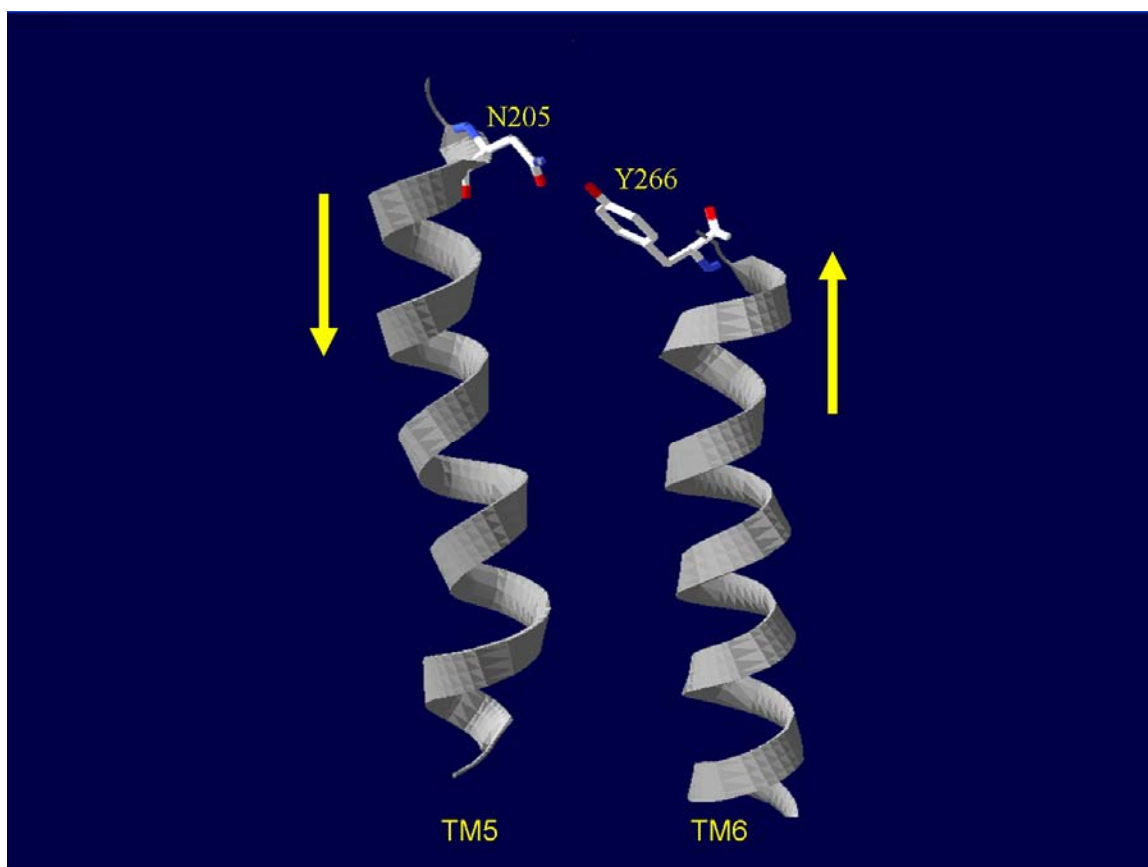


Figure 1. Helical movement of TM5 or TM6 during receptor activation.

This hypothesis was developed from the observations that there is a specific interaction between N205 and Y266 in an active state not in resting state of Ste2p despite the fact that N205 is accessible to thiol reagents whereas Y266 is not in the inactive state. The possible movement of TM5 and TM6 are indicated as yellow arrows.

Prominent among the changes in conformation of GPCRs is the change in tilt angle and/or counterclockwise rotation of TM6 (Ghanouni et al., 2001; Spalding et al., 1998). TM6 has been ascribed a role in terms of driving the activation mechanisms of GPCRs in terms of a movement possibly coupled with a counterclockwise rotation and a change in bending/kinking in this TM (Gether and Kobilka, 1998; Rasmussen et al., 1999). Similarly to the role of TM6 for GPCRs activation, the P258 and Y266 residues in TM6 of Ste2p have been known to be involved in receptor activation. The mutation of P258 resulted in a strong constitutively active receptor (Konopka et al., 1996) and the substitution of Y266 with non-aromatic amino acids impaired receptor function (Dosil et al., 1998; Leavitt et al., 1999; Lee et al., 2001; Lin et al., 2003). It has been proposed that general mechanism of receptor activation between rhodopsin and Ste2p are very similar even though their sequences are very divergent (Eilers et al., 2005). These results suggest that there is a general mechanism of GPCRs activation though there is sequence diversity among the GPCRs as well as ligand diversity.

CHAPTER 2

Future Studies

Despite the tremendous progress in understanding the structure of GPCRs over the last decade including the solving of the crystal structure of the ground state of rhodopsin (Palczewski et al., 2000) only partial information is known concerning conformational differences between active and inactive GPCRs due to the lack of crystal structure of any GPCR in the active state. Consequently, much less is known about the intramolecular interactions stabilizing an active state conformation compared to interactions stabilizing the inactive state conformation of GPCRs. This dissertation summarizes the first experimental demonstration of an interaction between specific residues in an active state, but not the resting state, of Ste2p.

Work to date has determined a specific interaction between N205 and Y266 in an active state (constitutively active Ste2p) not in resting state of Ste2p. In order for this interaction to take place, we hypothesized that there are conformational changes during receptor activation based on the previous result that these two residues are located in distinct environments (Lin et al., 2003). However, there is no experimental observation concerning the movement of TMs around N205 and Y266 residues. In addition, though we speculate that the interaction between N205 and Y266 occurs in the agonist-induced active state of Ste2p, we were only able to show this interaction in a constitutively-activated receptor, because the N205C/Y266C mutant in the WT background does not respond to α -factor. Therefore, further studies are needed to reveal conformational

changes and specific intramolecular interactions in order to understand the activation mechanism of Ste2p upon agonist binding. This final part of the dissertation outlines a few of the methods that could be applied, in our current model system, to elucidate the activation mechanism of Ste2p.

SCAM (substituted cysteine accessibility method) analysis of extracellular ends of TM5 and TM6 of Ste2p in the resting state and activated state:

The structure and function of GPCRs have been studied by SCAM using methane thiosulfonate (MTS) reagents (Javitch et al., 1997). For example, the relative movement of TM3 and TM6 in the β_2 adrenergic receptor was provided by monitoring the accessibility of cysteine residues to a MTS reagent during receptor activation (Javitch et al., 1997). In the SCAM analysis, receptor residues are mutated to cysteine that act as accurate reporters for the water accessibility of the corresponding residue in a wild-type receptor.

We propose to test the hypothesis that there are changes of water accessibility in extracellular ends of TM5 and TM6 between the resting state and active state using SCAM. This hypothesis was developed from our observations that there is a specific interaction between N205 and Y266 in an active state not in resting state of Ste2p despite N205 is accessible to thiol reagents whereas Y266 is not. This suggests that possible piston-like movement of either TM5 or TM6. The use of SCAM to probe the accessibility of an extracellular loop and TMs were successfully demonstrated using Ste2p (Lin et al., 2004; Lin et al., 2003).

We will make cysteine mutations in positions 205 through 210 (N205 to L210) of TM5 and in positions 261 through 266 (I261 to Y266) of TM6. It has been reported that cysteine substitution of these region did not affect the function of Ste2p except the N205C and Y266C mutations (Lin et al., 2004; Lin et al., 2003). We will determine if cysteine in these positions has the accessibility to MTS reagents similar to the previous report (Lin et al., 2004; Lin et al., 2003). Then, the SCAM analysis will allow us to carry out a complementary experiment to measure whether the presence of α -factor changes the reaction with specific residues. For these experiments, α -factor will be bound to Cys-substituted receptor, and then incubated with biotin-tagged MTS reagent for detection. If differences are observed with the various Cys-substituted receptors between in the presence of α -factor and in the absence of α -factor, then this would provide information about movement of TMs during receptor activation.

Alternatively, these experiments can also be carried out in the strong constitutively active background (P258L/S259L) to test whether the introduction of constitutive mutation can change the water accessibility of specific residues. If the trend of changes of water accessibility with specific residues are similar both in the α -factor-induced active receptor and constitutively active receptor, this would suggest the movement of TMs are conserved both in ligand induced active state and constitutively active state of receptor. This also would provide invaluable information about whether the conformation of a constitutively active receptor is similar or different from an active conformation induced by the sequential binding of agonist.

Determine intramolecular interaction of Ste2p in the resting and activated state:

It has been proposed that the resting state of GPCRs is constrained by a specific arrangement of the TMs, and activation is accompanied by the re-arrangement of inter-helical interactions induced by either ligand binding or introduction of constitutively active mutation (Gether, 2000). One way to test this hypothesis is to map key residues involved in intramolecular interactions in the resting state and activated state by using cysteine di-sulfide crosslinking. Several intramolecular interactions have been found in various GPCRs (Han et al., 2005; Ward et al., 2002; Yang et al., 1996) as well as Ste2p (Dube et al., 2000). In Ste2p, double mutant combinations were targeted to residues 222-229 in the intracellular end of TM5 and residues 246-252 in the intracellular end of TM6 of Ste2p. Study of crosslinking between cysteines residues engineered into these TMs showed that disulfide formation occurred only in the V223C/L247C mutant receptor (Dube et al., 2000).

In part III of this dissertation, we successfully identified a specific interaction between N205 and Y266 in an active state but not in the resting state of Ste2p using di-sulfide crosslinking (Figure 7 in Part III). Nevertheless, in no receptor system has a systematic mapping of intramolecular interactions in the resting state and activated state been achieved. However, since we know an intramolecular interaction point (between N205 and Y266) in an active state, it may be worthwhile to carry out a systematic mapping of intramolecular interactions around this interaction point both in the inactive state and active state of Ste2p. Single cysteine mutations in positions 205 through 210 (N205 to L210) of the extracellular ends of TM5 and in positions 261 through 266 (I261 to Y266) of the extracellular ends of TM6 will cover about 1.5 turns of TM5 and TM6

from N205 and Y266, respectively. Thirty six double –Cys mutations containing a combinations of one mutation in TM5 and one mutation in TM6 will be constructed by using the above single Cys mutant receptors. The biological activities and binding affinity of each of the mutants can be tested. As described in Part III, formation of a covalent bond between residues of the TM5 and TM6 can be detected by western blotting in which a larger peptide was apparent in non-reducing gels using an antibody to an epitope appended to TM7 as a result of formation of a disulfide bond between TM5 and TM6 after trypsin digestion. First, we can determine if di-sulfide crosslinking is formed in the absence of α -factor among the thirty-six double Cys mutations. If we find any residue forming a covalent bond to either N205C or Y266C, this would provide invaluable information about the conformational differences between the resting state and an activated state. This di-sulfide crosslinking analysis would allow us to carry out a complementary experiment to measure whether the presence of α -factor induces covalent bond if there was no crosslinking in the absence of α -factor or breaks the covalent bond if there was crosslinking in the absence of α -factor. If we find any change of di-sulfide crosslinking in the presence or absence of α -factor, this data would provide information to understand the Ste2p activation mechanism as well as to determine the inactive versus active state structure of Ste2p.

Similarly to the SCAM analysis, these experiments can also be carried out in the strong constitutively active background (P258L/S259L) to test whether the introduction of constitutive mutation can change covalent bond formation. This information could be

useful to determine whether or not the mechanisms of activation by either ligand induced or constitutively active mutation is similar.

The SCAM and di-sulfide crosslinking analysis will address major aspects of GPCRs biochemistry regarding the conformational changes in a receptor initiated by activation due to ligand binding or mutation to constitutive activity. Overall, these studies should provide fundamental information concerning the structure and function of GPCRs.

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