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Oligomerization and Endocytosis of the α-Factor Receptor: A Dissertation

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OLIGOMERIZATION AND ENDOCYTOSIS OF THE α-FACTOR RECEPTOR

A Dissertation Presented

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Abstract

α-Factor receptors from *Saccharomyces cerevisiae* are G-protein-coupled receptors containing seven transmembrane segments. The ability of α-factor receptors to form oligomeric complexes with each other and with other proteins was investigated. Both *in vivo* and *in vitro* evidence was obtained that suggests homo-oligomerization of receptors in the plasma membrane. When the membranes from cells coexpressing two differentially-tagged receptors were solubilized with detergent and subjected to immunoprecipitation, the antibodies specific for either epitope tag resulted in precipitation of both tagged species. Treatment of cultures with α-factor had little effect on the extent of oligomerization as judged by the sedimentation behavior of the receptor complexes and by the efficiency of coimmunoprecipitation. The ability of receptor complexes to undergo ligand-mediated endocytosis was evaluated by using membrane fractionation and fluorescence microscopy. Mutant receptors that fail to bind α-factor (Ste2-S184R) or lack the endocytosis signal (Ste2-T326) became competent for ligand-mediated endocytosis when they were expressed in cells containing wild-type receptors. Coimmunoprecipitation experiments indicated that the C-terminal cytoplasmic domain and intermolecular disulfide bonds were unnecessary for oligomer formation. Therefore, α-factor receptors form homo-oligomers and that these complexes are subject to ligand-mediated endocytosis.

A crosslinking and immunoprecipitation strategy was used to capture and characterize the transient complexes that contain the α-factor receptor Ste2. Tagged receptors were crosslinked to form at least three high molecular weight complexes and the complexes were immunoprecipitated with antibodies against the tag. Western

blotting analysis of the precipitated material revealed the presence of β and γ subunits of the heterotrimeric G protein, Ste4 and Ste18. Similar results were obtained when the cultures had been treated with α-factor prior to analysis. A truncated receptor missing most of the cytoplasmic C-terminal tail was also active in binding Ste4. Overall, these results constitute the first biochemical evidence for a physical association between the α-factor receptor and its cognate G-protein.

Endocytic signals in the C-terminal tail (residues 297-431) of the α-factor receptor were analyzed. One signaling element, SINNDAKSS, (residues 331-339) is known to be sufficient (but not necessary) for endocytosis. Internal deletions of the *STE2* gene were constructed that remove sequences encoding SINNDAKSS and selected regions of the C-terminal tail. Strains containing these alelles were then assayed for endocytosis in the presence and absence of α-factor. Residues from 360 to 431 were sufficient to mediate both constitutive and ligand-mediated endocytosis of the receptor even though 63 residues including the SINNDAKSS motif had been removed. Structural features of this region that were investigated further were the highly-ubiquitinated Lys374, the neighboring Lys387, and the GPFAD motif (residues 392-396). Lys374 and Lys387 were unnecessary for the element to promote exit from the plasma membrane; however, Lys374 may play some role in intracellular trafficking. The GPFAD motif was not sufficient to promote endocytosis, since the residues 360-399 provided no detectable endocytic activity. Overall, these results suggest that a new region in the C-terminal of the α-factor receptor, redundant with the SINNDAKSS motif, is sufficient to mediate the constitutive endocytosis as well as the ligand-mediated endocytosis of the receptor.

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CHAPTER I

INTRODUCTION

All cells need to sense changes in their environment and respond appropriately to extracellular cues for survival. Complex signal transduction networks couple the outside signals to cellular responses permitting them to alter the metabolic state, rate of growth, or morphology of the cell. The unicellular eukaryote *Saccharomyces cerevisiae* has proven to be a useful genetically tractable model organism to study signaling pathways as well as other aspects of basic cellular functions. *S. cerevisiae* exists in either of two forms in nature: haploid or diploid. Conjugation is initiated by the peptide pheromones **a**-factor and α-factor that are secreted by the haploid **a** and α cells, respectively. The farnesylated and carboxymethylated undecapeptide **a**-factor binds to the **a**-factor receptor the product of the *STE3* gene, that is expressed on the surface of α cells. Similarly, the α-factor receptor, the product of the *STE2* gene, on **a** cells is the target of the tridecapeptide α-factor. The binding of these pheromones to their specific receptors initiates the prerequisite events for mating. The **a** and α cells arrest early in G1 thereby synchronizing their respective cell cycles, and then undergo polarized morphological changes (e.g. they form cell-surface projections towards the opposite partner). In addition, transcription of the mating-specific genes that are required to carry out cell-cell adhesion and fusion is activated.

In this chapter, I will review briefly the current understanding of basic structural properties of the G-protein coupled receptor (GPCR) superfamily which includes the

mating pheromone receptors of *S. cerevisiae*. I will also examine the data regarding GPCR-G protein interactions; oligomerization of GPCRs and central features of plasma membrane protein endocytosis in yeast as the data pertain to my findings presented in the results chapters of this thesis.

G-protein-coupled receptors: structural and functional domains

The α-factor receptor (Ste2) and **a**-factor receptor (Ste3) are integral membrane proteins with a topology that is characteristic of members of the GPCR family. GPCRs comprise the largest and most diverse superfamily of cell-surface receptors (Bockaert and Pin, 1999; Hebert and Bouvier, 1998). Examples include, β-adrenergic, metabotropic glutamate, Ca^{2+}-sensing, dopamine, muscarinic, opioid, olfactory, taste, pheromone and light-sensing receptors in mammals, frizzled/smoothened family in *Caenorhabditis elegans*, and cAMP activated receptors in *Dictiosytelium*. About 5% of the *C. elegans* genome and perhaps 3% of the human genome encode for GPCRs (Bourne and Meng, 2000).

GPCRs possess a common seven-transmembrane domain topology with an extracellular glycosylated N-terminal segment, a cytoplasmic C-terminal segment and alternating intracellular and extracellular loops (Cartwright and Tipper, 1991). The high resolution crystal structure of rhodopsin, the light-sensing GPCR located in the rod cells of the retina has unequivocally demonstrated the boundaries, arrangement and orientation of the seven transmembrane α-helices and the intervening loops in this photoreceptor as well as the contact sites for the covalently attached chromophore, retinal, in its ground state (Palczewski K, 2000). The receptor transmembrane domains are tightly packed in a

bundle from helix one to seven in a clockwise direction when viewed from the cytoplasm. The second intracellular loop and an eighth α helix formed by the C-terminus lie parallel to the cytoplasmic face of the receptor creating a possible loading dock for transducin, the heterotrimeric G protein in the retina which is the effector molecule for rhodopsin. Overall the results are mostly in agreement with previous data obtained by cryo-electron microscopy, mutational analyses and other biochemical studies of rhodopsin (Bourne and Meng, 2000; Palczewski K, 2000). Most other members of the GPCR superfamily are predicted to have transmembrane helices and extracellular loops of comparable size to rhodopsin. Therefore, a similar transmembrane topology has been proposed for other GPCRs.

Despite their overall similarity in structure, GPCRs show little sequence conservation and respond to a variety of extracellular stimuli such as light, odorants, calcium, hormones and neurotransmitters. The smallest ligands (retinal, amines, nucleosides and lipid moieties) bind to a hydrophobic pocket formed by the core transmembrane helices, whereas small peptides bind both to the core and to extracellular loops. Higher molecular weight peptides and protein ligands usually bind to the N-terminus and the extracellular loops, whereas glycopeptides bind solely to the N-terminus (Ji et al., 1998). Most ligands bind the receptors reversibly whereas in the case of rhodopsin and thrombin receptors, the ligands are covalently associated with the receptor. Retinal is bound to Lys296 of rhodopsin by a protonated Schiff base linkage and stabilized by a salt bridge with Asp113 (Palczewski K, 2000). The N-terminal segment of thrombin family of receptors is cleaved by proteases, and the cleaved peptide acts as a ligand to activate the receptor.

One important aspect in GPCR activation is assembly of the right combination of receptor with the appropriate G protein subset from the cytoplasmic pool. Commonly, the intracellular loop 3 of a GPCR determines the Gαβγ selectivity (Bourne, 1997). In some instances intracellular loop 2 and rarely intracellular loop 1 have been implicated in establishing the specificity of the interaction with G proteins (Bourne, 1997). So far, it is not possible to predict the G protein trimer that will interact with a given GPCR based solely on its primary structure (Bourne, 1997). In some instances, the carboxy terminal tail of the receptor is also involved in binding to the G proteins. The C-terminal tail of Ste2 has been proposed to function in stabilizing the receptor-G protein interactions (Dosil et al., 2000), whereas the intracellular loop 3 has been proposed to bind the G proteins. The carboxy terminal tail is also implicated in adaptation, endocytosis and oligomerization (Cvejic and Devi, 1997; Rohrer et al., 1993; Schandel and Jenness, 1994).

Two conserved cysteines are present in many GPCRs, one in extracellular loop1 at the transmembrane helix 3 boundary and one in extracellular loop 2. The corresponding cysteines Cys187 and Cys110 in bovine rhodopsin form an intramolecular disulfide bond, covalently attaching these regions and perhaps stabilizing correct folding (Palczewski K, 2000). Cysteines present in some GPCRs, such as Ca^{2+}-sensing and metabotropic glutamate receptors, are required for oligomerization (Bai et al., 1998; Romano et al., 1996). The two cysteine residues in Ste2 do not seem to be required for receptor function since a receptor mutant that contains neither of these cysteines exhibits no defects in plasma membrane localization, signaling, endocytosis or oligomerization (Jeremy Thorner personal communication, (Yesilaltay and Jenness, 2000)).

G protein structure and functional domains

GPCRs rely on the action of heterotrimeric G proteins to relay the extracellular signals to the cell interior. Receptor stimulation by the ligand results in exchange of GDP for GTP in the α subunit of the G protein, and it is thought that the GTP-bound Gα dissociates from the Gβγ heterodimer. In mammalian cells, the GTP-bound Gα regulates downstream effectors such as adenylyl cyclases, phospholipases and ion channels (Meigs et al., 2001). In some cases, Gβγ subunits have been shown to interact with protein serine/threonine kinase KSR-1 and PI3Kγ. (Naor et al., 2000). Yeast cells elicit a response through the yeast homologs of the mammalian α, β and γ subunits of the heterotrimeric G protein (encoded by *GPA1*, *STE4* and *STE18* genes, respectively) that are coupled to the α- and **a**-factor pheromone receptors (Ste2 and Ste3, respectively) (Dohlman and Thorner, 2001). In yeast, it is the Gβγ heterodimer that activates the downstream effectors. Gα has a slow intrinsic GTPase activity. In addition, the GTPase activity of Gα is regulated by RGS proteins (regulator of GPCR signaling). Phosducins, on the other hand, modulate the function of Gβγ. When GTP is hydrolyzed, the GDP-bound Gα is likely to reassociate with the Gβγ heterodimer.

The high resolution crystal structure of the G protein has revealed the contact sites among subunits. The Gβ is shaped like a propeller with seven blades and a shaft domain (Lambright et al., 1996; Sondek et al., 1996; Wall et al., 1995). Gα and Gβ interact at two major sites, at the lipid-modified amino terminus and at the switch region of Gα. It is the association of the switch region of Gα with an electronegative region on Gβ that is

thought to regulate the activity of the switch region. However, it is still unclear where the receptor and the downstream effectors interact with the G protein.

Ste2-G protein interactions

A number of models have been proposed to explain the GPCR activation. According to the ternary complex model, GPCRs are thought to exist in an equilibrium between an inactive (R) state and an active (R*) state. In the absence of the ligand, most receptors are in the R state. Ligand binding stabilizes the R* state. The potency of a ligand is defined as a function of its ability to alter the equilibrium in favor of the active state. The ligand-bound receptor interacts transiently with the G protein causing a GDP-GTP exchange on the Gα subunit. A modified version of the ternary complex model (Samama et al., 1993) accounts for the existence of receptor-G protein complexes. In the modified model, receptors isomerize and assume many intermediate states (including the R* state) even in the absence of the ligand. Thus, a fraction of receptors (those in R* state) can associate with the G protein in the absence of the ligand. These precoupled receptor-G protein complexes are referred to as 'pre-activation complexes'. These complexes may play a role in enhancing the specificity and the efficiency of the receptor-G protein interactions.

In yeast, various indirect observations indicate a physical interaction between the receptor and its cognate G protein. For example the affinity of the receptor for the ligand is decreased in a *ste4Δ* strain (Jenness et al., 1987). Similarly, truncated receptors, which are thought to lack the ability to stabilize G-protein interactions, also have lower affinity for the ligand than full-length receptors (Dosil et al., 2000). In addition, α-factor

dissociates from receptors more rapidly when GTP analogs are present or when membranes are assayed from mutant cells that lack G protein subunits (Blumer and Thorner, 1990). Finally, the dominant negative phenotype associated with receptor mutants is reversed when the G proteins are overexpressed (Dosil et al., 1998). Genetic analyses also indicate that the third cytoplasmic loop of Ste2 is the site for the G protein interactions since mutations in this region either render the receptor constitutively active or abolish signaling. Consistent with this idea, the lethality phenotype associated with a temperature-sensitive allele of Gα (*gpa1-A345T*) resulting from constitutive activation of Gβγ, is rescued by a double mutation affecting the third cytoplasmic loop region of Ste2 (*ste2-Y266C-L236H*) (Dosil et al., 2000). Allele-specific interactions of this kind suggest a specific physical association. However, direct biochemical evidence for physical association of the receptor and G protein has not been described in this system.

Genetic evidence suggests the existence of pre-activation complexes in yeast, and the C-terminal tail of Ste2 is thought to stabilize the interaction between the receptor and the G protein (Dosil et al., 2000). First, ligand-binding-defective dominant negative mutants of Ste2 that interfere with signaling from the wild-type receptors failed to do so if they lacked the C-terminal cytoplasmic tail. Second, the C-terminal tail was also required for wild-type receptors to interfere with the signaling from a constitutively active receptor in the absence of the ligand. Finally, *GPA1-A345T* allele causes lethality at restrictive temperature in a *ste2Δ* strain or in a strain expressing a C-terminally truncated Ste2 mutant, however cells expressing Gpa1-A345T were viable when full-length receptors were expressed. These data indicate precoupling of the receptor and G protein in the absence of the ligand, and that the C-terminal tail of the receptor has a role in stabilizing the receptor-G protein interactions.

One reason why the receptor-G protein complexes have not been reported so far could be that these proteins are held together by weak interactions which dissociate during the immunoprecipitation procedure. In Chapter IV of this thesis, I present a crosslinking and immunoprecipitation approach to capture these putative complexes. I found that Ste4 crosslinks to and coimmunoprecipitates with Ste2 both in the absence and in the presence of the ligand suggesting a direct interaction between these two proteins and lending direct support to the existence of the pre-activation complexes in yeast. Furthermore, I was able to capture ligand-independent complexes between Gβ and truncated receptors, indicating that complexes can form, at least transiently, without the stabilizing influence of the C-terminal tail.

GPCR oligomerization

Early observations. While the current models of GPCR activation do not address the oligomeric state of the receptor, they do not theoretically exclude the possibility of receptor oligomers. In fact, receptor oligomerization may play an unforeseen role in function. Early biochemical and genetic findings suggest oligomerization of Ste2 and other GPCRs. At subsaturating concentrations of α-factor, Jenness and Spatrick observed that the rate of labeled α-factor endocytosed by the yeast cells was slower than the rate of α-factor receptor site loss from the plasma membrane (Jenness and Spatrick, 1986). One interpretation of this result is that unoccupied receptors internalize with the occupied ones in aggregates. Receptor aggregates were detected in SDS-PAGE analysis of both GPCRs (β adrenergic receptor, dopamine D2 receptor, muscarinic receptor, Ste2) (Avissar et al., 1983; Blumer et al., 1988; Conn and Venter, 1985; Hebert et al., 1996)

and other membrane proteins (glycophorin A, human erythrocyte band 3) (Furthmayr and Marchesi, 1976; Salhany et al., 1990) and named 'SDS-resistant' dimers. It is currently unclear whether these species represent physiologically relevant receptor dimers or non-specific aggregates that form during sample preparation.

Early genetic data also suggested oligomerization. Konopka and Jenness detected allele-specific complementation of mating-defective α-factor pheromone receptor mutants (Konopka and Jenness, 1991). Three signaling-defective *STE2* alleles that contained linker insertion mutations in or near transmembrane segments 1, 2 and 6 partially rescued the temperature-sensitive mating defect of the *ste2-3* allele upon coexpression, whereas two other alleles with mutations at transmembrane segments 4 and 6 failed to do so (Konopka and Jenness, 1991). Similarly, two receptor chimeras formed between α2 adrenergic and m3 muscarinic receptors which contained the first five transmembrane domains from one receptor and the last two transmembrane domains from the other receptor failed to bind the ligand for either receptor or to signal, unless the two chimeras were coexpressed, leading the investigators to argue that the two receptor chimeras oligomerized to form functional receptors (Maggio et al., 1993). More recently, a ligand-binding-defective phenotype of angiotensin receptor was complemented by coexpressing a second receptor containing a different defect in ligand binding (Monnot et al., 1996). Other allele-specific effects were observed with both V2 vasopressin and CCR5 receptors. Dominant-negative mutant receptors inhibited signaling from wild-type receptors when they were coexpressed, presumably by oligomerizing with the wild-type receptors and thereby causing the wild-type receptors to be retained in the intracellular compartments with the defective receptors (Benkirane et al., 1997; Zhu and Wess, 1998).

Detecting GPCR oligomers. Recent reports indicate that oligomerization may be a universal phenomenon for GPCRs. Evidence suggesting oligomerization was obtained through crosslinking and coimmunoprecipitation approaches. Examples include β2 adrenergic (Hebert et al., 1996), δ-opioid (Cvejic and Devi, 1997), D2 and D3 dopamine (Ng et al., 1996; Nimchinsky et al., 1997), m3 muscarinic (Zeng and Wess, 1999), metabotropic glutamate (Romano et al., 1996) and Ca^{2+}-sensing (Bai et al., 1998; Bai et al., 1999), V2 vasopressin (Hebert et al., 1996), bradykinin B2 (AbdAlla et al., 1999), and somatostatin receptors (Rocheville et al., 2000). In addition, various resonance energy transfer methods revealed homo-oligomers of β2 adrenergic, δ-opioid and SSTR5 somatostatin receptors in living cells (Angers et al., 2000; Kroeger et al., 2001; McVey et al., 2001). Furthermore, the high resolution crystal structure of the ligand binding domain of glutamate receptors demonstrated that the domain was a dimer both in ligand-bound and unbound states (Kunishima et al., 2000).

Hetero-oligomerization of various GPCRs was also reported. GPCR heterodimers were detected with receptors of the same subtype for κ- and δ opioid receptors; μ- and δ opioid receptors; somatostatin receptors SSTR1 and SSTR5; and $GABA_B$ R1 and R2 receptors (George et al., 2000; Jordan and Devi, 1999; Kuner et al., 1999; Rocheville et al., 2000). Recent reports indicate that hetero-oligomer formation does not require subtype specificity since D2 dopamine receptors oligomerize with SST5 somatostatin receptors (Rocheville et al., 2000). Type 1 angiotensin II receptors and B2 bradykinin receptors were found to form hetero-oligomers as well (AbdAlla et al., 2001).

The effect of ligands on oligomerization. Although receptor oligomerization is emerging as a common theme, the influence of agonists on receptor oligomerization has

been shown to vary among the GPCRs investigated. For example, agonists appeared to stabilize dimers of β2 adrenergic receptors prepared from COS-7 cell membrane extracts in a dose-dependent manner when the amount of SDS-resistant dimer formation was used as a criterion (Hebert et al., 1996). Similarly, a slight agonist-induced increase in oligomerization was reported in bioluminescence resonance energy transfer (BRET) experiments when fusion receptor constructs (β2 adrenergic receptor-luciferase and β2 adrenergic receptor-green fluorescent protein) were used (Angers et al., 2000). The oligomeric state of m3 muscarinic receptors is not altered when COS-7 cells expressing m3 receptors are treated with the muscarinic receptor agonist carbachol (Zeng and Wess, 1999). On the other hand, the monomeric state of δ-opioid receptor is apparently favored upon agonist binding (Cvejic and Devi, 1997). In this study, stably transfected Chinese hamster ovary cells were treated with a number of opioid agonists, and the oligomeric state was evaluated by comparing the monomer versus dimer ratio on SDS-PAGE analysis. However, McVey et al. detected no change in δ-opioid receptor oligomerization using two separate resonance energy transfer techniques (McVey et al., 2001). It should be noted that findings from resonance energy transfer techniques should be approached with caution, because the lack or ability to detect an interaction does not depend merely on the distance between two molecules but also on the relative alignment of the fluorophores. For example, even though no interaction is detected between two receptor constructs, agonist binding may induce a conformational change in the already-existing receptor homo-dimer thus generating improved energy transfer. Overall, whether these results reflect different experimental conditions or whether they represent bona fide ligand-mediated effects on GPCR oligomerization remains to be determined.

Mechanisms of oligomer formation. The structural determinants of the receptor that are important for oligomerization have also proven to be diverse. 15 amino acids in the C-terminal tail of δ-opioid receptor have been implicated in dimerization, since deleting these 15 amino acids prevents both receptor internalization and receptor oligomerization (Hebert et al., 1996). Interestingly, the $GABA_B$ R1 and R2 receptors (also referred to as GBR1 and GBR2) oligomerize through coiled-coil domains at their C-termini (White et al., 1998). However, the C-terminal tail of the δ-opioid receptor has no obvious coiled-coil domain (Milligan, 2001). A mutant bradykinin B2 receptor that is truncated at the N-terminus fails to oligomerize, underscoring the importance of this domain for oligomerization (AbdAlla et al., 1999). The Ca^{2+}-sensing receptor, the metabotropic glutamate receptor 5 and the muscarinic m3 receptor oligomerize through intermolecular disulfide bonds (Bai et al., 1998; Romano et al., 1996; Zeng and Wess, 1999). Transmembrane region VI is thought to mediate the association of β2 adrenergic receptors since peptides containing this sequence interfere with the recovery of receptor dimers (Hebert et al., 1996). On the other hand, peptides derived from various transmembrane domains of the dopamine D2 receptor inhibit receptor oligomerization (Ng et al., 1996).

Two models have been proposed to explain how the GPCRs form oligomers (Gouldson et al., 2000). Both models predict that the interaction between receptors involve multiple contacts between transmembrane helices V and VI. According to domain swapping model, GPCRs are comprised of two independently folding segments, the transmembrane helices I-V and VI-VII. These two domains can be split and coexpressed to form functional receptors (Barbier et al., 1998; Scarselli et al., 2000). This model suggests that the GPCR dimers form by exchanging the two independent

folding domains, that is the first five transmembrane helices are contributed from one receptors and the last two from the other. The contact dimer model proposes that the dimer formation is simply mediated by lateral interactions between transmembrane helices. Further experiments are needed to distinguish these two models.

Role for GPCR oligomerization in signal transduction. The functional significance of GPCR oligomerization is currently unclear. Agonist stimulation has led to increased oligomerization of β2 adrenergic and CCR5 receptors (Angers et al., 2000; Hebert et al., 1996; Vila-Coro et al., 2000). Hebert *et al.* suggest that the oligomerization of β2 adrenergic receptors regulates the signaling from these receptors since the peptide derived from the transmembrane region VI of β2 adrenergic receptor inhibited both oligomerization and adenylyl cyclase activity of the receptor, whereas another peptide derived from transmembrane region VII of the dopamine receptor failed to interfere with either process (Hebert et al., 1996). This suggestive evidence is contrasted by the work of George and coworkers who showed that a peptide derived from transmembrane VI of dopamine receptor D1 also inhibited the adenylyl cyclase activity without disrupting D1 receptor oligomers (George et al., 1998). In addition, when anti-β2 adrenergic receptor antibodies were allowed to interact with the receptors, bivalent antibodies but not monovalent Fab fragment antibodies, caused receptor activation (Mijares et al., 2000), consistent with a role for oligomerization in signal transduction. It is intriguing to note that cooperative interactions of G proteins have been reported (Wessling-Resnick and Johnson, 1989). Crystallographic data demonstrated $G\alpha_{i1}$ in oligomeric arrays (Chidiac and Wells, 1992; Mixon et al., 1995). This has prompted investigators to argue that GPCRs and G proteins may be organized in arrays in the plasma membrane facilitating signaling from these receptors (Rodbell, 1992).

Oligomers in chaperoning and transport. A potentially interesting insight into the role of oligomerization comes from the studies with GABA receptors. The first GABA receptor to be cloned was $GABA_B$ R1 (Kaupmann et al., 1997). However, this receptor bound agonist ligands poorly when expressed in recombinant systems, and when it was expressed in mammalian cells, it was retained in intracellular compartments as an immature glycoprotein. A closely related seven transmembrane protein, named GBR2 was simultaneously identified by a number of groups (Jones et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999; White et al., 1998). Expression of GBR2 inhibited adenylyl cyclase activity but only coexpression with GBR1 activated the outward potassium channels (Kuner et al., 1999). Therefore, the full physiological activity of GABA has been suggested to require the hetero-oligomerization of the two receptors. Coexpression of GBR2 with GBR1 rescued the intracellular retention defect of GBR1. Therefore, a role for oligomerization in proper folding and chaperoning of the receptors has also been proposed. Subsequently, an ER-retention signal was identified in GBR1, and the authors argue that the coiled-coil interactions in the C-terminal tails of GBR1 and GBR2 mask the ER-retention signal in GBR1, thereby regulating the ER export and plasma membrane trafficking of this receptor.

The ability of oligomers to form in the ER is also supported by studies with mutant vasopressin receptors. A vast majority of the clinically relevant mutations affecting the vasopressin receptors cause nephrogenic diabetes because the mutant receptors are retained in the ER. Dimers of these mutant receptors have been detected. These results suggest that oligomers form in the ER and that receptors are transported as oligomers to the plasma membrane. In addition, it has been proposed that a truncated

form of CCR5 causes wild-type receptors to be retained in the ER. Since CCR5 is a co-receptor for HIV entry, the slow onset of AIDS in patients carrying a single copy of this allele may be a consequence of the absence of the wild-type copy of the receptor on the plasma membrane.

Potential role for receptor hetero-oligomerization in altering the binding affinity and specificity of ligands. An extra level of regulation in receptor response may be achieved by altering functional properties of receptors through hetero-oligomerization. The δ- and κ-opioid receptors, as well as δ- and μ-opioid receptors, have been shown to form hetero-oligomers (George et al., 2000; Jordan and Devi, 1999). Cells coexpressing δ- and κ-opioid receptors exhibit binding specificities and affinities distinct from cells expressing the individual receptors (Jordan and Devi, 1999). Similar novel properties have been reported with regard to the coexpression of δ- and μ-opioid receptors (George et al., 2000). One caveat of both studies is that the experiments were performed with transfected cells that overproduced receptors. Interpreting the physiological relevance of these results will require knowledge of the relative levels of the receptor subtypes in normal cells. These findings may also have clinical importance since the heterodimer to homodimer ratio may affect the pharmacological properties of the response.

Oligomerization and endocytosis. A role for oligomerization in endocytosis has also been proposed. The monomerization of δ-opioid receptors by the agonist DAMGO precedes their endocytosis (Cvejic and Devi, 1997). Another opioid agonist morphine that does not induce endocytosis of these receptors also does not promote monomerization. In addition, when the C-terminal 15 amino acids responsible for oligomerization are deleted, the receptors fail to undergo ligand-mediated endocytosis.

Therefore the authors suggest that the monomeric species is actively endocytosed and that monomerization is required for internalization (Cvejic and Devi, 1997).

Chapter III of this thesis presents evidence indicating that Ste2 forms oligomers. Ste2 oligomers were also detected by fluorescence resonance energy transfer experiments (Overton and Blumer, 2000). The findings indicate that the formation of oligomers is unaffected by ligand and that receptors are endocytosed in oligomeric complexes suggesting that oligomerization potentially facilitates endocytosis rather than mediating signaling by Ste2.

Endocytosis of GPCRs

Upon agonist stimulation, GPCRs are phosphorylated at serine and threonine residues by G-protein-coupled receptor kinases (GRKs) (Krupnick and Benovic 1998). A special class of proteins called arrestins is involved in both desensitization and down regulation of receptors (Krupnick and Benovic 1998). Binding of arrestins to phosphorylated GPCRs prevents receptors from reassociating with G proteins. Furthermore, arrestins induce clathrin-mediated endocytosis of receptors by recruiting components of adaptor protein complex (AP-2). β-arrestins have been shown to bind clathrin and β2-adaptin of AP-2 complex (Krupnick et al. 1997; Laporte et al. 1999). A large GTPase, dynamin, is required for pinching off the endocytic vesicle containing receptors from the cell surface (McNiven, 1998). Following internalization, receptors are either recycled back to the surface or are transported to the lysosome for degradation.

Endocytosis of Ste2

The C-terminal cytoplasmic tail of Ste2 mediates its constitutive and ligand-mediated endocytosis (Fig. 1). Truncated receptors lacking 105 amino acids out of 134 tail residues cause 4–5 fold increased pheromone sensitivity, defects in recovery from pheromone and desensitization and defects in both constitutive and ligand-mediated endocytosis of the receptor (Konopka and Jenness, 1991). Nested deletions of the *STE2* gene have been constructed that result in consecutive truncations of the receptor starting from its C-terminus. Analysis of these mutants indicates that the first 39 amino acids immediately following the transmembrane region VII contain sufficient information to promote both constitutive and ligand-mediated endocytosis of the receptor, albeit at a reduced rate than the wild-type receptor (Rohrer et al., 1993). Further mutational analysis of the truncated receptor containing this 39 amino acid element, Ste2-T345, identified a nine amino acid motif SINNDAKSS required for residual endocytic activity of Ste2-T345. In addition, the SINNDAKSS sequence (even the DAKSS sequence) is sufficient to confer endocytosis of a severely truncated receptor (Rohrer et al., 1993). The substitution of Lys337 by either alanine or arginine abrogates the ability of this motif to promote endocytosis of the severely truncated receptor, and it blocks the ability of cells expressing this construct to recover from α-factor exposure. Further analysis of the truncation mutant indicated that Lys337 is ubiquitinated (Hicke and Riezman, 1996).

Ubiquitination of plasma membrane proteins. Ubiquitin, a well-conserved 76 amino acid protein, was originally identified as a tag that directs proteins to the proteasome for degradation (Hochstrasser, 1996). Ubiquitin forms an isopeptide bond through its C-terminal glycine and a lysine residue on a protein. The sequential action of

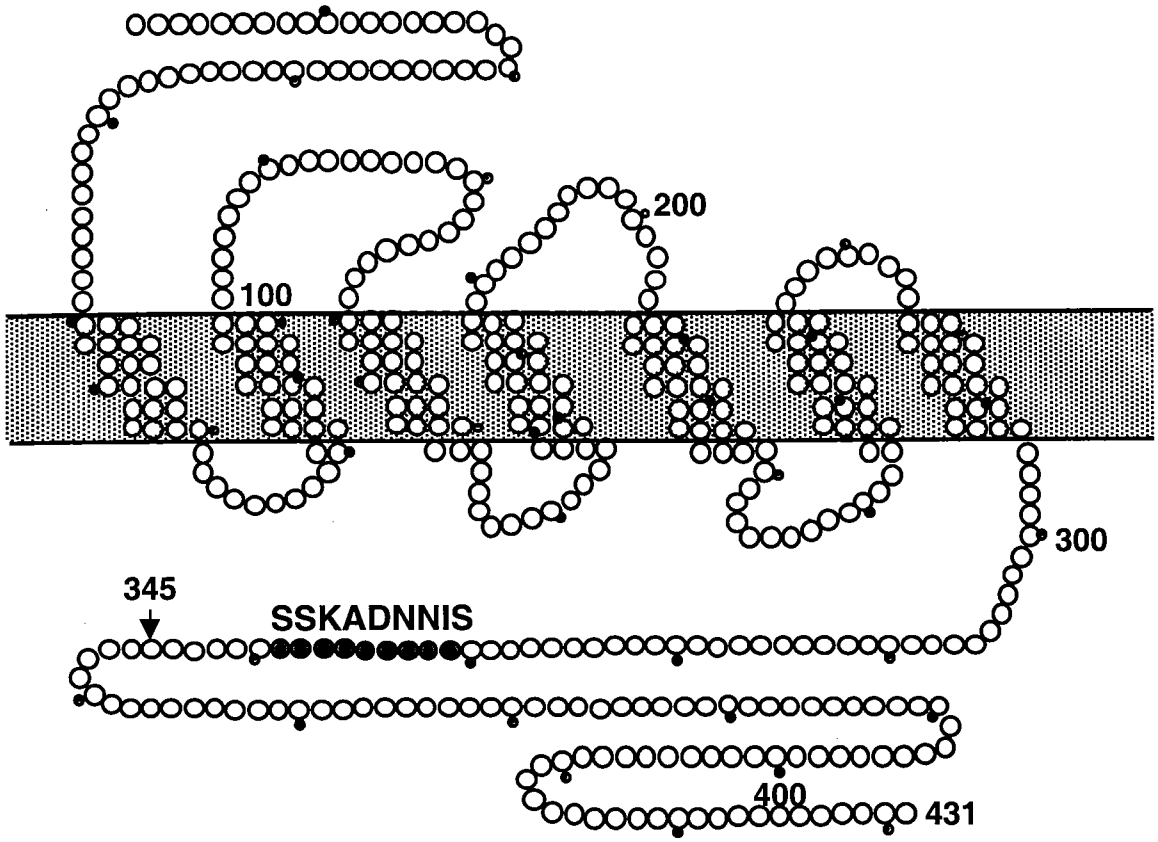


Figure 1. The predicted topology of the α–factor receptor. The endocytosis motif SINNDAKSS sequence is denoted in gray. The residue 345 is marked with an arrow.

three enzymes is required for the ultimate transfer of ubiquitin to the target protein: the ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin protein ligase (E3) (Hochstrasser, 1996). Cytoplasmic and nuclear proteins are marked for degradation with multiple ubiquitin chains. Each link in the chain is formed by the modification of the lysine residue at position 48 on one ubiquitin molecule with a second ubiquitin molecule. Following ubiquitination, proteins are targeted to the 26S proteasome. Ubiquitin also serves as an endocytic signal for several membrane proteins in yeast. Some examples include the pheromone receptors Ste2 and Ste3 (Hicke and Riezman, 1996; Roth and Davis, 1996), transporters Ste6 and Pdr5 (Egner and Kuchler, 1996; Kolling and Hollenberg, 1994) and permeases Fur4, Gap1, Gal2 and Mal61 (Galan et al., 1996; Horak and Wolf, 1997; Medintz et al., 1998; Springael and Andre, 1998). Ubiquitin was also shown to be important for the endocytosis of the growth hormone receptor in mammalian cells (Govers et al., 1997; Hicke, 1997; Strous et al., 1996).

Yeast contains ubiquitin chains that are linked through isopeptide bonds at Lys29, Lys48 or Lys63 (Arnason and Ellison, 1994; Hicke, 2001). It has been suggested that the position of the linkage determines the fate of the ubiquitinated proteins. Tetraubiquitin chains linked through Lys48 mark proteins as substrates for the proteasome, whereas diubiquitin chains linked through Lys63 have been implicated in endocytosis of Fur4 in yeast. So far, the proteins carrying ubiquitin chains linked through Lys29 have not been identified (Hicke, 2001).

Ubiquitination of Ste2. Ubiquitination is thought to be necessary for endocytosis of Ste2 in yeast (Hicke and Riezman, 1996). First, an *end4* strain which causes an overall defect in endocytosis of many cellular plasma membrane proteins as well as Ste2,

accumulates high molecular weight species of Ste2 which have been identified as ubiquitinated forms of the receptor using a sequential immunoprecipitation procedure when Ste2 antibody was followed with anti-ubiquitin antibody. In addition cells lacking ubiquitin conjugating enzymes *ubc1 ubc4*, and *ubc4 ubc5* also have defects in endocytosis. Furthermore, Ste2 turnover is apparently not mediated by the cytoplasmic proteasome but requires the action of vacuolar proteases. Deletion of two vacuolar proteases, Pep4 and Prb1, that cleave and result in the activation of other vacuolar hydrolases led to a marked increase in receptor half life and accumulation of ubiquitinated forms of the receptor. On the other hand, the half-life of Ste2 was unaffected in *pre1pre2* double mutant cells that lack two essential β type subunits of the proteasome (Hicke and Riezman, 1996).

Terrell and coworkers demonstrated that Ste2 is monoubiquitinated on multiple lysine residues and that monoubiquitination is sufficient to drive the endocytosis of the receptor (Terrell et al., 1998). To this end, the authors constructed a double mutation in the ubiquitin molecule, Ub-K48R/G76A. The lysine to arginine change at position 48 is commonly used to block poly-ubiquitin chain formation in the substrates of the proteasome. The alanine to glycine change at the C-terminus inhibits the removal of ubiquitin once it has been attached to the substrate. This mutant Ub-K48R/G76A has been previously shown to inhibit degradation of ubiquitinated substrates by the proteasome. Ste2 endocytosis is unaffected by the presence of this ubiquitin mutant. Moreover, other ubiquitin mutants that harbor different lysine to arginine substitutions also fail to affect the endocytosis of Ste2. Similar results were obtained when a ubiquitin mutant that harbors lysine to alanine substitutions of all of the lysines, Ub-NoLys, was expressed in a strain which lacks the deubiquitylation enzyme Doa4. The presence of

mono- and di-ubiquitinated forms of Ste2 as judged by SDS-PAGE analysis in the Ub-NoLys-expressing strain indicates monoubiquitination of Ste2 at one or two lysine residues rather than poly-ubiquitination (Terrell et al., 1998).

Phosphorylation of Ste2. Ste2 is phosphorylated at serine and threonine residues in its C-terminal tail. Upon binding α-factor, Ste2 becomes hyperphosphorylated (Reneke et al., 1988). Hyperphosphorylation causes Ste2 to migrate more slowly on SDS gels. Substituting serine residues for alanines in the SINNDAKSS motif of Ste2-T345 inhibited both endocytosis and the α-factor-induced mobility shift of the mutant receptor (Hicke et al., 1998). In addition, yeast cells carrying mutations affecting yeast casein kinase I homologues fail to internalize α-factor and do not phosphorylate or ubiquitinate their receptors (Hicke et al., 1998). Therefore, it has been proposed that phosphorylation may be a prerequisite for ubiquitination. Since ubiquitination mediates receptor endocytosis, phosphorylation may be required for endocytosis of Ste2. Currently, it is not clear whether the yeast casein kinases directly phosphorylate the tail or whether they regulate other kinases. Unlike **a**-factor receptors, the internalization of α-factor receptors is independent of pheromone response pathway since mutants defective for Gα subunit Gpa1, Gβ subunit Ste4 or the scaffolding protein Ste5 remain subject to α-factor-induced endocytosis (Blinder and Jenness, 1989; Jenness and Spatrick, 1986; Schandel and Jenness, 1994; Zanolari et al., 1992). Similarly, a receptor mutant that is defective in signaling is proficient in ligand-mediated receptor endocytosis (Schandel and Jenness, 1994). Therefore, the kinases responsible for Ste2 phosphorylation are unlikely to be the protein kinases regulated by the pheromone response pathway.

In Chapter V of this thesis, I report the identification of a new region of the C-terminal tail of Ste2 that is sufficient to mediate both the constitutive and ligand-mediated endocytosis of the α-factor receptor. I describe the endocytic properties of a mutant form of Ste2 that lacks the well-characterized SINNDAKSS endocytic motif. Specific mutants that remove other regions of the C-terminal tail in addition to SINNDAKSS were used to delineate the position of the second endocytic element.

CHAPTER II

MATERIALS AND METHODS

Plasmids. pJBK008 is a yeast centromeric plasmid that contains the *STE2* and *URA3* genes (Konopka et al., 1988). pYe(CEN3)30 is a yeast centromeric plasmid that contains the *TRP1* gene (Fitzgerald-Hayes et al., 1982). Plasmid pNED1(-Cys) (provided by Pam Torrance and Jeremy Thorner) is a derivative of plasmid pNED1 (David et al., 1997); it encodes a modified Ste2 that contains both the FLAG epitope and the 6His tag at the C-terminus, lacks Cys residues 59 and 252, and is expressed from the *TDH3* promoter. pDJ123 (provided by Kim Schandel) was constructed by cloning the 4.3 kb *Bam*HI fragment that contains the *STE2* gene into the *Bam*HI site of plasmid vector pYe(CEN3)30. pDJ323 (provided by Gul Bukusoglu) contains *Ste2-S184R* and was created by hydroxylamine mutagenesis of plasmid pJBK008; the *STE2* coding region was confirmed by DNA sequencing. Plasmid pEL36 carries the flanking STE4 sequences whereas the complete coding sequence has been replaced with URA3 (Leberer et al., 1992). Cutting pEL36 with *Eco*RI and transforming yeast results in the chromosomal deletion of the STE4 gene. Plasmid pDA6300 is a yeast episomal plasmid that contains the *MFα1* and *LEU2* genes. The yeast integrating plasmid pDJ338 contains the *URA3* gene, and it directs synthesis of a fusion protein that contains the α-factor receptor and the green fluorescent protein mutant GFP-S65T/V163A, (from here on referred to as GFP) under the control of the *STE2* promoter (Li et al., 1999). Integrating plasmid pDJ379 contains the *URA3* gene and the *STE2* codons 302 to 431 fused to the coding sequence for GFP (Li et al., 1999). Cleavage of pDJ379 with *Pst*I followed by

integration at the chromosomal *STE2* locus results in production of full-length Ste2p tagged at the C-terminus with GFP. Integrating plasmid pDJ466 contains the *TRP1* gene and *STE2* codons 302 to 431 fused to the coding sequence for the triple influenza hemagglutinin (HA) epitope. Cleavage of pDJ466 with *Pst*I followed by integration at the chromosomal *STE2* locus results in production of full-length Ste2p tagged at the C-terminus with the triple HA epitope. pDJ466 was constructed in two steps: first, the 0.4 kb *NsiI/SacII* fragment from pDJ338 (containing *STE2* codons 302 to 431) was subcloned into plasmid vector pRS304 (Sikorski and Hieter, 1989) that had been cut with *Pst*I and *Sac*II; and second, the resulting plasmid was digested with *Sac*I and *Sac*II and ligated with the *Sac*I/*Sac*II-digested product of a PCR reaction that contains the triple HA epitope DNA (from Mike Tyers) as template and oligonucleotide primers PO-140 (CGTGCCGAGCTCCCATGGTCAAGCAGCGTAATCTGGAACGTCATA) and PO-177 (GGCTCCCCGCGGTCTTTTACCCATACGATGTTCCTGACTAT). Integrating plasmid pDJ467 contains the *URA3* gene and *STE2* codons 156 to 326 fused to the GFP-coding sequence. Cleavage of pDJ467 with *Cla*I followed by integration at the chromosomal *STE2* locus results in production of Ste2-T326 tagged at the C-terminus with GFP. pDJ467 was constructed by ligating the 4.5 kb *Nsi*I/*Sac*II fragment (lacking *STE2*) from pDJ320 with the *Pst*I/*Sac*II-digested product of a PCR reaction that contained *STE2* DNA (pDJ320) as template and oligonucleotide primers PO-141 (GCGAAACTGCAGGGCGACAACTTCAAAAGGATAGGTTT) and PO-147 (CCACACCTACGAGTTCAA). pDJ469 (provided by Padhma Radhakrishnan) is a yeast centromere plasmid that contains *URA3* and directs synthesis of Ste2-T326 tagged at the C-terminus with GFP. pDJ469 was created by cloning the *Cla*I-*Xba*I fragment (containing *STE2* codons 259 to 326 and *GFP*) from pDJ467 into *Cla*I-*Spe*I sites in plasmid pDB02 (Dube and Konopka, 1998). Integrating plasmid pDJ470 contains *TRP1*

and *STE2* codons 156 to 326 fused to the triple HA-coding sequence. Cleavage of pDJ470 with *Eco*47III followed by integration at the chromosomal *STE2* locus results in production of Ste2-T326 tagged at the C-terminus with the triple HA epitope. pDJ470 was created in two steps. In the first step, primers PO-177 and PO-186 (GCGAAAGGTACCGGCGACAACTTCAAAAGGATAGGTTT) were used to amplify DNA encoding the triple HA epitope, and the PCR product was cloned between the *Sac*II and *Xba*I sites of plasmid pDJ467 replacing the GFP gene. In the second step, a 0.6 kb sequence in this plasmid (containing *STE2* codons 156 to 326 and the triple HA epitope) was PCR-amplified with oligonucleotide primers PO-140 and PO-141, and the *Pst*I/*Sac*I-digested product was cloned between the *Pst*I and *Sac*I sites of pRS304. Integrating plasmid pAY61 contains the *URA3* gene, and it directs synthesis of a fusion protein that contains the α-factor receptor lacking codons 297 to 360 and the green fluorescent protein (GFP) under the control of the *STE2* promoter. It was created in two steps. First, the *Cla*I/*Sac*II digest of the PCR product obtained from template pJBK063-12 with primers PO-141 and PO-92 (GCGGCCCGCGGCTAAATTATTATTATCTTCAGTCCAGAA) was cloned into the 5.3 kb *Cla*I/*Sac*II fragment of pDJ338. Then a mutation was corrected by cloning the *Cla*I/*Sac*II fragment of PCR product obtained the template from the previous step with primers PO-273 (GGTTCCATCGATAATATTCATCCTCGCATACAGTTTGAAACC) and PO-88 (GCGCGGCCGTCTAGATTATTTGTATAGTTCATCCATGCCAT) into the 5 kb *Cla*I/*Sac*II site into the plasmid from the previous step. Integrating plasmid pAY62 contains the *URA3* gene, and it directs synthesis of a fusion protein that contains the α-factor receptor lacking codons 297 to 391 and the green fluorescent protein (GFP) under the control of the *STE2* promoter. Plasmid pAY62 was created by cloning the *Cla*I/*Sac*II fragment of PCR product obtained from the template pJBK063-16 with

primers PO-141 and PO-92 into the 5 kb *Cla*I/*Sac*II fragment of pDJ338. Integrating plasmid pAY66 contains the *URA3* gene, and it directs synthesis of a fusion protein that contains the α-factor receptor lacking codons 297 to 360 and a lysine to arginine change at position 374 and the green fluorescent protein (GFP) under the control of the *STE2* promoter. Plasmid pAY66 was produced by cloning the *Pst*I/*Xba*I digest of PCR product obtained from template pAY61 with primers PO-271 (GAGACTGCAGATGATATAGAGAGAAATCAGTTTTATCAGTTGCCC) and PO-88 into the 5 kb *Pst*I/*Xba*I digestion fragment of pAY61. Integrating plasmid pAY67 contains the *URA3* gene, and it directs synthesis of a fusion protein that contains the α-factor receptor lacking codons 297 to 360 and a lysine to arginine change at position 387 and the green fluorescent protein (GFP) under the control of the *STE2* promoter. Plasmid pAY67 was produced with the Quik Change Site Directed Mutagenesis kit (Stratagene) according to manufacturer's instructions. The primers used were PO-269 (CACACCTACGAGTTCAAGAAATACTAGGATAGGACCG) and PO-270 (CGGTCCTATCCTAGTATTTCTTGAACTCGTAGGTGTG). The template was pAY61. The integrating plasmid pAY68 contains the *URA3* gene, and it directs synthesis of a fusion protein that contains the α-factor receptor lacking codons 297 to 360 and lysine to arginine changes at positions 374 and 387 and the green fluorescent protein (GFP) under the control of the *STE2* promoter. It was produced by cloning the *Pst*I/*Xba*I digest of PCR product obtained from template pAY67 with primers PO-271 and PO-88 into the 5 kb *Pst*I/*Xba*I fragment in pAY61. Integrating plasmid pAY83 contains the *URA3* gene, and it directs synthesis of a fusion protein that contains the green fluorescent protein (GFP) and a mutant version of the α-factor receptor that lacks codons 297 to 360 and is truncated at position 391 under the control of the *STE2* promoter. Plasmid pAY83 was created by cloning the *Cla*I/*Sac*II fragment of PCR product obtained from template

pAY61 with primers PO-274 (GCGGCCCGCGGCTATCCTAGTATTTTTTGAACTCGTAGGTGTGGGCAA) and PO-3 (GCAGTTCGATAGTTTCC) into the 5 kb *Cla*I/*Sac*II fragment of pAY61. Integrating plasmid pAY84 contains the *URA3* gene, and it directs synthesis of a fusion protein that contains the green fluorescent protein (GFP) and a mutant version of the α-factor receptor that lacks codons 297 to 360 and is truncated at position 399 under the control of the *STE2* promoter. Plasmid pAY83 was created by cloning the *Cla*I/*Sac*II fragment of PCR product obtained from template pAY61 with primers PO-275 (GCGGCCCGCGGCGTAACTTGCATCAGCAAACGGTCCTATCC) and PO-3 into the 5 kb *Cla*I/*Sac*II fragment of pAY61. Plasmid pDJ427 is an episomal plasmid, which carries GPD promoter inserted into the pRS426 plasmid (Mumberg et al., 1995). It also harbors the yeast *TRP1* marker. Plasmid pAY107 is a yeast episomal plasmid which carries the *TRP1* gene and a triple-tagged version of Ste2 (HA-Ste2-FT-HT) with an HA tag on the N-terminus and FLAG and His_6 tags on the C-terminus which is synthesized under the control of *TDH3* promoter. Plasmid pAY107 was created in two steps. First, the *Bam*HI digestion of pNED1(-Cys) was partially digested with SalI and the 1.4 kb product was cloned into the *Bam*HI/*Xho*I site in pRS426GPD. Second, the 1 kb *Sac*I/*Hpa*I digestion product of pDJ439 was cloned into the *Sac*I/*Hpa*I fragment of the vector obtained from the previous step.

Yeast strains. Yeast strains listed in Table 1 are congenic to strain 381G. Strains DJ1400-A, DJ1403-A, DJ1404-A, DJ1405-A, DJ1405-A, DJ1406-A, DJ1408-A, DJ1445, DJ1495, DJ1496 and DJ1414-A to DJ1417-A were derived from DJ211-5-3; strains DJ1402-A, DJ1407-A, and DJ1418-A were derived from AY1; strains DJ1410-A, DJ1411-A, DJ1413-A were derived from strain DJ1205-6-3; strains DJ1442, DJ1443,

TABLE 1. Yeast strains used in this study.

Strain[a]	Genotype[b]
381G	*MAT***a** *cry1 ade2-1 his4-580 lys2 trp1 tyr1 SUP4-3^{ts}*
DJ211-5-3	381G *leu2 ura3 bar1-1*
AY1	DJ211-5-3 *Ste2-S184R*
DJ1205-6-3	381G *ADE2*$^{+}$ *HIS4*$^{+}$ *LYS2*$^{+}$ *TYR1*$^{+}$ *ura3 bar1-1*
DJ1204-2	381G *ADE2*$^{+}$ *HIS4*$^{+}$ *LYS2*$^{+}$ *TYR1 TRP1*$^{+}$ *ura3 bar1-1ste2::LEU2*
DJ1400-A	DJ211-5-3 *STE2*::pDJ466
DJ1402-A	DJ211-5-3 *Ste2-S184R*::pDJ466 containing plasmid pJBK008
DJ1403-A	DJ211-5-3 *STE2*::pDJ466 containing plasmid pDJ323
DJ1404-A	DJ211-5-3 *STE2*::pDJ379
DJ1405-A	DJ211-5-3 *ste2-S184R*::pDJ379
DJ1406-A	DJ211-5-3 *STE2*::pDJ379 containing plasmid pDJ123
DJ1407-A	DJ211-5-3 *ste2-S184R*::pDJ379 containing plasmid pYe(CEN3)30
DJ1408-A	DJ211-5-3 *STE2*::pDJ466 containing plasmid pYe(CEN3)30
DJ1410-A	DJ1205-6-3 *STE2*::pDJ467 containing plasmid pYe(CEN3)30
DJ1411-A	DJ1205-6-3 *STE2*::pDJ467 containing plasmid pDJ123
DJ1413-A	DJ1205-6-3 *STE2*::pDJ379 containing plasmid pYe(CEN3)30
DJ1414-A	DJ211-5-3 *ura3*::pDJ338 *STE2*::pDJ466
DJ1417-A	DJ211-5-3 *STE2*::pDJ470 containing plasmid pDJ469
DJ1418-A	DJ211-5-3 *Ste2-S184R*::pDJ466 containing plasmid pDJ323
DJ1419-A	DJ211-5-3 *STE2*::pDJ466 containing plasmid pJBK008
DJ1442	DJ213-7-3 *ura3*::pDJ338-12
DJ1443	DJ213-7-3 *ura3*::pDJ338-16

TABLE 1. continued.

Strain[a]	Genotype[b]
DJ1445	DJ211-5-3 *ura3*::pDJ338-16
DJ1448	DJ213-7-3, containing plasmid pDJ427
DJ1449	DJ213-7-3, containing plasmid pAY107
DJ1460	DJ1204-2 *ura3*::pAY60
DJ1461	DJ1204-2 *ura3*::pAY61
DJ1462	DJ1204-2 *ura3*::pAY62
DJ1466	DJ1204-2 *ura3*::pAY66
DJ1467	DJ1204-2 *ura3*::pAY67
DJ1468	DJ1204-2 *ura3*::pAY68
DJ1483	DJ1204-2 *ura3*::pAY83
DJ1484	DJ1204-2 *ura3*::pAY84
DJ1494	DJ213-7-3 *ura3*::pDJ338
DJ1495	DJ211-5-3 *STE2*::pDJ466 *ste4*::pEL36
DJ1496	DJ211-5-3 *STE2*::pDJ439, containing plasmid pNED1(-Cys)
FY70/pDA6300	*Matα leu2Δ1,* containing plasmid pDA6300

[a] All strains are congenic with strain 381G (Hartwell, 1967).

[b] Mutation *bar1-1* inhibits α-factor degradation (Sprague and Herskowitz, 1981). Temperature-sensitive mutation *SUP4-3* suppresses amber mutations *his4-580* and *trp1* at 22°C. *STE2*::pDJ466, *ste2-S184R*::pDJ466, *STE2*::pDJ379, *ste2-S184R*::pDJ379, *STE2*::pDJ467, *STE2*::pDJ470, *ura3*::pDJ338, *ura3*::pDJ338-12, *ura3*::pDJ338-16,

TABLE 1. continued.

ura3::pAY61, *ura3*::pAY62, *ura3*::pAY66, *ura3*::pAY67, *ura3*::pAY68, *ura3*::pAY83, *ura3*::pAY84 result in the production of Ste2-HA, Ste2-S184R-HA, Ste2-GFP, Ste2-S184R-GFP, Ste2-T326-GFP, Ste2-T326-HA, Ste2-GFP, Ste2-Δ(297-360)-GFP, Ste2-Δ(297-391)-GFP, Ste2-Δ(297-360)-K374R-GFP, Ste2-Δ(297-360)-K387R-GFP, Ste2-Δ(297-360)-K374R/K387R-GFP, Ste2-Δ(297-360)-GFP-T391, Ste2-Δ(297-360)-GFP-T399, respectively. The CEN plasmids pJBK008 , pDJ323, pDJ123 and pDJ469 result in the production of Ste2, Ste2-S184R, Ste2, and Ste2-T326-GFP, respectively. pYe(CEN3)30 and pDJ467 are control plasmids that contain the *TRP1* marker. The yeast episomal plasmid, PDA6300, results in the production of α-factor.

DJ1448, DJ1449 and DJ1494 were derived from strain DJ213-7-3; strains DJ1461, DJ1462, DJ1466, DJ1467, DJ1468, DJ1483 and DJ1484 were derived from strain DJ1204-2 by transformation with the plasmids indicated in Table 1. Plasmids pDJ379 and pDJ466 were digested with *PstI* and plasmids pDJ467 and pDJ470 were digested with *ClaI* and *Eco47III*, respectively, prior to transformation to target the integration at the *STE2* locus. Plasmids pDJ338, pAY61, pAY62, pAY66, pAY67, pAY68, pAY83 and pAY84 were digested with *StuI* to target the integration at the *URA3* locus. Digestion of plasmid pEL36 with *Eco*RI resulted in the replacement of the *STE4* gene with URA3. Production of relevant proteins was confirmed by western blotting. Single integration events were confirmed by PCR. Strains DJ1410-A, DJ1412-A and DJ1413-A were constructed by standard genetic crosses. Yeast strains were transformed with plasmids by using standard techniques (Soni et al., 1993). Strain AY1 (provided by Amy Yang) was constructed by subcloning the *ste2-S184R* allele into integrating plasmid pDJ251 and then introducing it into the chromosomal locus of strain DJ211-5-3 by using the two-step gene replacement described previously (Schandel and Jenness, 1994).

Culture media. Liquid and solid media were prepared as previously described (Jenness et al., 1997). YM-1 is a rich liquid medium (Hartwell, 1967). Minimal selective media lacking uracil (-Ura+CAA) or lacking tryptophan (-Trp+CAA) are described elsewhere (Hirschman et al., 1997). The low sulfate medium has been described previously (Jenness and Spatrick, 1986).

Antisera and reagents. Rabbit polyclonal antisera were specific for GFP (Seedorf et al., 1999), for *Escherichia coli* aspartate transcarbamoylase (ATCase) (from Y. R. Yang and H. K. Schachman) or for the carboxy-terminal portion of the α-factor receptor, Ste2

(Konopka et al., 1988). Mouse monoclonal antibodies that recognize the yeast plasma membrane ATPase (Pma1) were from clone C56 (Aris and Blobel, 1988; Schandel and Jenness, 1994). Mouse monoclonal antibodies that recognize the *influenza* hemagglutinin epitope (HA.11) were from BAbCO, Berkeley Antibody Co., Richmond, CA. Peroxidase-conjugated goat anti-rabbit secondary antibodies were purchased from Life Technologies Inc., Baltimore, MD. Peroxidase-conjugated goat anti-mouse secondary antibodies, purified mouse immunoglobulin and n-dodecyl β-D-maltoside were from Sigma Chemical Co., St. Louis, MO. Purified bovine serum albumin (BSA) was purchased from Boehringer Mannheim Co., Indianapolis, IN. Peroxidase-conjugated goat anti-mouse and goat anti-rabbit $F(ab')_2$ fragment specific IgG were purchased from Jackson Immunoresearch Inc., West Grove, PA. The chemiluminescence kit Super Signal and Ultralink Immobilized Protein A beads were from Pierce Chemical Co., Rockford, IL.

Renografin density gradients. Cultures were grown in -Ura+CAA or in -Trp+CAA media depending on the plasmid markers used. Membranes were resolved in Renografin density gradients as previously described (Schandel and Jenness, 1994).

Immunoblotting and quantitation. Western blotting procedures and quantitation were carried out as previously described (Hirschman et al., 1997).

Immunoprecipitation. Cell lysates were prepared as described previously (Schandel and Jenness, 1994). Membranes were collected by centrifugation (Beckman airfuge at 28 psi for 20 min, or SW50 rotor at 40 krpm for 90 min). The pellet containing the membranes was suspended in ice-cold IP buffer (50 mM Tris-Cl [pH 7.4], 150 mM NaCl,

5 mM EDTA, 2 mg/ml n-dodecyl β-D-maltoside (DBM), 10% glycerol, 1 μg/ml PMSF, 0.1 μg/ml pepstatin A) and incubated on ice for 2 hours with occasional mixing. The solution was then centrifuged at 13,000×g for 5 minutes to remove insoluble material. In each experiment, equivalent number of cells was processed for each immunoprecipitation reaction, however, for the experiments in which the cultures had been treated with α-factor; the extracts were corrected for protein concentration. BCA (bicinchoninic acid) protein assay (Pierce Chemical Co., Rockford, IL) was used according to manufacturer's instructions. Precipitating antibodies were added to the supernatant, and the mixture was incubated at 4°C with gentle agitation for 2 hours. Protein A beads were added, and the mixture was incubated for 2 hours at 4°C. The beads were allowed to settle for 5 min, and then collected by centrifugation 1100×g for 5 seconds. Beads were washed 4 times with IP buffer and extracted with 2x SDS sample buffer for 10 min at 37°C. Samples were centrifuged at 13,000×g for 5 min. The proteins were resolved on 10% SDS-PAGE gels and detected by using immunoblotting methods. Peroxidase-conjugated goat anti-mouse or goat anti-rabbit $F(ab')_2$ fragment were used as the secondary reagent. The results were quantified by using laser-scanning densitometry (Molecular Dynamics).

Glycerol gradient sedimentation. 2×10^9 cells were collected from exponentially growing cultures that had been untreated or treated with 10^{-7} M α-factor for 5 min. Crude membranes were extracted with DβM as described in the previous section. The extract was cleared by centrifugation for 15 min at 13,000×g and mixed with 4.5 μg BSA (4.3S), 5 μg mouse IgG (7S), 10 μg ATCase (11.7S) as internal marker proteins. The mixture was applied to an 8–30% glycerol gradient in IP buffer and centrifuged in an

SW50 rotor at 40 krpm for 14 h at 4°C. Fourteen 350 µl fractions were collected assayed by using SDS-PAGE and immunoblotting methods.

Fluorescence microscopy. Cultures were grown overnight at 34° C to a density of 2×10^6 cells/ml in -Trp+CAA medium. These conditions provided selection of the plasmids bearing the *TRP1* gene. Cultures, which did not require selection by TRP1 gene, were grown to a density of 10^7 cells/ml in YM-1. Cells were collected and resuspended in the same volume of YM-1 media, and then cultured at 30°C to a density of 10^7 cells/ml. Cultures received cycloheximide (10 µg/ml) for 5 min and were then incubated at 30°C for 15 min in the presence or in the absence of α-factor (10^{-7} M). Endocytosis was terminated by chilling the cells and adding the metabolic poisons, NaN_3 (10 mM) and KF (10 mM). Cells were collected by centrifugation, washed with ice-cold phosphate-buffered saline (PBS) and suspended in 1/10 volume PBS. Epifluorescent images were obtained with a Nikon microscope equipped with a cooled CCD camera.

FM4-64 staining. Cultures (strain DJ1413-A) was grown overnight in -Trp+CAA medium as described above. 2×10^8 cells were collected, suspended in 1 ml ice-cold YM-1 containing the lipophylic dye FM4-64 (40 µM) with or without α-factor (10^{-7} M) and placed on ice water for 30 min to allow the dye to bind the cell surface. Cells were then collected by centrifugation, resuspended in ice-cold YM-1. Aliquots were removed from ice and placed in water at room temperature for the times indicated and then placed back on ice. Cells were collected, resuspended in ice-cold phosphate-buffered saline analyzed by fluorescence microscopy as described above.

Crosslinking assays. Cultures were grown overnight to a final density of $1\text{-}2\times10^{7}$ cells/ml. Cells were untreated or treated with α-factor as described in each figure. Cell lysates were prepared in phosphate-buffered saline supplemented with protease inhibitors (100 μg/ml PMSF, 10 μg/ml pepstatin A) by the method described previously (Schandel and Jenness, 1994). The protein amounts were determined by BCA (bicinchoninic acid) protein assay as mentioned above. Lysates containing equivalent amounts of protein were treated with 0, 2.5 or 5 mM of a thiol-reversible, water-soluble amine-reactive homobifunctional crosslinking agent DTSSP, dithio-*bis*-(sulfosuccinimidylpropionate) (Pierce Chemical Co.) which was freshly prepared as a 50 mM stock solution in 5 mM sodium citrate, pH 5. Following 2 hours of incubation on ice, the crosslinking reactions were quenched by the addition of 1/4 volume of 1 M Tris buffer, pH 7.4. The lysates were then diluted in SDS sample buffer in the presence or in the absence of reducing agents (50 mM DTT or 100 mM βME), subjected to SDS-PAGE and analyzed by western blotting as previously described (Hirschman et al., 1997).

Purification procedure for protein complexes containing tagged α-factor receptor. Cleared cell lysates were prepared as previously described (Schandel and Jenness, 1994). Protein concentrations were determined by BCA (bicinchoninic acid) protein assay, as described above. Crosslinking reaction was carried out as described in the previous section. 350 μl of the lysate with protein concentration of 0.4 mM was used. The DTSSP concentration was 4 mM. The crosslinked lysates were mixed with 76% Renografin to give a 1 ml layer with final Renografin concentration of 42%. 4 ml of 34% Renografin was layered on top. The two-step gradient was centrifuged in an SW-50 rotor at 40,000×rpm (4°C) for 15 hr. A 750 μl sample from the top, which contains the cellular membranes, was collected. The membrane fraction was diluted with 4 ml of

phosphate-buffered saline and pelleted by centrifugation at 40,000×rpm (4°C) for 2 hr. The pellet containing cell membranes was incubated in 0.2 ml IP buffer for 2 hr on ice to extract the membrane proteins. The reaction was centrifuged at 13,000×g in a microfuge, and 2 μg of anti-HA antibodies was added to the supernatant. The immunoprecipitation reaction was carried out as described above except after the last wash, the pelleted Ultralink Protein A Plus beads were washed with IP buffer without EDTA, and precipitated proteins were extracted by the addition of Urea-Ni^+ binding buffer (8 M urea, 500 mM NaCl, 5 mM imidazole, 20 mM Tris, pH 8.0) in three sequential applications (100 μl each). The extracted material was then incubated with Ni^+-NTA beads (Qiagen Inc.) that had been precycled in Urea-Ni^+ binding buffer. The reaction tube was rotated end-over-end for 5 h at room temperature. The beads were then washed four times with Urea-Ni^+ binding buffer, once with Urea-Ni^+ binding buffer with 150 mM NaCl and twice with Urea-Ni^+ binding buffer with no NaCl and no imidazole. The bound material was either eluted with SDS sample buffer and resolved on one-dimensional SDS-PAGE or processed as described below for two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis. The material obtained at the end of the 2-step purification procedure was processed for two dimensional electrophoresis by first extracting the bound proteins with 63 μl of solubilizing mixture (8 M urea, 2% CHAPS, 4% βME) and then by extracting the beads again with 190 μl of the solubilizing mixture supplemented with DTT (3 g/l) , Pharmalyte (1% v/v) and IPG buffer (1% v/v) (Pharmacia). The two extracts were pooled to obtain the 'rehydration mixture'. IPG strips with a pH range of 3-to-9 were used. Strips were each placed on top of 250 μl rehydration mixture. The rehydration was allowed to proceed for about 17 hr at room

temperature. The 2D gel electrophoresis was performed according to manufacturer's instructions (Pharmacia).

Silver staining. SDS gels were fixed for 30 min or overnight in 50% ethanol, 10% acetic acid, 40% Milli-Q water. Gels were incubated in 30% ethanol for 15 min and washed three times for 5 min with Milli-Q water. Gels were sensitized with freshly prepared 0.2 g/L sodium thiosulfate for 1.5 min and washed three times for 30 sec with Milli-Q water. In the staining step, gels were soaked in 2 g/L silver nitrate for 25 min and washed twice with 2 to 3 L of Milli-Q water. Gels were developed in 60 g/L sodium carbonate, 20 ml/L of the sodium thiosulfate solution from the sensitization step and 500 µl/L of 37% formaldehyde in Milli-Q. The development was stopped by removing the developing solution and adding 6% acetic acid in Milli-Q water for 10 min. Gels were then washed for a minimum of 4 changes of Milli-Q water with 15 min intervals each and the band of interest was cut out.

Purification of ^{35}S-labeled α-factor. The strain FY70/pDA6300 was grown overnight at 30°C to a density of 5×10^{6} cells/ml in 20 ml low sulfate medium (20 µM $NaSO_4$) or LSM+SO_4. 6×10^{7} cells were collected, washed with 12 ml LSM with no added sulfate and suspended in 12 ml LSM+20 µM $NaSO_4$. 25 mCi of ^{35}S-H_2SO_4 was added to the culture. The culture was incubated at 30°C with shaking until the cells incorporated 89% of the label. The cells were collected by centrifugation. The supernatant was supplemented with p-tosyl-L-arginine methyl ester (TAME) to a final concentration of 10 mM and β-mercaptoethanol (βME) to a concentration of 2 mM. The supernatant was then loaded onto a 2.5 ml Bio-Rex 70 column that had been precycled in 0.1 N acetic acid and 2mM β-mercaptoethanol (βME) at 4°C. The column was washed with 4 column

volumes of Wash1 (0.1 N acetic acid and 2mM βME) and then with 4 column volumes of Wash2 (50 % ethanol, 0.1 N acetic acid, 2 mM βME). The radiolabeled α-factor was eluted with 4 column volumes of 80 % ethanol, 0.01 N HCl, 2 mM βME. 60 µl of 1M Tris, pH8 was added to each of 2.5 ml fractions. 3 µl of each fraction was counted in a scintillation counter and the amount of label in each fraction was determined. Halo assays were performed on all fractions. Known concentrations of synthetic α-factor (Sigma Chemical Co.) were included as standards diluted in 80 % ethanol. A supersensitive strain (6360-17-2) was used. The percent binding activity was determined by incubating labeled α-factor with a large excess of a *STE2*$^+$ strain and separately with *ste2Δ* cells that had been poisoned with 10 mM NaN_3 and KF. The incubation time was 15 min at room temperature. Cells were collected by centrifugation and radioactivity in each supernatant was determined. The percentage of labeled material that was active was calculated as follows:

% active = [(cpm in *ste2Δ* sup) - (cpm in *STE2*$^+$ sup)] / (cpm in *ste2Δ* sup) × 100

Overall, I obtained 5 ml of 2×10^{-7}M ^{35}S-labeled α-factor with a specific activity of 63 Ci/mmol.

Downregulation assays. Cultures were grown overnight at 30°C to a density of 1-2$\times10^7$ cells/ml and then diluted to 5×10^6 cells/ml in the presence of 10 mM TAME. Cultures were then incubated with 10 µM cycloheximide in the presence or in the absence of 10^{-7}M unlabeled α-factor. At the times indicated, 25 ml aliquots were filtered on presoaked nitrocellulose filters and washed twice with 2 ml Inhibitory Medium (IM) which contained YM-1 in 10 mM NaN_3, 10 mM KF and 10 mM TAME. The cells that had been collected on the filter were suspended in 10 ml IM, and the bound unlabeled α-factor was allowed to dissociate. The cells expressing wild-type receptors were

incubated for 2 hours at room temperature, and the strains expressing Ste2 internal deletion mutants were incubated in a 30°C water bath for 4 h. It has been previously determined that incubation for 2 h at room temperature was sufficient for the dissociation of prebound α-factor from cells expressing Ste2, whereas strains expressing a severely-truncated receptor (Ste2-T326) required 4 hours at 30°C to allow for >90% dissociation (Schandel and Jenness, 1994). Cells were collected by centrifugation and resuspended in 0.5 ml IM and held on ice overnight. 90 µl samples of cells were added to four tubes. Two tubes contained 10 µl labeled α-factor (2×10^{-7} M). The other two tubes contained a mixture of labeled α-factor (2×10^{-7} M) with an excess of unlabeled α-factor (2×10^{-5} M). After 30 min of incubation at 25°C, 90 µl samples removed to 2 ml IM and immediately collected on prewetted GF/C filters. Filters were washed twice with 2 ml IM. Washing time was between 0.4-0.5 min. Dried filters were counted in toluene-omnifluor scintillant for 5 min with a liquid-scintillation counter. The counts obtained from duplicate filters were averaged. The binding activity at each time point was determined by subtracting the cpm values obtained from binding reactions that contained excess unlabeled α-factor from those that contained labeled α-factor-only. The percentage of receptor remaining on cell surface was determined by dividing the binding activity at each time point by the binding activity determined at time zero for the given strain.

Protein turnover assays. Cultures were grown overnight to a density of $1\text{-}2\times10^{7}$ cells/ml. Cycloheximide was added to a final concentration of 10 µg/ml. Samples were withdrawn at times indicated. Cell lysates were prepared, the amount of total protein in each sample was determined, and samples were processed for SDS-PAGE and western blotting as described above. Approximately 1 µg of protein per lane was

loaded onto duplicate gels per each sample. The samples obtained at the zero time point were loaded at 0.5 and 1 μg per lane to assess the linearity of the assay. Two separate autoradiographic exposures obtained from each gel were quantified by using laser-scanning densitometry (Molecular Dynamics). The scanning results obtained from the zero time point lanes loaded with 1 μg protein were compared with the results from the same samples that had been loaded with half the amount. The exposure in which the ratio was closest to 1/2 (within a range of 1/3 to 2/3) was used. Results obtained from the two independent experiments were averaged.

CHAPTER III

OLIGOMERIZATION OF THE α-FACTOR PHEROMONE RECEPTORS

Many cell surface receptors are organized in multimeric complexes. For certain receptors (e.g. JAK family receptors and receptor tyrosine kinases), such higher order structures provide higher ligand binding affinity and induce receptor activation and therefore contribute to the structure and function of these receptors. Until recently, members of G-protein-coupled receptor superfamily (GPCRs) were assumed to function as monomers. However, early observations suggested oligomerization of GPCRs. Some examples include allelic complementation of coexpressed mutant receptors (Konopka and Jenness, 1991), and the presence of large SDS-resistant receptor-protein aggregates on SDS-PAGE gels (Blumer et al., 1988; Herberg et al., 1984; Konopka et al., 1988; Ng et al., 1993). More direct evidence was obtained recently by using fluorescence resonance energy transfer (FRET), crosslinking and coimmunoprecipitation approaches, suggesting oligomerization of the β2 adrenergic (Hebert et al., 1996), δ-opioid (Cvejic and Devi, 1997), D2 and D3 dopamine (Ng et al., 1996; Nimchinsky et al., 1997), m3 muscarinic (Zeng and Wess, 1999), metabotropic glutamate (Romano et al., 1996) and Ca^{2+}-sensing receptors (Bai et al., 1998).

While oligomerization is emerging as a common theme for GPCRs, our understanding of this phenomenon is limited. The influence of agonists on receptor oligomerization and the structural determinants of the receptor that are important for oligomerization have been shown to vary among the GPCRs investigated. For example,

agonists appear to stabilize dimers of β2 adrenergic receptors (Hebert et al., 1996), whereas agonist binding favors the monomeric state of δ opioid receptors (Cvejic and Devi, 1997) and fails to alter the oligomeric state m3 muscarinic receptors (Zeng and Wess, 1999). Transmembrane region VI is thought to mediate the association of β2-adrenergic receptors since peptides containing this sequence interfere with the recovery of receptor dimers and interfere with signaling (Hebert et al., 1996). In contrast, a 15 amino acid sequence contained in the C-terminal tail of δ-opioid receptor has been associated with dimerization (Cvejic and Devi, 1997), and the Ca^{2+}-sensing receptor and metabotropic glutamate receptor 5 oligomers are linked covalently through disulfide bonds (Bai et al., 1998; Romano et al., 1996).

Functional significance of GPCR oligomerization is currently unclear. Functional interactions between oligomerized receptors have been inferred from the cooperative binding of subtype-specific ligands to receptor heterodimers containing δ- and κ-opioid receptors (Jordan and Devi, 1999). The relationship between oligomerization and signal transduction is controversial. A peptide derived from the transmembrane of the β2-adrenergic receptor results in the reduction of receptor dimers detected and the receptor signaling activity (Hebert et al., 1996). In contrast, the corresponding region of the D1 dopamine receptor inhibits receptor signaling activity without affecting oligomerization (George et al., 1998). For the δ opioid receptor, ligand-induced dissociation of receptor oligomers is found to precede ligand-mediated endocytosis (Cvejic and Devi, 1997). This correlation suggests that the dissociation of oligomers may be an essential step in the endocytic pathway.

Oligomerization of α-factor receptors in the plasma membrane was first proposed by Jenness and Spatrick (1986) since receptors were internalized more rapidly than bound

α-factor at subsaturating α-factor concentrations. In this chapter, I sought to determine whether yeast α-factor receptors form oligomers and whether oligomers are subject to ligand-mediated endocytosis.

Results

Immunoprecipitation of differentially-tagged receptors

Interactions between α-factor receptors were evaluated by performing coimmunoprecipitation experiments with differentially-tagged receptors that had been solubilized with the non-denaturing detergent n-dodecyl β-D-maltoside. We examined cells that express α-factor receptors tagged with the influenza hemagglutinin epitope (Ste2-HA) as well as α-factor receptors tagged with the green fluorescent protein (Ste2-GFP). Both epitope tags were fused to the receptor after the C-terminal residue. The genes encoding the two fusion proteins were present in single copy and utilized the native *STE2* promoter. Crude membranes were prepared and extracted with the detergent. Glycerol gradients were performed to evaluate the size and the homogeneity of the complexes containing receptors. Both Ste2-HA and Ste2-GFP sedimented as a single peak with sedimentation coefficient of roughly 8S in glycerol density gradients (Fig. 1A), although Ste2-HA sedimented slightly faster than Ste2-GFP. Similar results were obtained when the cultures had been exposed to α-factor (Fig. 1B). Both Ste2-HA and Ste2-GFP were found to coimmunoprecipitate, when the receptors in the detergent extract were precipitated with anti-HA antibodies and analyzed with immunoblotting methods using antisera specific for C-terminus of the receptor (Fig. 2A, lane 3). The two tagged species were resolved on the blot since Ste2-GFP is significantly larger than Ste2-HA (80 kDa versus 55 kDa, respectively). No precipitation of Ste2-GFP was detected in the analysis of the control cells that expressed only Ste2-HA or only Ste2-GFP (Fig. 2A, lanes 1 and 2, respectively).

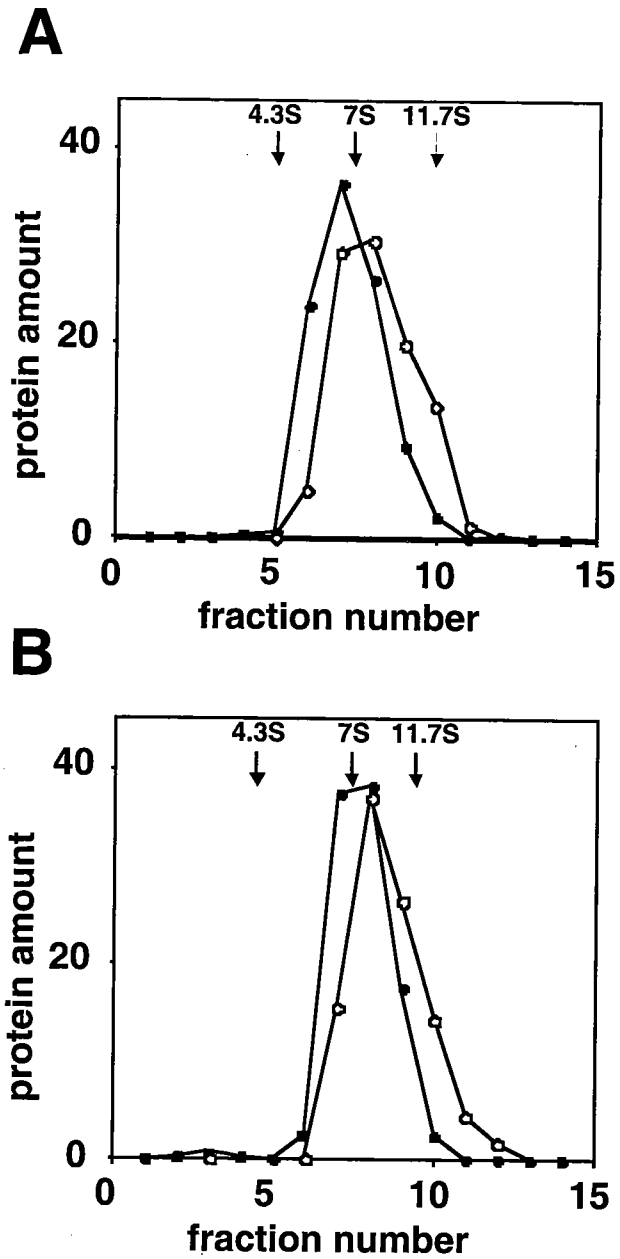


Figure 1. Glycerol gradient sedimentation. Membrane proteins from strain DJ1414-A were solubilized in IP buffer containing n-dodecyl β-D-maltoside and then resolved on a 8–30% glycerol gradient. Cultures were untreated **(A)** or treated with 10^{-7} M α-factor for 5 min **(B)**. Fractions were assayed for Ste2-HA (○) and Ste2-GFP (●) and for marker proteins, BSA (4.3S), mouse IgG (7S) and ATCase (11.7S).

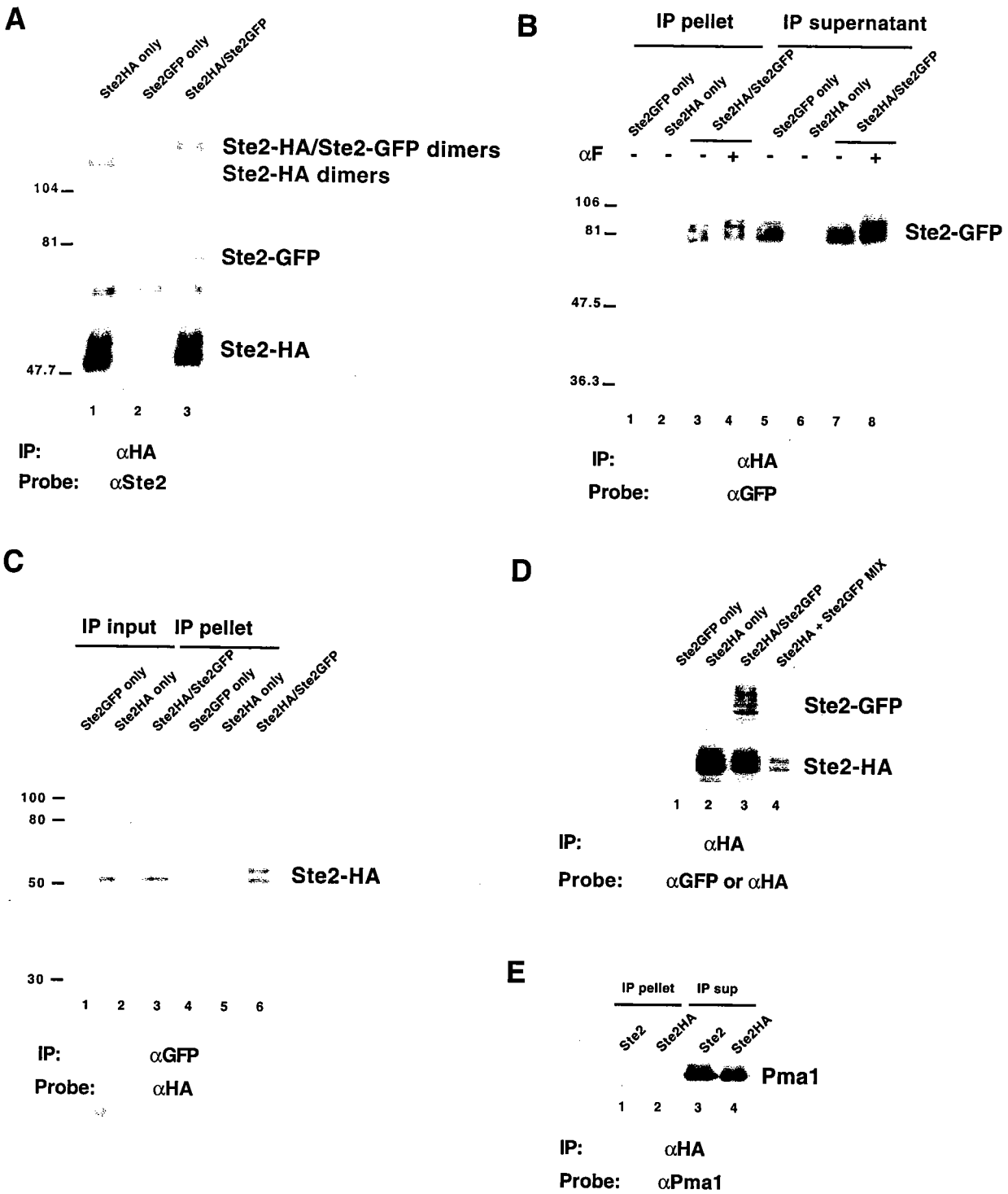

A
Ste2HA only
Ste2GFP only
Ste2HA/Ste2GFP
Ste2-HA/Ste2-GFP dimers
Ste2-HA dimers
104
81
Ste2-GFP
47.7
Ste2-HA
1 2 3
IP: αHA
Probe: αSte2
B
IP pellet
IP supernatant
Ste2GFP only
Ste2HA only
Ste2HA/Ste2GFP
Ste2GFP only
Ste2HA only
Ste2HA/Ste2GFP
αF - - - + - - - +
106
81
Ste2-GFP
47.5
36.3
1 2 3 4 5 6 7 8
IP: αHA
Probe: αGFP
C
IP input
IP pellet
Ste2GFP only
Ste2HA only
Ste2HA/Ste2GFP
Ste2GFP only
Ste2HA only
Ste2HA/Ste2GFP
100
80
50
Ste2-HA
30
1 2 3 4 5 6
IP: αGFP
Probe: αHA
D
Ste2GFP only
Ste2HA only
Ste2HA/Ste2GFP
Ste2HA + Ste2GFP MIX
Ste2-GFP
Ste2-HA
1 2 3 4
IP: αHA
Probe: αGFP or αHA
E
IP pellet
IP sup
Ste2
Ste2HA
Ste2
Ste2HA
Pma1
1 2 3 4
IP: αHA
Probe: αPma1

Figure 2. Immunoprecipitation of differentially-tagged receptors. Cells that expressed one or two tagged forms of the receptor (Ste2-HA and Ste2-GFP) were analyzed. Membrane proteins extracted with n-dodecyl β-D-maltoside were subjected to immunoprecipitation and then analyzed by using immunoblotting methods. The antibodies used for precipitation and for immunoblotting are denoted below each panel ("IP" and "Probe", respectively). As indicated above each lane, the cells analyzed expressed Ste2-HA, Ste2-GFP or both. **(A)** Anti-HA antibody precipitates Ste2-HA, Ste2-GFP and SDS-resistant dimers. **(B)** Efficiency of Ste2-GFP precipitation with anti-HA antibody. Lanes 1–4 contained 10% of the immunoprecipitated protein (pellet). Lanes 5–8 contained 2.5% of the unprecipitated protein (supernatant). Cells expressing both Ste2-HA and Ste2-GFP were either untreated or treated with 10^{-7} M α–factor for 5 min prior to analysis. **(C)** Efficiency of Ste2-HA precipitation with anti-GFP antibody. Lanes 1–3 contained 17% of the immunoprecipitated protein (pellet). Lanes 4–6 contained 1% of the total protein (input). **(D)** Coprecipitation does not result from processing of the samples after cell lysis. Lanes 1-3, processed as in panel B. Lane 4, the cells expressing only Ste2-HA were mixed prior to lysis with the cells expressing only Ste2-GFP. The same blot was sequentially probed with anti-GFP (top panel) and anti-HA antibody (bottom panel). **(E)** Plasma membrane ATPase is not present in immunoprecipitates containing Ste2-HA. Strains were DJ1400-A (Ste2-HA only), DJ1404-A (Ste2-GFP only), DJ1414-A (Ste2-HA/Ste2-GFP) and DJ211-5-3 (Ste2).

Two protein species containing Ste2 were detected that had molecular weights greater than 100 kDa (Fig. 2A, lanes 1 and 3). Such high molecular weight forms, designated "SDS-resistant dimers", are commonly observed when analyzing Ste2 and other GPCR proteins (Blumer et al., 1988; Hebert et al., 1996; Konopka et al., 1988). However, the proportion of receptors that migrated as SDS-resistant dimers was variable among different preparations. In earlier work, it has been unclear whether this high molecular weight species reflects the aggregation of Ste2 with itself or with other proteins, and it has also been unclear whether it represents receptor dimers present in the membrane or dimers that arise only after SDS extraction. Interestingly, a single high molecular weight protein was detected from cells expressing Ste2-HA alone (Fig. 2A, lane 1), consistent with Ste2-HA dimers, whereas cells expressing both Ste2-HA and Ste2-GFP produced bands consistent with Ste2-HA/Ste2-GFP hetero-dimers in addition to Ste2-HA homo-dimers (Fig. 2A, lane 3). The absence of Ste2-GFP homo-dimers is expected because the samples had been immunoprecipitated with anti-HA antibodies, therefore only Ste2-HA and Ste2-GFP heterodimers and Ste2-HA homodimer were expected to precipitate. This observation further implies that the SDS-resistant dimers did not form in the SDS sample buffer. If the SDS-resistant dimers were formed following extraction by SDS, then the Ste2-GFP molecules that dissociated from Ste2-HA would also have formed SDS-resistant homo-dimers. These observations indicate that an SDS-resistant dimer contains more than one molecule of Ste2.

As a more defined method of evaluating coimmunoprecipitation of Ste2-HA and Ste2-GFP, I performed reciprocal immunoprecipitation experiments. Antibodies against one epitope were used to precipitate receptors from the detergent extract, and then antibodies against the second epitope were used to test for the presence of the second tagged species in the immunoprecipitate. In Fig. 2B, anti-HA antibodies precipitated 11% of the Ste2-GFP

from the extracts of cell expressing both receptors (compare lanes 3 and 7), whereas no detectable Ste2-GFP was precipitated from the control cell extracts containing only Ste2-GFP or only Ste2-HA (lanes 1 and 2, respectively). Similar results were obtained when the cultures had been exposed to α-factor for 5 min (lanes 4 and 8, see also lanes 1 and 3) prior to the analysis. In the reciprocal experiment (Fig. 2C), anti-GFP antibodies precipitated about 6% of the Ste2-HA from the extracts of cell expressing both receptors (compare lanes 3 and 6), and no Ste2-HA was precipitated from either control extract (lanes 1 and 2). Again, prior exposure to α-factor for 5 min had no discernable effect. In both experiments, the antibody used for immunoprecipitation cleared all the antigen from the supernatant. Similar results were obtained using two other detergents, 1% TritonX-100 and 0.1% C12E8 suggesting that the interactions detected within the receptors are not specific to n-dodecyl β-D-maltoside (Fig. 3). To test whether the possibility that the oligomerization is induced at a later step, I performed immunoprecipitation experiments with cells that had been exposed to α-factor for up to 15 minutes. Receptors are normally depleted from the plasma membrane after 15 min under the conditions used (see Fig. 6A). Comparable amounts of Ste2-GFP coimmunoprecipitated with Ste2-HA from cells that had been treated with α-factor for 5, 10, 15 minutes (Fig. 4B). Similar results were obtained when anti-GFP antibodies were used and blots were probed with anti-HA antibodies (Fig. 4D). In both experiments, results from control blots indicated that equivalent amounts of Ste2-HA and Ste2-GFP precipitated with anti-HA and anti-GFP antibodies, respectively (Fig. 4A and 4C). Therefore, when the efficiency of coimmunoprecipitation of differentially-tagged receptors was used as a measure, the extent of receptor oligomerization was unchanged when the cultures had been exposed to α-factor for 15 minutes before analysis (Fig. 4).

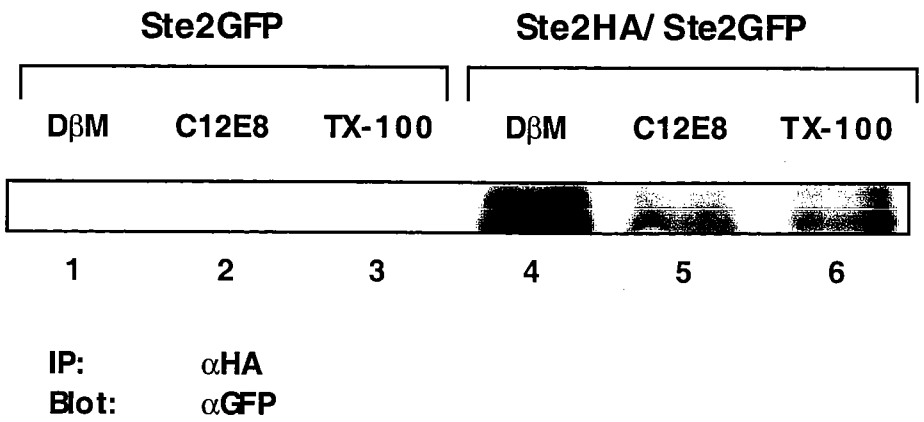


Figure 3: Coimmunoprecipitation of differentially tagged receptors in three different non-ionic detergents. Cells expressing one or both forms of the tagged receptor were analyzed. Membrane proteins extracted with 0.2% DβM (lanes 1 and 4), 0.1% C12E8 (lanes 2 and 5) or 1% Triton X-100 (lanes 3 and 6) were subjected to immunoprecipitation by using the anti-HA antibody. The coimmunoprecipitated protein was analyzed by using immunoblotting methods with the anti-GFP antibody from strains that expressed only Ste2-GFP (lanes 1-3) or both Ste2-HA and Ste2-GFP (lanes 4-6). Strains were DJ1404-A (Ste2-GFP) and DJ1414-A (Ste2-HA/Ste2-GFP).

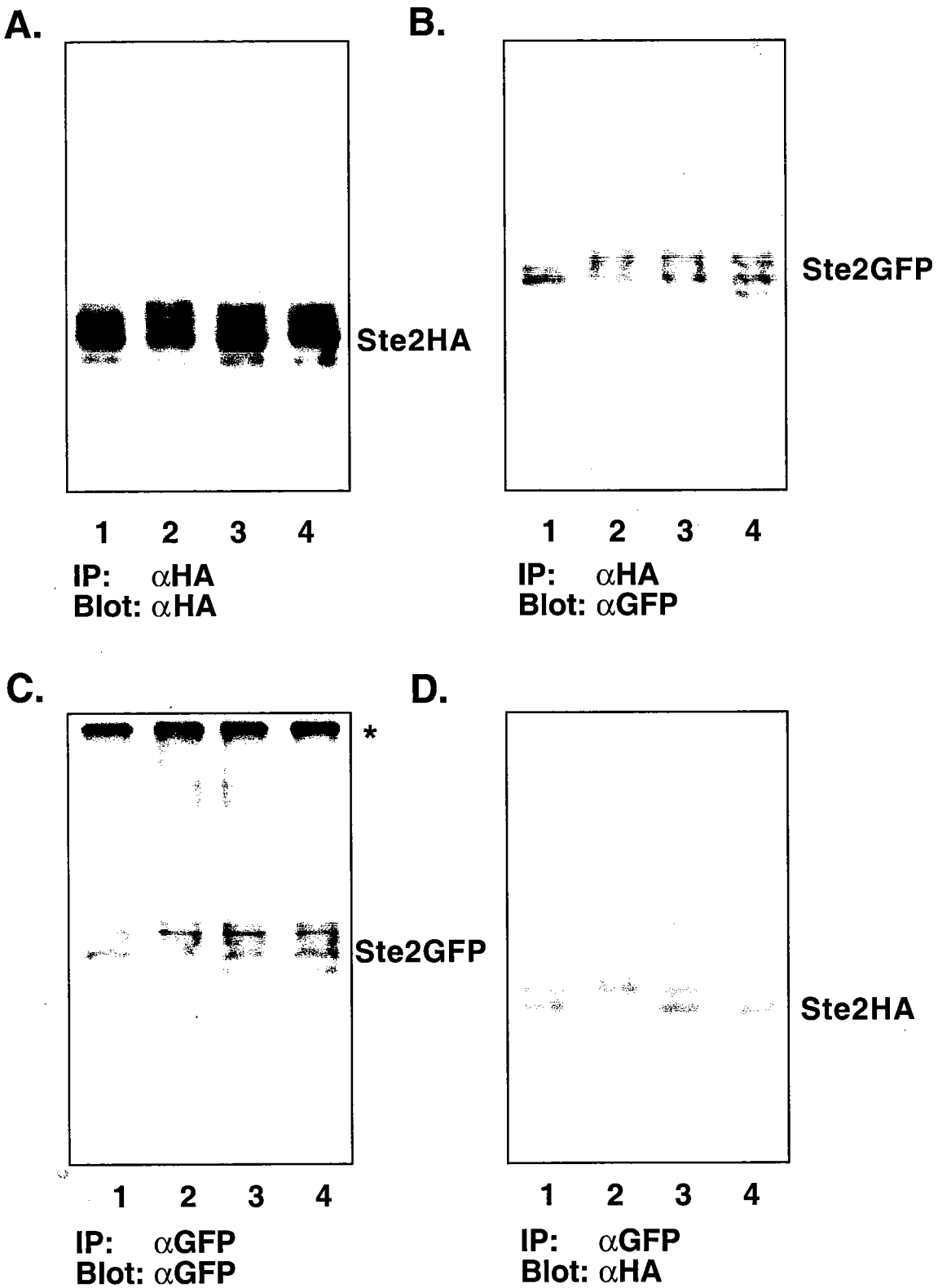

A.
B.
Ste2HA
Ste2GFP
1 2 3 4
IP: αHA
Blot: αHA
1 2 3 4
IP: αHA
Blot: αGFP
C.
D.
*
Ste2GFP
Ste2HA
1 2 3 4
IP: αGFP
Blot: αGFP
1 2 3 4
IP: αGFP
Blot: αHA

Figure 4. α-Factor treatment up to 15 minutes does not affect the levels of receptor oligomerization. Cultures of cells that expressed two tagged forms of the receptor (Ste2-HA and Ste2-GFP) were untreated (lane 1 in all four panels) or treated with 10^{-7} M α–factor for 5, 10 or 15 min (lanes 2, 3 and 4, respectively, for all panels). Membrane proteins that had been extracted with n-dodecyl β-D-maltoside were subjected to immunoprecipitation and then analyzed by using immunoblotting methods. The antibodies used for precipitation and for immunoblotting are denoted below each panel ("IP" and "Probe", respectively). **(A)** Anti-HA antibody precipitates equivalent amounts of Ste2-HA at each time point. **(B)** Anti-HA antibody precipitates equivalent amounts of Ste2-GFP at each time point. **(C)** Anti-GFP antibody precipitates equivalent amounts of Ste2-GFP at each time point. The asterisk shows the aggregates of Ste2-GFP that are typically detected upon precipitation of this protein with the polyclonal anti-GFP antibody. **(D)** Anti-GFP antibody precipitates equivalent amounts of Ste2-HA at each time point. The strain was DJ1414-A.

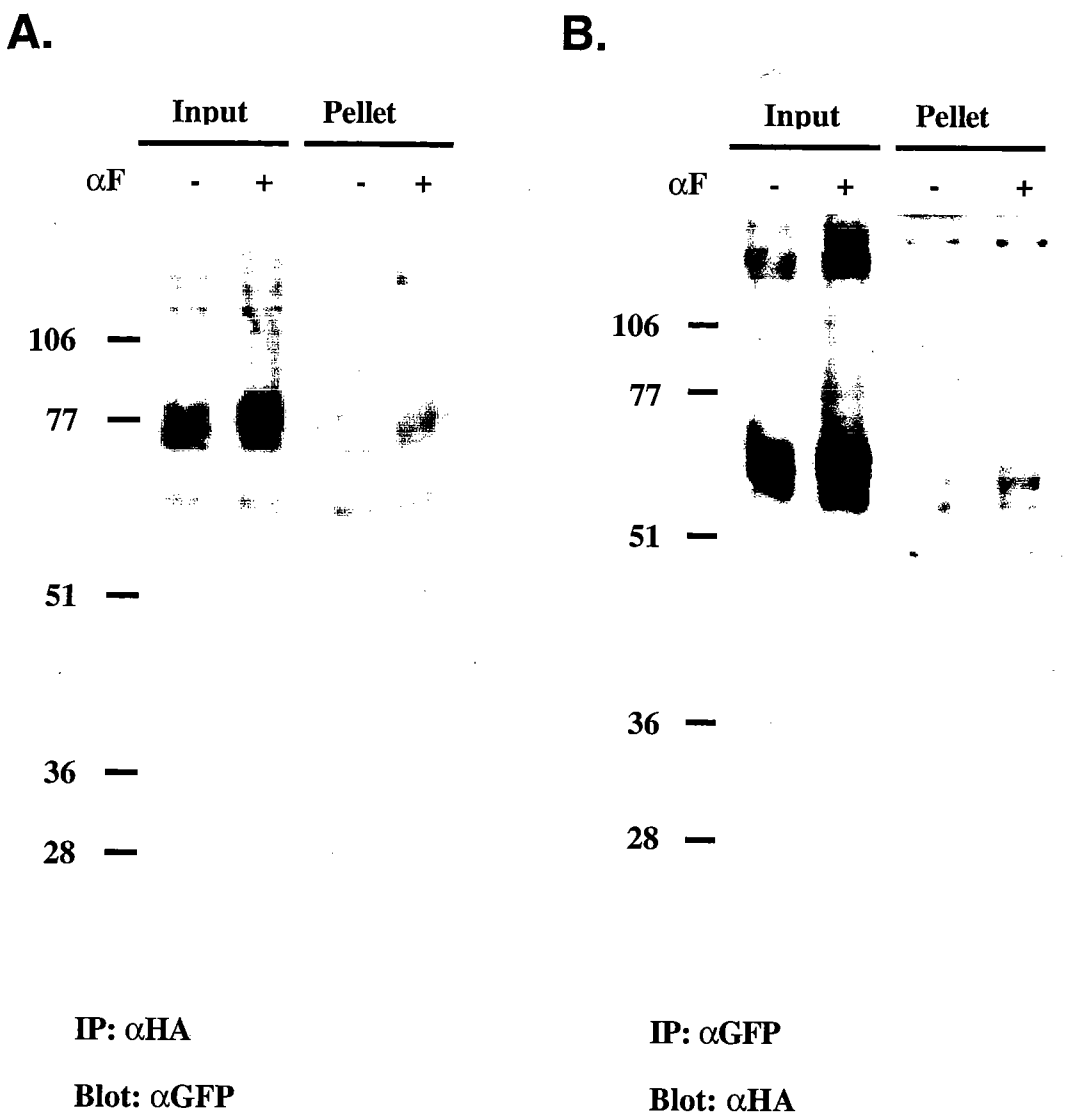


Figure 5. Immunoprecipitation of differentially-tagged receptors from glycerol gradients. The fractions 7 and 8 from the glycerol gradients in Fig. 1 were pooled, subjected to immunoprecipitation and then analyzed by using immunoblotting methods as in Fig. 2. **(A)** Anti-HA antibody precipitates Ste2-GFP from these fractions. **(B)** Anti-GFP antibody precipitates Ste2-HA. The strain was DJ1414-A.

Coprecipitation of Ste2-HA and Ste2-GFP was not a consequence of incomplete membrane solubilization or nonspecific aggregation of membrane proteins. The coprecipitated Ste2-HA and Ste2-GFP were apparently part of the 8S complex since essentially all of Ste2-HA and Ste2-GFP extracted under these conditions sediments approximately as an 8S species (Fig. 1A). Moreover when the 8S peak in Fig. 1A was pooled and analyzed according to the reciprocal immunoprecipitation method, about 5% of Ste2-GFP was precipitated with anti-HA antibody and 5% of Ste2-HA was precipitated with anti-GFP (Fig. 5). In addition, the protein complexes containing Ste2 do not appear to result from nonspecific aggregation of membrane proteins since the more abundant transmembrane protein, plasma membrane ATPase (Pma1) was not found in immunoprecipitates containing Ste2-HA (Fig. 2E).

I considered the possibility that receptor complexes that I observed had been formed only after the proteins were extracted from the membrane with detergent. To test this possibility, I mixed cells that only expressed Ste2HA with cells that only expressed Ste2-GFP and then processed the mixture for immunoprecipitation as above (Fig. 2D). Ste2-GFP and Ste2-HA coprecipitated only when both receptors were expressed in the same cells (Fig. 2D, lane 3) but not when the two cultures expressing Ste2-HA and Ste2-GFP receptors were mixed (Fig. 2D, lane 4). Similar results were obtained when the cultures had been treated with α-factor for 5 min. Therefore α-factor receptors are present in the plasma membrane as complexes containing two or more receptor molecules. Failure to detect any changes in the complexes induced by α-factor pretreatment does not rule out the possibility that α-factor influences higher order states of aggregation that were not stable in the solvent conditions used.

Ste2-S184R is internalized with the wild-type receptors upon α-factor exposure

Early work with α-factor receptor endocytosis suggested that receptors are internalized as oligomeric units (Jenness and Spatrick, 1986). When yeast cells are exposed to subsaturating concentrations of α-factor, the rate at which α-factor-receptor sites are lost from the plasma membrane is greater than the rate of α-factor uptake. This observation suggests that unoccupied receptors are internalized together with the occupied receptors. Three explanations (not mutually exclusive) account for these phenomena: (i) binding of α-factor may be necessary only to initiate the events that lead to the internalization, i. e., receptor internalization may proceed even after α-factor dissociates, (ii) the invaginations of the plasma membrane that occur during endocytosis may be large enough to include both occupied receptors and neighboring unoccupied receptors, (iii) the occupied and unoccupied receptors may exist as oligomeric units that remain coupled during endocytosis. To determine whether α-factor binding is required to initiate receptor internalization, wild-type Ste2 and the α-factor-binding-defective mutant, Ste2-S184R were coexpressed. In the presence of α-factor, these two receptors represent occupied and unoccupied receptors, respectively. If the internalization of the unoccupied receptors requires prior occupancy, then endocytosis of Ste2-S184R should not be induced by α-factor since it does not bind α-factor. Conversely, if internalization of unoccupied receptors reflects cointernalization, then Ste2-S184R internalization should be stimulated by α-factor.

Membrane fractionation was used to evaluate ligand-induced exit of Ste2-S184R from the plasma membrane in the presence and in the absence of the wild-type receptor. Strains that coexpress both wild-type Ste2 and mutant Ste2-S184R receptors were created.

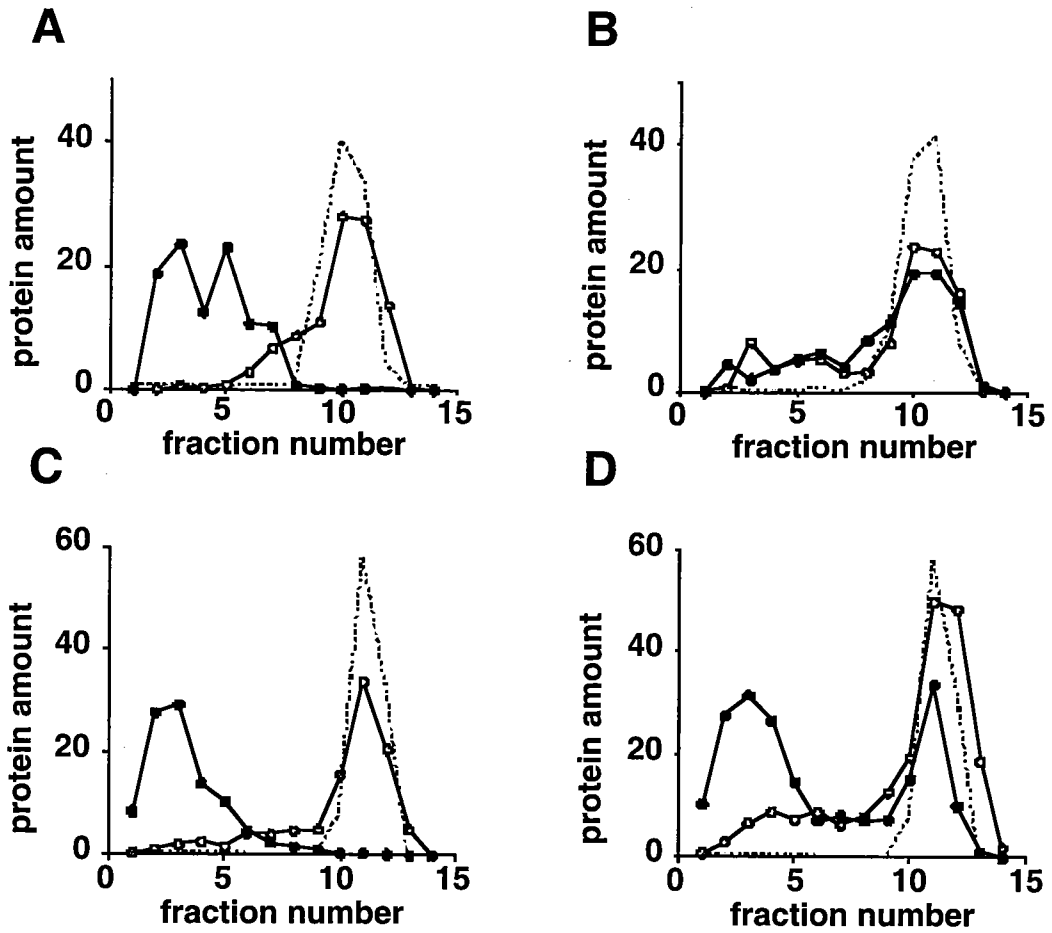


Figure 6. Binding-defective receptors, Ste2-S184R, undergo ligand-induced endocytosis when expressed with wild-type receptors. Each of the four strains analyzed expressed an HA-tagged receptor (Ste2-HA or Ste2-S184R-HA) encoded by a chromosomal allele and an untagged plasmid-encoded receptor (Ste2 or Ste2-S184R). Log-phase cultures in -Ura+CAA medium were treated with cycloheximide and then cultured for 15 min in the absence (○) or presence of α-factor (●). Membranes were fractionated by using Renografin density gradients. Fractions were assayed for HA-tagged receptors (○,●) and for plasma membrane ATPase (no plot symbol) by using immunoblotting methods. Plasma membrane marker shown for α-factor-treated cultures only. Protein amount is the

percentage of the total protein. **(A)** Cells expressing Ste2-HA and Ste2 (strain DJ1419-A). **(B)** Cells expressing Ste2-S184R-HA and Ste2-S184R (strain DJ1418-A). **(C)** Cells expressing Ste2-HA and Ste2-S184R (strain DJ1403-A). **(D)** Cells expressing Ste2-S184R-HA and Ste2 (strain DJ1402-A).

Genes encoding both receptors were present in a single copy and contained the native *STE2* promoter. In each experiment, the chromosomal allele directed synthesis of HA-tagged receptors, and a plasmid-born allele directed synthesis of untagged receptors. Exponentially growing cultures were treated with cycloheximide to block new receptor synthesis, incubated further either in the presence or in the absence of α-factor, and then after 15 min, the membranes were resolved on Renografin density gradients. Since essentially all of the receptors were on the cell surface prior to treatment (not shown), receptors detected in internal membrane fractions represent molecules that have exited the plasma membrane. As shown previously (Schandel and Jenness, 1994), the cells that expressed only wild-type receptors internalized essentially all their receptors in response to α-factor, as indicated by a shift of the Ste2 protein from the denser plasma membrane fractions to the more buoyant internal membrane fractions (Fig. 6A). In contrast, cells expressing only Ste2-S184R showed no α-factor-induced internalization (Fig. 6B). Upon α-factor exposure, a significant fraction of the tagged Ste2-S184R receptors were internalized in cells containing untagged wild-type receptors (Fig. 6D). This observation suggests that internalization of unoccupied receptors is not a consequence of prior α-factor occupancy since Ste2-S184R receptors do not bind α-factor. In the reciprocal experiment, internalization of tagged wild-type receptors was not influenced by the presence of the mutant Ste2-S184R receptors (Fig. 6C).

As a second assay for endocytosis of occupied and unoccupied receptors, I used fluorescence microscopy to monitor wild-type and mutant receptors that had been tagged with green fluorescent protein, GFP. Ste2-S184R-GFP was coexpressed with untagged wild-type receptors to test whether internalization of the wild-type receptors would cause the internalization of the Ste2-S184R-GFP receptors. As in Fig. 6, cultures that had been

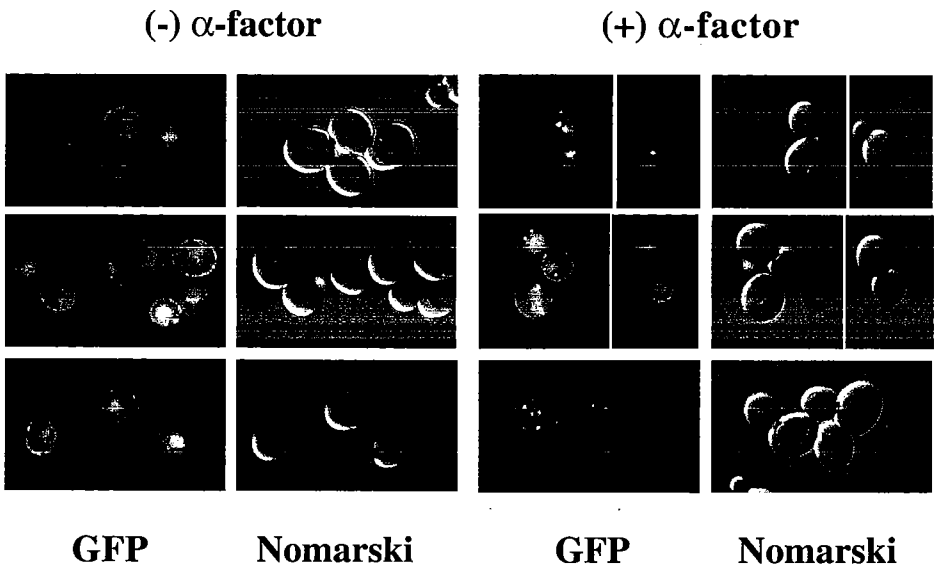


Figure 7. Ligand-induced internalization of GFP-tagged binding-defective receptors depends on the presence of wild-type receptors. Cultures were treated with cycloheximide and α-factor as described in Fig. 3. First two columns are controls lacking α-factor; the last two columns are α-factor-treated cells. GFP fluorescence images and Nomarski images are indicated below each column. Top row, cells expressing Ste2-GFP only (strain DJ1408-A). Middle row, cells expressing Ste2-S184R-GFP only (strain DJ1407-A). Bottom row, cells expressing both Ste2-S184R-GFP and untagged Ste2 (strain DJ1406-A).

TABLE 1. Quantitation of fluorescent foci from cells expressing Ste2 and Ste2-S184R

		percentage of cells[b]	
Ste2 forms expressed[a]	α-factor	n≥1	n≥3
Ste2-GFP	-	31 ± 3	7 ± 2
Ste2-GFP	+	94 ± 2	44 ± 3
Ste2-S184R-GFP	-	31 ± 3	6 ± 1
Ste2-S184R-GFP	+	57 ± 3	7 ± 2
Ste2-S184R-GFP and Ste2	-	33 ± 3	7 ± 2
Ste2-S184R-GFP and Ste2	+	72 ± 2	32 ± 2

[a] The forms of Ste2 expressed in each strain are shown. Strains used are, DJ1408-A (rows1, 2), DJ1407-A (rows 3, 4), DJ1406-A (rows 5, 6).

[b] Fluorescent foci were counted from GFP images of cells treated with cycloheximide and α-factor as in Fig. 3. The percentage of cells with one or more fluorescent foci (n≥1) and the percentage of cells with three or more fluorescent foci (n≥3) are indicated for the cells that had been cultured in the presence or absence of α-factor. More than 200 cells were examined for each entry.

treated with cycloheximide were challenged with α-factor. In the absence of α-factor, the cells containing GFP-tagged mutant and wild-type receptors exhibited fluorescence both at the plasma membrane and in the vacuole. Previous results (Li et al., 1999) indicate fluorescence in the vacuole reflects the free GFP that remains after the Ste2-GFP fusion protein has been endocytosed and the Ste2 portion of the protein degraded. In presence of α-factor, the wild-type Ste2-GFP was completely removed from the plasma membrane and appeared as punctate structures presumably corresponding to endocytic vesicles (Fig. 7, top row), whereas cells expressing only the Ste2-S184R mutant receptors showed very little internalization of cell-surface fluorescence (Fig. 7 middle row). In contrast, in cells expressing both Ste2-S184R-GFP with untagged Ste2, the plasma membrane fluorescence was significantly reduced and greater proportion of the fluorescence appeared in internal punctate structures (Fig. 7 bottom row). When analyzed quantitatively, 32% of the cells expressing both Ste2-S184R-GFP and Ste2 showed three or more fluorescent foci after α-factor treatment, whereas only 7% of the cells expressing Ste2-S184R-GFP alone showed more than three fluorescent foci (Table 1). In addition, coprecipitation of Ste2 and Ste2-S184R-GFP was observed (Fig. 10A). These observations are consistent with our results from the Renografin gradients indicating that Ste2-S184R receptors are internalized with the wild-type receptors in the presence of α-factor.

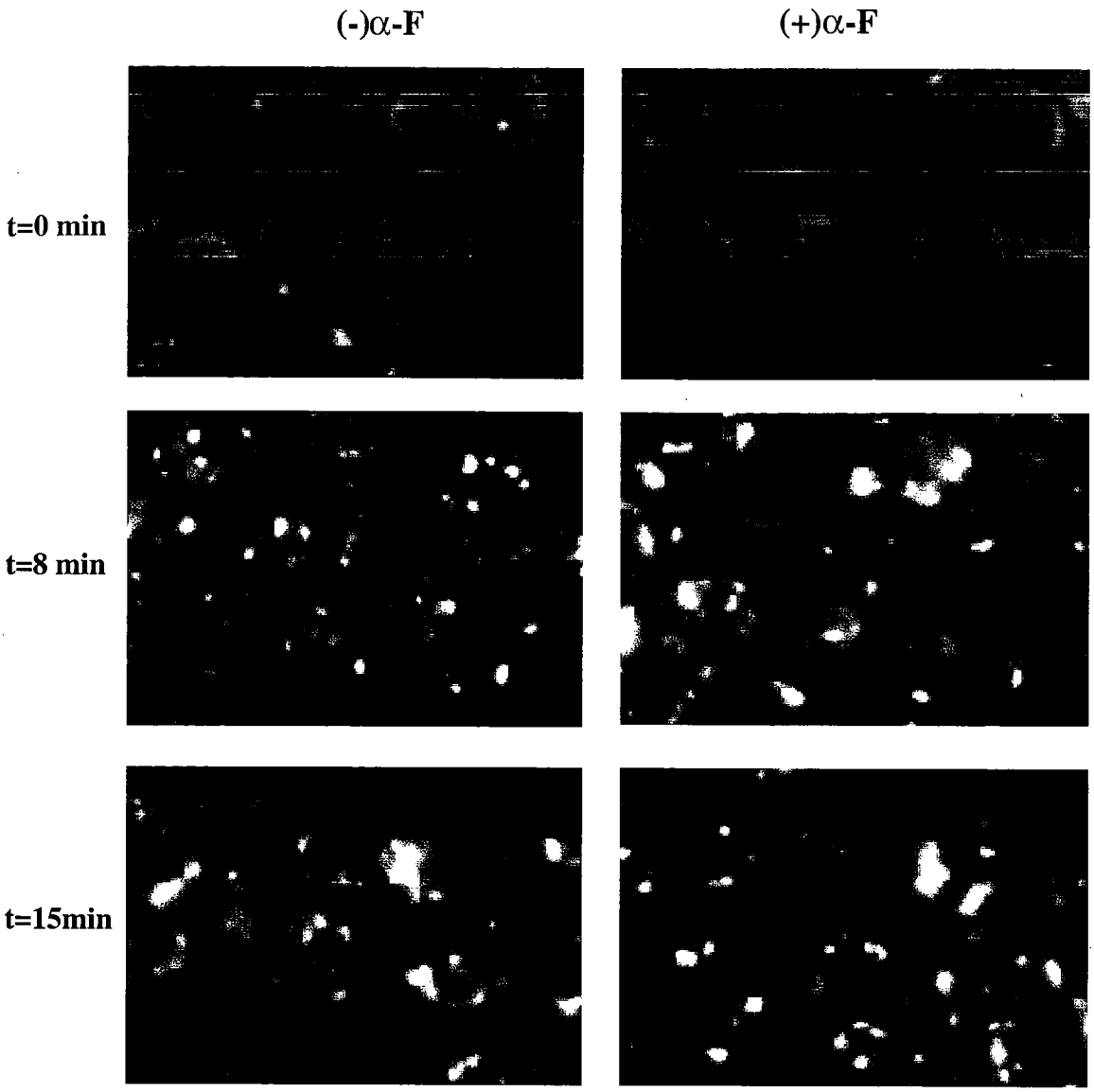
Two criteria were used to judge whether endocytosis of occupied receptors results in the internalization of a significant portion of surrounding plasma membrane and membrane proteins. First, essentially all of the abundant plasma membrane protein ATPase, Pma1, remained at the plasma membrane when the cells containing wild-type receptors were treated with α-factor (Fig. 6). Second, the bulk endocytosis of plasma membranes marked with the vital stain FM4-64 (Vida and Emr, 1995) was not influenced by α-factor (Fig. 8). At 8 min

FM4-64 was completely internalized in the presence and in the absence of α-factor (Fig. 8A, middle row) whereas for the same duration with α-factor, only about 50% of the α-factor receptors are depleted from the plasma membrane (Schandel and Jenness, 1994). Therefore, the rate of uptake of the dye is faster than the rate of internalization of the receptors. Similar results were obtained at 15 min time point (Fig. 8A, bottom row). I then wished to investigate the uptake of FM4-64 at earlier time points. A significant portion of the FM4-64 was internalized after only 2 min, however, the staining pattern was indistinguishable for the cells that were untreated or treated with α-factor (Fig. 8B). These results suggest that the endocytosis of unoccupied receptors does not simply reflect an increased rate of plasma membrane internalization in response to α-factor, and they are consistent with the hypothesis that unoccupied receptors are endocytosed because they form oligomeric complexes with occupied receptors. Immunoprecipitation experiments confirmed biochemically the formation of oligomers between wild-type and mutant receptors. When anti-GFP antibodies were used to precipitate Ste2-S184R-GFP receptors from cells coexpressing both mutant and wild-type receptors, Ste2 antisera against the carboxy terminus of the receptor detected the untagged wild-type receptors in the immunoprecipitate pellet.

Wild-type Ste2 causes internalization of endocytosis-defective receptors in the presence of α-factor

The C-terminal cytoplasmic tail of Ste2 contains sequence elements that are essential for both basal and ligand-induced endocytosis (Rohrer et al., 1993; Schandel and Jenness, 1994). The truncated receptor, Ste2-T326, binds α-factor normally, even though it lacks most of the C-terminal tail (Konopka et al., 1988). This domain contains the

A.



B.

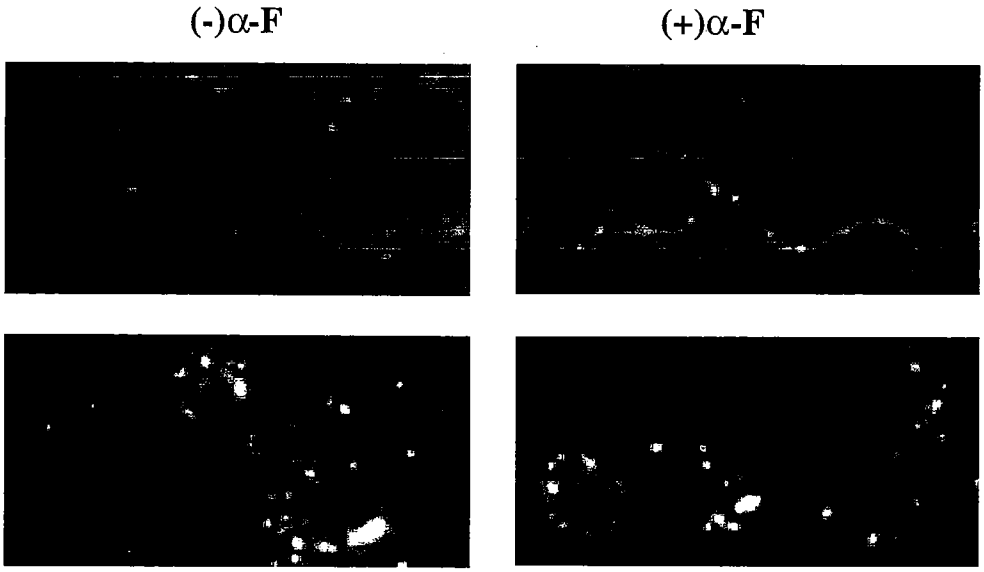


Figure 8. The dynamics of the lipophyllic dye uptake in α-factor-treated and untreated cells. The membrane permeable dye FM4-64 was simultaneously added to cell cultures with or without 10^{-7} M α–factor (left and right columns, respectively). The reactions were immediately placed on ice. Aliquots were taken out and incubated at room temperature for the durations indicated on the left and placed on ice with metabolic poisons. Then the samples were analyzed by fluorescence microscopy. **(A)** The dye uptake of cells that had been untreated or treated with α-factor for 0, 8 and 15 minutes. **(B)** The dye uptake of cells that had been untreated or treated with α-factor for 0 and 2 minutes. Strain was DJ1406-A.

well-characterized endocytosis motif (DAKSS) (Rohrer et al., 1993). My next goal was to determine whether the severe endocytosis defect associated with this mutant receptor could be overcome by forming oligomers with wild-type receptors. Coimmunoprecipitation of Ste2-HA and Ste2-T326 was observed when detergent extracts were analyzed according to the methods depicted in Fig. 1. (Fig. 10B, lane 1). Similar results were obtained when experiments were conducted with cells that had been exposed to α–factor for 5 minutes prior to analysis (Fig. 10B, lane 3). Renografin density gradients and fluorescence microscopy were used to monitor endocytosis of Ste2-T326 tagged with GFP. Table 2 summarizes α-factor-induced internalization of receptors, as judged by Renografin density gradients. Consistent with previous results (Schandel and Jenness, 1994), Ste2-T326-GFP levels increased in intracellular membranes due to increased protein synthesis. In Table 2 and in the previous study (Schandel and Jenness, 1994), a small number of the truncated receptors (17.6%) cofractionated with the internal membranes, and this quantity decreased slightly (to 13.5%) in the cells that had been exposed to α-factor. However, under these conditions, α-factor resulted in a nearly five-fold increase in the accumulation of Ste2-T326-GFP in the internal membrane fraction when Ste2-T326-GFP and wild-type Ste2 were coexpressed. This result is consistent with the endocytosis of oligomeric complexes containing truncated and wild-type receptors. It is currently unclear why some of the truncated receptors accumulate in the internal membrane pool, and why this quantity decreases when truncated and wild-type receptors are coexpressed. As has been proposed for CCR5 receptors (Benkirane et al., 1997), it is possible that the truncated α-factor receptors are partially retained in the ER and that the defect is overcome by forming oligomers with the wild-type receptors.

TABLE 2. Wild-type receptors cause truncated receptors to be internalized in presence of α-factor

		Percentage of receptors in internal membranes[b]	
Strain	Ste2 forms expressed[a]	(-) α-F	(+) α-F
DJ1413-A	**Ste2**	9.7 ± 2.2	86.2 ± 2.7
DJ1410-A	**Ste2-T326**	17.6 ± 6.7	13.5 ± 5.7
DJ1411-A	**Ste2-T326** and Ste2	4.7 ± 2.1	23 ± 2.1
DJ1411-A	Ste2-T326 and **Ste2**	24.7 ± 10.7	73.2 ± 8.1

[a] The Ste2 forms expressed in each strain are shown. For each entry, the Ste2 form that was assayed is marked in bold-type and underlined.

[b] Cells were processed as in Fig. 3. "The percentage of receptors in internal membranes" is the amount of receptor detected in Renografin gradient fractions 1–7 divided by the total amount of receptor assayed. Entries are the mean ± standard error for three independent experiments.

Internalization of Ste2-T326-GFP was also monitored by using fluorescence microscopy. In the control cells exposed to α-factor, wild-type Ste2-GFP was depleted from the plasma membrane (Fig. 9, top row), and all of the cells examined contained fluorescent foci (Table 3). When expressed alone, the vast majority of Ste2-T326-GFP was at the cell surface at all times, consistent with the endocytosis defect of this mutant (Fig. 9, middle row, and Table 3). However, when cells coexpressing both Ste2-T326-GFP and untagged wild-type receptors were exposed to α-factor, plasma membrane fluorescence was diminished and was accompanied by intracellular accumulation of fluorescent foci (Fig. 9, bottom row, and Table 3). A significant portion of the truncated receptor was internalized after addition of α-factor and appeared in punctate structures resembling endosomes (Fig. 2, bottom row). These results are in agreement with the Renografin density gradient experiments (Table 2) and suggest that oligomeric complexes containing internalization-defective truncated receptors and wild-type receptors are internalized in an α-factor-dependent fashion with high efficiency.

The C-terminal cytoplasmic tail and cysteine residues of Ste2 are not required for oligomerization

Coimmunoprecipitation experiments were used to test whether specific structural features of the receptor play an essential role in the formation of oligomers. To test whether the C-terminal cytoplasmic tail of the receptor is dispensable for oligomerization, I coexpressed two truncated forms of the receptor that were tagged differentially and performed coimmunoprecipitation tests on the detergent-solubilized receptors as described in Fig. 1. Coprecipitation of Ste2-326-GFP and Ste2-326-HA was observed when the precipitating antibodies were either anti-GFP or anti-HA (Fig. 11). This result suggests that

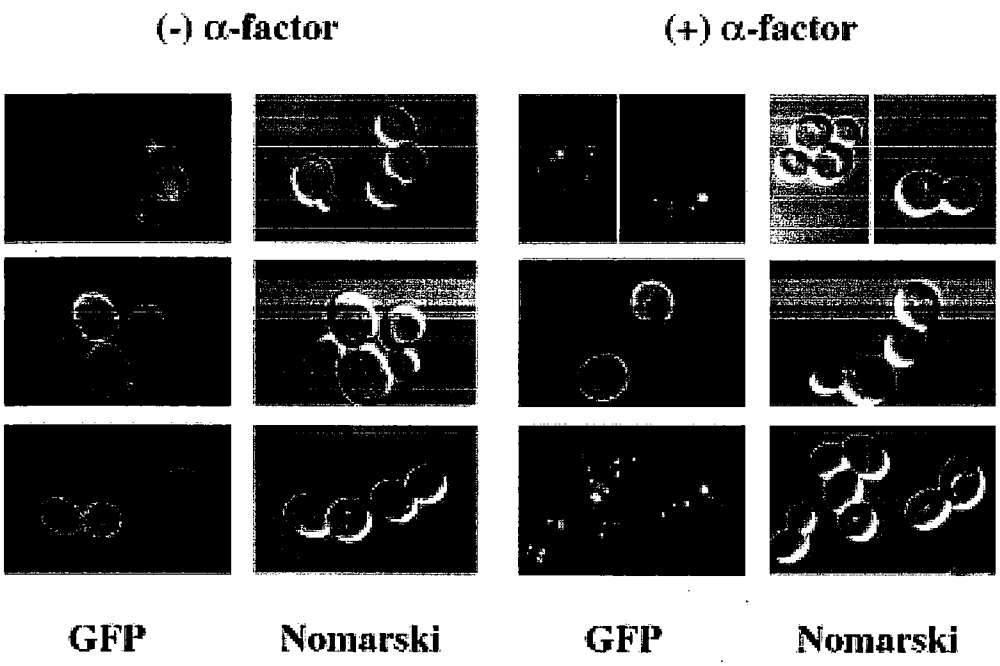


Figure 9. Ligand-induced internalization of GFP-tagged endocytosis-defective receptors depends on the presence of wild-type receptors. Cultures were treated with cycloheximide and α-factor as described in Fig. 3. First two columns are controls lacking α-factor; the last two columns are α-factor-treated cells. GFP fluorescence images and Nomarski images are indicated below each column. Top row, cells expressing Ste2-GFP only (strain DJ1413-A). Middle row, cells expressing Ste2-T326-GFP only (strain DJ1410-A). Bottom row, cells expressing both Ste2-T326-GFP and untagged Ste2 (strain DJ1411-A).

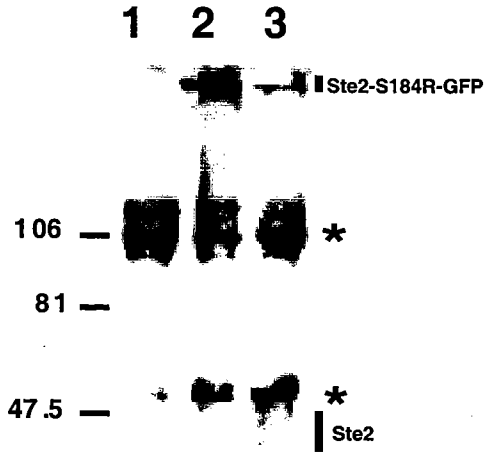
TABLE 3. Quantitation of fluorescent foci from cells expressing Ste2 and Ste2-T326

		percentage of cells[b]	
Ste2 forms expressed[a]	α-F	n≥1	n≥3
Ste2-GFP	-	42 ± 9	9 ± 5
Ste2-GFP	+	100	71 ± 11
Ste2-T326-GFP	-	5 ± 3	5 ± 3
Ste2-T326-GFP	+	5 ± 3	2 ± 2
Ste2-T326-GFP and Ste2	-	10 ± 5	7 ± 5
Ste2-T326-GFP and Ste2	+	74 ± 4	36 ± 5

[a] The forms of Ste2 expressed in each strain are shown on the leftmost column. Strains used are, DJ1413-A (rows 1, 2), DJ1410-A (rows 3, 4), DJ1411-A (rows 5, 6).

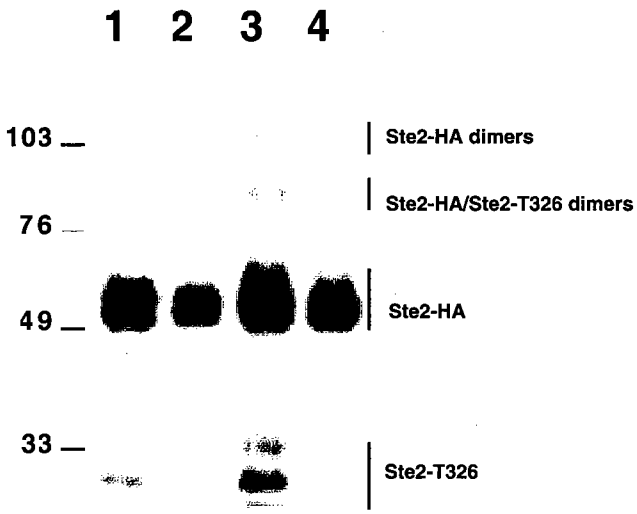
[b] Fluorescent foci were counted from GFP images of cells treated with cycloheximide and α-factor as in Fig. 3. The percentage of cells with one or more fluorescent foci (n≥1) and the percentage of cells with three or more fluorescent foci (n≥3) are shown in the presence or absence of α-factor.

A.



IP: α-GFP
Blot: α-Ste2-C'

B.



IP: α-HA
Blot: α-Ste2-N'

Figure 10. Immunoprecipitation of mutant and wild-type receptors. Cells coexpressed mutant and wild-type receptors. Membrane proteins extracted with n-dodecyl β-D-maltoside were subjected to immunoprecipitation and then analyzed by using immunoblotting methods. **(A)** Anti-GFP antibody precipitates Ste2-S184R-GFP and Ste2. Cells coexpressing α–factor-binding defective receptors Ste2-S184R-GFP with untagged wild-type receptors were analyzed. Anti-GFP antibody was used for precipitation and blots were probed with antisera against the carboxy terminus of Ste2. Cells expressing Ste2 (lane 1). Cells expressing Ste2-S184R-GFP (lane 2). Cells expressing Ste2-S184R-GFP and Ste2 (lane 3). Strains were DJ211-5-3 (Ste2), DJ1405-A (Ste2-S184R-GFP) and DJ1406-A (Ste2-S184R-GFP/Ste2). **(B)** Anti-HA antibody precipitates Ste2-HA and Ste2-T326. Cells coexpressing truncated receptors Ste2-T326 with HA-tagged wild-type receptors were analyzed. Anti-HA antibody was used for precipitation and blots were probed with antisera against the amino terminus of Ste2. Strain expressing Ste2-HA and Ste2-T326 was DJ1415-A (lanes 1 and 3). Strain expressing Ste2-HA was DJ1400-A (lane 2 and 4). The asterisks indicate the positions of the precipitating antibodies.

most of the C-terminal cytoplasmic tail of the receptor is not required on either partner for oligomerization. This extent of precipitation was unaltered when the cells were cultured for 5 min in α-factor before analysis. The Ste2-T326 contains 29 amino acids of the N-terminal region of the cytoplasmic tail. To rule out a possible interaction between these 29 residues, a mutant receptor Ste2-Δ(297-391)-GFP which lacks the 94 residues from the N-terminal region of the tail including the 29 amino acids that were present in Ste2-T326 was coexpressed with untagged wild-type receptors. Ste2-Δ(297-391)-GFP has a severe defect in both constitutive and ligand-mediated endocytosis (see Chapter V and Fig. 12, middle row). The mutant receptors remained on the plasma membrane in the absence and in the presence of α-factor (Fig. 12 middle row), whereas the wild-type receptors became completely endocytosed upon α–factor treatment (Fig. 12 top row). When cells coexpressing Ste2-Δ(297-391)-GFP and wild-type receptors were exposed to α-factor, cell surface fluorescence diminished and GFP-tagged mutant receptors appeared in endocytic vesicles (Fig. 12 bottom row). This result suggests that the entire cytoplasmic tail of the receptor is unnecessary for oligomerization.

Another consideration was whether either of the two cysteine residues in the receptor was essential for oligomer formation. The Ca^{2+}-sensing and metabotropic glutamate receptors have been proposed to dimerize through disulfide bonds (Bai et al., 1998; Romano et al., 1996). Two differentially-tagged forms of the receptor were coexpressed under the direction of the strong TDH3 promoter. Plasmid pNED1(-Cys) encodes a mutant form of the receptor (Ste2-C59S, C252A-Flag-His6) that lacks both cysteines and contains the Flag epitope, and this plasmid was introduced into strain 440-A that directs synthesis of wild-type receptors containing the T7 epitope. Ste2-C59S, C252A-Flag-His6 was precipitated with the anti-T7 antibodies (Fig. 13), indicating the presence of oligomers even though one of the receptors lacked cysteine residues. Therefore

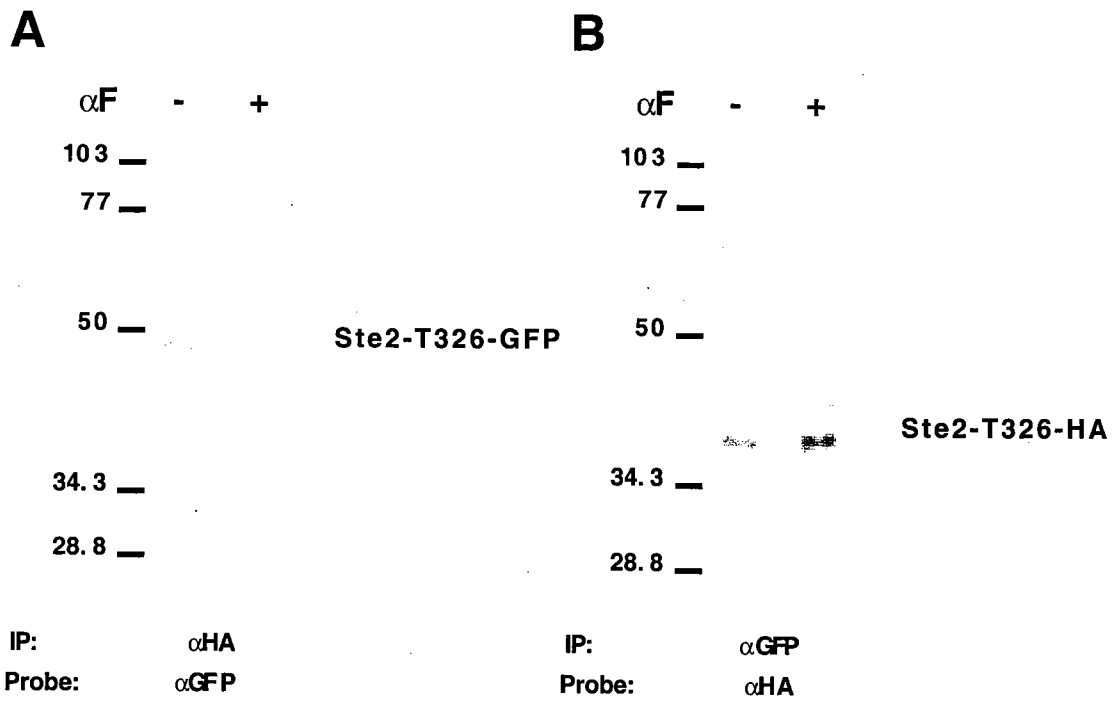


Figure 11. C-terminal tail of Ste2 is not required for coprecipitation. Cells expressing both Ste2-T326-HA and Ste2-T326-GFP (strain DJ1417-A) were treated with α-factor and processed for immunoprecipitation as in Fig. 2B. **(A)** Anti-HA antibody precipitates Ste2-T236-GFP. **(B)** Anti-GFP antibody precipitates Ste2-T326-HA.

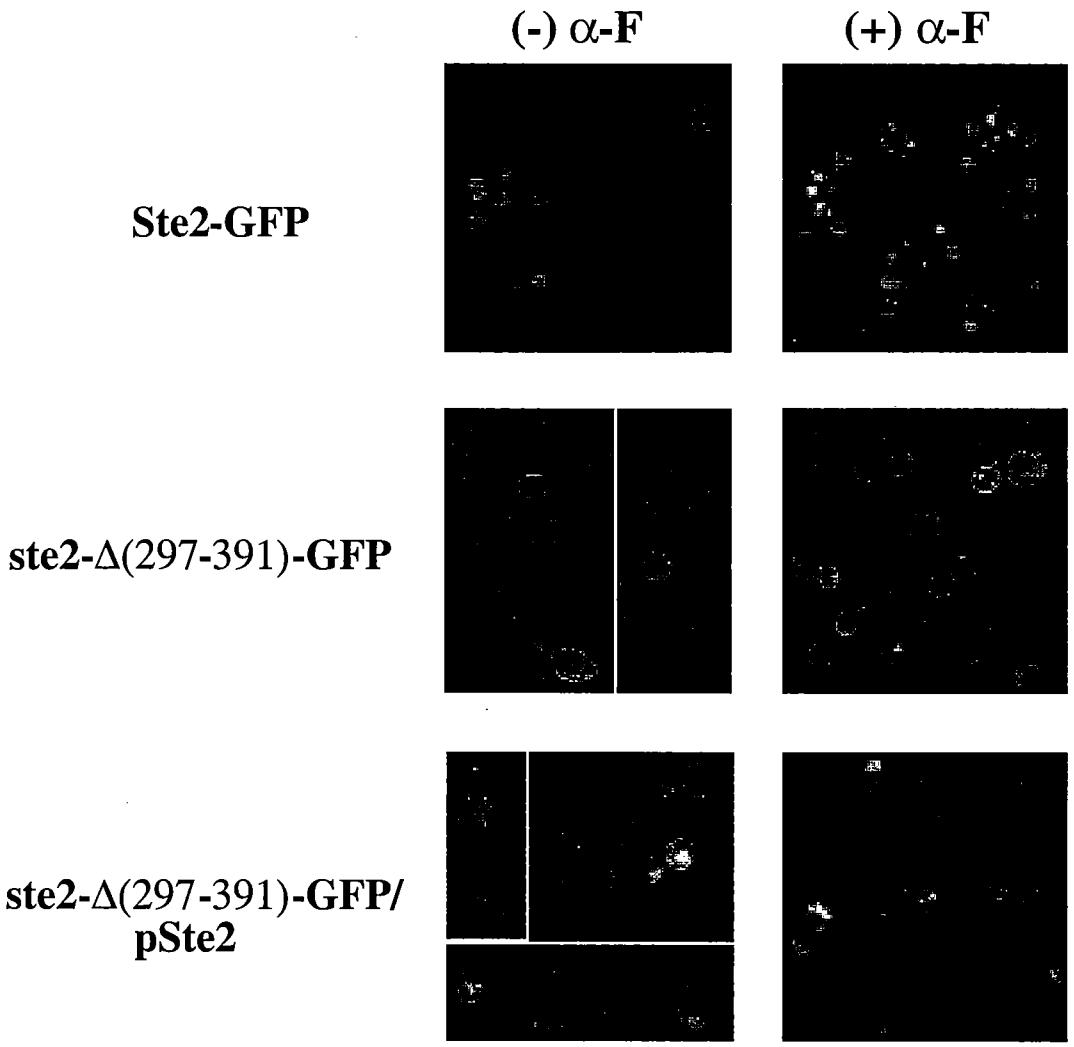


Figure 12. Ligand-induced internalization of other GFP-tagged endocytosis-defective receptors also depends on the presence of wild-type receptors. Cultures were treated with cycloheximide and α-factor as described in Fig. 3. The left column shows GFP fluorescence images of controls lacking α-factor; the right column shows the α-factor-treated cells. Top row, cells expressing Ste2-GFP only (strain DJ1494). Middle row, cells expressing Ste2-Δ(297-391)-GFP only (strain DJ1443). Bottom row, cells expressing both Ste2-Δ(297-391)-GFP and untagged Ste2 (strain DJ1445).

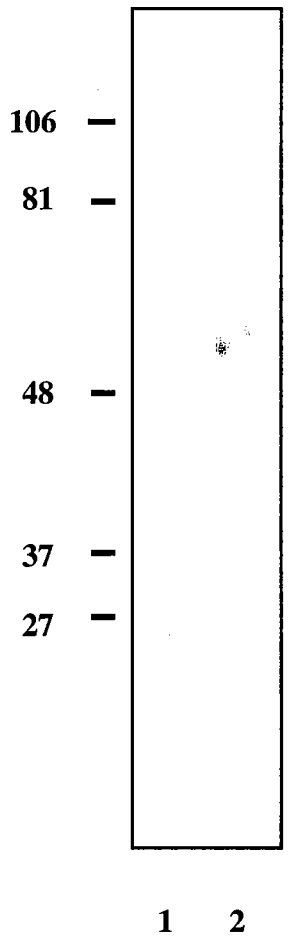


Figure 13. The two cysteines in Ste2 are not required for coprecipitation. Cells expressing only T7-Ste2 (lane 1) or both T7-Ste2 and Ste2-C59S, C252A-Flag-His6 (lane 2) were processed for immunoprecipitation as in Fig. 2B. Anti-T7 antibody was used for precipitation and blots were probed with anti-FLAG antibody. Strains were DJ440-A and DJ440-A/pNED1.

the C-terminal cytoplasmic domain and interchain disulfide bonds are unnecessary for the formation of receptor oligomers.

Discussion

In this chapter, I present evidence indicating that the α-factor receptors from *S. cerevisiae* form oligomeric complexes in the plasma membrane. Protein complexes containing the receptor were efficiently solubilized with the non-denaturing detergent, n-dodecyl β-D-maltoside, and, on glycerol density gradients, they sedimented as a monodisperse species with a sedimentation coefficient of about 8S. When the complexes containing differentially-tagged receptors were solubilized under these conditions and subjected to immunoprecipitation, both tagged species were precipitated with antibodies specific for either of the two tags. The efficiency of coprecipitation was not influenced by the presence of α-factor in the culture prior to extraction. Membrane fractionation and fluorescence microscopy indicated that oligomeric receptor complexes were subject to endocytosis and that unoccupied receptors could participate in these complexes. First, tagged mutant receptors, that lacked the SINNDAKSS endocytosis signal and were unable to undergo constitutive and ligand-induced endocytosis, became competent for endocytosis when they were coexpressed with untagged wild-type receptors. Second, unoccupied receptors were able to enter these endocytosis-competent complexes since tagged mutant receptors that were unable to bind α-factor also showed ligand-dependent endocytosis when they were coexpressed with untagged wild-type receptors. The complex formation between these receptors was confirmed biochemically since coimmunoprecipitation of mutant and wild-type receptors was observed.

A recently-published independent study also reports evidence for oligomerization of α-factor receptors [Overton, 2000 #67]. These authors used fluorescence resonance energy transfer between differentially-tagged receptors in whole cells as an indicator for

oligomerization. They also showed that tagged receptors lacking the SINNDAKSS endocytosis signal were endocytosed when coexpressed with wild-type receptors; however, they did not explore the endocytosis of unoccupied receptors, and they did not identify complexes in a membrane-free detergent-soluble system.

Little is known about the size and the structure of the oligomeric complexes that contain GPCRs. Although detergent-solubilized α-factor receptors (48 kDa) sedimented faster than the IgG marker protein (160 kDa), the extent to which detergent, shape, hydration and other proteins contribute to the sedimentation rate is not known. Two observations indicate that at least a portion of the α-factor receptor complexes contain more than one receptor molecule. First, the HA-tagged receptors sediment slightly faster than the GFP-tagged receptors even though the GFP-tagged receptors are larger, consistent with reduced stability of the GFP-tagged oligomers. Second, 6–11% coprecipitation of the differentially tagged receptors indicates that a minimum of 12–22% of these complexes is in oligomers (assuming two receptors per complex). This value is likely to be an underestimate since some complexes may disaggregate during analysis and since tighter-associating forms (i. e., Ste2-HA) may tend to reassociate into relatively stable homo-oligomers leaving the weaker interacting species (i. e., Ste2-GFP) to form less-stable oligomers. Based on the extensive cointernalization of mutant and wild-type receptors, it is likely that most of the receptors are in the oligomeric form *in vivo*. The structural determinants that bind α-factor receptors together are also unclear. Although metabotropic glutamate and Ca^{2+}-sensing receptors require the disulfide bonds of cysteines for oligomerization and δ-opioid receptors oligomerize through sequences in the C-terminal domain, I find that neither these structural features play an essential role in the oligomerization of α-factor receptors. Although transmembrane segment VI of β2

adrenergic receptors and D1 dopamine receptors are thought to play an essential role in aggregation, this possibility has not yet been explored for the α-factor receptor. These data do not exclude the possibility that a bridging protein mediates the association of receptor molecules.

Functional consequences of GPCR oligomerization are not well understood. The ability of one receptor to influence the activity of another receptor in the same oligomeric complex has been inferred from the cooperative binding of type-specific agonists to cells that coexpress δ- and κ-opioid receptors. Although the hypersensitivity phenotype of truncated α-factor receptors is reversed when they are coexpressed with wild-type receptors (Konopka et al., 1988; Reneke et al., 1988), this phenomenon is most readily explained by competition of the mutant and wild-type receptors for a common pool of G proteins (Dosil et al., 2000) rather than by direct aggregation of the two receptor forms. The functional consequences of disrupting GPCR oligomerization in vivo has been investigated by exposing cells to peptides corresponding to single transmembrane segments of the receptor. A peptide that comprises transmembrane VI of β2-adrenergic receptors inhibits both oligomerization and signal transduction activities of these receptors, suggesting that oligomerization may be essential for signaling (Hebert et al., 1996). However, the significance of this argument has recently been called into question since, for D1 dopamine receptors, a peptide containing transmembrane segment VI inhibits signaling without inhibiting oligomerization (George et al., 1998). It is possible that variations in the amount of oligomerized GPCRs mediate the response to agonists since the oligomeric state of some GPCRs is either increased or decreased by ligand binding (Cvejic and Devi, 1997; Hebert et al., 1996). For the δ-opioid receptor, the dimer-to-monomer transition has been associated with endocytosis since the natural agonists induce monomers prior to endocytosis and since

morphine does not induce receptor internalization and does not alter the oligomeric state (Cvejic and Devi, 1997). Disaggregation of α-factor receptors does not appear to control ligand-mediated endocytosis since the aggregation state is unaffected by the α-factor and since endocytosis-defective mutant receptors are internalized when they are associated with wild-type receptors. α-factor receptors provide a genetically-tractable model to study the role that oligomerization plays in the GPCR function.

CHAPTER IV

INTERACTIONS OF THE α-FACTOR PHEROMONE RECEPTOR WITH OTHER PROTEINS

G-protein-coupled receptors rely on the action of heterotrimeric G proteins to relay the extracellular signals to the cell interior. Receptor stimulation by the ligand results in a GDP/GTP exchange in the α subunit of the G protein, which is thought to cause the Gα to dissociate from the Gβγ heterodimer. Following activation, the Gα is thought to dissociate from Gβγ and the free Gα and Gβγ are then available to interact with the downstream effectors. There are four subfamilies of Gα subunits, which interact with different effectors in mammalian cells: G_s activates adenylyl cyclase, whereas G_i inhibits adenylyl cyclase and activates K^+ channels. $G_{q/11}$ regulates phospholipase C (Ostrom et al., 2000). Finally $G_{12/13}$ stimulates Bruton's tyrosine kinase and a ras GAP (Jiang et al., 1998; Naor et al., 2000). The Gβγ subunits have been shown to interact with protein serine/threonine kinase KSR-1 and PI3Kγ. (Naor et al., 2000).

In yeast, only one heterotrimeric G protein couples to the pheromone receptors (Ste2 and Ste3). The homologs of the mammalian α, β and γ subunits of the heterotrimeric G protein are encoded by *GPA1*, *STE4* and *STE18* genes, respectively. Another protein homologous to Gα subunit (*GPA2*) which is believed to associate with another yeast GPCR, Gpr1, has been implicated in pseudohyphal growth (Nakafuku et al., 1988; Pan and Heitman, 1999; Xue et al., 1998). Strains carrying a deletion of the GPA2 gene have no defects in the pheromone response pathway. For the pheromone receptors, the Gβγ subunit

interacts with the downstream effectors. The role of Gα is thought to be to maintain the heterotrimer in its inactive state. Upon activation, the free Gβγ heterodimer interacts with a scaffolding protein Ste5 that normally shuttles between the nucleus and the cytoplasm. This interaction causes the stable recruitment of Ste5 to the plasma membrane and brings three members of the mitogen-activated kinase family, Ste11, Ste7 and Fus3, bound to Ste5 in close contact with Ste20. Phosphorylation of Ste11 by Ste20 initiates that kinase cascade. When the GTP bound to Gα is hydrolyzed to GDP, Gα reassociates with the Gβγ rendering the complex inactive, thereby turning off the signal. The slow intrinsic GTPase activity of Gα is stimulated by the action of a regulator of G protein signaling protein, Sst2 (Apanovitch et al., 1998).

In recent years, high resolution crystal structures of all three G protein subunits and recently one GPCR have been solved (Lambright et al., 1996; Palczewski K, 2000; Sondek et al., 1996; Wall et al., 1995). While these and other studies have provided a framework for understanding the structure of G-proteins and GPCRs as well as the interactions among the G protein subunits, some basic questions regarding the receptor-G protein interactions remain unanswered. For example, it is currently unknown how G proteins are activated and what residues constitute the points of contact between receptors and G proteins. Ultimately, answering these kinds of questions will require solving the crystal structure of the extended receptor-heterotrimeric G protein complex. So far, studies analyzing receptor-G protein interactions mostly rely on indirect methods. Some examples include membrane binding studies employed with *in vitro*-translated Gα proteins (Onrust et al., 1997; Osawa and Weiss, 1995), measurement of signaling from receptor chimeras expressed with various mutants of Gα proteins (Liu et al., 1995) and crosslinking of receptor-derived peptides to G proteins (Taylor et al., 1994; Taylor et al., 1994; Taylor et al., 1996). In some cases,

receptor-G protein complexes have been successfully copurified (Brown and Schonbrunn, 1993; Law and Reisine, 1997; Law et al., 1993).

So far, the studies in yeast that point to an interaction between the receptor and its cognate G protein have been indirect. First, the affinity of the receptor for the ligand is decreased in a *ste4Δ* strain (Jenness et al., 1987). Second, the dissociation rate of α-factor is increased in the presence of GTP analogs (Blumer and Thorner, 1990). Additionally since overexpression of the G protein subunits relieves the phenotype due to interaction between dominant negative and wild-type receptors, it has been proposed that these receptors compete for binding to a limiting pool of G proteins (Dosil et al., 1998; Leavitt et al., 1999). Moreover, synthetic lethal interactions between *ste2Δ* and a temperature-sensitive allele of *GPA1* suggest direct interaction between Gα and the receptor (Dosil et al., 2000).

In this report, I have used a crosslinking- and immunoprecipitation-based approach to capture receptor-G protein interactions in yeast. I have crosslinked the α-factor pheromone receptor to form a higher molecular weight complex. The Gβγ subunits were then identified in the complexes that had been immunoprecipitated with an antibody directed against a tagged form of Ste2. These results constitute the first biochemical evidence for an interaction between the receptor and the G proteins in this system. It is also the first biochemical interaction of Ste2 with a protein other than itself and its ligand. This approach can be used as an assay to identify the critical residues required for receptor-G protein interactions. This type of interaction will be important for defining how G proteins are activated.

Results

Crosslinking of receptors

A chemical crosslinking and immunoprecipitation approach was undertaken to identify proteins that interact with the α-factor receptor. The purpose for using protein crosslinking was to capture the proteins that interact transiently or weakly with Ste2 by including them in a covalently-linked complex with Ste2. Moreover, weak interactions between the receptor and its binding proteins should be preserved during the subsequent processing of the samples. The goal was then to immunoprecipitate the complex with an antibody directed against a peptide tag fused with Ste2, to cleave the crosslinker and then to identify the contents of the complex by using either western blotting methods or mass spectrometry. A water soluble, amine-reactive crosslinking agent DTSSP, dithio-*bis*-(sulfosuccinimidylpropionate) was chosen. The advantages to using this crosslinker are several fold. First, it contains a disulfide bond between the two functional groups and therefore, following immunoprecipitation it can be cleaved with reducing agents thus permitting the separation and identification of the individual crosslinked proteins. Second, DTSSP has specificity for primary amines located on lysines or on the N termini of proteins. This property allows the HA epitope tag on Ste2 constructs to escape modification since HA is an internal epitope tag and contains no lysine residues. In addition, since DTSSP is water-soluble, it targets cytoplasmic domains of proteins and can be used for identification of cytoplasmic proteins interacting with Ste2. Ste2 contains 16 lysine residues most of which are found at the cytoplasmic face of the receptor making them candidates for modification by DTSSP (Fig. 1).

Lysates from cells expressing Ste2 tagged at the C-terminus with the triple influenza epitope (HA) were treated with DTSSP. The lysates were processed for SDS-PAGE under non-reducing conditions and analyzed by western blotting. One concern was 'overcrosslinking', that is, crosslinking unrelated cellular proteins into a few large complexes to a point that the distribution of the bulk protein bands on SDS gels would be lost. To test whether 'overcrosslinking' had occurred, I monitored the extent of crosslinking by using western blotting to judge the molecular weight of Ste2-containing aggregates (Fig. 2A) and by protein staining of the blots (Fig. 2C). The crosslinking did not alter the overall pattern of cellular protein bands on the SDS gels dramatically and treatment of the samples with reducing agents, βME and DTT reversed the effect (Fig. 2C). When the crosslinked lysates were analyzed by immunoblotting methods, three slower-migrating species (complexes 1, 2 and 3) were detected that resulted from chemical crosslinking (Fig 2A compare lane1 with lanes 2 and 3). The molecular weights of complexes 1 and 2 could not be determined since complex 1 barely entered the stacking gel and complex 2 barely entered the resolving gel. It is not clear whether the complexes 1 or 2 represent single species or multiple species of similar size. Increasing the crosslinker concentration resulted in an increase in the amount of the two larger species (complexes 1 and 2) compared with the amounts of the two smaller species (free Ste2 and complex 3) (Fig. 2A and 2B). Similar results were obtained when the lysates were prepared from cells that had been treated with α-factor (Fig 2A compare lane 6 with lanes 7 and 8) or when α-factor was added to the lysates prior to incubation with the crosslinker (Fig. 2A lanes 4 and 5).

Complexes 2 and 3 were not disrupted by β-mercaptoethanol or DTT, and might represent what are called 'SDS-resistant dimers' and tetramers of the receptor. SDS-resistant dimers are higher molecular weight aggregates that are detected during the

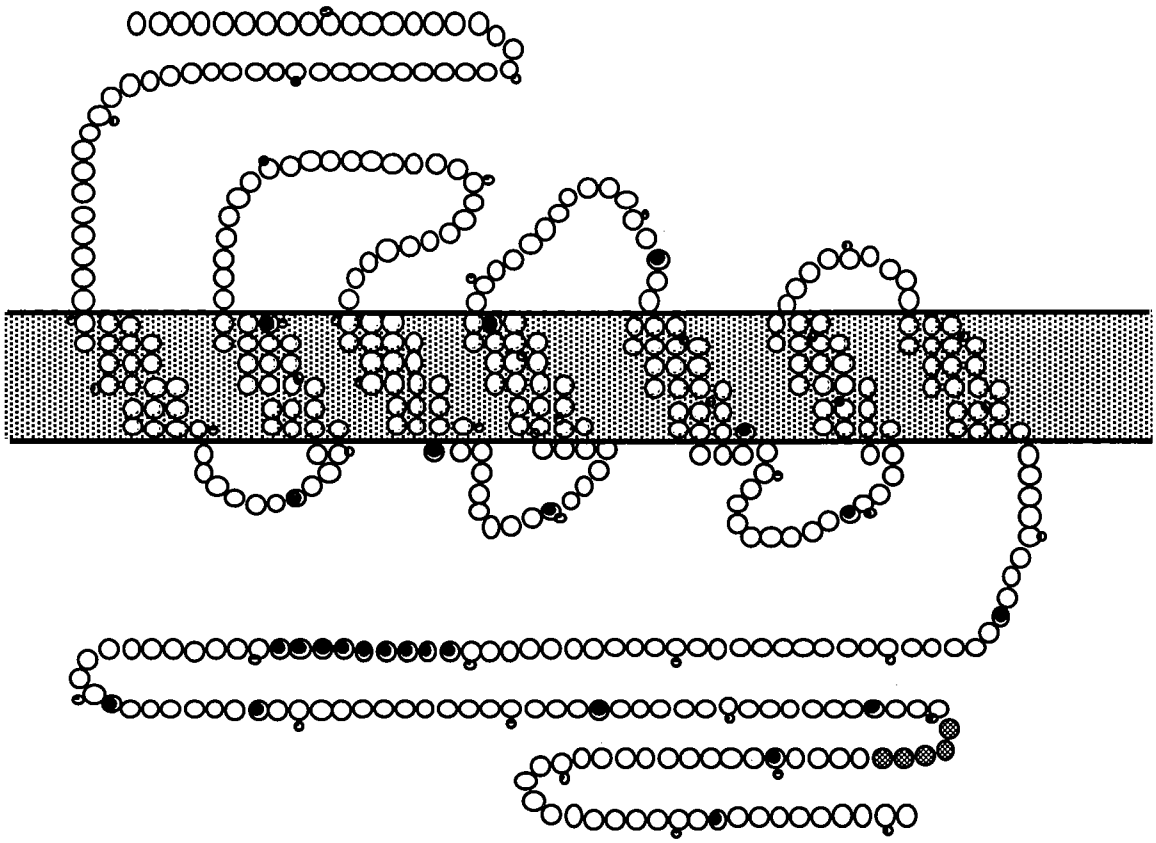
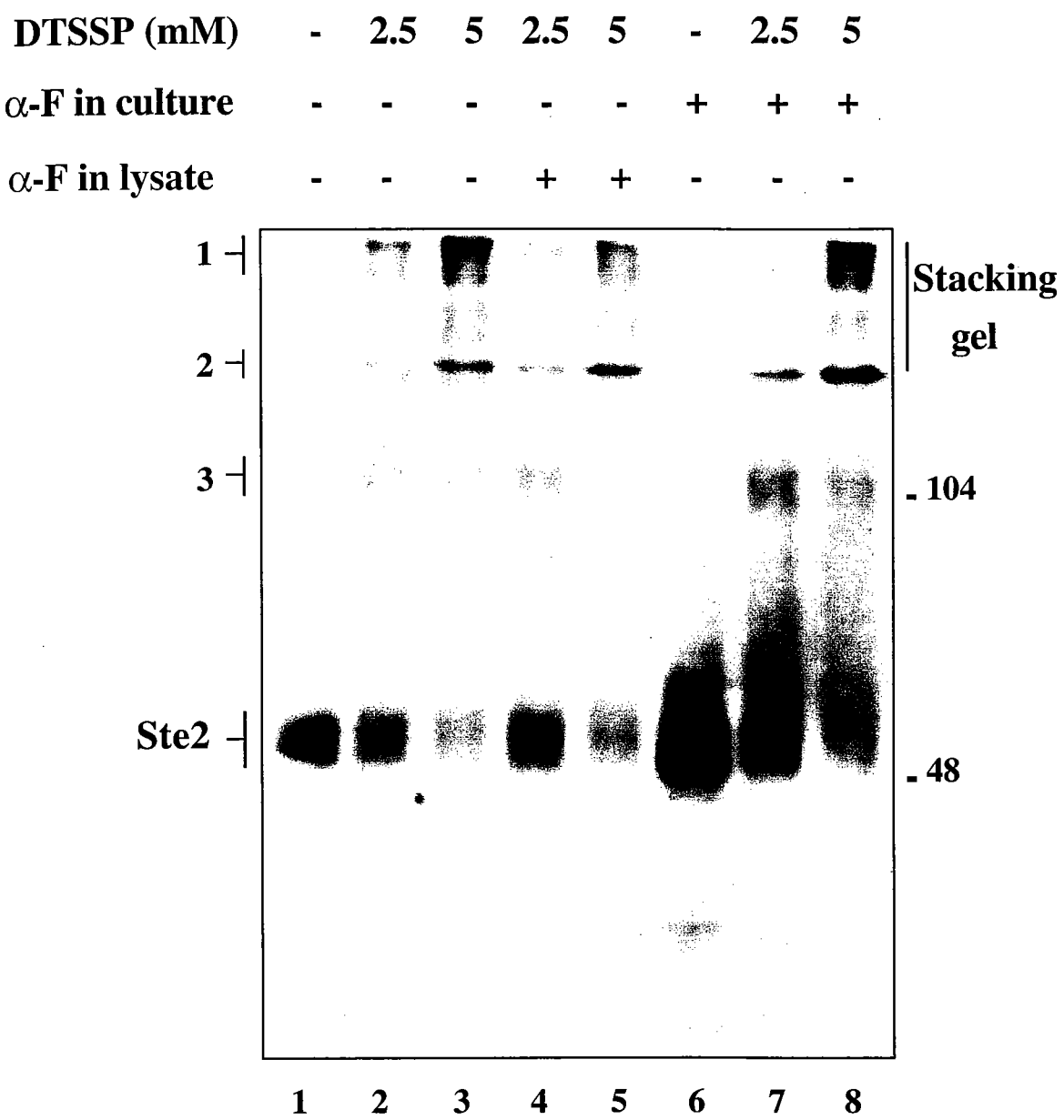


Figure 1 . Residues of interest in the α-factor receptor. The lysine residues are shown in black. The endocytosis motif SINNDAKSS sequence is denoted in gray. Putative endocytosis signal GPFAD sequence is marked with textured circles. The residue 326 is also numbered.

A.


DTSSP (mM) - 2.5 5 2.5 5 - 2.5 5
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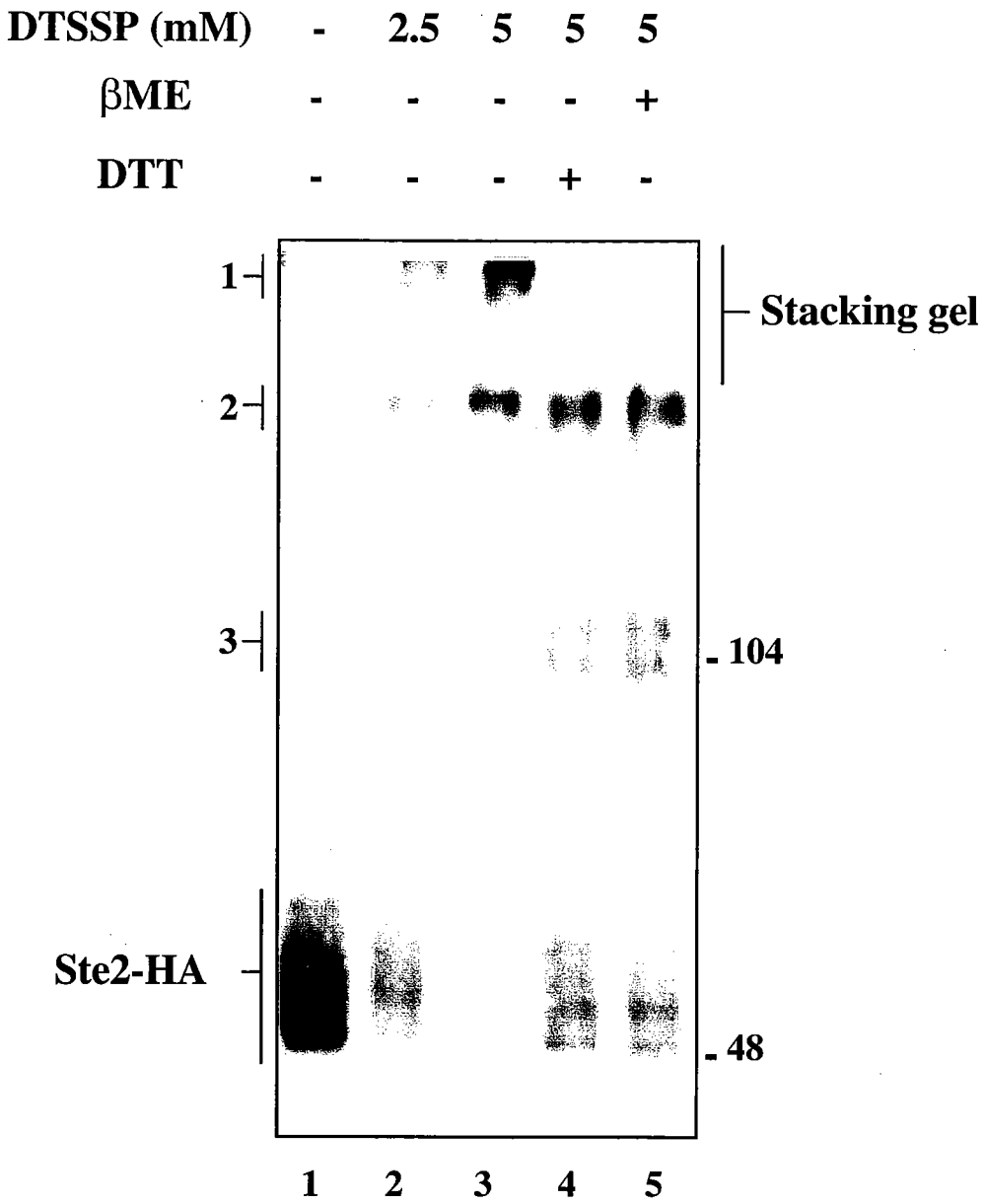


C.

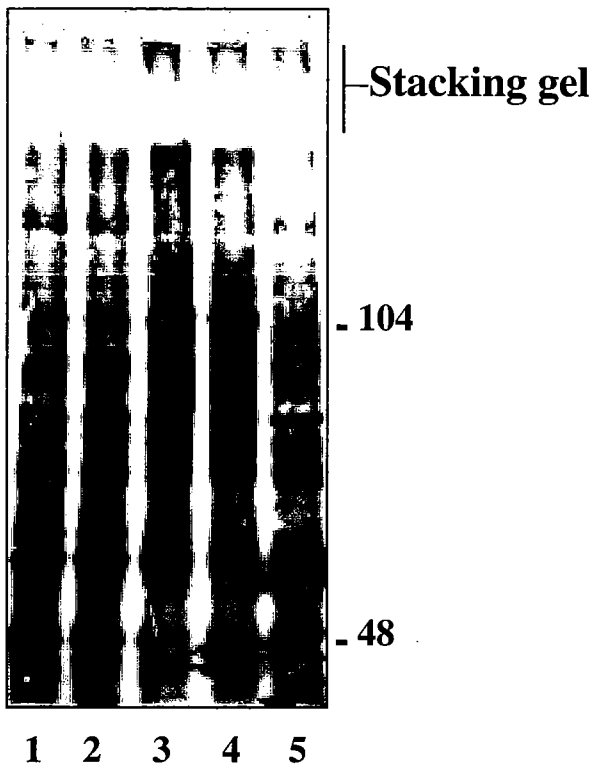


Figure 2. Crosslinking of the α-factor receptor. **(A)** Cells expressing Ste2-HA were untreated (lanes 1-5) or treated with 10^{-7} M α-factor (lanes 6-8). Lysates were prepared. Lysates prepared from cells that had been untreated, were treated with 10^{-7} M α-factor (lanes 4 and 5). Then all lysates were incubated for 2 hours on ice with 0, 2.5 or 5 mM DTSSP as designated above each lane. Total protein loaded in each lane was 1.6 μg. The crosslinking reactions were quenched by adding one-fourth volume of 1M Tris-Cl (pH 7.4). The samples were processed for SDS-PAGE analysis in non-reducing sample buffer. Samples were resolved on a 10% SDS gel. Ste2-HA was detected by immunoblotting methods with anti-HA antibody. Complexes 1, 2, 3 and free Ste2-HA are indicated on the left, whereas the boundaries of the stacking gel and molecular weight markers are indicated on the right. **(B)** Cultures expressing Ste2-HA had been treated with 10^{-7} M α-factor.

Whole-cell lysates were treated with DTSSP as for panel A. Protein complexes were resolved on an 8% SDS gel and detected by immunoblotting methods with anti-Ste2 antibodies. Complexes 1, 2 and 3 are marked as indicated in panel A. The samples in lanes 4 and 5 were identical to lane 3 except that they were treated with reducing agents, 100 mM βME or 50 mM DTT (lanes 4 and 5, respectively) immediately prior to loading.

(C) Reversible crosslinking of bulk cellular protein. The immunoblot that had been processed in panel B was subsequently stained with Colloidal Gold. The Colloidal Gold-stained blot is the mirror image of the immunoblot shown in Panel B. Molecular weight markers are shown in the right for all blots. The strain was DJ1400-A.

SDS-PAGE analysis of Ste2 and some other proteins with hydrophobic regions such as glycophorin A, tailspike protein from phage P22 and some GPCRs including β-adrenergic receptors (Blumer et al., 1988; Furthmayr and Marchesi, 1976; Hebert et al., 1996; Konopka et al., 1988; Yesilaltay and Jenness, 2000). The molecular weight and doublet migration pattern of complex 3 are consistent with receptor dimers. Similar dimer complexes have been observed upon treatment of other GPCRs, such as β-adrenergic and δ-opioid receptors, with membrane permeant crosslinking reagents; however, the authors did not report whether these SDS-resistant dimers are cleaved under reducing conditions (Cvejic and Devi, 1997; Hebert et al., 1996). In my experience, the SDS-resistant species were not cleaved completely under reducing conditions (Fig. 2B and data not shown). They were detected even in the absence of the crosslinker, and their occurrence and proportion varied among different preparations. Therefore they were not studied further.

According to three criteria, complex 1 represents a crosslinker-specific, covalent association of the receptor with itself or with other proteins: (i) it was formed only upon addition of the crosslinker, (ii) the crosslinker caused a dosage-dependent increase in the ratio of complex 1 to free receptor, and (iii) complex 1 disappeared upon cleaving the crosslinker with reducing agents DTT (fig. 2B, lane 4) and βME (lane 5). The complexes were not formed as a result of association of proteins with the HA tag since the HA epitope does not contain lysine residues or an α amino group.

Lysates were prepared from cells that carried single or multiple deletions in the genes encoding the components of the signal transduction cascade downstream of the receptor. Lysates were then treated with DTSSP and analyzed by western blotting methods. The purpose was to find out if a complex of small size would form in the absence of the

proteins that may link the receptor to the rest of the complex. A reduction in the size of the complex would suggest that the missing component was part of the original complex, and would simplify the identification of other proteins that remained in the small complex. However, similar size complexes were detected upon crosslinking and western blotting analysis of strains that harbored individual deletions in *STE4*, *STE5* and *SST2* genes and double deletions in *GPA1* and *STE5* or in *AKR1* and *STE5* genes or triple deletions in *AKR1*, *STE5* and *GPA1* genes (not shown). A truncated version of the receptor, Ste2-T326-HA, that lacks most of the carboxy terminal tail and has only about half the number of lysines on the cytoplasmic surface (see Fig. 1), also formed a complex that was comparable in size to complex 1 (not shown).

Identification of Gβγ heterodimer in the complex

The next goal was to test whether any subunits of the heterotrimeric G protein were a part of the complexes containing receptor. Crude membranes were prepared from crosslinked lysates of cells expressing Ste2-HA. Membrane proteins were extracted by the relatively non-denaturing detergent n-dodecyl β-D-maltoside (DβM), and the complexes were immunoprecipitated by using anti-HA antibodies. All three high molecular weight complexes that were previously detected (Fig. 2A) were present in the IP pellet (data not shown). The precipitated proteins were exposed to reducing agents, resolved by SDS-PAGE and analyzed by immunoblotting methods. To evaluate the presence of G proteins in the complex, I probed the blots with antisera specific for Ste4. Ste4 was found to coimmunoprecipitate with Ste2-HA only after the lysates were crosslinked (Fig. 3A top panel compare lanes 3 and 4). The coprecipitation of Ste4 depended on the presence of the HA-tagged Ste2, because it was absent in the immunoprecipitates obtained from control

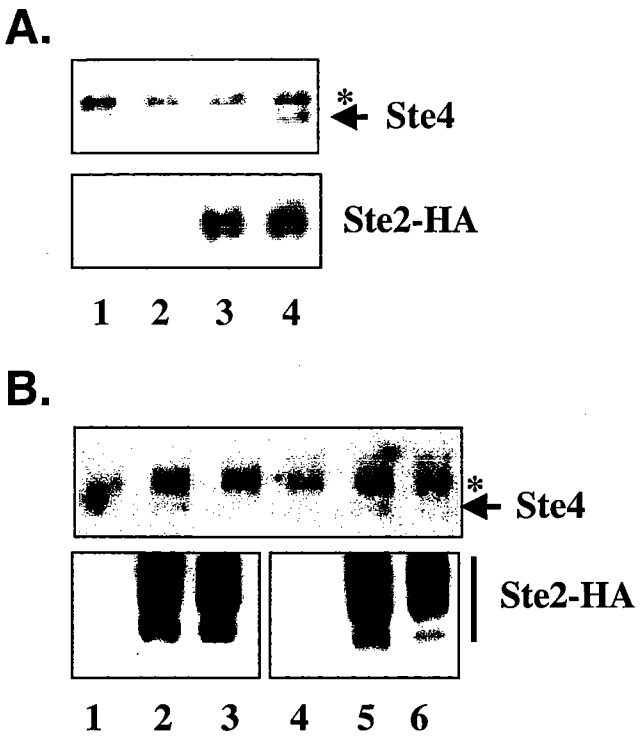


Figure 3 . Gβ subunit is crosslinked to and coprecipitates with the α–factor receptor. Cell lysates were treated with 5mM DTSSP as in Fig. 2 except protein to crosslinker concentration was approximately ten times higher. Ste2-HA in the detergent-solubilized membrane fraction was precipitated with anti-HA antibodies. The precipitated proteins were processed for SDS-PAGE with sample buffer containing reducing agent. Immunoblots were probed with anti-Ste4 antibody (upper blots in each panel) or anti-HA antibody (lower blots in each panel). The asterisks indicate the position of the immunoglobulin heavy chains. **(A)** Cells expressed untagged Ste2 (lanes 1 and 2) or Ste2-HA (lanes 3 and 4). Cells were untreated (lanes and 3) or treated with 5 mM DTSSP (lanes 2 and 4). **(B)** Cells expressed untagged Ste2 (lanes 1 and 4) or Ste2-HA (lanes 2, 3, 5 and 6). Strain used in lanes 3 and 6 also harbored a deletion of the STE4 gene. DβM (lanes 1-3) or SDS (lanes 4-6) was used to extract proteins from the membrane pellet. Strains were DJ211-5-3 (Ste2), DJ1400-A (Ste2-HA) and DJ1495 (Ste2-HA, ste4Δ).

cells expressing untagged Ste2 (Fig. 3A top panel, compare lanes 2 and 4). The band was positively identified as Ste4 since it was missing when experiments were repeated with *ste4Δ* cells. (Fig. 3B top panel, compare lanes 2 and 3). Ste4 appeared to be associated with the complex, because it also coprecipitated with Ste2-HA under denaturing conditions (Fig. 3B top panel, compare lanes 2 and 5).

Ste4 is known to become phosphorylated upon prolonged exposure to α-factor (Cole and Reed, 1991). The phosphorylated Ste4 migrates more slowly on SDS-PAGE. Coprecipitation of both the phosphorylated and the unphosphorylated forms of Ste4 was observed when the crosslinking and precipitation experiments were performed with cells that had been exposed to α–factor (Fig. 4, second row). *In vivo*, Ste4 and Ste18 form a heterodimer. As expected, Ste18 was also detected in the precipitates (Fig. 4, third row). The interaction detected was not the result of nonspecific crosslinking as suggested by the absence of other more abundant cytoplasmic or membrane proteins in the pellet, including the ribosomal protein, Tcm1, and abundant plasma membrane ATPase Pma1, respectively. These data suggest that the receptor and Gβ form a complex *in vivo* and provide the first biochemical evidence for a physical association of receptor and G proteins in this system. In addition, these results provide a biochemical tool to analyze these complexes further. In mammalian systems, a direct interaction between α2-adrenergic receptors and the Gβ subunit of the G_o/G_i protein family has been proposed based on binding studies with a receptor-derived peptide to Gβ (Taylor et al., 1994; Taylor et al., 1994). We cannot, however, exclude the possibility that the association we observe results from indirect interactions, that is, through a bridging protein between the receptor and Ste4.

The C-terminal tail of the α-factor receptor has been proposed to stabilize the interactions with the G protein (Dosil et al., 2000). I sought to determine whether a truncated version of the receptor, Ste2-T326-HA lacking most of the tail would form a complex with Ste4. Cells expressing Ste2-T326 are unable to endocytose their receptors but are proficient for signaling (Rohrer et al., 1993; Schandel and Jenness, 1994). Initial crosslinking analyses indicated that Ste2-T326-HA formed a reversible high molecular weight complex upon treatment with DTSSP (not shown). Subsequent crosslinking and coimmunoprecipitation experiments revealed that 10% of Ste4 was coprecipitated with the truncated receptor (Fig. 5), whereas about 1% of Ste4 was coprecipitated with the wild-type receptor typically. This difference may reflect the 3- to 4-fold higher accumulation of the truncated receptors at the cell surface. Higher levels of Ste4 were also found to coprecipitate when wild-type Ste2 was overexpressed (not shown).

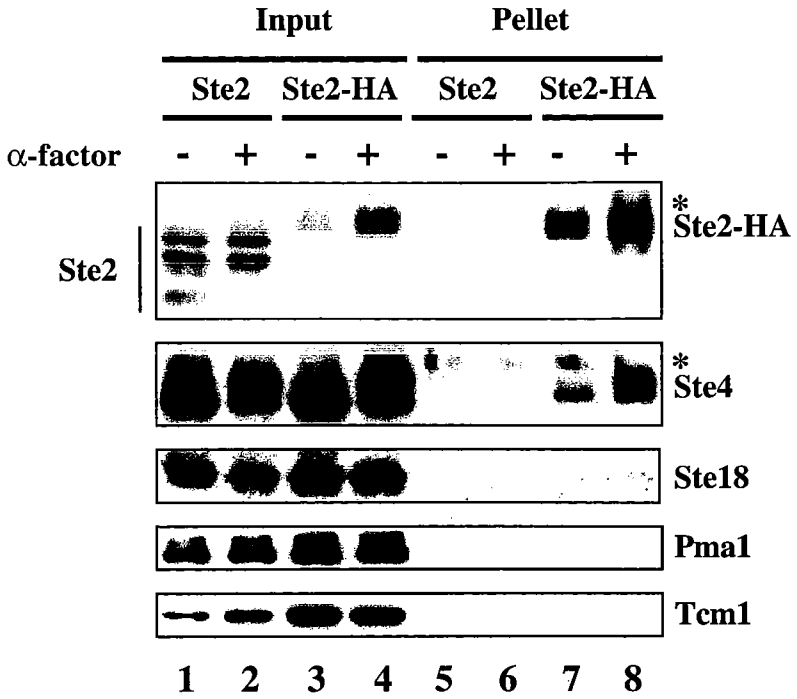


Figure 4. Both Gβ and Gγ crosslink to and coprecipitate with the α-factor receptor. Strains expressed untagged Ste2 (lanes 1, 2, 5 and 6) or Ste2-HA (lanes 3, 4, 7 and 8). Cultures were untreated (lanes 1, 3, 5 and 7) or treated with 10^{-7} M α–factor for 30 min (lanes 2, 4, 6 and 8). Cell lysates were treated with 5 mM DTSSP as in Fig. 3. The DBM-solubilized membrane fraction was subjected to immunoprecipitation with anti-HA antibodies. The input membrane fraction (lanes 1-4) and precipitated proteins from the immune pellets (lanes 5-8) were processed for SDS-PAGE in sample buffer under reducing conditions. Samples were analyzed by western blotting on three gels. The blots labeled Ste2 and Ste4 were obtained from 10% SDS gels and were reprobed with anti-Pma1 and anti-Tcm1 antibodies, respectively. The blot probed with anti-Ste18 was obtained from an 18% SDS gel. Immune blots were probed with anti-Ste2 antibody specific for Ste2 C-terminal domain (top panel), anti-Ste4 (second row), anti-Ste18 (third row), anti-Pma1

(fourth row) or anti-Tcm1 (fifth row) antibodies as indicated on the left. Strains were 211-5-3 (Ste2) and DJ1400-A (Ste2-HA).

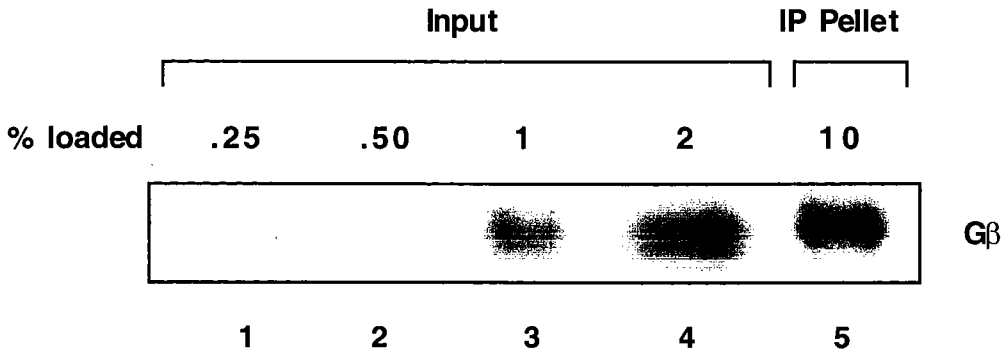


Figure 5. 10% of the Gβ subunit from the membrane fraction coprecipitates with the truncated α-factor receptor. Lysates were prepared from cells expressing ste2-T326-HA (Strain DJ1416-A). Crosslinking and precipitation experiments with the anti-HA antibody were carried out as described in Fig. 4. The whole cell membranes (lanes 1-4) and precipitated proteins from the immune pellets (lane 5) were processed for SDS-PAGE in sample buffer under reducing conditions. Percent loading for each sample is indicated above each lane.

Search for other proteins in the complex

An attempt was made to identify new proteins that associate with the α-factor receptor. To this end, lysates were prepared from a strain that carried a plasmid that results in the overproduction of a mutant form of Ste2 (designated HA-Ste2-FT-HT). HA-Ste2-FT-HT lacks the two cysteines and contains an HA epitope on the N-terminus and both a FLAG and His6 epitopes on the C-terminus. The two cysteine residues in Ste2 do not seem to be required for receptor function since a receptor mutant that lacks both cysteines shows no detectable defect in plasma membrane localization, signaling (Jeremy Thorner personal communication) or oligomerization (Yesilaltay and Jenness, 2000). The lysates were incubated with DTSSP and centrifuged at 100,000×g. Then the receptor complexes in the pellet were extracted with the non-denaturing detergent, DβM, immunoprecipitated with anti-HA antibody, and were purified further by using a Nickel column under protein denaturing conditions. The samples were eluted from the column by using SDS sample buffer, and the eluate was subjected to both SDS-PAGE and 2D gel electrophoresis under reducing conditions, and the proteins on the gels were detected with silver staining. As a preliminary experiment, I identified four prominent bands on one-dimensional SDS-PAGE gels. These bands were putative Ste2-interacting proteins based on the following criteria: first they were purified only in the presence of the tagged receptor, *i.e.*, they were missing from the immunoprecipitates obtained from control cells expressing untagged Ste2 (Fig. 6 compare lanes 1 and 2). Second, these proteins were not derived from the N-terminal or the C-terminal domain of Ste2 since, when the same samples were analyzed by using western blotting methods, the bands did not overlap with the signal that was obtained with anti-FLAG antibodies, anti-HA antibodies and anti-Ste2 antibodies specific for N- and C-termini of the receptor (not shown).

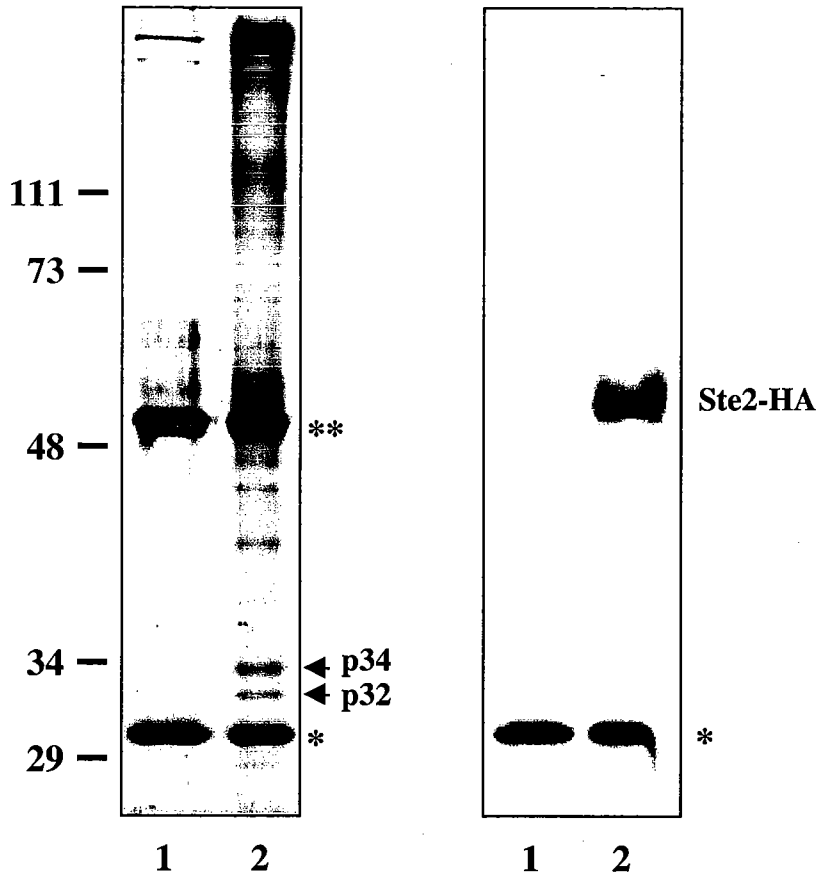
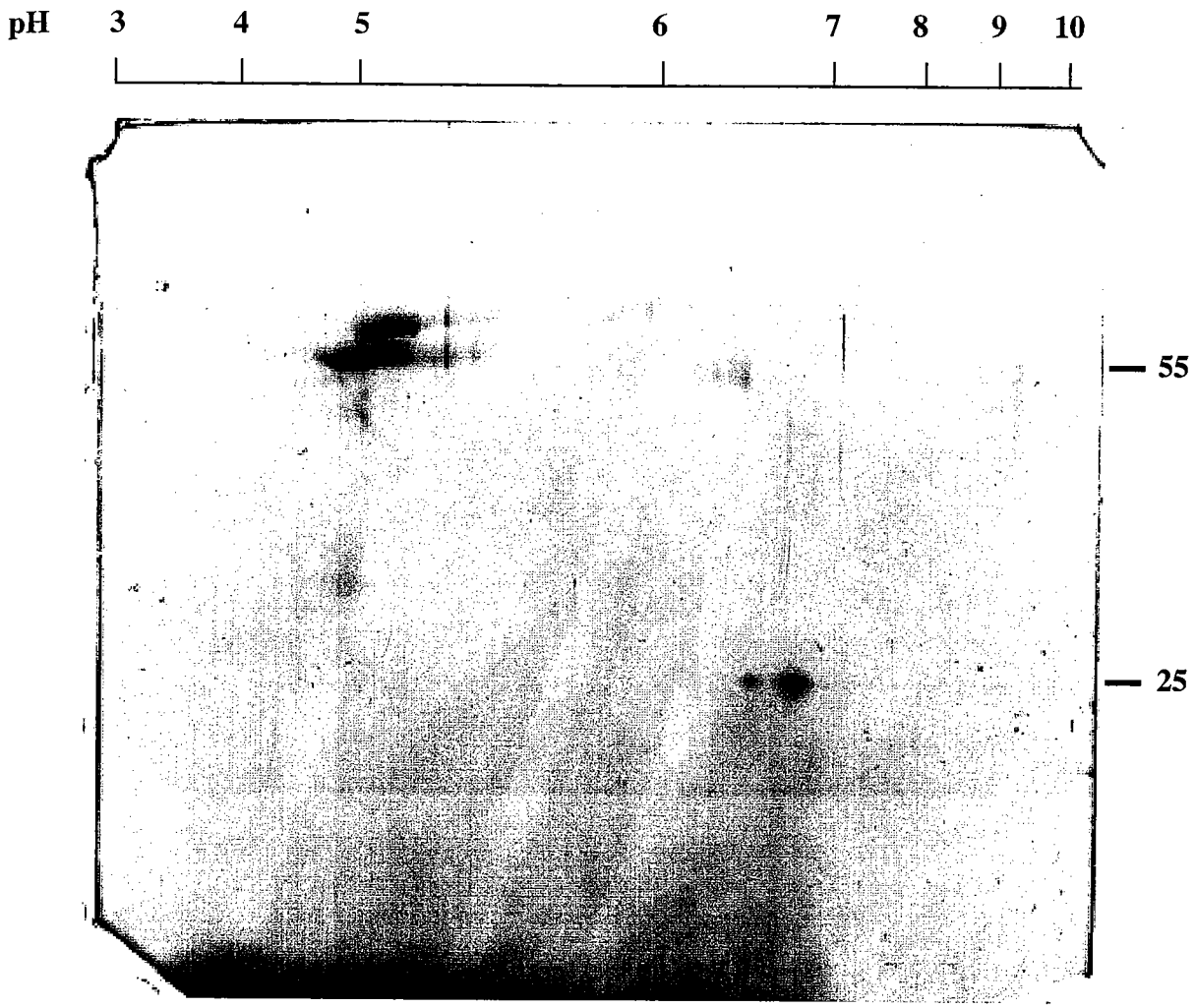


Figure 6 . Detection of the putative Ste2-interacting proteins. Lysates from cells lacking (lane 1) or carrying the plasmid expressing HA-Ste2-FT-HT (lane 2) were subjected to crosslinking and precipitation with anti-HA antibodies as described in Fig. 3. The precipitated material was then solubilized and bound to a Ni-column. The eluate from the column was processed for SDS-PAGE analysis in sample buffer under reducing conditions. The proteins on the gel were detected with silver staining (left panel). The immunoblot was probed with anti-HA antibodies (right panel). The arrows mark the two putative Ste2-interacting proteins p34 (the upper arrow) and p32 (the lower arrow). The single and double asterisks mark the immunoglobulin light and heavy chains, respectively. Strains were DJ1448 (ste2Δ) and DJ1449 (HA-Ste2-FT-HT).

A.



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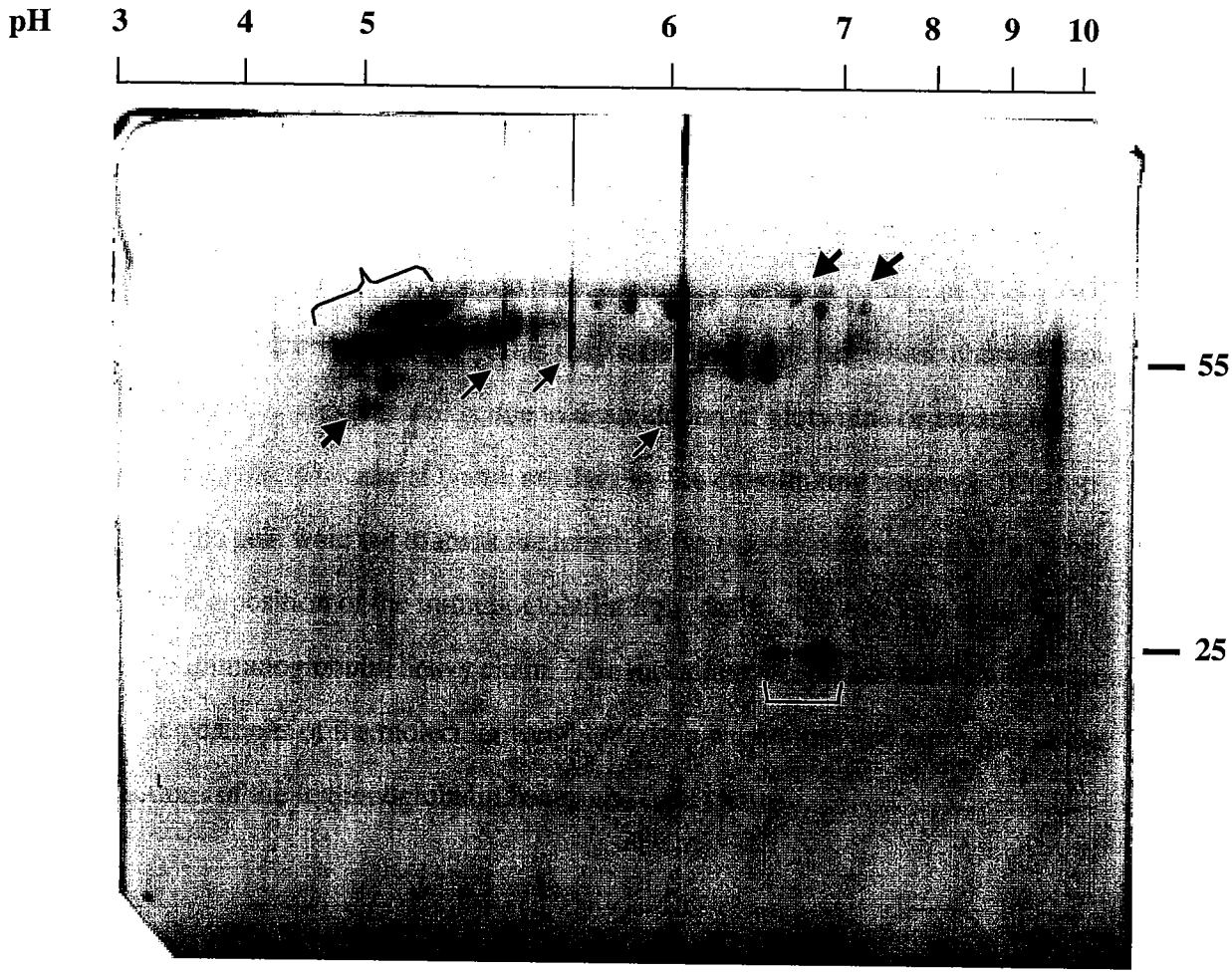
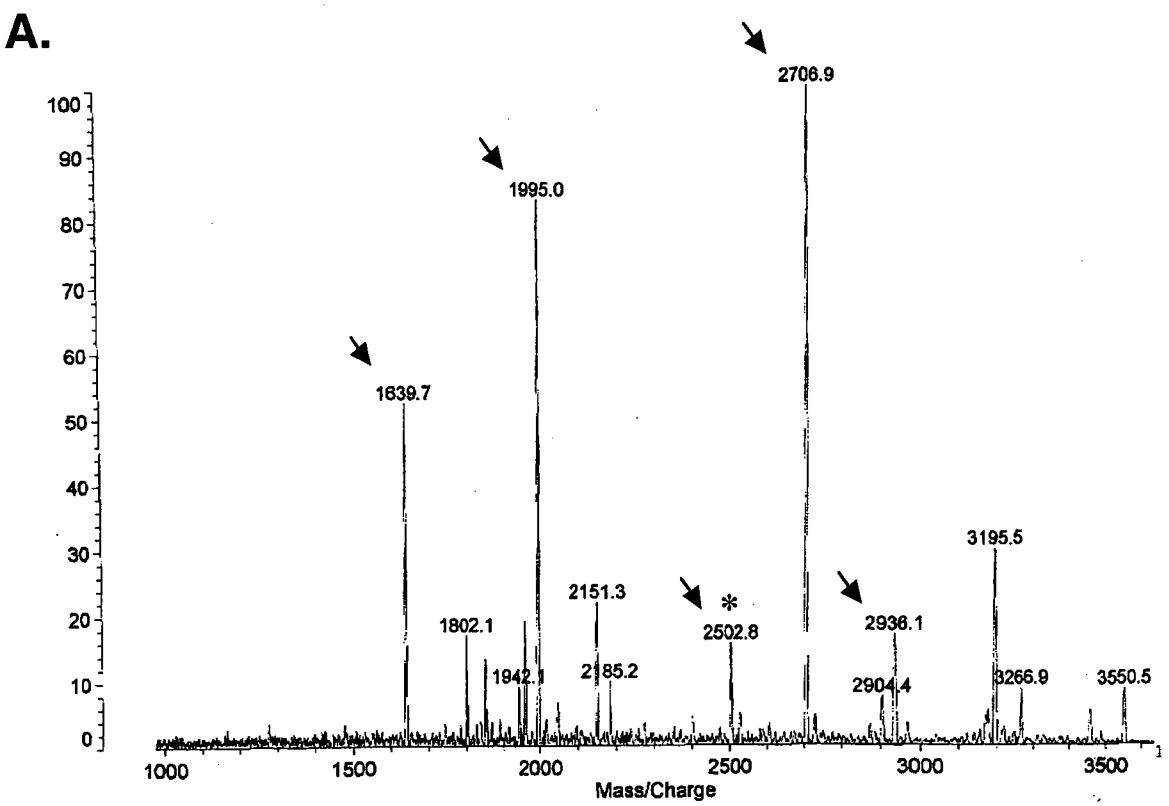


Figure 7. Two-dimensional electrophoretic detection of putative Ste2-interacting proteins. Lysates prepared from cells **(A)** lacking (strain DJ1448) or **(B)** carrying the plasmid that expresses the HA-Ste2-FT-HT (strain DJ1449) were processed for crosslinking, and then for immunoprecipitation with anti-HA antibodies. The proteins in the eluate were first incubated in a reducing sample buffer and then were resolved by 2D gel electrophoresis. Thick arrows in panel B point to spots that indicate putative Ste2-interactoing proteins. Thin arrows indicate three groups of spots with a similar migration pattern in SDS-PAGE. The difference in isoelectric focusing points may reflect Ste2 species whose charges were altered during sample preparation (e.g. due to deamidation of glutamine or asparagine residues, or due to modification of lysine residues by the crosslinking reagent). The top two corners of the gels were cut to mark the length of the isoelectric focusing strip. The brackets show the position of the immunoglobulin light chain. The brackets show the position of the immunoglobulin heavy chain. The molecular weight markers are shown on the right. The positions of the molecular weight markers were estimated according to the relative positions of the immunoglobulin heavy and light chains.

shown). Several putative interacting proteins were detected on the 2D gel analysis of the strain expressing the tagged Ste2 (Fig. 7B, arrows) that were not observed on the negative control gel (Fig. 7A). As expected, the immunoglobulin heavy and light chains were present in both gels (Fig. 7A and Fig. 7B). Three groups of spots (Fig. 7B, thin arrows) that had similar molecular weights were resolved along the isoelectric focusing dimension. These may represent Ste2 species whose charges were modified by the crosslinker or by the other modifications (*e.g.*, phosphorylation or deamidation). Ste2 typically migrates as a doublet on SDS-PAGE gels due to single and double glycosylation events that occur on its N-terminus. The receptor species that resolve in the second dimension may represent the glycosylated forms of Ste2. The putative Ste2 species are positioned between pH 5 and 6 with the darkest one approximately at pH 6. Using EMBOSS IEP program (Rice et al., 2000), the isoelectric point of Ste2 was calculated to be 6.15. This value suggests that the darkest groups of spots are unmodified Ste2 species. In addition, the same software was used to calculate the pI of Ste2 when a few lysines are changed to alanines in order to mimic the loss of positive charge on the protein when lysine residues are modified by the crosslinker. The following pI values were obtained for Ste2 with one, two and three lysine changes: 5.75, 5.40 and 5.17, respectively. The positions of the two additional putative Ste2 species indicate that their pI values are in agreement with these values, and therefore, they may represent Ste2 species that contain one or two modified lysines. In addition, the putative Ste2 spots have similar molecular weights compared to the immunoglobulin heavy chain spots (marked with braces) on the 2D gel. Therefore, the molecular weight, the migration pattern and the isoelectric points of these spots are consistent with these spots being unmodified and crosslinker-modified Ste2 species.

The next step was to prepare greater quantities of these putative interacting proteins and to analyze them by using mass spectrometry. The 2D analysis did not reveal many

more bands than the single dimension. Furthermore, it was not practical to analyze more material on the 2D method due to the limited capacity of the strips used for the isoelectric focusing step. Therefore, lysates were prepared from ten-fold more material and analyzed by using one-dimensional SDS-PAGE as in Fig. 6. Proteins were detected with silver staining methods. Two bands with molecular weights around 30 kD were cut out of the gel, subjected to in-gel trypsin digestion and analyzed by using mass spectrometry. The two proteins possessed a few digestion products with similar molecular mass (approximately 1640, 1995, 2707 and 2936 daltons, compare Fig. 8A and Fig. 8B) and therefore may have originated from the same protein. To rule out the possibility that the common products were cleavage products of trypsin itself, the mass spectrometry data of the two putative proteins were compared with data obtained from a trypsin-only experiment. Results indicated that only a few of the tryptic fragments obtained from the two bands appeared to be derived from tryptic self-cleavage (Fig. 8, asterisks in panels A and B). However, the molecular masses of the resulting tryptic digestion products did not match cleavage products predicted for any protein in the yeast or any other protein database. A computer program (Sherpa Light) was used to estimate the molecular weights of the partial and complete trypsin digestion products of Ste2. These computer-simulated trypsin digestion products were compared with the empirical data to test whether the fragments were degradation products of Ste2. I also considered the possibility that some peptides may contain the residue of the crosslinking reagent attached to an uncleaved lysine residue. The mass spectrometry data from the two bands did not match the predicted Ste2 fragments. Crosslinker-modified lysines are no longer substrates for trypsin digestion. A high protein-to-crosslinker concentration was picked to minimize modification of multiple lysines on a given protein. However, it is possible that the proteins were modified more severely than predicted. In this case, it may not be possible to identify novel proteins from the database because the complexity of the predicted peptides would be too high.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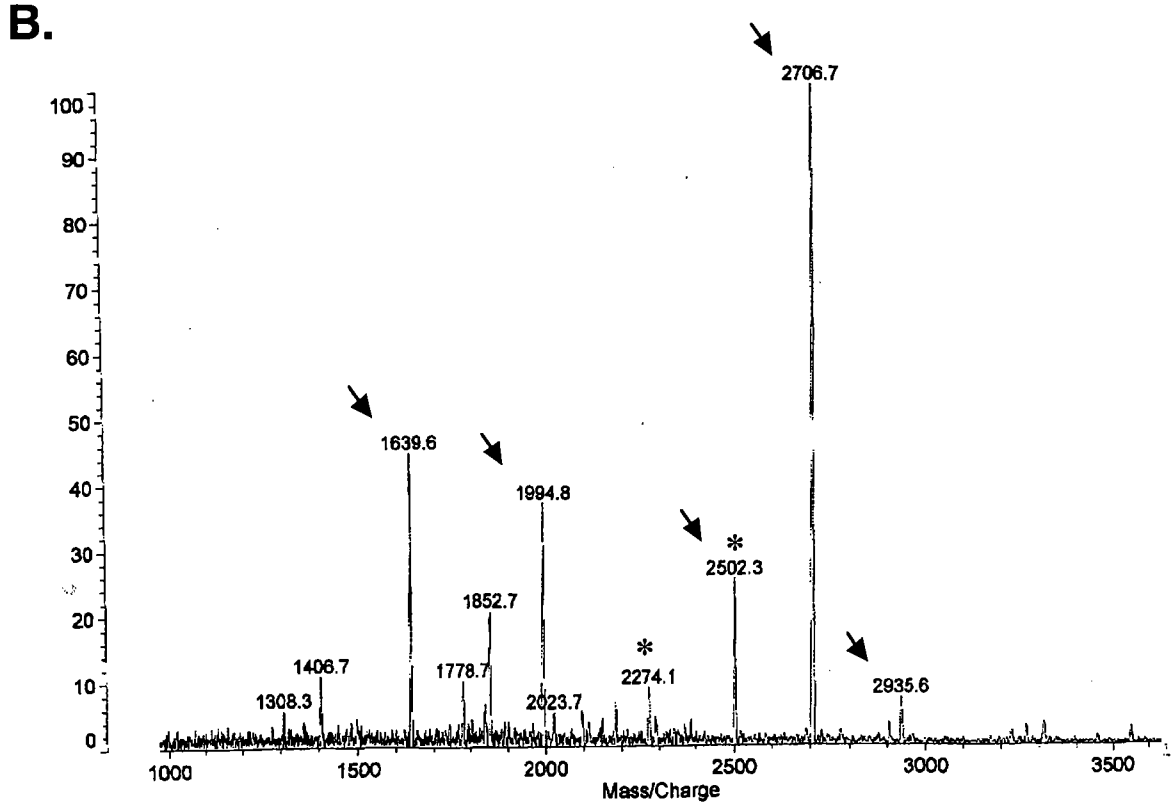
2502.8
2936.1
1942.1
2185.2
2904.4
3266.9
3550.5
1000
1500
2000
2500
3000
3500
Mass/Charge


B.
100
90
80
70
60
50
40
30
20
10
0
2706.7
1639.6
1994.8
2502.3
1852.7
1406.7
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C.

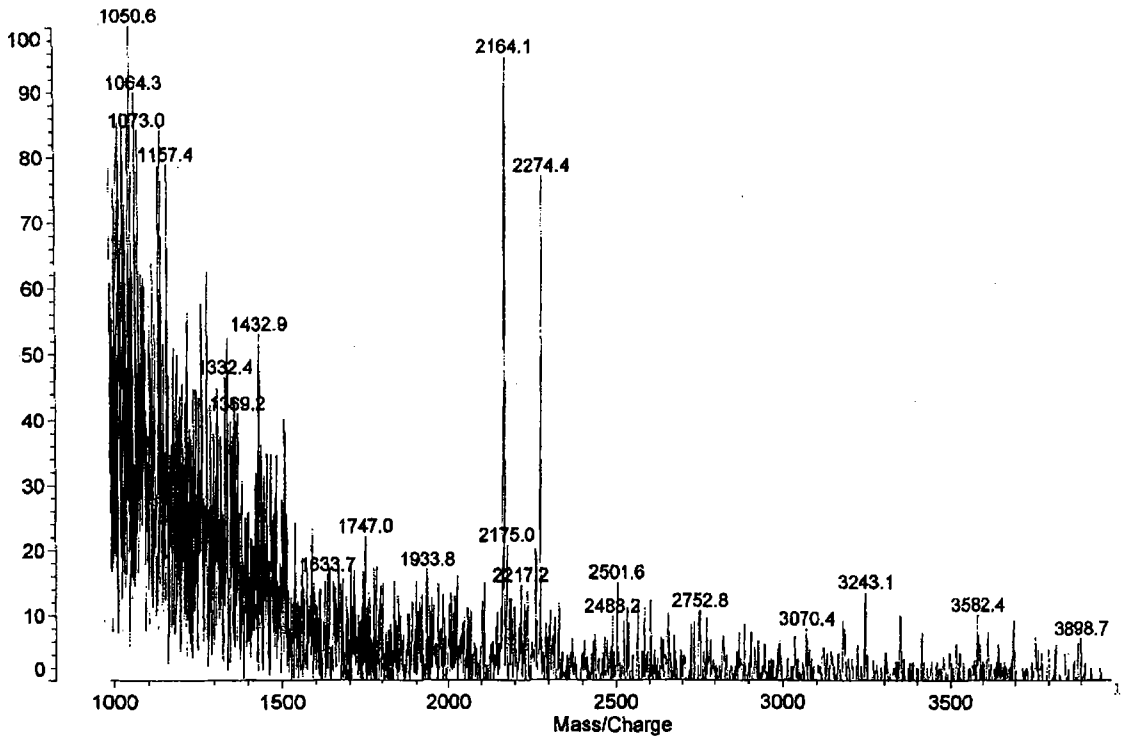


Figure 8 . The mass spectrometric analysis of the tryptic digestion products of putative Ste2-interacting proteins suggests that they are derived from the same protein. The mass spectrometric analysis of the **(A)** p34, **(B)** p32 **(C)** trypsin. Arrows in panels A and B indicate cleavage products. Asterisks indicate possible tryptic cleavage products in panels A and B. The strain was DJ1449.

Discussion

This chapter describes a crosslinking and immunoprecipitation strategy to capture and characterize the transient complexes that contain the α–factor receptor Ste2. Tagged receptors were crosslinked to form at least three high molecular weight complexes and the complexes were immunoprecipitated with antibodies against the tag. Western blotting analysis of the precipitated material revealed the presence of the β and γ subunits of the heterotrimeric G protein, Ste4 and Ste18. The interaction was specific as judged by the absence of two abundant proteins (plasma membrane ATPase Pma1 and a ribosomal subunit Tcm1) in the precipitate. Similar results were obtained when the cultures had been treated with α-factor prior to analysis. A truncated receptor missing most of the cytoplasmic C-terminal tail was also active in binding Ste4. Overall, these results constitute the first biochemical evidence for a physical association between the α-factor receptor and its cognate G-protein. An attempt was made to identify other components of the crosslinked complex. A number of putative Ste2-interacting proteins were detected when the crosslinked complexes were resolved by 1D and 2D-electrophoresis. Mass spectrometric analysis of tryptic fragments obtained from two of these proteins suggested that they originated from the same protein; however, it failed to identify a match in the yeast protein database as well as in other protein databases.

One factor that might provide efficiency and specificity in transmitting the signal is the precoupling of the G-protein-coupled receptors with their cognate G protein before exposure to ligand (Neubig, 1994; Shea and Linderman, 1997). The issue of specificity may be critical in mammalian systems since several different G proteins are available to interact with receptors (Insel et al., 1983; Milligan, 1996; Ransnas and Insel, 1988). This

point was previously overlooked for the most part because of the recognized promiscuity in receptor interactions with multiple G proteins (Brown and Schonbrunn, 1993; Milligan, 1996; Neubig, 1994). Early transfection studies in mammalian cells usually employed high-level expression of the components of the system. Thus, the issue of specific receptor-G protein coupling was avoided. Selective receptor interactions with specific G protein subtypes have only recently been appreciated. The light-sensing receptor rhodopsin associates much more efficiently with αt, transducin than with other Gα subunits present in the retina (Cerione et al., 1985; Kisselev and Gautam, 1993). Antisense RNA directed against the mRNA encoding the $G_o1\alpha$ subunit inhibited the activity from m4 muscarinic receptors in GH3 cells, whereas in the same cells specific disruption of somatostatin receptor activity has been achieved with antisense RNA that inhibits the mRNA for the $G_o2\alpha$ subtype (Kleuss et al., 1991). These results suggest that GPCRs selectively bind specific G protein subtypes from the cytoplasmic pool. Preactivation complexes would be one mechanism to achieve the specificity of receptor-G protein interactions. Engagement of δ-opioid and somatostatin receptors with a select number of Gα and Gβ subtypes in preactivation complexes has been confirmed by using coprecipitation studies (Law and Reisine, 1997; Law et al., 1993). In addition, *in vitro*-translated transducin interacts with rod-outer segment membranes containing rhodopsin both in the dark and in the light (Onrust et al., 1997; Osawa and Weiss, 1995). For all three receptors, activation by ligand enhanced the levels of receptor-G protein interactions detected in each assay.

Indirect genetic and biochemical evidence implies the existence of preactivation complexes in yeast. The basal levels of signaling is increased in cells deleted for pheromone receptors when reporter assays were performed using a pheromone-inducible promoter (Boone et al., 1993; Hasson et al., 1994) suggesting that wild-type receptors

sequester the G proteins prior to ligand occupancy. Similarly, for cells producing a constitutively active receptor, the basal signaling is decreased when wild-type receptors are coexpressed (Dosil et al., 2000). Finally, a mutation affecting Gpa1 causes lethality at high temperature in *ste2Δ* cells but not in cells expressing full-length receptors (Dosil et al., 2000). However, to date no biochemical data have been available to support this notion in yeast. As presented in this thesis, the ability to capture the interaction between Ste2 and Ste4 in the absence of α-factor suggests that receptor and G proteins are precoupled prior to stimulation by ligand and therefore lends support to the existence of preactivation complexes in yeast. Furthermore, α-factor had little effect on the Ste4 that coprecipitated with receptors. The slight increase in coprecipitated Ste4 observed with α-factor-treated cultures is consistent with higher level of Ste2 present in α-factor-treated cultures. Similarly enhanced levels of Ste4 coprecipitation were observed when an overproduced version of Ste2 was used in for crosslinking and immunoprecipitation (not shown). Moreover, more Ste4 coprecipitated with Ste2-T326; this truncated receptor results in a 4-5 fold accumulation of receptors on the plasma membrane due to its defect in endocytosis (Konopka et al., 1988).

Dosil and coworkers reported genetic findings that indicate a role for the cytoplasmic C-terminal tail of the receptor in stabilizing the receptor-G protein interactions (Dosil et al., 2000). Mutant receptors that cannot bind α-factor are thought to have a dominant negative influence on signaling because they sequester G proteins in preactivation complexes. However, these dominant negative receptors lose the ability to interfere with signaling from wild-type receptors when they are truncated. In addition, unoccupied full-length receptors interfere with basal levels of signaling from constitutively active receptors, whereas truncated receptors are unable to do so. Finally, the lethality associated

with a temperature-sensitive mutant of Gpa1 in *ste2Δ* background is reversed upon expression of full-length but not truncated receptors. Each of these examples is consistent with the C-terminal tail playing a central role in sequestering G proteins. However, crosslinking and coprecipitation studies presented in this chapter indicate that Ste2-T326 is able to interact with both Ste4 and Ste18. This apparent inconsistency might be due to the differences in the nature of the two assays. Genetic evidence indicates that the tail improves the ability of receptors to compete for a limiting pool of G proteins. Crosslinking results indicate that tail is not required for interaction in the absence of competition. In fact, it is not surprising to detect a physical interaction between the receptor and the G proteins since the truncated receptor is still proficient for signaling and therefore maintains its ability to interact with the downstream components of the signal transduction pathway. It is also plausible that the residues responsible for the interaction between Ste2-T326 and Ste4 detected in the crosslinking assay are different from those responsible for the interactions detected with the wild-type receptor and G proteins. The stabilizing effect imposed by the C-terminal tail might introduce some contacts that do not involve proximal lysines and therefore cannot be detected with the crosslinking reagent. Therefore, the *in vitro* results presented in this thesis complement the *in vivo* data obtained by Dosil and coworkers, and further suggest the existence of a preactivation complexes in yeast.

The α subunit, Gpa1 was not detected in the immune blots of the precipitated material. It should be noted that failure to detect crosslinking between two proteins in this assay does not mean the proteins do not associate with each other. Since the crosslinking reagent requires two amine groups positioned within 12Å to form a covalent bond efficiently, the inability to crosslink only indicates that the two proteins lack two lysines in close proximity. Additional experiments where both Ste2 and Gpa1 were tagged and

overproduced, showed that only a small fraction of Gpa1 coimmunoprecipitates with the receptor, and the presence of crosslinker did not interfere with or enhance the levels of precipitated Gpa1 (not shown).

Only a small fraction (about 1%) of Ste4 from the particulate fraction crosslinked to and precipitated with the HA-tagged receptor. Several factors may contribute to this low level of coprecipitation. First, the concentration of the crosslinking reagent and the protein:crosslinker ratio were determined so as to avoid extensive non specific crosslinking. Consequently, some of receptors and G proteins remained uncrosslinked. Second, the subcellular fractionation studies conducted in yeast have shown that less than half of Ste4 actually colocalizes with Ste2 on the plasma membrane (Hirschman et al., 1997). Authors of the study reported that roughly 40% of Ste4 was found in the plasma membrane fraction, 30% in other buoyant membranes and 30 % in denser fractions with no detectable membrane markers. The particulate fraction used in my experiments to collect the crosslinked material prior to immunoprecipitation, was reported to contain about 85% of the cellular Ste4 (Hirschman et al., 1997) suggesting that Ste4 from all three distinct locations was present in this fraction. Third, even though I was able to precipitate the majority of the crosslinked material, a small fraction of the complexes stayed in the supernatant. The HA tag was chosen since it lacks lysine residues and therefore minimizes the risk of modifying the epitope with the crosslinking reagent. It is possible that in some cases, the epitope tag is buried inside the complex and therefore is unavailable to the antibody in the precipitation reaction. Finally, the stoichiometry of the G protein:receptor has not been determined in yeast. In mammalian systems some quantitative estimates range from 10-to-100 fold excess of G protein over receptors (Insel et al., 1983; Ransnas and Insel, 1988). If there are more G proteins than receptors in yeast, this would readily explain why so little Ste4 would precipitate in the assay. It would also be consistent with the observation that more G

proteins are coprecipitated when receptors are overproduced. This hypothesis is in apparent contrast with the genetic data obtained from allelic interactions between mutant and wild-type receptors. Coexpression of signaling-defective dominant negative receptors with wild-type receptors inhibited signaling from these receptors, and overproduction of G protein subunits reversed this phenotype. (Dosil et al., 1998; Leavitt et al., 1999). If an excess of G proteins existed in yeast, such a titration effect might be hard to accomplish. This observation suggests that the G protein levels become limiting when receptor levels are increased about only two-fold, assuming that both mutant and wild-type receptors were produced in physiological levels. However, as mentioned previously not all of the G proteins reside in the plasma membrane (Hirschman et al., 1997). In addition, the fraction of G proteins located on the plasma membrane may not be active for coupling to the receptor.

It is not clear whether the Gβ or the Gγ makes direct contact with the receptor in the crosslinking assay. The data do not exclude the possibility that the interaction detected may be mediated by a bridging protein. As mentioned, when both the α subunit, Gpa1, and Ste2 were overproduced, a small fraction of Gpa1 coprecipitated with the receptor, and the coprecipitation was not dependent on the presence of the crosslinking reagent (not shown). Evidence from mammalian systems indicates a direct association between GPCRs and all three subunits of the heterotrimeric G proteins (Downes and Gautam, 1999; Milligan, 1996; Neubig, 1994). In addition, the crystal structure of rhodopsin reveals sufficient cytoplasmic surface area available for interaction with all G protein subunits (Bourne and Meng, 2000; Palczewski K, 2000). Therefore, it is plausible that all three G protein subunits interact with the receptor. Further experiments are needed to map the regions of contact between the receptor and the G protein.

Failure to identify other Ste2-interacting proteins can be attributed to the interference of the crosslinker with trypsin digestion and the production of partially digested products that carried a piece of the covalently attached crosslinker. The first immunoprecipitation protocol used the N-terminal HA tag and thereby eliminated the C-terminal degradation products. The use of Ni-column required the His6 tag at the C terminus of the protein and thus eliminated N-terminal degradation products. It is also possible that the crosslinker was not able to effectively introduce covalent bonds due to the constrained arrangement of receptor domains and lack of proximal lysines. The SDS-resistant dimers and perhaps tetramers of the receptor that were irreversible by the reducing agents may have made up the majority of complex 1.

In this chapter, I sought to identify new proteins interacting with the α-factor receptor. I have identified Ste4 and Ste18 in the crosslinked and precipitated complexes that contained Ste2. These results provide the first biochemical evidence for a physical association between Ste2 and Ste4/Ste18 in this system. In addition, my results provide biochemical evidence for the existence of preactivation complexes in yeast and complement the *in vivo* genetic studies.

CHAPTER V

INTERNALIZATION OF THE α-FACTOR PHEROMONE RECEPTOR

Peptide motifs mediate the endocytosis of cell-surface receptors by linking the protein to the internalization machinery in the cell. In mammalian cells, well-defined internalization signals include dileucine, dilysine or aromatic ring-containing motifs (containing tyrosine or phenylalanine) (D'Hondt et al., 2000). Two endocytic targeting motifs have been identified in yeast. One is NPFXD, which is present in Kex2, a resident Golgi enzyme, and in Ste3, the **a**-factor receptor (Tan et al., 1996). The other endocytic motif DAKSS (more specifically SINNDAKSS for Ste2), was identified in Ste2, the α-factor receptor (Rohrer et al., 1993). Two additional yeast proteins, ABC transporter, Ste6, and general amino acid permease, Gap1 have been proposed to contain DAKSS-like motifs that govern their endocytosis as well (Hein and Andre, 1997; Kolling and Hollenberg, 1994). Both of NPFXD and SINNDAKSS provide endocytic activity and subsequent vacuolar degradation of truncated versions of Ste3 and Ste2, respectively, in that they are sufficient for endocytosis of truncated Ste2 mutants that would otherwise lack endocytic activity (Rohrer et al., 1993; Tan et al., 1996). However, in the context of the full-length receptors, mutations affecting these signals lead to only marginal defects in internalization suggesting that Ste2 and Ste3 contain multiple endocytosis signals (Tan et al., 1996; Terrell et al., 1998).

The mechanism by which the NPFXD sequence mediates endocytosis is not known. In mammalian cells, analyses of aromatic ring-containing motifs were found to

associate with the AP2 adaptor complex (Backer et al., 1992; Bansal and Gierasch, 1991; Collawn et al., 1990; Eberle et al., 1991). AP2 is a component of the clathrin coat that mediates endocytic uptake at the plasma membrane (Beltzer and Spiess, 1991; Chang et al., 1993; Glickman et al., 1989; Nesterov et al., 1995; Ohno et al., 1995). Eps15, an AP2-interacting protein, was shown to bind the NPF sequence (Haffner et al., 1997). Three yeast proteins End3 (Benedetti et al., 1994; Raths et al., 1993), Pan1 (Tang et al., 1997; Wendland et al., 1996) and Ede1 (Gagny et al., 2000), which have been implicated in endocytosis, contain Eps15 homology (EH) domains. However, the role of adaptin homologs and clathrin in directing endocytic processes in yeast is unclear.

The lysine and serines in the SINNDAKSS motif have been shown to be critical for the endocytosis of a truncated form of Ste2 (Hicke and Riezman, 1996; Rohrer et al., 1993). Further analysis revealed that lysine 337 was ubiquitinated (Hicke and Riezman, 1996) and that phosphorylation of all three serines is required for ubiquitination of this lysine (Hicke et al., 1998). Ste2 is mostly mono- and diubiquitinated, and it is degraded in the vacuole (Hicke and Riezman, 1996; Schandel and Jenness, 1994; Terrell et al., 1998). Apparently, short ubiquitin chains mediate endocytosis of Ste2 rather than its degradation by the proteasome (Hicke and Riezman, 1996; Terrell et al., 1998). Ubiquitin is thought to be sufficient for endocytosis (Shih et al., 2000), since a genetic fusion construct that results in covalent attachment of a single ubiquitin moiety to the C-terminus of a tail-less receptor (lacking all endocytic signals) permits the fusion protein to be endocytosed (Terrell et al., 1998). Mutations affecting the SINNDAKSS sequence in the context of the full-length receptor have little effect on receptor endocytosis, presumably because the receptor tail contains multiple lysines that serve as redundant sites for ubiquitination. Changing all of the lysines in the receptor tail to arginines has

been found to reduce the internalization by six-fold but does not completely abolish receptor endocytosis (Terrell et al., 1998). Mutations affecting any of the three serines in the SINNDAKSS sequence in Ste2-T345 lead to loss of constitutive as well as ligand-mediated endocytosis. In contrast, the same mutations cause only a three-fold decrease in the rate of constitutive endocytosis in the context of the full-length receptor. It has therefore been proposed that other signals in the tail mediate receptor uptake and operate by a different mechanism (Terrell et al., 1998). One candidate signal in the Ste2 tail is the GPFAD sequence in the Ste2 tail since it resembles the NPFXD motif from Kex2 and Ste3 (Tan et al., 1996).

In this chapter, I summarize my genetic analysis of endocytic signals in the C-terminal tail of the α-factor receptor. Internal deletions of the *STE2* gene were constructed that remove sequences encoding selected regions of the C-terminal tail. Strains containing these alleles were then assayed for endocytosis in the presence and absence of α-factor. Residues from 360 to 431 were sufficient to mediate both constitutive and ligand-mediated endocytosis of the receptor even though 63 residues including the SINNDAKSS motif had been removed. These results suggest the presence of at least one alternative region in the tail that mediates endocytosis of the receptor. Another receptor mutant that only contained residues from 391 to 431 from the C-terminal tail was unable to undergo constitutive endocytosis as well as ligand-mediated endocytosis, suggesting that residues 360 to 391 are critical for endocytosis of the Ste2-Δ(297-360) receptor or they may be required for function of GPFAD. Further analysis indicated that Ste2-Δ(297-391) mutant did not undergo the α-factor-induced mobility shift characteristic of receptor hyperphosphorylation. Overall, these results suggest that a new region in the C-terminal of the α-factor receptor is sufficient to

mediate the constitutive endocytosis as well as the ligand-mediated endocytosis of the receptor.

Results

Internal deletions of the α-factor receptor tail

The C-terminal tail of the α-factor receptor is thought to contain multiple endocytosis signals, since the deletion of the well-characterized SINNDAKSS sequence alone has little effect on endocytosis. I sought to identify additional regions in the tail that mediate endocytosis. I used internal deletions of the *STE2* that remove portions of the tail including the SINNDAKSS sequence, and I tested these constructs for endocytosis in the presence and in the absence of α-factor. One problem in studying the down regulation of deletion constructs is that the remaining portions of the tail may become more susceptible to cellular proteases and may be degraded leaving a tail-less receptor that is proficient in binding the α-factor but is unable to undergo endocytosis. Consequently, the binding assays performed with strains harboring deletion constructs may be simply measuring binding to tail-less receptors (Konopka et al., 1988). In order to circumvent this potential problem, GFP was fused after the C-terminal residue of each construct. Therefore, detection of GFP ensures that the rest of the tail is intact. GFP did not alter the function of the receptor as evidenced from halo assays and down regulation assays. The C-terminal cytoplasmic domain of the receptor extends from residue 297 through the C-terminus at residue 431. Constructs that encode receptors with specific defects in the C-terminal tail are depicted in Fig. 1. These receptor-GFP fusions under the control of the native *STE2* promoter were integrated into the chromosomal *URA3* locus of a *ste2Δ* strain. The ability of these fusion proteins to undergo endocytosis was evaluated by fluorescence microscopy.

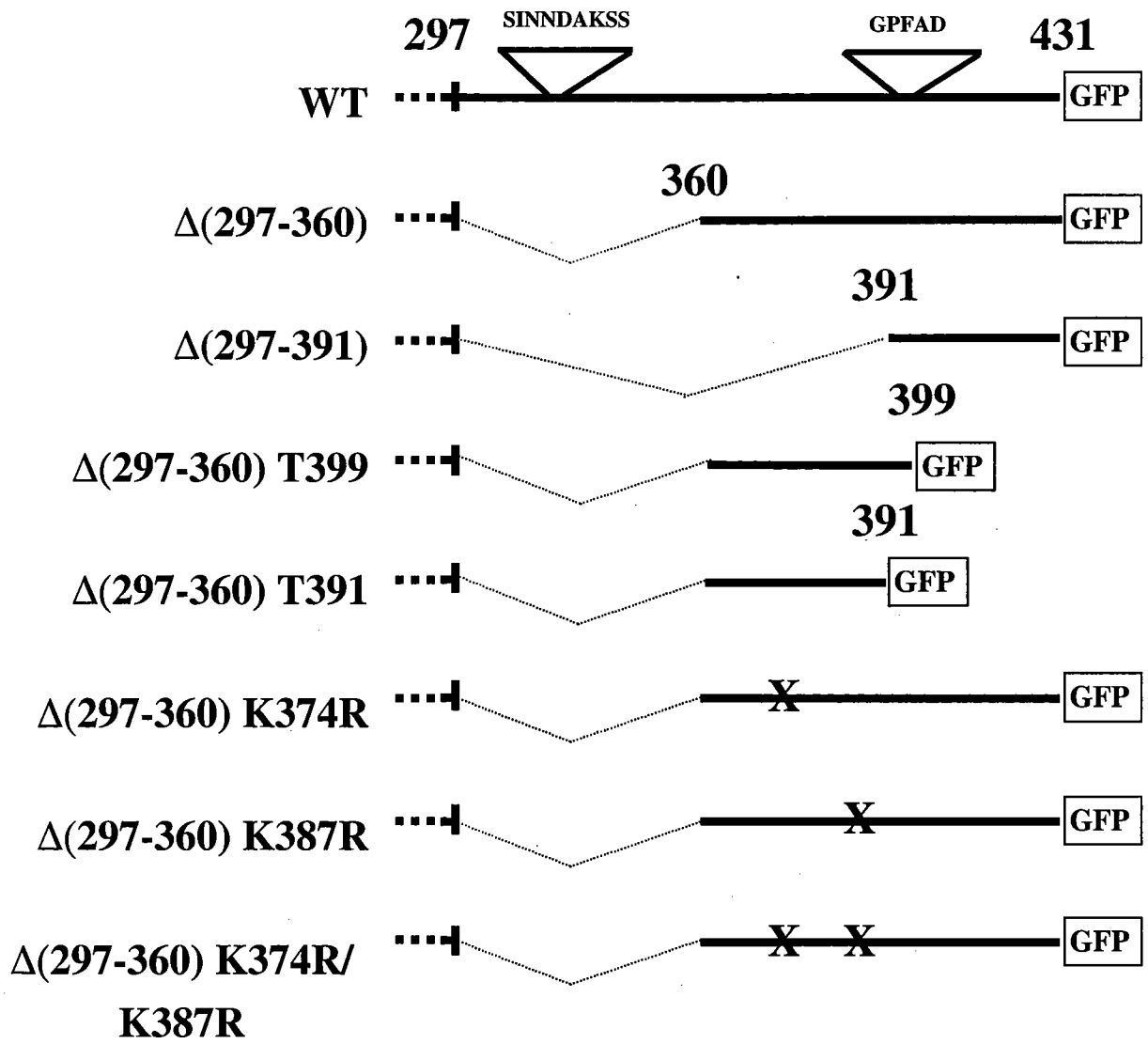


Figure 1. The schematic representation of the C-terminal cytoplasmic tails of various α-factor receptor-GFP fusion constructs. The positions of the lysine-to-arginine mutations are indicated as cross signs. The positions of SINNDAKSS an GPFAD sequences are shown for the wild-type construct.

I analyzed strains carrying deletion mutants of Ste2 tagged with GFP. Cells had been treated with cycloheximide to block new protein synthesis. The microscopic analysis of strains carrying GFP-tagged wild-type receptors typically revealed fluorescence on the cell surface and in the vacuole in the absence of α-factor (Fig. 2, top row, left panels). When the cells were treated with α-factor, the wild-type receptors were rapidly internalized and transported to the vacuole for degradation. As a result, the cell-surface fluorescence diminished (Fig. 2, top row, right panels). Cells expressing Ste2-Δ(297-360)-GFP were subject to endocytosis upon α-factor treatment as evidenced by a reduction in cell surface fluorescence (Fig. 2, middle row). Cells producing Ste2-Δ(297-391)-GFP did not endocytose their receptors since their cell surface fluorescence did not diminish following α-factor treatment (Fig. 2, bottom row). It also appeared that Ste2-Δ(297-360)-GFP was internalized more slowly than the wild-type receptors, since a faint surface fluorescence remained after α-treatment.

When endocytosed receptor-GFP fusions reach the vacuole, the receptor is degraded while the GFP portion resists degradation and accumulates in the vacuole (Li et al., 1999). Free GFP accumulation is, therefore, considered to indicate delivery of receptor-GFP to the vacuole. Even in the absence of α-factor, the cells expressing Ste2-Δ(297-360)-GFP fusion had vacuolar fluorescence that was similar to the cells expressing wild-type receptors (Fig. 2, top and middle rows, left panels). This result suggests that the Ste2-Δ(297-360)-GFP fusion is able to undergo constitutive endocytosis as well as ligand-induced endocytosis. On the other hand, the cells expressing the endocytosis-defective Ste2-Δ(297-391)-GFP had no detectable free GFP in the vacuole in the presence or in the absence of α-factor. Therefore, Ste2-Δ(297-391)-GFP is apparently defective for constitutive endocytosis in addition to its α-factor-mediated

endocytosis defect. Consistent with these results, western blotting analysis indicated very little free GFP from cells expressing the Ste2-Δ(297-391)-GFP (Fig. 3 lane 2). The Ste2-Δ(297-360)-GFP and the wild-type cells both produced larger amounts of free GFP (Fig. 3 lanes 3 and 4 respectively). The relative proportion of GFP that accumulates in the vacuole apparently depends on the growth rates of the cells, since cells that contained fewer auxotrophies showed lesser vacuolar signal (Fig. 4A).

Overall, these results suggest that 71 amino acids at the C-terminal end of the Ste2 cytoplasmic tail from residues 360 to 431 contain enough information to mediate both the constitutive and α-factor-mediated endocytosis of the receptor and define a new region in the tail that is critical for this process. In addition, more severe deletions including the residues 360 to 391 in the Ste2-Δ(297-391)-GFP mutant completely disrupt endocytosis. In other words, residues 391 to 431 are not sufficient to mediate endocytosis alone.

Additional deletion analysis of Ste2-Δ(297-360)-GFP

In order to determine whether residues 360-391 carry sufficient information to confer endocytosis, I evaluated a construct that fused these 31 amino acids to a severely truncated receptor generating Ste2-Δ(297-360)-T391-GFP. The fluorescence analysis of the resulting mutant indicated that the mutant receptors were incapable of mediating α-factor-mediated endocytosis, and receptors accumulated at the cell surface with no vacuolar GFP accumulation (Fig 4C). Therefore, the region between residues 360 to 431 requires sequences at both ends to function properly in endocytosis, since neither half of this region is sufficient to carry out endocytosis alone.

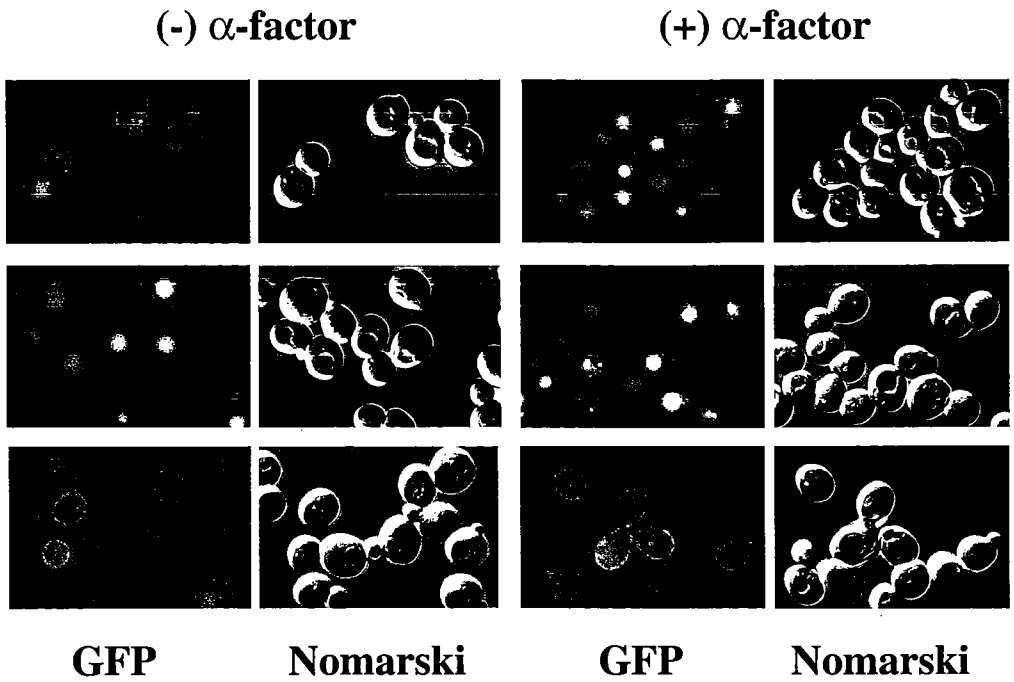


Figure 2. Ligand-induced internalization of GFP-tagged α-factor receptors harboring internal deletions in the C-terminal tail. Exponentially-growing cultures grown in rich YM1 medium were treated with cycloheximide and then cultured for 15 min in the absence or presence of 10^{-7} M α-factor. Further receptor endocytosis was inhibited by incubating the cells with metabolic poisons NaN_3 and KF. Cells were visualized by fluorescence and Nomarski microscopic methods. First two columns show the control cultures receiving no α-factor; the last two columns are α-factor-treated cells. GFP fluorescence and Nomarski images are indicated below each column. Top row, cells expressing Ste2-GFP (strain DJ1494). Middle row, cells expressing Ste2-Δ(297-360)-GFP (strain DJ1442). Bottom row, cells expressing Ste2-Δ(297-391)-GFP (strain DJ1443).

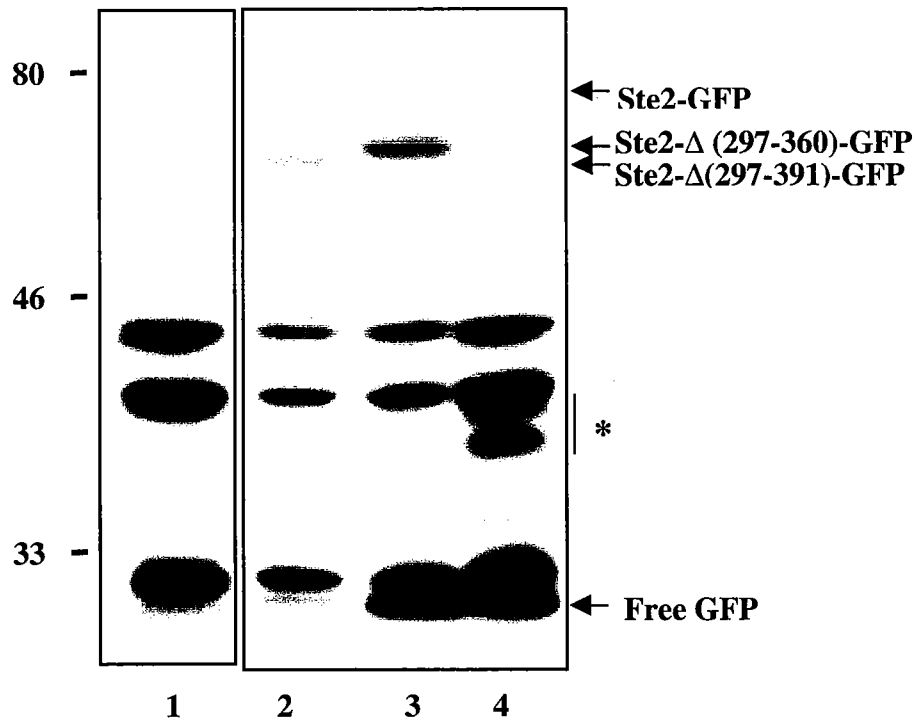
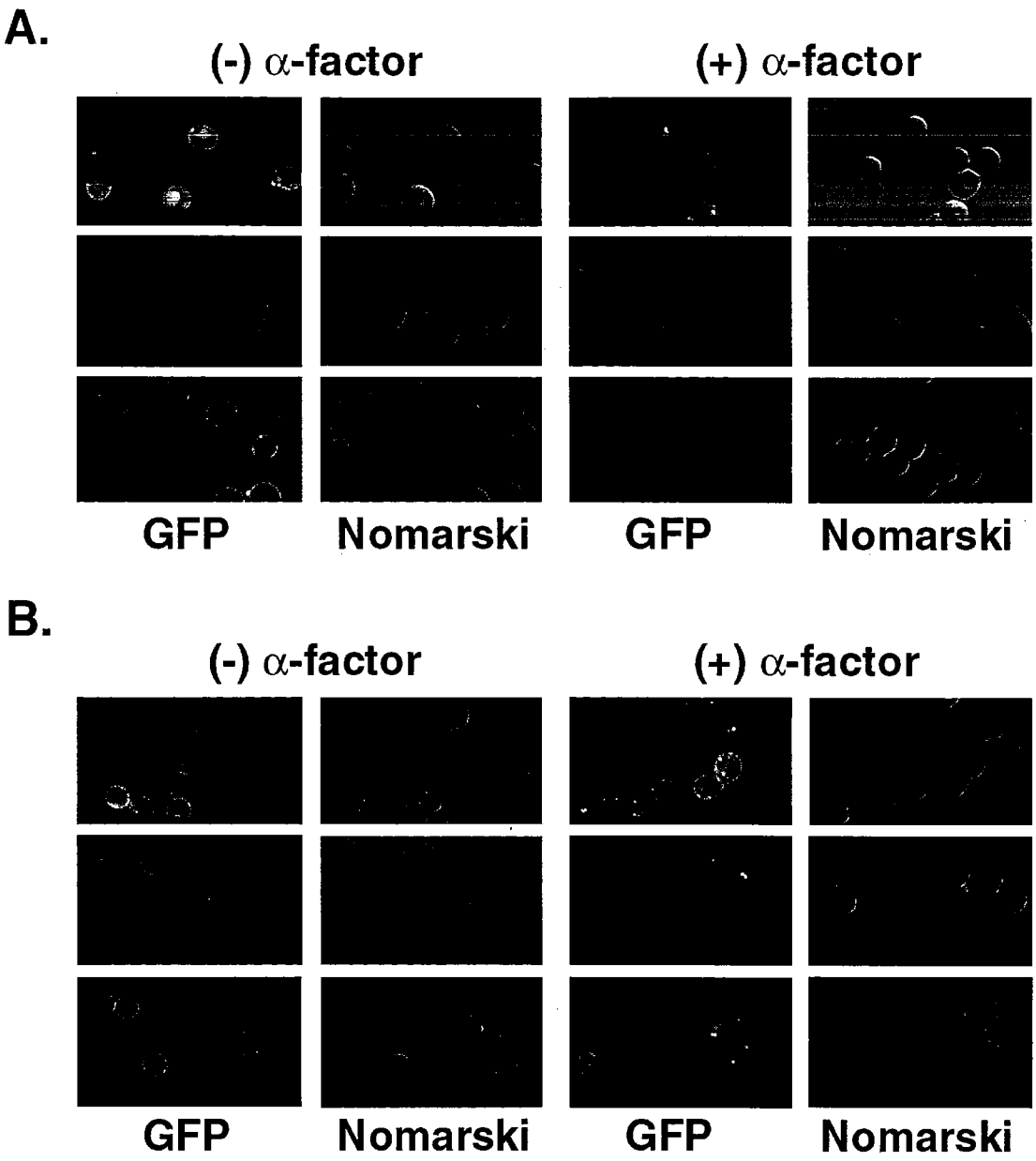


Figure 3. Western blotting analysis of the α–factor receptors harboring internal deletions in the C-terminal tail. Lysates were prepared from cells expressing Ste2-Δ(297-391)-GFP (lane 2), Ste2-Δ(297-360)-GFP (lane 3), Ste2-GFP (lane 4). Lane 1 is a negative control from cells expressing no GFP or Ste2. The samples were then processed for SDS-PAGE analysis and resolved on a 10% SDS gel. Proteins were detected with monoclonal anti-GFP antibodies and western blotting methods. Molecular weight standards are indicated at the left. The positions of the GFP fusion proteins and free GFP are indicated with arrows on the right. The asterisk marks a commonly-detected degradation product of Ste2-GFP (Li et al., 1999). Strains were DJ213-7-3 (ste2Δ), DJ1443 (Ste2-Δ(297-391)-GFP), DJ1442 (Ste2-Δ(297-360)-GFP) and DJ1494 (Ste2-GFP).


A.
(-) α-factor
(+) α-factor
GFP
Nomarski
GFP
Nomarski
B.
(-) α-factor
(+) α-factor
GFP
Nomarski
GFP
Nomarski

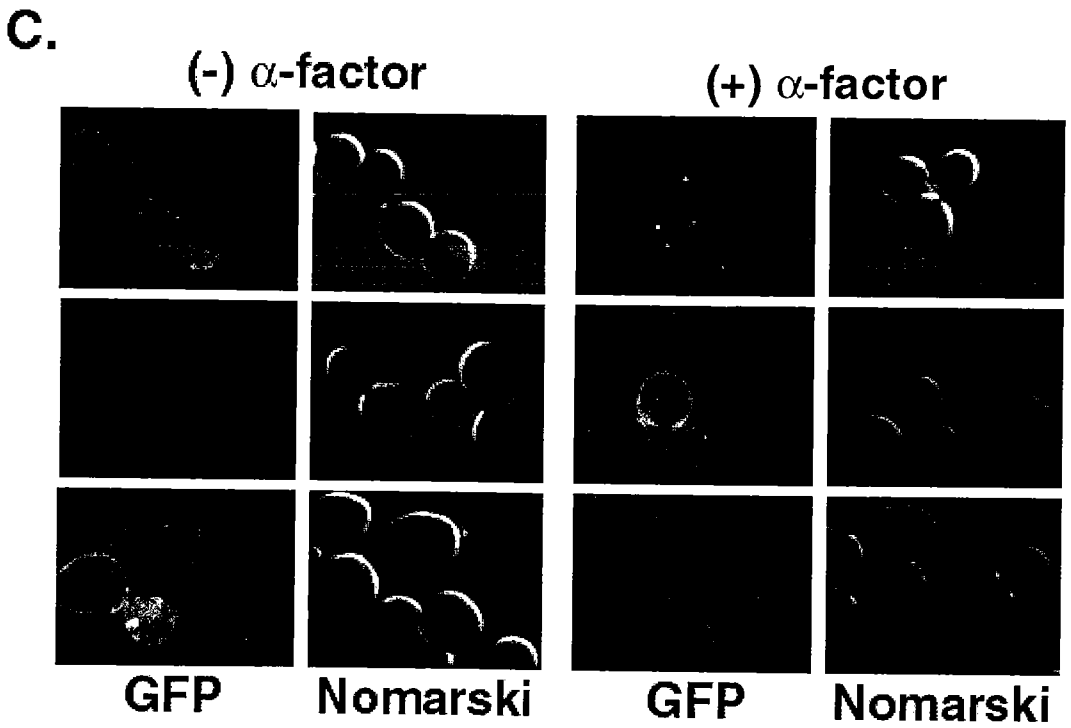


Figure 4. Ligand-induced internalization of GFP-tagged α-factor-receptors with internal deletions combined with lysine substitutions and truncations in the C-terminal tail. Exponentially-growing cultures in rich YM1 medium were treated with cycloheximide and α-factor as described in Fig. 2. The first two columns are controls lacking α-factor; the last two columns are α-factor-treated cells. GFP fluorescence images and Nomarski images are indicated below each column. The GFP images shown in panels A and B were derived from the same experiment, but divided into two panels for presentation purposes. **(A)** Top row, cells expressing Ste2-GFP (strain DJ1460). Middle row, cells expressing Ste2-Δ(297-360)-GFP (strain DJ1461). Bottom row, cells expressing Ste2-Δ(297-391)-GFP (strain DJ1462). **(B)** Top row, cells expressing Ste2-Δ(297-360)-K374R-GFP (strain DJ1466). Middle row, cells expressing Ste2-Δ(297-360)-K387R-GFP (strain DJ1467). Bottom row, cells expressing

Ste2-Δ(297-360)-K374R/K387R-GFP (strain DJ1468). **(C)** Top row, cells expressing Ste2-GFP (strain DJ1460). Middle row, cells expressing Ste2-Δ(297-360)-T391-GFP (strain DJ1483). Bottom row, cells expressing Ste2-Δ(297-360)-T399-GFP (strain DJ1484).

A five amino acid stretch on the tail has been postulated to be one of the weaker endocytosis signals, since this sequence, GPFAD, resembles another endocytosis motif NPFXD which is found on Ste3 and Kex2 (Tan et al., 1996). Another receptor mutant Ste2-Δ(297-360)-T399-GFP containing GPFAD in addition to the 360-391 sequence was created to address this possibility. However, this sequence in conjunction with the 360-391 sequence did not confer endocytosis to the receptor (Fig 4C). Therefore, the structural determinants constituting the C-terminal end of the endocytosis signal extend beyond residue 399. These elements may be required for direct recognition or for folding.

Substituting the lysines in the region between residues 360-391

Lysine 337 in the SINNDAKSS sequence has been shown to be required for internalization of Ste2-T345 (Hicke and Riezman, 1996; Rohrer et al., 1993). Furthermore, addition of the SINNDAKSS sequence to a severely truncated receptor restored endocytosis but not if the lysine is changed to an arginine (Rohrer et al., 1993). As mentioned previously, a receptor mutant that contains no lysines in the tail is able to undergo endocytosis, albeit six times more slowly (Terrell et al., 1998). There are two lysines in the 360-391 region of the tail at positions 374 and 387. It has been previously reported that Lys374 is the second most ubiquitinated lysine in the tail after the Lys337 of the SINNDAKSS sequence (Terrell et al., 1998). I wished to test whether Lys374 or Lys387 play a role in the activity of the endocytosis elements located in the C-terminal element. These two lysines were changed to arginines in the context of the Ste2-Δ(297-360)-GFP mutant (see Fig. 1), and the ability of these mutants to undergo endocytosis was evaluated. Cells carrying the mutant alleles were treated with

cycloheximide and α-factor as described above. Fluorescence microscopy analysis indicated that all of the lysine-to-arginine mutants were endocytosed upon α-factor exposure (Fig. 4B). Punctate fluorescent structures appear after α-factor treatment, presumably corresponding to endosomes. Therefore, the endocytic defect observed with Ste2-Δ(297-391)-GFP is not merely due to the absence of the two lysines in the 360-391 region.

Half-life assays and down regulation analyses of the deletion constructs

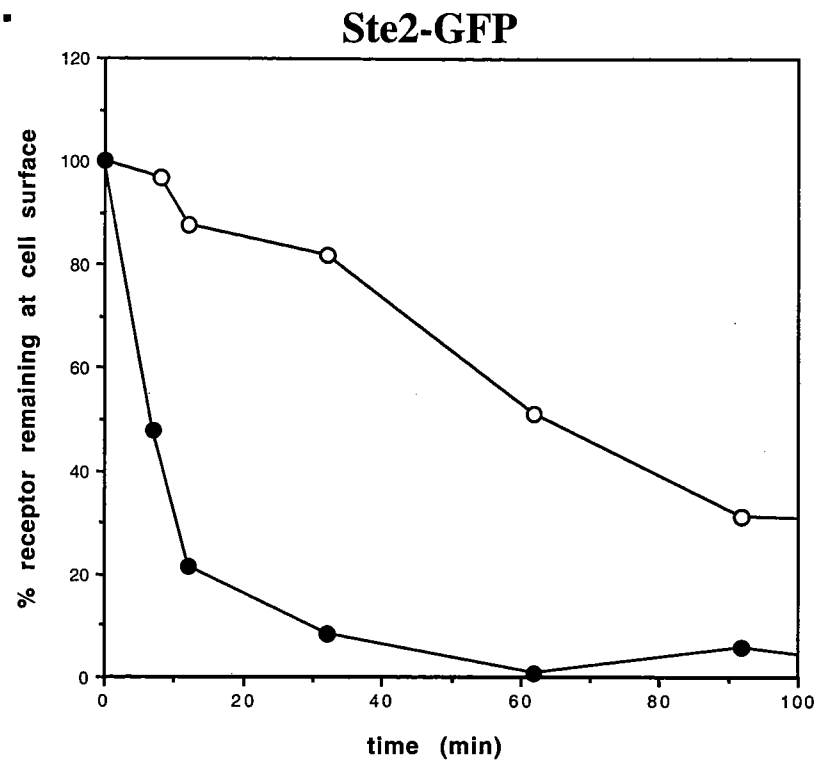
In order to obtain a quantitative evaluation of the effect of deletion and lysine-to-arginine mutants on constitutive endocytosis, I assayed the half-life of cell-surface receptor sites and total receptor protein. Briefly, the first assay utilizes radioactive α-factor binding to whole cells and therefore measures receptor exit from the plasma membrane. The second assay employs western blotting to determine the half-life of total receptor protein turnover, since endocytosed receptors are degraded in the vacuole. Thus the second assay measures receptor delivery to the vacuole.

Down regulation of receptor sites in the various mutants are depicted in Fig. 5. Cultures were treated with cycloheximide and cold α-factor as previously described (Jenness and Spatrick, 1986). Cells were collected on filters, and further receptor endocytosis was prevented by metabolic poisons. The bound cold α-factor was allowed to dissociate. The binding of radioactivity was used as a measure of the remaining receptor sites on the cell surface. Table 1 lists half-lives for receptor exit from the plasma membrane derived from the graphs in Fig. 5. Consistent with previous findings, the half-life for plasma membrane exit for the wild-type receptors was 7 minutes (Jenness

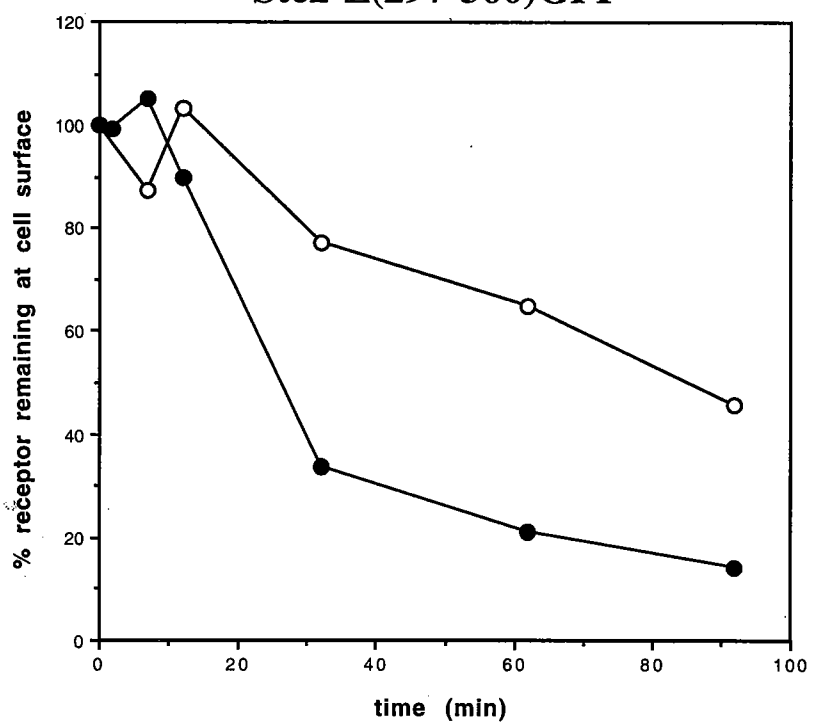
and Spatrick, 1986). The Ste2-Δ(297-360)-GFP mutant and all of the lysine-to-arginine mutants exited the plasma membrane more slowly than the wild-type receptors both in the presence and in the absence of α-factor. These results are in agreement with my previous observations with the fluorescence microscopy analysis (see Fig. 2, Fig. 4A and 4B). As expected, the Ste2-Δ(297-391)-GFP mutant had the most severe defect in both constitutive and ligand-mediated endocytosis. In fact, constitutive endocytosis was virtually abolished in the Ste2-Δ(297-391)-GFP mutant. Some residual loss of receptor sites was detected for this mutant receptor in the presence of α-factor.

To measure receptor delivery to the vacuole in the absence of α-factor, the half-life of total receptor protein was measured. Cultures were treated with cycloheximide, and aliquots were removed at various time points. The total receptor protein remaining at each time point was detected by using western blotting methods with anti-GFP antibody. The receptor bands were quantitated and plotted in Fig. 6. As expected, Ste2-Δ(297-360)-GFP underwent constitutive endocytosis more slowly than the wild-type control (Fig. 6A). The Ste2-Δ(297-391)-GFP mutant protein had the most severe defect in constitutive endocytosis. Of the two lysine-to-arginine mutant receptors, Ste2-Δ(297-360)-K387R-GFP and double mutant Ste2-Δ(297-360)-K374R/K387R-GFP were degraded at a rate that was similar to the Ste2-Δ(297-360)-GFP mutant (Fig. 6B). The turnover of Ste2-Δ(297-360)-K374R-GFP was apparently slower than the other two lysine-to-arginine mutants in this assay. However, radioactive α-factor binding assays indicated that Ste2-Δ(297-360)-K374R-GFP exits the plasma membrane faster than the other two lysine-to-arginine mutant receptors in the absence of α-factor (see Fig. 5 and Table 1). As mentioned above, protein turnover assay measures the remaining levels of

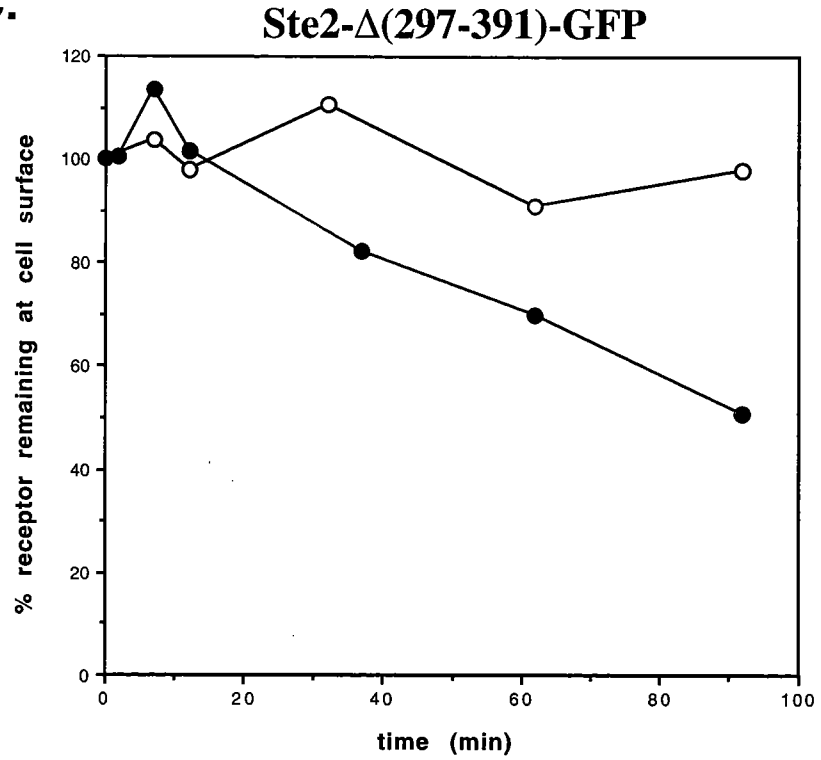
A.


Ste2-GFP
% receptor remaining at cell surface
time (min)
0
20
40
60
80
100
120

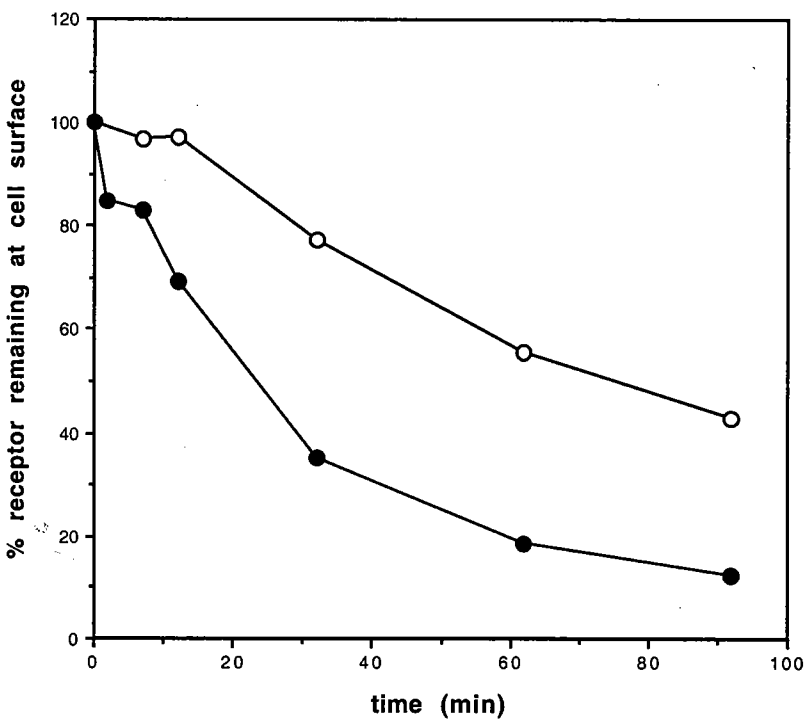
B.


Ste2-Δ(297-360)GFP
% receptor remaining at cell surface
time (min)
0
20
40
60
80
100
120

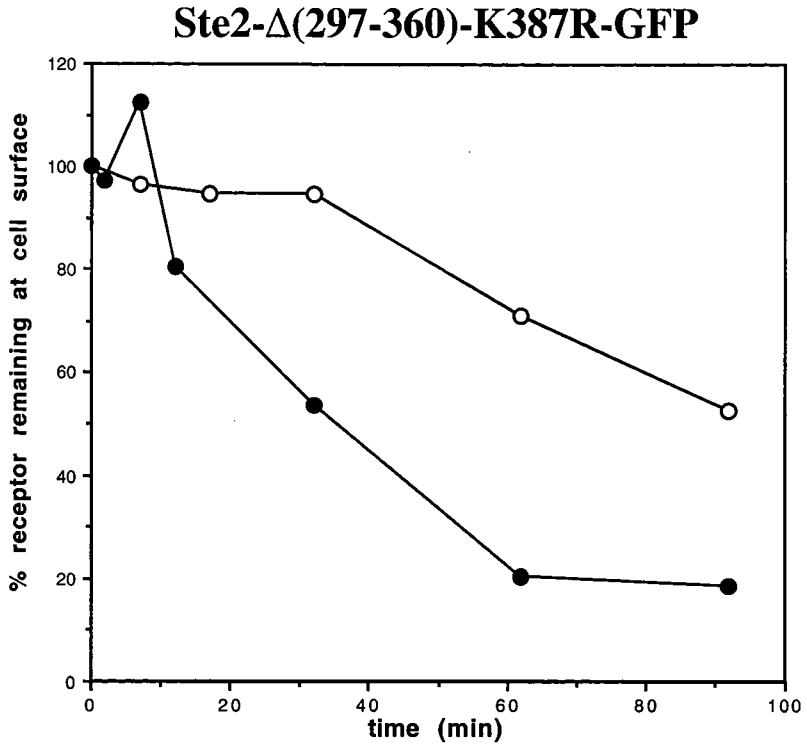
C.


Ste2-Δ(297-391)-GFP
% receptor remaining at cell surface
time (min)
0
20
40
60
80
100
120

D.


Ste2-Δ(297-360)-K374R-GFP
% receptor remaining at cell surface
time (min)
0
20
40
60
80
100
120

E.


Ste2-Δ(297-360)-K387R-GFP
% receptor remaining at cell surface
time (min)
0
20
40
60
80
100
120

F.

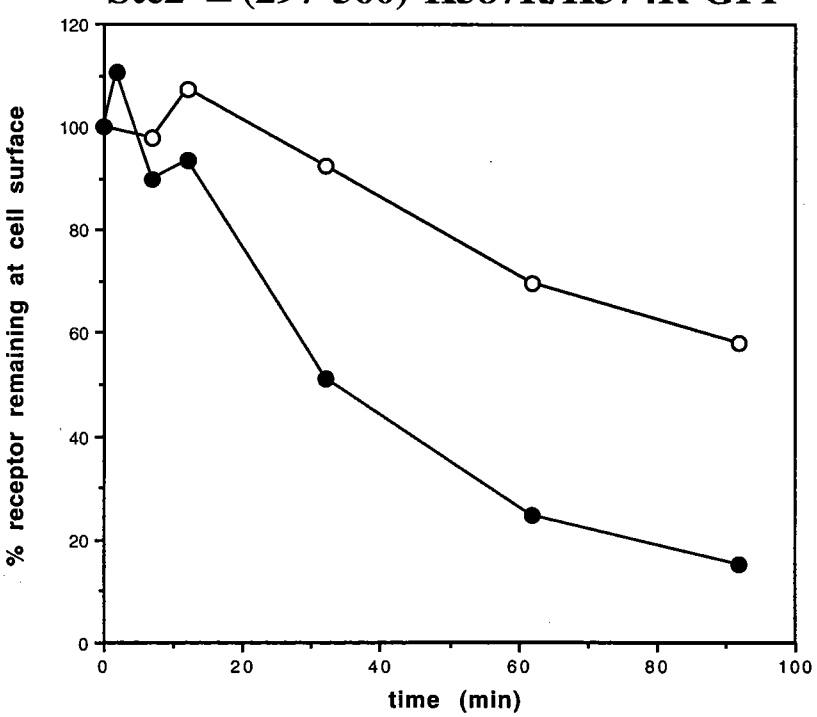

Ste2- Δ (297-360)-K387R/K374R-GFP
% receptor remaining at cell surface
time (min)
0
20
40
60
80
100
120

Figure 5. Down regulation of GFP-tagged α-factor-receptors harboring internal deletions and lysine substitutions in the C-terminal tail. Cultures received TAME and cycloheximide and were untreated (○) or treated (●) with unlabeled α-factor as described in Fig. 2. Aliquots of cells were removed at various time points. The bound unlabeled α-factor was allowed to dissociate. Cells were then incubated with ^{35}S-labeled α-factor, and the amount of radioactivity associated with the cells was measured. Cultures were **(A)** Ste2-GFP (strain DJ1460) **(B)** Ste2-Δ(297-360)-GFP (DJ1461) **(C)** Ste2-Δ(297-391)-GFP (DJ1462) **(D)** Ste2-Δ(297-360)-K374R-GFP (DJ1466) **(E)** Ste2-Δ(297-360)-K387R-GFP (DJ1467) **(F)** Ste2-Δ(297-360)-K374R/K387R-GFP (DJ1468).

TABLE 1. The rates of plasma membrane exit for different Ste2 deletion constructs

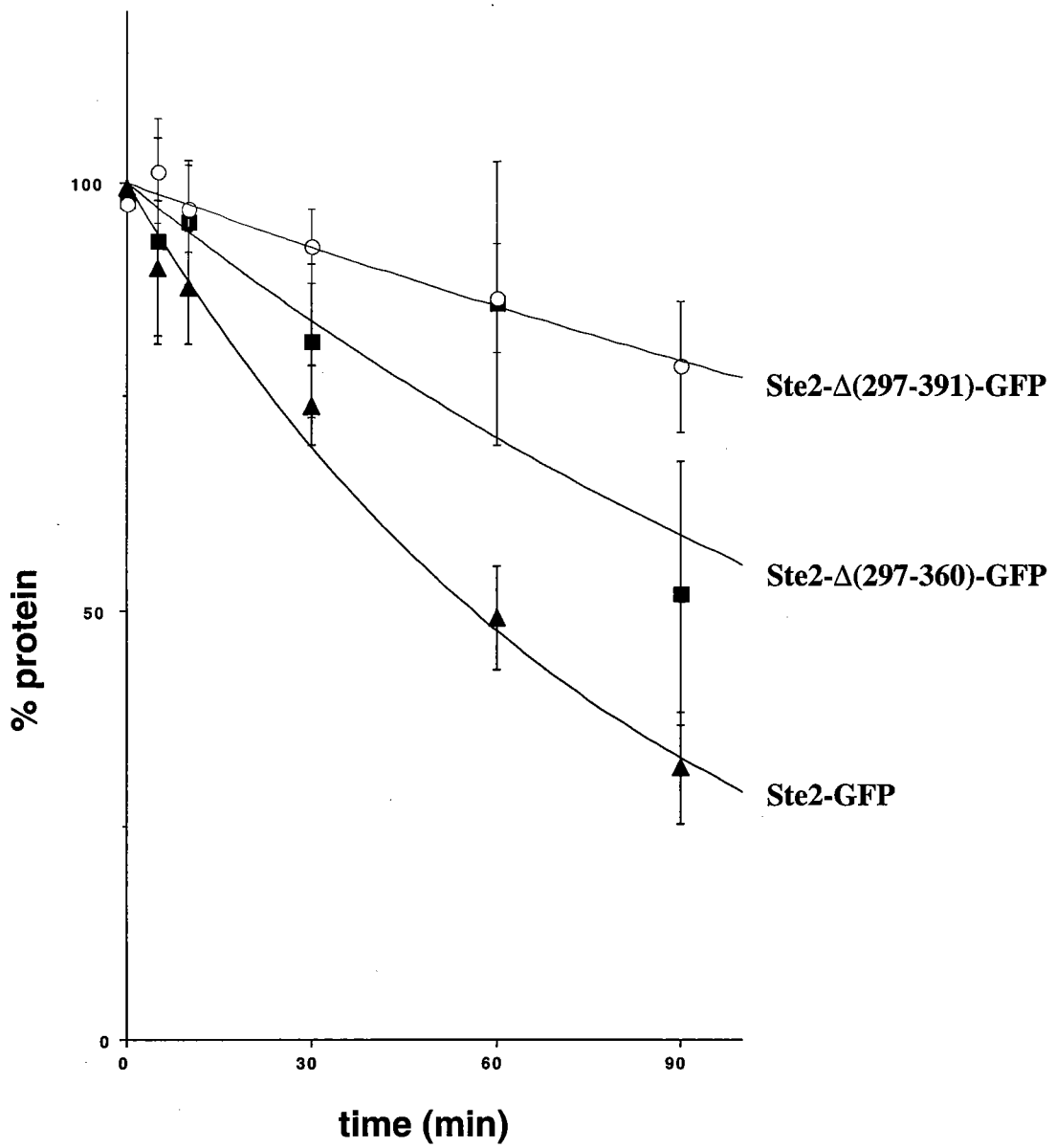
	Half-life for down regulation of receptors (min)[b]	
Ste2 allele expressed[a]	(-) α-factor	(+) α-factor
Ste2-GFP	60	7
Ste2-Δ(297-360)-GFP	90	27
Ste2-Δ(297-391)-GFP	stable[c]	90
Ste2-Δ(297-360)-K374R-GFP	76	25
Ste2-Δ(297-360)-K387R-GFP	>90	35
Ste2-Δ(297-360)-K374R/K387R-GFP	>90	34

[a] The strains used were the same ones as in Fig. 5.

[b] The half-life values for plasma membrane exit of each receptor was derived from the corresponding graph in Fig. 5.

[c] No loss of receptor sites was detected for the 90 min duration of the assay.

A.


100
50
0
% protein
0
30
60
90
time (min)
Ste2-Δ(297-391)-GFP
Ste2-Δ(297-360)-GFP
Ste2-GFP

B.

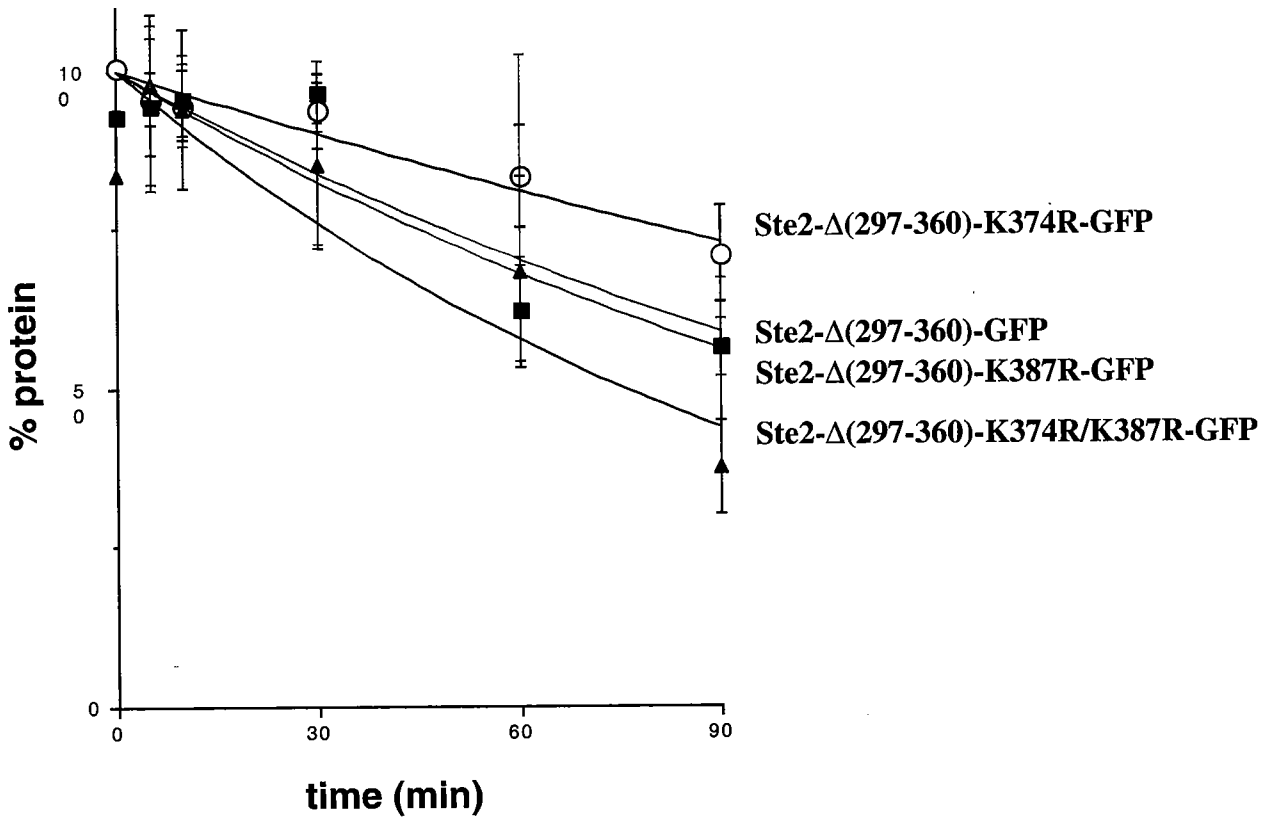


Figure 6. Turnover of GFP-tagged α-factor-receptors harboring internal deletions and lysine mutations in the C-terminal tail. Cultures were treated with cycloheximide and aliquotes were removed at various time points and placed on ice with metabolic poisons NaN3 and KF to prevent further receptor endocytosis. The samples were analyzed by SDS-PAGE and western blotting methods. **A.** Samples were Ste2-GFP (closed triangles), Ste2-Δ(297-360)-GFP (closed squares), Ste2-Δ(297-391)-GFP (open circles), **B.** Ste2-Δ(297-360)-GFP (no plot symbol), Ste2-Δ(297-360)-K374R-GFP (open circles), Ste2-Δ(297-360)-K387R-GFP (closed squares) and Ste2-Δ(297-360)-K374R/K387R-GFP (closed triangles).

receptors in the cell and therefore provides a measure of the combined efficiency of receptor down regulation, transport to the vacuole and degradation in the vacuole. Therefore, taken at face value these results indicate that Ste2-Δ(297-360)-K374R-GFP exits the plasma membrane faster than the other two lysine-to-arginine mutants, but it is delivered to the vacuole at a slower rate.

As a second measure for delivery of the mutant receptors to vacuole, I took advantage of appearance of free GFP that results from delivery of receptor-GFP to the vacuole. The free GFP can be detected by western blotting analysis. I quantified the free GFP to the receptor-fusion protein bands from western blots of untreated and α-factor-treated mutants in Fig 7A. The ratios of the receptor-fusion protein to the free GFP are listed in Table 2 for each of the mutants. As expected, free GFP accumulation in strains expressing Ste2-GFP and Ste2-Δ(297-360)-GFP was higher than the amount of free GFP in strains expressing Ste2-Δ(297-391)-GFP. This result is in agreement with the western blotting experiment performed in a different strain (see Fig. 3). The ratio of free GFP to receptor-fusion protein was lower in the strain expressing Ste2-Δ(297-360)-K374R-GFP than the other two lysine-to-arginine mutants. This finding is in agreement with the results obtained from the receptor turnover assay (Fig. 6) and may reflect a defect in the trafficking of this mutant to the vacuole. Interestingly, Lys374 is known to be ubiquitinated in wild-type receptors , and thus ubiquitination of Lys374 may play a role in intracellular trafficking events. However, because of the technical limitations in the ability to measure these events accurately, it is not possible to make a definitive conclusion at this time.

Phosphorylation of the α-factor receptor

Receptors contain phosphorylated serine and threonine residues in the absence of α-factor and then become hyperphosphorylated upon α-factor exposure (Reneke et al., 1988). α-Factor-treatment causes a slight decrease in the mobility of Ste2 on SDS gels (Hicke et al., 1998) (Fig. 7, compare lanes 1 and 7 in each panel). A similar mobility shift in wild-type receptors is thought to reflect phosphorylation because treatment with phosphatases reverses the ligand-induced shift (Hicke et al., 1998). The western blotting analysis of the untreated and α-factor-treated mutants indicated that the Ste2-Δ(297-391)-GFP mutant did not undergo the α-factor-induced mobility shift whereas wild-type receptors and all other mutants did (Fig. 7). A previous study (Chen and Konopka, 1996) utilized 2D gel analysis of tryptic fragments obtained from this mutant and indicated that the Ste2-Δ(297-391) receptor maintains basal levels of phosphorylation. They found that α-factor causes only a slight increase in the phosphorylation status of this receptor (Chen and Konopka, 1996). I have not detected any increase in the levels of phosphorylation. This discrepancy may be due to the difference in sensitivity of the two assays. Since phosphorylation is required for ubiquitination and the subsequent constitutive and ligand-mediated internalization of the receptor, the internalization defect of Ste2-Δ(297-391)-GFP may result from the lack of sufficient levels of phosphorylation or the lack of phosphorylation of specific residues.

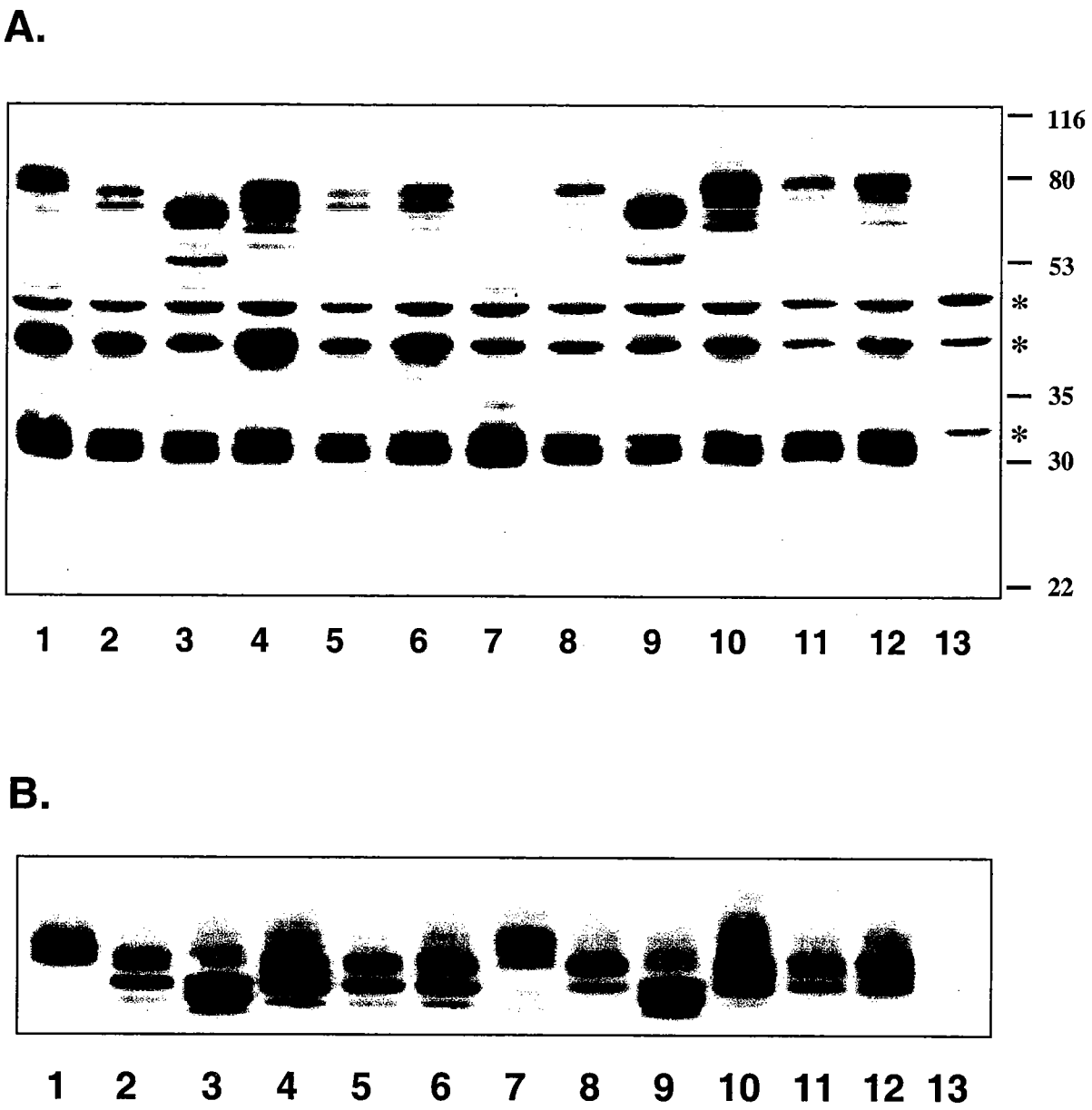


Figure 7. Western blotting analysis of GFP-tagged α-factor-receptors harboring internal deletions and lysine mutations in the C-terminal tail. Lysates were prepared from cells

untreated or treated with α-factor as described in Fig. 2. The samples were analyzed by SDS-PAGE and western blotting methods with either **(A)** monoclonal or **(B)** polyclonal anti-GFP antibodies. Lanes 1-6 show lysates from untreated cells and lanes 7-12 show cells treated with α-factor for 15 min. Lane 13 shows the untreated negative control cells that do not express GFP or Ste2. The asterisks mark the position of the nonspecific bands that are present in the control lane. Samples were Ste2-GFP (lanes 1 and 7), Ste2-Δ(297-360)-GFP (lanes 2 and 8), Ste2-Δ(297-391)-GFP (lanes 3 and 9), Ste2-Δ(297-360)-K374R-GFP (lanes 4 and 10) Ste2-Δ(297-360)-K387R-GFP (lanes 5 and 11) and Ste2-Δ(297-360)-K374R/K387R-GFP (lanes 6 and 12). Strains were Ste2-GFP (strain DJ1460), Ste2-Δ(297-360)-GFP (DJ1461), Ste2-Δ(297-391)-GFP (DJ1462), Ste2-Δ(297-360)-K374R-GFP (DJ1466), Ste2-Δ(297-360)-K387R-GFP (DJ1467) and Ste2-Δ(297-360)-K374R/K387R-GFP (DJ1468).

TABLE 2. The ratio of free GFP to the receptor-fusion protein for various Ste2 mutants

Ste2 allele expressed[a]	Ratio of free GFP to full-length receptor-fusion[b]
Ste2-GFP	1.00
Ste2-Δ(297-360)-GFP	1.28
Ste2-Δ(297-391)-GFP	0.34
Ste2-Δ(297-360)-K374R-GFP	0.37
Ste2-Δ(297-360)-K387R-GFP	1.52
Ste2-Δ(297-360)-K374R/K387R-GFP	0.78

[a] The strains used were the same ones as in Fig. 7.

[b] The ratios of receptor-fusion protein were quantified from the immunoblot in Fig. 7A. The values were then normalized to the value obtained from the wild-type receptor.

Discussion

In this chapter, I have utilized a deletion strategy to delineate an endocytic signal in the cytoplasmic tail of the α-factor receptor. GFP fusions of internal deletions in the Ste2 tail that lack the well-characterized endocytosis motif SINNDAKSS were studied. The analysis revealed that the last 71 amino acids in the tail are able to mediate both constitutive and α-factor-mediated internalization of the receptor. Structural features of this region that were investigated further were the highly ubiquitinated Lys374, the neighboring Lys387 and the GPFAD motif (residues 391-395). Lys374 and Lys387 were unnecessary for the element to promote exit from the plasma membrane; however, Lys374 may play some role in intracellular trafficking. The GPFAD motif was not sufficient to promote endocytosis, since the residues 360-399 provided no detectable endocytic activity. Further analysis suggested that Ste2-Δ(297-391)-GFP was unable to undergo hyperphosphorylation after α-factor treatment.

It has been proposed that the efficiency of endocytosis by the ubiquitination pathway is a function of the number of the ubiquitinated lysine residues present in a receptor mutant (Terrell et al., 1998). Substituting all the lysines by arginines in the context of the full-length receptor causes a six-fold reduction in the rate of plasma membrane depletion of these receptors compared to the wild-type (Terrell et al., 1998). There are a total of eight lysine residues in the Ste2 tail. Four of these lysines remain in the Ste2-Δ(297-360)-GFP. The Ste2-Δ(297-360)-GFP mutant was endocytosed roughly four fold slower than the wild-type receptors in the presence of the α-factor. In addition, loss of Lys387 or both Lys374 and Lys387 caused a further reduction in the rate of receptor removal from the plasma membrane compared to the Ste2-Δ(297-360)-GFP

mutant that contained no lysine substitutions. These results are, therefore, in agreement with the aforementioned view that the number of ubiquitinated lysines may affect the efficiency of endocytosis. Multiple ubiquitinated lysines may provide more attachment points for the endocytic machinery that recognizes these signals. Oligomerization of the receptors may further enhance the efficiency of the process (Hicke, 2001; Yesilaltay and Jenness, 2000).

Since lysine substitutions do not completely abolish the internalization of the receptors, it has been speculated that lysines in the cytoplasmic loops may be ubiquitinated in a level that is undetected but may be sufficient to carry out the low level of endocytosis that is observed for the mutant that lacks lysines (Terrell et al., 1998). This seems unlikely because a truncated receptor that has all of its cytoplasmic loops intact and that signals and binds the ligand with wild-type affinity is unable to become endocytosed. On the other hand, it is possible that these mutant receptors are endocytosed through the action of an alternative mechanism. The NPFXD motif is one of the lysine-free endocytosis signals in yeast. Additionally, PEST-like sequences were indicated to play a role in the endocytosis of the **a**-factor receptor and uracil permease (Marchal et al., 1998; Roth et al., 1998).

Phosphorylation precedes ubiquitination (Hicke et al., 1998). Phosphorylation of the three serines of the SINNDAKSS sequence was shown to be required for the ubiquitination and subsequent internalization of a truncated receptor (Hicke and Riezman, 1996; Hicke et al., 1998). In addition, strains which were deleted for the yeast homologues of casein kinase I had defects in internalizing **a**-factor- and α-factor receptors (Hicke et al., 1998; Panek et al., 1997). The lack of extensive phosphorylation

in Ste2-Δ(297-391)-GFP could result from the absence of the critical serine and threonine residues that are potentially required for ubiquitination of lysines in the rest of the tail. Alternatively, but not mutually exclusive, the absence of the region 360-391 might cause a conformational change that inhibits the exposure of the serines and threonines in the rest of the tail to protein kinases. Therefore, the 360-391 region may either affect the folding of the receptor tail so some other signal on the tail such as GPFAD is no longer recognized by the endocytic machinery, or may directly be involved in recognition by the endocytic machinery. Chen and Konopka (Chen and Konopka, 1996) showed that Ste2-Δ(297-391)-GFP is phosphorylated in the absence of the pheromone. Addition of pheromone caused a slight increase in the levels of phosphorylation (Chen and Konopka, 1996). Mutating the four serine and threonine residues to alanines in the remainder of the tail of Ste2-Δ(297-391)-GFP completely abolishes the basal levels of phosphorylation, as detected in a phosphate labeling assay, and results in enhanced signaling activity (Chen and Konopka, 1996). Even though constitutive phosphorylation of these residues, observed in Ste2-Δ(297-391)-GFP, was apparently insufficient to confer endocytosis to the receptor, I can not exclude the possibility that these residues play a role in the full-length receptor.

Overall, these results define a new region in the cytoplasmic tail of the α-factor receptor that mediates both the constitutive and ligand-mediated endocytosis of the receptor.

CHAPTER VI

DISCUSSION

The work presented in this thesis aims to extend the current understanding of the regulation of GPCR activity by analyzing the proteins that interact with the α-factor pheromone receptor Ste2, a GPCR from *Saccharomyces cerevisiae*. In addition, the role of the cytoplasmic C-terminal domain of Ste2 in receptor interactions and in endocytosis has been investigated.

Previous findings have implicated oligomerization of several mammalian GPCRs. In this study, I present evidence indicating that the α-factor receptors from *S. cerevisiae* also form oligomeric complexes in the plasma membrane (Chapter III). When membranes containing two differentially-tagged receptors were solubilized with the non-denaturing detergent n-dodecyl β-D-maltoside and subjected to immunoprecipitation, both tagged species were precipitated with antibodies specific for either of the two tags. The formation of receptor oligomers was unaffected by ligand. When two receptor mutants, one defective for ligand binding and one defective for internalization, were individually coexpressed with wild-type receptors, and analyzed by membrane fractionation and fluorescence microscopy, each mutant became competent for endocytosis indicating that oligomeric receptor complexes were subject to endocytosis and that unoccupied receptors could participate in these complexes. Due to the extensive cointernalization of these complexes, it is plausible to argue that all of the receptors are found in oligomers in the plasma membrane.

The Gβ and Gγ subunits of the heterotrimeric G protein in yeast, Ste4 and Ste18 were also found to associate with the α-factor receptor upon immunoprecipitation of the crosslinked complexes containing the receptor (Chapter IV). The points of contact between receptor and G protein are not known, but most likely involve the residues located at the third cytoplasmic loop of the receptor. Mutations in this region impair receptor signaling. The C-terminal tail of Ste2 is not required for the receptor G protein interactions as evidenced by the coprecipitation of Ste4 with a truncated mutant of Ste2 that lacks most of the tail. However, genetic evidence suggests that the C-terminal tail may play a role in stabilizing the association between the receptor and the G protein. The C-terminal tail may, therefore, be involved in forming additional contacts with the Gβ subunit. These contacts may become critical when other contact points fail to associate.

The receptor-G protein interactions were detected both in the absence and in the presence of the ligand. Since the extent of receptor oligomerization is unaffected by ligand, it is unlikely that the receptor-G protein interaction is mediated by a change in the oligomerization state of the receptor. However, these data do not exclude the possibility that a single heterotrimeric G protein associates with multiple receptors present in a complex and that this interactions between the receptors and the G proteins require receptor oligomerization.

Alternatively, heterotrimeric G proteins may also be present in multimeric complexes that may interact with individual or multiple receptors. Such oligomerized G protein arrays have been observed during the analysis of the high resolution crystal structure of Goi. Unfortunately, the methods used previously to study the interactions between the GPCRs and G proteins, such as mutational studies or peptide binding

experiments, do not address the possibility for requirement of receptor oligomers in these interactions. The crystal structure of a GPCR bound to its cognate G protein would answer such questions and identify the exact points of contact between these proteins. However, crystallization of protein complexes including components with vastly different properties like a multiple transmembrane receptor and soluble G protein subunits may not be possible. On the other hand, a mutational analysis approach could be undertaken to obtain an oligomerization-defective GPCR to study the role of oligomerization on receptor function including the receptor G protein interactions.

Yeast provides an amenable model for genetic manipulations. However, since the role of oligomerization on receptor function is unknown, the oligomerization mutants cannot be selected for phenotypes resulting from altered receptor functions. A visual screen can help to identify the oligomerization-defective mutants of Ste2 (Mike Chang and Duane Jenness, unpublished observations). The screen makes use of the observation that an internalization-defective Ste2 mutant, Ste2-T326, becomes competent for endocytosis when coexpressed with wild-type receptors (Chapter III, Table 2 and Figure 9). The interaction between Ste2-T326 and wild-type receptors has been confirmed by coimmunoprecipitation experiments, indicating that the cointernalization is the result of direct physical association (Chapter III, Figure 10). To this end, random mutagenesis of the *Ste2-T326* gene can be performed and the mutagenized receptors can be expressed as GFP fusion proteins with wild-type receptors. Fluorescence microscopy analysis of the cells harboring mutagenized receptors would help to identify receptor mutants that cannot oligomerize with the coexpressed wild-type receptors since they would remain on the plasma membrane following treatment of cells with α-factor. On the other hand, the oligomerization-proficient receptors would cointernalize with the wild-type receptors.

Once such mutations are isolated, they can be tested in the context of the full-length receptor for formation of oligomeric complexes in coimmunoprecipitation experiments. This approach may help to identify the receptor segments involved in oligomerization and to investigate the biological significance of receptor oligomers.

Oligomerization may also affect receptor functions by protein-protein interactions, that is, the oligomerization partners may induce conformational changes in each other. For example, when α-factor-binding defective receptors were coexpressed with wild-type receptors, and treated by α-factor, the activated wild-type receptors may induce an active receptor conformation on the oligomerized partner. This possibility can be addressed by coexpressing two receptor mutants, with one defective in ligand binding (such as Ste2-T326) and the other defective in endocytosis (such as Ste2-S184R), and by evaluating whether either receptor species is internalized upon treatment with α-factor. Cointernalization would suggest that the activated Ste2-T326 could induce an active conformation in the Ste2-S184R and therefore could cause Ste2-S184R and also the oligomerized Ste2-T326 to be endocytosed.

The C-terminal cytoplasmic tail of the receptor includes redundant endocytic signals. The well-characterized SINNDAKSS motif exclusively mediates the endocytosis of a truncated form of Ste2, Ste2-T345 but it has little effect on endocytosis in the context of the full-length receptor. SINNDAKSS, however, is sufficient to confer endocytosis to an endocytosis-defective tail-less receptor. A novel region in the Ste2 tail that is sufficient to mediate endocytosis was discovered by deletion analyses (Chapter V). This region extends from amino acid residues 360 to 431. Such redundant signals may be required to provide avidity in interactions with the components of the endocytic

machinery. Another way to achieve avidity is through oligomerization of receptors, and thereby creating a high local concentration of endocytic signals. Thus, oligomerization may have a broader role of facilitating all membrane trafficking events that involve the receptors, including endocytosis and exit from the ER. Interestingly, when truncated receptors were coexpressed with wild-type receptors, both forms of receptors affected the intracellular buoyant membrane localization of each other, suggesting that oligomers may be forming in the ER. For example, the membrane gradient analysis indicated that while most of Ste2-T326-GFP is on the plasma membrane, about 18 % of receptors accumulate in some intracellular compartments (Chapter II, Table 2). Fluorescence microscopy images suggest that some of truncated receptors are not localized to the vacuoles and may therefore be in the ER. When coexpressed with wild-type receptors, truncated receptors were found to a lesser extent in these compartments, suggesting that the presence of the wild-type receptors may have facilitated the exit of truncated receptor from the ER. Similar effects of oligomerization in facilitating receptor exit from the ER have been reported between $GABA_B$ GBR1 and GBR2 receptors and between wild-type and truncated forms of CCR5 (Kuner et al., 1999; Benkirane et al., 1997).

More experiments are needed to assess at which compartment the oligomers form inside the cell. To this end, pulse-chase experiments may be performed to analyze oligomerization status of differentially-tagged receptors by coimmunoprecipitation. The receptors may be tagged with GFP and HA, and therefore different species can be resolved on SDS gels. To commit receptors to a specific cellular location, strains producing temperature-sensitive mutant forms of proteins that mediate membrane trafficking events from from ER to Golgi (*sec12*) or from Golgi to the plasma membrane (*sec1*) can be used.

Alternatively, Ste2-3, a temperature-sensitive mutant form of Ste2, may be useful to assess the affect of receptor oligomerization on cellular localization. Ste2-3 is transported directly to the vacuole without reaching the plasma membrane at non-permissive temperature (Jenness et al., 1997). GFP-tagged Ste2-3 and HA-tagged wild-type receptors can be coexpressed in cells and can be grown at non-permissive temperature, and the effect of each receptor species on the localization of the other can be assessed by fluorescence microscopy and membrane fractionation studies. The localization of GFP-tagged Ste2-3 on the plasma membrane at non-permissive temperature would indicate formation of receptor oligomers in the ER.

The possible role for receptor oligomerization in relationship to endocytosis and signal transduction is unclear and controversial (Hebert et al. 1998). Interestingly, recent evidence suggests a link between endocytosis and signaling in mammalian cells (Di Fiore and De Camilli, 2001). For example Trk receptors migrate from nerve terminals to the cell body upon stimulation by nerve growth factor, and failure to endocytose Trk receptors impede the ability of the nerve cell to induce a transcriptional response to the hormone (Kuruvilla et al., 2000). Moreover, a scaffolding protein, arrestin, interacts with the phosphorylated cytoplasmic tails of activated GPCRs as well as with parts of endocytic machinery, such as clathrin heavy chain and clathrin adaptor AP-2, and components of signaling machinery, such as c-Src and JNK3, linking endocytosis and signal transduction (Hall et al., 1999). In yeast cells, for the α-factor receptor, these two events seem independent since receptor mutants that are defective for endocytosis are able to signal, and mutations in components of the signal transduction machinery are competent for endocytosis. Even though signaling and endocytosis are separate events,

both events may require oligomerization. Oligomerization of receptors may provide avidity and therefore facilitate interactions among components of both the signaling and the endocytic machinery by causing a localized increase in the density of signals.

Another unanswered question is how many receptors are present in the oligomeric complexes. The receptors form a complex that is approximately 8S in size in glycerol gradient centrifugation. However, the relative contribution of the solubilizing detergent to the size of the receptor complex is unknown. The molecular weight of the detergent-solubilized complex can be determined from the Svedberg equation after measuring the partial specific volume and the diffusion coefficient. The hydrodynamic properties of another GPCR have been studied by a similar approach (Peterson et al, 1986). The association of the G proteins with the receptors is detected only in the presence of a crosslinking reagent, so the receptor oligomers may be the only protein species in the solubilized complexes. In that case, the calculated molecular weight would reflect the number of receptors in the complex.

In conclusion, the findings presented in this study indicate that the α-factor pheromone receptors form oligomeric complexes and that these complexes are functional units of endocytosis. The role of receptor oligomerization on receptor function remains to be elucidated. In addition, biochemical evidence for a physical association between α-factor receptors and heterotrimeric G proteins has been provided. Finally, a novel region in the receptor tail that is sufficient to mediate endocytosis has been described.

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