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Mechanisms Governing the Activation and Trafficking of Yeast G Protein-coupled Receptors

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We have addressed the mechanisms governing the activation and trafficking of G protein-coupled receptors (GPCRs) by analyzing constitutively active mating pheromone receptors (Ste2p and Ste3p) of the yeast *Saccharomyces cerevisiae*. Substitution of the highly conserved proline residue in transmembrane segment VI of these receptors causes constitutive signaling. This proline residue may facilitate folding of GPCRs into native, inactive conformations, and/or mediate agonist-induced structural changes leading to G protein activation. Constitutive signaling by mutant receptors is suppressed upon coexpression with wild-type, but not G protein coupling-defective, receptors. Wild-type receptors may therefore sequester a limiting pool of G proteins; this apparent "precoupling" of receptors and G proteins could facilitate signal production at sites where cell surface projections form during mating partner discrimination. Finally, rather than being expressed mainly at the cell surface, constitutively active pheromone receptors accumulate in post-endoplasmic reticulum compartments. This is in contrast to other defective membrane proteins, which apparently are targeted by default to the vacuole. We suggest that the quality-control mechanism that retains receptors in post-endoplasmic reticulum compartments may normally allow wild-type receptors to fold into their native, fully inactive conformations before reaching the cell surface. This may ensure that receptors do not trigger a response in the absence of agonist.

INTRODUCTION

G protein-coupled receptors (GPCRs) are integral membrane proteins that are inserted into the membrane of endoplasmic reticulum (ER), folded into their native, inactive conformations, and transported through the secretory pathway to the cell surface where they can be activated by hormones, neurotransmitters or sensory stimuli. Mechanisms that control the activation or biogenesis of GCPRs therefore have critical roles in governing cellular responsiveness to an array of extracellular signals.

GPCR activation has been investigated in many systems, leading to the following model (Baldwin, 1993; Lefkowitz *et al*., 1993; Coughlin, 1994). In the absence of ligands, GPCRs are thought to exist in equilibrium between inactive and active conformations, usually favoring the inactive state. Agonists bind and stabilize receptors in their active conformations, leading to G protein activation. Inverse agonists bind and stabilize the inactive conformation of GPCRs, precluding receptor activation by agonists. Antagonists bind receptors without significantly affecting the equilibrium distribution between inactive and active conformations, which also blocks agonist-induced signaling.

Recent studies have begun to reveal structural changes that distinguish the active and inactive states of GPCRs. Mutations affecting cytoplasmic loops I, II, or III, or transmembrane segments (TMS) I, II, VI, or VII, constitutively activate GPCRs by destabilizing the inactive state or stabilizing the active state (Kjelsberg *et al*., 1992; Robinson *et al*., 1992; Parma *et al*., 1993; Robbins *et al*.; 1993, Samama *et al*., 1993; Shenker *et al*., 1993; Konopka *et al*., 1996; Scheer *et al*., 1996). Indeed, conformational changes accompanying GPCR activa-

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tion occur in cytoplasmic loops, near the cytoplasmic terminus of TMS III or VII and within TMS VI (Ganter *et al*., 1992; Farahbakhsh *et al*., 1993; Bukusoglu and Jenness, 1996; Lin and Sakmar, 1996). Furthermore, the distance between TMS III and VI increases when rhodopsin is activated (Farrens *et al*., 1996; Yang *et al*., 1996). However, the specific kinds of secondary or tertiary structural changes that occur in activated GPCRs are poorly understood because high-resolution structural information is unavailable.

Less is understood about the mechanisms governing the biogenesis and trafficking of GCPRs, although insights are emerging from studies of visual opsins. Opsin biogenesis is facilitated by the action of cyclophilin-related proteins, which apparently function as prolyl isomerases and chaperones in the ER (Colley *et al*., 1991, 1995; Baker *et al*., 1994; Ferreira *et al*., 1996). However, the specific steps in the folding, assembly, and transport of opsins that are facilitated by cyclophilin homologs or other components of the quality control apparatus in the secretory pathway have not been clearly established.

Receptors for the oligopeptide mating pheromones, α-factor and **a**-factor of the yeast *Saccharomyces cerevisiae*, are useful models with which to study the function and biogenesis of GPCRs (Dohlman *et al*., 1991; Sprague and Thorner, 1992). Mating pheromones trigger a G protein-linked signal transduction pathway that induces expression of mating-specific genes, arrests cells in the G1 phase of the cell cycle, and alters cell morphology, culminating in cell and nuclear fusion. Mating pheromone receptors use their third cytoplasmic loops to couple with heterotrimeric G proteins (Boone *et al*., 1993; Weiner *et al*., 1993; Clark *et al*., 1994; Stefan and Blumer, 1994); they use their C-terminal cytoplasmic domains to promote receptor endocytosis and desensitization (Konopka *et al*., 1988; Reneke, *et al*., 1988; Rohrer *et al*., 1993), indicating that yeast and mammalian GCPRs function in similar ways.

Here we describe mutations that constitutively activate the receptors for the pheromones α-factor and **a**-factor. Characterization of these constitutively active receptors suggests that a conserved proline residue in transmembrane segment VI has a critical role in governing the activity and trafficking of GPCRs and provides genetic evidence that pheromone receptors and G proteins are precoupled before agonist stimulation.

MATERIALS AND METHODS

Materials, Media, and Isotopes

Enzymes used for recombinant DNA methods were purchased from commercial sources and used according to the suppliers' recommendations. Sources of growth media for yeast and bacterial cells have been described previously (Blumer *et al*., 1988; Reneke *et al*., 1988). [^{35}S]H_2SO_4 (carrier free) was obtained from Du Pont-New England Nuclear (Boston, MA). Sources of antibodies were as follows: rabbit polyclonal antibodies specific for Kar2p (Rose *et al*., 1989) (S. Wente of this department); rabbit polyclonal antisera specific for Gda1p (Berninsone *et al*., 1995) (C. Hirschberg, (University of Massachusetts, Amherst, MA); mouse monoclonal antibody C56 specific for the plasma membrane ATPase (Pma1p) (Aris and Blobel, 1988; Schandel and Jenness, 1994) (D. Jenness [University of Massachusetts] and J. Aris [University of Florida, Gainesville, FL]); mouse monoclonal antibodies specific for dolichol phosphate mannose transferase (Dpm1p), and the vacuolar ATPase (Vph1p) (Molecular Probes, Eugene, OR); peroxidase-, fluorescein isothiocyanate- and rhodamine-conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse) (Organon Teknika, Durham, NC).

Plasmids and Yeast Strains

A plasmid that was used to create an unmarked chromosomal deletion of the *STE2* gene was constructed by inserting a 1.6-kilobase (kb) *Eco*RI–*Hin*dIII fragment from pRS314*STE2* (Weiner *et al*., 1993) into YIp5 that had been cleaved with *Eco*RI and *Hin*dIII, creating YIp5*STE2*-5′-UTR. Polymerase chain reaction (PCR) was used to generate a 0.7-kb *Hin*dIII–*Sph*I fragment containing sequences downstream of the *STE2*-coding region. This fragment was digested with *Hin*dIII and *Sph*I and inserted into YIp5*STE2*-5′-UTR that had been cleaved with *Hin*dIII and *Sph*I to create YIp5*ste2*Δ. Thus, in plasmid YIp5*ste2*Δ the entire *STE2*-coding region was removed from a *Hin*dIII site 538 base pairs (bp) upstream of the start codon to a *Hin*dIII site 195 bp downstream of the stop codon.

To facilitate construction of plasmids that express various *STE2* alleles, we deleted the *Pst*I site in the polylinker of pRS314*STE2* to create a plasmid (pRS314ΔP-*STE2*) with a unique *Pst*I site in the *STE2*-coding region. For coexpression of various *STE2* alleles, plasmid pRS313*STE2* was constructed by isolating a 3.6 kb *Eco*RI–XbaI fragment encompassing the *STE2* locus from pRS314*STE2* and inserting it into pRS313 that had been cleaved with *Eco*RI and *Xba*I. Plasmid pRS313*ste2*L236R was constructed by isolating a 3.6-kb *Eco*RI–*Xba*I fragment containing the *ste2*L236R allele and inserting it into pRS313 that had been cleaved with *Eco*RI and *Xba*I. To overexpress various *STE2* alleles, we inserted 4.3-kb *Apa*I–*Sac*I fragments carrying either the wild-type allele or various codon 258 mutations into the high copy plasmid pRS424 that had been cleaved with *Apa*I and *Sac*I. To express various *STE3* alleles, we constructed plasmid pRS425*STE3* by isolating a 2.4-kb *Hpa*I–*Sac*I fragment encompassing the *STE3* locus and inserting it into pRS425 that had been cut with *Eco*RV and *Sac*I.

To remove the C-terminal cytoplasmic domain of the α-factor receptor, we used PCR to create a nonsense mutation at codon 300 in the *STE2*-coding sequence and to introduce unique *Bgl*II and *Pst*I sites immediately upstream of this stop codon. This PCR product was cloned, sequenced, digested with *Aat*II and *Sac*I, and introduced into pRS314ΔP-*STE2* that had been cleaved with *Aat*II and *Sac*I to create pRS314*ste2*-300ter. Plasmid pRS314*ste2*P258L,300ter was created by inserting a 0.4-kb *Aat*II–*Pst*I fragment containing the *ste2*P258L allele into pRS314*ste2*-300ter that had been cleaved with *Aat*II and *Pst*I.

To detect α-factor receptors by immunological methods, we constructed plasmids that express wild-type and constitutively active receptors containing three c-*myc* epitopes at their extreme C termini. PCR was used to change the *Bcl*I site overlapping the natural stop codon of the *STE2*-coding sequence to a unique *Bgl*II site, destroying the translational stop codon. This PCR product was cloned, sequenced, digested with *Pst*I and *Sac*I, and inserted into pRS314ΔP-*STE2* that had been cleaved with *Pst*I and *Sac*I to create pRS314*STE2*Δter. Plasmid pRS314*STE2*-3x*myc* was created by inserting a *Bam*HI fragment containing three c-*myc* epitopes in the appropriate reading frame into the *Bgl*II site of pRS314*STE2*Δter. A 0.6 kb *Aat*II-*Pst*I fragment carrying various mutations affecting codon 258 of the *STE2* gene was inserted into pRS314*STE2*-3x*myc* that had been cleaved with *Aat*II and *Pst*I to generate plasmids that express *myc*-tagged constitutively active receptors.

The *S. cerevisiae* strains used in these studies were: KBY16 (*MAT**a** ura3–52 trp1–903 his3-Δ200 ade2–101 leu2–3, 112 lys2–801 mfα1::LYS2 mfα2::LEU2 ste2Δ::HIS3 sst1-Δ5)* (Stefan and Blumer, 1994), KBY17 (same as KBY16 but *sst2*Δ), KBY18 (same as KBY16 but *far1*Δ), KBY20 (same as KBY16 except it contains an unmarked *ste2*Δ allele), KBY22 (same as KBY16 except *ste4::URA3*), and SY1985 (*MATα ste3*Δ*::URA3 ste2*Δ *mf**a**1*Δ *mf**a**2*Δ*::FUS1-lacZ FUS1::HIS3 ura3–52 leu2–3, 112 ade1 sst2*Δ). They were constructed as follows. KBY17 was constructed by using *Nhe*I-cut pBC14 (Dohlman *et al*., 1996) to disrupt the *SST2* gene in KBY16 by two-step gene replacement. Plasmid pFC13 (Chang and Herskowitz, 1990) digested with *Not*I was used to disrupt the *FAR1* gene in KBY16; a 5-fluoroorotic acid-resistant derivative of this *far1*Δ strain was selected to create KBY18. KBY22 was constructed by using pAG3 (Grishin *et al*., 1994) cut with *Pst*I and *Xho*I to disrupt the *STE4* gene in KBY16. An unmarked deletion of *STE2*, *ste2*Δ, was made in KBY16 by two-step gene deletion using *Cla*I-cut YIp5*ste2*Δ to create KBY20; this disruption was confirmed by loss of *HIS3*. Strain SY1985 is a *ste3*Δ*::URA3 sst2*Δ derivative of SY1937 (Boone *et al*., 1993), which was provided by G.F. Sprague Jr. (University of Oregon, Eugene, OR).

Mutagenesis and Genetic Screening

Generation of mutations throughout the *STE2*-coding region was performed by hydroxylamine treatment (Sikorski and Boeke, 1991) and low fidelity PCR (Kocher *et al*., 1989) of pRS314*STE2*. Mutations isolated by genetic screens were identified by using primers to sequence the region encoding the Ste2p polypeptide. Site-directed mutagenesis of codon 258 in *STE2* and codon 222 in *STE3* was performed by PCR as described previously (Kjelsberg, *et al*., 1992). PCR products were digested with *Aat*II and *Pst*I to generate 0.6-kb fragments carrying various *STE2* mutations and were inserted into pRS314ΔP-*STE2* that had been cleaved with *Aat*II and *Pst*I. In experiments involving *STE3*, PCR products were digested with *Nhe*I and *Nde*I to generate 0.8- kb fragments carrying the various codon 222 mutations, which were inserted into pRS425*STE3* that had been cleaved with *Nhe*I and *Nde*I. The resultant plasmids were sequenced across the relevant regions of the *STE2*- or *STE3*-coding regions to confirm the presence of codon 258 or 222 mutations and the absence of secondary mutations.

A library of hydroxylamine-treated plasmids (pRS314*STE2*) carrying mutations in sequences coding for Ste2p was introduced by transformation into a *ste2*Δ*::HIS3 far1*Δ *mfα1::LYS2 mfα2::LEU2* strain (KBY18) containing *FUS1-lacZ* on plasmid pSL307 (McCaffrey, *et al*., 1987). In addition, four pools of fragments carrying random mutations in the *STE2* gene that had been generated by low-fidelity PCR were independently introduced into KBY18 (containing pSL307) by gap repair of pRS314*STE2* that had been cleaved with *Nde*I and *Aat*II. Cells were plated on selective media (SD-tryptophan and uracil) lacking pheromone. Transformant colonies were replica plated onto filters impregnated with X-gal and assayed for expression of β-galactosidase as described previously (Fields and Song, 1989). Under the assay conditions employed (1 h incubation at 30°C), cells expressing the wild-type *STE2* gene remained white. Plasmids isolated from transformants that were blue (expressed *FUS1-lacZ*) were transferred to *Escherichia coli* and introduced again into KBY18 containing the *FUS1-lacZ* plasmid. These transformants were subjected to quantitative assays to measure the strength of the constitutive signal, as described below.

Pheromone Response Assays and Dominance Tests

The level of pathway activation was determined by measuring the expression of the pheromone-inducible *FUS1-lacZ* reporter gene in plasmid pSL307. Cells carrying pSL307 and expressing various *STE2* alleles were grown in selective media to a density of 10^7 cells/ml. Cultures were split into aliquots: one was a control, and the other received α-factor (1 μM final concentration). After a 2-h incubation at 30°C, cells were permeabilized and assayed for β-galactosidase activity (McCaffrey *et al*., 1987). Dominance tests were performed by using centromeric plasmids to coexpress various constitutively active α-factor receptors (pRS314 derivatives) and the wild type *STE2* or *ste2*L236R alleles (pRS313 derivatives) in a *ste2*Δ mutant (KBY20) that also carried the *FUS1-lacZ* gene on pSL307; pathway activation in the absence or presence of α-factor was determined as described previously.

Ligand Binding and Receptor Internalization Assays

Methods used to purify [^{35}S]α-factor and perform ligand- binding assays with inviable, intact cells have been described (Blumer *et al*., 1988). Assays of cells expressing wild-type receptors employed [^{35}S]α-factor (20 Ci/mmol) at concentrations ranging from 0.1 to 10 nM, and those of cells expressing constitutively active receptors used α-factor concentrations from 0.05 to 20 nM. Assays of cells overexpressing various *STE2* alleles from high-copy plasmids used [^{35}S]α-factor (15 Ci/mmol) at concentrations ranging from 0.05 to 30 nM. Ligand-binding data were plotted according to the method of Scatchard and fitted by nonlinear least mean square regression. Nonspecific binding was determined in the presence of a 500-fold excess of unlabeled α-factor.

Rates of ligand-independent and ligand-induced loss of α-factor binding sites from the cell surface were measured as previously described (Stefan and Blumer, 1994), with the following modifications. Cultures were grown at 22°C in selective media (SD-tryptophan) to a density of 10^7 cells/ml and treated with cycloheximide (20 μg/ml) for 5 min. Basal rates of receptor internalization were determined in the absence of α-factor. Pheromone-induced rates of receptor internalization were determined by adding unlabeled α-factor to a final concentration of 50 nM. Aliquots of cells were removed at various times, treated with 10 mM NaN_3 and 10 mM KF, and washed in YP (Blumer *et al*., 1988) containing 100 mM H_3PO_4, pH 2.5, to remove cell surface-bound α-factor. After cells were washed in 10 mM PIPES (pH 6.0), 1 mM $MgCl_2$, 0.1 mM EDTA, 10 mM NaN_3, 10 mM KF in YP media (Blumer *et al*., 1988), they were incubated with [^{35}S]α-factor (10 nM, 30 Ci/mmol) with or without a 250-fold excess of unlabeled α-factor, which was used to determine levels of nonspecific binding. To determine whether α-factor–binding sites were preserved by these manipulations, we treated control cells with metabolic inhibitors (NaN_3 and KF) immediately after treatment with cycloheximide and before addition of unlabeled pheromone and treated them as described above.

Immunoblotting and Indirect Immunofluorescence

Cultures were grown to a density of 2×10^7 cells/ml in synthetic medium (SD-tryptophan) to select for plasmid pRS314ΔP*STE2*-3x*myc* and its derivatives encoding *myc*-tagged constitutively active α-factor receptors. Methods used to detect *myc*-tagged Ste2p in yeast whole-cell extracts by immunoblotting were based on those previously described (Blumer *et al*., 1988). The protein concentration of yeast whole-cell lysates was determined by the Bradford method and adjusted to 2 mg/ml with Laemmli sample buffer before SDS-PAGE.

Preparation of cells for antibody incubations and immunofluorescence was performed essentially as described (Pringle *et al*., 1991). Cultures were grown at 30°C in selective medium (SD-tryptophan) to a density of 10^7 cells/ml. Formaldehyde was added to a final concentration of 3.7%. Cells were incubated 5 min at room temperature, washed, and suspended in 0.1 M potassium phosphate buffer, pH 7.0, containing 1 M sorbitol (buffer A). Spheroplasts were generated by incubating cells with glusulase and zymolyase 20T and washed with buffer A. Spheroplasts were bound to polylysine-coated slides, washed with Tris-buffered saline (TBS) containing 0.02% Tween 20, 0.01% Triton X-100, and 2% nonfat milk. Samples were incubated with antibodies for 16 h (9E10 tissue culture supernatant and/or Kar2p antibodies diluted 1:2 or 1:200, respectively, in TBS containing 0.01% Tween-20 and 2% nonfat milk [dilution buff-

er]). Slides were washed seven times with dilution buffer and incubated 2 h with rhodamine-conjugated goat anti-rabbit IgG and/or fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody diluted 1:500 or 1:1000, respectively, in TBS containing 0.01% Tween 20 and 1% bovine serum albumin (BSA). Slides were washed 10 times with TBS containing 0.01% Tween 20 and 1% BSA and incubated 5 min with 2.5 μg/ml 4′,6-diamino-2-phenylindole in TBS containing 0.01% Tween 20 and 1% BSA. Slides were washed once with TBS containing 0.01% Tween 20 and 1% BSA, and cells were observed under an Olympus epifluorescence microscope.

Subcellular Fractionation

Subcellular fractionation was carried out by equilibrium density gradient centrifugation essentially as described previously (Kölling and Hollenberg, 1994). Cells were grown in selective medium (SD-tryptophan) to a density of 10^7 cells/ml. Cultures were treated with 10 mM sodium azide and 10 mM KF. Cells were collected by centrifugation and washed once with 25 ml of sorbitol buffer (10 mM Tris, pH 7.6, 0.8 M sorbitol, 10 mM NaN_3, 10 mM KF, 1 mM EDTA, pH 8.0). Cells were collected by centrifugation and washed once with 1 ml sorbitol buffer, once with 1 ml sucrose buffer (10 mM Tris pH 7.6, 1 mM EDTA, 10% [wt/vol] sucrose), and suspended in 1 ml sucrose buffer containing protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 20 μM tosyl-phenylalanine chloromethyl ketone, 5 μM pepstatin A, and 5 μM leupeptin). Glass beads were added, and the cells were lysed by mechanical disruption. Unbroken cells were removed from the lysate by centrifugation at 300 $\times$ *g* for 5 min. The supernatant fraction (0.5 ml) was mixed with 0.5 ml of 50% (wt/vol) sucrose in 10 mM Tris, pH 7.6, 1 mM EDTA, and layered on top of a 4 ml, 35–60% linear sucrose gradient prepared in 10 mM Tris, pH 7.6, 1 mM EDTA. Gradients were centrifuged 20 h at 150,000 $\times$ *g* in a SW50.1 rotor at 4°C. Fractions (350 μl) were collected from the top of the gradient and diluted 1:2 with 2$\times$ Laemmli sample buffer containing 8 M urea. Samples were heated for 10 min at 37°C before SDS-PAGE. Ste2p-myc, Vph1p, Gda1p, Dpm1p, and Pma1p were detected by immunoblotting.

RESULTS

Identification of Constitutively Active α-Factor Receptors

To identify mutations in the α-factor receptor structural gene (*STE2*) that activate the response pathway in the absence of pheromone, we used a genetic screen to identify cells that constitutively express a pheromone-inducible reporter. The yeast strain used for this purpose (KBY18) had the following important features: a *ste2*Δ mutation, which ensures that cells express receptors only from mutagenized plasmids; deletions of the two α-factor structural genes (*MF*α*1*, *MF*α*2*), which ensure that apparent constitutive signaling is not due to autocrine stimulation of a hypersensitive mutant receptor; a deletion of the gene (*FAR1*) encoding a cyclin-dependent kinase inhibitor, which prevents growth arrest from occurring in cells expressing strongly constitutively active receptors; and a plasmid-borne pheromone-inducible reporter (*FUS1-lacZ*), which enables the identification of cells that signal constitutively (stain blue with X-gal in the absence of α-factor). This strain was transformed with pools of a mutagenized (hydroxylamine treatment or error-prone PCR) single-copy plasmid in which the *STE2* gene is expressed from its normal promoter. Approximately 60,000 transformants were screened in the absence of pheromone for elevated expression of *FUS1-lacZ* by staining colony filter lifts with X-gal. Plasmids from 10 transformants that were positive in this assay were recovered in *Escherichia coli* and rescreened in yeast for the ability to induce *FUS1-lacZ* in the absence of pheromone. Eight of the 10 plasmids passed this test. The *STE2*-coding regions of these eight plasmids were sequenced.

Three classes of mutations were obtained. The first class (four plasmids) contained a mutation that resulted in substitution of proline-258 for leucine (P258L) in transmembrane segment six (TMS VI). This *ste2*P258L allele was chosen for further study because it caused the strongest constitutive signaling phenotype (C. Stefan, unpublished data). Furthermore, the P258L substitution was particularly interesting because proline residues in transmembrane domains have been proposed to control the activity of receptors, ion channels, and transporters (Williams and Deber, 1991), and a proline residue is present in TMS VI in >90% of all GPCRs (Table 1; Baldwin, 1993), suggesting that it may have a conserved function. The second class (one plasmid) carried a single mutation that resulted in a serine-to-proline substitution at position 259 in TMS VI, and a third class (three plasmids) contained several mutations within the *STE2* gene; analysis of these two classes will be described elsewhere.

Other investigators have shown previously that the P258L substitution in TMS VI constitutively activates the α-factor receptor (Konopka *et al*., 1996). This finding led Konopka and colleagues to propose that a conserved proline residue at this position is required to stabilize the inactive conformation of the α-factor receptor, and perhaps other GCPRs as well, possibly by inducing a kink in TMS VI. However, there were several reasons why we believed it was important to test this model further. First, these investigators did not determine whether a proline residue is specifically required at position 258 in the α-factor receptor or whether other amino acids at this site can preserve wild-type receptor function. Second, the analogous proline residue has been mutated in other GCPRs, but constitutive activity was not reported (Wess *et al*., 1993; Kaushal and Khorana, 1994; Kolakowski *et al*., 1995). Third, a significant number of GPCRs (~10%) lack a proline at this position (Table 1 lists some examples), indicating that a proline is not always required. Accordingly, to address these points we have determined whether changing proline-258 to any other amino acid constitutively activates the α-factor receptor, investigated cellular regulatory mechanisms that influence detection of a constitutive signal, and determined whether changing the equivalent proline

Table 1. TMS VI sequences of representative GPCRs

Receptor	TMS VI sequence
AChM3 bovine (493-513)	SAILLAFIITWT**P**YNIMVLV
α1B-AR rat (296-319)	LGIVVGMFILCWL**P**FFIALPLGSL
β2AR human (275-298)	LGIIMGTFTLCWL**P**FFIVNIVHVI
BK-1R human (248-272)	TTALILTLVVASLVCWA**P**YHFFAFL
FMLF-R human (243-266)	LSFVAAAFFLCWS**P**YQVVALIATV
FSH-R human (574-597)	MAMLIFTDFLCMA**P**ISFFAISASL
δOpioid R-1 human (262-284)	MVLVVVGAFVVCWA**P**IHIFVIVW
Rhodopsin bovine (253-276)	MVIIMVIAFLICWL**P**YAGVAFYIF
PGE2-R2 human (263-286)	LILLAIMTITFAVCSL**P**FTIFAYM
NK1R rat (249-270)	MMIVVVCTFAICWL**P**FHVFFLL
ETR1 bovine (307-328)	TVFCLVVIFALCWF**P**LHLSRIL
THR-R rat (319-341)	LFLSAAVFCIFIVCFG**P**TNVLLI
MGR1 rat (768-808)	YIAFTMYTTCIIWLAFV**P**IYFGS
CASR rat (806-828)	ICFFFAFKSRKL**P**ENFNEAKFIT
GUSB bovine (242-265)	LFTVVIVFIVTQL**P**YNIVKFCQAI
STE2 *S. cerevisiae* (244-266)	FHILLIMSCQSLLV**P**SIIFILAY
STE3 *S. cerevisiae* (206-228)	FARLLIFCFIIILVMF**P**FSVYTF
CALR human (360-377)	ATMILV**P**LL**G**IQFVVF**P**W
GIP-R human (342-362)	STLTLV**P**LL**G**VHEVVFA**P**VTE
PTR-R human (410-428)	TLVLM**P**LF**G**VHYIVFMATP
OLF1 rat (237-260)	IFSTCGSHLSVVSLFY**G**TII**G**LYL
VN2 rat (241-259)	ILMLRSLF**G**LMSIFDSIAS
srb-1 *C. elegans* (238-260)	FTLIVSFTHILFI**G**WYLGVTIFI
CAR1 *D. discoidium*. (206-224)	FKLINYIIVFLVCWVFAVV
VN1 rat (241-259)	ILMLMSLFVLMSVFDSIVC
srg-5 *C. elegans* (236-260)	LCFASFYMSAAFFSAALFQSYFAFF

TMS VI sequences from the indicated receptors are shown; members of the first group (which constitutes ~90% of GPRCs) contain proline residues in TMS VI, those of the second contain glycine and/or proline residues, while those of the third lack proline and glycine residues. The N- and C-terminal boundaries of TMS VI of each receptor (indicated in parentheses) were assigned according to data obtained from the GPCR database (http://receptor.mgh.harvard.edu/GCRDBHOME.html). The single letter amino acid code is used.

residue in another GPCR results in constitutive signaling.

Role of Proline-258 in TMS VI of the α-Factor Receptor

We constructed a set of mutations that change proline-258 in the α-factor receptor to all other amino acids. Each allele was expressed from the normal *STE2* promoter on single-copy plasmids in a *ste2*Δ mutant that contained a pheromone-inducible reporter (*FUS1-lacZ*) on a high-copy plasmid (pSL307). We found that several substitutions of proline-258 increased agonist-independent reporter gene expression (Table 2, column 2). Substitution of proline-258 with methionine caused the strongest constitutive signaling phenotype (basal expression of *FUS1-lacZ* was increased approximately 50-fold above wild-type basal levels).

However, not all substitutions of proline-258 resulted in a detectable constitutive signal (Table 2, column 2). Although there were several possible explanations, one obvious possibility was that these mutant receptors transduce a weak constitutive signal that is attenuated by mechanisms that normally promote desensitization to pheromone.

To determine whether desensitization mechanisms reduce the apparent strength of the constitutive signal, we expressed mutant receptors in two types of desensitization-defective mutants. First, we blocked phosphorylation-dependent receptor desensitization and endocytosis by removing the C-terminal cytoplasmic domain of receptors bearing the P258L substitution (*ste2*P258L, 300ter). Combining this truncation mutation with the P258L substitution increased agonist-independent reporter gene expression eightfold over that observed when the P258L substitution was present in the full-length receptor (Table 2, column 2), consistent with the expectation that the apparent strength of the constitutive signal is negatively regulated at the receptor level. Second, we expressed each of the 19 mutant receptors in an *sst2*Δ mutant, which lacks a regulator of G-protein signaling homolog that apparently promotes desensitization by stimulating the guanosine triphosphatase activity of the yeast G protein α subunit (Gpa1p) (Dohlman *et al*., 1996; Dohlman and Thorner, 1997; Dohlman, personal communication). In an *sst2*Δ mutant, substitution of proline-258 in the α-factor receptor with any other amino acid resulted in a detectable constitutive signal (2- to 40-fold above wild-type receptor controls) (Table 2, column 4). This allowed us to compare the phenotypes conferred by various mutations, leading to the following observations. Substitution of proline-258 with aliphatic amino acids generally gave the strongest constitutive signal. Substitution of proline-258 with a charged residue resulted in intermediate constitutive activity and somewhat impaired responses to pheromone. Substitution of proline-258 with uncharged hydrophilic residues resulted in weak constitutive activity and strongly impaired responses to pheromone. Thus, the conserved proline residue at position 258 is essential for normal function of the α-factor receptor, consistent with the suggestion that it is required for the receptor to adopt or maintain a native, fully inactive conformation and to be activated normally by agonist.

Mutations Affecting the Conserved Proline Residue in TMS VI of the a-Factor Receptor

To determine whether the conserved proline residue of TMS VI may generally control the activity of

Table 2. Substitutions of proline-258 in TMS VI of the α-factor receptor: effects on receptor signal transduction, agonist binding affinity, and cell surface expression

	FUS1-lacZ expression (% wild type + α-factor)				α-Factor binding sites	
	SST2		*sst2*Δ		*SST2*	
STE2 allele	−α-factor	+α-factor	−α-factor	+α-factor	K_d (nM)	B_{max} (sites/cell)
*ste2*Δ	0.2	0.2	1.6	1.4	–	–
wild type	0.2	100	1.3	100	3.3	15,000
P258A	2.7	78	14	100	0.37	1,000
P258I	4.2	110	18	120	0.44	1,100
P258L	5.3	140	13	99	0.42	1,700
P258M	14	120	53	110	1.0	480
P258V	2.8	110	21	70	0.34	1,500
P258D	1.0	23	7.1	58	0.58	260
P258E	0.2	18	6.0	51	0.56	70
P258H	0.2	0.5	6.1	6.9	n.d.	n.d.
P258K	0.2	7.2	5.3	28	1.2	40
P258R	0.4	16	3.6	17	0.68	60
P258C	0.5	23	13	56	0.45	210
P258G	0.4	20	5.6	110	1.0	170
P258N	1.7	3.2	11	12	n.d.	n.d.
P258Q	0.6	10	3.5	9.4	0.58	40
P258S	0.3	0.6	4.6	7.3	n.d.	n.d.
P258T	0.3	0.7	6.8	7.8	n.d.	n.d.
P258Y	13	47	25	63	n.d.	n.d.
P258F	3.7	83	27	91	2.5	170
P258W	1.6	3.2	5.3	6.9	n.d.	n.d.
300ter	1.0	79				
P258L,300ter	45	92				

The indicated *STE2* alleles were expressed from centromeric plasmids (pRS314 derivatives) in isogenic *MAT***a** *ste2*Δ *mfα1*Δ *mfα2*Δ strains that expressed (KBY16) or lacked (KBY17) the *SST2* gene. Cells also contained the pheromone-inducible *FUS1-lacZ* gene on plasmid pSL307. Cells were treated as indicated with synthetic α-factor (1 μM, 2 h at 30°), and activation of the pheromone response pathway was quantified by performing β-galactosidase assays. Data are expressed as the percent of the activity detected in α-factor–treated cells that expressed the wild-type *STE2* gene. Data shown for each *STE2* allele are the average obtained from assays of at least four independent transformants, each of which was assayed in duplicate; standard errors were 10-30% of the values shown. Radioligand binding assays were performed using [^{35}S]α-factor and KBY16 cells expressing the indicated *STE2* alleles from centromeric plasmids (pRS314 derivatives). The K_d and B_{max} values shown for cells expressing each *STE2* allele were calculated by nonlinear regression of data obtained from two to three independent transformants assayed in duplicate; standard errors for these determinations were 5-15% of the values shown. n.d., Specific binding was not detected.

GPCRs, we generated substitutions of the equivalent proline residue (proline-222) in TMS VI of the **a**-factor receptor of *S. cerevisiae* (*STE3* gene product), which is unrelated in sequence to the α-factor receptor. These experiments employed a strain of a different genetic background with the following key features: a *ste3*Δ mutation, which ensures that **a**-factor receptors are expressed only from mutated plasmids; deletions of both **a**-factor structural genes (*MF**a**1, MF**a**2*), which preclude autocrine stimulation of mutant receptors; an *sst2*Δ mutation, which eliminates RGS-stimulated guanosine triphosphatase activity of G_α subunits that might otherwise attenuate a weak constitutive signal; and a chromosomally integrated pheromone-inducible reporter gene (*FUS1-lacZ*), which allows constitutive signals to be detected by performing β-galactosidase assays. Using this strain we examined the effects of substituting proline-222 of the **a**-factor receptor with leucine (*ste3*P222L) or tyrosine (*ste3*P222Y); the analogous substitutions affecting the α-factor receptor gave readily detectable constitutive signals. Although expression of these *ste3* alleles from their normal promoters on single-copy plasmids did not result in a detectable constitutive signal (Stefan, unpublished

Table 3. Effects of substitutions of proline-222 in TMS VI of the **a**-factor receptor

	FUS1-lacZ expression (% wild type +**a**-factor)	
STE3 allele	−**a**-factor	+**a**-factor
*ste3*Δ	40 ± 6	40 ± 6
wild-type	32 ± 7	100 ± 9
P222L	60 ± 10	72 ± 8
P222F	60 ± 11	76 ± 3

The indicated *STE3* alleles were overexpressed from their normal promoters on high copy plasmids (pRS425 derivatives) in a *ste3*Δ *mfa1*Δ *mfa2*Δ *sst2*Δ strain (SY1985) that contained an integrated *FUS1-lacZ* reporter. Where indicated, cells were diluted 1:1 with culture fluid from *MAT***a** cells (source of **a**-factor), incubated 2 h at 30° and assayed for β-galactosidase activity. Data are expressed as a percent of the activity detected using **a**-factor-treated cells that expressed the wild-type *STE3* gene. At least four independent transformants of each type were assayed in duplicate; standard errors are indicated.

data), expression from high-copy plasmids did result in a twofold increase in constitutive expression of the reporter, relative to wild- type receptor controls (Table 3, column 2). This constitutive signal was significant because it is 50% of the maximal pheromone-stimulated signal in cells expressing wild-type receptors (Table 3, column 3). We also noted that **a**-factor receptors bearing either substitution of proline-222 did not respond to **a**-factor (Table 3, column 3), possibly because these substitutions interfere with ligand binding, receptor folding, or cell surface expression (see below). Despite these complex effects on receptor function, the results support the hypothesis that a conserved proline residue in TMS VI helps establish or maintain the inactive conformation of GPCRs.

Other Functions of the α-Factor Receptor Influenced by Proline-258

Because various substitutions of proline-258 in the α-factor receptor constitutively activated the response pathway to different degrees, and because some of these substitutions impaired further activation of the pathway by pheromone, it was likely that proline-258 has complex roles in governing receptor function. Therefore, we analyzed other properties of mutant receptors to investigate the mechanisms that may underlie these phenotypic differences.

Level and Affinity of Cell-Surface α-Factor–Binding Sites. Radioligand binding experiments employing intact, inviable cells revealed differences among the 19 mutant receptors (Table 2, columns 6 and 7). One striking difference was the level of α-factor–binding sites expressed at the cell surface. Six mutants displayed undetectable levels of agonist-binding activity.

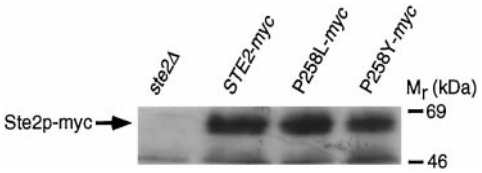

Figure 1. Expression of wild-type and constitutively active α-factor receptors. Various *myc*-tagged α-factor receptors were expressed from their normal promoters on centromere-containing plasmids (pRS314 derivatives) in a *ste2*Δ mutant (KBY16). Equivalent amounts of protein extract (50 μg) prepared from cells carrying a control plasmid (lane 1), or plasmids expressing wild-type *STE2-myc* (lane 2) or constitutively active α-factor receptors (*ste2*P258L-*myc*, lane 3; *ste2*P258Y-*myc*, lane 4) were resolved by SDS-PAGE. Immunoblotting was performed by using 9E10 antibodies specific for the c-*myc* epitope and a chemiluminescence detection system.

Relative to wild-type cells, the remaining mutants expressed 10- to 400-fold fewer ligand-binding sites per cell. A second difference was that mutant receptors displayed increased affinity for α-factor, ranging from a 50% increase (P258F) to nearly 10-fold (P258A, P258I, P258L, P258V, P258C, P258Q), similar to the properties of constitutively active GPCRs in mammalian cells (e.g., Kjelsberg *et al.*, 1992). These differences in α-factor–binding affinity could reflect the extent that various amino acid substitutions destabilize the inactive conformation of the receptor, affecting agonist-binding affinity indirectly; alternatively, they could be due to alterations of the ligand-binding site, directly affecting pheromone-binding affinity. Further experiments will be needed to address these questions.

Receptor Protein Expression and Trafficking. Substitutions of proline-258 could reduce the expression of cell-surface α-factor–binding sites by affecting receptor endocytosis, degradation, retention within the cell, or folding to form an active ligand-binding site. To address these possibilities we performed several experiments with a subset of the mutant receptors.

Initially, immunoblotting was used to examine the expression of wild-type and constitutively active receptor polypeptides. These experiments employed receptors that were tagged at their C termini with three tandem copies of the c-*myc* epitope, a modification that did not alter the signaling, ligand-binding, or internalization properties of the receptors (Stefan, unpublished data). The results indicated that although substitutions of proline-258 caused severe reductions in the level of cell-surface ligand-binding sites (P258L, eightfold reduction; P258Y, undetectable ligand-binding activity), they had relatively little effect on receptor protein expression levels (Figure 1).

Subsequently, we examined whether reduced cell surface expression of receptors was due to increased rates of receptor internalization. This was studied in two ways. First, we examined rates of receptor internalization from the cell surface. These experiments

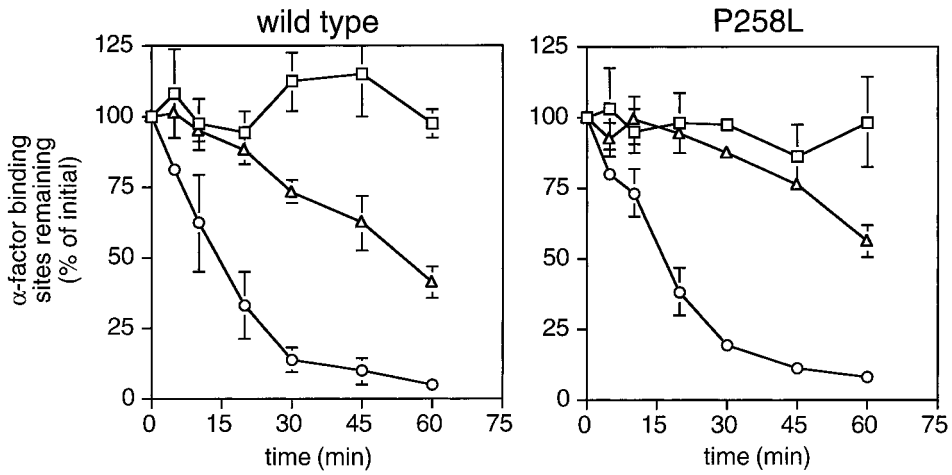

Figure 2. Internalization of wild-type and constitutively active α-factor receptors. The wild-type *STE2* or *ste2*P258L alleles were expressed from their normal promoters on centromere-containing plasmids (pRS314 derivatives) in a *ste2*Δ mutant (KBY16). Assays of basal and agonist-induced internalization of wild-type receptors (left panel) and constitutively active α-factor receptors (expressed from the *ste2*P258L allele; right panel) were performed by determining the number of cell surface α-factor–binding sites remaining as a function of time. Rates of receptor internalization were determined in the absence of α-factor (triangles) and in response to unlabeled α-factor (circles) that was stripped from cells before radioligand binding assays were performed. As a control, the stability of cell surface receptors (squares) was determined by inhibiting internalization (with NaN_3 and KF) and determining the number of ligand-binding sites remaining over time. Data shown are the average of duplicate assays of two independent transformants expressing wild-type or constitutively active receptors; standard deviations are indicated.

were done by following the loss of cell-surface α-factor–binding sites over time under conditions in which new receptor synthesis is blocked. The results indicated that the P258L substitution did not increase the basal or α-factor–stimulated rates of receptor internalization (Figure 2). However, this does not necessarily rule out that mutant receptors have increased basal internalization rates. During the course of the internalization experiment there could be two balancing, competing processes occurring simultaneously: increased basal internalization of mutant receptors from the cell surface, and the delivery of mutant receptors from intracellular pools to the cell surface. If so, removal of the C-terminal domain of mutant receptors, which is required for endocytosis (Reneke *et al*., 1988), should restore cell surface expression of mutant receptors to wild-type levels. Accordingly, we truncated wild-type and mutant receptors bearing the P258L substitution immediately after TMS VII by changing codon 300 to a nonsense codon, creating the *ste2*-300ter and *ste2*P258L,300ter alleles, respectively. We found that the level of ligand-binding sites on the surface of cells expressing truncated constitutively active receptors (encoded by the *ste2*P258L, 300ter allele) was 10-fold lower than that on the surface of cells expressing truncated wild-type receptors (*ste2*-300ter) (5,000 sites/cell and 49,000 sites/cell, respectively). Thus, there were no indications that increased basal rates of receptor internalization are responsible for reducing the levels of mutant receptors at the cell surface.

Because decreased protein expression or increased endocytosis rates appeared insufficient to account for the low level of cell-surface ligand-binding sites in cells expressing constitutively active receptors, we examined the subcellular localization of wild-type and constitutively active receptors by performing indirect immunofluorescence experiments. These experiments employed the full-length *myc*-tagged wild-type and constitutively active receptors described previously. Cells expressing wild-type *myc*-tagged receptors displayed intense cell-surface staining (Figure 3B), and less extensive staining of intracellular compartments, consistent with previous studies using untagged wild-type receptors (Jackson *et al*., 1991). In contrast, cells expressing *myc*-tagged receptors harboring either the P258L or P258Y substitution displayed weak or undetectable cell-surface staining (Figure 3, C and D); however, staining of intracellular compartments was observed. Therefore, the low level of α-factor–binding sites detected in cells expressing constitutively active receptors is correlated with the retention of receptor polypeptides in intracellular organelles.

To characterize the intracellular compartment(s) where mutant α-factor receptors accumulate, we performed subcellular fractionation and double-label immunofluorescence experiments. The former experiments used sucrose density gradients to fractionate lysates prepared from cells expressing *myc*-tagged wild-type or mutant (P258Y) receptors. Immunoblotting was used to detect *myc*-tagged receptors and various marker proteins in gradient fractions (Figure 4).

As expected, the fractionation of wild-type receptors most closely resembled that of the plasma membrane ATPase (Pma1p). In contrast, the fractionation of mutant receptors most closely resembled that of the Golgi-localized guanosine diphosphatase (Gda1p). This conclusion was further supported by the results of double-label immunofluorescence experiments using anti-Kar2p antibodies and anti-*myc* monoclonal antibodies (Figure 5). Kar2p immunofluorescence was restricted mainly to perinuclear rings characteristic of the ER. In contrast, staining of *myc*-tagged mutant receptors was more widely distributed in a punctate pattern that did not overlap considerably with that of Kar2p. These results therefore suggested that mutant α-factor receptors bearing substitutions of proline-258 accumulate in post-ER compartments.

The intracellular accumulation of constitutively active receptors could occur for various reasons. One possibility is that receptor retention is caused directly or indirectly by activation of the pheromone-response pathway. To test this possibility we inactivated the signal transduction pathway by deleting the *STE4* gene, which encodes the G protein β subunit required for receptor-G protein coupling and signal propagation (Whiteway *et al*., 1989; Grishin *et al*, 1994), and examined the expression and localization of wild-type and constitutively active receptors by performing radioligand binding and immunofluorescence experiments. In the *ste4* mutant, wild-type and constitutively active (P258L) receptors were expressed at 2500 sites/cell and 350 sites/cell, respectively. Similarly, immunofluorescence experiments indicated that wild-type *myc*-tagged receptors were present primarily at the cell surface in *ste4* mutants, whereas myc-tagged constitutively active receptors (bearing the P258L substitution) were localized in intracellular compartments (Stefan, unpublished data). Therefore, activation of the signaling pathway was not required for intracellular localization of constitutively active receptors.

A second possibility is that mutant receptors are retained in intracellular compartments because they have folding defects. However, the folding status of the intracellular pool of mutant receptors cannot be determined by performing ligand-binding assays because the ligand-binding site should be lumenally disposed, and because receptors are inactivated upon detergent treatment of membranes (Blumer, unpublished data). Nevertheless, a folding defect seems likely because similar defects appear to occur when conserved proline residues in other GPCRs are substituted with other amino acids (Wess *et al*., 1993; Kolakowski *et al*., 1995).

To determine whether defects in receptor expression at the cell surface limit the strength of the constitutive signal, we examined the effects of overexpressing various *STE2* alleles. Overexpression of *STE2* alleles (wild type, P258D, P258L, P258Y) from high-copy plasmids increased the levels of cell-surface α-factor binding sites 3- to more than 10-fold (compare Table 4, column 4, with Table 2, column 7). Whereas overexpression of wild type receptors did not increase the basal signal, overexpression of mutant receptors caused a threefold increase (up to 50% of the fully induced level) in the constitutive signal (compare Table 4, column 2, with Table 2, column 2). Furthermore, receptor overexpression completely corrected the defects in agonist-induced signaling of some of these proline-258 substitutions (P258D and P258Y; compare Table 4, column 3 with Table 2, column 3). Thus, defects in receptor expression at the cell surface probably account for the relatively weak constitutive signal and impaired agonist-induced signaling caused by certain substitutions of proline-258.

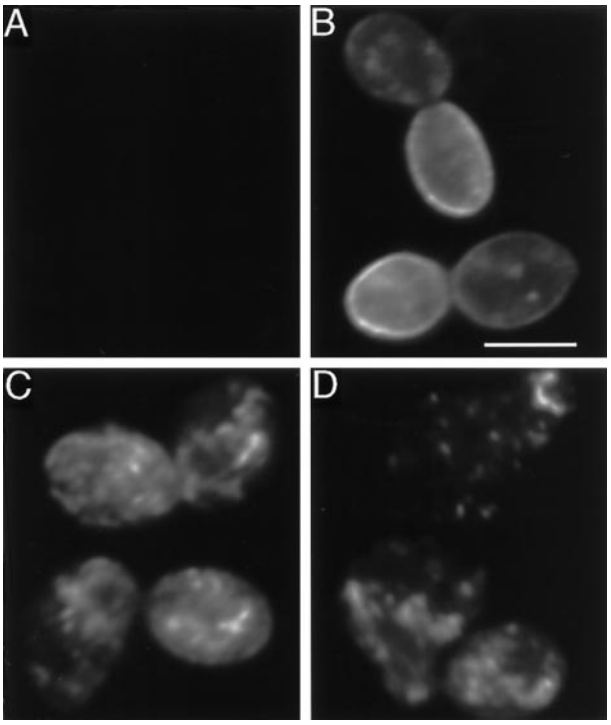

Figure 3. Immunofluorescence localization of *myc*-tagged wild-type and constitutively active α-factor receptors. Various *myc*-tagged α-factor receptors were expressed from their normal promoters on centromere-containing plasmids (pRS314 derivatives) in a *ste2*Δ mutant (KBY16). Cells carrying a control plasmid (expressing untagged α-factor receptors; panel A), or plasmids expressing *myc*-tagged wild-type (panel B) or constitutively active α-factor receptors (*ste2*P258L-*myc*, panel C; *ste2*P258Y-*myc*, panel D) were prepared for indirect immunofluorescence using 9E10 monoclonal antibodies specific for the *c-myc* epitope. Cells expressing the *ste2*P258Y-*myc* allele were misshapen, resembling the morphology of pheromone-treated wild-type cells. Bar, 5 μm.

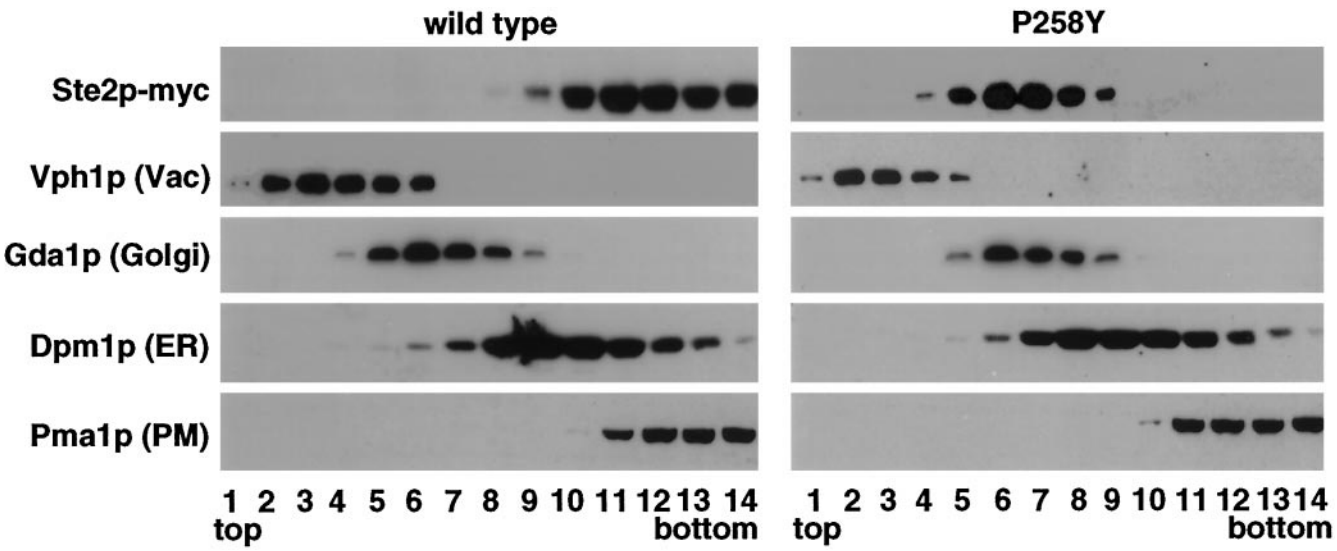

Figure 4. Subcellular fractionation of wild-type and constitutively active α-factor receptors. *Myc*-tagged forms of wild-type and constitutively active (P258Y) α-factor receptors were expressed from their normal promoters on centromere-containing plasmids (pRS314 derivatives) in a *ste2*Δ mutant (KBY16). Cell extracts were fractionated by sucrose gradient centrifugation. Gradient fractions (1 = top; 14 = bottom) were analyzed by immunoblotting using anti-*myc* antibodies and antibodies specific for the following marker proteins: Vph1p (vacuole), Gda1p (Golgi), Dpm1p (ER), and Pma1p (plasma membrane).

Expression of Wild-Type Receptors Suppresses Constitutive Signaling by Mutant Receptors

Because α-factor receptors bearing substitutions of proline-258 are constitutively active, we anticipated that they would signal constitutively when they are coexpressed with wild-type receptors. Contrary to this expectation, when constitutively active (P258L or P258Y) and wild-type α-factor receptors were coexpressed from their normal promoters on single-copy plasmids, a significant constitutive signal was not detected (Table 5), indicating that mutations resulting in constitutively active receptors are nearly completely recessive. Similarly, Konopka and colleagues showed that the presence of wild-type receptors reduces ability of constitutively active receptors to transduce a signal in the absence of α-factor (Konopka *et al*., 1996); however, the magnitude of this inhibitory effect was less than we observed, which led these investigators to conclude that the *ste2*P258L allele is partially dominant. Differences in strain background might account for these quantitative differences (for example, our strains were deleted for the α-factor structural genes, whereas those used by others were not), but this has not been examined directly. Nevertheless, our results agree qualitatively with those published previously (Konopka *et al*., 1996).

Wild-type receptors could interfere with the ability of constitutively active receptors to signal by various mechanisms. For example, wild-type and mutant receptors could interact to form oligomers having low agonist-independent activity similar to that of wild-type receptors alone; however, evidence that α-factor receptors form oligomers in the membrane has not been reported. Alternatively, in the absence of agonist, wild-type receptors could associate with and sequester G protein heterotrimers that are present in limiting amounts (i.e., receptors and G proteins are "precoupled"), thereby preventing constitutively active receptors from transmitting a signal. Overexpressing the three G protein subunits could overcome this effect, but this would be difficult to accomplish experimentally because the subunits must be overproduced stoichiometrically. As an alternative, we determined whether receptors that interact inefficiently with G proteins are unable to interfere with the ability of constitutively active receptors to signal. Accordingly, we coexpressed constitutively active receptors with receptors that bear a substitution affecting the third cytoplasmic loop (*ste2*L236R, which reduces coupling efficiency 10-fold without affecting ligand-binding affinity, receptor cell surface expression, or endocytosis; Weiner *et al*., 1993). In this situation, constitutively active receptors were able to transduce a constitutive signal (Table 5). Assuming mutations that uncouple receptors do not affect receptor oligomerization or other aspects of receptor function, these results suggest that α-factor receptors and G proteins are precoupled in the absence of pheromone stimulation.

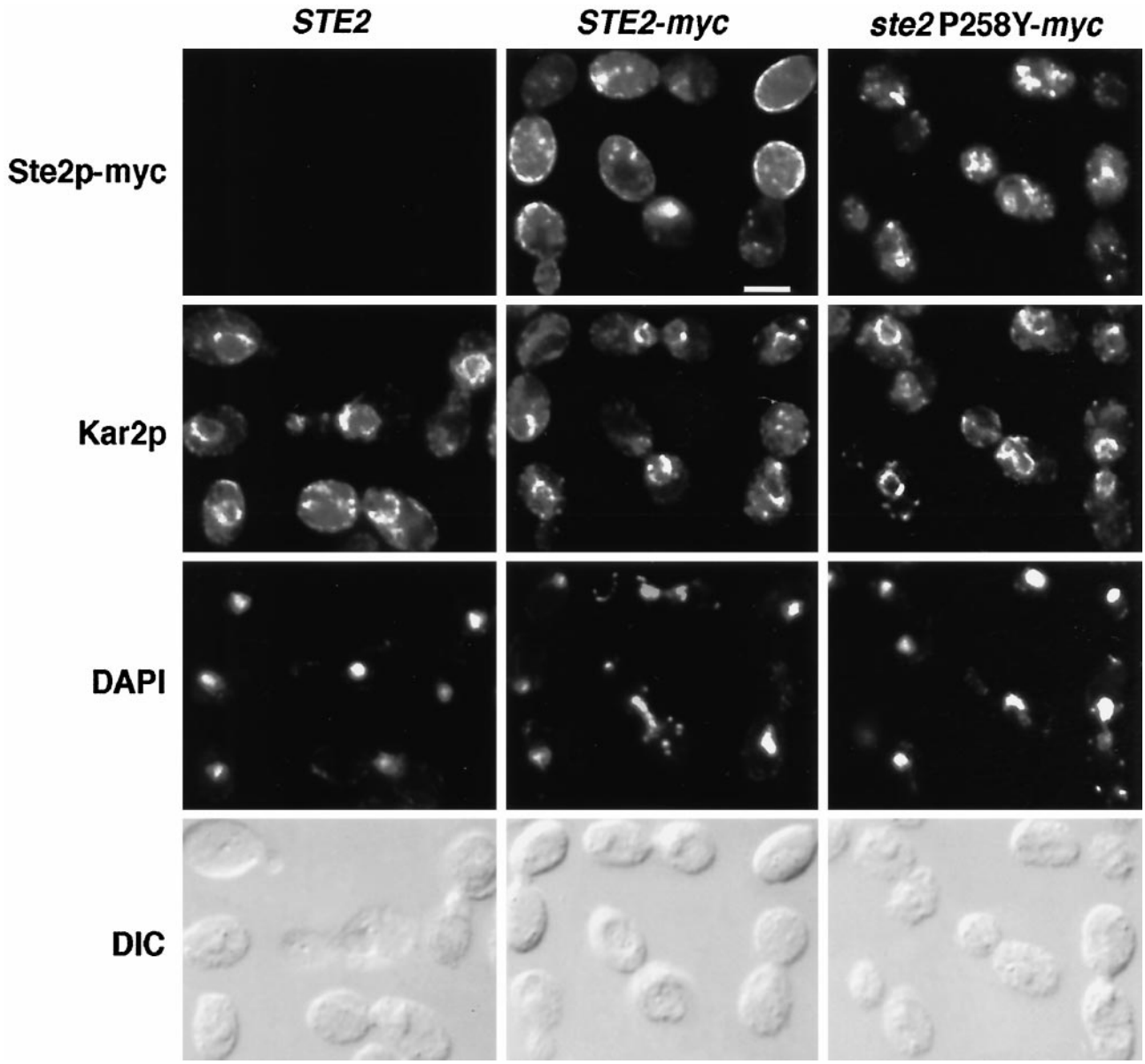

Figure 5. Double-label immunofluorescence localization of α-factor receptors and Kar2p. Untagged wild-type receptors (*STE2*; first column) and *myc*-tagged forms of wild type (*STE2-myc* ; second column) and constitutively active (*ste2*P258Y-*myc*; third column) α-factor receptors were expressed from their normal promoters on centromere-containing plasmids (pRS314 derivatives) in a *ste2*Δ mutant (KBY16). Cells were costained with anti-*myc* monoclonal antibodies (first row), a rabbit antisera specific for the ER marker protein Kar2p (second row), and 4′,6-diamino-2-phenylindole (third row). Bar, 5 μm.

DISCUSSION

GPCR Activation Mechanisms

Our results and those of others (Konopka *et al*., 1996) indicate that a conserved proline residue in TMS VI controls the equilibrium between the inactive and active states of the α-factor and **a**-factor receptors. We suggest that the same mechanism is likely to control the activity of other GPCRs, even though substitutions affecting the equivalent proline residues in rhodopsin, m_3-muscarinic acetylcholine receptors, and C5a receptors reportedly do not cause a constitutive signal (Wess *et al*., 1993; Kaushal and Khorana, 1994; Kolakowski *et al*., 1995). These negative results may be due to the type of amino acid used to replace the proline residue in these receptors, reductions in receptor expression at the cell surface, or the action of RGS proteins, all of which strongly influence the apparent strength of

Table 4. Effects of overexpressing *STE2* alleles on constitutive activation of the pheromone response pathway and expression of cell-surface α-factor binding sites

STE2 allele expressed	*FUS1-lacZ* expression (% wild type + α-factor)		Receptor expression (α-factor binding sites/cell)
	−α-factor	+α-factor	
*ste2*Δ	0.7	1.0	−
Wild type	0.4	100	34,000
P258L	18	96	18,000
P258D	3.9	97	700
P258Y	54	103	250

The indicated *STE2* alleles were expressed from their normal promoters on high-copy plasmids (pRS424 derivatives) in a *ste2*Δ *mfα1*Δ *mfα2*Δ mutant (KBY16) that contained the *FUS1-lacZ* gene on a plasmid (pSL307). Where indicated, cells were treated 2 h at 30°C with 1 μM synthetic α-factor (+α-factor); β-galactosidase assays were performed to monitor activation of the pheromone response pathway. Data are expressed as a percent of the activity detected with α-factor–treated cells overexpressing the wild-type *STE2* gene. Values shown are the average of a least four independent transformants, each assayed in duplicate; standard errors were 5-30% of the values shown. Radioligand binding assays using [^{35}S]α-factor were performed to determine the number of cell-surface ligand-binding sites (B_{max}) in cells (KBY16) overexpressing various *STE2* alleles from high-copy plasmids (pRS424 derivatives). The values shown for each *STE2* allele were calculated from nonlinear regressions of data obtained from two independent transformants, each of which was assayed two to four times. Standard errors for these determinations were 5-15%

Table 5. Effects of coexpressing *STE2* alleles on constitutive activation of the pheromone response pathway

STE2 alleles coexpressed		*FUS1-lacZ* expression (% wild type + α-factor)	
		−α-factor	+α-factor
Wild type	Wild type	0.4	100
Wild type	P258L	0.9	128
Wild type	P258F	0.5	103
L236R	Wild type	0.7	123
L236R	P258L	4.6	114
L236R	P258F	7.0	104

The wild-type *STE2* gene or the *ste*2L236R allele, which causes a specific defect in receptor coupling with G proteins, was coexpressed with the wild-type *STE2* gene, or the *ste*2P258L or *ste*2P258F alleles. These genes were coexpressed from their normal promoters on centromeric plasmids (pRS313 and pRS314 derivatives) in a *ste2*Δ *mfα1*Δ *mfα2*Δ mutant (KBY20) that contained the *FUS1-lacZ* gene on a plasmid (pSL307). Where indicated, cells were treated 2 h at 30°C with 1 μM synthetic α-factor (+α-factor); β-galactosidase assays were performed to monitor activation of the pheromone response pathway. Values are expressed as a percent of the activity detected with α-factor–treated cells expressing the wild-type *STE2* gene. Values shown are the average of at least four independent transformants, each assayed in duplicate; standard errors were 5-30% of the values shown.

the signal transduced by constitutively active receptors in yeast.

There are several ways, which are not mutually exclusive, whereby the conserved proline residue in TMS VI could control the activity of the α-factor receptor or other GPCRs. It could facilitate the initial folding of the receptor into its native, inactive conformation, stabilize the inactive conformation once it forms, and/or participate directly in the process of agonist-induced activation. It could control these processes by affecting the secondary and/or tertiary structure of the receptor. For example, the conserved proline residue may allow TMS VI to switch between kinked and more helical secondary structures, consistent with studies of artificial proline-containing transmembrane segments that suggest secondary structural changes of this kind could involve relatively modest changes in free energy (Polinsky *et al*., 1992). Alternatively, the conserved proline residue could allow TMS VI to adopt a relatively fixed secondary structure that favors formation of a native, inactive tertiary structure. Changing the secondary structure of TMS VI by substituting the proline with another amino acid could therefore destabilize the inactive tertiary structure of the receptor, leading to G protein activation.

These hypotheses are consistent with recent biochemical and biophysical studies of rhodopsin. In rhodopsin the region of helix F (TMS VI) containing the conserved proline residue (proline-267) is located near the β-ionone ring of retinal when the chromophore exists in the *cis* isomer (Nakanishi *et al*., 1995), which maintains the inactive conformation of rhodopsin. When rhodopsin is activated by light, the environment of tryptophan-265 in helix F changes (Lin and Sakmar, 1996) and a rigid-body motion of helix F relative to helix C (TMS III) appears to occur (Farrens *et al*., 1996).

The conserved proline residue of TMS VI is probably not the sole determinant governing GPCR activation. We have found that none of the substitutions of proline-258 appear to result in full constitutive activation. Instead, pheromone stimulation was needed to elicit a maximal signal. Mutations affecting domains other than TMS VI of various mammalian GPCRs also result in constitutive activation (Robinson *et al*., 1992; Parma *et al*., 1993; Robbins *et al*., 1993; Samama *et al*., 1993; Shenker *et al*., 1993). Thus, the activation process probably involve various subdomains of GPCRs.

GPCR Trafficking

Our results indicate that the conserved proline residue in TMS VI is required for efficient expression of α-factor receptors at the cell surface. Receptors bearing substitutions of proline-258 accumulate in intracellular compartments, achieving steady state levels similar

to those of wild-type receptors expressed at the cell surface; however, it is possible that a small proportion of the mutant receptor population is targetted to the vacuole and degraded. Mutant receptors may accumulate in intracellular compartments because they are folded incompletely, although this remains to be established experimentally. Intracellular accumulation of mutant receptors apparently occurs by a mechanism that does not involve receptor internalization from the cell surface. Instead, mutant receptors accumulate mainly in post-ER compartments. Consistent with post-ER accumulation of receptors, loss of Cne1p, a calnexin homolog that is a component of the ER quality control machinery (Parlati *et al*., 1995), does not suppress the cell surface expression defects of constitutively active α-factor receptors (Stefan, unpublished results).

Various mutant forms of the α-factor receptor appear to have distinct targetting defects. Whereas constitutively active receptors accumulate in post-ER compartments without undergoing extensive degradation, temperature-sensitive receptors are targetted relatively efficiently to the vacuole and degraded (Jenness *et al*., 1997). Although the mechanisms responsible for achieving these different fates are unknown, there are several possibilities. For example, cells may possess two types of trafficking receptors, one that recognizes more grossly misfolded membrane proteins, such as temperature sensitive α-factor receptors, targetting them to the vacuole, and a second type of trafficking receptor that binds more completely folded membrane proteins, such as constitutively active α-factor receptors, preventing them from reaching the cell surface until folding is complete. In a second model, a single type of trafficking receptor recognizes relatively grossly misfolded membrane proteins and targets them to the vacuole, whereas more completely folded membrane proteins accumulate in the Golgi because they are not packaged or concentrated efficiently into secretory vesicles destined for the plasma membrane. In a third model, a single type of trafficking receptor or chaperone binds membrane proteins that are folded nearly normally, allowing them to be retained in post-ER compartments until folding is complete, whereas grossly defective membrane proteins are not bound and are targetted by default to the vacuole. Of these models, the latter is somewhat more consistent with the general view that protein targetting to the vacuole is the default pathway for defective membrane proteins (e.g., Chang and Fink, 1995; Jenness *et al*., 1997) or proteins that fail to be retained normally in the ER or Golgi (e.g., Roberts *et al*., 1992; Wilcox *et al*., 1992; an alternate interpretation is expressed by Rayner and Pelham, 1997). Regardless of the specific mechanisms involved, these quality control processes may ensure that wild-type pheromone receptors are retained intracellularly until they fold into their native, fully inactive conformations. This may prevent partially folded wild-type receptors, which may have some degree of constitutive activity, from reaching the cell surface and inappropriately triggering a signal in the absence of pheromone.

Similar quality control mechanisms may govern the trafficking and biogenesis of GPCRs in mammalian cells because normal biogenesis of certain mammalian GPCRs appears to require the conserved proline residue in TMS VI. For example, a leucine substitution of the conserved proline residue in TMS VI of human rhodopsin causes autosomal dominant retinitis pigmentosa (Fishman, *et al*., 1992), which can be caused by defects in rhodopsin biogenesis (Sung *et al*., 1993, 1994; Kaushal and Khorana, 1994; Colley *et al*., 1995). Similarly, substitutions affecting the equivalent residues in m_3-muscarinic acetylcholine and C5a receptors cause defects in receptor expression at the cell surface (Wess *et al*., 1993, Kolakowski *et al*., 1995), although the effects of these mutations on the stability, endocytosis, or transit of these receptors through the secretory pathway have not been established. However, GPCR-targetting defects do not always result from substitutions affecting the conserved proline residue in TMS VI (Hong *et al*., 1997), suggesting that targetting defects can be receptor- and/or cell type-specific.

Precoupling of Pheromone Receptors and G Proteins?

We have found that coexpression of wild-type, but not G protein coupling-defective receptors, effectively suppresses the ability of constitutively active α-factor receptors to signal in the absence of agonist. Based on this finding, our current working hypothesis is that wild-type pheromone receptors associate with and sequester a limiting pool of G proteins. This "precoupling" model is consistent with pharmacological and biochemical evidence in mammalian systems (Neubig *et al*., 1988; Siciliano *et al*., 1990; Tian and Deth, 1993; Shi and Deth, 1994), whereas other potential mechanisms, such as receptor oligomerization, are less well substantiated biochemically. Precoupling of receptors and G proteins may enable cells to respond efficiently and rapidly to low levels of signal and/or facilitate signal propagation at specific sites on the cell surface. In yeast, precoupling could be important for sensing and responding chemotropically to pheromone gradients (Segall, 1993), as is thought to occur during mating partner discrimination (Jackson *et al*., 1991).

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