Purification and Characterization of a Recombinant G-Protein-Coupled Receptor, *Saccharomyces cerevisiae* Ste2p, Transiently Expressed in HEK293 EBNA1 Cells†

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ABSTRACT: The production of milligram quantities of purified, active, folded membrane protein from heterologous expression systems remains a general challenge due to intrinsically low expression levels, misfolding, and instability. Here we report the overexpression and purification of milligram quantities of functional *Saccharomyces cerevisiae* G-protein-coupled receptor, Ste2p, from transiently transfected human embryonic kidney 293 EBNA1 cells. Fluorescent microscopy indicates localization of Ste2p-GFP and Fc-Ste2p-GFP fusion receptors to the cell membrane. Up to 2 mg (∼10 pmol/million cells) of the Fc-Ste2p-GFP fusion and 1 mg of a Ste2p-Strep-TagII/$(His)_8$-tagged version were purified per liter of culture following protein A−Sepharose and Talon metal affinity chromatography, respectively. Two distinct fluorescent labels, the hydrophobic 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) and the more hydrophilic fluorescein-5-maleimide (FM), were individually attached to the C-terminus of the α-mating factor ligand by addition of a reactive cysteine residue to produce active fluorescent pheromones. *In vitro* fluorescent ligand binding assays demonstrated that a high percentage of the recombinant purified receptor is correctly folded and able to bind ligand. K_D values of 34 ± 3 and 300 ± 20 nM were observed respectively for the CPM- and FM-labeled ligands. These results combined with blue-shifted emission peaks and loss of fluorescent quenching observed for both fluorescent-labeled Cys α-factors when bound to receptor support a model in which the C-terminus of the ligand is packed in a hydrophobic pocket at the interface between the transmembrane and extracellular loop domains. Overall, we present an efficient system for recombinant production of milligram quantities of purified Ste2p in a biologically active form with applications to future structure and functional studies.

G-protein-coupled receptors (GPCRs)[1] comprise one of the largest known superfamilies of receptors with in excess of 2000 genes identified across taxa (*1*). These heptahelical receptors play crucial roles in the transduction of extracellular (EC) signals (such as light, calcium, amines, peptides, nucleotides, and hormones) to intracellular responses through conformationally mediated interactions with downstream heterotrimeric G-proteins (*2*). They represent a major class of pharmaceutical targets in pain perception, viral pathogenesis, growth, and blood pressure regulation (*3*).

Despite the widespread occurrence of GPCRs and the various proposed modes of action currently available in the literature, fundamental information about the molecular details of their mechanisms of action including structures of ligand binding sites, conformational changes associated with activation events, and the specific interactions with downstream regulatory proteins including G-proteins, GPCR kinases, and arrestins are still not well understood (e.g., refs *4*−*6*). A variety of factors have limited progress in this field. Of particular note are the overall low expression levels and general instability of the GPCRs in both native and recombinant systems. As well, loss of activity upon detergent solubilization/purification and general incompatibilities between these amphipathic molecules and classical biophysical methods of protein analysis further complicate studies. However, some recent successes have been achieved with the prototypical receptor rhodopsin, including the elucidation of a high-resolution crystal structure of the inactive state (*7*). Modeling other GPCRs based on this crystal structure has provided some evidence of a general activation mechanism associated with the helical bundle, but the lack of high-resolution data and the overall low homology in crucial

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[1] Abbreviations: GPCR, G-protein-coupled receptor; Ste2p, α-factor receptor; Ste3p, a-factor receptor; EC, extracellular; TM, transmembrane; HEK293, human embryonic kidney; 293E, human embryonic kidney 293 Epstein−Barr virus nuclear antigen 1 cells; CPM, hydrophobic 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin; FM, fluorescein-5-maleimide; GFP, green fluorescent protein; PEI, polyethylenimine; BCS, bovine calf serum; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; DM, *n*-dodecyl β-D-maltoside; WT, wild type; PMF, peptide mass fingerprinting; MS, mass spectrometry; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight.

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ligand and protein binding loop regions have limited the applicability (*8*, *9*). The lack of homology in the EC and cytoplasmic loop regions makes the acquisition of additional structure/function information, not only for rhodopsin but also for a cross section of GPCRs representing the different classes, absolutely essential.

The *Saccharomyces cerevisiae* class D α-factor receptor (Ste2p) is a GPCR that, upon the binding of tridecapeptide pheromone (WHWLQLKPGQPMY), transduces a mating signal via G-protein-mediated downstream mitogen-activated protein kinase cascades, much as in mammalian systems (*10*−*12*). Although the amino acid sequence of Ste2p does not share high homology to other GPCRs, mutational and homology modeling analyses highlight underlying structural and functional similarities between yeast and mammalian GPCRs (reviewed in ref *9*). In addition, experiments have demonstrated activation of mammalian G-proteins by Ste2p and the reciprocal activation of the yeast pheromone responsive pathway by mammalian GPCRs (*13*−*16*). Together, these observations emphasize the applicability of Ste2p as a model par excellence with which to study GPCR signal transduction mechanisms toward understanding human GPCR structure and related drug design.

The biophysical and structural characterizations of GPCRs require large quantities (tens of milligrams) of pure protein. Natural sources are limited due to low expression levels, and thus recombinant technology is necessary to increase yields and facilitate purification (reviewed in ref *17*). The overexpression of GPCRs in prokaryotic hosts such as *Escherichia coli* is limited by the lack of posttranslational modification and often results in the accumulation of the receptor in inclusion bodies (*18*). Yeast provides a eukaryotic environment and ease of handling; however, previous overexpression of Ste2p yielded intermediate levels of heterogeneously glycosylated receptor forms that upon detergent solubilization and purification yielded mainly inactive receptor in the absence of lipid extracts (*19*). While insect cells have also been applied to GPCR heterologous expression (*20*, *21*), mammalian cells are proving to be the ideal system for preservation of structural and functional integrity of the recombinant receptors for high-yield overproduction. Human embryonic kidney 293 (HEK293) cells in particular have been used to successfully express many GPCRs including dopamine receptors (*22*), prostanoid receptors (*23*), α- and β-adrenergic receptors *(24*, *25*), rhodopsin (*26*), and most recently yeast Ste2p (*16*).

In this work, overexpression of yeast pheromone receptor Ste2p was performed using suspension-adapted HEK293 cells stably expressing the Epstein−Barr virus nuclear antigen 1 (293E cells). The protein was produced by transient transfection using polyethylenimine (PEI) as the transfection reagent (*27*). Fluorescent microscopy observation indicates that the bulk of the GFP-fused receptor is localized to the cell membrane. Following solubilization in *n*-dodecyl β-D-maltoside (DM) detergent, affinity purification methods including protein A−Sepharose, immobilized metal affinity chromatography (IMAC), and Strep-Tactin affinity chromatography were compared. Yields of purified receptor reach up to 2 mg/L of media (∼10 pmol/million cells). Correct folding and activity of recombinant Ste2p was confirmed by binding of fluorescent-labeled α-factor to the receptor *in vivo* and kinetic characterization of ligand binding after its purification in vitro. Overall, these results indicate that this purified recombinant receptor is amenable to future biophysical analyses.

MATERIALS AND METHODS

Chemicals. Protease inhibitor substitute, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), was obtained from Roche Diagnostics Corp. *n*-Dodecyl β-D-maltoside (DM) was obtained from Anatrace. Horseradish peroxidase (HRP) conjugated anti-HisG antibody was obtained from Invitrogen (Burlington, Ontario, Canada). Rabbit polyclonal HRP-conjugated anti-GFP antibody was obtained from Santa Cruz Biotechnology. Geneticin (G418) was obtained from Invitrogen. Linear 25 kDa polyethylenimine (PEI) was obtained from Polysciences Inc. Bovine calf serum (BCS) was obtained from Hyclone (Logan, UT). Talon resin was from BD Biosciences (Palo Alto, CA) and Strep-Tactin Sepharose was from Qiagen (Mississauga, Ontario, Canada). 7-(Diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) and fluorescein-5-maleimide (FM) were purchased from Biotium. All other reagents were from Sigma.

α-*Factor Peptide*. Wild-type α-factor (WT-α-factor, Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr) was purchased from Zymo Research. α-Factor with cysteine at the C-terminus (Cys α-factor, Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr-Cys) was synthesized and purified by HPLC in the peptide synthesis laboratory, PBI/NRC and Peptide Research Institute/University of Alberta, and characterized by matrix-assisted laser desorption ionization−time-of-flight mass spectrometry (MALDI-TOF-MS). The modification of Cys α-factor with FM (FM-Cys α-factor) or CPM (CPM-Cys α-factor) was performed as follows: 100 μL of 2 mg/mL Cys α-factor in PBS, pH 7.0, was mixed thoroughly with 100 μL of 1 mg/mL FM (resuspended in 50% *N*,*N*-dimethylformamide:50% water) or CPM (resuspended in 80% *N*,*N*-dimethylformamide:20% water), respectively, for 30 min on ice. The reaction was stopped by adding 10 μL of 10% (v/v) β-mercaptoethanol (β-ME). The labeled α-factor was prepared for kinetic binding analyses by dialysis against 50 mM Tris-HCl buffer, pH 7.0, using a 1000 Da molecular mass cutoff membrane (Spectra/Pro) to get rid of excess unbound fluorescent label and then subsequently characterized by MALDI-TOF-MS.

Strains and Plasmids. Strain DH5α was used to amplify plasmids for transient transfection. Plasmids were purified by a Qiagen plasmid mega kit. Yeast strain BY4741 (YIL015W−MATa Δhis3 Δleu2 Δmet15 Δura3 Δbar1) was from American Type Cultue Collection. The *STE2* gene was amplified from genomic DNA with forward (5′-TCAGCTAGCATGTCTGATGCGGCTCCT-3′) and reverse (5′-TGAGCTAGCAGGATCCAAATTATTATTATCTTC-3′) primers and cloned in-frame into the *Nhe*I sites of the pTT5/Fc-GFP vector to yield the pTT/Fc-Ste2p-GFP plasmid (Figure 1) or amplified with forward (5′-AGCAGTGGATC-CGCCACCATGTCTGATGCGGC-3′) and reverse (5′-ACT-GCTTGGATCCTCTAAATTATTATT-3′) primers and introduced in-frame into the *Bam*HI position of either the pTTSH8Q1 vector (*28*) to make pTT/Ste2p-SH8Q1 [pTT/Ste2p-Strep-TagII/$(His)_8$] or a modified version of pTT/GFP (second 3′ *Bam*HI site deleted by mutagenesis) to make pTT/

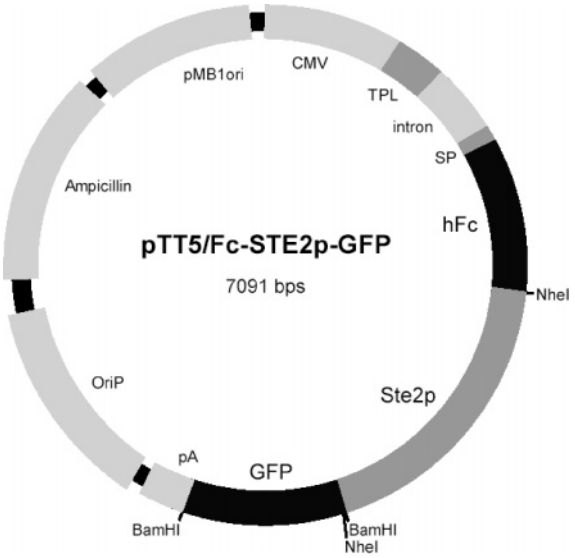

FIGURE 1: Map of the pTT5 vector with the Fc-Ste2p-GFP fusion construct. Abbreviations: CMV, CMV promoter; TPL, adenovirus tripartite leader sequence; SP, human IgG κ signal peptide; hFc, human IgG Fc fragment; pA, rabbit β-globin polyadenylation site; OriP, Epstein−Barr virus origin of replication fragment. See ref *27* for more details on the pTT vector.

Ste2p-GFP. See ref *27* for more information about the pTT vectors.

Cell Culture and Transient Transfection. 293E cells were maintained in low-calcium hybridoma serum-free medium (LC-SFM) supplemented with 1% BCS and 50 μg/mL G418 as previously described (*27*). Cells were maintained in agitated (110−130 rpm) shake flasks under standard humidified conditions (37 °C and 5% CO_2). One day prior to transfection, cells were diluted at a density of 0.25×10^6/mL in LC-SMF growth medium, supplemented with 1% BCS and transfected 18−24 h later when the density reached approximately 0.5×10^6 cells/mL. Plasmid DNA and PEI were mixed together in LC-SFM medium at 1 and 2 μg/mL of culture to be transfected, respectively. Upon the addition of PEI to the DNA, the mixture was vortexed, incubated for 15 min, and added to the cells. Cells were harvested 72−100 h post-transfection (hpt) by centrifugation at 1500*g* for 10 min, followed by a wash with ice-cold PBS and a subsequent centrifugation step at 1500*g* for 15 min. The cell pellets were frozen at −80 °C.

Receptor Solubilization and Purification. The expressed Ste2p was extracted either from the whole cell or from the membrane fraction. For the whole cell extraction, the pelleted cells were thawed and resuspended in extraction buffer (50 mM PBS buffer, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM AEBSF) with gentle swirling on ice. The solution was homogenized by a 1500 psi French press. DM (1%) and 5 mM β-ME were added to the lysed sample, stirred on ice for 1 h, and then centrifuged for 1 h at 14000*g* to remove insoluble cellular material. For preparation of membrane fractions, the cell pellet was French pressed at 1500 psi and centrifuged at 3000*g* to remove debris. The remaining suspension was centrifuged at 100000*g* for 60 min. The final membrane pellet was washed and resuspended in solubilization buffer (50 mM PBS buffer, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM AEBSF, 1% DM, 5 mM β-ME), stirred for 1 h, and centrifuged at 14000*g*. The solubilized samples either from the whole cell extraction or from the membrane fraction were then applied to affinity resin. Fc-Ste2p-GFP-containing samples were loaded on protein A−Sepharose, washed by 100 mM Tris-HCl buffer, pH 7.0, containing 100 mM NaCl, 0.05% DM, 5 mM $MgCl_2$, and 20% glycerol (v/v) for 10 column volumes, eluted by 50 mM pH 3.0 glycine buffer containing 100 mM NaCl, 0.05% DM, 5 mM $MgCl_2$, and 20% glycerol (v/v), and immediately neutralized by 20% (v/v) 1 M Tris-HCl, pH ~7.0. Ste2p-Strep-TagII/$(His)_8$ was loaded on Talon affinity resin or Strep-Tactin resin. The Talon affinity column was washed with 10 column volumes of buffer A [50 mM PBS, pH 8.0, 100 mM NaCl, 0.05% DM, 5 mM $MgCl_2$, 20% glycerol (v/v)] containing 50 mM imidazole and eluted with buffer A containing 250 mM imidazole. The Strep-Tactin affinity column was washed with 10 column volumes of buffer B [50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.05% DM, 5 mM $MgCl_2$, 20% glycerol (v/v), 1 mM EDTA] and eluted with buffer B containing 2.5 mM desthiobiotin.

Protein Analysis. Protein was analyzed by SDS−PAGE on 8% acrylamide gels, stained by Coomassie Brilliant Blue R-250, or transferred to PVDF membrane for western blot. The purity of protein samples was determined by scanning of Coomassie-stained gels with "Scion" imaging software for Windows. Quantitation of purified samples was by either the Bio-Rad BCA protein assay method or Coomassie-stained gel visual comparison using purified glutathione *S*-transferase (GST) as a standard (GST was quantified by estimating absorption at 280 nm = 1 for 0.5 mg/mL protein). The enhanced chemiluminescence method was used for western blot of the crude and purified protein. Purified protein band sequences were identified by peptide mass fingerprinting (PMF) involving tryptic cleavage, combined with MALDI-TOF analysis (at PBI/NRC Mass Spec Facility) using Findmod Tool analysis.

Fluorescent Localization of Ste2p in 293E Cells. The location of the expressed Ste2p GFP fusions in 293E cells was determined by fluorescent microscopy (Leica DMR). At 72 hpt, 293E cells expressing either Fc-Ste2p-GFP or Fc-Ste2p fusions were excited at 480 nm, and emission light (510 nm) was captured by a digital camera. 293E cells expressing cytosolic GFP were used as a control.

Halo Assay of Growth Arrest. The activities of wild-type and modified α-factor ligands were analyzed by halo assay. The yeast strain BY4741 (YIL015W) was used to determine growth arrest. Ten microliters of an overnight culture grown in YPD media at 30 °C was mixed with 4 mL of 0.5% agar at 50 °C and poured over a prewarmed YPD plate. Five microliters respectively of 500, 200, 100, 50, 20, and 10 μM wild-type α-factor, Cys α-factor, CPM-Cys α-factor, or FM-Cys α-factor were pipetted onto the cool plate. The halo results were scanned with black background after 24−48 h of incubation at 30 °C.

α-Factor Binding in Vivo and in Vitro. The *in vivo* binding of modified α-factors to Ste2p was performed using fluorochrome-modified α-factor and imaged by fluorescent microscopy using a GFP filter (480/510 nm) and UV filter (360/420 nm) to observe the binding of FM-Cys α-factor and CPM-Cys α-factor, respectively. Cells binding modified α-factor were prepared as follows: 1 mL of 293E cells 96 hpt was centrifuged at 100*g* and resuspended in 100 μL of prechilled PBS buffer, pH 7.0. Either 0.1 μL of FM-Cys α-factor or 0.1 μL of CPM-Cys α-factor was then added

with mixing and incubated for 30 min on ice. The solution was then centrifuged at 100*g*, and the pellet was washed three times with 1 mL of PBS, pH 7.0, at 4 °C and resuspended in a final volume of 100 μL of PBS. Cells not expressing Ste2p were used as a control.

Saturation ligand binding assays were performed on membranes extracted from cells expressing Ste2p-Strep-TagII/$(His)_8$. Membranes from untransfected cells were used as a control for subtraction of nonspecific binding values. Equal quantities of membrane samples ($OD_{600nm} = 0.4-0.5$ mL) were individually mixed with different concentrations of modified α-factor and incubated on ice for 30 min. The membranes were then pelleted by centrifugation (16000*g*), washed, and resuspended in 400 μL of PBS buffer. The presence of ligand was determined by a fluorescent spectrometer. Competition assays were performed by mixing the same quantity of modified α-factor in the presence of different concentrations of WT α-factor and treated as described above. Assays were carried out in triplicate for both the saturation and competition binding experiments.

The on-column binding assay was carried out as follows: Ste2p-Strep-TagII/$(His)_8$ was loaded on Talon columns and then washed with 10 column volumes of binding buffer. An approximately 10-fold excess of fluorescent-labeled α-factor was then added. Each column was again washed with 10 column volumes of binding buffer and eluted as described above.

In Vitro Ligand Affinity Assay. Dissociation constants (K_D) between labeled factors and Ste2p were determined by fluorescent methods. Purified Ste2p-Strep-TagII/$(His)_8$ samples were mixed with different concentrations of CPM-Cys α-factor or FM-Cys α-factor (200 μL each). Samples were then loaded into 25000 kDa MW cutoff dialysis membranes and dialyzed against 20 mL of buffer (50 mM Tris-HCl buffer, pH 7.0, containing 5 mM $MgCl_2$, 100 mM NaCl, 5 mM β-ME, 0.05% DM, and 20% glycerol). Fluorescent signals were determined after 15 h dialysis (excitation at 384 nm and emission at 469 nm for CPM-labeled factor; excitation at 490 nm and emission at 520 nm for FM-labeled factor). The K_D values were calculated as follows:

$$K_D = [L]_F \frac{[Ste2]_U}{[Ste2]_B} \quad (1)$$

$$K_D = \left(\frac{F_{out}}{\epsilon_1}\right)\frac{[Ste2T] - [(F_{in} - F_{out})/\epsilon_2]}{(F_{in} - F_{out})/\epsilon_2} \quad (2)$$

where ϵ_1 is the emission intensity of 1 M samples of CPM-labeled α-factor (excited at 384 nm) and FM-labeled α-factor (excited at 490 nm) observed at 469 and 520 nm, respectively; ϵ_2 is the emission intensity of 1 M CPM-labeled α-factor after binding with Ste2p (excited at 384 nm) and 1 M FM-labeled α-factor after binding with Ste2p (excited at 490 nm) observed at 469 and 520 nm, respectively; F_{in} is the fluorescent intensity in the dialysis bag; F_{out} is the fluorescent intensity outside the dialysis bag; $[Ste2]_B$ is the concentration of α-factor-bound Ste2p, equal to $(F_{in} - F_{out})/\epsilon_2$; $[Ste2]_U$ is the concentration of unbound Ste2p, equal to $[Ste2T] - (F_{in} - F_{out})/\epsilon_2$, where [Ste2T] is the total concentration of Ste2p in the assay. Rearrangement of eq 2 gives

$$\frac{F_{in} - F_{out}}{F_{out}} = [Ste2T]\frac{\epsilon_2}{\epsilon_1}\left(\frac{1}{K_D}\right) - \frac{1}{\epsilon_1}\left(\frac{1}{K_D}\right)(F_{in} - F_{out}) \quad (3)$$

Using the experimental data F_{in} and F_{out}, the slope $-1/\epsilon_1 K_D$ can be obtained. ϵ_1 was calculated by fluorescent intensity using known concentrations of labeled factor.

Fluorescent Quenching. The exposure of the FM and CPM labels to the external environment was evaluated using the collision quencher potassium iodide. A 2 M stock of potassium iodide with 200 mM sodium thiosulfate was used for all of the reactions. Quenching of free CPM-Cys α-factor and free FM-Cys α-factor was compared to quenching within the complexes of Ste2p/FM-Cys α-factor and Ste2p/CPM-Cys α-factor. Complexes were prepared by mixing purified Ste2p-Strep-TagII/$(His)_8$ with a 5-fold excess of either CPM-Cys α-factor or FM-Cys α-factor and purified by Talon affinity as described above. The eluted complexes were washed by Millipore ultracentrifuge (MWCO 10,000 Da) concentration three times to replace the elution buffer by pH = 8.0, 50 mM PBS containing 5 mM $MgCl_2$, 100 mM NaCl, 5 mM β-ME, and 0.05% DM (w/v). Fluorescent intensities were measured in the presence of different I^- concentration.

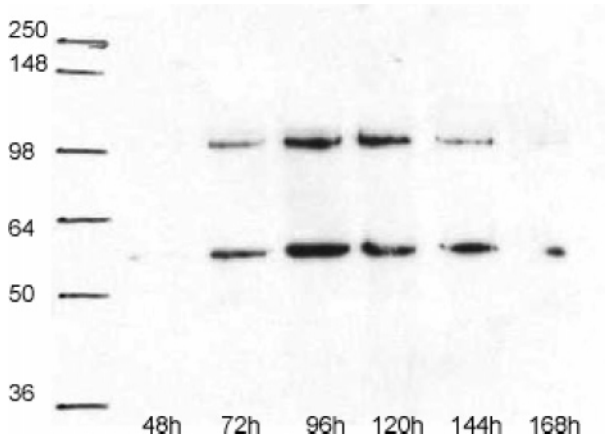

FIGURE 2: Western blot analysis of Ste2p-Strep-TagII/$(His)_8$ expression time course in 293E cells. The samples were harvested 48, 72, 96, 120, 144, and 168 h post-transfection, respectively. Equal quantities of total protein were loaded on 8% SDS−PAGE and transferred to PVDF membrane. Ste2p was detected using anti-HisG HRP antibody.

RESULTS

Recombinant Expression and Localization of Ste2p in 293E Cells. Expression of the Ste2p-Strep-TagII/$(His)_8$ fusion protein was quantified by western detection of its C-terminal $(His)_8$ tag. Expression levels reached a maximum 4−5 days post-transfection and subsequently decreased until day 7 post-transfection (Figure 2). Two discrete bands were observed in the crude membrane when examined by western analysis: a lower band at 55 kDa matching the expected molecular mass for the glycosylated form of Ste2p-Strep-TagII/$(His)_8$ monomer and a higher band above 100 kDa, representing glycosylated dimer. Incubation of total cell lysates (data not shown) or purified Ste2p (see below and Supporting Information, Figure S1) with PNGaseF prior to western analysis

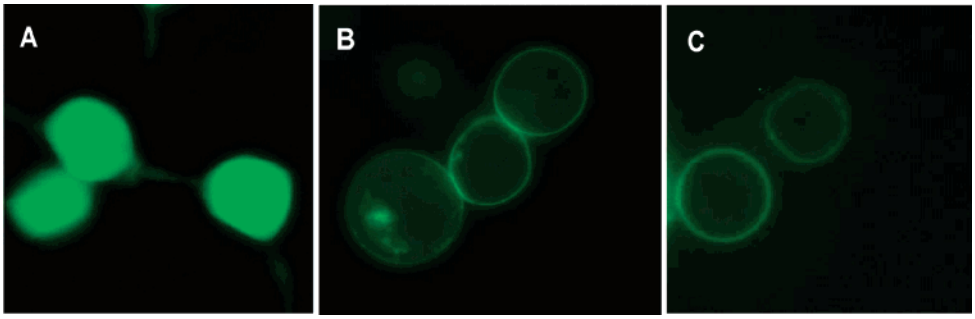

FIGURE 3: Localization of Fc-Ste2p-GFP and Ste2p-GFP fusion proteins in 293E cells. (A) 293E cells transfected with the pTT/GFP vector. (B) 293E cells transfected with the pTT/Ste2p-GFP fusion construct. (C) 293E cells transfected with the pTT5/Fc-Ste2p-GFP fusion construct. Pictures were taken 72 h post-transfection.

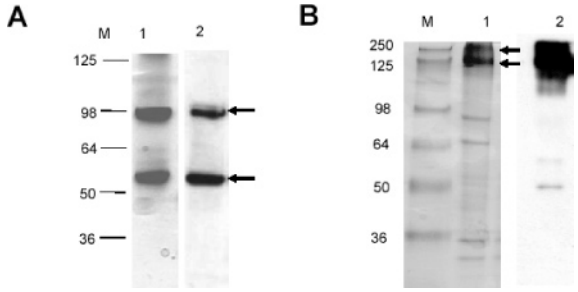

FIGURE 4: Purification of tagged Ste2p by Talon and protein A−Sepharose. (A) Purification of Ste2p-Strep-TagII/$(His)_8$ using the Talon affinity column. The 250 mM imidazole elution fraction is shown. The yield is about 1 mg/L of cell culture after purification. (B) Purification of Fc-Ste2p-GFP fusion by protein A−Sepharose. The yield is about 2 mg/L of cell culture after purification. Lanes: M, protein molecular mass standards; 1, purified fraction stained by Coomassie; 2, western blot of the same fractions using anti-HisG HRP or anti-GFP-HRP. Identities of bands were confirmed by peptide mass fingerprinting.

yielded lower bands at 47 and 98 kDa, confirming the glycosylated nature of the recombinant protein. Reduction of the growth temperature from 37 to 30 °C led to significant decreases in levels of expressed protein. On the basis of these results the optimal time of harvest was set to 96 h at 37 °C to maximize Ste2p yields. Similar results were obtained for expression of the fusion protein Fc-Ste2p-GFP in 293E cells (data not shown).

Both GFP (C-terminal) fusion and Fc (N-terminal)/GFP (C-terminal) fusion versions of Ste2p were used to determine localization of recombinant expressed Ste2p in vivo. Transiently transfected cells expressing either the Fc-Ste2p-GFP or the Ste2p-GFP fusions were imaged by fluorescent microscopy. 293E cells expressing cytoplasmic GFP were used for comparison. Both Ste2p fusions were found mainly localized to the cell membrane (Figure 3).

Affinity Purification of Recombinant Ste2p. Strep-TagII/$(His)_8$-tagged Ste2p was purified by one-step Talon affinity or Strep-Tactin affinity chromatography. SDS−PAGE analysis of the 250 mM imidazole elution fraction from the Talon affinity column gave rise to heavy bands at 55 and 100 kDa as expected (Figure 4A). In instances where receptor expression was less than optimal, primarily associated with lower cell viability, two weaker bands at 47 and 98 kDa were also observed, corresponding to the unglycosylated monomeric and dimeric forms of Ste2p-Strep-TagII/$(His)_8$ fusion (see Supporting Information, Figure S2). Western blot analysis with anti-HisG HRP antibody indicated that all four bands maintained the His_8 tag, and PMF analysis further confirmed that the four bands were all Ste2p. Glycosylated Ste2p was highly purified (95% homogeneity) after only one-step Talon affinity chromatography. About 1 mg of Ste2p-Strep-TagII/$(His)_8$ was purified from 1 L of culture. In comparison, after one-step Strep-Tactin affinity chromatography only a single weak band at 47 kDa was observed, and the overall yield was only ~50 μg/L (Supporting Information, Figure S3). Using protein A−Sepharose, Fc-Ste2p-GFP was purified to 90% homogeneity at a level of ~2 mg/L of culture (Figure 4B). Two bands were observed by SDS−PAGE again matching the expected molecular masses for the glycosylated monomeric and dimeric forms of the fusion protein: ~125 and 250 kDa, respectively. Identity was confirmed by western analysis using anti-GFP HRP antibody. On the basis of a final cell density of 2×10^6/mL at the time of harvest, over 5 million Fc-Ste2p-GFP molecules were expressed and purified from each 293E cell, corresponding to ~10 pmol of receptor/million cells.

Binding of Fluorescent-Labeled α-Factor to Recombinant Ste2p. To confirm correct folding and ligand binding activity of the recombinant purified Ste2p, a novel method for fluorescent labeling of α-factor was developed. Panels A and B of Figure 5 show the fluorescent reagents and peptides used in the synthesis. The specific reaction between maleimide and cysteine hydrosulfide groups was used to attach the fluorescent group to the Cys-modified peptide. MALDI-TOF-MS was used to confirm the final product and purity of the modified factors (Figure 5C). The observed molecular masses of 1786.86, 2190.97, and 2238.14 Da correspond exactly with the expected masses of Cys α-factor (1787.1), CPM-Cys α-factor (2190.6), and the sodium adduct of FM-Cys α-factor (2215.1 + 23.0 = 2238.1). While peaks representing single and double sodium adducts of the respective molecules are present in the spectra, there is no evidence of any residual WT α-factor (1684.0) in the Cys α-factor sample or unlabeled Cys α-factor in the modified pheromone samples. *In vivo* yeast halo assays were applied to assess the ability of these modified ligands to stimulate the pheromone response. Figure 5D shows the halo assay results comparing WT α-factor (I) to Cys α-factor (II), CPM-Cys α-factor (III), and FM-Cys α-factor (IV). Halo diameters of all three modified α-factors are smaller than those of WT α-factor at identical concentrations (see Figure S6 for details of the halo diameters). CPM-Cys α-factor has higher activity compared to Cys α-factor and FM-Cys α-factor at the lower concentrations tested. This difference becomes negligible at higher concentrations (500 μM) due to the lower solubility and lower diffusion rate in agar of CPM-Cys α-factor. The

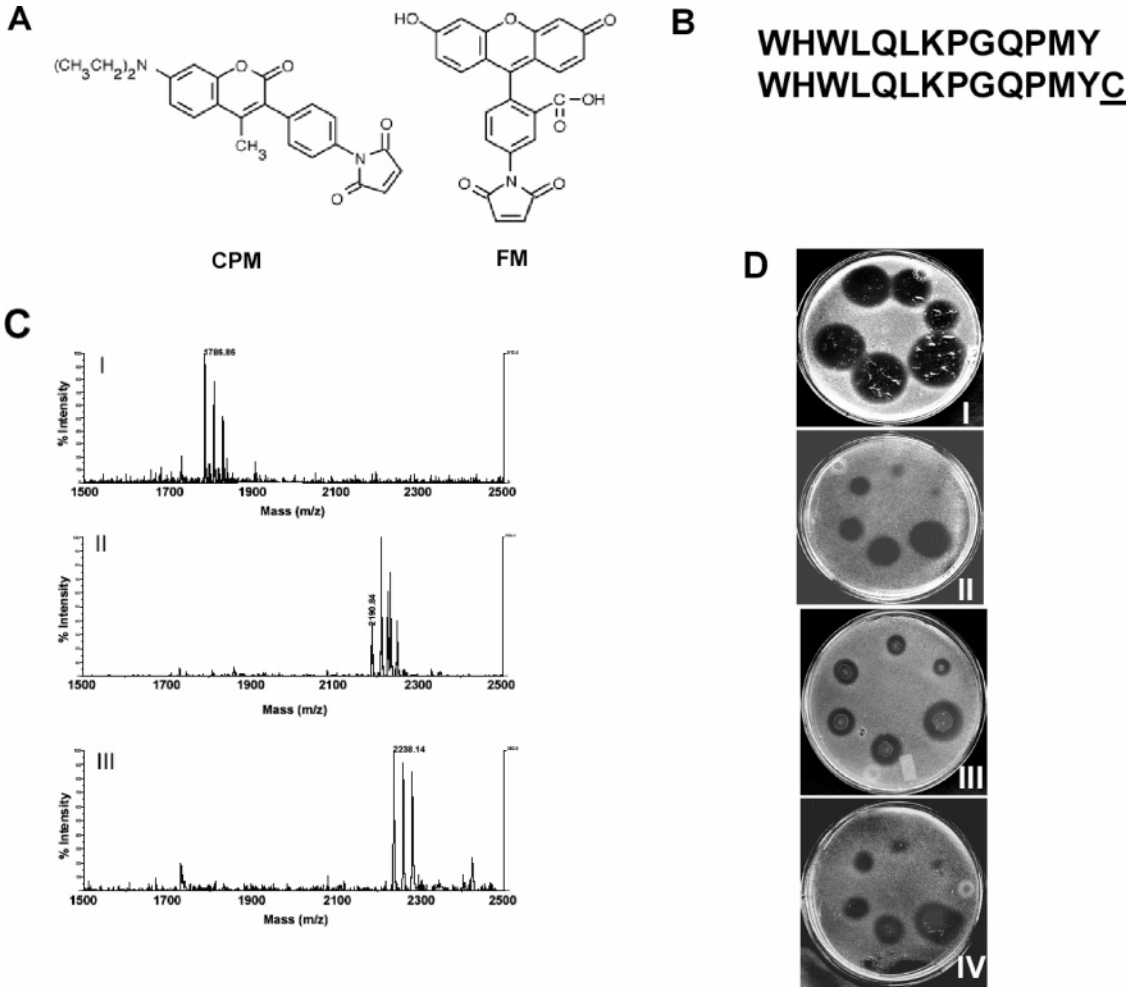

FIGURE 5: Synthesis and signaling of fluorescently labeled α-factors. (A) Structures of the two cysteine-specific fluorescent labels. (B) Sequence of WT α-factor (top) and Cys α-factor (bottom). (C) MALDI-TOF-MS characterization of (I) Cys α-factor, (II) CPM-Cys α-factor, and (III) FM-Cys α-factor. (D) Halo assay of wild-type α-factor (I), Cys α-factor (II), CPM-Cys α-factor (III), and FM-Cys α-factor (IV). The concentrations of WT and modified α-factors were 10, 20, 50, 100, 200, and 500 μM from top right to bottom right via counterclockwise rotation.

higher apparent activity of the CPM-Cys α-factor compared to the Cys α-factor and FM-Cys α-factor suggests that the C-terminus of the pheromone is localized to a hydrophobic pocket of the receptor upon binding. Buffer only, CPM only, and FM only controls all showed no signaling activity (data not shown). Overall, the biological activity response of fluorescent-modified α-factor indicates that they maintain the ability to transduce signals via Ste2p. It thus appears that modification of the C-terminal carboxyl group of the ligand does not affect general downstream signaling events.

In vitro binding assays were performed to demonstrate binding activity in crude membranes and of purified recombinant receptor. Panels A and B of Figure 6 show the results obtained when fluorescent-labeled CPM-Cys α-factor or FM-Cys α-factor was mixed *in vitro* with membranes from 293E cells expressing Ste2p-Strep-TagII/$(His)_8$ or an equal quantity of membrane from 293E cells not expressing Ste2p as a control. Saturable binding was observed for both modified labeled factors after subtracting nonspecific binding (Figures 6A,B, left panels). In the case of the FM-labeled ligand, the lack of complete saturability is a reflection of its lower overall affinity for the receptor (see experiments below). Competition of fluorescent-modified α-factors by WT α-factor gave rise to concentration-dependent decreases in fluorescent ligand binding, emphasizing the specificity of the ligand binding event (Figures 6A,B, right panels). Figure 6C shows fluorescent spectra of isolated purified fluorescent α-factors/Ste2p-Strep-TagII/$(His)_8$ complexes produced by adding the modified ligand to the receptor immobilized on affinity resin. The emission peaks arising from the eluted complexes were blue shifted by 6 and 10 nm compared to free CPM-Cys α-factor and FM-Cys α-factor, respectively, further supporting localization of the α-factor C-terminus in a hydrophobic domain after binding Ste2p. As an additional control for specificity of *in vitro* ligand binding, identical on-column ligand binding experiments were carried out using immobilized Ste3p-Strep-TagII/$(His)_8$ (*S. cerevisiae* a-factor receptor) in place of Ste2p-Strep-TagII/$(His)_8$ [see Supporting Information, Figure S4, for expression/purification of Ste3p-Strep-TagII/$(His)_8$], which further confirmed the specific nature of the ligand binding events occurring within this *in vitro* assay system (dotted lines, Figures 6C). Similar experiments using Fc-Ste2p-GFP were carried out in an attempt to estimate the percentage of active receptor in the sample (see Supporting Information, Figure S5, and Discussion). Finally, the fluorescent-labeled ligands were also used to determine ligand binding of the recombinant Ste2p fusions *in vivo*. The application of CPM-labeled α-factor to 293E cells expressing either Fc-Ste2p-GFP or Ste2p-Strep-TagII/$(His)_8$ fusions gave clear blue fluorescent signals at

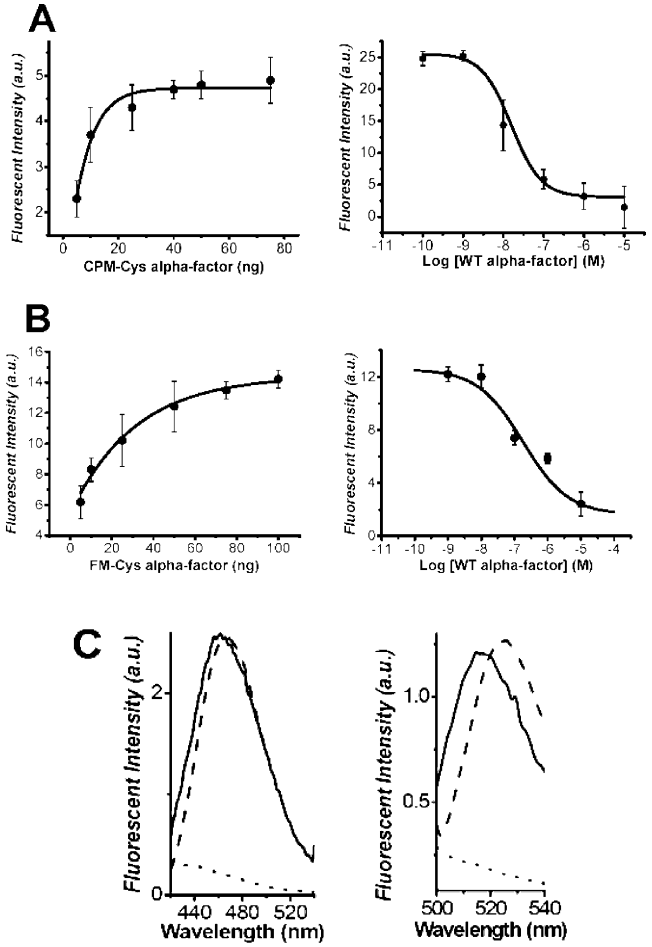

FIGURE 6: *In vitro* binding of fluorescent-modified α-factors to recombinant Ste2p. (A) Saturation (left) and competition binding assays (right) with CPM-Cys α-factor. (B) Saturation (left) and competition binding assays (right) with FM-Cys α-factor. Membranes from 293E cells expressing Ste2p or control membranes (not expressing Ste2p) were prepared as described in Materials and Methods. (C, left panel) Isolation of the purified CPM-Cys α-factor/Ste2p-Strep-TagII/$(His)_8$ complex by on-column binding. The emission spectra of eluted CPM-Cys α-factor bound to Ste2p (solid line), free CPM-Cys α-factor (thick dashed line), and the eluted Ste3p control (dotted line). The excitation wavelength was 384 nm. (C, right panel) Isolation of the purified FM-Cys α-factor/Ste2p-Strep-TagII/$(His)_8$ complex by on-column binding. The emission spectra of eluted FM-Cys α-factor bound to Ste2p (solid line), free FM-Cys α-factor (thick dashed line), and the eluted Ste3p control (dotted line). The excitation wavelength was 480 nm.

the cell membrane, a signal which overlapped exactly with the green fluorescent signal of the Fc-Ste2p-GFP-expressing cells (data not shown).

Affinities of the Fluorescent α-Factors for Recombinant Purified Ste2p. Using a sensitive fluorescent dialysis method, the dissociation constants between the fluorescent-labeled factors and purified recombinant Ste2p were determined. Identical samples of known concentration (0.325 mg/mL) of purified Ste2p-Strep-TagII/$(His)_8$ fusion protein were mixed with excess fluorescent Cys α-factors and the systems allowed to equilibrate by dialysis overnight. The necessary length of dialysis was determined previously by the time required for the free ligand to reach equal concentration (as determined by fluorescence) inside and outside of the dialysis membrane in a separate ligand-only control experiment. Determination of the difference between the fluorescence remaining in the dialysis membrane with receptor and outside

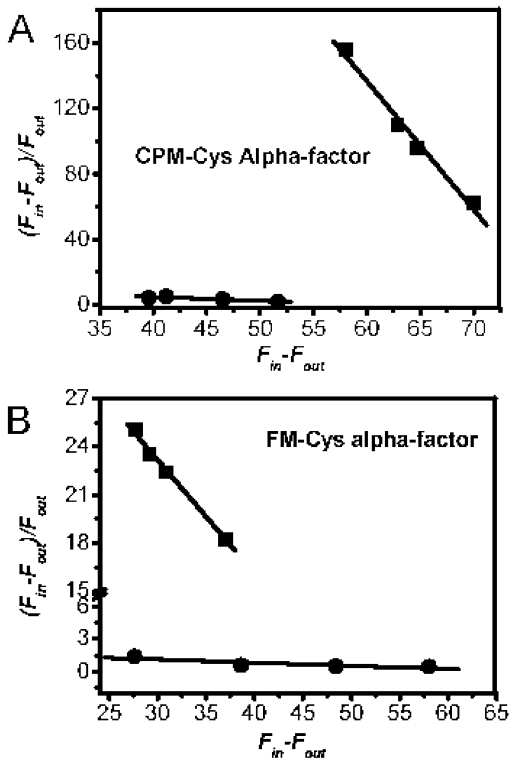

FIGURE 7: Quantitation of *in vitro* ligand binding affinities. (A) K_D assay for CPM-Cys α-factor interaction with Ste2p-Strep-TagII/$(His)_8$ (■). The K_D value was determined to be 34 ± 3 nM. (B) K_D assay for FM-Cys α-factor interaction with Ste2p-Strep-TagII/$(His)_8$ fusion protein (■). The K_D value was determined to be 300 ± 20 nM. Linear relationship coefficients were (A) 0.997 and (B) 0.992. Control experiments were performed on equal quantities of purified Ste3p-Strep-TagII/$(His)_8$ (●).

the membrane provided values for the amount of ligand bound at a given concentration. The K_D values were determined by evaluation of slopes from kinetic plots and calculated to be 34 ± 3 nM for CPM-Cys α-factor and 300 ± 20 nM for FM-Cys α-factor (Figure 7). Nonspecific binding can be ignored on the basis of the lack of any detectable binding occurring in control kinetic experiments where identical quantities of purified Ste3p-Strep-TagII/$(His)_8$ were used in place of Ste2p-Strep-TagII/$(His)_8$ inside the dialysis bag (Figure 7).

Determination of the Pheromone C-Terminal Environment When Bound to Ste2p. Potassium iodide fluorescent quenching experiments were carried out probing the localization of the C-terminus of the FM-Cys α-factor and CPM-Cys α-factor in complex with Ste2p-Strep-TagII/$(His)_8$. Stern−Volmer plot analyses indicate that quenching of the fluorescent signal was reduced in the complexed forms for both modified ligands (Figure 8). This confirms that the FM and CPM fluorescent groups attached to the ligands are protected from the environment in the complexed form compared to free FM-Cys α-factor and CPM-Cys α-factor in solution, where the fluorescent group is highly accessible.

DISCUSSION

GPCRs are an important superfamily of integral membrane proteins, for which we have only limited knowledge of structure and mechanism of action. This is in part due to limitations in the ability to produce sufficient (milligram) quantities of folded, biologically active, and purified recom-

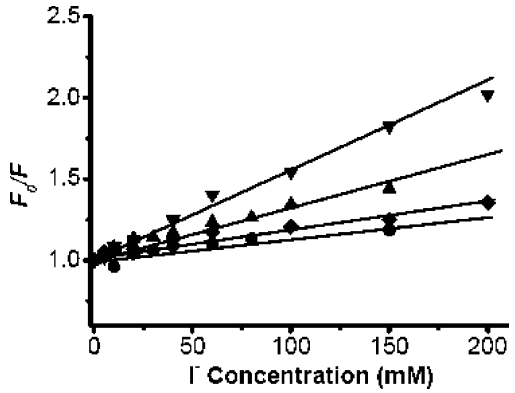

FIGURE 8: Stern−Volmer plot for I^- fluorescent quenching of FM-Cys α-factor and CPM-Cys α-factor bound to Ste2p. The complexes were obtained by on-column binding of ligand to immobilized Ste2p-Strep-TagII/$(His)_8$. The eluted complex was washed by Millipore ultracentrifuge (MWCO 10000 Da) concentration three times to replace the elution buffer with 50 mM PBS, pH 8.0, containing 5 mM $MgCl_2$, 100 mM NaCl, 5 mM β-ME, and 0.05% DM (w/v). Key: CPM-Cys α-factor (●); FM-Cys α-factor (◆); CPM-Cys α-factor/Ste2p complex (▲); FM-Cys α-factor/Ste2p complex (▼).

binant forms of the receptors for biophysical analyses. Crucial steps in the biological production of membrane-bound proteins are not restricted to transcription and translation but include post-translational modifications and the insertion into and concomitant folding of the protein within the membrane. The two latter processes are often the rate-limiting steps in production. Inactive and partly aggregated protein will be produced and accumulate within cells if correct insertion occurs but folding fails (*29*). In the work reported here, observation of fluorescent signals *in vivo*, arising from GFP-tagged Ste2p constructs, demonstrates that recombinant Ste2p is being transported to the 293E cell membrane where it accumulates. This localization of Ste2p suggests correct folding of the overexpressed receptor. This was further confirmed by *in vivo* and *in vitro* fluorescent-based ligand binding experiments.

Yields of the receptor reached as high as 2 mg/L of cell culture after purification of the Fc-Ste2p-GFP fusion. This corresponds to approximately 10 pmol of purified receptor/million cells, a value that is 200 times higher than that recently reported by Yin et al. (*16*). These represent the highest levels of recombinantly produced Ste2p achieved to date and further extend the application of the large-scale transfection of 293E cells to the efficient production of GPCRs.

Very often, isolated membrane proteins tend to form aggregates even in the presence of detergents, which reduces the efficacy of most separation techniques. It is therefore essential to select an effective but nondenaturing detergent for receptor solubilization (*29*). Ten detergents were tested for the solubilization of Ste2p, including *n*-dodecyl-*N*,*N*-dimethylamine *N*-oxide, 1-*S*-octyl β-D-thioglucoside, 1-*S*-octyl-β-D-thiomaltose, *n*-octyl β-D-glucoside, *n*-decyl β-D-glucoside, *n*-nonyl β-D-glucoside, *n*-decyl β-D-maltoside, *n*-nonyl β-D-maltoside, DM, and CHAPS. No clear difference was observed between the effective solubility of Ste2p in any of these at a standard concentration of 1% detergent as detected by western blot analysis (data not shown). DM was ultimately selected as the detergent to solubilize Ste2p from the cell membrane, as it is a mild sugar-based detergent, with a low critical micelle concentration of 0.01% (0.2 mM), which has been repeatedly shown to increase the probability of isolating active forms of membrane proteins in general and has previously been applied to Ste2p (*19*, *29*). The salt concentration was set to 100 mM, and no other salt concentrations were tested as there is generally little discrimination between low and high salt effects in the presence of high concentrations of neutral detergents (*29*). Following solubilization in DM, protein A−Sepharose was shown to purify Fc fusion Ste2p to >90% homogeneity in a single step. Similarly, purification of Ste2p-Strep-TagII/$(His)_8$ by Talon affinity yielded slightly lower levels of protein purified to >95% homogeneity. In contrast, purification of Ste2p-Strep-TagII/$(His)_8$ by Strep-Tactin affinity columns yielded only very low levels of nonglycosylated receptor. As the K_D for the Strep-Tactin/Strep-TagII interaction is ~1 μM (*30*), the Ste2p concentration in the solubilized samples described herein may possibly have been too low to efficiently bind to the affinity resin, a situation that may have been further exacerbated by the observed heavy glycosylation of the receptor.

Over the last 20 years, many different methods have been developed to assay the binding of α-mating factor to Ste2p such as iodinated α-factor (*31*, *32*), [^{3}H]-α-factor (*33*−*35*), ^{35}S-labeled α-factor (*36*, *37*), and most recently fluorescent-labeled factor (*38*−*40*). The novel fluorescent-labeled pheromones used in this current study were designed on the basis of the following rationale. The terminal carboxyl group (COOH) of the pheromone has not been implicated in mediating ligand interactions with receptor, as such blocking of this group by the addition of a small 14th amino acid at the C-terminus of the pheromone peptide was not anticipated to have a significant impact on ligand binding. At the same time, while the aromatic ring of the WT α-factor Tyr^{13} residue has been shown to play an essential role in mediation of the interaction with Ste2p (*32*), it has also been shown that a much larger benzene derivative, *p*-benzoyl-L-phenylalanine, can be accommodated at this site, albeit with significantly reduced affinity and activity (*41*). Together, these suggested the possibility of attaching fluorescent labels not directly at position 13 but through reaction with a C-terminal Cys^{14} to produce active fluorescently labeled pheromone. The MEROPS database indicated that there is no known specific protease that cleaves at X-Tyr-Cys, so the peptide was anticipated to be stable. MALDI analyses of the reaction products indicated that labeling of the Cys α-factor went to completion, and assays demonstrated the ability of these modified pheromones to stimulate signal responses in yeast. The growth arrest activity of the CPM-Cys α-factor was higher than the FM-Cys α-factor although both were somewhat lower than wild type. These differences were later attributed to subtle differences in the affinities of the two fluorescent-labeled factors. The CPM-labeled ligand showed a 5-fold lower affinity for the receptor than the wild-type ligand (*33*). However, it is important to keep in mind the impact that even very small populations of inactive receptors (within the larger purified receptor sample) might have on decreasing apparent affinities in these in vitro assays. Nonetheless, this apparent affinity is comparable to that of an alternate fluorescent peptide ligand ([K7(NBD),Nle12] K_i 26 nM) recently characterized by Ding et al. (*38*) and subsequently used to study Ste2p mutants in vivo (*39*). The

FM-labeled factor showed 10-fold less affinity than the CPM-labeled factor, and complete saturable binding was difficult to demonstrate. However, it still maintains greater affinity than other factors fluorescently labeled at the C-terminus by replacement of Tyr^{13} [$K_i > 1$ μM (*39*)].

A previous report on the overexpression and purification of Ste2p from yeast indicated that only 6% of the total purified DM-solubilized receptors were active (*19*). The addition of yeast lipid extracts to the purified samples restored as much as 80% activity. In contrast to this, a very high overall binding activity for DM-solubilized, 293E-derived, Ste2p is estimated herein on the basis of fluorescent binding studies. While the exact binding activity cannot be quantitated due to the lack of a known quantum yield value for CPM (FM cannot be used as its profile overlaps directly with that of GFP), the relative fluorescent intensities in the profile arising from a purified Fc-Ste2-GFP/CPM-Cys α-factor complex (5:1 peak ratio; see Supporting Information, Figure S5) suggest that the ligand remains bound to >30% of the receptors even after extensive washing (20 column volumes over ~30 min) and elution [assuming approximately comparable quantum yields (60%) for GFP and CPM, with extinction coefficients of 55000 and 33000, respectively]. Taking into consideration the lack of nonspecific binding demonstrated for this system, the calculated K_D, dilution effects, and time of washing, >80% of the purified DM-solubilized, HEK293-derived, receptor is estimated to be active. Various subtle differences between the yeast protocol and the mammalian system reported here could account for the differences in ligand binding activities. One of the most likely is the degree of delipidation that occurs between solubilizing with 1% DM (this report) and 2% DM (yeast report). Alternatively, perhaps at 2% DM the receptor is being monomerized or reversibly denatured from an otherwise oligomeric or active state, which is retained at 1% DM. Another possible factor is the difference in cellular lipid content and therefore receptor-bound lipid populations between yeast and mammalian systems, which could impact on the overall delipidation and general receptor stability. The difference of using French press versus glass bead vortexing to lyse cells could have an impact. Even the physiological context of expressing high levels of Ste2p in its native host could lead to identification and internalization of the receptor away from the cell membrane lipids. This is in contrast to the mammalian system where high levels of Ste2p have been shown to accumulate preferentially in the cell membrane. The contribution of these and other factors remains to be determined.

Quenching experiments reported herein support a model in which the ligand C-terminal label is not accessible to solvent when bound to Ste2p. As well, the discrepancy between the observed affinities and biological activities of the CPM and FM modified factors, respectively, and the blue-shifted emission peaks of receptor-bound fluorescent-Cys α-factors suggests that the labels are likely located in a relatively hydrophobic environment when bound to receptor. These results support a model in which the C-terminus of the ligand is packed in a hydrophobic environment at the transmembrane (TM)/EC interface. Models based primarily on site-directed mutagenesis and ligand labeling experiments have placed the C-terminus of the ligand in close proximity to the TM/EC interface near helices 5 and 6 (*10*, *42*, *43*). In particular, interaction with Phe^{204} is proposed. More recently, *in vivo* cross-linking studies have provided a more accurate model in which the N-terminus interacts at the TM/EC interface at helices 5 and 6, while the C-terminal region of the ligand is localized close to helix 1 of the receptor in proximity to Arg^{58} (*41*). According to this model, the replacement of receptor Tyr^{98} with ligand Tyr^{13} in a cation$-\pi$ interaction with Arg^{58} mediates the conformational change for downstream signal transduction. This latter model still places the backbone of the C-terminal region of the ligand near the TM/EC interface.

Overall,we have successfully developed and characterized a system for the efficient recombinant expression and purification of milligram quantities of biologically active Ste2p. This includes design of novel fluorescent ligand probes for facilitated analysis of structure/function relationships of this receptor. Further optimization of the system is being carried out, including assessment of low-glycosylation and inducible stable cell line technologies (*44*, *45*). These modifications should further improve sample yield and quality for future applications of intact receptor to biophysical analyses.

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SUPPORTING INFORMATION AVAILABLE

Six figures showing PNGaseF digestion of purified glycosylated Ste2p, western blot comparing glycosylation of purified Ste2p, purification of Ste2p-Strep-TagII/$(His)_8$, expression and purification of a-factor receptor Ste3p, on-column binding between Fc-Ste2p-GFP and CPM-Cys α-factor, and relationship between the biological activity of WT and modified α-factor. This material is available free of charge via the Internet at http://pubs.acs.org.

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