Expression and Purification of the *Saccharomyces cerevisiae* α-Factor Receptor (Ste2p), a 7-Transmembrane-segment G Protein-coupled Receptor*

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Nathaniel E. David‡, Melanie Gee§, Birgitte Andersen¶, Fred Naider**, Jeremy Thorner‡, and Raymond C. Stevens§ ‡‡

From the ‡Department of Molecular and Cell Biology and the §Department of Chemistry, University of California at Berkeley, Berkeley, California 94720, the ¶Department of Plant Biology, The Royal Veterinary and Agricultural University, DK-1871 Frederiksberg C, Denmark, the ¶¶Life Sciences Division of Lawrence Berkeley National Laboratory, Donner Laboratory, Berkeley, California 94720, and the **Department of Chemistry, College Of Staten Island, Staten Island, New York 10314

A plasmid vector was developed that permitted high-level expression of a functional form of the *Saccharomyces cerevisiae* α-factor receptor (the STE2 gene product) tagged at its C-terminal end with an epitope (FLAG) and a His₆ tract. When expressed in yeast from this plasmid, Ste2p was produced at a level at least 3-fold higher than that reported previously for any other 7-transmembrane-segment receptor expressed in the same cells. For purification, isolated cell membranes containing the overexpressed receptor were solubilized with detergent under specific conditions and subjected to immobilized metal affinity chromatography. Yields as high as 1 mg of nearly homogeneous (95%) receptor were routinely obtained even from relatively small scale preparations (60 g of frozen cell paste). The purified receptor was reconstituted into artificial phospholipid vesicles. Radioligand binding studies demonstrated that the purified receptor, in the reconstituted vesicles, bound its tridecapeptide ligand (α-factor) with a Kᵣ (155 ns) consistent with the affinity expected for this receptor in the absence of its associated G protein. Efficient restoration of ligand binding activity upon reconstitution required the addition of solubilized membranes prepared from a yeast strain lacking the receptor. Sufficient amounts of active material can be obtained by this procedure to allow physical studies of this receptor and other 7-transmembrane-segment receptors expressed in this system.

The α-factor receptor (Ste2p) from *Saccharomyces cerevisiae* belongs to the family of G protein-coupled receptors (GPCRs)¹ that, upon the binding of a ligand, transduce a signal via an associated guanine-nucleotide binding protein (G protein) (1, 2). GPCRs function in physiological processes ranging from vision (the rhodopsins), to smell (the olfactory receptors), to neurotransmission (e.g. muscarinic acetylcholine, dopamine, and adrenergic receptors) (1, 2). In *S. cerevisiae*, the binding of the peptide pheromones (α-factor and α-factor) to their receptors initiates the cascade of events that lead to the mating of haploid yeast cells (3, 4).

GPCRs are integral membrane proteins ranging in size from 400 to 1000 amino acids. Hydrophobicity analysis of the GPCRs reveals the presence of 7 hydrophobic regions predicted to form membrane-spanning α-helices, suggesting a similar structural arrangement of these proteins in the membrane (1). Several amino acids in the hydrophobic domains are conserved among all GPCRs, suggesting their importance in the proper folding of the protein within the membrane. Other amino acids are conserved only within a particular GPCR subclass and are therefore thought to confer binding specificity for a certain class of ligands (5–8).

The hydrophilic domains of GPCRs (the extracellular and intracellular loops and the cytosolic tails) exhibit a large variation in size and amino acid composition. Site-directed mutagenesis and biochemical characterization of the GPCRs has demonstrated that for the smaller ligands (like catecholamines) the transmembrane helices 3, 4, 5, 6, and 7 carry determinants for ligand recognition (6, 8). The binding of larger ligands, such as peptides or glycoproteins, is less well characterized, but the extracellular hydrophilic domains of the receptor appear to be involved in ligand binding to some extent (8, 9).

The third cytoplasmic loop of the receptors is implicated in the interaction with and activation of the associated heterotrimeric G protein (10, 11).

Detailed biophysical studies of GPCRs (such as structure determination by x-ray or electron crystallography) have been hampered by the difficulties in obtaining large quantities of purified receptors from natural sources. One exception is bovine rhodopsin, available in large quantities from bovine rod cells. Recently, Schertler and Hargrave (12) succeeded in forming well diffracting two-dimensional crystals of rhodopsin. The electron diffraction data permitted the production of a projection map of rhodopsin at 9 Å resolution, showing high density areas corresponding to the seven putative membrane-spanning α-helices. The successful determination of a high resolution structure of bacteriorhodopsin (13) and light-harvesting com-

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¹ The abbreviations used are: GPCR, G protein-coupled receptor; BAP, bacterial alkaline phosphatase; DBM, N-dodecyl-β-D-maltoside; ECL, enhanced chemiluminescence; FT, FLAG tag; G protein, guanine nucleotide-binding protein; HT, His₆ tag; Ni-NTA, nickel-nitritotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PNGase F, peptide N-glycosidase F; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol.

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plex II (14) by electron diffraction of two-dimensional crystals suggests that a high resolution structure of rhodopsin may be available in the future.

Overexpression in heterologous expression systems (such as insect cells, yeast, or E. coli) has been used to produce GPCRs. Several GPCRs have been expressed in a functional form using the baculovirus system, including the D1 and D2 dopamine receptors (15, 16), the M1 and M2 muscarinic acetylcholine receptors (17, 18), and the β2-adrenergic receptor (17). The D2S dopamine receptor has been functionally expressed in both S. cerevisiae and Schizosaccharomyces pombe (19, 20), and an E. coli expression system has also been successfully used for expression of GPCRs (21, 22).

Yeast-based expression systems for GPCRs have several advantages over other systems. First, the cost of growing yeast in large fermentors is small compared with the costs of insect cell maintenance and growth. Second, a yeast-based system is genetically flexible. It is possible to easily construct, express, and purify mutants versions of receptors without the complications of recombinant virus selection and viral amplification encountered in the baculovirus system. Third, certain vertebrate GPCRs heterologously expressed in S. cerevisiae are able to elicit growth arrest in response to their natural agonists (23, 24). As a result, mutants of these GPCRs (truncations or point mutants useful in biophysical studies) can be functionally characterized in S. cerevisiae cells.

Here we describe overexpression, purification, and initial in vitro characterization of Ste2p, the α-factor receptor from S. cerevisiae. Because the purification requires only a single column and yields approximately 1 mg of receptor protein, degeneration of the nucleotide sequence of the entire domain of Ste2p was inserted in frame into the pET15b expression vector (Novagen, Madison, WI) and introduced into the BL21 DE3 E. coli strain by transformation. The pET15b expression vector allows for the rapid expression of recombinant proteins, provided that cloning into the vector introduces an N-terminal polyhistidine tag. The His-tagged 131-aminio Ste2p subdomain was purified to homogeneity by immobilized metal affinity chromatography. Rabbits were immunized essentially as described by Harlow and Lane (31). Bleeds were tested for immunoreactivity by immunoblot analysis and visualized by enhanced chemiluminescence (ECL). Antibodies generated in this work showed no cross-reactivity with whole cell extracts from strain DK102, which carries a deletion mutation (ste23) of the α-factor receptor gene.

Protease Inhibitors—All manipulations with membranes and all purification steps were carried out in the presence of the following protease inhibitor mixture: 1.0 μg/ml leupeptin, 1.0 μg/ml pepstatin A, and 17.4 μg/ml phenylmethylsulfonyl fluoride.

Expression and Purification of the α-Factor Receptor—To elicit growth arrest in response to their natural agonists (23, 24). As a result, mutants of these GPCRs (truncations or point mutants useful in biophysical studies) can be functionally characterized in S. cerevisiae cells.

Growth of Yeast Cells—DK102, RC629, DK102 [pNED1], and BJ2168 [pNED1] were grown to midexponential phase (A600 = 1). DK102 and RC629 were grown at 30 °C in YPD medium (32), while the strains transformed with pNED1 were grown at 30 °C in a synthetic medium without tryptophan (32) to maintain selection for the plasmid. For the large-scale culture of cells for the purification of Ste2p, FT.HT, BJ2168 cells carrying the pNED1 plasmid were grown at 26 °C to A600 = 3 in medium lacking tryptophan using a 200 liter fermentor. Cells were harvested, frozen as cakes (60 g), and stored at −80 °C until use.

Membrane Preparation—All steps were performed at 4 °C, and all buffers were supplemented with protease inhibitors. For small-scale experiments, cells equivalent to A600 = 5 were resuspended in 50 mM Tris-HCl (pH 7.0) and lysed by vortexing with glass beads. Unlysed cells were removed by centrifugation at 700 × g, and the membrane fraction was collected by centrifugation at 150,000 × g. For large scale preparation of membranes for purification of Ste2p, FT.HT, approximately 60 g of yeast cell paste (1 frozen cake) were thawed and resuspended in 90 ml of 10% (w/v) sucrose in buffer A (5 mM HEPES (pH 7.5), 5 mM EDTA, protease inhibitors, and 500 mM α-factor). The cells were lysed by vigorous shaking with glass beads for three 2-min pulses in a Braun Scientific (Allentown, PA) cell homogenizer. Unlysed cells were removed by centrifugation at 700 × g, and the membrane fraction was collected by centrifugation at 186,000 × g for at least 1 h.

Measurement of Protein Expression Level by Immunoblot—Cell lystate corresponding to a suspension of A600 = 5 (in 100 μl) was solubilized in sample buffer (50 mM Na2CO3, 50 mM dithiothreitol, 15% (w/v) sucrose, 600 μM Na2EDTA, protease inhibitors, and 500 mM α-factor) and subjected to electrophoresis on a 12% SDS-polyacrylamide gel. For immunoblot analysis, the proteins were transferred to nitrocellulose and analyzed using a rabbit polyclonal antibody directed against the C-terminal 131 amino acids of wild-type Ste2p. The dilution factors were chosen so as to give comparable signals when the immune complexes were visualized by ECL.

Bacterial Assay for Growth of Ste2p—The response of cells carrying pNED1 to α-factor was analyzed by halo assay (33). Different amounts of α-factor (100 ng, 500 ng, and 1000 ng) were spotted on filter paper disks and placed on agar plates containing DK102, RC629, or DK102 [pNED1]. Plates were grown at 30 °C for 48 h, and the sizes of the halos were determined.

Purification of Ste2p, FT.HT—All steps were performed at 4 °C, and all buffers were supplemented with protease inhibitors and 500 mM α-factor. Yeast membranes from 60-g cells were homogenized in 90 ml of 10% (w/v) sucrose in the buffer A described under “Membrane Preparation.” Discontinuous sucrose gradients were made from 14 ml of 45% (w/v) and 6 ml of 53% (w/v) sucrose in buffer A. Aliquots (15 ml) of the homogenized membranes were layered on the gradients and centrifuged overnight at 27,000 rpm in a Beckman SW28 rotor. The band between 10–43% (w/v) sucrose was collected, diluted with D2O, and centrifuged at 186,000 × g for 1 h. The pellet was resuspended in 25 ml of 50% glycerol and added to 25 ml of 2% n-dodecyl-β-D-maltoside (DBM) (Anatrace, Maumee, OH), 50 mM glycerol, 50 mM HEPES (pH 7.5), and 150 mM NaCl. After 1 h of incubation, unsolubilized material was removed by centrifugation at 186,000 × g for 30 min. The soluble fraction was diluted with 12 ml of 99% glycerol and 200 mM buffer B (0.075% DBM, 15% glycerol, 50 mM NaCl, 50 mM imidazole, 50 mM HEPES (pH 7.5), 0.4 mg/ml soybean tα-α-actin (type II-S from Sigma). The sample was loaded at 12 ml/h onto a 4 ml Ni-NTA-agarose column (Qiagen, Chatsworth, CA) pre-equilibrated with buffer B. The column was then washed with 80 ml of buffer B. The concentrations of NaCl and glycerol were reduced by washing with 20 ml of buffer C.
The concentration of Ste2p.FT.HT in Liposomes—The concentration of Ste2p.FT.HT present in reconstituted liposomes was determined by quantitative immunoblot. The accuracy of this measurement technique was confirmed by comparing the values determined this way with those determined by the Bradford method (36). This normalization method was motivated by the observation that membrane lipids interfered with accurate assessment of protein concentration. By using aliquots of the membrane suspension, a more reasonable approximation of bulk membranes could be made as a means to normalize the total amount of membrane protein from strain to strain.

For reconstitutions in the presence of solubilized yeast membranes, the above protocol was modified as follows. Total DK102 (ste2Δ) membranes were isolated as described under “Membrane Preparation” and solubilized at a final protein concentration of about 10 mg/ml in 1% DBM, 45% glycerol, 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, and protease inhibitors. After mixing for 1 h at 4 °C, unsolubilized material was removed by centrifugation at 229,000 × g for 45 min. The solubilized DK102 (ste2Δ) extract (2 ml) was incubated with a 1-ml aliquot of the pooled Ni-NTA fractions containing purified Ste2p.FT.HT (10 mg/ml of purified protein) for 1 h at 4 °C. The detergent-saturated proteoliposomes was separated from the Bio-Beads and then collected by centrifugation at 229,000 × g for 1 h. The pellet was washed with the HEPES-buffered solution before resuspension and use in radioligand binding assays.

For reconstitutions, the number of binding sites per cell (a) was measured by the overexpressed receptor, a concentrated suspension of membranes from strain DK102 (pNED1) was diluted to 10 mg/ml. To determine dissociation constants (KD) and Bmax for the purified receptor, Ste2p.FT.HT was reconstituted into lipid vesicles, resuspended in YM1 + i medium, and then subjected to ligand binding measurements. The reactions were incubated for 1 h at 25 °C, diluted with 1.5 ml of ice-cold YM1 + i medium, and rapidly filtered through GF/F glass microfiber filters (Fisher, Pittsburgh, PA) presoaked in 0.3% polyethyleneimine (Sigma) (3). The reaction tubes and filters were washed three times with 1.5 ml of ice-cold YM1 + i medium. The amount of bound [3H]α-factor was determined by scintillation counting of the glass filters in ScintiVerse BD scintillation mixture (Fisher) after overnight solubilization. Nonspecific binding was determined as described above. KD and Bmax were determined for each experiment using least squares fitting analysis to the Langmuir isotherm equation (Bound) = Bmax([1 + KD]/ [Frel]). All manipulations in these studies were carried out using minisorb tubes (Fisher) and siliconized pipette tips (Fisher) to reduce adsorption of [3H]α-factor to plasticware.

Assessment of Stereospecificity of Ligand Binding—For the determination of competition binding curves using isolated membranes containing the overexpressed receptor, a concentrated suspension of membranes from strain DK102 (pNED1) was diluted to a final A280 of 0.001 (approximately 10 μg/ml) of membrane protein. This concentration was chosen to minimize ligand depletion that would invalidate the estimation of KD from IP50. [3H]α-Factor was added to the diluted membrane suspension to a final concentration of 4 nM. Two peptides were compared for their ability to displace the bound [3H]α-factor, [α-Ala]α-factor and [α-Ala]α-factor. Concentrations of the α-factor analogs were determined using A280 for each peptide as determined by Raths et al. (30). The dissociation constants (KD) for each analog were estimated using the equation KD, Analog = IC50/1 + [Tracer]/KD, Tracer, where IC50 is the concentration of analog required to compete 50% of specifically
bound \(^{3}H\)α-factor. Incubations were carried out at room temperature for 90 min and then collected on glass filters as described above. 

[3H]α-Factor competition studies on the purified receptor were similar to those for membranes except that the concentration of [3H]α-factor used in the assays was 40 nM.

RESULTS

Ste2p.FT.HT Is Functional in Vivo—The sequence modifications made to the C terminus of the receptor did not compromise its ability to mediate growth arrest in response to α-factor, as demonstrated in an in vivo bioassay (see Fig. 2). DK102 (which does not express Ste2p) showed no response to α-factor, as expected. RC629 and DK102 [pNED1] (Fig. 1) showed similar halo patterns (Fig. 2), demonstrating that the FLAG and His\(_6\) tags appended to the C terminus of the receptor do not compromise either ligand binding or signal transduction.

BJ2168 [pNED1] Overproduces Ste2p at Least 80-fold—Expression of Ste2p was monitored using two approaches, immunoblot analysis (Fig. 3) and [3H]α-factor binding to whole cells (Fig. 4A) and to isolated cell membranes (Fig. 4B). As found by immunoblotting (i.e. as judged by comparing the amounts of membranes required to give equivalent ECL signals), when the expression plasmid (pNED1) was propagated in a MATa strain lacking any endogenous Ste2p (DK102), the receptor was produced at least 50-fold above the endogenous level, relative to a MATa strain (RC629) expressing Ste2p from the chromosomal STE2 locus. A protease-deficient strain (BJ2168) carrying pNED1 produced about 80-fold more receptor than the wild-type MATa strain (RC629).

Radioligand binding is more quantitative than immunoblot-
tinting and has the added advantage of detecting only active receptor molecules. Radioligand binding on whole cells (Fig. 4A) demonstrated that the two overexpression strains have similar levels of cell surface-expressed Ste2p, approximately 20-fold higher than the level on a wild-type cell (RC629). This value was lower than the estimate obtained by immunoblotting, suggesting the possibility that not all of the receptor was present on the cell surface (perhaps contained in an internal membrane compartment) and was thus inaccessible in the whole cell binding assay. Indeed, when cells were lysed and membranes isolated from DK102 [pNED1] were assayed for binding, it was found that they contained 79-fold more binding sites than membranes from RC629. Membranes isolated from BJ2168 [pNED1] cells had a similar number of binding sites as DK102 [pNED1] (Fig. 4B). These results indicate that essentially all of the receptor molecules produced retain their ligand binding ability.

Ste2p,FT,HT and Wild-type Ste2p Have Similar Affinities for \(^{3}H\)-a-Factor—All saturation binding data performed on whole cells (Fig. 4A) and on cell membranes (Fig. 4B) were transformed into Scatchard plots and straight lines were obtained, suggesting that each data set represented a population of receptors of a single ligand affinity. For whole cells, a \(K_D\) of \(-6\) nM was obtained for all strains examined (Table I). This value is very similar to that previously reported by Jenness, et al. (37). The \(K_D\) values observed for the membranes from RC629, DK102 [pNED1], and BJ2168 [pNED1] were 5 nM, 14 nM, and 24 nM, respectively (Table I). These values are also in agreement with previous reports of the affinity of a-factor for Ste2p in cell membranes (37).

Milligram Quantities of Ste2p,FT,HT Can Be Purified to 95% Homogeneity—Due to its extremely high level of expression, purification of the receptor could be achieved using only two steps (as described in detail under “Materials and Methods”). Isolated intracellular membranes were collected on a discontinuous sucrose gradient, solubilized with detergent, and the solubilized receptor was then subjected to immobilized metal affinity chromatography (Fig. 5A, lane 2). The yield of purified Ste2p,FT,HT was determined using the Bradford protein assay (36). About 1 mg of purified Ste2p,FT,HT could be routinely obtained with a purity estimated to be 95% (as judged on an overloaded Coomassie-stained gel, see Fig. 5A). If one also includes receptor solubilized from the membrane material that collects between the 43 and 53% (w/v) sucrose steps, as much as 2 mg of receptor could be obtained, but at a lower purity (approximately 80%, data not shown).

Receptor Microheterogeneity Is Due in Part to Receptor Glycosylation—Purified Ste2p,FT,HT (as well as wild-type Ste2p) migrates as at least four bands by SDS-PAGE. At least some of this complexity is the result of N-linked oligosaccharides added to the N terminus of the protein (38). Digestion of the purified receptor with PNGase F resulted in a collapse of the four blurry bands into two sharp bands (Fig. 5B), indicating that glycosylation accounts for some of the polydispersity present in the receptor preparation.

Purified Ste2p,FT,HT Has an Affinity for Its Ligand Consistent with the Absence of Its Cognate G Protein—Purified Ste2p,FT,HT was reconstituted into synthetic liposomes as described under “Materials and Methods.” Steady-state binding of \(^{3}H\)-a-factor to purified, 60:40 POPC:POPG-reconstituted Ste2p indicates that the purified receptor binds \(^{3}H\)-a-factor with a \(K_D\) of 155 nM and a \(B_{max}\) of 1064 pmol/mg (Fig. 6). This affinity is similar to previous reports of the affinity of a-factor for Ste2p in the absence of its associated G protein (150 nM) (3).

Efficient Restoration of Ligand Binding Activity Required the Addition of Solubilized Yeast Membranes—Based on the amount of purified Ste2p,FT,HT reconstituted into the artificial vesicles, and assuming all of the receptor molecules were properly oriented in these liposomes, only a fraction (\(-6\%\)) of the total expected ligand binding capacity was observed. We found that ligand binding could be restored by adding solubilized membranes from DK102 (ste2Δ) before vesicle reconstitution. The resulting Ste2p,FT,HT-containing proteoliposomes displayed a specific activity of 14,500 pmol/mg Ste2p,FT,HT (Fig. 6), suggesting that most of the purified receptor (at least 80%) is capable of binding ligand after vesicle reconstitution.

Receptor in Cell Membranes and Purified Ste2p,FT,HT Discriminate Similarly between Stereoisomeric Analogs of a-Factor—The ability of a receptor to discriminate between different ligands is a measure of the authenticity of ligand binding. \([\text{D-Ala}^9\text{]}\)a-Factor is a pheromone analog that binds Ste2p with an affinity somewhat greater (\(>1.5\)-fold greater) than that of authentic a-factor. \(\text{[L-Ala}^9\text{]}\)a-Factor can bind to Ste2p expressed on the surface of whole cells approximately 300-fold better than \([\text{L-Ala}^9\text{]}\)a-factor. We found that crude membranes containing Ste2p as well as purified and reconstituted Ste2p,FT,HT displayed similar differences in affinities for these stereoisomers. In control studies, authentic a-factor competed approximately as well as did \([\text{D-Ala}^9\text{]}\)a-factor (data not shown). For membranes isolated from DK102 [pNED1], a 433-fold difference in \(K_D\) was observed between the \([\text{L-Ala}^9\text{]}\)a-factor and \([\text{D-Ala}^9\text{]}\)a-factor (Fig. 7A). Likewise, for purified and 60:40 POPC:POPG-reconstituted Ste2p,FT,HT, a 396-fold difference in \(K_D\) was observed between the \([\text{L-Ala}^9\text{]}\)a-factor and \([\text{D-Ala}^9\text{]}\)a-factor (Fig. 7B). Ste2p,FT,HT reconstituted in the presence of solubilized DK102 (ste2Δ) membranes displayed at least a 200-fold difference in affinity for the stereoisomers (data not shown). These results indicate that the purified receptor maintains its correct ligand recognition characteristics.

### DISCUSSION

The system used to express Ste2p was designed to maximize production of receptor protein and to introduce two different

### TABLE I

| Source       | Preparation                                                | \(K_D\) (nM) | \(B_{max}\) (pmol/mg) |
|--------------|------------------------------------------------------------|--------------|-----------------------|
| RC629 (STE2+) | Cells                                                      | 7 ± 1        | 13 ± 1 fmo1/10^6 cells |
| DK102 (ste2Δ) | Cells                                                      | 6 ± 1        | 265 ± 7 fmo1/10^6 cells |
| BJ2168 [pNED1] | Cells                                                      | 7 ± 0.4      | 236 ± 4.0 fmo1/10^6 cells |
| RC629 (STE2+) | Membranes                                                 | 5 ± 1        | 2250 ± 90 fmo1/500 unit |
| DK102 (ste2Δ) | Membranes                                                 | 14 ± 1       | 1.7 ± 10^2 ± 4100 fmo1/500 unit |
| BJ2168 [pNED1] | Membranes                                                 | 24 ± 3       | 1.8 ± 10^3 ± 6700 fmo1/500 unit |
| BJ2168 [pNED1] | Purified Ste2p,FT,HT reconstituted with a 60:40 POPC:POPG lipid mixture | 155 ± 31    | 1604 ± 65 pmol/mg Ste2p,FT,HT (80% active) |
| BJ2168 [pNED1] | Purified Ste2p,FT,HT reconstituted with a 60:40 POPC:POPG lipid mixture and DK102 extract | 185 ± 13    | 14,500 ± 23 pmol/mg Ste2p,FT,HT (80% active) |
Expression and Purification of the α-Factor Receptor

affinity tags on the C terminus of the protein to facilitate receptor purification and detection (Fig. 1). The expression vector was derived from pG3, a high-copy number plasmid designed to constitutively express proteins in S. cerevisiae (28). The vector contains a strong, constitutively active promoter, the glyceraldehyde-3-phosphate dehydrogenase (TDH3) promoter, and an efficient terminator, the phosphoglycerate kinase (PGK1) terminator. The plasmid exists in high copy number due to the presence of the origin of replication derived from a DNA plasmid.

Procedures have been described for modifications that can be introduced at the beginning and the end of open reading frames in S. cerevisiae to increase protein expression (39). These modifications were introduced into the STE2 gene by PCR. First, the -3, -2, and -1 positions with respect to the initiating ATG were changed to ATA (Fig. 1). Procedures have also been described for modifications that can be introduced into the primary sequence of a GPCR to facilitate its purification (40), but these procedures result in an expression level at least one order of magnitude less than that reported here.

Fig. 5. A, purification of Ste2p.FT.HT. Lane 1, a sample (0.6 μg) of the BJ2168 [pNED1] DBM-solubilized membranes; lane 2, a sample (0.6 μg) of the eluate from the Ni-NTA-agarose; lane 3, immunoblot of lane 2 probed with an anti-FLAG M2 monoclonal antibody and visualized by ECL. Proteins in lanes 1 and 2 were stained with Coomassie Brilliant Blue R-250. B, deglycosylation of Ste2p.FT.HT. Lane 1, purified Ste2p.FT.HT (0.17 μg); lane 2, purified Ste2p.FT.HT (0.17 μg) digested with 30 units of PNGase F at room temperature for 1 h. The immunoblot was probed with an anti-FLAG M2 monoclonal antibody and visualized by ECL.

Fig. 6. [3H]α-Factor binding measurements (Fig. 4) provided a similar yet more quantitative estimate of Ste2p overexpression than did the immunoblot results. In the whole cell binding assay, 19-fold (BJ2168 [pNED1]) to 21-fold (DK102 [pNED1]) more binding sites were observed on the surface of each of the overexpression strains than on a wild-type MATa strain (RC629). Though the dilution factor chosen was 1:25, the ECL signal was more than 2-fold stronger in lane 2, suggesting that the apparent 50-fold difference is a minimum estimate of overexpression. Introducing the same plasmid into the protease-deficient strain BJ2168 further increased the overexpression of Ste2p to at least 80-fold relative to RC629 (compare lanes 4 and 5). Again, this estimate is based on the comparison of the dilution factors required to give comparable signals. It should be noted that both the wild-type Ste2p and the overexpressed Ste2p.FT.HT appeared as several closely-spaced bands on the immunoblot, some of the heterogeneity being due to differing degrees of glycosylation (Fig. 5B). It should also be noted that the Ste2p.FT.HT receptor migrates slightly slower than the wild-type receptor by SDS-PAGE, as expected from its increased molecular mass. This mobility shift is consistent with the presence of the additional amino acids from the FLAG and His6 tags appended to the C terminus of the protein.

To assess the effectiveness of our expression system, the expression level of the Ste2p.FT.HT receptor was monitored by immunoblotting (Fig. 3) and [3H]α-factor binding measurements (Fig. 4, Table I). The specificity of the antibody was demonstrated by the absence of detectable cross-reaction when DK102 (ste2Δ) membranes were analyzed (Fig. 3, lane 1). Comparison of lane 5 (RC629) with 2 (DK102 [pNED1], diluted 1:25) in Fig. 3 indicates that the pNED1 expression vector transformed into DK102 gives approximately 50-fold overexpression of Ste2p relative to a wild-type MATa strain (RC629). Though the dilution factor chosen was 1:25, the ECL signal was more than 2-fold stronger in lane 2, suggesting that the apparent 50-fold difference is a minimum estimate of overexpression. Introducing the same plasmid into the protease-deficient strain BJ2168 further increased the overexpression of Ste2p to at least 80-fold relative to RC629 (compare lanes 4 and 5). Again, this estimate is based on the comparison of the dilution factors required to give comparable signals. It should be noted that both the wild-type Ste2p and the overexpressed Ste2p.FT.HT appeared as several closely-spaced bands on the immunoblot, some of the heterogeneity being due to differing degrees of glycosylation (Fig. 5B). It should also be noted that the Ste2p.FT.HT receptor migrates slightly slower than the wild-type receptor by SDS-PAGE, as expected from its increased molecular mass. This mobility shift is consistent with the presence of the additional amino acids from the FLAG and His6 tags appended to the C terminus of the protein.

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We estimate the expression level of Ste2p.FT.HT in BJ2168 [pNED1] to be approximately 352 pmol/mg membrane protein. This value is at least 3-fold higher than that reported for any other 7-transmembrane-segment receptor expressed in yeast. The level of expression of the rat M5 muscarinic acetylcholine receptor was approximately 0.1 pmol/mg protein (41). The level of D2 dopamine receptor expression was reported to be 1–2 pmol/mg protein (20). The highest expression previously reported came from King et al. (23), who observed 115 pmol/mg protein for a fusion protein of the N terminus of Ste2p and the human \( \beta_2 \) adrenergic receptor.

Having determined that our expression system was effective, it was necessary to demonstrate that the modified receptor was functional. Cells containing the overexpressed Ste2p.FT.HT responded to \( \alpha \)-factor (arrested their growth in a halo assay) with a sensitivity comparable with that of the wild-type MATa strain (Fig. 2), demonstrating that the introduction of the FLAG and His6 tags at the C terminus of the protein does not compromise receptor function. The halo assay measures the ability of a yeast strain to undergo growth arrest in response to \( \alpha \)-factor. The larger the halo of non-growth, the more sensitive the strain. All strains used in the halo assays performed here are disrupted at the SST1 locus (42), which encodes a protease responsible for \( \alpha \)-factor degradation. Thus, all strains tested in this work should possess the same intrinsic sensitivities to \( \alpha \)-factor. Even though cells carrying pNED1 overproduce Ste2p substantially, it has been observed previously that halo size is not detectably increased by receptor overexpression (38), suggesting that components downstream of the receptor are limiting in the growth arrest response. Thus, it is not surprising that receptor overexpression in DK102 [pNED1] has no measurable effect on halo size.

Although the halo assay demonstrated that the modified receptor was able to mediate growth arrest, it was also necessary to demonstrate that the receptor had a similar affinity as the wild-type receptor for \( \alpha \)-factor. The affinity of \( [\text{3H}]\alpha \)-factor for all three yeast strains used in this work (Table I) is in very close agreement with the value determined by Jenness et al. (37) as measured on whole cells (6 nM). Additionally, the values measured for the membranes of each of the strains are similar to previously observed results (Table I). Blumer et al. (38) observed an affinity of 2 nM in membranes expressing a wild-type level of Ste2p (comparable with the value of 5 nM determined for wild-type cell membranes in this work) and an affinity of 12 nM in membranes derived from a strain overexpressing Ste2p (comparable with the values of 14 nM and 24 nM determined in this work). Thus, the sequence modifications made to

**Fig. 7. Competition for \([\text{3H}]\alpha \)-factor binding by different stereoisomeric \( \alpha \)-factor derivatives.** Competition of the \([\text{3H}]\alpha \)-factor was performed as described under “Materials and Methods.” All data points were collected in triplicate. The competition curves shown are representative of a minimum of three independent experiments. IC\(_{50}\) values were determined by least squares fitting analysis using the TableCurve 2D computer program (Jandel Scientific). Total binding was determined with six samples in the absence of any competitor peptide, and nonspecific binding was determined with six samples in the presence of a 1000-fold molar excess of non-radioactive \( \alpha \)-factor. Specific binding was obtained by subtracting nonspecific from total binding. For the generation of the competition curves, relative percentage occupancies for binding were determined for each analog at each concentration relative to the specific binding. A, isolated membranes; B, purified Ste2p.FT.HT reconstituted with 60:40 POPC:POPG alone. +, [L-Ala\(_9\)]\( \alpha \)-factor; \( \triangle \), [D-Ala\(_9\)]\( \alpha \)-factor.
the receptor did not affect either its signaling functions or ligand binding properties.

To achieve the purity reported here (approximately 95%), Ste2p.FT.HT was purified only from a sucrose gradient fraction thought to be highly enriched for intracellular membranes (the 10–43% (w/v) interfacial band). This choice, while yielding a slightly purer receptor preparation, recovers only half of the total Ste2p.FT.HT produced by the cells. By purifying the receptor from both sucrose gradient bands (the 10–43% (w/v) and the 43–53% (w/v)), one doubles the yield of receptor, but the preparation is less pure (approximately 80%, data not shown).

The yield of Ste2p.FT.HT purified from internal membranes alone is 0.8–1.0 mg from 60 g of cell paste (corresponding to approximately 20 liters of cell culture grown to 3 A600). If one purifies Ste2p.FT.HT from both sucrose gradient bands, the yield is approximately 2 mg of Ste2p.FT.HT. While the FLAG tag is not necessary to purify Ste2p.FT.HT, the epitope tag is useful when this expression and purification system is applied to receptors that express less well or receptors for which one lacks antibodies.3

Digestion of the purified receptor with enzymes that should remove certain types of post-translational modifications helped clarify the nature of the multiple species present in our purified receptor preparation. Deglycosylation of the purified Ste2p.FT.HT with PNGase F collapsed the multiple protein bands around 50 kDa from four diffuse bands to two sharp bands (Fig. 5, A and B). Further treatment of the doublet with endoglycosidase Hf (another deglycosylation enzyme) or alkaline phosphatase had no detectable effect (data not shown). Since deglycosylation of the wild-type Ste2p in solubilized membranes revealed the same shift in mobility as the overexpressed receptor (data not shown), it can be ruled out that the appearance of the multiple protein bands is an artifact of overexpression. The reason for the presence of the two bands even after deglycosylation might be enzyme-resistant glycosylation (O-linked glycosylation), partial proteolytic processing of the receptor, or perhaps post-translational modifications other than glycosylation or phosphorylation.

To demonstrate that the purified, reconstituted receptor in vesicles had the pharmacological properties of the wild-type receptor, ligand binding studies were performed. [3H]-α-factor binding studies yielded a Kd consistent with the notion that the receptor has been purified away from its associated G protein (Fig. 6). For many mammalian GPCRs, the physical removal of G proteins from their cognate receptors (either by receptor purification or through the addition of a non-hydrolyzable GTP analog) results in a decreased affinity for ligand. This same phenomenon has been seen previously with Ste2p in yeast cell membranes (3). If any of the three G protein subunits is absent, or if the G protein is dissociated from the receptor by the addition of a non-hydrolyzable GTP analog, the affinity of the receptor for α-factor is decreased to 150 nM, which is in agreement with the value of 155 nM that we found for the purified and reconstituted receptor.

The purified and reconstituted receptor also maintains its ability to discriminate between atomically identical (but stereochromically distinct) α-factor analogs (Fig. 7B), as does the normal receptor in its native membrane environment (Fig. 7A). Competition studies using crude membranes showed that Ste2p.FT.HT has a 433-fold greater affinity for [α-Ala6]α-factor than for [L- Ala6]α-factor. Likewise, purified and reconstituted Ste2p.FT.HT has a 396-fold greater affinity for [α-Ala6]α-factor than for [L-Ala6]α-factor. The stereoisomers were chosen because they have identical atomic compositions (and thus identical hydrophobicities), yet they are reported to vary at least 300-fold in their affinities for Ste2p expressed on the surface of whole cells.4

Efforts to develop an α-factor binding assay using the detergent-solubilized receptor were hindered by the observation that most detergents interfere with α-factor binding to Ste2p expressed on the surface of whole cells (data not shown). This phenomenon motivated the development of a detergent-free binding assay using artificial vesicles. The specific activity of the purified and 60:40 POPC:POPG-reconstituted receptor (1064 pmol/mg) is, however, notably less than might be expected for a receptor properly oriented in liposomes. This specific activity suggests that perhaps only 6% of the receptor is available for ligand binding.

One possible explanation for the low specific activity is that some essential membrane component (perhaps a particular lipid or protein) is required to help Ste2p maintain its proper shape in the membrane. To test this hypothesis, solubilized membranes from a yeast strain lacking Ste2p (DK102) were added back to the purified receptor before reconstitution into artificial phospholipid vesicles. While DK102 (ste2Δ) membranes possessed no ligand binding ability of their own (data not shown), the addition of the solubilized extract to purified Ste2p.FT.HT restored most of the expected ligand binding activity (at least 80%) based on the amount of receptor present. Also, addition of the DK102 (ste2Δ) extract had almost no effect on the affinity of α-factor (or any of the α-factor analogs) for the purified and reconstituted receptor (Fig. 6). Our laboratory is currently attempting to identify the co-factors responsible for this effect.

Despite the hundreds of GPCRs cloned to date, very few are available in the quantities required for physical studies. Using the system described here, as much as 1 mg of Ste2p.FT.HT could be purified using relatively small-scale techniques. These quantities are sufficient for a concerted structural effort. Our laboratory has also successfully used this system to express and purify the human D1A dopamine receptor,5 suggesting that the system may be widely applicable to GPCR expression and purification. The system has also been used to express a catfish olfactory receptor, albeit at lower levels than either Ste2p or the D1A dopamine receptor.6

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