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Successful expression of a functional yeast G-protein-coupled receptor (Ste2) in mammalian cells

Dezhong Yin [a], Shai Gavi [a], Elena Shumay [a], Ken Duell [b], James B. Konopka [b], Craig C. Malbon [a,*], Hsien-yu Wang [c]

[a] *Department of Molecular Pharmacology, University Medical Center, SUNY/Stony Brook, Stony Brook, NY 11794-8651, USA*
[b] *Department of Molecular Genetics and Microbiology, Diabetes and Metabolic Diseases Research Program, University Medical Center, SUNY/Stony Brook, Stony Brook, NY 11794-8661, USA*
[c] *Department of Physiology and Biophysics, Diabetes and Metabolic Diseases Research Program, University Medical Center, SUNY/Stony Brook, Stony Brook, NY 11794-8661, USA*

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Abstract

G-protein-coupled receptors (GPCRs) are membrane-embedded cell signaling devices transducing ligand binding to activation of heterotrimeric G-proteins, providing a paradigm for signaling for yeast and mammals alike. Probing the extent to which yeast GPCRs may couple to mammalian G-proteins has been problematic. In the current work, we explored conditions that enable the cell-surface expression of a yeast α-factor pheromone receptor (Ste2). When expressed in human HEK293 cells, Ste2 is shown to bind its ligand α-factor, to be functional and catalyze activation of the mitogen-activated protein kinase cascade, and to demonstrate agonist-induced internalization. In response to agonist Ste2 as maintained intracellularly for several hours and avoids the degradation process observed for Ste2 in yeast cells. This is the first successful demonstration of the ability to express a functional yeast GPCR in mammalian cells.

Keywords: G-protein-coupled receptor; yeast; Ste2; α-mating factor; Mammalian cell; Expression; MAP kinase; Internalization; Signaling; Mitogen-activated protein kinase

G-protein-coupled receptors (GPCRs) are heptahelical membrane proteins that likely constitute >5% of the human genome and enjoy wide distribution in nature, from yeast, to flies, worms, fish, and mammals [1]. In yeast, GPCRs play a key role in mating. Yeast harboring mutation in the STE2 gene, encoding a member of the superfamily of GPCRs, lack the ability to respond to the peptide pheromone alpha-mating factor (α-factor) and do not display sexual conjugation [2–5]. The STE2 gene product is a membrane protein with heptahelical hydropathy plots and binds the α-factor pheromone [6,7]. Although many properties of GPCRs are conserved among all multicellular organisms [8], the ability of the Ste2, which is very divergent from mammalian GPCRs, to operate in the context of a mammalian cell has not been reported. The ability to express Ste2 in animal cells would provide a new cellular context for understanding the protein domains in mammalian GPCRs that participate in signaling and regulation. Yeast cells, for example, are devoid of tyrosine kinases making the Ste2 an ideal target for insertion studies of various GPCR motifs in mammalian cells replete with such kinases. In the current work, we explore the ability to express Ste2 in a variety of mammalian cells and succeed in optimizing the conditions for cell-surface expression

* Corresponding author. Fax: +1 631 444 7696.
E-mail address: craig@pharm.sunysb.edu (C.C. Malbon).

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of Ste2 in human embryonic kidney 293 cells. We report that the expressed Ste2 binds α-factor, activates a mitogen-activated protein kinase pathway involving Erk1,2, and undergoes agonist-induced internalization. Unlike Ste2 in yeast cells, Ste2 once internalized in response to agonist in mammalian cells lacks the ability to be rapidly degraded.

Materials and methods

Plasmids. The STE2 gene was amplified by PCR by using plasmid pDB02 [9] as the template and a pair of primers designed with *Nhe*I or *Bam*HI linkers. The PCR product was digested with *Nhe*I and *BamH*I, and cloned into the unique *Nhe*I/*Bam*HI sites of peGFP-N1 expression vector (Clontech). The plasmid encoding the enhanced GFP-tagged human β_2AR (in pCDNA3) was a generous gift from Dr. Jeffrey Benovic (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA) and eGFP-tagged β_2AR has been fully characterized in a variety of mammalian cell lines [10,11].

Cell culture and transfections. The human embryonic kidney HEK293, human epidermoid A431, and human carcinoma HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, HyClone), penicillin (60 μg/mL) plus streptomycin (100 μg/mL), and grown in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Chinese hamster ovary (K-strain, CHOK) cells were maintained in DMEM supplemented with 5% FBS, penicillin (60 μg/mL) plus streptomycin (100 μg/mL), and grown in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. For transfections, cells were seeded at a density of 2×10^6 cells/100-mm dish, cultured for 24 h, and transiently transfected using LipofectAMINE (Invitrogen) according to the manufacturer's recommendations. The cells then were cultured in the growth medium for 48 h.

Assay of Erk1,2 activity by immunoblotting. Cells were stimulated with α-mating factor (α-factor, Sigma) or α-factor antagonist (des-Trp, Ala-3 analog of α-factor, Bachem) for the indicated times and then lysed in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM NaF, 40 mM sodium pyrophosphate, 50 mM KH_2PO_4, 10 mM sodium molybdate, 2 mM sodium orthovanadate, 20 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.5% Nonidet P-40, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride). Whole-cell lysates (50 μg) were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting was performed, as previously described [12]. Detection of phosphorylated, active forms was performed with anti-phospho-Erk1,2 antibodies, whereas determination of the total pool of these MAP kinases was performed with anti-Erk1,2 antibodies (Santa Cruz Biotechnology), as previously described [13].

Assay of Ste2 expression by Immunoblotting. For preparation of crude membrane fractions, cells were washed twice with phosphate-buffered saline (PBS) and collected by centrifugation at 1000*g* for 5 min. The cell pellet was resuspended in HME buffer (20 mM Hepes, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, supplemented with 5 μg/mL leupeptin, 5 μg/mL aprotinin, and 0.2 mM PMSF), disrupted with a Dounce homogenizer (10 strokes), and centrifuged at 2000*g* for 5 min at 4 °C to remove nuclei. The supernatant fraction then was centrifuged at 10,000*g* for 30 min at 4 °C. The pellets (i.e., "crude membrane fractions") were resuspended in HME buffer and subjected to SDS–PAGE and immunoblotting by using anti-Ste2 antibody to an N-terminal domain of the Ste2 [14].

Radioligand binding assays of Ste2 and β2-adrenergic receptors. The ability of Ste2 expressed in mammalian cells to bind ligand was performed by use of an α-factor binding assay, performed essentially as described for such measurements of binding in yeast [9]. Briefly, yeast and HEK293 cells alike were incubated in the presence of 10 mM NaN_3 and 10 mM KF to inhibit receptor internalization. HEK293 cells were resuspended at a density of 5×10^6 cells/50 μL and incubated with 20 nM [^{35}S]methionine-metabolically labeled α-factor prepared as previously described [9] for 30 min at room temperature. Cells were then collected on a Whatman GF/C filter and washed to remove unbound α-factor. Non-specific binding assays were performed in the presence of a 100-fold molar excess of non-radiolabeled, synthetic α-factor. The expression of β_1AR and β_2AR on plasma membrane of HEK293 cells transiently transfected with an expression vector harboring the human β_1AR or β_2AR cDNA was quantified using the water-soluble, membrane-impermeant βAR-specific radioligand CGP-12177 (50 nM), as described [11]. Cells were incubated with [^{3}H]CGP-12177 for 6 h at 4 °C. The cells were rapidly washed and collected on GF/C membranes. The radioligand bound to the washed cell mass on the filter was counted by liquid scintillation spectrometry.

Confocal microscopy. For the confocal microscopy studies, cells expressing GFP-tagged receptors were grown on Nunc chamber slides. Following an overnight serum starvation, cells were treated as indicated and fixed with ice-cold methanol for 2 min at −20 °C. Images were taken with Zeiss510 inverted confocal microscope (60×, oil immersion). Images were imported as tiff.files, processed, and prepared in Adobe Photoshop 5.5.

Results

Our initial attempts to express the yeast α-factor pheromone receptor (Ste2) were performed in human epidermoid A431 cells (Fig. 1A), under conditions that typically yield robust expression of the human β_2-adren-

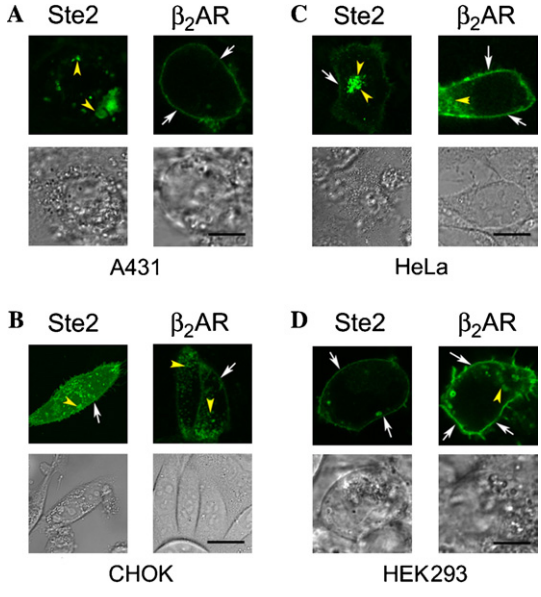

Fig. 1. Localization of the yeast GPCR Ste2 in mammalian cells. Confocal microscopy was used to analyze expression of Ste2-GFP or β_2AR-GFP in the indicated cell lines, following transient transfection: human epidermoid A431 (A), Chinese hamster ovary (K-strain) CHOK (B), human carcinoma HeLa (C), and human embryonic kidney HEK 293 (D) cells. White arrows highlight plasma membrane-localized receptors; yellow arrowheads highlight receptors in the intracellular, perinuclear compartment of the cells. Confocal (top) and phase contrast (bottom) images for each cell line are provided. Scale bar, 10 μm.

ergic receptor (β_2AR). Both receptors were expressed as fusion proteins in which the enhanced green fluorescent protein (GFP) was fused to the C-terminus to permit analysis of the receptor localization in the cell by confocal microscopy (Fig. 1A). Whereas β_2AR-GFP was largely confined to the plasma membrane (white arrows), the Ste2-GFP was essentially localized to intracellular, perinuclear areas (yellow arrowheads), lacking appreciable cell-surface localization. This intracellular, perinuclear disposition of Ste2 was not influenced by adjustments in the amount of DNA used to transfect the cells, the cell density at the time of transfection, or the time period for which the cells were transfected (not shown). We were unable to improve the relative amounts of plasma membrane localized Ste2, even when we transfected cells with a Ste2/β_2AR chimera in which the first 20-amino acid N-terminal stretch of the β_2AR was spliced onto the N-terminus of Ste2 (not shown). Thus, the addition of the β_2AR N-terminal domain did not act as a "leader sequence" to improve transit of the Ste2 to the plasma membrane.

To determine if other cell lines might permit better cell-surface expression, expression of Ste2-GFP and β_2AR-GFP was analyzed in several additional cell lines, including Chinese hamster ovary (CHO, K-strain) cells, human carcinoma HeLa cells, and the human embryonic kidney (HEK) 293 cells (Figs. 1B–D). CHOK cells lack expression of β_2AR and are therefore widely employed for the study of adrenergic and other GPCR subtypes [15–18]. In contrast to the situation observed in the A431 cells, Ste2-GFP and β_2AR-GFP could be detected in the plasma membrane of CHOK cells (Fig. 1B). However, under these conditions Ste2 was localized largely to the intracellular, perinuclear area. This marked intracellular localization of the Ste2 in CHOK cells essentially precluded use of these cells for probing the receptor internalization in response to agonist. HeLa cells also were capable of expressing both Ste2 and β_2ARs, but again receptor localization was predominantly intracellular, with sparingly little receptor localized to the plasma membrane (Fig. 1C).

HEK293 cells were examined next, since they are well known for their ability to express relatively high level of GPCRs, under a variety of conditions [19–29]. The levels of expression of mammalian GPCRs achieved in HEK293 cells often are orders of magnitude greater than those observed in other cell lines, suggesting that these cells have a robust capacity to chaperone and to mediate trafficking of GPCRs to the plasma membrane. Similar results were obtained in HEK293 cells for the more divergent Ste2, in that the Ste2-GFP displayed a cellular localization very similar to that obtained for β_2AR-GFP, expressed under these same conditions (Fig. 1D). Thus, use of the HEK293 provided the first expression system to permit further analysis of Ste2 in a mammalian cell.

To determine whether Ste2-GFP expressed in HEK293 cells was properly folded (i.e., functional) and to probe further that the receptors were localized to the plasma membrane, their ability to bind cognate ligands was studied (Fig. 2A). We tested the ability of the expressed Ste2 to bind ^{35}S-labeled α-factor under conditions employed to measure binding to Ste2 in yeast cells. Such studies have not been reported for yeast GPCR expressed in a mammalian cell. The HEK293 cells expressing Ste2-GFP displayed the ability to bind ^{35}S-α-factor in a manner that was sensitive to competition with 100-fold molar excess concentrations of synthetic, unlabeled α-factor. The HEK293 cells express approximately 50 fmol ^{35}S-α-factor binding per 10^6 cells. Likewise we assayed the ability of HEK293 cells to express the β_1AR (Fig. 2B) and β_2AR (not shown) on the plasma membrane through use of a hydrophilic, cell-impermeant β-adrenergic ligand CGP-12177[30]. The expression of cell-surface localized β_1AR-GFP was sensitive to blockade by the β-adrenergic antagonist propranolol (10 μM) and accounted for ~800 fmol per 10^6 cells.

Since expression of yeast GPCRs in animal cells had not been reported, we examined the molecular nature of the Ste2 expressed in mammalian cells in comparison to Ste2 expressed in yeast, using SDS–PAGE and immuno-

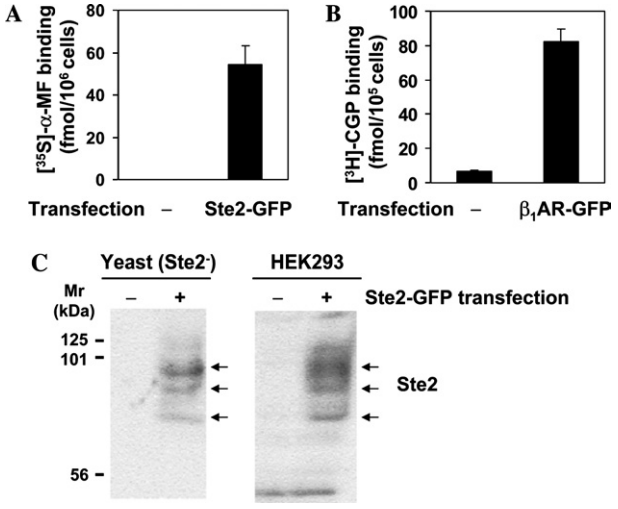

Fig. 2. Characterization of expression of yeast Ste2 in HEK293 cells. (A), binding of ^{35}S-α-factor to Ste2 in HEK293 cells transiently transfected with an expression vector harboring the Ste2-GFP cDNA. (B) Binding of hydrophilic, cell-impermeable β-adrenergic antagonist [^{3}H]CGP-12177 to HEK293 cells transiently transfected with an expression vector harboring the β_1AR-GFP cDNA. The data are presented as mean values ± SEM from triplicate determinations obtained from three separate experiments. (C) Analysis of M_r on SDS–PAGE. Whole-cell lysates of an Ste2 deletion strain (*Ste2*delta, strain YLG123) transformed with expression vector harboring the Ste2-GFP cDNA (+) or the empty vector (−) and crude membrane fractions (200 μg) prepared from HEK293 cells transiently transfected with either the expression vector harboring the Ste2-GFP cDNA (+) or the empty vector (−) were resolved on 10% SDS–PAGE.

blotting (Fig. 2C). When subjected to SDS–PAGE, the Ste2-GFP expressed in yeast resolves into three distinct bands with M_r of ~74, ~82, and ~88 kDa. Ste2, like most GPCRs, is a glycoprotein [7]. The multiple forms of Ste2 expressed in yeast cells likely represent differential glycosylation [31], as commonly observed in mammalian GPCRs [31,32,32]. When expressed in HEK293 mammalian cells, Ste2-GFP appeared in immunoblots to display a similar pattern of heterogeneity and a similar pattern of relative electrophoretic mobilities (i.e., M_r of ~74, ~82, and and 86–92 kDa). Thus, on the basis of cellular localization, the ability to bind agonist ligand, and the molecular nature as revealed by SDS–PAGE, Ste2-GFP expressed in mammalian cells displays all of the properties that we would expect for Ste2 expressed in yeast or that would be expected for mammalian GPCRs expressed in these same cells.

Having established the successful expression of a yeast GPCR in mammalian cells that binds the agonist ligand (α-factor), is expressed on the plasma membrane, and displays the same M_r as authentic Ste2 expressed in yeast cells, we explored the ability of the receptor to undergo agonist-induced internalization, a hallmark of mammalian GPCRs. Agonist-induced internalization of Ste2 was examined in HEK293 cells and compared to that observed for β_2AR (Figs. 3A and B). Confocal microscopy of the GFP-tagged Ste2 following a challenge with α-factor for up to 30 min revealed a rapid internalization of the receptor (Fig. 3A). Within 5–10 min of incubation with agonist, Ste2 leaves the plasma membrane and accumulates in the perinuclear region of the cells. The β_2AR displays a similar agonist-induced internalization in response to stimulation with the β-adrenergic agonist isoproterenol (Fig. 3B). Thus, the yeast Ste2 expressed in a mammalian cell appears to behave much like the β_2AR following activation by its cognate agonist.

The effects of longer-term treatment of Ste2 with agonist ligand on receptor internalization were studied. The effect of incubation with agonist ligand for 3 h on agonist-induced internalization in Ste2-expressing cells was examined and compared to agonist-induced internaliza-

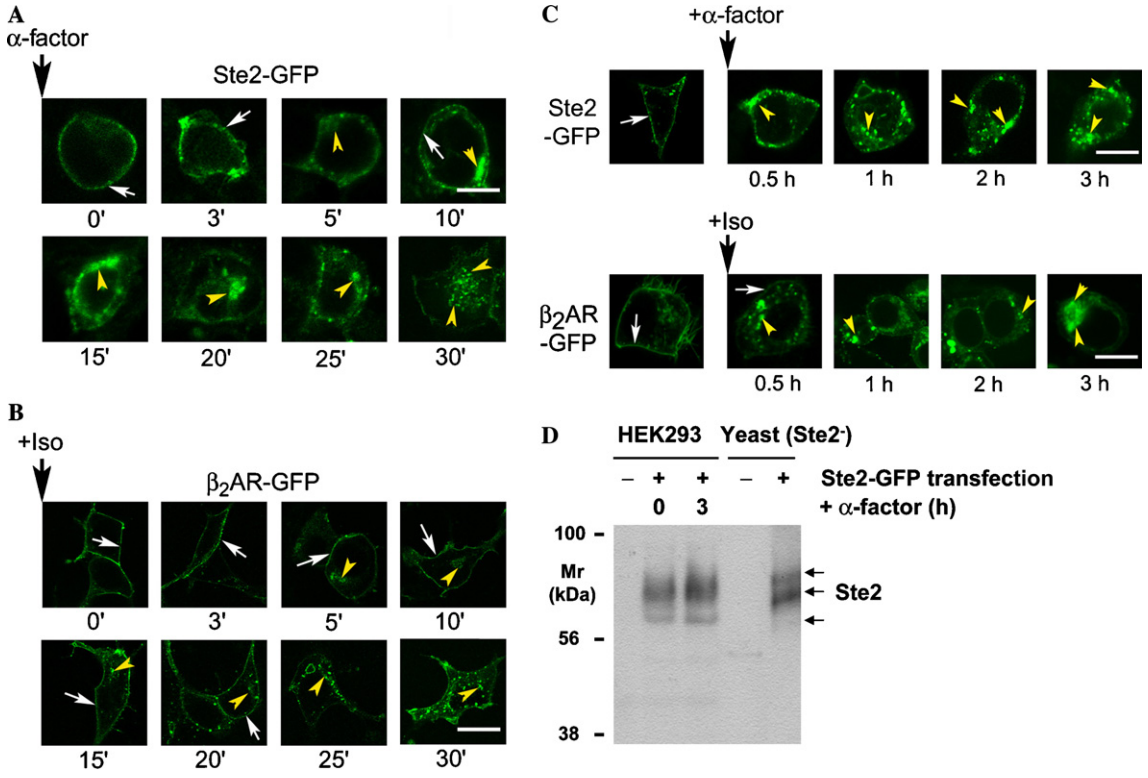

Fig. 3. α-factor stimulates agonist-induced internalization of Ste2 in mammalian cells. HEK293 cells transiently transfected with Ste2-GFP or β_2AR-GFP were serum-starved overnight and challenged with α-factor (10 μM) or isoproterenol (Iso, 10 μM) for the time indicated. (A) Time-course of α-factor-induced receptor internalization of Ste2-GFP. (B) Time-course of Iso-induced receptor internalization of β_2AR-GFP. (C) Extended time-courses of agonist-induced internalization of either Ste2-GFP or β_2AR-GFP expressed in HEK293. Transiently transfected HEK293 cells were pretreated with cycloheximide (20 μg/mL) for 30 min and then treated with agonists for either Ste2 (α-factor) or β_2AR (Iso). White arrows highlight plasma membrane-localized receptors; yellow arrowheads highlight perinuclear-localized receptors. Scale bar, 10 μm. (D) SDS–PAGE analysis of Ste2 degradation. HEK293 cells transiently transfected with the empty vector (−) or with expression vector harboring the Ste2-GFP cDNA (+) were serum-starved overnight and challenged with α-factor (10 μM) for 0 or 3 h in the presence of cycloheximide (20 μg/mL). Crude membrane fractions (50 μg) prepared from the HEK293 cells treated as above and whole-cell lysates of an Ste2 deletion strain (*Ste2*delta, strain YLG123) transformed with expression vector harboring the Ste2-GFP cDNA (+) or the empty vector (−) and were resolved on 10% SDS–PAGE.

tion of β_2AR (Fig. 3C). From 1 to 3 h of challenge with agonist ligand, Ste2 accumulates most prominently in large punctate structures in the intracellular, perinuclear regions of the cells (Fig. 3C). This pattern of longer-term agonist-induced internalization was remarkably similar to that observed for β_2AR in cells treated with the β-agonist isoproterenol (Iso). Although the β_2ARs display little degradation of internalized receptors over this time frame [33], it was of interest to determine if yeast Ste2 displays the agonist-induced sequestration and degradation typically observed in yeast cells [3,34]. Although only a qualitative observation, it was clear from the confocal images that the cellular abundance of Ste2 and β_2AR does not change significantly over 3 h of exposure to agonist ligands. Performing these experiments in the presence or absence of the protein synthesis inhibitor cycloheximide did not appreciably alter this pattern of internalization (data not shown). Binding studies making use of ^{35}S-α-factor to assay Ste2 binding sites on the plasma membrane of HEK293 cells confirmed the observed loss of cell-surface Ste2 in response to treatment with the agonist that was observed by confocal microscopy (data not shown).

To quantitatively study whether there was agonist-induced degradation of Ste2 in HEK293 cells, HEK293 cells transiently expressing Ste2-GFP were treated with α-factor for 3 h and in the presence of cycloheximide and then crude membrane fractions were analyzed by Western immunoblotting for determination of possible degradation of Ste2-GFP. Ste2 displays rapid turnover (i.e., $t_{1/2}$ ~30 min) in yeast [35]. Treating HEK293 cells with α-factor for 3 h internalized Ste2 but did not significantly lead to degradation of Ste2-GFP (Fig. 3D). These biochemical determinations agree well with the observations made by confocal microscopy, i.e., when expressed in mammalian cells, the yeast GPCR does not display the rapid α-factor-induced degradation observed in yeast cells.

With the successful expression of Ste2 in mammalian cells, we were able to examine whether (or not) Ste2, when activated by α-factor, couples to a mammalian signaling pathway. We examined first the mitogen-activate protein (MAP) kinase regulatory network [36], based upon the ability of Ste2 to activate MAPK signaling in yeast. Of the three MAP kinase pathways examined (i.e., JNK, p38, and Erk1,2), only the Erk1,2 displayed significant activation in response to treatment of Ste2-expressing HEK293 cells with α-factor (Fig. 4). Activation of Erk1,2 was determined using phospho-specific antibodies that recognize only the fully activated forms of Erk1,2. Total cellular levels of Erk1 (p44) and of Erk2 (p42) were unaffected by treatment of cells with α-factor, whereas the levels of dually phosphorylated, activated p42, and p44 were increased several fold in the cells expressing Ste2, but not in control HEK 293 cells transfected with the empty vector. The time-course for Erk1,2 activation was quite rapid, reaching maximal levels (3–4-fold stimulation) within 3 min of stimulation with α-factor, but not with α-factor antagonist (Fig. 4).

Discussion

The mechanisms by which cells regulate the trafficking of GPCRs are not fully understood. To determine the nature of protein motifs perhaps responsible for GPCR regulation, we attempted to express in mammalian cells a yeast GPCR, Ste2, that is highly divergent from mammalian GPCRs. Initially, many problems were encountered in attempting to express the yeast

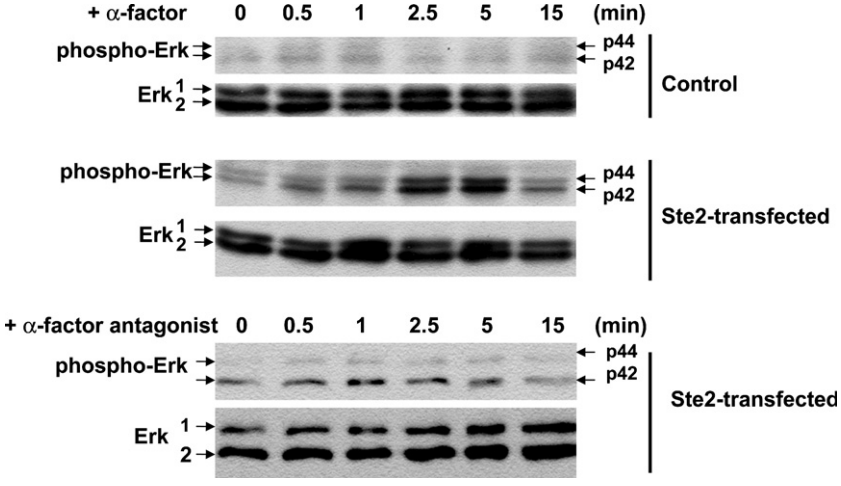

Fig. 4. Activation of Erk1,2 by treating Ste2-expressing mammalian cells with α-factor. HEK293 cells transfected with the empty vector (control) or HEK293 cells stably transfected to express Ste2-GFP were serum-starved overnight and challenged with α-factor (10 μM) or with α-factor antagonist (10 μM) for the times (min) indicated. The Western blots were prepared from whole-cell lysates (50 μg protein/lane) and then probed with activation-specific phospho-Erk1,2 antibodies. Loading equivalence was verified by probing with an antibody that recognizes Erk1,2.

GPCR Ste2 in a variety of mammalian cells. Ste2 production was apparent in all of the cell lines studied, but in the initial cell lines tested the bulk of the receptor failed to transit to the plasma membrane, likely a reflection of differences in protein folding that results in retention in the endoplasmic reticulum. Confocal microscopy of most of the cell lines tested demonstrated the existence of large, punctate aggregates of receptors in the intracellular, perinuclear compartment and little apparent transit of Ste2 to the plasma membrane. Perhaps this results, in part, from differences in protein folding in a membrane differing from that of yeast in lipid composition or may result from the absence of proper chaperones. Attempts to rectify this problem by fusing the N-terminus of the β_2AR onto Ste2 to create a β_2AR/Ste2 chimera to attempt to improve trafficking to the plasma membrane were not successful. The HEK293 cells, in contrast, proved to be a successful target for the expression of Ste2. The HEK293 cell line was investigated based upon the previous reports that these cells can achieve high levels of expression of various mammalian GPCRs. Consistent with previous studies of the expression of mammalian GPCRs, the HEK293 cells were able to express and transit yeast Ste2 to the plasma membrane.

Expression of the yeast Ste2 at the plasma membrane of animal cells was suggestive that these heterologous receptors were functional, but demonstration that the Ste2 was properly folded and embedded in the alien membrane required further study. The features of the Ste2 and its regulation were compared to these properties of the β_2AR, a prototypic mammalian GPCR. The results of these studies provide compelling evidence for a functional Ste2 operating in a mammalian cell, in spite of differences of the lipid composition between animal and plant cells. When expressed in the context of a mammalian plasma membrane, Ste2 displayed properties as follows: Ste2 was largely confined to the plasma membrane of HEK293 cells; the Ste2 displayed a pattern (M_r) on SDS–PAGE that was very similar to that observed for Ste2 expressed in native yeast cells; stimulation of Ste2 with α-factor led to activation of downstream Erk1,2 in HEK293 cells; and activation of Ste2 with α-factor promoted rapid, agonist-induced sequestration of the receptor to intracellular, perinuclear areas of the cell.

The observation that Ste2 produced in mammalian cells responds to its ligand to activate MAP kinase signaling and undergoes ligand-induced internalization is significant in that Ste2 does not share significant sequence similarity with any of >1500 mammalian GPCRs. Ste2 does, however, share a similar overall seven-transmembrane domain architecture common to mammalian GPCRs. Although a number of mammalian GPCRs enable agonist-activated G-protein signaling in yeast to induce mating responses [37,38], the present report is the first to succeed in expression of a yeast GPCR in an mammalian cell and demonstrate cell-surface localization and function. Taken altogether, these results indicate that aspects of the seven-transmembrane domain structure in Ste2, an evolutionarily distant receptor from a simple unicellular organism, contain underlying features that are similar to those of mammalian GPCRs.

Further analysis of Ste2 trafficking in mammalian cells identified a major difference between Ste2 and β_2AR in that there was no detectable recycling of Ste2 from the intracellular compartment to the plasma membrane following ligand-induced internalization. Once Ste2 is trafficked to the intracellular compartment in response to stimulation of the cell with α-factor, washing away the agonist ligand failed to provoke any appreciable recycling of the Ste2 to the plasma membrane (not shown). In yeast, α-factor binding causes Ste2 to undergo ubiquitination that promotes endocytosis and transit of the receptor to the vacuole (i.e., yeast lysosome) where the Ste2 is degraded shortly. However, α-factor treatment did not lead to quick degradation of Ste2 in mammalian cells. Perhaps, it is the lack of proper recognition sites for mammalian ubiquitin ligases or other elements involved in receptor recycling or degradation that spares the Ste2 from either fate. The ability to express a functional GPCR lacking in these response may open up the opportunity to employ the Ste2 as a framework for construction of chimera GPCR into which interesting protein motifs/modules can be spliced and receptor biology examined.

Acknowledgments

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