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Functional Expression of the Yeast α-Factor Receptor in *Xenopus* Oocytes*

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The *STE2* gene of the yeast *Saccharomyces cerevisiae* encodes a 431-residue polypeptide that has been shown by chemical cross-linking and genetic studies to be a component of the receptor for the peptide mating pheromone, α-factor. To demonstrate directly that the ligand binding site of the α-factor receptor is comprised solely of the *STE2* gene product, the *STE2* protein was expressed in *Xenopus* oocytes. Oocytes microinjected with synthetic *STE2* mRNA displayed specific surface binding for ^{35}S-labeled α-factor (up to 40 sites/μm^2/ng RNA). Oocytes injected with either *STE2* antisense RNA or heterologous receptor mRNA (nicotinic acetylcholine receptor α, β, γ, and δ subunit mRNAs) showed no binding activity (indistinguishable from uninjected control oocytes). The apparent K_D (7 nM) of the α-factor binding sites expressed on the oocyte surface, determined by competition binding studies, agreed with the values reported for intact yeast cells and yeast plasma membrane fractions. These findings demonstrate that the *STE2* gene product is the only yeast polypeptide required for biogenesis of a functional α-factor receptor. Electrophysiological measurements indicated that the membrane conductance of oocytes injected with *STE2* mRNA, or with both *STE2* and *GPA1* (encoding a yeast G protein α-subunit) mRNAs, did not change and was not affected by pheromone binding. Thus, the α-factor receptor, like mammalian G protein-coupled receptors, apparently lacks activity as an intrinsic or ligand-gated ion channel. This report is the first instance in which a membrane-bound receptor from a unicellular eukaryote has been expressed in a vertebrate cell.

In the yeast *Saccharomyces cerevisiae*, *MAT***a** and *MAT*α haploid cells each secrete an oligopeptide mating pheromone (**a**-factor and α-factor, respectively) that acts specifically upon the opposite haploid cell type (1, 2). Haploid cells exposed to mating pheromone display a series of physiological responses that are preparatory for conjugation, including rapid changes in gene expression, arrest of cell growth in the G1 phase of the cell cycle, and alterations in cell surface and nuclear morphology (3, 4). It has also been reported that pheromone response in *MAT***a** cells is accompanied by both rapid (5) and sustained (6) increases in Ca^{2+} uptake, although it is uncertain whether these Ca^{2+} fluxes are an obligatory part of the cellular response to mating pheromone.

The mating response pathway in yeast is mediated through a G protein-linked signal transduction system. Haploid *MAT***a** cells possess on their surface saturable binding sites for α-factor, which have been shown genetically and biochemically to be encoded, at least in part, by the *STE2* gene. Cells carrying temperature-sensitive *ste2* mutations display thermosensitive α-factor binding activity *in vitro* (7, 8). Chemical cross-linking to radiolabeled α-factor (9), and the changes in ligand binding specificity observed when a *STE2* homolog from another yeast species (*Saccharomyces kluyveri*) was expressed in *S. cerevisiae* (10), have demonstrated that the *STE2* product contributes to the ligand recognition activity of the α-factor receptor. Hydropathy analysis indicates that the predicted *STE2* gene product (11, 12) contains seven hydrophobic segments followed by a hydrophilic C-terminal region, a structural motif common to mammalian G protein-linked receptors. Indeed, immunological and biochemical analyses have confirmed that the *STE2* gene product is a plasma membrane-associated glycoprotein and that its C-terminal hydrophilic domain is disposed on the cytosolic side of the membrane (9, 13, 14).

Genetic evidence indicates that pheromonal signals are transduced, directly or indirectly, from the receptor to a guanine nucleotide-binding protein (G protein). Inactivation of the *GPA1* gene, which encodes a protein homologous to mammalian G_α subunits, results in constitutive induction of the pheromone response pathway (15–18). Null mutations in the *STE4* and *STE18* genes (which encode G_β and G_γ subunit homologs, respectively) block response to mating pheromone (19).

In this report, the *Xenopus* oocyte expression system and direct α-factor binding assays were employed to determine if the yeast *STE2* gene product was sufficient, as well as necessary, to form a functional pheromone receptor. Electrophysiological measurements were used to explore the potential consequences of expressing functional α-factor receptors in amphibian oocytes. The results obtained have significant implications for understanding the subunit composition of the α-factor receptor, the potential biochemical function of the receptor in transmembrane signaling, and the evolutionary conservation of G protein-coupled signal transduction systems.

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals—Materials were obtained from the following sources: T7 RNA polymerase from the United States Biochemical Corp.; the RNase inhibitor RNasin from Promega Biotec; restriction endonucleases from New England Biolabs, Boehringer Mannheim, and Bethesda Research Laboratories; mRNA cap analog, $GpppG_{OH}$ from Pharmacia LKB Biotechnology Inc.; nucleotides from Sigma;

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carrier-free [^{35}S]H_2SO_4, for preparation of ^{35}S-labeled α-factor from Du Pont-New England Nuclear; and synthetic α-factor from Peninsula Laboratories.

Plasmids—Plasmid pT7STE2, in which expression of *STE2* mRNA can be driven by a phage T7 promoter, has been described previously (9). To place the *STE2* coding region in the antisense orientation under transcriptional control of a T7 promoter, the 2.2-kilobase pair *Bam*HI fragment of pT7STE2 was inserted into the *Bam*HI site of pGEM1 (Promega Biotec). The structure of the resultant plasmid, pGEM1-STE2, was verified by appropriate restriction endonuclease mapping. The *GPA1* gene was obtained by screening a yeast genomic library (20) with a synthetic 25-base oligonucleotide derived from the published gene sequence (16, 21). To place the *GPA1* coding region under transcriptional control of a phage SP6 promoter, a 1.9-kilobase pair *Eco*RI fragment containing the entire *GPA1* gene was inserted into the *Eco*RI site of pGEM2 (Promega Biotec). Appropriate restriction endonuclease mapping confirmed the structure of the resultant plasmid, pGEM2-GPA1.

Template Preparation and in Vitro Transcription—The procedure of White *et al.* (22) was used with some modifications (23) for *in vitro* transcription of mRNA or antisense RNA. T7 or SP6 RNA polymerases were employed at a final concentration of 600 units/ml. To monitor RNA synthesis, [α-^{32}P]CTP (Du Pont-New England Nuclear) was included in transcription reactions at a specific activity of 40 mCi/mmol, and a portion of each reaction mixture was treated with glyoxal, subjected to gel electrophoresis (24), and examined by autoradiography. *STE2* mRNA was synthesized by transcription of pT7 *STE2* that had been digested with *Hin*dIII. *STE2* antisense RNA was produced by transcription of pGEM1-STE2 cleaved with *Xba*I. *GPA*1 mRNA was made by transcription of pGEM2-GPA1 also cleaved with *Xba*I. In the resultant mRNA, the *GPA1* initiation codon is preceded by 210 nucleotides that lack other AUG codons. Translation of this mRNA in a rabbit reticulocyte lysate (Promega Biotec) yielded a 49-kDa protein, in agreement with the predicted size (16, 21) of the *GPA1* gene product.[1] The mRNA corresponding to the α, β, γ, and δ subunits of *Torpedo* nicotinic acetylcholine receptor were prepared from plasmids pMARA, pMARB, pMARG, and pMARD (25), as described before (23).

Preparation of Oocytes, RNA Injection, and Electrophysiology—Mature female *Xenopus* frogs were obtained from commercial suppliers (Xenopus I, Inc.). Preparation of oocytes and RNA injections were performed as described previously (23). Oocytes were typically injected with 50 nl of a 1 mg/ml solution of RNA synthesized *in vitro* from the desired plasmids. Voltage clamp experiments were performed on oocytes to monitor any transmembrane ionic currents in response to α-factor stimulation, using a two-microelectrode voltage clamp as described previously (23)

Preparation of Purified ^{35}S-Labeled α-Factor—Isolation of ^{35}S-labeled α-factor from the culture fluid of yeast cells labeled with carrier-free [^{35}S]H_2SO_4 has been described in detail previously (9).

α-Factor Binding Assay—Binding assays employing oocytes were conducted at room temperature (20 °C) in 24-well dishes (Falcon 3047) containing Barth's medium (pH 7.4) supplemented with antibiotics (either 0.5 mg/ml gentamycin sulfate, or 100 units/ml potassium penicillin G and 0.1 mg/ml streptomycin sulfate) and with 1 mg/ml bovine serum albumin ("oocyte solution"). Oocytes were prewashed for 5 min and then incubated for 60 min with orbital shaking at 150 rpm in oocyte solution containing various concentrations of ^{35}S-labeled α-factor. Oocytes then were washed four times (1 min/wash) with oocyte solution lacking α-factor. Individual oocytes were transferred to scintillation vials and submerged in 3 ml of scintillation fluid (Safety-Solve, Research Products International Corporation). Radioactivity was quantified at room temperature using a scintillation spectrometer (Packard) with the energy windows set for ^{14}C (^{35}S) and ^{3}H. Because binding of ^{35}S-labeled α-factor to the oocyte surface resulted in little quenching of radioactivity (<5%), correction of the raw data was not performed. To measure total surface binding sites, ^{35}S-labeled α-factor was present in binding reactions at a concentration of 5–10 nM. In competition experiments, ^{35}S-labeled α-factor was present at 0.5 nM, and the labeled α-factor and various concentrations of unlabeled synthetic α-factor were added simultaneously.

Immunological Detection of Proteins Expressed in Oocytes—To detect production of the yeast G_α subunit in oocytes injected with *GPA1* mRNA, three batches of 5 oocytes each (uninjected, saline-injected, or *GPA1* mRNA-injected) were homogenized in 100 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (9), and heated at 95 °C for 5 min. The extracts were clarified by centrifugation (10,000 × *g*, 1 min), and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9). Proteins were transferred electrophoretically to nitrocellulose sheets and probed as described (37) using affinity-purified *GPA1*-specific antibodies.[2]

[1] K. J. Blumer, unpublished data.

RESULTS AND DISCUSSION

To test whether the ligand binding capacity of the yeast α-factor receptor is contributed solely by the *STE2* gene product, and to explore potential modes of action of this receptor, *STE2* mRNA prepared *in vitro* was microinjected into *Xenopus* oocytes. At various intervals after injection (1–7 days), oocytes were assayed for their ability to bind radiolabeled α-factor (see "Experimental Procedures") at concentrations near the K_D reported for the α-factor receptor on yeast cells (7–9). One to two days after injection, oocytes showed surface binding sites for α-factor at levels of 1.5–10 fmol/oocyte (data not shown). When expressed as the number of binding sites per unit area of membrane surface, up to 40 sites/μm^2/ng RNA were obtained; this level is approximately 5-fold greater than the density of α-factor receptors present on the surface of wild-type *MAT***a** cells (7–9). Expression of α-factor binding activity was not a transient phenomenon because >70% of the initial binding activity remained even 7 days after RNA injection (data not shown). The number of α-factor binding sites per oocyte did vary, however, among oocytes obtained from different animals (0.5–10 fmol/oocyte) and depended on the particular preparation of *STE2* mRNA injected; similar variations have been observed with oocyte expression of other membrane-bound proteins (23, 26). Nevertheless, when a single mRNA preparation was used, the expression level of α-factor binding sites was reasonably consistent (coefficient of variation <0.35) among oocytes obtained from a single frog. Because of these observations, for the individual experiments described below, oocytes were obtained from a single animal and were analyzed 2 days after injection.

Two approaches were used to determine if the α-factor binding activity expressed in oocytes exhibited the characteristics of the authentic yeast pheromone receptor. First, oocytes injected with no RNA, *STE2* antisense RNA, or *Torpedo* nicotinic acetylcholine receptor α, β, γ, and δ subunit mRNAs were assayed for pheromone binding activity. All three types of control oocytes displayed at least 10-fold lower binding activity than oocytes injected with *STE2* mRNA (Fig. 1). Thus, α-factor binding activity was elicited only upon injection of *STE2* mRNA and was not due to nonspecific binding resulting from the presence of another membrane-bound receptor or resulting from perturbation of the oocyte surface due to the injection process itself. Second, competition binding experiments employing increasing concentrations of unlabeled synthetic α-factor were used to estimate the equilibrium dissociation constant (K_D) of the α-factor binding sites expressed on the oocyte surface. When the data were corrected for the effects of isotope dilution and expressed according to the method of Klotz (27) (Fig. 2), a value of 7 nM was calculated for the K_D. This value is in good agreement with that determined for the authentic α-factor receptor expressed on the surface of intact yeast cells and in isolated yeast membrane fractions (8, 9).

The high level and stable expression of α-factor binding sites exhibiting a ligand affinity essentially identical to that of the authentic yeast pheromone receptor demonstrates that *STE2* protein is the only yeast gene product required for the synthesis, membrane targeting, and insertion of a functional

[2] K. J. Blumer and J. Thorner, submitted for publication to *Proc. Natl. Acad. Sci. U. S. A.*

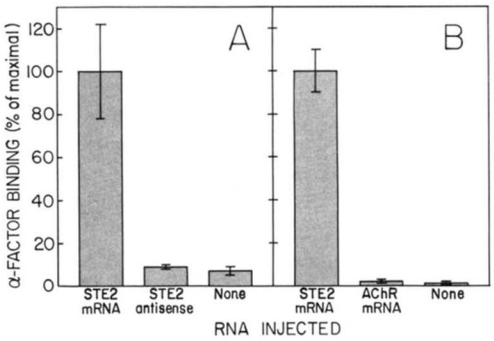

FIG. 1. **α-Factor binding to *Xenopus* oocytes microinjected with different RNA species.** Oocytes (n = 6–8) were microinjected with 50 nl of desired RNA solutions at 1 mg/ml, or left uninjected (n = 10–18) as controls. After incubation at 20 °C for 2 days, injected and control oocytes were subjected to analysis of surface binding for ^{35}S-labeled α-factor (see "Experimental Procedures"). The results were normalized to the mean of surface binding of *STE2* mRNA-injected oocytes and were expressed as percentages. The *error bar* indicates S.E. Each panel represents the data obtained using oocytes from a single frog. *A*, comparison of the binding activity of oocytes injected with *STE2* mRNA or antisense RNA. *B*, comparison of the binding activity of oocytes injected with *STE2* mRNA and oocytes injected with a mixture of mRNAs encoding the α, β, γ, and δ subunits of the nicotinic acetylcholine receptor (*AChR*) (23, 25).

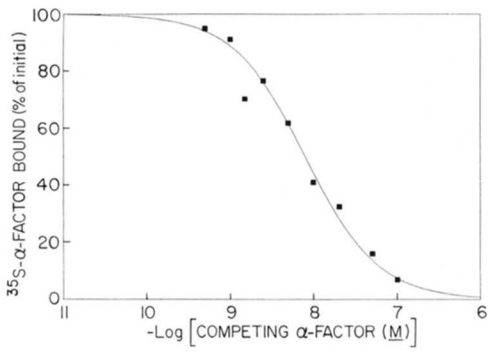

FIG. 2. **Determination of the dissociation constant for ^{35}S-labeled α-factor binding by competition with unlabeled synthetic α-factor.** Oocytes (n = 9–11 for each data point) were injected with 50 nl of 1 mg/ml *STE2* mRNA, incubated at 20 °C for 1–2 days, and their capacity to bind ^{35}S-labeled α-factor at a concentration of 0.5 nM in the presence of various concentrations of unlabeled α-factor was measured. Data are corrected for the effects of isotope dilution and then plotted according to the method of Klotz (27). The *smooth line* represents a computer-derived best fit of the data, assuming that displacement of labeled ligand is first order with respect to the concentration of unlabeled synthetic α-factor.

α-factor binding site. The simplest interpretation of these results is that the yeast α-factor receptor is composed solely of the *STE2* gene product, consistent with the view that G protein-coupled receptors, in general, consist of single polypeptide chains that are responsible for ligand recognition (28). It is also interesting to note that membrane localization of the yeast receptor occurred efficiently in a heterologous host cell.

Although pheromonal signaling in yeast is transduced via G protein-coupled receptors and involves a common pathway in both *MAT***a** and *MAT*α cells (29, 30), the identity of the intracellular second messenger(s) has been elusive. Oocytes have been used to detect receptor-mediated activation of various second messenger systems. Ligand binding to membrane receptors that have an intrinsic ion channel activity can be readily detected by changes in ionic conductance (23, 31). In addition, ligand binding to surface receptors coupled to a G protein-phospholipase C-phosphatidylinositol 4,5-bisphosphate pathway can elicit a robust chloride current in denuded (follicle cell-less) oocytes (32, 33). Similarly, potassium currents, although much smaller in magnitude, are detected in follicle cell-associated oocytes upon ligand binding to G protein-coupled receptors that stimulate adenylate cyclase. In an effort to identify potential second messenger systems through which the yeast pheromone receptor is capable of acting, we looked for any changes in the electrophysiological properties of both denuded and folliculated oocytes in which the *STE2* protein was expressed. In voltage clamp experiments, no reproducible changes in membrane conductance were observed in either the absence or presence of α-factor (at concentrations from 1 to 100 nM) over the voltage range between −100 and +80 mV (data not shown).

Although it appeared that agonist binding to the yeast receptor failed to stimulate either the phosphatidylinositol 4,5-bisphosphate- or adenylate cyclase-dependent signaling pathways, the absence of detectable electrophysiological responses could simply have been due to the inability of the yeast receptor to be recognized by amphibian G proteins resident in the oocyte. In an attempt to circumvent this potential problem, both the yeast α-factor receptor and its cognate G_α subunit (*GPA1* gene product) were expressed simultaneously by coinjection of both *STE2* and *GPA1* mRNAs. Such an approach assumes, of course, that the yeast G_α subunit, which shares about 45% sequence identity with mammalian $G_{s\alpha}$, $G_{i\alpha}$, and $G_{o\alpha}$, is capable of coupling to vertebrate G_β and G_γ subunits and capable of modulating either vertebrate phospholipase C or vertebrate adenylate cyclase. Oocytes injected with both the *STE2* and *GPA1* mRNAs expressed a level of α-factor binding activity equivalent to that seen in oocytes injected with *STE2* mRNA alone (data not shown). Moreover, these oocytes also synthesized the *GPA1* gene product as detected by immunoblotting of oocyte extracts with affinity-purified *GPA1*-specific antibodies (data not shown). The apparent mass of this species (~43 kDa) was slightly less than that observed for the product of *in vitro* translation of the synthetic *GPA1* mRNA (~49 kDa)[2] due to the high concentration of interfering proteins in this size range in the oocyte extracts. Thus, the yeast G_α subunit is expressed in oocytes injected with *GPA1* mRNA, but it is either incapable of coupling with vertebrate second messenger systems or it is acting upon a second messenger pathway that does not modulate the activity of any well characterized ion channel. Alternatively, if the yeast G_α subunit cannot form a complex with frog G_β and G_γ subunits, the resultant free yeast G_α subunit may be incapable of interacting with its cognate receptor (*STE2* protein).

Certain quantitative considerations argue that, if the yeast pheromone receptor itself functions as any sort of ionic channel, its average probability of opening must be exceedingly low compared with well characterized ion channels in animal (34, 35) and yeast cells (36). At a standard holding potential of −60 mV (which we employed in some of our electrophysiological experiments), the lower limit of current detection (10 nA) corresponds to a whole cell conductance of 1.7×10^5 picosiemens. Given that high levels of expression of the α-factor receptor were achieved (6×10^9 binding sites/oocyte), if the *STE2* protein functions as an ionic channel with a unit conductance as small as 1 picosiemens, its mean open probability must be less than 2.8×10^{-5} in order to account for our inability to detect any significant current changes. Thus, it seems extremely unlikely that the *STE2* protein possesses intrinsic ion channel activity or is an α-factor-gated channel for any common ion; therefore, the *STE2* protein itself does not directly mediate the pheromone-induced influx of Ca^{2+}

reported for *MAT***a** cells challenged with α-factor (5, 6).

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