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A role for a complex between activated G protein-coupled receptors in yeast cellular mating

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Cell-cell fusion is a fundamental process that facilitates a wide variety of biological events in organisms ranging from yeast to humans. However, relatively little is actually understood with respect to fusion mechanisms. In the model organism *Saccharomyces cerevisiae*, mating of opposite-type cells is triggered by pheromone activation of the G protein-coupled receptors, α -factor receptor (Ste2p) and a-factor receptor (Ste3p), leading to mitogen-activated protein kinase signaling, growth arrest, and cellular fusion events. Herein we now provide evidence of a role for these receptors in the later cell fusion stage of mating. *In vitro* assays demonstrated the ability of the receptors to promote mixing of proteoliposomes containing phosphatidylserine, potentially based on a pheromone-dependent interaction between Ste2p and Ste3p that was confirmed by tandem affinity purification and cellular pull-down assays. The cellular mating activity of Ste2p was subsequently probed *in vivo*. Notably, a receptor-null yeast strain expressing N-terminally truncated Ste2p yielded a phenotype demonstrating wild-type signaling but arrested mating. The arrested prezygotes showed evidence of some cell wall erosion but no membrane juxtaposition at the fusion site. Further, *in vitro* analyses correlated this mutation with loss of the interaction between Ste2p and Ste3p and inhibition of related lipid mixing. Overall, these results support a role for a complex between activated yeast pheromone receptors in later cell fusion stages of mating, possibly mediating events at the level of cell wall digestion and membrane juxtaposition before membrane fusion.

Ste2p | Ste3p | protein-protein interaction | membrane juxtaposition

Exoplasmic cell-cell fusion is a fundamental process with roles in a wide variety of biological events, including sperm-egg fertilization, fiber-cell fusion in lens formation, macrophage fusion in immune surveillance and bone resorption, trophoblast fusion in placenta development, myocyte fusion in muscle formation, stem cell fusion, and yeast mating (1–3). However, exoplasmic fusion mechanisms are poorly understood compared with vesicular fusion and virus-cell fusion events.

Saccharomyces cerevisiae presents a system par excellence to study eukaryotic cell fusion with the possibility of combining genetic, biochemical, and cell biological approaches. Identified steps in yeast cell mating include induction of pheromone response, prezygotic signaling, osmotic sensing, polarization of growth, cell wall dissolution, plasma membrane juxtaposition, remodeling, and fusion (4). Specifically, it is known that Ste2p (α -factor receptor) and Ste3p (a-factor receptor) are G protein-coupled receptors (GPCRs) presented in the cell membranes of opposite yeast-mating haploid types (MATa and MAT α), respectively (5–10). Activation of these receptors by pheromones leads to well characterized G protein-mediated mitogen-activated protein kinase signal transduction events, G₁ cell cycle arrest, and polarized cell growth before cellular fusion events. To date, only a small number of cell fusion facilitators, including Fus1p (11), Fus2p (12, 13), and Prm1p (14, 15), have been characterized with roles proposed in cell wall dissolution, osmotic stability, and pore formation.

Interestingly, immunolocalization work has demonstrated localization of Ste2p specifically to the site of cell fusion (16), and models have demonstrated that pheromone concentration gradients are involved in opposite mating-partner recognition (17, 18). High pheromone concentrations have also been shown to be required for initiation of cell fusion in prezygotes, further supporting a signaling-independent role for the pheromones at the fusion step (19, 20). As well, receptor-null yeast mutants that retained signaling by overexpression of Ste12p not only demonstrated poor partner discrimination but also were defective in prezygote formation (21). Similarly, an *S. cerevisiae* receptor-null mutant overexpressing a heterotrimeric G protein β -subunit yielded a phenotype that maintained signal transduction and G₁ arrest but displayed only very low ($\approx 5\%$ of wild type) mating efficiency (22). Together, these findings emphasize the possibility of roles for the GPCRs not only in signaling but also in downstream events such as chemotropic responses (21) and cellular mating.

In this work, we provide evidence of a role for an activated Ste2p/Ste3p complex in yeast mating. Specifically, lipid mixing of proteoliposomes containing phosphatidylserine demonstrated the activated receptors' abilities to promote membrane juxtaposition before membrane fusion events, based on a pheromone-dependent interaction between Ste2p and Ste3p, the formation of which was confirmed by tandem affinity purification (TAP) and cellular pull-down assays. *In vivo* signaling, mating, and phenotypic characterizations of a receptor-null yeast strain transformed to express mutant versions of Ste2p led to the identification of a mutation (truncation of N-terminal 20 amino acids) that specifically isolates the receptor's signaling activities from its cell fusing functionalities. In particular, mating arrest was observed by electron microscopy to occur in a prezygotic state showing some cell wall erosion at the fusion site but no membrane juxtaposition. Further TAP analysis and proteoliposome mixing assays correlated this observed arrest with loss of the interaction between Ste2p and Ste3p and loss of lipid mixing functionality. Consideration of these results supports a role for a complex between activated yeast GPCRs in later cell fusion stages of mating, possibly at the level of mediating cell wall degradation and juxtaposition of opposite membranes before membrane fusion.

Results

Reconstituted Ste2p and Ste3p Mediate Lipid Mixing in the Presence of Pheromone. To directly test the ability of the activated yeast GPCRs to promote lipid mixing, we applied a classical liposome

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Abbreviations: GPCR, G protein-coupled receptor; Ste2p, α -factor receptor; Ste3p, a-factor receptor; 293E, human embryonic kidney cells stably expressing the 293 Epstein-Barr virus nuclear antigen 1; TAP, tandem affinity purification; PS, phosphatidylserine.

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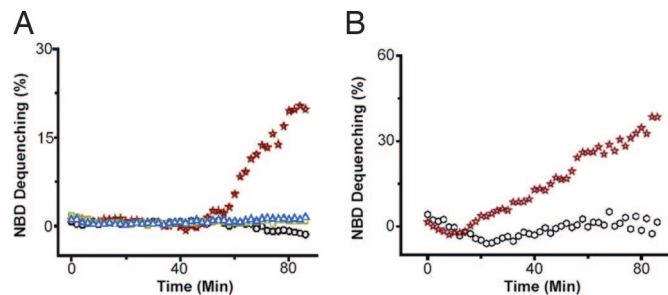


Fig. 1. Activated Ste2p and Ste3p mediate lipid mixing *in vitro*. (A) Fluorescent liposomes containing Ste2p-(His)₈ were mixed with nonfluorescent liposomes containing Ste3p-(His)₈ in the presence (red star) or absence (black hexagon) of a- and α-factor. 7-Nitro-2-1,3-benzoxadiazol-4-yl dequenching was detected at 2-min intervals. Fusion in the absence of Ste2p-(His)₈ and Ste3p-(His)₈ was carried out in the presence (blue triangle) or absence (green square) of a- (125 μM) and α-factor (500 μM) to exclude the possibility of lipid mixing mediated by pheromones alone. (B) Fluorescent liposomes containing Ste2p-(His)₈ were mixed with nonfluorescent liposomes containing Ste3p-(His)₈ in the presence (red star) or absence (black hexagon) of a- and α-factor with 10 mM Ca²⁺ included in the system.

fusion assay, used extensively to study soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (23, 24) and viral fusion. It must be understood that this experimental method does not necessarily provide evidence supporting minimal membrane fusion machinery (25, 26). Rather, it may serve as an indicator of the proteins ability to mediate membrane juxtaposition.

Recombinant Ste2p-(His)₈ purified from human embryonic kidney 293E cells (293E) as reported [supporting information (SI) Fig. 5A; ref. 27], was reconstituted into 82% phosphatidylcholine (PC)/15% phosphatidylserine (PS) lipid vesicles containing a quenched mixture of fluorescent phospholipids. Upon subsequent mixing and incubation of the fluorescent Ste2p-(His)₈ vesicles with nonfluorescent Ste3p-(His)₈-containing vesicles (PC:PS 85:15 molar ratio) in the presence of pheromones, a dequenching in fluorescence was observed starting at ≈50 min (Fig. 1A) or ≈10 min when 10 mM Ca²⁺ was included (Fig. 1B). No dequenching was observed when any one (or more) of the GPCRs or pheromones was omitted (Fig. 1 and SI Fig. 5B), indicating essential roles for each of the four components. Similar results were obtained in reciprocal experiments (SI Fig. 5C). Finally PC-only liposomes did not support any lipid mixing regardless of the presence of GPCRs, pheromones, or calcium (data not shown). Together, these results emphasize that pheromone activation of the reconstituted GPCRs cooperate with PS to drive the lipid mixing reaction in this *in vitro* system and, although not essential, Ca²⁺ mediates the initiation of observable dequenching in a PS-dependent manner. Overall, these results indicate a role for the pheromone-inducible GPCRs in membrane juxtaposition before membrane fusion.

In these lipid mixing assays and in subsequent experiments included in this work, pheromones are applied in the millimolar concentration range. Although this far exceeds what are considered to be physiologically relevant concentrations for signal transduction events, it is documented that much higher concentrations of pheromones are required for initiation of cell fusion in prezygotes (19, 20). Such high concentrations are expected to occur naturally in close proximities to cells secreting the pheromones and may play a role in stabilizing the GPCR–GPCR complex (17).

It should also be noted that compared with soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (23, 24), a slower initiation of events is indicated for activated GPCR-mediated lipid mixing. This may in part arise from the increased complexity of the pheromone inducible

four-component GPCR system. However, the observation that initiation occurs sooner in the presence of Ca²⁺ suggests that Ca²⁺-induced increases in membrane curvature (28) or decreases in electrostatic repulsion between phospholipid head groups by the formation of Ca²⁺-phospholipid complexes (29) may also play an important role.

Finally, it is also important to emphasize that in the SNARE system, protein reconstitution alone is sufficient to mediate lipid mixing, which thereby introduces uncertainty related to differences in liposome physical characteristics when comparing to empty liposome controls (25, 26). In contrast, lipid mixing is not induced by reconstitution of the GPCRs into vesicles alone but rather depends on pheromone activation of the already reconstituted receptors. Thus, within the context of this GPCR system, physical characteristics of the liposomes (and any effects introduced by the addition of 10 mM Ca²⁺) remain constant relative to controls, eliminating much of the uncertainty associated with this assay.

Ste2p and Ste3p Make a Specific Pheromone-Dependent, Extracellular-Domain Mediated Interaction *in Vitro*. The lipid reconstitution experiments suggest a mechanism that includes the formation of a ligand-dependent extracellular domain-mediated interaction between Ste2p and Ste3p. To further test this mechanism, we applied TAP, again using receptors recombinantly expressed and purified from 293E cells (27). SDS/PAGE and Western blot analyses of TAP elution fractions indicated coelution of Ste3p with Ste2p in the presence of both pheromones together (Fig. 2A and B, lane 3) but not in the absence of either one or both pheromones (Fig. 2A and B, lanes 5, 7, and 9). As a control for nonspecific binding, we confirmed that no interaction was detected by TAP between immobilized Ste2p and an alternate GPCR, bovine rhodopsin (data not shown). It is also important to emphasize the ligand-dependent nature of this interaction, which excludes the possibility of this interaction arising through nonspecific associations between hydrophobic domains of the receptors.

We further confirmed formation of the pheromone-dependent interaction complex between Ste2p and Ste3p by using a surface plasmon resonance based biosensor (Fig. 2C), which indicated relatively rapid complex formation in GPCR solutions preincubated with pheromones. This is in contrast to the slower initiation kinetics observed for in lipid mixing studies, highlighting that GPCR complex formation itself is not likely a primary rate-limiting step in the lipid mixing experiment. Other time-dependent factors, including diffusion and binding of pheromones to the receptors, diffusion of receptors within liposomes, diffusion of liposomes to enable GPCR–GPCR recognition, and subsequent lipid-dependent rearrangements (stalk formation, etc.), all contribute to and are represented in the lipid mixing kinetics.

Finally, to demonstrate that the interaction between Ste2p and Ste3p is mediated by extracellular regions of the receptor domains, a cellular pull-down assay was carried out by using Ste3p-(His)₈ presented on the surface of 293E cells and purified Fc-Ste2p-GFP in 0.02% *n*-dodecyl-β-D-maltopyranoside (DM) buffer (which does not lyse 293E cells or introduce purified GPCR into the cell membrane; data not shown). In the presence of a- and α-factor together, an anti-GFP reactive band at ≈125 kDa, representing Fc-Ste2p-GFP, was observed upon Western blot analysis of the pull-down fraction (Fig. 2D Left). A reciprocal experiment similarly yielded two anti-His sensitive bands, representing the monomer and dimer forms of Ste3p-(His)₈ (Fig. 2D Right). No bands were found in the absence of pheromones for both experiments, which again excludes the possibility of nonspecific binding of the purified receptors to the cell surfaces.

ever, when mated with MAT α (SCY060 genotype MAT α his3-11, 15 leu2-3, 112 trp1-1 ura3-1 kan1-100 ade2-1 are1::His-3), only very low levels of cell fusion were observed (Fig. 3B, lane 2) similar to those observed for the receptor null MAT α strain (YFL026W) overexpressing Ste4p (G β) (Fig. 3B, lane 4, and ref. 20). Quantitation of cell fusion for the Ste2p Δ N20-GFP mutant, following the protocol of Sprague *et al.* (34) indicated a reduction to 2.5% of the total cell population compared with 55% of the total cell population for the same system overexpression wild-type Ste2p-GFP (SI Fig. 8A). Subsequently, when DAPI staining was used to differentiate prezygote from zygote formation, comparison to the levels observed for overexpression of Ste2p-GFP, overexpression of Ste2p Δ N20-GFP was found to lead to a 2.9-fold increase in prezygote accumulation (wild type, 2.0%; Δ N20, 7.8%), with a corresponding 11-fold decrease in zygotes (wild type, 26.8%; Δ N20, 2.2%) (SI Fig. 8B). Overall, these results support a role for the receptor in mediation of cellular fusion events later in mating.

Additional phenotypic characterization of the cell fusion arrest mediated by the strain expressing Ste2p Δ N20-GFP was initially performed by colabeling with 5-(and-6)-carboxy-2',7'-dichloro fluorescein diacetate (FM4-64) to visualize cellular membranes and DAPI to visualize DNA (Fig. 3C), and in a separate experiment by labeling with fluorescent brightener 28 [FB28 (35)] to visualize cell walls and emphasize the opposite mating types of the fused prezygotic cells (SI Fig. 9). The observation of the formation of the distinctive neck structure, combined with the maintained isolation of the DNA and membrane regions, is clearly indicative of two cells joined in a sonication-resistant prezygotic state. However, the apparent continued presence of some cell wall (chitin) in this neck region raised questions related to the actual stage of arrest.

EM examination of the arrested prezygotes showed evidence of cell wall erosion at the fusion site but no juxtaposition of membranes (Fig. 3D). This supports a model for the GPCRs potentially mediating stages of plasmogamy before and including membrane juxtaposition. The lack of complete cell wall digestion in the arrested state suggests other additional role(s) for the activated GPCR complex in mediating more extensive cell wall digestion, most likely through transactivation signaling associated with a synchronous cell wall digestion/membrane juxtaposition model (4, 36).

Finally, it is important to note that these results do not confirm, disprove, or conflict with the previously proposed chemotropic response role for the receptors (19). Rather, herein additional late mating cell fusion related activities for an activated GPCR complex are proposed to function downstream of chemotropic responses.

The N-Terminal Region of Ste2p Mediates the Receptors Interaction with Ste3p and Lipid Mixing Activities. To establish that the observed Ste2p Δ N20-mediated prezygotic arrest, although likely linked to mediation of cell wall erosion, is also related to inhibition of the interaction between Ste2p and Ste3p and membrane juxtaposition promoting activity, an Fc-Ste2p Δ N20-GFP mutant receptor was recombinantly produced in 293E cells and purified as described for Fc-Ste2p-GFP (27). Ligand-binding assays indicated the recombinant receptor maintains saturable and specific pheromone binding characteristics (SI Fig. 10). Subsequent TAP analysis of the interaction of Fc-Ste2p Δ N20-GFP with Ste3p-(His) $_8$ in the presence of pheromone, indicated that deletion of the N-terminal 20 amino acids of Ste2p inhibits its interaction with Ste3p in the presence of pheromones (Fig. 4A). As well, we observe complete loss of the receptor ligand-mediated lipid mixing functionality when assayed by our proteoliposome fusion assay (Fig. 4B).

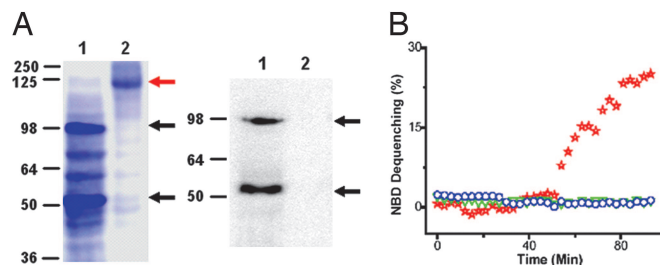


Fig. 4. The Ste2p Δ N20 mutant does not support Ste3p complex formation or lipid mixing. (A) Deletion of the N-terminal 20 amino acids of Ste2p eliminates interaction with activated Ste3p *in vitro*. (Left) SDS/PAGE analysis of TAP elution fractions. (Right) Western blot of Left probing for the presence of Ste3p-(His) $_8$ by using anti-HisG HRP as the primary antibody. Lanes 1, control samples of Ste3p-(His) $_8$ purified from Ni-NTA for TAP. Lanes 2, elution of Fc-Ste2p Δ N20-GFP after incubation with purified Ste3p-(His) $_8$ in the presence of α - and α -factor. The arrows indicate the expected positions of the Fc-Ste2p Δ N20-GFP (\approx 120 kDa) band and the monomer (\approx 52 kDa) and dimer (\approx 100 kDa) Ste3p-(His) $_8$ bands. (B) Deletion of the N-terminal 20 amino acids of Ste2p eliminates lipid mixing activity. Fluorescent liposomes containing Ste2p Δ N20-(His) $_8$ were mixed with nonfluorescent liposomes containing Ste3p-(His) $_8$ in the presence (blue hexagon) or absence (green inverted square) of α - and α -factor. 7-Nitro-2-1,3-benzoxadiazol-4-yl dequenching was detected at 3-min intervals. A positive-control experiment for the liposome fusion with Ste2p-(His) $_8$ and Ste3p-(His) $_8$ in the presence of pheromones (red star) was repeated for comparison.

Discussion

In summary, we herein present *in vitro* and *in vivo* data indicating a role for an activated Ste2p/Ste3p complex in yeast late-mating cellular fusion events. Specifically, evidence supporting functionalities at the level of cell wall dissolution and membrane juxtaposition before membrane fusion events are presented.

With respect to a possible role in membrane juxtaposition, electrostatic repulsion between bilayers and the presence of protein restricts distances between biological membranes to no less than 10–20 nm (37, 38). This restriction helps prevent promiscuous membrane fusion and maintains the individuality of intracellular compartments and the cell itself. However, in order for two opposed membranes to fuse, they must come into much closer proximity. The energetically costly process of bringing two membranes together is mediated by proteins, which either pull or push the opposite membranes toward each other and exclude water at the fusion site by formation of intermembrane protein–protein interactions (37, 38). Only a few proteins have been implicated in this membrane juxtaposition role to date. In vesicular fusion, SNAREs have been identified as likely candidates (39). In virus-cell fusion systems, HIV gp120/41 is activated at neutral pH by complex interactions with cellular receptors to mediate viral infection (40, 41) and viral Semliki Forest E1 protein is activated by low pH to infect cells (30). Finally in exoplasmic fusion, although no protein has been definitively identified, a sperm-specific protein called Izumo is hypothesized to interact with CD9 from egg membrane to initiate the sperm-egg membrane fusion processes (42) and cell-cell fusion in *Caenorhabditis elegans* has been shown to require the EFF-1 protein (43).

Although in the past a number of other *S. cerevisiae* proteins have been proposed to function in promoting and stabilizing cell fusion events, none of these has met the criteria of functionalities associated with a membrane juxtaposition role (2, 4). In particular, Fus2p has been shown to be involved in cell wall dissolution (12, 13). Matings between Δ *prml* mutants gave rise to a phenotype that arrested following membrane juxtaposition (14, 15), and Fus1p has been shown to regulate the opening and expansion of fusion pores also after membrane juxtaposition has already occurred (11). There has also been speculation that the

farnesylated a-factor might remain tethered to MAT α cells and in the process of binding to Ste3p in MAT α cells bring the two cell types together (44). However, this possibility has also been eliminated by the demonstration of complete release of the a-factor from the MAT α cells (45). Although we do not think the yeast GPCR system represents the membrane fusion machinery, it does meet many of the expected characteristics associated with membrane juxtaposition functionality including: opposite membrane localization, formation of protein–protein interactions and mediation of lipid mixing of PS-containing liposomes.

Deletion of the N-terminal 20 amino acids of Ste2p was found not only to inhibit the GPCR-GPCR interaction and related lipid mixing functionalities but also to isolate the receptor's early signaling from late cellular mating functionalities *in vivo*, thus confirming the role of this complex in cellular fusion events in particular at the level of membrane juxtaposition before membrane fusion. Additional phenotypic characterization of the associated arrested prezygote also indicated limited cell wall digestion along with loss of membrane juxtaposition. This observation does not negate the possibility of an *in vivo* membrane juxtaposition role for the Ste2p/Ste3p complex, which would occur after more extensive cell wall degradation, but rather adds another putative role for the receptor complex in mediation of cell wall dissolution. Although more detailed analysis of any additional roles occurring before membrane juxtaposition goes beyond the scope of this paper, it is clear that receptor functionalities in late-mating cellular fusion events are likely to be far more complex than originally anticipated. Thus, we conclude that an activated Ste2p/Ste3p complex plays a direct role in mediating later mating cell fusion events, possibly at the level of cell wall dissolution and membrane juxtaposition.

Materials and Methods

Chemicals. HRP-conjugated anti-HisG antibody and FM4–64 were obtained from Invitrogen (Burlington, ON, Canada). Rabbit polyclonal HRP-conjugated anti-GFP antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). *n*-Octyl- β -D-glucopyranoside (OG) and *N*-dodecyl- β -D-maltoside (DM) were from Anatrace (Maumee, OH). All lipids were from Avanti Polar Lipids (Alabaster, AL). α -Factor was from Zymo Research (Hornby, ON, Canada). All other reagents were from Sigma (St. Louis, MO).

Strains and Plasmids. SCY060 and SCY061 were a gift from S. Sturley (Columbia University, New York), and all other yeast strains were obtained from American Type Culture Collection (Bethesda, MD). All PCR amplifications were carried out by using Taq polymerase and cloned into pYES2.1/V5-His-Topo (Invitrogen, Carlsbad, CA) for expression in yeast MAT α YFL026W. All constructs were sequenced before transformation. STE2-GFP was amplified from pTT/Ste2p-GFP (27) with forward primer 1 (see [SI Table 1](#) for full primer sequences) and reverse primer 2. ste2 Δ N20-GFP and ste2 Δ N30-GFP were amplified with forward primers 3 or 4 and reverse primer 5. STE4 was amplified from genomic DNA with forward primer 6 and reverse primer 7. For expression in 293E cells, ste2 Δ N20 was amplified with forward primer 8 and reverse primer 9, digested by NheI, and ligated into pTT/Fc-GFP to form pTT/Fc-ste2 Δ N20-GFP plasmid. ste2 Δ N20-(HIS) $_8$ was amplified with forward primer 10 and reverse primer 11, digested by BamHI, and then ligated into pTT to form pTT/ste2 Δ N20-(HIS) $_8$ plasmid.

Proteoliposome Fusion Assay. Lipid mixing was assayed by using the method of Weber *et al.* (23, 24). Nonfluorescent liposomes were prepared by mixing 1-palmitoyl, 2-oleoyl phosphatidylcholine (POPC), and 1,2-dioleoyl phosphatidylserine (DOPS) at an 85:15 molar ratio and fluorescent liposomes by mixing POPC:DOPS: 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl): 1,2 dipalmitoyl-*sn*-glycero-3-

phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) in chloroform/methanol (2:1) at an 82:15:2:1 molar ratio, respectively. The lipids were dried under nitrogen gas and rehydrated to obtain a 20 mM lipid concentration in HBS buffer (10 mM Hepes, pH 7.4/150 mM NaCl) and then extruded through a 0.45- μ m syringe-driven filter. The purified Ste2p-(His) $_8$ or Ste2p Δ N20-(His) $_8$ and Ste3p-(His) $_8$ were washed three times by ultracentrifugation in 10 ml of HBS buffer containing 1% (wt/vol) OG. Detergent-saturated liposome suspensions [0.2 ml; 10 mM lipid and 1% (wt/vol) OG] were mixed with an equal volume of 0.5 mg/ml purified receptor as designated and shaken for 60 min. The concentration of OG was decreased in 0.1% increments to a final concentration of 0.4% by sequential dilution at 4°C with HBS (30-min intervals), followed by extensive dialysis against 4 liters HBS to remove residual detergent. The prepared liposomes were then extruded through a 0.45- μ m syringe-driven filter. Fluorescent proteoliposomes were mixed with nonfluorescent proteoliposomes (1:4 ratio) and incubated at 4°C for 5 min. a- (5 μ l, 2.5 mM) and α -factor (5 μ l, 10 mM) with or without 10 mM Ca $^{2+}$ or 10 μ l of HBS buffer were added to the liposome mixtures to a final 100- μ l volume. Increased fluorescence was monitored for 2 h by spectrofluorimetry at 37°C. Excitation and emission wavelengths of 450 and 535 nm were used, respectively.

TAP. Ste3p-(His) $_8$ was purified from a 400-ml culture of transfected 293E cells by Talon Ni-NTA columns, as described (27), and then concentrated by Millipore (Billerica, MA) ultracentrifugal filtration (molecular weight cutoff 10,000) with the removal of imidazole by washing with 10 ml of Buffer A [50 mM PBS, pH 8.0/100 mM NaCl/0.05% DM/5 mM MgCl $_2$ /20% glycerol (vol/vol)/1 mM 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride] followed by reconcentration to \approx 0.2 mg/ml. A total cell lysate from a 100-ml culture of transfected 293E expressing either Fc-Ste2p-GFP or Fc-Ste2p Δ N20-GFP was loaded on a protein A Sepharose column, which was subsequently washed with 10 volumes of Buffer B [100 mM Tris, pH 7.0, containing 100 mM NaCl, 0.05% DM, 5 mM MgCl $_2$, and 20% glycerol (vol/vol)]. The purified Ste3p-(His) $_8$ sample was then added to the Ste2p or Ste2p Δ N20 immobilized protein A Sepharose resin in the presence or absence of a- (5 μ M/ml) and α -factor (10 μ M/ml) and incubated at 4°C for 30 min. The resin was subsequently washed by an additional 10 volumes of Buffer B to remove any unbound Ste3p. The sample was eluted with Buffer C [100 mM NaCl/0.05% DM/5 mM MgCl $_2$ /20% glycerol (vol/vol)] and immediately neutralized by 20% (vol/vol) 1 M Tris-HCl, pH 7.0.

Surface Plasmon Resonance. All experiments were conducted on a Biacore-X instrument (Biacore, Uppsala, Sweden) at the Saskatchewan Structural Science Center/University of Saskatchewan. NTA sensor chips were prepared per the factory instructions. Purified Ste3p-(His) $_8$ (0.5 mg) was prepared as described for TAP and mixed with a-factor (67 μ g/ml). The resulting solution was injected into the Biacore instrument and immobilized in one cell on an NTA chip at a flow rate of 10 μ l/min over 3 min using the second cell as a control. Both cells were washed with Buffer C to establish a stable baseline. Purified Fc-Ste2p-GFP was prepared as described (27) and washed 3 times with Buffer C by Millipore [ultracentrifugal filtration (molecular weight cutoff 10,000)], mixed with 38 μ M a- and 333 μ M α -factor, and applied at two different sample concentrations (800 and 200 nM) to the Ste3p-(His) $_8$ chip and control cell at a flow rate 10 μ l/min.

Cellular Pull-Down Assay. Purified 0.1 mg/ml Fc-Ste2p-GFP or Ste2p-(His) $_8$ in 50 mM Tris-HCl, pH 7.0, containing 0.02% DM was mixed with 1 ml of fresh 293E cells presenting Ste3p-(His) $_8$

or Fc-Ste2p-GFP respectively on their cell surfaces in the presence and absence of both α - (5 μ M/ml) and α -factor (10 μ M/ml) and 0.02% DM for 10 min on ice. The cells were then centrifuged at $100 \times g$ for 1 min and carefully washed twice with 50 mM Tris-HCl, pH 7.0, containing 0.02% DM to remove any unbound solubilized receptor. The cells were then lysed and any pulled-down receptor enriched by affinity chromatography and visualized by Western blot analysis.

Halo Assays. The pheromone responses of transformed yeast strains (MATa YFL026W) were analyzed by halo assay. The strains were cultured overnight in SC minimal medium (-Ura) with 2% galactose and 1% raffinose. A 5- μ l sample of each resulting culture was mixed with 0.5% agar at 55°C and plated on the surface of a yeast peptone dextrose plate with galactose. After solidification of the agar, the pheromone was added (2, 1, 0.5, or 0.25 μ l of 10 mM). The plates were scanned after 2–3 days incubation at 30°C.

S. cerevisiae Mating Experiment. All transformed strains were cultured overnight in 2% glucose and then, where relevant, in 2% galactose SC minimal medium (-Ura) for 3 h. Cells were harvested by centrifugation at 2,500 rpm (Eppendorf centrifuge 5810R) for 5 min and washed with sterile TE buffer (100 mM Tris buffer, pH 7.5, with 10 mM EDTA), and resuspended in SC minimal medium (-Ura and -His or -Ura according to the gene markers). For Ste2p mutant mating experiments, 5×10^6 cells of the pYES2.1 transformed MATa YFL026W strains were mixed with 5×10^6 of the MAT α SCY060 strain (100 μ l total) in -His and -Ura 2% galactose minimal medium. Cells (5×10^4) as well as 3-fold and 9-fold dilutions were spotted on -His and -Ura minimal medium plates and incubated for 2–4 days at 30°C.

Microscopy. Mating products stained by DAPI and FM28 for confocal microscopy (see *SI Text* for detailed sample preparation) were imaged with a Zeiss (Oberkochen, Germany) META 510 laser-scanning confocal microscope using a Plan Apochromat 63 \times , N.A. 1.2 water-immersion objective equipped with differential interference contrast optics. FM4–64 images were collected by using excitation from the 488-nm line of the argon laser (10% of 25 mW), beam splitters HFT 405/488/543 and NFT545, and emission filter LP650. The electron microscopy specimens (see *SI Text* for sample preparation details) were sectioned (70-nm thickness) and mounted on 200 mesh formula-coated grids. The grids were stained for 30 min with aqueous 2% uranyl acetate with Triton X-100, rinsed with water, and then stained with Rorald's lead citrate for 10 min and again rinsed with water. Grids were viewed with a Philips 410LS (Eindhoven, The Netherlands) transmission electron microscopy at 80 kV with 4,500 and 24,000 \times amplification.

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