Gradient-directed cell migration (chemotaxis) and growth (chemotropism) are processes that are essential to the development and life cycles of all species. Cells use surface receptors to sense the shallow chemical gradients that elicit chemotaxis and chemotropism. Slight asymmetries in receptor activation are amplified by downstream signaling systems, which ultimately induce dynamic reorganization of the cytoskeleton. During the mating response of budding yeast, a model chemotropic system, the pheromone receptors on the plasma membrane polarize to the side of the cell closest to the stimulus. Although receptor polarization occurs before and independently of actin cable–dependent delivery of vesicles to the plasma membrane (directed secretion), it requires receptor internalization. Phosphorylation of pheromone receptors by yeast casein kinase 1 or 2 (Yck1/2) stimulates their internalization. We showed that the pheromone-responsive Gβγ dimer promotes the polarization of the pheromone receptor by interacting with Yck1/2 and locally inhibiting receptor phosphorylation. We also found that receptor phosphorylation is essential for chemotropism, independently of its role in inducing receptor internalization. A mathematical model supports the idea that the interaction between Gβγ and Yck1/2 results in differential phosphorylation and internalization of the pheromone receptor and accounts for its polarization before the initiation of directed secretion.

INTRODUCTION

Polarized cellular growth in response to a chemical gradient (chemotropism) is a fundamental process that is required for a broad range of phenomena, including angiogenesis, axon growth cone guidance, pollen tube guidance, fungal life cycles, and fungal pathogenicity. Similar to chemotaxing cells, chemotroping cells must be able to determine the direction of dynamic chemical gradients, establish a stable axis of polarity, and realign that axis as they track changes in the gradient.

The ability of the budding yeast, *Saccharomyces cerevisiae*, to mate efficiently depends on what is to date the best-understood chemotropic response. During the sexual reproduction phase of their life cycle, haploid yeast cells of the opposite mating types MATα and MATa signal their position to one another by secreting peptide pheromones. The binding of pheromone to heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors (GPCRs) on the surface of each cell type—Ste2 in MATa cells and Ste3 in MATα cells—and the consequent activation of their cognate G proteins induce cell cycle arrest, changes in gene expression, and the formation of mating projections, commonly called shmooes. Cells grow up the pheromone concentration gradient toward a potential mating partner, leading to the eventual fusion of the partners at their growth tips (1). The polarization of cell growth (morphogenesis) is accomplished by the directed movement of secretory vesicles along actin cables oriented toward the growth site (2, 3). The Gβγ subunit of the heterotrimeric G protein serves as a positional determinant for the chemotropic growth site by linking the pheromone receptor to the machinery that nucleates actin cables (4, 5).

As the most upstream elements in the mating response pathway, the pheromone receptor and its G protein are the primary gradient sensors. Unlike most chemotaxing cells, whose surface receptors are uniformly distributed on the plasma membrane, pheromone-stimulated yeast cells polarize their GPCR. In response to isotropic pheromone treatment, the receptor concentrates around the predetermined bud site, which is also known as the default polarity site. In mating mixtures, the receptor polarizes toward proximal mating partners, presumably where the pheromone concentration is greatest, and this phenomenon is detectable before morphogenesis occurs (6). The phenomenology and regulation of pheromone-induced receptor polarization in *MATa* cells is well documented. During vegetative growth, the *MATa*-specific receptor Ste2 appears uniformly distributed on the plasma membrane. When activated by ligand, however, the receptor is sequentially phosphorylated and ubiquitylated on its C-terminal cytoplasmic domain (7, 8). This stimulates its global internalization, after which it reappears as a polarized crescent on the cell surface (9, 10). Although receptor polarity could arise from the directed delivery of nascent receptors to the incipient shmoo site, several observations argue for a distinct mechanism. First, Ste2 becomes polarized before morphogenesis occurs. Second, establishment of receptor polarity does not depend on the directed delivery of vesicles along actin cables (6). Third, the genesis of receptor polarity absolutely depends on receptor internalization (6).

**Gβγ promotes pheromone receptor polarization and yeast chemotropism by inhibiting receptor phosphorylation**

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How does the receptor polarize toward the gradient source before and independently of actin cable-directed delivery of secretory vesicles? One possibility is that the pheromone-induced global internalization of the receptor is slower on the up-gradient side of the cell such that relative receptor density increases at the future shmoo site. This is consistent with the observation that the redistribution of the receptor depends on its internalization, but at the same time, it introduces a paradox: Because only activated receptors are phosphorylated, ubiquitylated, and internalized, the rate at which the receptor is removed from the cell surface should be greatest where the pheromone concentration is highest. In principle, therefore, the establishment of the chemotrophic growth site through differential internalization of the receptor requires a mechanism to locally inhibit receptor internalization.

In a genetic screen for proteins that interact directly with Gβ (11), we identified yeast casein kinase 1 (Yck1), one of a pair of sister casein kinases (CKs) (12) that are essential for the phosphorylation of the pheromone receptor on its C-terminal cytoplasmic domain (8) and hence for its internalization (7, 8). Here, we showed that Gβ interacted with Yck1 at the plasma membrane and that Gβ locally inhibited phosphorylation of the receptor, consistent with the idea that differential phosphorylation and internalization of the receptor play a role in the establishment of polarity. In addition, imaging and genetic data suggest that differential receptor phosphorylation contributes to chemotropic function. A mathematical model that incorporates the Gβ-dependent inhibition of receptor phosphorylation mimics key aspects of gradient-induced receptor polarization.

**RESULTS**

Gβ interacts with Yck1 at the plasma membrane and is a candidate Yck substrate

To identify candidate interactors of Gβ, we previously conducted an allelenic-specific dosage suppressor screen in yeast in which we took advantage of the observation that the overexpression of Gβ induces the mating signal and thereby confers cell cycle arrest (13, 14). A high-copy yeast complementatory DNA (cDNA) library was screened for plasmids that could rescue the overexpression of wild-type Gβ, but not that of an adaptive-defective mutant form of Gβ, encoded by STE4^A405V. Ten genes were identified, including those encoding Rho1 (11), Dse1 (15), and Yck1. In addition to genetic evidence of Gβ-Yck1 interaction, here we found that a myc-tagged form of Yck1 expressed in yeast specifically bound to a Gβ affinity column, as evidenced by both Western blotting and mass spectrometric analyses (Fig. 1A and B, and Table 1). Moreover, a direct physical interaction between Gβ and Yck1 in yeast was shown by bimolecular fluorescence complementation (BiFC) assay (Fig. 1C and Table 2). The Gβ-Yck1 BiFC signal was detectable on the plasma membrane of pheromone-treated cells before morphogenesis occurred and was concentrated on the plasma membrane of mating projections in shmooing cells.

Because Gβ is phosphorylated on multiple sites in spermotrope-treated cells (16) and because its sequence contains potential CK1 sites (17–19), we asked whether Gβ phosphorylation depended on Yck activity. Pheromone did not induce full phosphorylation of Gβ in cells lacking functional Yck1 and Yck2 (hereafter, Yck1/2) (Fig. 1D). Previous genetic analyses suggested that Gβ residues Thr^230 (T230), Thr^232 (T232), and Ser^335 (S335) are phosphorylated and that full Gβ phosphorylation is dependent on the pheromone-responsive mitogen-activated protein kinase (MAPK) Fus3 (20). Mass spectrometric analysis of Gβ phosphorylated peptides purified from pheromone-treated cells confirmed these phosphorylation sites and revealed an additional site at Thr^118 (Fig. 1E and fig. S1). Study of mammalian CK1s suggests that they require a phosphorylated substrate and
strain YDB111 to isotropic pheromone treatment (membrane enriched in hypophosphorylated receptor arises in response to receptor phosphorylation. Consistent with this, a region of the plasma membrane was identified by mass spectrometry as specifically binding to the G protein Gα and Ste27XR, a mutant form of the regulator of G protein signaling (RGS) protein Sst2, by both Western blotting and mass spectrometric analyses (Fig. 1, A and B). Sst2-GFP polarized localization for Gα overexpression inhibits phosphorylation of the Yck1/2-dependent phosphorylation, which inhibits polymerization of actin) and pheromone cannot redistribute the receptor; nevertheless, they exhibit asymmetric receptor phosphorylation (6). To determine whether Gβ could inhibit receptor phosphorylation, we examined pheromone- and LatA-treated YDB111 cells overexpressing either Gβ or Gβγ, the weaker Yck1/2-binding form of Gβγ (Fig. 2, A to F). Overexpression of Gβγ correlated with an increase in both the amount of Sst2-GFP that was localized to the plasma membrane and the extent of Sst2-GFP polarity. In contrast, overexpression of Gβγ did not affect the plasma membrane localization of Sst2-GFP. To further test the effect of weakening the Gβ-Yck1/2 interaction on receptor phosphorylation, we replaced STE4 (Gβγ) with ste4T320A/S335A (Gβγ) in the YDB111 strain. Treatment with pheromone and LatA stimulated statistically significantly less plasma membrane localization of Sst2-GFP in cells expressing the native amount of Gβγ, compared to that in control cells (Fig. 2, F and G). Together, these data support the idea that Gβγ protects the pheromone receptor from Yck1/2-dependent phosphorylation, and that it does so most effectively in a discrete region of the plasma membrane.

Local inhibition of receptor phosphorylation by Gβγ helps to establish and maintain the chemotropic growth site

In time-lapse images of mating mixtures, we found that the polarized receptor crescent frequently realocated from the default polarity site to the chemotropic site in cells that had not yet shmooed (Fig. S2). Could anisotropic receptor phosphorylation play a role in this process? In mating mixtures of YDB111 cells, the Sst2-GFP crescent was almost always visible before morphogenesis occurred and was either formed initially at the eventual shmoo site or relocated from the presumptive default site to an apparent chemotropic site before morphogenesis (Fig. 3). Thus, the position of minimal receptor phosphorylation anticipated the eventual site of morphogenetic events.

Table 1. Mass spectrometric quantification of Yck1 and Yck2 pull-downs by Gβγ affinity beads. Total protein (225 μg, low input) from cells expressing endogenous Yck2 and overexpressing myc-Yck1 were incubated with the indicated Gβγ affinity beads or negative control beads (empty). Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), processed, and analyzed by mass spectrometry as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Detected protein</th>
<th>Affinity bead type</th>
<th>Intensity, 70 to 55 kD</th>
<th>Intensity, 55 to 45 kD</th>
<th>Total intensity</th>
<th>Gβ/Gβγ ratio</th>
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<tr>
<td>Yck1</td>
<td>Gβγ</td>
<td>3.16 × 10⁷</td>
<td>2.25 × 10⁷</td>
<td>5.42 × 10⁷</td>
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<tr>
<td></td>
<td>Gβγγ</td>
<td>6.88 × 10⁷</td>
<td>1.72 × 10⁷</td>
<td>2.41 × 10⁷</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Empty</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Yck2</td>
<td>Gβγ</td>
<td>2.39 × 10⁷</td>
<td>0</td>
<td>2.39 × 10⁷</td>
<td>2.09</td>
</tr>
<tr>
<td></td>
<td>Gβγγ</td>
<td>1.15 × 10⁷</td>
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<td>1.15 × 10⁷</td>
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<td></td>
<td>Empty</td>
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Table 2. Quantification of BiFC results. No background signal was seen in the VF1-YCK1 + VF2 negative control cells. Detectable fluorescence was observed on the tips of a substantial percentage of the shmooing Gβ-VF2 + VF1-negative control cells. This was attributable to the direct, irreversible interaction between the VF1 and VF2 fragments, where they were concentrated together by directed secretion, and was clearly distinguishable from the signal from the interaction between Gβ-VF2 and VF1-Yck1 by the measures shown here. PM, plasma membrane; n.d., not determined.

<table>
<thead>
<tr>
<th>% Cells with PM signal*</th>
<th>PM/Cyto ± SEM†</th>
<th>% PM ± SEM ≥ 1.25× Cyto‡</th>
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<tr>
<td></td>
<td>Pre-shmoo</td>
<td>Shmoood</td>
</tr>
<tr>
<td>Gβγ-VF2 + VF1-Yck1</td>
<td>28.5</td>
<td>15.2</td>
</tr>
<tr>
<td>Gβγ-VF2 + VF1</td>
<td>3.4</td>
<td>27.4</td>
</tr>
</tbody>
</table>

*Cells with a clear plasma membrane signal were scored; n ≥ 45 cells. †Plasma membrane signal was normalized to the cytoplasmic signal in each cell by dividing the mean plasma membrane fluorescence by the mean cytoplasmic fluorescence. ‡Percentage of the plasma membrane that showed a signal at least 25% greater than the mean cytoplasmic signal in each cell was determined. §As compared to the corresponding measure for Gβ-VF2 + VF1 cells; P < 0.0001 (n ≥ 19 cells).
Fig. 2. Effects of Gβ and GβP− on receptor phosphorylation. (A to C) Overexpression of Gβ, but not GβP−, inhibits phosphorylation of the pheromone receptor. (A) YDB111 cells expressing native Gβ protein, excess Gβ, or excess GβP− (excess indicated by double arrows) were treated with pheromone and LatA at time zero. The cells were then imaged by epifluorescence microscopy to visualize the indicated proteins. The intensity of the Sst2-GFP signal on the plasma membrane corresponds to the location and quantity of the unphosphorylated pheromone receptor, whereas that of the Ste27XR-mCherry signal corresponds to the location and quantity of total pheromone receptor. The arrowhead indicates a polarized Sst2-GFP crescent. Relative signal intensities are indicated by the color bar. Images are representative of three or two (for the control strain) experiments. (B) Western blotting analysis of the relative amounts of Gβ and GβP− in lysates of 15Dau cells expressing the indicated constructs after 0 and 2 hours of exposure to galactose to induce GAL1 expression. The topmost band corresponds to Gβ; asterisks indicate Gβ degradation products. Western blots are representative of four experiments. (C) Normalized quantification of the amount of Sst2-GFP localized to the plasma membrane. The relative amount of Sst2-GFP localized to the plasma membrane at the indicated times after treatment with pheromone and LatA at time zero was normalized to total receptor abundance by dividing the mean plasma membrane Sst2-GFP signal by the mean plasma membrane Ste27XR-mCherry signal. Data are mean ratios ± SEM of three or two (for the GβP-overexpressing strain) experiments. *P ≤ 0.003 compared to control. n ≥ 44 cells for each time point for control and Gβ-overexpressing strains; n = 15 cells for each time point for the GβP−-overexpressing strain. (D and E) Overexpression of Gβ enhances polarity of unphosphorylated receptor in pheromone- and LatA-treated cells. (D) YDB111 cells expressing the native Gβ, excess Gβ, or excess GβP− (excess indicated by double arrows) were treated with pheromone and LatA at time zero. The cells were then imaged by epifluorescence microscopy to visualize Sst2-GFP. Arrowheads indicate polarized Sst2-GFP crescents. (E) The degree of polarization of Sst2-GFP to the plasma membrane of each cell is indicated by the polarity index: the mean fluorescence in the brightest third of the plasma membrane divided by the mean signal in the rest of the plasma membrane. Data are means ± SEM of at least two experiments per strain. *P < 0.02, **P = 0.002, compared to cells expressing excess Gβ. n ≥ 20 cells for each strain and time point. (F and G) The pheromone receptor is hyperphosphorylated in GβP− cells. (F) YDB111 cells expressing endogenous Gβ or GβP− were treated with pheromone and LatA at time zero. The cells were then imaged by epifluorescence microscopy to visualize the indicated proteins. (G) Quantification of the extent of Sst2-GFP localization to the plasma membrane. Data are means ± SEM of 15 cells for each strain and time point. *P = 0.005 for GβP− compared to Gβ cells at 90 min. Plasma membrane Ste27XR-mCherry amounts did not differ significantly in the control and experimental cells.
of polarized growth. Although the Sst2-GFP crescent in YDB111 cells expressing Gβ also anticipated the shmoo site, the Gβ cells shmooed about one time point earlier (15 min) than did the Gβ cells (n ≥ 30 cells; P = 0.05) and did so markedly closer to the presumptive default site (Fig. 3). These data suggest two conclusions. First, cells that are unable to internalize receptors, and thereby polarize receptor distribution, are nevertheless able to establish a chemotropic growth site by local inhibition of receptor phosphorylation. Second, the role Gβ plays in positioning and stabilizing the chemotropic growth site correlates with its effect on receptor phosphorylation.

To further examine the possibility that receptor phosphorylation plays a role in chemotropism apart from triggering receptor internalization, we replaced native STE2 with STE2^{7XR}, STE2^{27XR 6SA}, or STE2^{27XR 6SD} in an otherwise wild-type MATa strain. These alleles all encode forms of the receptor that cannot be internalized (7XR) (23) and that either are barely phosphorylated (6SA) or have phosphomimetic substitutions (6SD) (24).

We then assessed the effects of the mutant receptors on chemotropism in time-lapse images of mating mixtures (Fig. 4). Cells that were fully competent to mate typically shmooed directly toward their mating partner, grew chemotropically about the same distance to their point of contact, and ultimately fused at an angle near 0° (Fig. 4, B and D). The resulting zygotes were straight and symmetrical. In contrast, cells defective in gradient sensing or chemotropism, or wild-type cells confused by exogenous pheromone (fig. S3), did not orient directly toward their partners and ultimately formed angled zygotes (21).

When chemotropism was assayed by observing mating pairs directly rather than by quantifying the efficiency of diploid formation using genetic markers, as performed by McClure et al. (25), it was apparent that STE2^{7XR} conferred a defect in initial orientation (Fig. 4B). The mean orientation angle of the mutant cells was considerably smaller than that of the control cells, and the mean fusion angle and the mean time to fusion were markedly greater in the wild-type MATa × MATa STE2^{7XR} mating mixtures than in the wild-type × wild-type crosses (Fig. 4, C to E). Note that STE2^{27XR} conferred an increase in the mean fusion angle that was equivalent to the addition of exogenous pheromone to wild-type mating mixtures (fig. S3). We infer that the inability to internalize and thereby redistribute the mutant receptor compromised directional sensing. When cells could neither polarize the distribution of the pheromone receptor nor locally affect its phosphorylation state, they were unable to grow chemotropically. In mating mixtures, the MATa STE2^{7XR 6SA} cells that ultimately formed zygotes with wild-type MATα cells always polarized their growth adjacent to their last bud site (Fig. 4B). Similarly, all MATa STE2^{27XR 6SA} cells that ultimately formed zygotes polarized their growth—or mated without undergoing morphogenesis—proximal to their last bud site. Thus, MATa STE2^{7XR 6SA} and MATa STE2^{27XR 6SD} cells could not switch from the presumptive default polarity site to a chemotropic growth site regardless of the position or proximity of potential mating partners (Fig. 4C).

In addition to eliminating gradient sensing in situ, the STE2^{7XR 6SA} and STE2^{27XR 6SD} alleles conferred pronounced defects in mating projection formation (Fig. 4F). Although most of the mating MATa STE2^{7XR 6SA} cells and all of the mating MATa STE2^{27XR 6SD} cells exhibited robust polarized growth, a substantial fraction of the mutants apparently could not stabilize their axis of polarity well enough to generate a tapered shmoo tip. Such cells elongated along very broad growth zones, the shape of which was easily distinguished from that of a normal mating projection. Notably, the broadly polarized MATa STE2^{7XR 6SA} and MATa STE2^{27XR 6SD} cells persistently turned in one direction as they elongated (Fig. 4B and fig. S4), a behavior also seen in sst2Δ cells (26). Because some of these cells eventually mated, albeit over abnormally long times (Fig. 4E), their curving growth might be mistaken for chemotropism. Is the persistent turning toward the eventual fusion site a marked example of reorientation? Our data indicate that it is not. The broadly polarized and curving MATa STE2^{7XR 6SA} and MATa STE2^{27XR 6SD} cells showed no sign of gradient sensing. Their initial direction of growth was determined by default polarity (Fig. 4C), and they continued to elongate and turn until they encountered a mating-competent MATα cell directly in their path, often after having failed to mate with nearer potential partners (Fig. 4B). Only 26% of the MATa STE2^{7XR 6SA} and MATa STE2^{27XR 6SD} cells that elongated and turned, and 42% of those that were initially positioned within 1 μm of one or more potential partners, formed zygotes within the 6-hour time course (fig. S4). On the basis of our analysis of cells expressing mutant receptors in physiological gradients, we conclude that receptor internalization is required for full chemotrophic function and that receptor phosphorylation plays a critical role in chemotropism that is distinct from its established function as a stimulus for internalization.

The pheromone receptor becomes polarized before Cdc42 is activated

In pheromone-treated cells that were first allowed to internalize the receptor for 15 min and then treated with LatA, a polarized receptor crescent subsequently formed on the plasma membrane within about 30 min, even though ongoing actin-dependent secretion and endocytosis were blocked (Fig. 6, A and B) (6, 27). A likely explanation for this is that, at the time of LatA treatment, a preexisting polarity site—in this case, one that was generated as the receptor was internalized—was amplified by uniform delivery of secretory vesicles together with biased docking and fusion at the plasma membrane, as proposed by Sahin et al. (28). The receptor itself could be the primary determinant of this site. Although the membrane localization of the Ste2-GFP reporter was undetectable after 15 min of induced internalization (6), a concentrated receptor patch was seen when such cells were labeled with Alexa Fluor 594-tagged pheromone (fig. S5). Alternatively, cortical polarity could be generated by actin-independent, Cdc42-dependent exocytosis, as described by Bendezú and Martin (29), or by endocytosis and recycling of Cdc42, as proposed by Yamamoto et al. (30). To distinguish between these possibilities, we investigated whether pheromone-induced receptor (Ste2-GFP) polarity was detectable before...
Fig. 4. Effects of receptor internalization and phosphorylation on chemotropism. (A) Illustration of how the initial orientation angles (left, δ) and the fusion angles (right, γ) were measured. Polarized growth initiated within 45° of the cytokinesis site (CKS, red bar) was presumed to be at the default polarity site, demarcated by dotted blue lines. (B) Time-lapse DIC and fluorescent images of the indicated mating mixtures taken at 15-min intervals after spreading on agarose pads. The GFP-Bud1 signal at the plasma membrane was used to distinguish the MATα cells and as a marker for cell fusion. The initial orientation, reorientation, and fusion angles are indicated by δ, δ′, and γ, respectively. (i) Representative wild-type (WT) × WT mating. The MATα cell shmooed opposite to its CKS. (ii) Example of a mating cell reorienting by initiating a second projection in a WT × WT cross. (iii) Representative WT MATα × MATα STE27XR mating showing defective initial orientation and the formation of an angled zygote after 90 min. (iv) Two representative WT MATα × MATα STE27XR 6SA matings showing a MATα cell fusing adjacent to the CKS of a mutant cell that failed to polarize its growth (top) and a mutant cell that broadly polarized its growth in the default region but failed to fuse with an adjacent partner until it had elongated over an abnormally long time and distance (bottom). (v) Two representative WT MATα × MATα STE27XR 6SD matings showing mutant cells that broadly polarized their growth at the default site (top) or presumptive default site (bottom) and elongated while persistently turning until their growth zones were opposed to a partner. (C) Quantification of initial orientation angles [purple lines in (A)]. *P < 0.06, **P < 0.0001. (D) Quantification of fusion angles [aqua lines in (A)]. *P < 0.0001. (E) Time to fusion. Diffusion of GFP-Bud1 from the MATα partner to the MATα partner was used as a marker for the completion of cell fusion. *P = 0.01, **P < 0.0001. (F) Effect of receptor mutants on morphogenesis. MATα cells that mated were placed in three classes on the basis of their ability to form mating projections: tapered growth (normal shmoo), broadly polarized growth, and no polarized growth, as indicated. The proportion of normal shmooes was compared by χ² test. *P < 0.0001. The indicated n values represent two experiments for the STE27XR 6SA and STE27XR 6SD strains and one experiment for the WT and STE27XR strains. NS, not significant.
Cdc42-guanosine 5′-triphosphate (GTP) [Gic2–protein binding domain (PBD)–red fluorescent protein (RFP)] polarity occurred or vice versa. Before they were treated with pheromone, about half of the cells in G1 exhibited Gic2-PBD-RFP polarity, but not Ste2-GFP polarity, whereas none showed receptor polarization alone, consistent with the essential role of Cdc42-GTP in bud emergence (Fig. 5, A and B). Conversely, about half of the cells in G1 that were not yet shifting after 60 min of pheromone treatment exhibited receptor crescents before Cdc42-GTP polarity was detectable, even though the reporter signals were of similar intensity. No pheromone-treated cells were observed that exhibited polarized Gic2-PBD-RFP before exhibiting polarized Ste2-GFP (Fig. 5, A and B).

Changes in the localization of the receptor also preceded the redistribution of active Cdc42 and morphogenesis in gradient-stimulated cells (Fig. 5, C and D). In time-lapse images of MATα cells that switched from their presumptive default polarity site to a presumptive chemotrophic growth site in mating mixtures, both Ste2-GFP and Gic2-PBD-RFP moved slowly from their initial sites to final sites of polarization, consistent with results reported by Hegemann et al. (31). The pheromone receptor was clearly detectable and centered around the eventual polarized growth site before Gic2-PBD-RFP was similarly redistributed in 13 of 19 scored cells (68%) and was the first to fully relocalize in 13 of 16 scored cells (81%). Redistribution of Gic2-PBD-RFP was never seen to precede that of Ste2-GFP. These data suggest that pheromone-induced polarity of the receptor determines the position of the chemotrophic shmoos site upstream of active Cdc42.

**Gββ’-cells are defective in receptor polarization**

Our biochemical data suggest that Gββ’ has a reduced affinity for Yck1/2 compared to that of Gβ. We also previously showed that the pheromone-induced polarization of GFP-Gββ’ before morphogenesis occurs is moderately defective (21). If either early Gβ polarization or the Gβ-Yck1 interaction plays an important role in establishing receptor polarity, then Gββ’ would be expected to adversely affect the formation of receptor crescents. To test this, we compared the localization of GFP-tagged receptor in Gβ and Gββ’-cells treated with LatA 15 min after pheromone stimulation. We found that Gββ’-cells exhibited reduced receptor polarization as compared to that of Gβ cells (Fig. 6, A and B), suggesting a role for Gβ phosphorylation in the establishment of the polarity site as the receptor undergoes global internalization. A similar result was observed under physiological conditions: Gββ’-cells exposed to pheromone gradients in mating mixtures also exhibited a defect in receptor polarization (Fig. 6, C and D).

**Pheromone-induced internalization can result in the polarization of activated receptors**

To follow the internalization of ligand-bound receptors as distinct from the trafficking of inactive receptors, we treated cells with fluorescent pheromone. Cells were labeled on ice in medium lacking essential nutrients and were imaged at room temperature 1 to 2 min after being refed. Although the cells were not exposed to a pheromone gradient, about 25% exhibited an asymmetry in the rate of receptor internalization, leading to enhanced receptor polarity (fig. S6). Thus, the surface distribution of the activated receptor changed as the receptors were internalized. We speculate that the cell cycle–dependent polarization of the G protein (6) mimics the early response of cells to stimulation by a gradient, revealing the possibility of polarization through differential internalization.

**The GβY-Yck interaction is critical in a reaction-diffusion model that mimics gradient-induced receptor polarization upstream of directed secretion**

The observations presented earlier are suggestive of a previously uncharacterized mechanism for the establishment of pheromone-induced receptor polarity. By interacting with Yck1/2, GβY inhibits phosphorylation of the pheromone receptor, which in turn slows receptor internalization. The interaction between GβY and Yck1/2 would also be expected to inhibit endocytosis of the G protein because we previously found that both Gα and GβY internalize with the receptor (6). Initially, a shallow gradient of pheromone is mirrored by a similarly shallow gradient of occupied receptor across the cell. This slight differential in activated receptor should lead to a corresponding differential of activated Gα and free GβY. Because the receptor and G protein are less likely to be internalized where the concentration of free G-βγ is highest, a positive feedback loop is generated, which, in combination with other mechanisms, rapidly amplifies the intracellular signaling gradient before actin cables become nucleated (Fig. 7A). Previously published data suggest that Gα recruits the activated MAPK Fus3 to phosphorylate cortical Gβ (32, 33) and demonstrate roles for the Gα-Fus3 interaction in directional sensing and chemotrophic shmoos (21, 33, 34). Given the data reported here, phosphorylation of Gβ by a MAPK would be expected to enhance its interaction with Yck1/2. This raises the possibility that two positive feedback loops act synergetically to promote receptor polarization: (i) Gα/Fus3-mediated phosphorylation of Gβ and (ii) inhibition of the Yck-dependent internalization of the receptor and G protein by phosphorylated Gβ.

To determine whether our postulated feedback mechanisms could explain how receptor polarity is generated in response to a pheromone gradient, we developed a reaction-diffusion model that, unlike other mathematical models of yeast chemotropism (35–37), is based on mechanisms that are independent of Cdc42 (Fig. 7, B to D, fig. S7, and tables S3 to S6). We first generated a core network that included only the best-characterized components. Network 1 consists of the receptor-pheromone interaction, the G protein cycle, and the Yck-dependent internalization of the receptor and G protein. Network 2 adds the GβY-Yck interaction, whereas network 3 adds the Gα/Fus3 interaction. A 10 to 5 nM pheromone gradient induced a substantial reduction in the number of receptors on the plasma membrane of a computational yeast cell lacking downstream regulation of receptor internalization (Fig. 7E), with no resulting polarity (network 1). In contrast, a computational cell that differed only in the addition of the GβY-Yck interaction (network 2; Fig. 7F) responded to the same gradient by robustly polarizing its receptor. Addition of the Gα/Fus3 feedback loop (network 3; Fig. 7G) resulted in faster receptor polarization. Note that the rate of receptor internalization was greatest on the side of the cell facing the gradient immediately after it was stimulated with pheromone, which was expected because of the higher receptor occupancy in this area (fig. S8). Within 12 min (network 3) or 15 min (network 2), however, iteration of the feedback loops reversed the differential so that receptor internalization was slowest at the front of the cell, where the receptor eventually became polarized. Output from the mathematical model also yielded testable predictions about the spatiotemporal dynamics of other key signaling elements in the response networks. Whereas the heterotrimeric G protein remained largely uniform in distribution on the surface of the gradient-stimulated computational cell, activated Gα and free GβY polarized together with the receptor (fig. S9). At the same time, inverse intracellular gradients of free Yck (back) and GβY-bound Yck (front) were generated (fig. S10).

**DISCUSSION**

Although both chemotactic and chemotropic cells use GPCRs to detect and respond to chemical gradients, they differ in how they deploy their surface receptors. With few exceptions, chemotactic cells exhibit a uniform surface distribution of receptors. This enables them to rapidly sense changes in ligand concentration in three dimensions. Although the surface
Fig. 5. Localization of receptor and Cdc42-GTP reporters in vegetative and pheromone-stimulated G1 cells. (A and B) Sequential polarization of the receptor and active Cdc42 in cultured cells. Strain AIY301 was grown to log phase, and G1 (round, unbudded) cells were scored for polarization of the Ste2-GFP (receptor) and Gic2-PBD-RFP (Cdc42-GTP) reporters before and after pheromone treatment. (A) Representative images 60 min after treatment with pheromone. The exposures and processing were the same for all images. (B) Relative proportions of cells showing Ste2-GFP and Gic2-PBD-RFP polarity before and at the indicated times after pheromone-induced morphogenesis. RNP: round cell, neither reporter polarized; RAC: round cell, activated Cdc42 polarized; RR: round cell, receptor reporter polarized; RB: round cell, both reporters polarized; SB: shmooing cell, both reporters polarized. n = 20, 35, and 56 cells at the 0-, 1-, and 2-hour time points, respectively, from two experiments. (C and D) Sequential polarization of the receptor and active Cdc42 in mating cells. (C) Time-lapse images of representative MATa cells that switched from their presumptive default polarity site to a presumptive chemotropic growth site in mating sites. Polarity of the Ste2-GFP and Gic2-PBD-RFP reporters is categorized in one of three ways: polarity established at the initial site (PE; dotted arrowheads), polarity switching to the future growth site (PS; solid arrowheads), and polarity fully switched and fixed at the final site (PF; filled arrowheads). (D) Percentages of cells that showed sequential relocalization of the Ste2-GFP and Gic2-PBD-RFP reporters in mating mixtures. PS corresponds to the solid arrowheads in (C) (n = 19 cells). PF corresponds to the filled arrowheads in (C) (n = 16 cells). Two experiments were performed.
distribution of receptors is uniform, the distribution of activated receptors reflects changes in the gradient within seconds. Local receptor activation is amplified by downstream signaling mechanisms, which leads to polarized cellular outputs (38, 39). By comparison, chemotropism is a slower and more two-dimensional process. As exemplified by budding yeast, chemotropic growth occurs on a time scale of tens of minutes rather than seconds and on solid surfaces rather than in fluid or tissues. These spatiotemporal differences may underlie the redistribution of receptor to the region of polarized growth in pheromone-stimulated cells, as compared to the invariant receptor display on the surface of chemotactic cells.

The formation of polarized receptor crescents in shmoosing yeast was first reported in 1991 (40), and its importance has been debated ever since. Is receptor polarization simply the result of global internalization followed by actin cable–directed delivery of secretory vesicles to the plasma membrane, or does the local concentration of receptor determine the position of the chemotropic growth site? A number of results indicate that the initial polarization of surface receptor is established independently of actin cable–directed secretion. Polarized receptor crescents are easily visualized before morphogenesis (6, 31). Pheromone-treated cells in which actin cable–directed secretion is disabled by mutation or drug nevertheless form robust receptor crescents (6, 31). Here, we report the formation of receptor crescents before the polarization of active Cdc42 in pheromone-treated G1 cells (Fig. 5), which implies that the receptor polarizes before the actin cables are nucleated.

In contrast to the robust receptor polarization that we observed in the absence of directed secretion, the receptor cannot polarize if its internalization is blocked by mutation or by treatment with LatA together with pheromone (6). However, when pheromone-induced receptor internalization was allowed to continue for 15 min before actin was depolymerized with LatA, robust receptor crescents subsequently appeared (Fig. 6) (6). We infer that a polarity site is established while the receptor is being internalized, and that the position of this site is most likely determined by the receptor itself. A mutant form of the receptor that cannot be internalized, and is thus unable to polarize (Ste27XR), conferred a defect in gradient sensing, as was measured in low-density unilateral mating mixtures (Fig. 4). Moreover, the receptor crescent shifted from the default polarity site to the chemotropic growth site in wild-type cells that had not yet changed shape (fig. S2). Together, these data indicate that receptor polarization occurs earlier than directed secretion and contributes to gradient sensing.

In addition to suggesting that the distribution of the receptor and G protein can be polarized by differential internalization, our data indicate that differential phosphorylation of the receptor alone can support a chemotropic response. In mating cells that could not internalize the pheromone receptor, the unphosphorylated receptor crescent was often seen to move from the presumptive default polarity site to an apparent chemotropic response in advance of morphogenesis; that is, the unphosphorylated receptor crescent responded to the gradient and anticipated the eventual shmoo shape (Fig. 3). Because the distribution of unphosphorylated receptor
the Gα-Far1-Cdc24-Bem1 protein complex was established early in the study of chemotropism that is distinct from its role as a stimulus for receptor phosphorylation of Gβγ (GbgP) to Yck inhibits receptor and G protein internalization. (D) Network 2. With the addition of the Gβγ-Yck interaction, which slows the internalization of receptor and G protein, the phe- nomenon gradient induces receptor polarity. (G) Network 3. The recruitment of Fus3 by Gα leads to locally enhanced phosphorylation of Gβγ and, conse- quently, faster receptor polarization.

could not be determined with the Sst2-GFP reporter in wild-type cells, we measured the effects of preventing receptor internalization and phosphorylation on chemotropism in otherwise wild-type cells stimulated by natural gradients. In contrast to the moderate orientation defect seen in Ste27XR cells, Ste27XR 6SA cells and Ste27XR 6SD cells that could neither internalize nor phosphorylate their receptor were unable to switch from the default polarity site to the chemotropic polarity site and were compromised in their ability to form mating projections (Fig. 4 and fig. S4). These data suggest that receptor phosphorylation plays an essential role in chemotropism that is distinct from its role as a stimulus for receptor internalization, and that the localized concentration of unphosphorylated receptor is critical to establishing the chemotropic growth site.

The processes that enable yeast cells to align their mating projection sites with gradient and amplitude of growth are not well understood; however, two relevant mechanisms have been identified. The binding of free Gβγ to the Far1-Cdc24-Bem1 protein complex was established early in the study of yeast chemotropism (4, 5). It was subsequently shown that disrupting either the Gβγ-Far1 interaction (4) or the Gβγ-Cdc24 interaction (5) prevents the internalization of inactive receptors (R) and active receptors (RL), represented by the green and blue bars, respectively. One heterotri- meric G protein is removed along with each receptor. (C) Network 2. Phos- phorylation of Gβγ and the Gβγ-Yck interaction are included. The binding of Gβγ-GbgP to Yck inhibits receptor and G protein internalization. (D) Network 3. The Gα-Fus3 feedback loop is included. Active Gα recruits active Fus3 to phosphorylate Gβγ, which augments the interaction of Gβγ with Yck and enhances the Gβγ-dependent activation of Fus3 through the MAPK cas- cade (not shown). (E to G) Comparison of network outputs. The graphs show the spatiotemporal dynamics of the total receptor (both active and inactive). (E) Network 1. Without downstream regulation, the pheromone gradient induces complete removal of the receptor from the plasma membrane; no polarity is generated. (F) Network 2. With the addition of the Gβγ-Yck inter- action, which slows the internalization of receptor and G protein, the phe- nomenon gradient induces receptor polarity. (G) Network 3. The recruitment of Fus3 by Gα leads to locally enhanced phosphorylation of Gβγ and, conse- quently, faster receptor polarization.

As the primary determinant of the chemotropic growth site downstream of the receptor, Gβγ must be locally concentrated where the pheromone concentration is greatest. Polarization of Gβγ is required to constrain the polarity patch and nucleate actin cables. There are two challenges to
explaining how Gβγ polarizes before polarity patch stabilization and actin cable nucleation occur. First, physiological gradients are shallow. It has been estimated that a 1% difference in receptor occupancy across the 5-μm length of a yeast cell in a pheromone gradient is sufficient to elicit robust orientation of cell growth toward the pheromone source (40), and microfluidic studies suggest an even greater acuity (36). Thus, although there is a slight excess of free Gβγ on the side of the cell exposed to the highest concentration of pheromone, there is almost as much on the opposite side. Second, because activated receptors are targeted for phosphorylation and internalization, the density of receptor and G protein at the plasma membrane is expected to be least where the pheromone concentration is greatest, the opposite of what would be expected to drive chemotropism. The Gβγ-Yck interaction that we have demonstrated here (Fig. 1, A to C, and Tables 1 and 2) provides a means to invert the relationship between receptor activation and receptor internalization. From first principles, the proportion of activated receptors on the surface of a cell subjected to a pheromone gradient should increase as a function of pheromone concentration. On the basis of the established model of pheromone-induced receptor modification and endocytosis, the initial rate of receptor internalization is expected to be greatest where the density of activated and phosphorylated receptors is greatest, as is the initial rate of heterotrimeric G protein activation. We postulate that free Gβγ protects proximal receptors from Yck-dependent phosphorylation and internalization, which in turn preserves heterotrimeric G proteins at the plasma membrane (6). Thus, the slightly greater burst of Gβγ released from Go on the up-gradient side of the cell triggers a feedback loop that amplifies signaling at the incipient shmo site. As yet, the most upstream event in this pathway is the induction of anisotropic receptor phosphorylation such that the most concentrated region of activated, unphosphorylated receptor marks the direction of the gradient source.

On the basis of published results and the observations presented here, we also postulate a feedback loop that promotes the Gβγ-Yck interaction. Activated Go is thought to recruit active Fus3 to the cell cortex (32, 33, 41), where it phosphorylates Gβ (20) on one or more MAPK consensus sites (Fig. 1E). Given the CK1 consensus motifs in the Gβ sequence (17–19), phosphorylation of either Thr318 or Thr320 by a MAPK could convert Gβ to a Yck substrate. Indeed, Gβ was hypophosphorylated in cells lacking Yck1/2 function (Fig. 1D). These various points can be unified as follows. Yck has a basal affinity for unphosphorylated Gβ, which is increased when Gβ is phosphorylated by Go-recruited Fus3. Thereby marked as a Yck substrate, Gβ acts as a competitive inhibitor of receptor phosphorylation. Alternatively, Gβ might allosterically decrease the catalytic activity of Yck. Although it will be of interest to distinguish between these possibilities, the protection of proximal receptors by Gβ could involve either or both mechanisms.

We conclude that, similar to chemotactic cells, yeast cells unable to polarize the distribution of receptor can nevertheless orient in a gradient, albeit not as well as do wild-type cells. How do they do this? A likely explanation is that, as the distribution of the phosphorylated receptor becomes polarized, G protein activation is favored where the active, unphosphorylated receptor is concentrated. This requires that the G protein can freely diffuse along the plasma membrane and be subject to active internalization by coupling with ligand-bound receptors, as has been reported for chemotactic cells. In Dictyostelium, for example, activated Go and free Gβγ are thought to be concentrated on the leading edge of chemotaxing amoebae when freely diffusing heterotrimeric G proteins couple with ligand-bound receptors on the side of the cell facing the gradient (42, 43). The output from our mathematical model is consistent with this hypothesis: Although the heterotrimeric G protein is almost uniformly distributed on the surface of the gradient-stimulated computational cell, activated Go and free Gβγ markedly polarize together with the active receptor (Figs. S7 and S8). It remains to be determined whether the ligand-bound, unphosphorylated receptor activates the G protein more effectively than does the ligand-bound phosphorylated receptor and, if so, how it does so. If confirmed, however, a confined G protein activation center would add another powerful feedback mechanism to our proposed networks. In this scenario, activated receptor would generate activated Go and free Gβγ, which would increase the local density of activated, unphosphorylated receptor and G protein, which would further increase the local density of activated Go and free Gβγ, and so on. As discussed earlier, the rapid localization of Gβγ toward the source of the gradient is essential for positioning the chemotrophic growth site.

Together, our data suggest that polarity is first established in response to a pheromone gradient by differential phosphorylation and internalization of the receptor, which depends, in turn, on anisotropic protection of the receptor by its G protein. At least two interconnected positive feedback loops are at the heart of the localized amplification mechanism underlying the yeast chemotropic response. Similar processes may contribute to directional sensing in other systems. For example, a role for GPCR phosphorylation other than desensitization has been reported in Dictyostelium chemotaxis (44).

MATERIALS AND METHODS

Molecular and microbiological techniques
Standard methods were used for microbial culture and molecular manipulation, which were performed as described previously (45–47).

Yeast strain construction
The yeast strains used in this study are listed in table S1. Unless otherwise noted, otherwise, they were derived by transformation from strain 15Dau (MATa bar1Δ ade1 his2 leu2-3, -112 trp1 ura3Δ), which is congenic with strain BY264-15D (48). To generate strains that could be heavy-labeled for mass spectrometric analysis, ARG5.6 was knocked out in strains 15Dau and RDI114 by transplacement with a fragment containing KanMX4 G418, PCR-amplified from pFA6-kanMX4 (49), and flanked by ends homologous to ARG5.6 with the oligomers 5′-TCCAAATCTCCAAAACTTTTCTCTCTATACACCAAACTTCCATAGGCTGCTTCGAC… and 5′-TCAGGGATACCAGCATACTCTCCATAACCCATAGCAAGATTAATTTTTGATCGTAATTGCAGCTCG-3′. Integrants were selected with medium containing geneticin (G418, 200 μg/ml). LYSI was then replaced with a TEF promoter–hpl–TEF terminator fragment amplified from the pAG32 plasmid (EUROSCARF) and flanked by ends homologous to LYSI with the oligomers 5′-GCTGCGTCACATTACATCAAGAGCTGAAACFTAAACCCCCTAGAGGCTCTTTGATGCTTGCCTTGTC-3′ and 5′-GTACCAGAAGCCTAGTGTATTTGTGTAACACAGTAGC-CACAGTGTATATGCTGTTTTCGACACTGGAT-3′. Integrants were selected on medium containing hygromycin B. To enable exclusive expression of 6-His-Stel18 and 3′-HA-Stel18, native STE18 (Gy) was replaced with a fragment containing URA3 as described previously (50). Strain AIY109 was generated by integrating ste4Δ320Δ Δ335Δ into strain YDB111 (22) in situ with Msc1-cut Yplac128-ste4Δ320Δ Δ335Δ (pAI130). Strain AIY197 was generated by integrating Hph1-cut LHP1921 (51) into RDI114 (21). Strain AIY301 was generated by integrating Apal-digested Yplac211-Gic2-PBD-RFP (52) into DMY169 (6). Strains XWW005, XWW008, and XWW018 were generated by integrating Cla1-digested DLB3850, DLB3784, and DLB3851, respectively, into strain DSY257. The fluorescent-marked MATa mating tester strain, XWW027, was generated by integrating Bsu361-digested pRS406-GFP-BUD1 at the BUD1 locus of DSY129. All integrations intended to cause gene deletion, replacement, or in situ tagging were checked by genomic sequencing.

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Plasmid construction

The plasmids used in this study are listed in table S2. YCplac22/GAL1-3×HA-STE18 was constructed by PCR-amplifying STE18 from pGEX-KG-STE18 (MCB35) with the oligomers 5′-TCTATCCGATCGACATGTTACCAGCTCCACGATCTTACCATCATGTTCCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTG TTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATCACCCATGCATCAGCTCCACGATCTGTTACATGCATGACGATC

Gßγ and GßPγ′ affinity beads and pull-down analysis.

To prepare Gßγ affinity beads and GßPγ′ affinity beads, strains NWY069, NWY068, and NWY071 were grown to mid-log phase in selective medium containing 2% sucrose and 0.1% dextrose, induced with 2% galactose for 5 hours, and then treated with 150 nM α-factor for 1 hour. Cells were harvested at 2300g at room temperature, washed once with ice-cold deionized water, and frozen in dry-ice ethanol. Cell pellets were lysed at 4°C with 0.5-mm silica beads in 1× tris-buffered saline (TBS) buffer containing protease inhibitors [150 mM NaCl, 10 mM tris-HCl (pH 8), 100 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM aprotinin, 2 mM pepstatin, and 2 mM leupeptin]. Crude lysates were centrifuged at 16,100g at 4°C for 20 min, and protein concentrations were determined with the Pierce 660nm Assay kit (Thermo Fisher). Each cell lystate (7.3 mg) was mixed with 30 μl of anti-HA agarose beads (Thermo Fisher), adjusted to a total volume of 1.25 ml with 1× TBS buffer containing protease inhibitors, and incubated for 2 hours at 4°C with end-over-end rotation. The beads were washed three times with 1× TBS-T buffer [150 mM NaCl, 10 mM tris-HCl (pH 8), and 0.1% Tween 20]. To obtain cell lysates containing myc-Yck1, strain NWY073 was cultured and processed as described earlier except that it was induced with galactose for 3 hours. Low (L; 225 μg) and high (H; 900 μg) amounts of total protein from the NWY073 lysate were added to the Gßγ or GßPγ′ affinity beads. Volumes were adjusted to 1.25 ml with 1× TBS buffer containing protease inhibitors, and the slurries were incubated at 4°C for 1 hour with end-over-end rotation. The beads were washed three times with 1× TBS-T buffer and then were reduced in 1× SDS sample buffer. The supernatant was split into two fractions for Western blotting and mass spectrometric analyses. For Western blotting analysis, myc-Yck1 was detected with a horseradish peroxidase (HRP)–conjugated mouse anti-c-myc antibody (1:900, Thermo Fisher); 3×HA-Gy was detected with a primary mouse anti-HA antibody (1:900, Covance) and an HRP-conjugated secondary goat anti-mouse immunoglobulin G antibody (1:400,000, Jackson ImmunoResearch Inc.). Densitometric analysis of scanned Western blotting films was performed with the ImageJ gel analysis tool, which provided AI values for each band. The relative amount of myc-Yck1 binding to Gßγ as compared to GßPγ′ was determined by subtracting the corresponding negative control signal (L or H) and then taking the ratio of the resulting SAN values. Normalization to HA-Gy was not necessary because the signal detected with the anti-HA antibody did not vary from lane to lane. For the mass spectrometric analysis, 225 μg of total protein from NWY073 lysates was incubated with the affinity beads. Eluted proteins were alkylated and resolved by SDS-PAGE. The ~55- to 70-kD (for myc-Yck1 and Yck2) and 45- to 55-kD (myc-Yck1 cleavage products; Gßβ′) regions of the gel were then excised, the proteins were in-gel digested with sequencing-grade modified trypsin (Promega), and the resulting trypsin fragments were analyzed by nanoscale liquid chromatography combined with mass spectrometry (LC-MS/MS), as previously described (55, 56).

Analysis of Gß phosphorylpeptides

NWY052 cells were grown to mid-log phase in selective synthetic 2% sucrose medium containing natural arginine and lysine (normal Arg/Lys) and induced with 2% galactose for 5.5 hours (light culture). A parallel culture of NWY052 cells was labeled in medium containing heavy Arg/Lys (20 mg/liter; L-lysine:2HCl, U-13C6 and L-arginine:HCl, U13C6, U-15N2; Cambridge Isotope Laboratories) and treated with 150 nM α-factor for 1 hour after galactose induction. The light and heavy cultures were prepared for mass spectrometric analysis in parallel. Cells were harvested at 2300g at room temperature, washed once with ice-cold deionized water and lyophilized. The proteins were digested with sequencing-grade modified trypsin (Promega), and the tryptic digests were analyzed by nanoscale liquid chromatography and mass spectrometry (LC-MS/MS), as previously described (55, 56).

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water, and frozen in dry-ice ethanol. Cell pellets were lysed at 4°C with 0.5-mm silica beads in 1× TBS buffer containing protease and phosphatase inhibitors ([150 mM NaCl, 10 mM tris-HCl (pH 8), 100 mM PMSF, 2 mM aprotonin, 2 mM pepstatin, 2 mM leupeptin, 1 mM sodium orthovanadate, 50 mM NaF, 10 mM sodium pyrophosphate, and 10 mM β-glycerol phosphate]). Crude lysates were centrifuged at 16,100g at 4°C for 20 min, and protein concentrations were determined with the Pierce 660nm Assay kit. Total protein (25 mg) from each lysate was mixed with 100 μl of Ni-NTA beads (Qiagen). Volumes were adjusted to 1.25 ml with 1× TBS buffer, containing protease and phosphatase inhibitors, and the slurries were incubated at 4°C with end-over-end rotation for 16 hours. The beads were washed 10 times with 1× TBS-T buffer ([150 mM NaCl, 10 mM tris (pH 8), and 0.1% Tween 20] and then reduced in 1× SDS sample buffer. Equal volumes of supernatant from heavy and light cultures were mixed. The proteins were alkylated and separated by SDS-PAGE. The ~45- to 50-kD region of the gel was excised and subjected to in-gel digestion with sequencing grade trypsin. Peptides were concentrated under vacuum and enriched for phosphopeptides with a TiO2 phosphopeptide enrichment and clean-up kit (Pierce Biotechnology). Phosphopeptides were concentrated under vacuum and analyzed by nanoscale LC-MS/MS as previously described (53–57). A peptide search engine using a probabilistic scoring model (Andromeda) was used to analyze the resulting spectra and obtain assignments for sites of phosphorylation (58). 

Analysis of the localization of Sst2-GFP, Ste22XR-mCherry, and Ste2-GFP in G1-synchronized cells

G1-synchronized cells were purified by elutriation as previously described (6). Cells used to study the localization of Ste2-GFP and Ste2XR-mCherry (strains AIY100, AIY101, and AIY221) were spun down and resuspended in selective synthetic medium containing 2% galactose, cultured for 1 hour at 30°C, and then treated with 1.2 μM α-factor and 200 μM LatA. Cells used to study the localization of Ste2-GFP (strains DMY169 and AIY197) were spun down and resuspended in synthetic medium containing 1.5% sucrose and 0.5% dextrose and treated with 9 nM α-factor at time zero and with 200 μM LatA 15 min later. All cultures were maintained at 30°C. Images were acquired at 15-min intervals with an Axioskop 2 microscope (Carl Zeiss) with a 100× oil immersion objective and a digital AxioCam camera. Laser excitation was at 488 nm. Images were processed with Zeiss AxioVision software (Carl Zeiss). 

Time-lapse analysis of Sst2-GFP and Ste2-GFP localization in mating mixtures

Wild-type Matα cells and experimental Matα cells (AIY301) were grown to mid-log phase in Synthetic 2% dextrose medium, mixed at a ratio of 1:1, and spread at a density of 14,000 cells/mm² on agarose pads made from synthetic dextrose medium. Mating mixtures were maintained at 30°C with a DeltaVision environment control chamber (GE Healthcare Biosciences). Images were acquired from 20 fields at 15-min intervals with a DeltaVision Elite microscope (GE Healthcare Biosciences) with a 60× oil immersion objective and a front-illuminated sCMOS digital camera. To follow details of cell growth and zygote formation, DIC images were acquired in 31 z sections over 6 μm. To identify the Matα cells, fluorescent images (461- to 489-nm excitation) were acquired at the center slice. Images were processed with ImageJ software. Initial orientation angles, fusion angles, time of fusion, and morphologies of Matα cells were quantified and analyzed as described in Fig. 4. 

Analysis of Ste2-GFP and Gic2-PBD-RFP localization in asynchronous cells

AIY301 cells were grown to mid-log phase at 30°C in selective medium containing 2% sucrose and were treated with 15 nM α-factor. Images were acquired 1 and 2 hours later with an Axioskop 2 microscope as described earlier. RFP and GFP were visualized with laser excitations of 561 and 488 nm, respectively. Exposures and processing were identical for all images. 

Time-lapse analysis of Ste2-GFP and Gic2-PBD-RFP localization in mating mixtures

Wild-type Matα (DSY129) cells and experimental Matα cells (AIY301) were grown to mid-log phase in synthetic 2% dextrose medium, mixed at a ratio of 1:1, and spread at a density of 14,000 cells/mm² on agarose pads made from synthetic dextrose medium. Mating mixtures were maintained at 30°C with a DeltaVision environment control chamber. Images were acquired from 15 fields at 7.5-min intervals with a DeltaVision Elite microscope with a 60× oil immersion objective and a front-illuminated sCMOS camera. To follow the localization of the Ste2-GFP and Gic2-PBD-RFP reporters, fluorescent images were acquired in 3 z sections over 1 μm with light-emitting diode excitations of 461 to 489 nm and 529 to 556 nm, respectively. The exposures were the same for all images. Images were processed with ImageJ software. Processing was the same for all Ste2-GFP images in a given series, and all Gic2-PBD-RFP in a given series. 

Receptor labeling and internalization assays

The surface-expressed α-factor receptor Ste2 was labeled with Alexa Fluor 594–conjugated α-factor according to the method of Toshima et al. (60). To determine how the density and distribution of the receptor changed over time in response to pheromone, DMY169 cells were G1-synchronized by elutriation and treated with 6 nM α-factor at 30°C in YEPD. Aliquots were taken at 5-min intervals, treated with 10 mM sodium azide, and incubated with the labeled α-factor (a gift from D. King, University of California, Berkeley). The Matα ste2Δ strain (EDY208) was used as a negative control for receptor labeling. Images were acquired with an Andor Revolution WD spinning disc laser confocal system with a motorized Olympus IX81 microscope, a Yokogawa CSU-W1 spinning disc unit, a Prior motorized stage, and a Neo sCMOS camera controlled by Andor iQ2 software. A UPLSAPO 60× silicon immersion objective (NA, 1.3) was used, and nine z sections were acquired over 4.8 μm with a laser excitation of 561 nm; one center slice was imaged with DIC. Images were sum-projected with ImageJ. To assay the relative rate of receptor internalization as a function of position on the plasma membrane, Matα cells (RDY126) were grown to mid-log phase in YEPD medium, labeled with Alexa Fluor 594–conjugated α-factor, and imaged at 1-min intervals at room temperature as described earlier. Nine z sections were acquired over 5.6 μm with a laser excitation of 561 nm, and one center slice was imaged with DIC. Images were sum-projected and membrane fluorescence was obtained with the segmented line tool of ImageJ.
Relative rates of signal decay for labeled receptors at the plasma membrane

The variation in fluorescence intensity measured by ImageJ from pixel to pixel along the plasma membrane was smoothed using a moving average function with the sliding window equal to 1/10th of the membrane perimeter. For position $x$ at time $t$ in cell $c$ with a window size $w$, the fluorescence intensity was calculated by the following formula:

$$F_{I}(x, t, c) = \text{avg} \left[ F_{I_{0}} \left( x - \frac{w}{2}, t, c \right), \ldots, F_{I_{0}} \left( x + \frac{w}{2}, t, c \right) \right]$$

in which $F_{I_{0}}$ denotes the new value after filtering, and $F_{I_{0}}$ represents the original raw value. The background was removed by subtracting the minimum value from each data set:

$$F_{I}(x, t, c) = F_{I_{0}}(x, t, c) - \min_{x,t} F_{I_{0}}(x, t, c)$$

Assuming that the kinetics of internalization resembled a first-order chemical reaction, we calculated the relative fluorescence intensity of time point $t$ compared to time point 0 to determine how membrane receptor density changed with time using the following formula:

$$F_{I}(x, t, c) = F_{I_{0}}(x, t, c) / F_{I_{0}}(x, 0, c)$$

Finally, we used the data set for each cell to determine whether a discrete region of the plasma membrane exhibited a statistically significantly slower rate of signal loss than did the remainder of the plasma membrane. This was determined by fitting the 10 values for each pixel (time points 0 to 9) to the exponential decay formula:

$$F_{I}(x, t, c) = A \exp(-\lambda t)$$

in which the decay rate $\lambda$ and the initial quantity $A$ are the two parameters to be fitted. A putative area of receptor protection was defined as the points that were in the 30th percentile for decay rate (that is, the slowest 30%). We calculated the average $F_{I}$ of the “protected region” and compared it to the average of the rest of the cell. In some cases, the protected region was fragmented. The regions were connected if the distance between them was less than 1/10 of the perimeter of the cell and the average decay rate of region after connecting was still above the threshold.

Computational model of pheromone-induced receptor polarization

To model the plasma membrane of a yeast cell, we used a sphere, the surface of which was partitioned into patches by uniformly spaced $n$ latitude and $m$ longitude lines (fig. S7). The center of each patch was used to represent its position. The surface distances between neighboring patches are given by Eqs. 1 and 2 (table S3). The $j$th patch in the $i$th band can be denoted by an index pair $(i, j)$. The position of the $(i, j)$ patch $(x_{i}, y_{i}, z_{i})$ is given by Eq. 3 (table S3). In each patch, we stimulated the reaction network model with the pheromone concentration appropriate to its position. Because the pheromone gradient is aligned along the x axis and the pheromone concentration is assumed to change linearly with $x$, only $x_{i}$ is needed to determine the local pheromone concentration of the $(i, j)$ patch (Eq. 4). All proteins were assumed to diffuse laterally because only the plasma membrane was modeled in this analysis. If the effect of surface curvature is ignored, the diffusion of each molecular species can be obtained from Eqs. 5 to 7 (table S3). To model pheromone-induced receptor polarization that occurs before directed secretion and to evaluate how our postulated feedback mechanisms affect the establishment of receptor polarity, we first created a core network that includes only the most basic and best-characterized components. Network 1 comprises the receptor-pheromone interaction, G protein cycle, and Yck-dependent internalization of the receptor and G protein (Fig. 4B). Network 2 adds the Gβγ-Yck interaction (Fig. 4C), and network 3 adds the Gα-For3 interaction (Fig. 4D). The mathematical representation of these networks is detailed in tables S4 to S6. A common set of coupled partial differential equations (PDEs) was derived from their corresponding reaction formulae. Each PDE describes how the concentration of a given molecule changes over time and space. Differences in the topology of the three networks were accounted for by varying the initial values of the relevant parameters (color-coded in table S4).

SUPPLEMENTARY MATERIALS

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Fig. S1. Examples of mass spectra used to map the sites of pheromone-induced Gβγ phosphorylation.

Fig. S2. Dynamic localization of the pheromone receptor as cells orient toward mating partners.

Fig. S3. Exogenous pheromone induces the formation of angled zygotes.

Fig. S4. Time-lapse images of elongating and turning cells in mating mixtures.

Fig. S5. Surface distribution of the pheromone receptor as assayed by Alexa Fluor 594–conjugated α-factor binding.

Fig. S6. Receptor internalization assay.

Fig. S7. Spatial model of the computational yeast cell.

Fig. S8. Computational model of receptor dynamics.

Fig. S9. Computational model of G protein dynamics.

Fig. S10. Computational model of Yck dynamics.

Table S1. Yeast strains used in this study.

Table S2. Plasmids used in this study.

Table S3. Equations used for the spatial model of the yeast cell.

Table S4. Definitions and parameters.

Table S5. Reaction formulae.

Table S6. Partial differential equations.

References (61–65)

REFERENCES AND NOTES


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Protected pheromone receptors signal
In budding yeast, opposite mating types signal to each other by secreting distinct pheromones, which bind to and activate receptors on the surface of compatible cells. The stimulated cell produces a mating projection (shmoo), which is enriched in pheromone receptors, that grows along the pheromone gradient to fuse with the shmoo of a mating partner. Ismael et al. imaged pheromone-treated yeast cells and found that the G protein β subunit, which is activated by ligand-bound pheromone receptors, competed with a kinase for access to receptors at the site closest to the highest amount of pheromone. In this way, the phosphorylation and internalization of receptors occurring in the rest of the cell was inhibited at this local site, which enabled the receptor to persist at the cell surface and stimulate growth of the shmoo.