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 MATα cells and Ste3 in MATa 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 MATα cells is well documented. During vegetative growth, the MATα-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).
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 chemotropic 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) (I2) 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 allelle-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 complementary 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 STE4A and TE4B. 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 beads. The relative amount of myc-Yck1 that bound to Gβ versus Gβγγ is expressed as the Gβγγ/Gβγ ratio. Data are representative of three experiments. (C) Pheromone-treated 15Dau cells expressing the indicated constructs were analyzed by BiFC before morphogenesis (top) and after mating projection formation (bottom). Some fluorescence was detectable at the tips of shmooing negative control cells (bottom middle), but this was of lower intensity and distinct localization compared to that in the experimental cells (see Table 2). Images are representative of two experiments. (D) YCK1 YCK2 control cells and yck1A yck2A mutant cells were left untreated or were exposed to pheromone (aF) at a permissive temperature [room temperature (RT)] or a restrictive temperature (37°C). The indicated cell lysates were then analyzed by Western blotting to detect Gβ. Lysate from ste4A cells (ΔGβ) provided a negative control for antibody specificity. The bracket indicates the positions of the Gβ bands. The lowest band is unphosphorylated Gβ; the higher bands are the phosphorylated species. The asterisks indicate two background bands that cross-react with the Gβ antibody. Western blots are representative of three experiments. (E) Map of phosphorylation sites among amino acid residues 317 to 336 of Gβ. Sites in blue and green indicate sites identified by mass spectrometric analysis (see fig. S1) or genetic analysis (20), respectively, whereas residues in red indicate sites identified by both methods. Arrows indicate putative MAPK and CK1 sites.
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>
</tr>
</thead>
<tbody>
<tr>
<td>Yck1</td>
<td>Gβγ</td>
<td>3.16 × 10^7</td>
<td>2.25 × 10^7</td>
<td>5.42 × 10^7</td>
<td>2.25</td>
</tr>
<tr>
<td></td>
<td>Gβγ−γ</td>
<td>6.88 × 10^6</td>
<td>1.72 × 10^7</td>
<td>2.41 × 10^7</td>
<td>1.94</td>
</tr>
<tr>
<td></td>
<td>Empty</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Yck2</td>
<td>Gβγ</td>
<td>2.39 × 10^7</td>
<td>0</td>
<td>2.39 × 10^7</td>
<td>2.09</td>
</tr>
<tr>
<td></td>
<td>Gβγ−γ</td>
<td>1.15 × 10^7</td>
<td>0</td>
<td>1.15 × 10^7</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Empty</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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 Gβγ-VF2 + VF1 negative control cells. This was attributable to the direct, irreversible interaction between the Gβγ and Yck1, which inhibits polymerization of actin and pheromone cannot redistribute the receptor; nevertheless, they exhibit asymmetric receptor phosphorylation (6).

To determine whether Gβγ could inhibit receptor phosphorylation, we examined pheromone- and LatA-treated YDB111 cells overexpressing either Gβ or Gβγ−γ, the weaker Yck1/2-binding form of Gβ (Fig. 2, A to F). Overexpression of Gβ 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−γ 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 (Gβ) with ste4Δ120A Sst2–335A (Gβγ−γ) 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−γ, 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 relocated 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 S/TP (where n is any amino acid residue) is the consensus motif (17–19). If this holds true for yeast, the phosphorylation of Thr18 or Thr320 would convert Gβ to a Yck1 substrate (Fig. 1E). Conversely, unphosphorylated Gβ would be expected to interact with Yck1 to a lesser degree than would the partially phosphorylated species. We tested this with a mutant form of Gβ, ste4Δ120A Sst2–335A (henceforth Gβγ−γ), which cannot be phosphorylated (20). In pull-down experiments in which the relative amount of Yck1 that bound to Gβγ or Gβγ−γ beads was quantified by both Western blotting and mass spectrometric analyses (Fig. 1, A and B, and Table 1), the apparent affinity of Yck1 for Gβγ−γ was less than that for Gβ. Note that endogenous Yck2 (that is, untagged native protein) was also identified by mass spectrometry as specifically binding to the Gβγ beads and, like Yck1, it showed a weaker apparent affinity for Gβγ−γ than for Gβ (Table 1).

Gβγ overexpression inhibits phosphorylation of the pheromone receptor and promotes its polarized localization

Because Gβγ adopts a polarized localization before pheromone-induced morphogenesis occurs (21), its direct interaction with Yck1 raises the possibility that Gβγ promotes receptor polarization by locally inhibiting receptor phosphorylation. Consistent with this, a region of the plasma membrane enriched in hypophosphorylated receptor arises in response to isotropic pheromone treatment (6, 22). This was demonstrated with strain YDB111 (22), which expresses a green fluorescent protein (GFP)–tagged form of the regulator of G protein signaling (RGS) protein Sst2, and Ste2ΔNKR, a mutant form of the MATα7 receptor that cannot be internalized. The Sst2-GFP reporter binds specifically to the unphosphorylated form of the Ste2 receptor (22). YDB111 cells treated with latrunculin A (LatA, which inhibits polymerization of actin) and pheromone cannot redistribute the receptor; nevertheless, they exhibit asymmetric receptor phosphorylation (6).

% Cells with PM signal*  PM/Cyto ± SEM†  % PM ± SEM ≥ 1.25× Cyto‡
<table>
<thead>
<tr>
<th></th>
<th>Pre-shmoo</th>
<th>Shmoood</th>
<th>Pre-shmoo</th>
<th>Shmoood</th>
<th>Pre-shmoo</th>
<th>Shmoood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gβγ-VF2 + VF1-Yck1</td>
<td>28.5</td>
<td>15.2</td>
<td>n.d.</td>
<td>1.46 ± 0.09§</td>
<td>n.d.</td>
<td>50.5 ± 5.4§</td>
</tr>
<tr>
<td>Gβγ-VF2 + VF1</td>
<td>3.4</td>
<td>27.4</td>
<td>n.d.</td>
<td>0.95 ± 0.03§</td>
<td>n.d.</td>
<td>17.0 ± 1.9</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 Gβγ-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 Ste2mCherry 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 Ste2mCherry 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 Ste2mCherry 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 chemotrophic 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 STE27XR, STE27XR 6SA, or STE27XR 6SD in an otherwise wild-type MATα 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 STE27XR 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 always larger in the wild-type MATα × MATα STE27XR mating mixtures than in the wild-type × wild-type crosses (Fig. 4, C to E). Note that STE27XR 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 affect its phosphorylation state, they were unable to grow chemotropically. In mating mixtures, the MATα STE27XR 6SD cells that ultimately formed zygotes with wild-type MATα cells always polarized their growth adjacent to their last bud site (Fig. 4B). Similarly, all MATα STE27XR 6SA cells that ultimately formed zygotes polarized their growth—or mated without undergoing morphogenesis—proximal to their last bud site. Thus, MATα STE27XR 6SD and MATα STE27XR 6SA 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 STE27XR 6SA and STE27XR 6SD alleles conferred pronounced defects in mating projection formation (Fig. 4F). Although most of the mating MATα STE27XR 6SA cells and all of the mating MATα STE27XR 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 MATα STE7XR 6SA and MATα STE7XR 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 MATα STE7XR 6SA and MATα STE7XR 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 MATα STE7XR 6SA and MATα STE7XR 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
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 shmoos), broadly polarized growth, and no polarized growth, as indicated. The proportion of normal shmoos 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 shnoosing after 60 min of pheromone treatment exhibited 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 MATa 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 shmoo site upstream of active Cdc42.

**Gβg**-cells are defective in receptor polarization

Our biochemical data suggest that Gβg has a reduced affinity for Yck1/2 compared to that of Gβ. We also previously showed that the pheromone-induced polarization of GFP-Gβg 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 Gβg would be expected to adversely affect the formation of receptor crescents. To test this, we compared the localization of GFP-tagged receptor in Gb and Gβg cells treated with LatA 15 min after pheromone stimulation. We found that Gβg 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: Gβg 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 Gβγ–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, Gβγ inhibits phosphorylation of the pheromone receptor, which in turn slows receptor internalization. The interaction between Gβγ and Yck1/2 would also be expected to inhibit endocytosis of the G protein because we previously found that both Gα and Gβγ 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 Gu and free Gβγ. 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 shmooshing (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 synergistically 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 Gβγ–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), without resetting polarity (network 1). In contrast, a computational cell that differed only in the addition of the Gβγ–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 Gu and free Gβγ polarized together with the receptor (Fig. S9). At the same time, inverse intracellular gradients of free Yck (back) and Gβγ-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 AY301 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 $MA7a$ cells that switched from their presumptive default polarity site to a presumptive chemotropic growth site in mating mixtures. 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.
Fig. 6. Effect of Gβ− on receptor polarization. (A and B) Isotropic conditions. Gβ cells and Gβ− cells expressing the receptor reporter Ste2-GFP were treated with pheromone at time zero and with LatA after global receptor internalization (15 min). (A) Representative images of the indicated cells to visualize Ste2-GFP. Arrowheads indicate Ste2-GFP crescents. (B) Polarity indices for the cells represented in (A) were measured as described for Fig. 2D. Data are means ± SEM of at least 30 cells of each strain from three experiments. *P < 0.03. (C and D) Mating mixtures. (C) Time-lapse images of mating Gβ cells and Gβ− cells showing Ste2-GFP localization. Insets show heat map images analyzed with the BudPolarity Matlab program (59), which quantifies signal intensity along the long axis of the cell. (D) Output BudPolarity trace of the pictured cell (inset). Receptor polarization in the cells represented in (C) was quantified by dividing the peak fluorescence signal at the plasma membrane by the minimum fluorescence at the plasma membrane before morphogenesis became apparent. Mean polarization values ± SEM were 1.76 ± 0.14 and 1.28 ± 0.07 for Gβ cells and Gβ− cells, respectively (P < 0.007). Data are means ± SEM of at least 17 cells of each strain from two independent experiments. a.u., arbitrary units.

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 thus unable to polarize (Ste2NKR), 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 site in advance of morphogenesis; that is, the unphosphorylated receptor crescent responded to the gradient and anticipated the eventual shmoo site (Fig. 3). Because the distribution of unphosphorylated receptor...
the yeast chemotropism (Far1-Cdc24-Bem1 protein complex was established early in the study of relevant mechanisms have been identified. The binding of free G$_{bg}$ to the gradient of pheromone (loop 2). The two loops act synergistically to promote local signaling while protecting G$_{bg}$ from internalization, thereby generating a concentration gradient of free G$_{bg}$. Ultimately, the localized increase in G$_{bg}$ is sufficient to stimulate the nucleation of actin cables. Directed secretion then reinforces the spatial signal and drives mating projection formation. (B to D) Network diagrams. (B) Network 1. Without downstream regulation, the pheromone gradient induces complete removal of the receptor from the plasma membrane; no polarity is generated. (E) Network 2. The addition of the G$_{bg}$-Yck interaction, which slows the internalization of receptor and G protein, the pheromone gradient induces receptor polarity. (G) Network 3. The recruitment of Fus3 by Ga$_{a}$ leads to locally enhanced phosphorylation of G$_{bg}$ and, consequently, 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 pheromone gradients are not well understood; however, two relevant mechanisms have been identified. The binding of free G$_{bg}$ 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 G$_{bg}$-Far1 interaction (4) or the G$_{bg}$-Cdc24 interaction (5) prevents chemotropic shmooing. More recently, Dyer et al. (35) and Hegemann et al. (31) used live-cell imaging to show that a dynamic polarity complex, which is likely composed of the core constituents Bem1, Cdc24, Cdc42, and Bni1, moves in a random walk around the cortex of vegetative cells. When cells are exposed to pheromone, the movement of the polarity complex is biased toward the incipient shmoo site through the interaction of Far1-Bem1-Cdc24 with free G$_{bg}$ (31, 35). In principle, therefore, yeast chemotropism can be explained as follows: A gradient of pheromone induces a gradient of activated receptor on the surface of the cell, which, in turn, is mirrored by a gradient of activated G protein. Free G$_{bg}$ then restricts the movement of the polarity patch along the cell cortex on the side of the cell exposed to the highest concentration of pheromone and ultimately stabilizes its position at the incipient growth site. Actin cables are subsequently nucleated at this site. As the primary determinant of the chemotropic growth site downstream of the receptor, G$_{bg}$ must be locally concentrated where the pheromone concentration is greatest. Polarization of G$_{bg}$ 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 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 shmoo 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 cannot nevertheless orient in a gradient, albeit not as well as 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 activation 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 chemotropc 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, they were derived by transformation from strain 15Dau (MATa bar1Δ ade1 his2 leu2-3, -112 trp1 ura3Δ), which is congenic with strain BF264-15D (48). To generate strains that could be heavy-labeled for mass spectrometric analysis, ARG5.6 was knocked out in strains 15Dau and RDY114 by transplacement with a fragment containing KanMX4 G418, PCR-amplified from pFA6-kanMX4 (49), and flankd by ends homologous to ARG5.6 with the oligomers 5′-TCCAAATTTCACCCAAATTGTTGCT-TCAATTAAACATTCACCAGCGGGTACCGTGCGAGGC-3′ and 5′-TCAGGGATATCCGATCATTCATTCAACCACCACGAGAAT-3′. Integrants were selected with medium containing geneticin (G418, 200 μg/ml). LYS1 was then transformed with a TEF promoter–hpl–TEF terminator fragment amplified from the pAG32 plasmid (EUROSCARF) and flankd by ends homologous to LYS1 with the oligomers 5′-GCTCGCGTACATTACATCTAGAGC-CTGAACTAAACCCCTAGAGCTCTGTTTACGTGCTGGTC-3′ and 5′-GTACCAGAGAGGTAGGTTTGTGTTAACACGAGTGTC- CACAGTGTTATAGCTGGTATCCTGGACACTGGAT3′. Integrants were selected with medium containing histidine (YM15, 50 μg/ml) or histidine and leucine (YM15, 50 μg/ml) B. To enable exclusive expression of 6×His-Ste18 and 3×HA-Ste18, native STE18 (Gy) was replaced with a fragment containing URA3 as described previously (30). Strain AY109 was generated by integrating ste4Δ320D S354 into strain YDB111 (22) in situ with Msc1-cut Yplac128/ste4Δ320A S35A NA112 (pAI1B130). Strain AY197 was generated by integrating Hp1-1apal-digested Yplac211-GIC2-PBD-RFP (32) into DMY169 (6). Strains XWH005, XWH008, and XWH018 were generated by integrating Apal-digested Yplac211-GIC2-PBD-RFP (32) into DMY169 (6). Strains XWH005, XWH008, and XWH018 were generated by integrating Clai-digested DLB3850, DLB3784, and DLB3851, respectively, into strain DSY257. The fluorescent-marked MATA mating tester strain, XWH027, was generated by integrating Bsu36I-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.
Plasmid construction
The plasmids used in this study are listed in table S2. YCplac22/GAL1-3-α-STE18 was constructed by PCR-amplifying STE18 from pGEX-KG-STE18 (MCB35) with the oligomers 5'-GTGATCCAGCTGCACATGATCCAATGACATCGTTAACAGTACGCAGTACATGTAACCAGTCGTTAACCAGTACAGTCGCTATGACATCGCTATACATAGCTTAAATC-3' and 5'-TGGACCGCCAAGAGCTTTTACATAAGCGTACAACAAA-3', in which the underlined sequence encodes the N-terminal 3×HA tag, and 5'-TGGACCGCCAAGAGCTTTTACATAAGCGTACAACAAA-3' and 5'-TGGACCGCCAAGAGCTTTTACATAAGCGTACAACAAA-3'. The Sal I–Hind III–digested PCR product was inserted into YCplac22/GAL1 (53). Y plac128/ste4 T203A S35A Nα112 was constructed by PCR-amplifying ste4 T203A S35A Nα112, minus the first 112 bases of the coding region, from RDY120 (21) with the oligomers 5'-CGCGAAATTCTCGCTTCCACAAGAATAGG-3' and 5'-CGCGATCTCAATGACAGGCGGCAAGACAGA-3'. The PCR product digested with Eco RI and Bam HI was subcloned into Yplac128 (53) to generate AIB130. pRS415/ADH1-STE4-VF2 was constructed by PCR-amplifying genomic STE4 lacking the stop codon with the oligomers 5'-GGCAGATCTGGAGACCACTCAGATGG-3' and 5'-GGTGGATCCTTGATAACCTGGAGAC-3'. The Bam HI–digested PCR fragment was subcloned into p455-VF2 (54) in frame with the C-terminal split-Venus fragment under the control of the ADH1 promoter to generate AIB201.

Gβγ-Yck1 split-Venus BiFC assay
Because constitutive overexpression of Gβ causes permanent cell cycle arrest, the Gβ-Yck1 BiFC assay was performed in a strain that enabled conditional expression of STE7. MA7a STE7A::KanMX YCplac22/GAL1-STE7 cells transformed with pRS416/ADH1-VF1-YCK1 and pRS415/ADH1-STE4-VF2 (A1Y273), pRS416/ADH1-VF1-YCK1 and pRS415/ADH1-STE4-VF2 (A1Y276), or pRS415/ADH1-STE4-VF2 and pRS416/ADH1-VF1 (A1Y275) were grown to mid-log phase in selective medium containing 2% sucrose and were induced with 2% galactose for 5 hours, and then treated with 150 nM α-factor for 1 hour. Cells were harvested at 2300 g 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 aprotonin, 2 mM pepstatin, and 2 mM leupeptin]. Crude lysates were centrifuged at 16,100g for 20 min at 4°C. Protein concentrations were determined with the Pierce 660nm Assay kit (Thermo Fisher). Each cell lysate (7.3 µg) was mixed with 30 µl of anti-HA agarose beads (Thermo Fisher); 3×HA-Gβγ or Gβγ 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 [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 Gβγ or Gβγ 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-Gγy 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 Gβγ as compared to Gβγ 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-Gγy 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; Gβγ) 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 (35, 56).

Analysis of Gβ phosphopeptides
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, U-13C6, 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 2300 g at room temperature, washed once with ice-cold deionized gel (Bio-Rad), and the amount of Gβ was assayed by Western blotting as described earlier.

Gβγ and Gβγ affinity beads and pull-down analysis.
To prepare Gβγ affinity beads and negative control 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 2300 g 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 aprotonin, 2 mM pepstatin, and 2 mM leupeptin]. Crude lysates were centrifuged at 16,100 g at 4°C for 20 min. Protein concentrations were determined with the Pierce 660nm Assay kit (Thermo Fisher). Each cell lysate (7.3 mg) was mixed with 30 µl of anti-HA agarose beads (Thermo Fisher); 3×HA-Gβγ or Gβγ 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-Gγy 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 Gβγ as compared to Gβγ 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-Gγy 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; Gβγ) 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 (35, 56).

Analysis of Gβ phosphopeptides
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, U-13C6, 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 2300 g 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× TBS buffer containing protease and phosphatase inhibitors [150 mM NaCl, 10 mM tris-HCl (pH 8), 100 mM PMSF, 2 mM apro- tinin, 2 mM peptatin, 2 mM leupeptin, 1 mM sodium orthovanadate, 50 mM NaF, 10 mM sodium pyrophosphate, and 10 mM β-glycerol phos- phate]. 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 slurry was 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 ana- lyzed 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, Ste2XR-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 A1Y100, A1Y101, and A1Y221) 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 A1Y197) 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 MATά cells (AIY301) were grown to mid-log phase 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.

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

A1Y301 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 MATά 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 micro- scope 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 con- trol 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, MATata 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:

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

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

\[
F(x, t, c) = F_{ln}(x, t, c) - \min_{x,t} F_{ln}(x, t, c)
\]

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:

\[
F_t(x, t, c) = F(x, t, c) / F(x, 0, c)
\]

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:

\[
F(x, t, c) = A \exp(-\lambda t)
\]

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 lowest 30%). We calculated the average \( F_t \) 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 \((x_{ij}, y_{ij}, z_{ij})\) 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 \( x_{ij} \) 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 Gq-Fus3 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

www.sciencesignaling.org/cgi/content/full/9/423/ra38/DC1

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 MATa STE2h/STE12h and MATa STE2h/STE12h 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


ARC grant SF120121205755 (to R.A.A.), NIH grant GM079804 and NSF grant DBI 1062328 (to J.L.), and NIH grant 1RO3CA150131 (to M.V.M.). This work was also funded by the Chicago Biomedical Consortium with support from the Searle Funds at The Chicago Community Trust.

**Author contributions:** D.S. and E.B. performed experiments; A.I., N.W., X.W., and M.V.M. performed experiments and analyzed data; Y.C., W.T., and J.L. did the mathematical modeling and analyzed data; D.E.S. and R.A.A. analyzed data; D.E.S. wrote the manuscript.

**Competing interests:** The authors declare that they have no competing interests.

Submitted 14 September 2015
Accepted 29 March 2016
Final Publication 12 April 2016
10.1126/scisignal.aad4376

**Citation:** A. Ismael, W. Tian, N. Waszczak, X. Wang, Y. Cao, D. Suchkov, E. Bar, M. V. Metodiev, J. Liang, R. A. Arkowitz, D. E. Stone, Gβ promotes pheromone receptor polarization and yeast chemotropism by inhibiting receptor phosphorylation. Sci. Signal. 9, ra38 (2016).
Phosphorylation promotes pheromone receptor polarization and yeast chemotropism by inhibiting receptor phosphorylation

Amber Ismael, Wei Tian, Nicholas Waszczak, Xin Wang, Youfang Cao, Dmitry Suchkov, Eli Bar, Metodi V. Metodiev, Jie Liang, Robert A. Arkowitz and David E. Stone

Sci. Signal. 9 (423), ra38.
DOI: 10.1126/scisignal.aad4376

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.