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Shelly Catherine Strickfaden
University of Massachusetts Medical School

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Distinct Roles for Two Ga–Gβ Interfaces in Cell Polarity Control by a Yeast Heterotrimeric G Protein

Shelly C. Strickfaden and Peter M. Pryciak

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA 01605

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Saccharomyces cerevisiae mating pheromones trigger dissociation of a heterotrimeric G protein (Gaβγ) into Ga-guanosine triphosphate (GTP) and Gβγ. The Gβγ dimer regulates both mitogen-activated protein (MAP) kinase cascade signaling and cell polarization. Here, by independently activating the MAP kinase pathway, we studied the polarity role of Gβγ in isolation from its signaling role. MAP kinase signaling alone could induce cell asymmetry but not directional growth. Surprisingly, active Gβγ, either alone or with Ga-GTP, could not organize a persistent polarization axis. Instead, following pheromone gradients (chemotropism) or directional growth without pheromone gradients (de novo polarization) required an intact receptor–G protein–coupling. These signaling events induced cell polarization requires continuous receptor–Gaβγ communication but not modulation of MAP kinase signaling. To explore regulation of Gβγ by Ga, we mutated Gβ residues in two structurally distinct Ga–Gβ binding interfaces. Polarity control was disrupted only by mutations in the N-terminal interface, and not the Switch interface. Incorporation of these mutations into a Gβ–Ga fusion protein, which enforces subunit proximity, revealed that Switch interface dissociation regulates signaling, whereas the N-terminal interface may govern receptor–Gaβγ coupling. These findings raise the possibility that the Gaβγ heterotrimer can function in a partially dissociated state, tethered by the N-terminal interface.

INTRODUCTION

Cell polarization involves the asymmetric distribution of intracellular materials in a way that is usually directed toward localized instructional cues, which can be internal or external to the cell. External cues govern chemotaxis in cells such as Dictyostelium discoideum and mammalian neutrophils, such that a gradient of chemoattractant from a localized source stimulates cell polarization and directed cell movement along the gradient (Iijima et al., 2002; Bagorda et al., 2006; Franca-Koh et al., 2006). The ability to coordinate the direction of cell movement with the location of the external signal implies that the initial sensing of chemoattractant and at least some of the subsequent intracellular signal transduction events do not occur uniformly within the cell but instead occur in an asymmetric, spatially restricted manner.

The mating reaction of the budding yeast Saccharomyces cerevisiae provides a model system to study how cells generate an asymmetric response to polarization stimuli. Here, the two haploid cell types (a or α) secrete cell type-specific mating pheromones (a factor or α factor, respectively), which serve as chemoattractants that stimulate mating responses in cells of the opposite type (Dohlman and Thorner, 2001). Each pheromone binds a specific G protein-coupled receptor (GPCR) (Ste2 or Ste3, which recognize α factor and a factor, respectively) that couples to a heterotrimeric G protein (Gaβγ, composed of Gpa1 [Ga], Ste4 [Gβ], and Ste18 [Gγ]), which is not cell type specific. Binding of pheromone to the receptor catalyzes exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) on the Ga subunit, causing dissociation of Ga-GTP from the Gβγ dimer. Once released from the inhibitory Ga subunit, Gβγ can trigger signaling through a mitogen-activated protein (MAP) kinase cascade; this involves membrane recruitment of the scaffold protein Ste5, which leads to activation of the Ste5-associated kinases, Ste11 (MAP kinase kinase kinase), Ste7 (MAP kinase kinase), and Fus3 (MAP kinase [MAPK]) (Pryciak and Hunte, 1998; Mahanty et al., 1999; van Droogenbroeck et al., 1999, 2000, 2001; Winters et al., 2005). These signaling events induce cell cycle arrest and the transcription of mating specific genes (Dohlman and Thorner, 2001).

Mating pheromones also regulate cell morphology. The actin cytoskeleton and membrane trafficking systems are rearranged to form a polarized mating projection, which then grows toward the partner in a process termed chemotropism (Segall, 1993; Arkowitz, 1999; Pruyne and Bretscher, 2000). These polarization events highlight another role for Gβγ. Current evidence suggests that Gβγ recruits the polarity proteins Far1 and Cdc24 (Valtz et al., 1995; Butty et al., 1998; Nern and Arkowitz, 1998, 1999) to the region of plasma membrane receiving the highest pheromone dose. Cdc24 is the guanine-nucleotide exchange factor (GEF) for the Rho-family GTPase Cdc42, which controls actin organization and cell polarity (Chant, 1999; Etienne-Manneville, 2004). Far1 is an adaptor protein that binds both Gβγ and Cdc24 (Butty et al., 1999; Nern and Arkowitz, 1999). Hence, communication between Gβγ and Far1/Cdc24 is thought to direct Cdc42 activity to the proper site to allow polarized growth along the gradient of pheromone. This process may be facilitated...
by additional interactions between Ga and Fus3 (Metodiev et al., 2002) or between Gβγ and Rho1 (Bar et al., 2003).

For chemotactic cells (Dictyostelium and neutrophils) and chemotropic cells (yeast), a gradient of chemotactic emptor normally serves as a spatial cue for the direction of polarization. However, these cells will still polarize when exposed to a uniform concentration of chemotactic emptor, implying the existence of “symmetry breaking” mechanisms that can generate asymmetric responses to symmetric signals (Sohrmann and Peter, 2003; Weddich-Soldner and Li, 2003). In yeast, prior work reveals two ways in which a cell can polarize when pheromone is provided uniformly rather than as a gradient, which we will refer to as “default” and “de novo” polarization. Default polarization uses preexisting polarity information provided by the bud side selection proteins as a spatial cue, resulting in the formation of a mating projection at the presumptive bud site (Madden and Snyder, 1992; Dorer et al., 1995; Nern and Arkowitz, 1999). This default polarization is independent of Far1–Cdc24 communication, but it depends on bud site selection proteins such as Rsr1/Bud1 (Nern and Arkowitz, 1999). When these default sites are absent, cells can still polarize in a uniform field of pheromone, but now the axis of polarization forms de novo at random positions that bear no relationship to previous polarization sites. This de novo polarization is independent of bud site selection proteins, but it requires Far1–Cdc24 binding; when this binding is disrupted, cells initiate polarization but fail to organize a stable axis of polarized growth (Nern and Arkowitz, 1999, 2000). Thus, interactions that are required for asymmetric response to a gradient of pheromone are also required when asymmetric growth is initiated de novo.

To better understand how external signals couple with intracellular factors to generate an asymmetric response, we investigated the regulation of yeast Gβγ activity in response to a gradient or uniform field of pheromone. We show that in addition to Gβγ, the receptor and Ga are required not only to sense pheromone gradients during chemotropism but also for directionally persistent growth during de novo polarization. In addition, by using a series of mutations in Gβ (Ste4) to disrupt regulation of Gβγ by Ga, we observed qualitatively different polarity phenotypes depending on which Ga–Gβ interaction interface is disrupted. Our results suggest that one Ga–Gβ interface controls signaling, whereas the other facilitates directional responses by allowing receptor–Gaβ coupling.

MATERIALS AND METHODS

Yeast Strains

Yeast strains are listed in Table 1. P_{GAL1–STE4}×ΔCTM was integrated at the HIS3 locus by transformation with Nhel-digested pPP1266 to create strains PPY1303, PPY1304, PPY1306, and PPY1307. P_{GAL1–STE7}×ΔCTM-ST7 was integrated at the HIS3 locus by transformation with Nhel-digested pPI1270 to create strains PPY1309, PPY1310, PPY1311, PPY1312, PPY1313, PPY1314, PPY1395, and PPY1952.

Plasmids

Plasmids used in this study are listed in Table 2. Ste4 mutants defective at binding Gpa1 (see next section) were originally isolated as variants of the two-hybrid construct pPP268 (identical to pGAD-STE4 except for an extra SpH site following STE4) and then were transferred as EcoRI–BamHI fragments to a two-hybrid construct with a stronger promoter, pGADXP-STE4. All mutations were also transferred into pPP268 (CEN URA3 STE4) as PstI–XhoI or BglII–BsaI (for W411R) fragments; pPP268 contains STE4. All mutants were transferred as MscI–XhoI fragments from the PGAL1–STE4–GPA1 fusion construct pST4-GPA1-b (Klein et al., 2000). These STE4–GPA1 fusions were then placed under control of the native STE4 promoter by transferring MscI–BspEII fragments from these PGAL1–STE4–GPA1 constructions into pPP226, which contains STE4 on a 4-kb EcoRI fragment, from +2045 to +2003, in the EcoRI site of pRS316.

To construct STE4–GPA1 fusions harboring the GPA1–Q323L mutation, the Q323L mutation was first transferred on a SpHl–SpHl fragment from pRS316–GPA1–Q232L (Papanovich et al., 1998) into the STE4–GPA1 fusion construct pPP1341, creating pPP1859. The GPA1–Q323L fusion was then transferred into pPP3343 to create pPP2801. To place these mutant STE4–GPA1 fusions under control of the GAL1 promoter, the MscI–BspEII fragments from pPP1859 and pPP2801 were transferred into pSTE4-GPA1-b (Klein et al., 2000), creating pPP2907.

Plasmid pPP2711 (CEN TRP1 GPA1) was created by polymerase chain reaction (PCR) amplification of GPA1 (–201 to +1707) and ligation as a SacI–KpnI fragment into pSI314 (Sikorski and Hieter, 1989). The GPA1 mutation Q323L and K216E were transferred into pPP2711 as BglII–BspEII fragments from pRS316–GPA1–Q323L (Papanovich et al., 1998) and YCplac22–GPA1–K216E (Metodieva et al., 2002), to create pPP2802 and pPP2743, respectively. The GPA1 mutants E206K and E208A were generated by site-directed mutagenesis of YCplac22–GPA1 (Wilson and Botstein, 1994) and YCplac22–GPA1 (Harris et al., 1996), creating plasmids pPP1501, pPP1502, pPP1503, and pPP1505. The P_{GAL1–STE7} construct pPP2773 was made by transferring STE7 as a BamHII–PstI fragment from pG7 (Harris et al., 2001) into the CEN TRP1–STE7×ΔCTM vector pPP449 (Pryciak and Hartwell, 1998).

To create epitope-tagged forms of Ste4 and Gpa1, we first introduced unique NolI sites into their coding sequences. For STE4, the NolI site was added between a repeat of the first two codons (ATG GCA ggc ggc cgc ATG GCA) in a myc13–NotI cassette, which we used to transform pPP226 and pPP2743. To create the myc13–P_{GAL1–STE4}–GPA1 fusion. Although these derivatives were functional in vivo, the myc13 epitope caused reduced signaling in FUS1-lacZ and halo assays, and thus they were used only to test effects of Ste4 mutants on protein levels and the mating competence pathway. Finally, the intact N and C termini of the protein are functionally important. Hence, the NolI site was added within a yeast-specific insert region (Lambright et al., 1996), overlapping codons 161–163 (Agc CGc CGc) and introducing a conservative residue change (T161S). An NIAc–NolI cassette was then inserted, creating pPP275. These derivatives were fully functional in vivo.

Isolation of Ste4 Mutants

1. A genetic screen was conducted using a two-hybrid assay to isolate Ste4 mutants with specific defects in binding Gpa1. Libraries of ste4 mutants (made by error-prone PCR) were created in a two-hybrid activation-domain vector (pGAD424) as described previously (Pryciak and Hartwell, 1996), and they were transformed into a MATa two-hybrid reporter strain (L40). Transformants were mated by replica plating to a MATa strain (AMR70) harboring DNA-binding domain (DBD) fusions to either Gpa1 (pHTM-GPA1) or Ste4 (pHTM-STE4). Clones identified that showed reduced interaction with Gpa1 but not with Ste18. Secondary screens tested interaction with Ste5 (pM276/p16) and Far1 (pPP743). All isolated sequences harbored mutations in residues that contact Ga in crystal structures (Lambright et al., 1996; Sondek et al., 1996). Eighty-two isolates (and their mutations) were as follows: Gm3–E41 (L154S), Gm3–C7 (K126E), Gm3–B47 (K126E D370G), Gm5–E73 (L117R), Gm5–H124 (D224V), Gm5–J38 (D224E D402E), and Gm5–I22 (S164W K213D). The D224A mutation in Gm4-I22 was separated from the other isolates (and their mutations) were as follows: Gm3–E41 (L154S), Gm3–C7 (K126E), Gm3–B47 (K126E D370G), Gm5–E73 (L117R), Gm5–H124 (D224V), Gm5–J38 (D224E D402E), and Gm5–I22 (S164W K213D). The D224A mutation in Gm4-I22 was separated from the other isolates by swapping restriction fragments; although largely responsible for the Gpa1-binding defect, the single D227A mutation was not as severe as the original quadruple mutant, and therefore all assays reported here for D227A used the stronger quadruple mutant. The isolate Gm3–E41 (L154S) was briefly reported previously (Pryciak and Hartwell, 1996). Because this L154S mutant did not show the strong chemotropism defects seen with N-terminal interface mutants (see Results), and because both Leu154 and the neighboring Asn156 residue are predicted to contact Gpa1 (Lambright et al., 1996; Sondek et al., 1996), we made an additional mutant (L154S N156K) in an attempt to cause a more severe disruption. In fact, this mutant and the original L154S mutant were effectively indistinguishable from each other and only the mutants with the more drastic substitution (L154R N156K) is analyzed here. In addition, for comparison to the phenotypically strong mutants L117R and K126E, we made less drastic substitutions at the same positions (L117A, L117G, L117V, K126A, and K126N).

2. Another screen was designed to isolate Ste4 mutants with reduced mating efficiency but normal pathway signaling. A ste4 mutant library was created in a pGAL1–STE4 vector (pYES-R) as described previously (Pryciak and Hartwell, 1996), and they were transformed into a MATa–MATa strain. Transformants were mated (on galactose medium) to a mixed lawn of pheromone-producing and pheromoneless MATa cells, and screened for clones showing reduced mating only with the pheromone-
producing MATα partner. The same clones were also screened for intact FUS1-lacZ induction (on galactose plates), by using a filter-based β-galactosidase assay (Bartel and Fields, 1995). Five isolates with the strongest mating defects were sequenced. One had multiple mutations and was not pursued further. Each of the other harbored mutations at Lys126 (three were K126E, one was K126N). One isolate, called m3-D10 was not pursued further. The other mutations had weak effects on Gpa1 binding (see Results), consistent with their absence from genetic screens.

3. A Ste4 mutant with Gpa1-binding defects, W136R L138F, which was isolated in a previous screen (Whiteley et al., 1994), was also studied here. This mutation was transferred by PCR from its original context (CEN HIS3 Pgal-SCZ::STE5(H131) FUS1-lacZ). The D97G K380E mutation disrupts binding to the Gα N-CTM (CEN HIS3 Pgal-SCZ::STE5(H131) FUS1-lacZ). The T181R Y183N mutant was defective in all assays, and it was close to a predicted Gα-binding partner; each mutation lies near nine regions. Therefore, the other three regions (composed of 6 residues) were mutated, using drastic residue changes to disrupt Gpa1 binding as severely as possible. These included the triple mutation N92G K94E S96A, the double mutation T181R Y183N, and the single mutation W411R. The T181R Y183N mutant was defective in all assays, and it was defective at binding all partners tested (including Ste18), suggesting it may be misfolded, and so it was not pursued further. The other mutations had only weak effects on Gpa1 binding (see Results), consistent with their absence from genetic screens.

5. Additional mutants used as controls were isolated in screens for Ste4 mutants with Ste18-binding defects (e.g., CEN HIs3 F30 [D97K K380E]) (Pryciak and Hartwell, 1996) or with defects in binding Ste4 and Farc1 (e.g., Gm3-J70 [L49P]). The D97K K380E mutation disrupts binding to the Gγ subunit Ste18 and hence to Gγ-binding partners; each mutation lies close to a predicted Gγ-contact residue (W100 and L379) (Sondek et al., 1998) but that was not known which mutation disrupts Ste18 binding (or whether both contribute). The L49P mutant was isolated in a screen to be described elsewhere, but it harbors a mutation identical to a mutation found previously (Dowell et al., 1998).
Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Alias</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td>pPP120</td>
<td>pRD-STE11-H3</td>
<td>CEN URA3 P\textit{GAL1}\text{-GST-STE11}\text{ΔN}</td>
<td>Neiman and Herskowitz (1994)</td>
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<td>pPP126</td>
<td>YCPGPA1</td>
<td>CEN LEU2 GPA1</td>
<td>Stone and Reed (1990)</td>
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<td>pPP134</td>
<td>pNC252</td>
<td>2 μm URA3 P\textit{GAL1}-ST12</td>
<td>Pryciak and Hartwell (1996)</td>
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<td>pPP226a</td>
<td>p316-ST4-a</td>
<td>CEN URA3 P\textit{STE4}-STE4 (-2045 to +2003)</td>
<td>This study</td>
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<td>pGAD424</td>
<td>2 μm LEU2 GAL4-AD vector</td>
<td>Bartel and Fields (1995)</td>
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<td>pPP247</td>
<td>pBTH-GPA1</td>
<td>2 μm TRP1 lexA-DBD-GPA1</td>
<td>Pryciak and Hartwell (1996)</td>
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<td>pPP249</td>
<td>pGAD-STE4</td>
<td>2 μm LEU2 GAL4-AD-STE4</td>
<td>Pryciak and Hartwell (1996)</td>
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<td>pPP255</td>
<td>pBTH-STE18</td>
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<td>Pryciak and Hartwell (1996)</td>
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<td>pM276p16</td>
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<td>Pryciak and Huntress (1998)</td>
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<td>pPP479</td>
<td>p14-G55AN-CTM</td>
<td>CEN HIS3 P\textit{GAL1}-ste5AN-CTM (= Snc2 TM domain)</td>
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<td>pRS316</td>
<td>CEN URA3 vector</td>
<td>Sikorski and Hieter (1989)</td>
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<td>pPP1151a</td>
<td>pGT-STE4</td>
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<td>Klein et al. (2000)</td>
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<td>p316-P-ST4AB</td>
<td>CEN URA3 P\textit{GAL1}-ST4(-780 to +1616)</td>
<td>This study</td>
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</table>

a.a., amino acids; TM, transmembrane.

These plasmids, containing wild-type \textit{STE4}, serve as the parental constructs for multiple mutant derivatives used in this study encompassing 71 additional plasmids, which consist of 15 mutant derivatives each of pPP268 and pPP643, 13 derivatives of pPP2968, six derivatives each of pPP1150 and pPP1340, and four derivatives each of pPP226, pPP1151, pPP2838, and pPP2839.

Microscopy

For de novo and default polarization assays, transformants were grown in selective rafinose media, and expression of \textit{P\textit{GAL1}}-driven constructs was induced by addition of galactose to 2%; where indicated, 10 μM α factor was also added simultaneously. After 2–8 h induction, cells were visualized without fixation. Differential labeling of old and new cell surface growth with fluorophore-conjugated concanavalin A (ConA) used methods described previously (Nern and Arkowitz, 2000; Matheos et al., 2004). Cells were grown first in synthetic rafinose medium, labeled with 0.1 mg/ml fluorescein isothiocyanate (FITC)-ConA, labeled with 2% galactose ≤ 10 μM α factor for 4 h, and then labeled with 0.05 mg/ml tetramethylrhodamine B isothiocyanate (TRITC)-ConA.

Mating Assays

For patch mating assays, cells were patched directly onto a lawn of partner \textit{PT2a} cells, mated overnight at 30°C, and then diploids were selected by replication to minimal media. After an immediate (1°) replica was made, more dilute replicas were made by repeating the replication of the master mating plate twice more, using a fresh velvet each time, to create 2° and 3° replicas (Harris et al., 2001). Replicates were incubated for 2 d at 30°C. For pheromone confusion assays, α factor was spread on one plate to give a final concentration of 30 μM (unless indicated otherwise) and allowed to dry before the PT2a lawn was spread.

Quantitative mating assays followed previous methods (Pryciak and Huntress, 1998; Lamson et al., 2002). In brief, plasmid-transformed cells were grown overnight in selective rafinose medium, and then 0.5–5 × 10^7 cells were mixed with an equal number of wild-type α cells (strain PT2a) and collected onto filters. The filters were placed on SC/rafinose/galactose plates and incubated for 6–7 h (unless indicated otherwise) at 30°C. Afterward, filters were suspended in phosphate-buffered saline (PBS), and serial dilutions were plated on minimal media to select for diploids. Mating efficiency was calculated as the percentage of input haploid cells (before mating) that formed diploid colonies (after mating). Where indicated, a factor was spread on the mating plate (to give a final concentration of 30 μM) before filters were added.

To analyze zygote formation, mated cells were suspended in PBS, sonicated, and fixed in 5% formaldehyde. Cells were imaged by differential
interference contrast microscopy (DIC) and green fluorescent protein (GFP) microscopy.

**Pheromone Response, β-Galactosidase, and Two-Hybrid Assays**

Halo assays of growth arrest were performed by plating cells on -Trp-Ura plates and overlaying with filters containing 20 μl of 1 mM α factor (Lamson et al., 2002). Plates were incubated for 2 d at 30°C. For FUS1-lacZ transcriptional induction assays were performed as described previously (Pryciak and Huntress, 1998; Lamson et al., 2002). Induction by pathway-activating constructs under control of the GAL1 promoter was measured 4 h after addition of 2% galactose ± 10 μM α factor to cultures grown in raffinose medium. For FUS1-lacZ assays comparing mutant derivatives of P<sub>GAL1</sub>-STE4 in steΔ cells, or STE4 derivatives in steΔ ste7Δ + P<sub>GAL1</sub>-STE7 cells, cultures in raffinose medium were pretreated with 2% galactose for 1 h and then treated for 2 h with 2% galactose ± 10 μM α factor.

β-Galactosidase and two-hybrid assays were performed as described previously (Pryciak and Hartwell, 1996; Lamson et al., 2002).

**Cell Lysates and Protein Analysis**

To test coimmunoprecipitation of myc-13-Ste4 with HA-3-Gpa1, clarified cell lysates were prepared by glass bead lysis, incubated with mouse anti-myc (9E10; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-hemagglutinin (HA) (HA.11; Covance Research Products, Princeton, NJ) antibodies and precipitated with protein G-agarose beads by using methods described previously (Lamson et al., 2002). After separation by SDS-polyacrylamide gel electrophoresis (PAGE) and transfer to polyvinylidene difluoride membranes, blots were probed with rabbit anti-myc (A-14; Santa Cruz Biotechnology) or rabbit anti-HA (HA.11; Covance Research Products). To analyze steady-state levels of Ste4 and Ste4-Gpa1 fusion proteins, crude whole-cell extracts were prepared by pelleting 1.5 ml of culture (OD<sub>600</sub> = 0.5), resuspension in 45 μl of SDS-PAGE loading buffer supplemented with protease inhibitors (Lamson et al., 2002), and boiling for 10 min. After pelleting cell debris for 2 min, 10 μl was analyzed by SDS-PAGE, blotted, and probed with rabbit anti-myc (A-14; Santa Cruz Biotechnology).

**RESULTS**

**Separating the Polarity Role of Gβγ from Its Signaling Role**

The Gβγ dimer normally performs two roles in the mating pathway: it activates MAP kinase cascade signaling and it regulates proteins that control cell polarity. Our goal in this work was to study the polarity role of the receptor–Gβγ module in isolation from its requirement in activating the MAP kinase cascade. Therefore, we used a variety of methods to activate signaling independent of pheromone and Gβγ, and then studied how perturbing Gβγ function affects chemotropism and cell polarization. This strategy is an extension of one used previously in which overexpression of the transcription factor Ste12 was used to study the mating role of various MAP kinase pathway components while bypassing their role in transcriptional induction (Schrick et al., 1997). Here, we used several newer reagents such as membrane-targeted versions of Ste5 (Ste5ΔN-CTM and Ste5ΔN-Sec22), which can promote robust MAP kinase cascade signaling, wild-type levels of mating, and normal polarized morphogenesis (Pryciak and Huntress, 1998; Harris et al., 2001). To ensure that communication between Gβγ and Ste5 was severed, these reagents used a truncated form of Ste5 that lacks the Gβγ-binding site (Ste5ΔN), and all assays were performed in strains where the genomic STE5 locus was deleted (ste5Δ). For comparison, we also used a constitutively active form of Ste11 (Ste11ΔN) (Cairns et al., 1992; Neiman and Herskowitz, 1994) and overexpressed Ste12 (Dolan and Fields, 1990; Schrick et al., 1997). When signaling output was measured by induction of a transcriptional reporter construct (FUS1-lacZ), we found that some bypass methods caused stronger activation than others, but all were independent of Ste4 (Gβγ) and pheromone (Figure 1A, right).

We then used these signaling bypass methods to address the role of Gβγ in chemotropic mating. Chemotropism was monitored using a “pheromone confusion” assay (Dorer et al., 1995; Nern and Arkowitz, 1998), in which mating success is compared in the absence versus presence of excess exogenously added pheromone (α factor), which obscures natural pheromone gradients emanating from partner cells. Chemotropically proficient cells can use pheromone gradients to locate mating partners, and thus they mate with higher efficiency when pheromone gradients are left intact (−α factor) than when gradients are obscured (+α factor); cells defective at chemotropism cannot follow pheromone gradients, and thus they mate at the lower efficiency under either condition. Thus, this assay does not directly monitor directional growth, but rather it infers the ability of cells to detect and use pheromone gradients. Unlike earlier measures of “mating partner discrimination” (Jackson and Hartwell, 1990; Jackson et al., 1991), chemotropically proficient in the pheromone confusion assay requires both Far1 and Far1-Cdc24 binding (Dorer et al., 1995; Valtz et al., 1995; Nern and Arkowitz, 1998), and it accurately reflects the ability of cells to establish a new polarization axis along pheromone gradients (Valtz et al., 1995; Nern and Arkowitz, 1998). Using this assay, we found that cells expressing Ste4 were proficient at chemotropism, whereas those lacking Ste4 were defective (Figure 1A, left). Among the different bypass methods, the membrane-targeted Ste5 reagents promoted the most efficient mating, but a consistent behavior emerged regardless of the bypass method or the absolute signaling level; namely, removal of Ste4 or addition of excess pheromone disrupted chemotropic mating without altering MAP kinase pathway signaling.

Gβγ activates MAP kinase cascade signaling via interactions with Ste5 and Ste20 (Whiteway et al., 1995; Inouye et al., 1997; Feng et al., 1998; Leeuw et al., 1998; Pryciak and Huntress, 1998). To unequivocally determine whether these interactions are dispensable for the chemotropic role of Gβγ, we performed quantitative assays of chemotropic mating in ste5Δ ste20Δ cells, by using activated Ste11 (Ste11ΔN) or excess Ste12 to induce signaling or transcription. Indeed, these cells were proficient at chemotropism, whereas strains that also lacked Ste4 (ste4Δ ste5Δ ste20Δ) were not (Figure 1B). Thus, the chemotropism role of Gβγ does not require it to interact with Ste5 or Ste20. These findings provided a framework from which to further probe the chemotropism and polarity functions of the receptor–Gβγ module without concern for their effects on MAP kinase pathway signaling.

**The Pheromone Receptor and All Three Gα Subunits Are Required for Chemotropism**

The ability of Gβγ to mediate chemotropism without regulating MAP kinase cascade signaling is consistent with the fact that Gβγ interacts directly with polarity proteins via Far1 (Butty et al., 1998; Nern and Arkowitz, 1998, 1999). However, because chemotropism is a directional phenomenon, it would be logical that Gβγ–Far1 binding could help guide polarization in the proper direction only if Gβγ was activated in a spatially asymmetric manner, congruent with the pheromone gradient. Because Gβγ activation is regulated by the receptor and Gα subunit, we directly compared the requirement for the receptor and all three G protein subunits in chemotropic mating assays. As mentioned above, MAP kinase signaling, transcription, or both were activated independent of Gβγ, so that genetic perturbation of the receptor–Gαβγ module would affect only chemotropism. We found that only the cells with an intact receptor–Gαβγ module could use pheromone gradients to increase...
is important to note that under these signaling bypass conditions, receptor-Gαβγ and receptor is separate from signaling. (A) Chemotropism requires Ste4 even when its signaling role is bypassed. Strains PYY858 (ste5Δ) and PYY886 (ste5Δ ste4Δ) harbored galactose-inducible forms of Ste5 (pPP452), Ste5Δ-CTM (pPPS13), Ste5Δ-Ste2 (pPP524), Ste5Δ-CTM (pPP575), or Ste12 (pPP741) or PYY8271). Chemotropic proficiency (left) was assessed by patch matings performed in the absence (−) or presence (+) of exogenous α factor. The mating results using Ste5 derivatives show the more-dilute 2° replica, whereas the others show the 1° replica (see Materials and Methods). Results were similar in both a and α cells, and in both W303 and 381G strain backgrounds (unpublished observations). Transcriptional activation of FUS1-lacZ (right) is shown for the same strains and plasmids after galactose induction (± α factor). Bars, mean ± SD (n = 3). To emphasize that transcription levels were not the primary determinant of mating efficiency, results are shown using Ste12 overexpression constructs that yield high (hi) or low (lo) levels of transcriptional induction (due to different Gαβγ constructs) (B). Ste4 can perform its chemotropism role without Ste5 and Ste20. Quantitative matings were performed ± exogenous α factor. Strains PYY863 and PYY842 harbored galactose-inducible Ste5Δ-CTM (pPP513), Ste5Δ-CTM (pRD-STE11-H3) or Ste12 (pNC252). Bars, mean ± range (n = 2). (C) An intact receptor-Gαβγ module is required for chemotropism. Signaling was activated by galactose-inducible constructs (pPP513, pPP575, and pPP741) in the indicated strains (PYY858, PYY979, PYY978, PYY886, and PYY989). Chemotropism was monitored by patch or quantitative mating assays in the absence (−) or presence (+) of α factor. Bars, mean ± SD (for Ste5Δ-CTM; n = 4) or mean ± range (n = 2). Transcriptional activation of FUS1-lacZ by the same constructs after galactose induction ± α factor was similar in all strains (data not shown). (D) Zygote formation. Strains from C. harboring galactose-inducible GFP-Ste5Δ-CTM (pPP513), were mated with PT2α partner cells for 5.5 h. Representative fields of DIC and fluorescence (GFP) images are shown. We counted 500 cells for each mixture, and the percentage that were zygotes is shown.

their mating success, whereas cells lacking the receptor (ste5Δ ste2Δ) or any one of the G protein subunits (ste5Δ gpa1Δ, ste5Δ ste4Δ, or ste5Δ ste18Δ) could not (Figure 1C). It is important to note that under these signaling bypass conditions, the receptor–Gαβγ module can only contribute to mating success when pheromone gradients are intact, whereas it performs no detectable role when gradients are absent (i.e., the wild-type and null alleles of each component are indistinguishable).

Microscopic analysis (Figure 1D) confirmed that the intact receptor–Gαβγ module allows cells to locate and fuse with mating partners, as judged by the formation of dumbbell-shaped zygotes, although the mating-defective cells could still form polarized mating projections. Therefore, under these conditions the receptor-Gαβγ module is required for polarization per se, but for properly guiding cell polarization toward a mating partner. Note that these findings are consistent with the expectation that polarization in the “correct” direction (i.e., toward the source of pheromone) should require spatial regulation of Gβγ activity, and so they do not necessarily imply that the receptor and/or Go perform separate polarization functions.

Free Gβγ Is Insufficient for De Novo Polarization

To study the polarity function of the receptor–Gαβγ module in a setting where cells do not have to detect the direction of a localized stimulus, we assayed de novo polarization in response to a uniform field of pheromone. Polarization was restricted to the de novo pathway by using rsr1Δ mutant strains, in which the default pathway is inactivated (Nern and Arkowitz, 1999, 2000). First, we tested whether pheromone had a role in de novo polarization beyond activating the MAP kinase cascade. Pathway signaling was activated by expressing either Ste5Δ-CTM (P\text{GAL1-STE5Δ-CTM}) or an activated form of Ste11 fused to Ste7 (P\text{GAL1-STE11Δ-STE7}), which permits normal mating morphology by reducing cross-activation of other signaling pathways (Harris et al., 2001). Despite being able to trigger default polarization (i.e., in RSR1 cells), pathway activation by Ste5Δ-CTM or Ste11Δ-Ste7 could not trigger de novo polarization (i.e., in rsr1Δ cells) (Figure 2A). This inability was not due to interference from excess MAP kinase pathway signaling, as cells harboring these activators (ste5Δ + P\text{GAL1-STE5Δ-CTM} or P\text{GAL1-STE11Δ-STE7}) could undergo de novo polarization when pheromone was added (Figure 2B). Importantly, we found that de novo polarization requires Gβγ activity, as cells lacking the Gβ subunit (ste5Δ + P\text{GAL1-STE11Δ-STE7}) did not polarize even when pheromone was added (Figure 2B). Notably, however, communication between Gβγ and Ste9 was not required, because pheromone could stimulate polarization in cells lacking Ste5 (ste5Δ + P\text{GAL1-STE11Δ-STE7}) (Figure 2B). As expected, transcriptional
with chemotropism, the ability of pheromone and Gβγ to regulate de novo polarization is separable from any regulatory effects on the MAP kinase cascade.

Because de novo polarization does not require cells to sense the direction from which pheromone emanates, and because Gβγ interacts directly with Far1 and Cdc24, (Butty et al., 1998; Nern and Arkowitz, 1998, 1999), it seemed possible that Gβγ alone would be sufficient to promote de novo polarization, with pheromone serving only to generate free Gβγ by dissociating the Gαβγ heterotrimer. To test this view, Gβγ was activated without using pheromone, by deletion of GPA1 or by overexpression of STE4 (PGAL1-STE4). To avoid persistent growth arrest due to constitutive MAP kinase pathway signaling, GPA1 was deleted in a steΔ strain harboring PGAL1-STE11ΔN-STE7 (Figure 2B) or in a steΔ strain harboring PGAL1-STE4 (Figure 2C). Remarkably, although each method of Gβγ activation (i.e., gpa1Δ or PGAL1-STE4) could induce cell cycle arrest and cell polarization by the default pathway (i.e., in RSR1 cells), neither method could induce de novo polarization (i.e., in rsr1Δ cells) (Figure 2, B and C). Furthermore, the ability of pheromone to trigger de novo polarization was actually eliminated by the gpa1Δ mutation (Figure 2, B and C, right columns), and thus it requires Gα in addition to Gβγ. Therefore, although Gβγ can directly communicate with polarization proteins, free Gβγ is not sufficient for de novo polarization. This deficiency might reflect a separate role for receptors or GTP-loaded Gα, or it might indicate that receptors and Gα can promote an asymmetric distribution of Gβγ activity even when external pheromone is distributed uniformly.

To follow the pattern of cell surface growth, we used fluorophore-conjugated ConA to differentially label cell wall formed before versus during the period of mating pathway activation. It was previously shown that when de novo polarization failed due to disruption of Far1–Cdc24 interaction, cells could initiate polarized growth, but the polarization axis could not be maintained; consequently, it wandered about a broad region of the cell periphery (Nern and Arkowitz, 2000). Similarly, we observed that when cells failed de novo polarization due to absence of pheromone, Gpa1, or Ste4, they still showed asymmetric cell surface growth, but it was broadly distributed across one hemisphere (Figure 2, B and C, right). Thus, consistent with the requirement for Fus3 (Matheos et al., 2004), our results suggest that activation of the MAP kinase cascade is sufficient to initiate asymmetric growth, even in the absence of Gα or Gβγ. Nevertheless, organization of a well-focused and persistent polarization axis requires both Far1–Cdc24 interaction and an intact receptor–Gαβγ module.

Gβ Mutants Reveal Distinct Roles for Two Gα–Gβ Binding Interfaces

To further investigate the requirement for the intact receptor–Gαβγ module in chemotropism and de novo polarization, we used a series of Gβ mutants to disrupt regulation of Gβγ by the Gα subunit. Crystal structures of mammalian Gαβγ heterotrimers reveal two contact surfaces between Gα and Gβ, termed the “switch interface” and the “N-terminal interface” (Wall et al., 1995; Lambright et al., 1996). The switch (Sw) interface involves a region of Gα that undergoes a conformational switch upon GTP binding, whereas the N-terminal (Nt) interface involves an N-terminal helix of Gα that protrudes away from the globular GTPase domain (Figure 3A). The Gα and Gβ residues contacting one another in each interface are well conserved between mammalian and yeast counterparts (Lambright et al., 1996; Sondek et al.,
were derived from multiple sources; briefly, mutations in six gpa1 (S. C. Strickfaden and P. M. Pryciak, 1999) with Gpa1. HA-tagged Gpa1 (pPP2775) or vector (pRS316) was coexpressed with myc-tagged Ste4 (derivatives of pPP2838; vector/pH11005 was expressed from the native STE4 promoter). (B) Effects of Ste4 mutations on Gpa1 binding. Mutated Ste4 residues are listed alongside their homologous residues in mammalian Gβ1. Activation domain fusions (AD-Ste4) were expressed in a gpa1Δ ste11Δ two-hybrid tester strain (PPY1158), with DNA-binding domain fusions to Gpa1 (pPP3047) or to the Gpa1-binding regions of Ste5 (pPP305) or Far1 (pPP743). Binding was detected by growth on -His plates ± 3-aminotriazole (3-AT; an inhibitor of His3), or by quantitative β-galactosidase assay (bars, mean ± SD; n = 4). To reveal the full range of effects on Gpa1 binding, quantitative assays used AD-Ste4 fusions expressed from either a strong promoter (derivatives of pPP643) or a weak promoter (derivatives of pPP268); plate growth assays used pPP643 derivatives. As controls (at bottom), two additional Ste4 mutations outside the Ga–Gβ interfaces (see Materials and Methods) were analyzed in parallel; these disrupt binding to Ste5 and Far1, but not to Gpa1. (C) Ste4 mutations cause deregulated signaling. To avoid constitutive growth arrest, the Ste4 mutants were tested in ste4Δ ste7Δ cells (PPY1662) harboring P4A1/H1 STE7 (pPP2773). Fus1-lacZ induction (mean ± SD; n = 4) was measured 2 h after addition of galactose ± α factor. Ste4 was expressed from the native STE4 promoter (derivatives of pPP2968; vector = pRS316). (D) Ste4 mutations disrupt coimmunoprecipitation with Gpa1. HA-tagged Gpa1 (pPP2775) or vector (pRS314) was coexpressed with myc-tagged Ste4 (derivatives of pPP2838; vector = pRS316) in strain PPY1230 (gpa1Δ ste4Δ ste5Δ). Cell extracts were analyzed by immunoprecipitation (IP) and immunoblotting ( blot).
Figure 4. Ste4 mutations in the Nt interface, but not the Sw interface, disrupt polarity control. (A) Chemotropism proficiency was assessed by patch mating assays performed in the absence (−) or presence (+) of exogenous α factor. PYP867 (ste4Δ ste5Δ) harbored P<sub>GAL1</sub> STE5ΔN-SEC22 (pPP524) and either vector (pRS316) or Ste4 variants expressed from the native STE4 promoter (derivatives of pPP2968).

The effects of the Ste4 mutations on Gpa1 binding are summarized for reference (see Figure 3B). (B) Analysis of additional Nt interface mutants. Left, two-hybrid binding assays (as in Figure 3B) using AD–Ste4 fusions (derivatives of pPP643; vector=pPP636) and DBD-Gpa1 (pPP247) in PYP1158. (Note: in quantitative assays using the lacZ reporter, the Gpa1 interaction signal of Ste4-K126A was measurably reduced to 27% of Ste4-WT.) Right, quantitative assays of Ste4 function during chemotropic mating. PYP876 (ste4Δ ste5Δ) harbored P<sub>GAL1</sub> STE5ΔN-SEC22 (pPP524) and either vector (pRS316) or Ste4 variants expressed from the PGAL1-STE4 promoter (derivatives of pPP1151) in PYP794 (ste4Δ rsr1Δ) or PYP1248 (ste4Δ rsr1Δ), and cell morphology was examined after induction with galactose ± α factor.

The Ste4 mutations change Lys126 to Glu (K126E), thereby reversing the charge. To make a compensatory charge-reversal mutation in Gpa1, we changed Glu28 to Lys (E28K). This Gpa1–E28K mutation, but not a control mutation (Gpa1–E28A), restored measurable binding with Ste4-K126E (Figure 5B), although not to wild-type levels. Also, the Gpa1 E28K and E28A mutations each reduced binding to wild-type Ste4, although by a mild degree that was most noticeable when Ste4 was expressed at lower levels from a weak promoter (Figure 5B). It was not entirely surprising that Gpa1–E28K only partially restored binding to Ste4-K126E and that Ste4-K126E caused a stronger binding defect than Gpa1–E28K, because the mammalian Gβ residue homologous to Ste4 Lys126 contacts Ga not only through this ion pair but also through hydrogen bonding and van der Waals interactions (Lambright et al., 1996). Nevertheless, these binding effects were enough to confer informative phenotypes in mating assays.

Indeed, the chemotropism defect of the Ste4-K126E mutant was at least partially suppressed by the Gpa1–E28K mutant, because mating of cells harboring Ste4-K126E was more efficient when coexpressed with Gpa1-E28K than with Gpa1–wild type (WT) (Figure 5, C and D). Whereas the Gpa1–E28K mutation reduced mating when combined with Ste4–WT, it increased mating by an average of 4.6-fold when combined with Ste4–K126E (Figure 5D). Although mating was not restored to wild-type levels, it would be unreasonable to expect this given the incomplete restoration of Gpa1–Ste4 binding. Notably, the observed suppression was allele-specific, because Gpa1–E28A did not suppress Ste4–K126E, and neither Gpa1–E28K nor Gpa1–E28A could suppress the
other Nt mutant, Ste4-L117R (Figure 5, C and D). Furthermore, although the Gpa1–E28K mutation improved mating by Ste4-K126E, it reduced mating by Ste4-WL/RF and Ste4-LN/RK, such that these Sw mutants were actually more defective than Ste4-K126E in cells expressing Gpa1–E28K (Figure 5C). This finding makes it highly unlikely that the phenotypic differences between Ste4 Nt and Sw mutations can be explained by their different impact on binding between Gβγ and an unknown factor. Instead, the pattern of allele-specific suppression and enhancement found with the Gpa1–E28K mutation supports a special role for Gα–Gβ binding via the Nt interface in chemotropism and cell polarization, and it suggests that this interface can remain functional when the Sw interface is dissociated by mutation. Because the Gpa1–E28K mutant is sensitized to disruption of the Sw interface, an intact Sw interface may help maintain Gα–Gβ association when the Nt interface is mildly disrupted.

**Differences between Nt and Sw Interface Mutants Are Not Due to Altered Gpa1–Fus3 Interaction**

Gpa1 can interact with the MAPK Fus3 via a docking motif in its N terminus, and mutations in this motif (Gpa1-K21E R22E, denoted Gpa1-EE) disrupt Fus3 binding and reduce mating (Metodiev et al., 2002). This raised the possibility that an intact Nt interface is required mainly to allow proper interaction between the Gpa1 N terminus and Fus3. To test this notion, we used the Gpa1-EE mutant to disrupt interaction between Gpa1 and Fus3, and we found that the Nt and Sw mutations in Ste4 still showed their characteristic phenotypic differences (Figure 6A). We also tested the Ste4 mutants in cells lacking Fus3 (ste4Δ fus3Δ). Here, mating by the Sw interface mutants was not as efficient as that of wild-type Ste4, but it was still more efficient than that of the Nt interface mutants (Figure 6A). Thus, both approaches suggest that the role of the Nt interface, and the different behavior of the Nt versus Sw mutants, cannot be explained by indirect effects on the Gpa1–Fus3 interaction.

It is notable that we did not detect a strong mating defect for the Gpa1-EE mutant alone, suggesting that the Gpa1–Fus3 interaction may not be required for chemotropism. To address this issue further, we compared the roles of Fus3 and Far1. Loss of either protein caused a mating defect, but a much greater defect occurred when both Fus3 and Far1 were absent (Figure 6B), indicating that although both proteins are required for maximum mating efficiency, each protein can perform its function without the other. Moreover, an important distinction between the roles of Fus3 and Far1 was apparent: the fis3Δ cells were proficient at chemotropism (i.e., sensitive to whether pheromone gradients were present or absent), whereas the far1Δ cells were defective (Figure 6B). To rule out the possibility that chemotropism proficiency in fis3Δ cells reflects redundancy between Fus3 and Kss1, we performed additional mating assays using fis3Δ kss1Δ cells (in which sterility was suppressed by PGAL1–STE12). Despite low overall mating efficiency, the fis3Δ kss1Δ cells could still use pheromone gradients, and this behavior required Far1 (Figure 6C). The simplest overall interpretation is that detecting gradients and using gradient information to locate mating partners does not require Fus3, whereas the role of Fus3 in polarized morphogenesis (Matheos et al., 2004) is distinct from gradient sensing per se. These results also imply that, unlike cell cycle arrest (Gartner et al., 1998), phosphorylation of Far1 by Fus3 is dispensable for chemotropism.
The findings described above suggest that proper communication between Gaβγ and the receptor is necessary for directionally persistent polarization even when the pheromone stimulus is provided uniformly. In theory, this receptor-Gaβγ communication could be required solely to promote GTP–GDP nucleotide exchange on Ga, perhaps allowing GTP-bound Ga to perform a polarization role that acts synergistically with Gaβγ. Alternatively, receptor–Gaβγ communication might be required to generate asymmetry in the distribution of Gaβγ activity (and/or Ga-GTP), which otherwise would remain symmetric (e.g., in gaαΔ cells or with constitutively active Ste4 mutants). To address these possibilities, we used an activated mutant form of the Gaα subunit, Gpa1-Q323L (herein referred to as Gpa1-QL), which is defective at GTP hydrolysis (Dohlman et al., 1996; Apano-vitch et al., 1998). We found that simultaneous activation of both Gaα and Gaβγ, by coexpressing Gpa1-QL with Ste4, was still not sufficient for de novo polarization in the absence of pheromone (Figure 8, A and B). This was true regardless of whether Gpa1-QL was expressed with Ste4-WT or with the constitutively active Sw interface mutant, Ste4-WL/RF. In fact, we found that trapping Gpa1 in the GTP-bound state...
was detrimental, because cells expressing Gpa1-QL could not polarize even after exposure to pheromone (Figure 8, A and B, + α factor). Therefore, polarization requires more than just the acquisition of both GTP-bound Ga and active Gβγ.

It seemed possible that activated Ga and Gβγ subunits might have to remain in close mutual proximity and that this might be accomplished during receptor-mediated activation but not by coexpression of mutationally activated subunits. Therefore, to force GTP-bound Ga to remain associated with Gβγ, we incorporated the Gpa1-QL mutation into the Ste4–Gpa1 fusion. In signaling assays, either the Ste4-WL/RF or Gpa1-QL mutations (or both) caused constitutive activity (Figure 8C); yet, none of these fusions could induce de novo polarization in the absence of pheromone (Figure 8, A and B, − α factor). Notably, the fusions containing the Gpa1-QL mutation did promote a detectable increase in elongation (and more so than when the same subunits were expressed as separate polypeptides), but these elongated cells did not form the pointed, pear-shaped shmoo seen during pheromone treatment. Thus, the cells were still missing some aspect of pheromone-induced polarization that can focus morphogenesis to a restricted portion of the cell perimeter.

Furthermore, the ability of pheromone to trigger de novo polarization remained intact when Ste4 was fused to Gpa1, but this was disrupted by the Gpa1-QL mutation (Figure 8, A and B, + α factor). Consistent with these findings, the fusions containing the Gpa1-QL mutation were also defective in chemotropic mating assays (Figure 8D), and the Gpa1-QL mutant (expressed as a separate polypeptide) eliminated the mating advantage of Sw interface mutants over Nt interface mutants (Figure 8E). Thus, interfering with GTP hydrolysis activates Gβγ signaling, but it disrupts cell polarization and chemotropism, regardless of whether Ga is fused to Gβ or kept separate. Finally, it should be noted that the chemotropism and polarity defects shown by the Gpa1-QL mutant are recessive to wild-type Gpa1 (unpublished observations). Together, these findings suggest that the ability of pheromone-bound receptor molecules to guide cell polarization, either along a de novo polarization axis or along pheromone gradients, requires normal coupling to the Ga GTP hydrolysis cycle.

**DISCUSSION**

This work examines how a GPCR and its coupled Gaβγ heterotrimeric coordinate an asymmetric response to external stimuli. Our findings indicate that although yeast Gβγ is known to interact with downstream polarity factors (e.g., Far1, Cdc24), proper control of cell polarity in either the presence or absence of pheromone gradients requires an intact receptor-Gaβγ module and a normal GTP–GDP cycle on Ga. Furthermore, our results suggest distinct roles for the two Ga–Gβ interaction interfaces, whereby the Sw interface controls signaling and the Nt interface governs coupling to the receptor. As such, pheromone control of polarity requires maintenance of the Nt interface but not the Sw interface. Overall, these results suggest that continuous communication between the receptor and Gβγ is important for chemotropism and polarized growth, perhaps to regulate Gβγ in a spatially quantized manner.

**Distinctions between Signaling and Polarity Roles of Gβγ**

Bypassing the role of Gβγ in signaling revealed several interesting points regarding mating and cell polarization. First, the ability of pheromone and Gβγ to regulate chemotop
Figure 8. Polarity control requires GTP hydrolysis by Gpa1. (A and B) De novo polarization was monitored using ste4Δ gpa1Δ rsr1Δ cells (PPY1380) expressing Ste4 and Gpa1 variants as either separate or fused polypeptides, after 4-h induction with galactose ± α factor. Separate Ste4 and Gpa1 subunits were expressed from pPP1151, pPP1228 (= WL/RF derivative of pPP1151), pPP2711, and pPP2802. Ste4–Gpa1 fusions were expressed from derivatives of pPP1150. The Gpa1-QL mutant is defective at GTP hydrolysis. (A) Representative images of the predominant morphologies. (B) Cell morphologies were quantified by blind counting of 200 cells per condition in three separate experiments. Cells were scored as “polarized” if they formed pear-shaped shmoos or elongated to a point at one end; cells were scored as “elongated” if one axis was clearly longer than the other but neither end was pointed. Bars, mean ± SD (n = 3). (C) Transcriptional induction. PPY856 (ste4Δ) cells harboring the indicated Pcal-STE4-GPA1 plasmids (derivatives of pPP1150) were induced with galactose ± α factor. Results for the WT–WT and WL/RF–WT fusion proteins are identical to those for the Pcal–driven constructs in Figure 7E (bottom, middle and right), and they are shown here for comparison. Bars, mean ± SD (n = 4). (D) Fusion proteins harboring the Gpa1-QL mutation are defective at chemotropism. Mating was assayed ± 20 μM α factor by using strain PPY1230 (ste4Δ gpa1Δ ste5Δ) expressing Pcal-GPA1 and Ste4-Gpa1 fusions (as in C). (E) Gpa1-QL eliminates the chemotropic advantage of Sw interface mutants over Nt interface mutants. Mating was assayed in strain PPY1230 (ste4Δ gpa1Δ ste5Δ) expressing Pcal-GPA1 and the indicated combination of Gpa1 and Ste4 (derivatives of pPP226; vector = pRS316).

Heterotrimeric G Protein Dissociation

Several of our findings are relevant to the mechanism of Gαβγ dissociation. Although it is generally accepted that nucleotide exchange causes Gα-GTP to dissociate from Gβγ (Gilman, 1987; Neer, 1995; Cabrera-Vera et al., 2003), some studies suggest that complete dissociation may not be necessary (Rebois et al., 1997; Klein et al., 2000; Levitzki and Klein, 2002; Bunemann et al., 2003; Gales et al., 2006). Indeed, prior work showed that the yeast heterotrimer remained functional when Gα and Gβγ were expressed as a Gβγ–Gα fusion protein (Klein et al., 2000), which should restrict dissociation. We extended these results by showing that this Gβγ–Gα fusion protein performs both signaling and polarity functions indistinguishably from the wild-type (unfused) proteins, even when expressed at native levels. Moreover, the chemotropic and de novo polarization proficiency we observed with the Sw interface mutants indicates that despite being active for signaling, they can still mediate a response to the pheromone ligand whereas the Nt interface mutants cannot. This implies that the Sw mutants maintain a functional Nt interface and hence raises the possibility that the Gαβγ heterotrimer may remain tethered by the Nt interface, allowing continued communication with the receptor (Figure 9). By extension, it is conceivable that this “partial dissociation” state applies not only to the Sw interface mutants but also to receptor-activated Gαβγ. That is, GTP-induced conformational changes in the Switch region of Gα may trigger an initial state in which the Sw interface dissociates but the Nt interface does not.

Several structural considerations are pertinent to this partial dissociation model. First, the N-terminal helix of Gα is implicated in receptor recognition, and it is thought to lie tangential to the membrane (Taylor et al., 1994; Bourne, 1997; Hamm, 1998, 2001; Itoh et al., 2001; Cabrera-Vera et al., 2003). Second, Gαβγ is anchored to the membrane by addition of lipophilic groups to the Gα N terminus and the Gγ C terminus (Wedegaertner et al., 1995), which lie adjacent to each other at one end of the Nt interface (Wall et al., 1995; Lambright et al., 1996) (Figure 9). Membrane insertion of these groups stabilizes the intact heterotrimer (Bigay et al., 1994); thus, it may also stabilize the weak interaction at the Nt interface enough to keep the partially dissociated structure intact in vivo but not in detergent-solubilized cell extracts. Third, the behavior of the Gβγ–Gα fusion protein is compatible with the partial dissociation model, because the link formed between the Gβγ C terminus and the Gα N terminus would constrain separation of the Nt interface but not the
Symmetry Breaking and De Novo Polarization

Symmetry breaking, in which cells or molecular modules self-polarize without a localized cue, is thought to involve the amplification of an initial asymmetry that occurs through stochastic variation (Kirschner et al., 2000; Wedlich-Soldner and Li, 2003). In yeast, symmetry breaking can produce polarized buds in the absence of directional cues (Chant and Herskowitz, 1991; Irazoqui et al., 2003). This has been proposed to result from stochastic variation in the local amount of activated (GTP-bound) Cdc42, which is then amplified by positive feedback loops via two possible pathways. One pathway involves self-assembly of the adaptor protein Bem1, which links Cdc24 and Cdc42 (Butty et al., 2002; Irazoqui et al., 2003; Wedlich-Soldner et al., 2004). The other pathway involves feedback between Cdc42-induced actin polymerization and polarized transport of secretory vesicles carrying Cdc42 as cargo (Gulli et al., 2000; Wedlich-Soldner et al., 2003; Wedlich-Soldner and Li, 2003).

Analogous mechanisms may apply during pheromone response to allow de novo polarization. In principle, breaking the symmetry inherent in a uniform field of pheromone could begin with small differences in receptor–Gαβγ activation. However, our results indicate that activation of the MAP kinase cascade is sufficient to trigger some asymmetry even in the absence of pheromone or G protein subunits. Furthermore, previous studies show that establishment and maintenance of the polarity axis are separable (Nern and Arkowitz, 2000) and that the MAP kinase Fus3 is required to establish asymmetry (Matheos et al., 2004). Hence, the most parsimonious model may be that the MAP kinase pathway imparts the polarity machinery with some inherent organizational ability, whereas the receptor–Gαβγ module controls orientation and directional persistence, either by influencing the initial directional choice or by providing continuous reinforcement of the initial asymmetry.

Our findings implicate a role for continuous communication between pheromone-bound receptors and Gαβγ, because activated Gαβγ and Gα alone cannot promote directionally persistent polarization. Because pheromone receptors are rapidly internalized and redelivered by secretory transport along actin filaments (Jenness and Spatrick, 1986; Schandel and Jenness, 1994; Ayscough and Drubin, 1998), initial asymmetries might become amplified by polarized delivery of new pheromone receptors to the initial landmark, resulting in heightened pheromone response in that location which would reinforce the initial directional choice. The mutations that disrupt de novo polarization (i.e., gpa1-Q323L, or ste4 Nt interface mutations) may disable this positive reinforcement by disrupting the ability to coordinate receptor localization with the activity/localization of Gα, Gβγ, or both. In contrast, the behavior of the Sw interface mutants suggests that, despite constitutively active Gβγ signaling, these mutant heterotrimers remain in regulatory communication with the receptor. Although it remains unclear what this communication achieves in molecular terms, we envision three speculative scenarios: 1) interaction with asymmetrically localized receptors might influence localization of the heterotrimer, thereby causing asymmetrically localized Gβγ activity; 2) the Sw mutants might allow nucleotide exchange on the Gα subunit at sites of pheromone-bound receptors, potentially promoting localized Gα-GTP, which could control polarization in synergy with Gβγ; or 3) pheromone-bound receptors might interact directly with downstream polarity factors, but normal affinity of receptors for ligand might require coupling to a Gαβγ heterotrimer with an intact Nt interface and normal GDP-GTP exchange properties. Distinguishing among these scenarios will require further study.

GTP Hydrolysis and Localized Signaling

GTP hydrolysis is important for polarity control by Cdc42 in both yeast and mammalian cells (Stowers et al., 1995; Irazoqui et al., 2003). Similarly, we found that chemotropism and de novo polarization require GTP hydrolysis by the Gα subunit Gpa1. To provide a directional cue, GTP loading of Gα and the resultant activation of Gβγ may have to occur in a localized manner, which would be obscured if all Gα molecules were locked in the GTP-bound state and distrib-
uted uniformly. Thus, the spatial distribution of active signaling molecules is likely to be critical to their function in guiding cell polarity. To focus this spatial distribution, the activities of these proteins may also need to be rapidly extinguished by mechanisms such as by GTP hydrolysis, phosphorylation, or endocytosis. Indeed, chemotaxis is thought to depend on a combination of local excitation and global inhibition of polarization activities (Iijima et al., 2002). Endocytic clearing allows directionally transported membrane proteins to maintain a polarized distribution (Valdez-Taubas and Pelham, 2003; Marco et al., 2007), and pheromone-regulated polarization in yeast is facilitated by endocytosis signals in receptor C termini (Konopka et al., 1988; Vallier et al., 2002). Thus, localized signaling and delivery may generally work in concert with down-regulatory mechanisms to ensure proper polarity control.

CONCLUSIONS

The findings reported here reveal several unexpected lessons that may be applicable to other examples of cell polarity control, heterotrimeric G protein function, or both. First, the activated Gaβγ heterotrimer (and the resulting downstream signaling) is not inherently able to organize a directionally persistent polarization axis. Second, the requirements of the receptor–Gaβγ module are similar for gradient-controlled polarization and de novo polarization. Third, the two structurally distinct Ga–Gβ interfaces have functionally distinct roles. Fourth, the yeast heterotrimer may be able to function in a partially dissociated state that allows continued regulatory communication with the receptor. If future studies, it will be interesting to compare these behaviors shown by the yeast system to those of other systems.

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REFERENCES


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The protein kinase homologue Ste20p is required to link the yeast pheromone response pathway and contributes to control of both cell shape and signal transduction. Mol. Cell Biol. 16, 2614–2626.


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