# *Dictyostelium* Ric8 is a nonreceptor guanine exchange factor for heterotrimeric G proteins and is important for development and chemotaxis

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Heterotrimeric G proteins couple external signals to the activation of intracellular signal transduction pathways. Agonist-stimulated guanine nucleotide exchange activity of G-protein-coupled receptors results in the exchange of G-protein-bound GDP to GTP and the dissociation and activation of the complex into Gα-GTP and a Gβγ dimer. In *Dictyostelium*, a basal chemotaxis pathway consisting of heterotrimeric and monomeric G proteins is sufficient for chemotaxis. Symmetry breaking and amplification of chemoattractant sensing occurs between heterotrimeric G protein signaling and Ras activation. In a pull-down screen coupled to mass spectrometry, with Gα proteins as bait, we have identified resistant to inhibitors of cholinesterase 8 (Ric8) as a nonreceptor guanine nucleotide exchange factor for Gα-protein. Ric8 is not essential for the initial activation of heterotrimeric G proteins or Ras by uniform chemoattractant; however, it amplifies Gα signaling, which is essential for Ras-mediated symmetry breaking during chemotaxis and development.

Chemotaxis is a dynamic cellular process that leads to the directed movement of cells along extracellular gradients (1, 2). Chemotaxis plays important and diverse roles in different organisms, from tracking down food sources in prokaryotes to helping mediate immune response, tissue maintenance, and organization of embryos in metazoa (3–6). The free soil-living amoeba *Dictyostelium discoideum* has been an important model system for studying the mechanism by which cells sense and respond to chemoattractants (7).

In *Dictyostelium*, the signal cascade for chemotaxis begins with the binding of a chemoattractant (cAMP or folic acid) to specific receptors, resulting in the activation of the associated G proteins Gαβγ and downstream signaling pathways. This results in cytoskeletal rearrangement, with Filamentous-actin (F-actin) at the front and myosin filaments at the rear of the cell (7). *Dictyostelium* contains one Gβ subunit and one Gγ subunit, both of which are essential for chemotaxis to cAMP or folate. Of the 12 identified Gα subunits, Gα2 is coupled to the cAR1 receptor and is essential for cAMP-mediated chemotaxis (8–10), whereas Gα4 is essential for mediating chemotaxis toward folate (11). *Dictyostelium* cells can detect very shallow spatial gradients of ~1% concentration difference across the cell (12). Previously, a basal signaling module was identified that provides Ras activation at the leading edge, which is sufficient for chemotaxis (13). The four Ras-activated pathways, PI3K, TorC2, PLA2, and sGC, are not required for Ras activation and chemotaxis to folate or to steep gradients of cAMP, but they do provide directional memory and improved orientation of the cell, which together allows chemotaxis in more shallow cAMP gradients. Because chemotaxis and Ras activation are completely lost in cells lacking chemoattractant receptors (14), Gβγ (15), and RasC/G (16), the basal signaling module has to consist of at least surface receptors and heterotrimeric and monomeric G proteins (13). The activation of cAMP receptors and dissociation of their associated

G protein, Gα2βγ, is approximately proportional to the steepness of the gradient (17, 18). In contrast, activation of RasC and RasG is much stronger in the front than in the rear of cells undergoing chemotaxis (19–21). This reveals that Ras is the most upstream component of the signaling cascade, which shows stronger activation at the leading edge than the steepness of the gradient, suggesting that symmetry breaking occurs between heterotrimeric G protein signaling and Ras activation.

To gain further insight into the mechanism of symmetry breaking and chemotaxis, we have used a proteomic approach to identify regulators of G-protein signaling. The G proteins Gα2 and Gα4 were used as bait in pull-down screens, and interacting proteins were identified by mass spectroscopy. One of the binding partners, resistant to inhibitors of cholinesterase 8 (Ric8), was characterized as a nonreceptor guanine exchange factor (GEF) for Gα2 and Gα4 protein. Deletion studies reveal that Ric8 is critical for G-protein activation, development, and symmetry breaking of Ras and chemotaxis.

## Results and Discussion

**Ric8 Interacts Specifically with Gα Proteins.** To identify regulators of heterotrimeric G protein signaling, we performed pull-down screens from *Dictyostelium* lysates with purified Gα proteins as bait (Fig. S1*A*). By using mass spectrometry, a *Dictyostelium* homolog of human Ric8 (16% homology) was identified as a potential binding partner of Gα2 and Gα4. Ric8 belongs to a family of proteins that is conserved in fungi and animals but that is absent in plants and does not share conserved domains with other proteins (22). Ric8 has been implicated in the activation of a subset of Gα proteins, including mammalian Gα$_q$, Gα$_i$1, and Gα$_o$ (23, 24). A reverse pull-down mass-spectroscopy experiment with purified GST-fused Ric8 as bait in *Dictyostelium* lysate confirmed its binding to Gα2 and Gα4 and, in addition, revealed binding of Ric8 to Gα1, Gα7, Gα9, and Gα12. Ric8 does not bind to Gβγ (Fig. S1 *A* and *B*).

To confirm that *Dictyostelium* Ric8 can directly interact with Gα, the proteins were expressed and purified from *Escherichia coli* and subsequently used in GSH pull-down experiments (Fig. 1*A*). Using recombinant GST-Gα1, GST-Gα2, or GST-Gα4 as bait, we were able to pull down recombinant Ric8 protein, whereas GST does not bind to Ric8 (Fig. 1*A*). Pull-down experiments in a lysate of *Dictyostelium* cells expressing GFP-tagged Ric8 with the
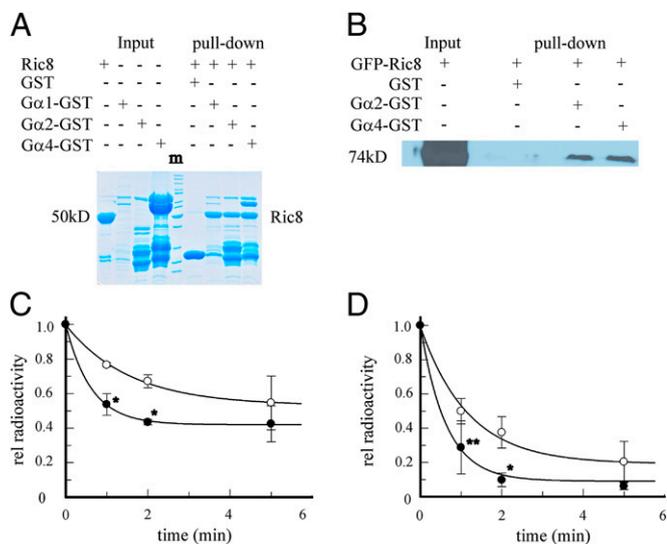
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**Fig. 1.** Ric8 interacts with Gα proteins. (*A*) Pull-down with recombinant purified GST, GST-Gα1, GST-Gα2, or GST-Gα4 as bait and recombinant Ric8 as prey. (*B*) Recombinant GST, GST-Gα2, and GST-Gα4 bound to GSH beads were incubated with GFP-Ric8 cell lysate. Beads were precipitated, and the amount of GFP-Ric8 was detected by Western blotting, using antibody specific for GFP. The figure is representative of three independent experiments. Ric8 is a GEF for Gα proteins. Gα2 (*C*) and Gα4 (*D*) were loaded with $^{3}$H-GDP and incubated with (closed circles) and without (open circles) Ric8 in the presence of excess GDP, and nucleotide release was plotted as the decay of radioactivity with time. Data are mean and SD of at least three independent experiments; significantly different from control without Ric8 at *$P < 0.01$ and **$P < 0.05$, Student $t$ test.

recombinant purified GST-fused Gα proteins as bait (Fig. 1*B*) show that the interaction between Ric8 and Gα proteins can occur in vivo. To determine whether Ric8 binds specifically to heterotrimeric G proteins, we performed a GSH pull-down experiment with recombinant small G proteins as bait. None of the tested GST-tagged Ras, Rap, or Rac proteins showed interaction with Ric8 (Fig. S2 *A* and *B*).

Together, these results demonstrate that *Dictyostelium* Ric8 binds directly and specifically to Gα proteins.

**Ric8 Is a Nonreceptor GEF for Gα Proteins.** G proteins are molecular switches that cycle between an inactive GDP and active GTP bound state. This G-protein cycle is regulated by guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP. Heterotrimeric G proteins are activated by G-protein-coupled receptors. Upon ligand binding, these receptors undergo a conformational change that enables them to catalyze the exchange of inactive GDP-bound to active GTP-bound Gα (25–27). However, recently, nonreceptor GEFs, including Ric8, also have been identified (28–31). To investigate whether *Dictyostelium* Ric8 also acts as a GEF for Gα proteins, we performed in vitro nucleotide exchange assays. For these experiments, recombinant Gα2 and Gα4 proteins were loaded with $^{3}$H-GDP. The exchange reaction was started by the addition of excessive GDP in the presence or absence of purified Ric8. Nucleotide exchange was measured as decay of protein-associated radioactivity caused by the release of $^{3}$H-GDP from Gα2 or Gα4 (Fig. 1 *C* and *D*). In the presence of Ric8, the nucleotide exchange of both Gα2 and Gα4 is ~2.5-fold faster compared with the intrinsic dissociation rate. Consistent with the absence of interaction between Ras proteins and Ric8 in pull-down assays, Ric8 does not stimulate the nucleotide exchange of small G proteins (Fig. S2 *C* and *D*), indicating that Ric8 acts as a GEF specific for Gα proteins.

**Ric8 Is Essential for Development.** To study the role of Ric8 in *Dictyostelium*, the phenotype of cells lacking *ric8* and cells expressing

N-terminal GFP-tagged Ric8 (Ric8$^{OE}$) was analyzed. The knockout strain was generated by homologous recombination, and successful gene disruption was confirmed by PCR (Fig. S3*A*). GFP-Ric8 is uniformly distributed in the cytosol, which does not alter when stimulated with cAMP (Fig. S3*B*). Because both *Dictyostelium* Gα2 and Gα4 are important for multicellular development (9, 11), the Ric8 mutants were tested in an aggregation assay. In wild-type (AX3), aggregation centers are formed after 6 h, Mexican hats are visible after 16 h, and after 24 h, cells culminate into fruiting bodies (Fig. 2*A*). In contrast, *ric8*-null cells completely failed to form streams and aggregates. This phenotype is completely reverted on reexpression of Ric8 (Fig. 2*A*).

A possible explanation for the phenotype of *ric8*-null cells could be a defect in cAMP relay and the accompanied defect in expression of developmental genes. To address this, we studied the expression of the developmental marker cAR1 in starved wild-type and *ric8*-null cells (Fig. 2*B*). Starved *ric8*-null cells totally lack expression of cAR1. However, in *ric8*-null cells stimulated exogenously with cAMP pulses, cAR1 expression is comparable to that of wild-type cells (Fig. 2*B*). Consistent with a defect in signal relay, *ric8*-null cells mixed with wild-type in a ratio of 50:50, or cAMP-pulsed *ric8*-null cells, showed normal development when plated on nonnutrient agar. Previous experiments have shown that correct expression of cAR1 requires functional Gα2 (9). In contrast, phosphorylation of cAR1 is independent of heterotrimeric G proteins (32). Phosphorylation of cAR1 can be analyzed using a gel-mobility shift assay (33). As indicated in Fig. 2*C*, *ric8*-null and wild-type cells show a similar cAMP-induced shift in the mobility of cAR1.

Together, these results suggest that Ric8 is essential for the regulation and function of Gα during development.

**Ric8 Is Essential for Chemotaxis Toward Folate and Shallow Gradients of cAMP.** The role of Ric8 in chemotaxis toward cAMP was investigated using a micropipette assay. All cells were pulsed with cAMP during starvation. In a steep gradient of cAMP ($>10$ nM/μm), wild-type cells were very polarized and robustly moved toward the pipette (Fig. 3*A*). Under these conditions, *ric8*-null cells and Ric8$^{OE}$ cells migrate with an efficiency similar to that of wild-type cells; however, Ric8$^{OE}$ cells have decreased speed, which could be a result of the various small pseudopods that the cells makes at the leading edge (Fig. 3*A*).

The input signal for chemotaxis is a spatial gradient of cAMP (dC/dx). Because the gradient applied in our described pipette experiment is much stronger than the gradient to which cells are exposed during natural waves (34, 35), we determined the gradient that induces half-maximal chemotaxis, $(dC/dx)_{50}$ (see ref. 34 for the equations that define the spatial gradient at different distances from a pipette). Cells lacking *ric8* fail to chemotax in shallow gradients ($<100$ pM/μm). Mutant cells require a ~50-fold steeper gradient and have a ~10% lower maximal response than wild-type cells (Fig. 3 *A* and *B*). This suggests that Ric8-mediated regulation of Gα is critical to allow chemotaxis in more shallow cAMP gradients.

Folate chemotaxis requires activation of Gα4βγ (11) and Ras activation at the leading edge (36). To determine the contribution of Ric8 to folate chemotaxis, we applied gradients of folate to wild-type, *ric8*-null, and Ric8$^{OE}$ cells (Fig. 3*A* and Movies S1, S2, and S3). Although Ric8$^{OE}$ cells have a comparable chemotaxis index and speed compared with wild-type cells, they respond faster and start moving earlier toward the folate pipette (Movie S3). In contrast, *ric8*-null cells move more slowly and randomly and do not show any folate-directed cell movement, indicating that Ric8 is essential for chemotaxis toward folate.

**Ric8 Amplifies Ras Activation at the Leading Edge.** To investigate the role of Ric8 in symmetry breaking and directional movement in more detail, we obtained quantitative data on Ras activation, which can be detected as the translocation of RBD-Raf-GFP from the cytoplasm to Ras-GTP at the membrane (13). On uniform stimulation with cAMP (Fig. 4 *A* and *C*) or folate (Fig. 4 *B*

**Fig. 2.** Ric8 is essential for multicellular development. (*A*) Cells were plated on nonnutrient agar and allowed to develop. Pictures were taken at the indicated times after starvation. Whereas AX3 and Ric8[OE] formed fruiting bodies after 24 h, *ric8*-null cells completely failed to aggregate (Scale bar: 1 mm.) (*B*) The level of expression of cAR1 was determined by Western blot with antibody specific for cAR1. Contrary to wild-type cells, starved *ric8*-null cells do not express cAR1, whereas on pulsing exogenously with cAMP, expression of cAR1 returns to normal. (*C*) The phosphorylation of cAR1 was determined using a mobility shift assay. Western blot with α-cAR1 antibody shows similar cAMP-stimulated cAR1 phosphorylation in *ric8*-null cells compared with wild-type cells.

and *D*), RBD-Raf-GFP transiently translocates uniformly to the cell boundary. Cells lacking *ric8* and Ric8[OE] exhibit essentially the same maximal response as wild-type cells; however, *ric8*-null cells require higher cAMP and folate concentrations (Fig. 4 *E* and *F*).

On application of a chemoattractant gradient to wild-type cells, initially RBD-Raf-GFP transiently translocates uniformly to the cell boundary, which is then followed by RBD-Raf-GFP localization at the side of the cell facing the gradient (Figs. 3*C* and 4*A*). In a steep gradient of cAMP, *ric8*-null cells also exhibit strong localization of RBD-Raf-GFP at the leading edge and perform good chemotaxis (Fig. 3 *B* and *C*). However, *ric8*-null cells are less polarized and have a broader leading edge, with RBD-Raf-GFP enriched in a larger crescent ($14.2 \pm 2.4$ μm) compared with wild-type ($7.5 \pm 0.6$ μm). Application of a shallow gradient of cAMP or folate induces a short transient uniform Ras activation, but compared with wild-type cells, the specific upgradient translocation of RBD-Raf-GFP is strongly reduced in *ric8*-null cells, and *ric8*-null cells show little (cAMP) or no (folate) directional movement to the pipet (Fig. 3).

**Ric8 Amplifies Heterotrimeric G-Protein Signaling.** To study the exact role of Ric8 in the heterotrimeric G-protein cycle, we obtained quantitative data on Gα2 expression and activation. Mammalian Ric8 has GEF activity to convert Gα-GDP back to the activated Gα-GTP form, but in some cases it also acts as a chaperone to improve the expression and folding of G proteins (37). Western blot analysis shows that the level of expression of Gα2 in starved *ric8*-null cells is similar to that of wild-type cells (Fig. 5*A*), indicating that *Dictyostelium* Ric8, in contrast to mammalian Ric8 (37), does not play a role in the expression or folding of G proteins.

In *Dictyostelium* cells, binding of cAMP to the receptor and subsequent activation and dissociation of Gα2 leads to a decreased affinity of cAR1 for cAMP (38). The interaction between cAR1 and Gα2 can be monitored in vitro as the inhibition of cAMP binding induced by full activation of Gα2 with nonhydrolysable GTPγS. In both wild-type and *ric8*-null cells, GTPγS

reduces cAMP binding by 24% and 28%, respectively, indicating that Ric8 is not necessary for Gα2 activation by GTPγS.

Dissociation of Gαβγ can be monitored in vivo by measuring the cAMP-induced FRET change between FRET pairs (Gα2–Cerulean and/or Gβ–Venus) (Fig. 5*B* and ref. 39). The FRET loss on exposure of uniform cAMP is presented as an increased ratio of Cerulean and Venus intensities ($I_{\text{cerulean}}/I_{\text{Venus}}$). The maximum FRET change in *ric8*-null cells, both at high and low cAMP concentration, is ~40% of that seen in wild-type cells (Fig. 5 *C* and *D*). Half maximum activation for wild-type and *ric8*-null cells is reached after ~6 and 10 s, respectively. These results indicate that *ric8*-null cells have both a decreased and slower Gα dissociation response compared with wild-type cells.

Because of the intrinsic Gα-associated GTPase activity, GTP is hydrolyzed to GDP; the produced Gα-GDP then rapidly associates with Gβγ to form the inactive heterotrimer (25–27). Thus, both Ric8 and Gβγ interact with Gα in a GDP-dependent manner, suggesting they use partly overlapping binding pockets. To address whether Ric8 can inhibit the association of Gα-GDP to Gβγ, pull-down studies with cells expressing GST-Gα and GFP-Gβγ were performed in the presence and absence of Ric8 that was expressed and purified from *E. coli* (Fig. 5*E*). Consistent with our hypothesis, an increasing concentration of Ric8 results in decreased GFP-Gβγ binding to GST-Gα2.

**Model for the Function of Ric8.** Binding of chemoattractants to G-protein-coupled receptors enables them to activate heterotrimeric G proteins by exchanging the G-protein-bound GDP to GTP. This exchange promotes disassociation of the Gαβγ complex into Gα-GTP and a Gβγ dimer, both of which can regulate a diverse set of downstream effectors (Fig. 6). Because of the intrinsic Gα-associated GTPase activity, GTP is hydrolyzed to GDP; the produced Gα-GDP rapidly associates with Gβγ to form the inactive heterotrimer (25–27).

We have shown that *Dictyostelium* Ric8 binds to free Gα-GDP and that the GEF activity of Ric8 stimulates the exchange of

**Fig. 3.** Ric8 is essential for chemotaxis toward folate and shallow gradients of cAMP. (*A*) Chemotaxis index and speed of AX3, *ric8*-null, and Ric8[OE] cells moving toward a micropipette filled with the indicated concentration of cAMP or folate. Data are the means and SD of at least eight cells. (*B*) Chemotaxis of *ric8*-null cells toward cAMP is dose-dependent. The chemotaxis index of wild-type (open circle) and *ric8*-null (closed circle) cells was measured at different distances from micropipettes that contained different concentration of cAMP (0.1 μM to 10 nM). Using equations for the gradient formed at different distances from the pipette, the spatial gradient (dC/dx, in nM/μm) was calculated. Data are the means and SD of at least eight cells. (*C*) Altered Ras activation in *ric8*-null cells in cAMP gradients. Representative images of RBD-Raf-GFP expressing cells. (*I*) Cells in shallow gradients (approximately 0.1 nM/μm). (*II*) Cells in steep gradients (10 nM/μm). (Scale bar: 5 μm.)

Gα-GDP back to the active GTP-bound form. In this way, Ric8 enhances receptor-mediated activation of heterotrimeric G proteins. Ric8 does not alter localization on stimulation, however, suggesting that it is uniformly activated or regulated by local activation, rather than translocation. To date, we cannot exclude that there is local binding of Gα2 to Ric8, but because of the background of Ric8 binding to multiple other uniform localized Gα subunits, the cAMP-mediated Ric8 translocation is below the detection limit.

Regulators of G-protein signaling proteins are potential candidates to catalyze the reverse reaction by acting as a GTPase activating protein (GAP) on Gα-GTP (40). In this model, Ric8 is not essential for the initial activation of heterotrimeric G proteins or Ras by uniform stimulation with chemoattractants. However, cells lacking *ric8* require higher uniform concentrations of cAMP and folate for maximal activation, and Ric8 is important for persistent Ras activation in a gradient of chemoattractant. In the absence of Ric8, persistent Ras activation in a cAMP gradient is impaired, but not absent, because G proteins are still partly activated. Therefore, *ric8*-null cells can only perform chemotaxis in steep gradients of cAMP. In contrast, cells lacking *ric8* do not show any up-gradient activation of Ras or movement toward folate.

Chemotaxis of unpolarized wild-type cells to folate requires steeper gradients and is less efficient than chemotaxis of polarized cells to cAMP. Previously, it was shown that the four amplification pathways do not contribute to folate chemotaxis, and consistent with the current data, folate chemotaxis depends more on the basal heterotrimeric and monomeric signaling pathway than cAMP chemotaxis (13). Furthermore, cells lacking *gα4* still have a significant Ras response to uniform folate, but similar to *ric8*-null cells, they do not show the specific up-gradient response or directional movement in a folate gradient (Fig. S4). This suggests that like Ric8, Gα4 is not essential for uniform Ras

activation but is critical for symmetry breaking of Ras in a folate gradient.

We speculate that *Dictyostelium* Gα2 potentiates at least two positive feedback loops that are essential for cAMP relay and chemotaxis, respectively. During cAMP relay, extracellular cAMP induces the formation of intracellular cAMP that is secreted, thereby inducing more cAMP production. This system leads to autonomous cAMP oscillations with a period of ~5 min. The fundamental theoretical properties of cAMP relay have been well established: it requires an excitable system and threshold cAMP stimulation to obtain autocatalytic activation (41–44). The impaired activation of Gα2 in *ric8*-null cells leads to reduced excitability and a higher threshold for cAMP to induce oscillations. The experiments reveal that free-running cAMP oscillations do not occur in *ric8*-null cells, and therefore cell development is inhibited. Consistent with the notion that Gα2 can be activated to some extent in *ric8*-null cells, exogenously applied cAMP pulses can fully restore this developmental defect. Gα2 also activates a second autocatalytic feedback loop that is involved in chemotaxis. A gradient of ~10% concentration difference of cAMP across the cell leads to a strong intracellular gradient of Ras activation that is at least 10-fold stronger in the front than in the rear of the cell. The molecular mechanism of this symmetry breaking of Ras activation is not well understood but includes tight regulation of RasGEFs and RasGAPs (21, 45) and most likely involves actin and myosin feedback loops on Ras activity at the front and rear of the of the cell, respectively (19, 46).

The presented experiments suggest that Ric8 and Gα2 can initiate the activation of this loop. Cells lacking *ric8* require higher uniform concentrations of cAMP for maximal activation of Ras, and much steeper cAMP gradients are required to obtain Ras activation at the leading edge and chemotaxis. The mechanism
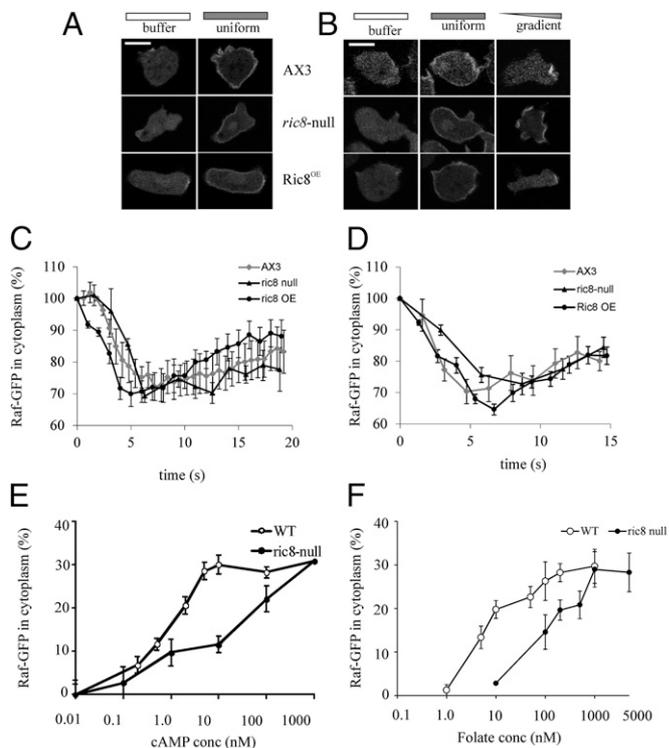


**Fig. 4.** Images of RBD-Raf-GFP expressing cells in buffer, 4–6 s after uniform stimulation with 1 μM cAMP (*A*) and on uniform or gradient stimulation with 1 μM folate (*B*). (Scale bar: 5 μm.) Time-course of RBD-Raf-GFP translocation from cytoplasm to the membrane on stimulation with 1 μM cAMP (*C*) and 1 μM folate (*D*), respectively. Dose-response curve for the uniform cAMP-stimulated (*E*) and folate-stimulated (*F*) Ras response. Shown is the maximum depletion of RBD-Raf-GFP from cytoplasm as the mean and SEM of at least 5 cells.

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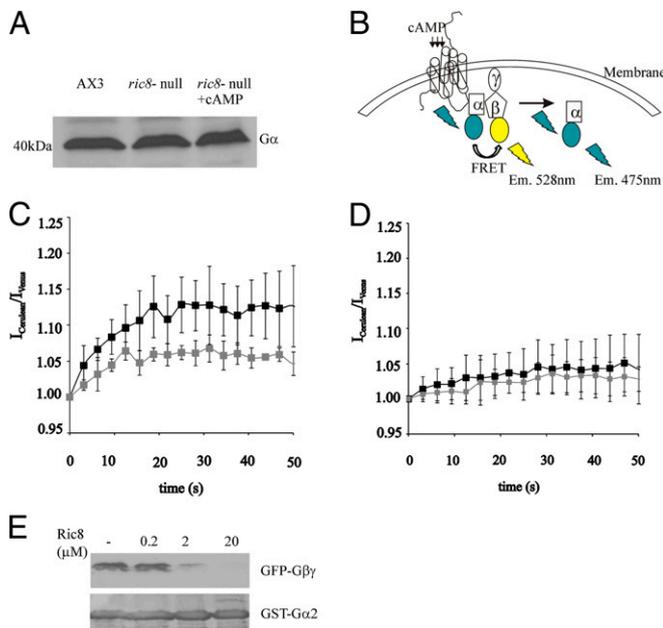
**Fig. 5.** Ric8 amplifies heterotrimeric G protein signaling. (*A*) Ric8 is not a chaperone for Gα proteins. Expression of Gα2 in AX3 and *ric8*-null cells starved in shaking culture for 6 h with or without pulsing with cAMP was detected by Western blotting with specific antibodies against Gα. (*B*) cAMP binding to the receptor leads to dissociation of G proteins, which can be monitored by measuring FRET loss between CFP-Gα and YFP-Gβ. AX3 (*C*) and *ric8*-null (*D*) cells were transformed with the FRET pair, Gα2-Cerulean and Gβ-Venus. FRET change is presented as the intensity ratio of Cerulean and Venus ($I_{Cerulean}/I_{Venus}$) on stimulation with 10 μM cAMP (black) and 10 nM cAMP (gray). (*E*) Ric8 inhibits Gβγ interaction with Gα. GST-Gα2-overexpressing cells were lysed and incubated with increasing concentrations of Ric8, followed by incubation with GFP-Gβγ lysate. The amount of Gβγ bound to Gα2 was detected by Western blotting, using anti-GFP antibodies.

by which Gα2 protein induces symmetry breaking of Ras activation is the most critical in chemotaxis. Therefore, it will be important to identify further regulators and downstream effectors of Gα signaling; our mass pull-down screen could be important in this enterprise.

Experiments on the function of Ric8 in mammalian cells have suggested an implication in the regulation of olfactory signaling via Gαolf (47), Gq-mediated ERK activation (48), and G-protein-mediated neural function and embryogenesis (49, 50). This illustrates the relevant functions of mammalian Ric-8 in the regulation of G-protein signaling and suggests a similar mechanism of Ric8-mediated excitation of Gα-protein signaling as described here for *Dictyostelium*. Furthermore, it will now be important to see whether the same role and mechanism hold true for other critical mammalian G-protein-mediated processes, such as Ca$^{2+}$ oscillations and the migration of lymphocytes during immune response.

## Materials and Methods

**Cell Culture.** AX3 was used as a parent strain to make all cell lines. The indicated *Dictyostelium ric8* (GenBank accession number: DDB_G0292036) overexpressing and knock-out constructs were constructed (*SI Materials and Methods*) and transformed to *Dictyostelium* cells. Cells were grown in HL5-C medium including glucose (ForMedium) and containing, for selection, the respective antibiotics at a concentration of 10 μg/mL.

**Protein Purification.** Ric8, Gα2, and Gα4 were expressed from a pGEX4T1 plasmid containing an N-terminal GST and Tobacco Etch Virus (TEV) cleavage side. Proteins were isolated, as previously described, by GSH affinity, cleavage, and size-exclusion chromatography (51). The purified proteins were analyzed by SDS/PAGE, and the concentration was determined by Bradford's method (Bio-Rad).

**Gα Interaction Screen.** A detailed protocol for the Gα pull-down screen and mass spectrometry is provided in the *SI Materials and Methods*.

**GSH Binding and Activation Assays.** Detailed protocols are provided in the *SI Materials and Methods*. In short, binding of Ric8 to Gα was tested with GSH pull-down experiments, in vitro exchange activity was measured as the decay in radioactivity of $^3$H-GDP-Gα, and to detect in vivo Gα activity, the previously described FRET assay was used (39).

**cAR1 and Gα Expression.** Expression of cAR1 and Gα was determined by Western blot, using anti-cAR1 antibodies and anti-Gα antibodies (Gramsch Laboratories), respectively. cAR1 phosphorylation was analyzed using a gel-mobility shift assay (33).
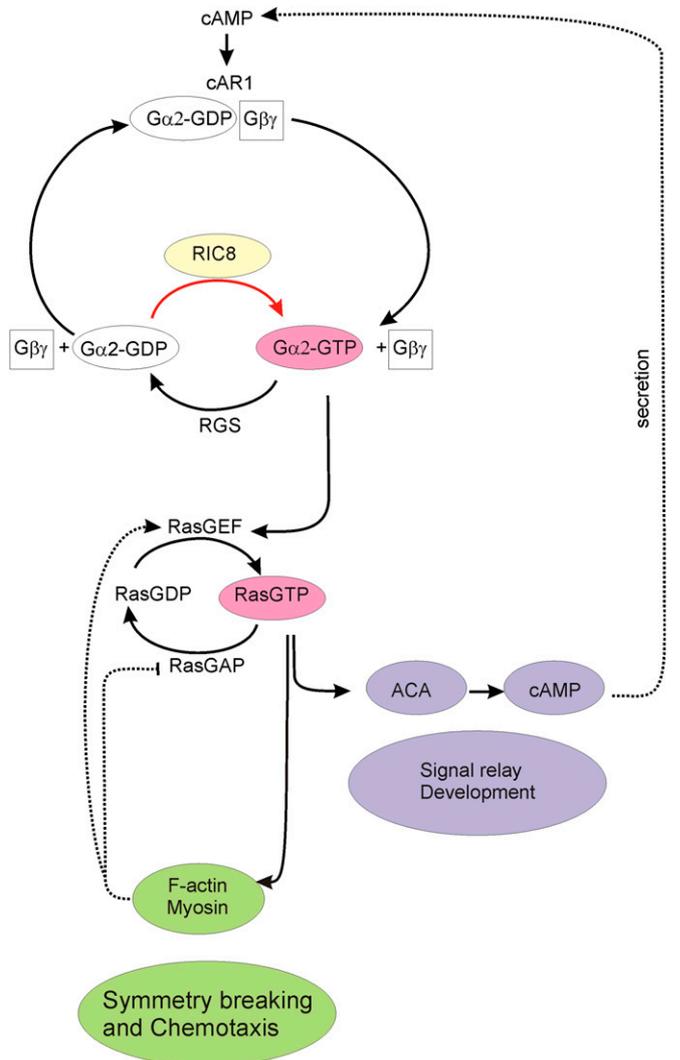


**Fig. 6.** Ric8 is a regulator of G-protein signaling. Binding of cAMP to the cell-surface receptor, cAR1, results in the activation and disassociation of the Gα2-GDP/Gβγ complex into Gα2-GTP and Gβγ. Because of the intrinsic Gα2-associated GTPase activity, GTP is hydrolyzed to GDP, and the inactive heterotrimer rapidly reassociates. Ric8 binds, amplifies, and extends Gα2 activation by stimulating the exchange of Gα2-GDP back to the active GTP-bound form (red arrow). In this way, Ric8 lowers the thresholds for cAMP and increases the excitability of Gα2 signaling, which is essential for processes with autocatalytic loops. In *Dictyostelium*, active Gα2 regulates the activity of Ras-GEF and/or Ras-GAPs, and thereby activates at least two Ras-mediated pathways consisting of autocatalytic feedback loops (dotted lines) that are essential for cAMP relay (purple) and symmetry breaking during chemotaxis (green).

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**Development, Chemotaxis, and Confocal Imaging.** *Dictyostelium* cells ($2 \times 10^7$) were collected, washed, and resuspended in PB (10mM KH2PO4/Na2HPO4, pH 6.5). After washing, the cells were placed on nonnutrient agar plates (15 g/L agar in PB), and pictures were taken at the indicated times with a Zeiss Stemi SV11 microscope, 2× objective, equipped with DCM130 camera.

To obtain cells that are responsive to cAMP, cells were either starved for 6 h on nonnutrient agar plates or pulsed for 6 h with 0.1 μM cAMP in PB. For chemotaxis to cAMP, we used pipettes with a tip opening of 0.5 μm containing cAMP at concentrations varying between 0.1 mM and 10 nM cAMP. For folate chemotaxis, vegetative cells were washed twice with PB; the conditions used were 1 μM folate, a pipette tip opening of 3 μm, and a pressure of 2 hPa (Femtojet, Eppendorf). Chemotaxis data were recorded at different distances from the pipette. The actual gradient was measured with the fluorescent dye Alexa Fluor 594 (Invitrogen), which was added to the chemoattractant solution; the local concentration was recorded in the red channel of the confocal microscope (34). These experiments revealed that the spatial gradient at a minimum distance of 30 μm and a maximum distance of 100 μm from the pipette is stable at 5–10 s after application of the pipette. It holds that the concentration is given by $C(x) = \alpha dC/dx$ (34), where α is a constant.

Movies were recorded with an Olympus microscope equipped with JVC TK-C1381 camera. The chemotaxis index and speed were determined as previously described, using ImageJ (National Institutes of Health, Bethesda), with the position of the centroid of the cells determined every 1 min (52).

Confocal images were recorded using a Zeiss LSM 510 METANLO confocal laser scanning microscope equipped with a Zeiss plan-apochromatic X63 numerical aperture 1.4 objective. The quantification of fluorescence intensity was done as described before (13).

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