Microtubule pulling forces that govern mitotic spindle movement of chromosomes are tightly regulated by G-proteins. A host of proteins, including Gα subunits, Ric-8, AGS3, regulators of G-protein signalings, and scaffolding proteins, coordinate this vital cellular process. Ric-8A, acting as a guanine nucleotide exchange factor, catalyzes the release of GDP from various Gα-GDP subunits and forms a stable nucleotide-free Ric-8A:Gα complex. AGS3, a guanine nucleotide dissociation inhibitor (GDI), binds and stabilizes Gα subunits in their GDP-bound state. Because Ric-8A and AGS3 may recognize and compete for Gα-GDP in this pathway, we probed the interactions of a truncated AGS3 (AGS3-C; containing only the residues responsible for GDI activity), with Ric-8A:Gα and that of Ric-8A with the AGS3-C:Gα-GDP complex. Pulldown assays, gel filtration, isothermal titration calorimetry, and rapid mixing stopped-flow fluorescence spectroscopy indicate that Ric-8A catalyzes the rapid release of GDP from AGS3-C:Gα-GDP. Thus, Ric-8A forms a transient ternary complex with AGS3-C:Gα1-GDP. Subsequent dissociation of AGS3-C and GDP from Gα1 yields a stable nucleotide free Ric-8A:Gα1 complex that, in the presence of GTP, dissociates to yield Ric-8A and Gα1-GTP. AGS3-C does not induce dissociation of the Ric-8A:Gα1 complex, even when present at very high concentrations. The action of Ric-8A on AGS3:Gα1-GDP ensures unidirectional activation of Gα subunits that cannot be reversed by AGS3.

Canonical G-protein signaling pathways are activated when agonist-bound heptahedral receptors, acting as guanine nucleotide exchange factors (GEFs), promote the exchange of GDP for GTP on Gα subunits present in Gα-GDP:Gβγ heterotrimers (1–3). Upon binding GDP, conformational changes in the switch regions of Gα subunits destabilize the heterotrimer and allow Gα-GTP to dissociate from Gβγ subunits (4, 5). Downstream regulatory molecules such as the regulators of G-protein signaling (RGS) accelerate Gα-catalyzed GTP hydrolysis, allowing the Gα subunits to revert to their resting GDP-bound conformation and priming them for the next receptor-induced G-protein cycle (6–8). Receptor-mediated signaling accounts for the majority of G-protein-regulated cellular control mechanisms. However, during the past few years evidence has emerged that, in both lower and higher eukaryotes, multicomponent G-protein signaling systems, operating outside the realm of membrane-bound receptors, play significant roles in various biological processes (9). These include control of the generation of microtubule pulling forces during cell division (10–16), synaptic signaling processes (17), and cardiovascular function (18). A receptor-independent G-protein-mediated signaling pathway, regulating a fundamental event such as asymmetric cell division, may involve proteins that can modulate G-protein nucleotide exchange in a manner that resembles the action of agonist-bound receptors and Gβγ subunits.

In nematodes, asymmetric cell division is a result of eccentric positioning of the mitotic spindle apparatus and the generation of cortical pulling forces on the posterior spindle poles by astral microtubules (13). Studies of fertilized eggs from Caenorhabditis elegans show that mutational inactivation of Gα subunits GOA-1 and GPA-16 leads to defective astral microtubule motion, indicating that these two proteins are vital for the proper positioning of the mitotic spindle (19–21). Along with Gα subunits, GPR1/2 proteins that contain GoLoco/G protein regulatory (GPR) motifs, operating in conjunction with “resistance to inhibitors of cholinesterase” (Ric-8) and RGS7, are necessary and sufficient for regulation of these events (22–27). In dividing Drosophila neuroblasts, Gα1 subunits complexed with the GPR/GoLoco motif-containing protein PINS (PINS indicates Partner of Inscutable) binds to Inscutable or MUD to control asymmetric cell division (28, 29). In dividing sensory precursor cells of Drosophila, Ric-8 has been shown to positively regulate Gα1 activity and is implicated in the membrane targeting of both PINS and Gα1 subunits (29–31). Biochemical characterization of Ric-8 and GPR/GoLoco motif proteins indicates that they respectively exhibit GEF and guanine nucleotide dissociation inhibitory (GDI) activity toward Go1.

The mammalian 60-kDa protein Ric-8A, identified as a Gα-binding protein by yeast two-hybrid analysis, has been

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**Ric-8A Catalyzes Guanine Nucleotide Exchange on Gα11 Bound to the GPR/GoLoco Exchange Inhibitor AGS3**

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shown in vitro to bind to resting state (GDP-bound) Gαi1, Gαi2, and Gαo, and catalyze the exchange of GDP for GTP (and slowly hydrolyzing analogs of GTP) (32). Ric-8A forms a stable complex with nucleotide-free Gαi1, that dissociates in the presence of GTP, releasing Gαi1-GTP (32). Although the action of Ric-8A on Go subunits resembles that of a G-protein-coupled receptor, Ric-8A does not directly compete with the receptor for Gαi1-GDP as it does not recognize Gαi1-GDP in a Gβγ-bound heterotrimeric state (32). However, human embryonic kidney cells (292T) transfected with Ric-8A-specific short interfering RNA exhibited a suppression of Gαi1-coupled/receptor-mediated extracellular signal-regulated kinase (ERK) activation, indicating that Ric-8A may also potentiate Gαi1-mediated signaling (33).

A recently identified class of regulators, typified by "activator of G-protein signaling 3" (AGS3), which bears four GPR/GoLoco signature motifs, has been shown to possess GDI activity similar to that of Gβγ subunits (34–38). The N-terminal portion of AGS3 consists of seven tetratricopeptide repeats that are implicated in cellular trafficking of protein binding partners (34, 35). An N-terminally truncated variant of AGS3 produced by alternative or trans-mRNA splicing, comprising only the GPR/GoLoco motifs of AGS3, has been found in mammalian heart (39). This variant of AGS3 possesses the C-terminal three GPR/GoLoco motifs but encodes only half of the first GPR/GoLoco motif present in the full-length AGS3. For this work, we have used a synthetic short form of AGS3 (AGS3-C) that encodes all four GPR/GoLoco motifs (35). Earlier thermodynamic studies have shown that all four GPR/GoLoco motifs of AGS3-C bind cooperatively to Gαi1-GDP and form a stable AGS3-C:[Gαi1-GDP]4 complex (40). For simplicity, we henceforth refer to this complex as AGS3-C.Gαi1-GDP. The structure of the complex between the GPR/GoLoco peptide of RGS14 and Gαi1-GDP reveals that GPR/GoLoco motifs function as GDIs by interacting with the switch II regions and the α3 helix of the Gαi1 Ras-like domain (41). The arginine residue located in the highly conserved Asp-Gln-Arg (DQR) triad in the GPR/GoLoco motif inserts into the GDP-binding site and interacts with the β-phosphate of GDP, thereby preventing the release of GDP from Gαi1 subunits.

The interplay between the GEF activity of Ric-8A and the GDI effect of GPR/GoLoco proteins on Go subunits was recently demonstrated (42). LGN, a paralog of AGS3, and microtubule-binding nuclear mitotic apparatus protein (NuMA) were shown to interact with and stabilize Gαi1-GDP in a Gαi1-GDP:LGN:NuMA complex (43). The Gαi1-GDP subunits in this complex are proposed to be substrates for the GEF activity of Ric-8A. Although this study provides evidence that Ric-8A forms a transient complex with Gαi1-GDP:LGN:NuMA, it does not preclude the possibility that Ric-8A is a direct competitor of LGN for a common binding site on Gαi1-GDP. These alternative models could have different implications for the roles of Ric-8A and GPR/GoLoco proteins in the putative receptor-independent regulatory events mediated by Go (42).

In this biophysical study we show that Ric-8A acts catalytically on Gαi1-GDP complexed with AGS3-C to effect nucleotide exchange. We utilize fluorescence labeling and thermodynamic and enzyme kinetic analysis to show that Ric-8A forms a transient multimeric complex with AGS3-C:Gαi1-GDP. This intermediate then decays to a stable, nucleotide-free Ric-8A:Gαi1 heterodimer after liberating AGS3-C and GDP. The inhibitory effect of AGS3-C on the interaction between Ric-8A and Gαi1-GDP is not strong enough to abrogate Ric-8A GEF activity. Further experiments indicate that AGS3-C cannot reassociate with the nucleotide-free Ric-8A-Gαi1 complex, indicating that this reaction pathway proceeds in a unidirectional manner.

**EXPERIMENTAL PROCEDURES**

**Molecular Cloning and Protein Expression**—The open reading frame of rat Ric-8A, encoding amino acid residues 12–492 (Ric8-At), was amplified by PCR using attB-modified forward primer 5'-GGGGACAGTTTGTACAAAAAAACAGGCTACGAAACCTATCTTTGCAGGG-3' encoding a TEV site N-terminal to residue 12 and 3'-CACATGTACAAACTTGTCCAGGTGGTCACCCTCTGGTGCAGAAGACATGTTCACCCGGGG-5' as the reverse primer. The resulting PCR product was cloned into the pDEST-15 destination vector to be expressed as a GST fusion protein using the Gateway cloning system (Invitrogen). The expression vector was transformed into Escherichia coli BL21(DE3)-RIPL cells and grown in LB media containing ampicillin (120 mg/liter) and induced with 300 μM of isopropyl β-D-thiogalactopyranoside at 20 °C. After overnight growth at 20 °C, cells were suspended in lysis buffer (50 mM Tris, pH 8.0, 250 mM NaCl, 2 mM DTT, and 2 mM PMSF) and lysed in a French press (Avanti cell disrupter). The cell lysate was clarified by centrifugation and loaded onto a packed column containing 5 ml of glutathione-Sepharose 4B resin (GE Healthcare). After extensive washing with lysis buffer, Ric-8At was cleaved from the resin overnight at 4 °C using a quantity of TEV protease corresponding to 10% of the total GST fusion protein estimated to be on the resin. TEV-digested protein was eluted from the GST-Sepharose 4B resin with elution buffer (50 mM Tris, pH 8.0, 2 mM DTT, and 2 mM PMSF) and dialyzed against the same buffer. The dialysate was loaded onto an UNO-Q matrix (Bio-Rad) and eluted with a 0–500 mM NaCl gradient on an AKTA FPLC system (GE Healthcare). Pure Ric-8At eluted from the matrix at 165–175 mM of NaCl. The protein was later found to be pure by SDS-PAGE analysis.

The open reading frame of rat Gαi1 was amplified by PCR using attB-modified forward primer, encoding a TEV site N-terminal to the Gαi1 sequence, 5'-GGGGACAGTTTGTACAAAAAAACAGGCTACGAAACCTATCTTTGCAGGG-ATGTACTCTTTCTGCTGAA-, and 3'-TTTTTATTAGAATTTCTAACCAGAAAAAATCTGGTGAAAGAC-ATGTTTCACCAGGGG-5' as reverse primer. The resulting PCR product was cloned into the pDEST-15 destination vector to be expressed as a GST fusion protein using the Gateway cloning system (Invitrogen). Expression and TEV digestion protocols were similar to those used for Rat Ric-8At except that Gαi1 eluted at 130–140 mM of NaCl from the UNO-Q matrix. Hexahistidine-tagged Gαi1 encoded in a pQE60 vector was expressed in E. coli strain JM-109 and purified as described earlier (44). The (W211A)Gαi1 mutant was generated using the QuikChange kit according to the manufacturer's protocol.
RIC-8A Catalyzes GEF on Gαi1 Bound to AGS3

(Stratagene) using the pQE60 vector harboring wild type Gαi1 as a template. After sequencing to confirm the presence of the mutation, (W211A)Gαi1 was expressed and purified using the protocol developed for the wild type protein. Gαi1 and the (W211A)Gαi1 mutant proteins were utilized in nonmyristoylated form for all subsequent experiments.

AGS3-C, comprising residues 465–650 of rat AGS3, was cloned into the pDEST-15 destination vector for a GST fusion protein using the Gateway cloning system (Invitrogen). A tobacco etch virus protease cleavage site was inserted between coding regions for GST and AGS3-C regions. AGS3-C was purified as described earlier (40).

Yellow fluorescent protein (YFP) was appended onto the 5’ end of human M19T Ric-8A by PCR sewing and inserted into the E. coli expression vector, pET28a (Novagen). This construct contained the following features: 5’-His6-Nhel-YFP-EcoRI-Ric-8A-Notl-3’. His6-YFP-Ric-8A protein was expressed in E. coli similarly to Ric8-At (above). The protein was purified by successive nickel-nitrilotrisacetic acid (Qiagen), Hi-trap Q and Superdex gel filtration chromatographies (GE Biosciences).

To synthesize the cyan fluorescent protein (CFP)-AGS3-C fusion protein, DNA encoding the C-terminal four GPR/GorLoco domains of rat AGS3 were amplified by PCR using the pDEST-15 vector that encoded AGS3 residues 465–650 (40). The PCR product was cloned into the Notl/XbaI sites following the CFP coding region of CFP pCDNA3.1. A 24-amino acid linker was engineered between the CFP and AGS3-C sequences. The regions encoding CFP, the linker, and residues 465–650 of AGS3 were amplified by PCR and cloned into the pDEST-15 destination vector for expression as a GST fusion protein using the Gateway cloning system (Invitrogen). A tobacco etch virus protease cleavage site was inserted between coding regions of GST and CFP. The expression vector was transformed into Bl21DE3.RIPL cells, grown in LB media containing ampicillin (120 mg/liter), and expression was induced from single batches of E. coli cells.

Competition Assays—To assay displacement of AGS3-C from the AGS3-C:His6-Gαi1-GDP complex by Ric-8A, gel-filtered AGS3-C:His6-Gαi1-GDP complex (25 μM, dissolved in 50 mM Tris, pH 8.0, 250 mM NaCl, 1 mM imidazole, 1 mM DTT, and 50 μM GDP) was incubated for 2 h at 20 °C with Ric-8A (100 μM) in a total volume of 150 μl and then allowed to bind to 50 μl of Ni2+ IMAC resin (Bio-Rad). The beads were washed three times with 400 μl of buffer (50 mM Tris, pH 8.0, 250 mM NaCl, 10 mM imidazole, 1 mM DTT, and 50 μM GDP), boiled in SDS-PAGE loading buffer, and run on an SDS-polycrylamide gel. Samples of purified Ric-8At, AGS3-C, AGS3-C:His6-Gαi1-GDP, and Ric8-At:Gαi1 were run as standards. Proteins were visualized by staining with Coomassie Brilliant Blue dye. To assay displacement of Ric-8At from the Ric-8At:Gαi1 complex by AGS3 gel-filtered Ric-8At:Gαi1 (25 μM suspended in 50 mM Tris, pH 8.0, 250 mM NaCl, 10 mM imidazole, 1 mM DTT, and 50 μM of GDP) complex was incubated for 2 h at 20 °C with AGS3-C (25 μM or 250 μM) and analyzed as described above.

Gel Filtration Assays—AGS3:Gαi1-GDP complex (14 μM complex in 20 mM Tris, pH 8.0, 250 mM NaCl, 1 mM DTT, and 10 μM of GDP) was incubated for 2 h at 20 °C with Ric-8At (100 μM) in a total volume of 1 ml and loaded on a tandem Superdex 200/75 gel filtration matrix pre-equilibrated with running buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM DTT). The column was run at a constant flow rate of 0.4 ml/min, and fractions were collected. In a separate experiment, Ric-8At:Gαi1 complex (100 μM, suspended in 20 mM Tris, pH 8.0, 250 mM NaCl, and 2 mM DTT) was incubated with AGS3-C (200 μM) in a total volume of 1 ml for 2 h at 20 °C and gel-filtered under the same conditions. Peak fractions of both runs were boiled with SDS-PAGE loading buffer and run on an SDS-polyacrylamide gel. The proteins were visualized by Coomassie Brilliant Blue dye.

Isothermal Titration Calorimetry (ITC)—ITC experiments were performed using a VP-ITC (MicroCal) instrument. Fixed aliquots (8 μl) of Gαi1-GDP (200 μM) suspended in ITC buffer (20 mM Tris, pH 8.0, 250 mM NaCl, 2 mM DTT, and 25 μM GDP)
were injected into the calorimeter cell containing 1.43 ml of AGS3-C (6 μM) in ITC buffer, and the heats at binding were recorded. Alternatively, 8-μl aliquots of AGS3-C (350 μM) in ITC buffer were injected into the calorimeter cell containing 1.43 ml of Ric-8At:Gαi1 (10 μM) in ITC buffer. As a control experiment, 8-μl aliquots of Gαi1-GDP (200 μM) or 8-μl aliquots of 350 μM AGS3-C, both in ITC buffer, were injected into the calorimeter cell containing 1.43 ml of ITC buffer to measure the heats of protein dilution.

**Ric-8A Concentration-dependent Kinetics of Gαi1 Binding to GTPγS**—Kinetics of Ric-8A-assisted binding of GTPγS to Gαi1 were followed by monitoring the change in intrinsic fluorescence of Gαi1 at 340 nm upon exchange of GDP with GTPγS. Gαi1-GDP (1 μM) in buffer composed of 20 mM HEPES, pH 8.0, 100 mM NaCl, 10 mM MgCl2, 1 mM DTT, and 0.05% C12E10 in a reaction volume of 400 μl was allowed to equilibrate for 10–15 min at 30 °C in a quartz fluorescence cuvette. GTPγS (10 μM) was added to the reaction mixture in the absence or presence of Ric-8At (0.125 μM-1 μM), and the increase in fluorescence at 340 nm change was monitored upon excitation at 290 nm (45). Similar experiments were conducted using (W211A)Gαi1 in the presence of 1 μM Ric-8At. Fluorescence measurements were conducted using an LS55 spectrofluorometer (PerkinElmer Life Sciences) attached to a circulating water bath to maintain a steady sample temperature. The excitation and emission slit widths were set at 2.5 nm. All exciting light was eliminated by use of a 290 nm cut-off filter positioned in front of the emission photomultiplier.

**Kinetic Analysis of Ric-8A GEF Activity**—Initial rates of Ric-8A-mediated Gαi1 guanine nucleotide exchange were determined at different concentrations of Gαi1-GDP in the presence or absence of AGS3-C. Ric-8A (50 μM) was added to buffer (20 mM HEPES, pH 8.0, 100 mM NaCl, 10 mM MgCl2, 1 mM DTT, and 0.05% C12E10) containing various concentrations of Gαi1-GDP (0.1–1 μM). The reactions were incubated at 30 °C for 10 min; GTPγS (10 μM) was added, and fluorescence was monitored at 340 nm upon excitation at 290 nm as described above. Initial rates were determined as a function of Gαi1-GDP concentration. The same set of experiments was performed in the presence of different concentrations of AGS3-C (0.2, 0.3, and 0.4 μM). Lineeweaver-Burk plots were constructed to obtain Vmax and Km values for Ric-8A-stimulated Gαi1 GTPγS binding in the absence or presence of AGS3-C.

**Stopped-flow Fluorescence Assay**—A stopped-flow based assay was performed to study the formation and decay of FRET because of interaction of YFP-Ric-8A with CFP-AGS3-C:Gαi1-GDP. Equal volumes of YFP-Ric-8A (50 μl of 55 μM protein) and CFP-AGS3-C:Gαi1-GDP (50 μl of 12 μM protein) in stop flow buffer (20 mM HEPES, pH 8.0, 300 mM NaCl, 10 mM MgCl2, 1 mM DTT, and 0.1% C12E10) were rapidly mixed into a 20-μl optical cell of a pneumatically driven stopped-flow apparatus (SFM-18MV, Applied PhotoPhysics, Leatherhead, UK). FRET emission at 527 nm upon excitation at 415 nm using a nitrogen-purged xenon arc lamp was measured for 300 s. Elimination of scattered exciting light entering the fluorescence photomultiplier was achieved using a second monochromator unit set at 527 nm. The dead time of the instrument was estimated to be 1.34 ms when used with a 20-μl cell. A control assay was performed by rapidly mixing equal volumes of YFP-Ric-8A (50 μl of 12 μM protein) with CFP-AGS3-C (50 μl of 12 μM protein) using the same experimental conditions. The basal YFP emission generated by CFP wavelength excitation light was used as the base line to quantify the FRET signal.

A stopped-flow double mixing experiment was performed to observe reassociation of YFP-Ric-8At:Gαi1 with CFP-AGS3-C upon addition of GTP. The reaction mixture was composed of the products from the reaction of YFP-Ric-8A with CFP-AGS3-C:Gαi1-GDP as described above. Equal volumes of YFP-Ric-8A (110 μl of 55 μM protein) and CFP-AGS3-C:Gαi1-GDP (110 μl of 12 μM protein) in stop flow buffer (20 mM HEPES, pH 8.0, 300 mM NaCl, 10 mM MgCl2, 1 mM DTT, and 0.1% C12E10) were rapidly mixed into an aging loop, and the FRET formation/decay reaction was allowed to proceed for 150 s. GTP (110 μl of 300 μM) in the same reaction buffer was then sequentially mixed with the aging loop product into an optical cell of the stopped-flow apparatus. FRET at 527 nm was measured for 1300 s upon excitation at 415 nm using a nitrogen-purged xenon arc lamp.

The rate of GDP release from the AGS3-C:Gαi1-GDP complex was measured by rapidly mixing equal volumes of AGS3-C:Gαi1-GDP (50 μl of 12 μM protein) with Ric-8At (50 μl of 55 μM protein), both suspended in release buffer composed of 20 mM HEPES, pH 8.0, 150 mM NaCl, 10 mM MgCl2, 1 mM DTT, and 0.1% C12E10, and monitoring fluorescence emission at 340 nm upon excitation at 290 nm. A control experiment was performed by rapidly mixing equal volumes of AGS3-C (50 μl of 12 μM protein) with Ric-8At (50 μl of 12 μM protein), both suspended in release buffer in the absence of Gαi1-GDP. To estimate the rate of GDP release by Gαi1-GDP that is not in complex with AGS3-C, upon binding to Ric-8At, equal volumes of Ric-8At and Gαi1-GDP (50 μl of 55 μM protein each) in release buffer were rapidly mixed into a stopped-flow cell, and the fluorescence was monitored at 340 nm upon excitation at 290 nm. All data reported are averages of 12–15 independent experimental traces performed under identical conditions. Sequential mixing experiments were averages of 5–8 independent experimental traces performed under identical conditions. Reactions were performed at 25 °C.

**RESULTS**

Preliminary experiments, aimed to identify regions of Ric-8A that bind Gαi1-GDP, established that residues 1–492 or 12–492 of Rat Ric-8A were sufficient to exhibit full GEF activity toward Gαi1-GDP. Protein expression trials indicated that, unlike the full-length rat Ric-8A (residues 1–530) that could be expressed in Sf9 cells (32) but not at high yield in E. coli, the Ric-8A fragment (residues 12–492, referred to as Ric-8At; Fig. 1A) could be expressed in E. coli to produce moderate yields (5–7 mg of purified protein/liter of LB media) of protein for biophysical studies. Nonmyristoylated Gαi1 was used for the studies reported here.

We found that the rate of Ric-8At-stimulated Gαi1 exchange of GDP for GTPγS (0.45 min−1) is comparable with that catalyzed by full-length Ric-8A purified from insect cell culture.

3 G. G. Tall and C. J. Thomas, unpublished observations.
RIC-8A Catalyzes GEF on Goi, Bound to AGS3

![Image](60x298 to 288x733)

**Figure 1.** Ric-8At induces dissociation of AGS3-C:Goi,-GDP, whereas AGS3-3 does not disrupt the Ric-8At:Goi,- complex. A, schematic representation of the protein constructs used in this study. Vertical hatched boxes represent tetra-tricopeptide repeats; gray boxes, GPR/GoLoco repeats in AGS3. Residue numbers at N- and C-terminal boundaries of constructs are indicated. B, gel-filtered AGS3-C-His6:Goi,-GDP complex (25 μM) was incubated for 2 h at 20 °C with Ric-8At (100 μM) and then bound to 50 μl of Ni2+-IMAC resin. The resin was run on an SDS-polyacrylamide gel, and the proteins were visualized by staining with Coomassie Brilliant Blue dye (lane 1). Gel-filtered Ric-8At:Hii,-Goi,- complex (25 μM) was incubated for 2 h at 20 °C with AGS3-C (250 μM) and analyzed as above (lane 2). Similar results were obtained in the presence or absence of excess GDP. Lanes 3–7 indicate the proteins AGS3 (lane 3), His6:Goi,- (lane 4), and Ric-8 (lane 5) and protein complexes AGS3-C-His6:Goi,-GDP complex (lane 6) and Ric-8At:Hii,-Goi,- (lane 7) used in this study. C, AGS3-C:Goi,-GDP complex (14 μM) was incubated for 2 h at 20 °C with Ric-8At (100 μM) in a total volume of 1 ml, and the reaction mixture was resolved using a tandem Superdex 200/75 gel filtration matrix (profile I). Conversely, Ric-8At:Goi,- (100 μM) complex was incubated with AGS3-C (200 μM) for 2 h at 20 °C and treated under similar gel filtration conditions (profile II). *Inset,* the peak fractions for both experiments were analyzed by SDS-PAGE to visualize the proteins. *Insets* show Coomassie stained SDS-polyacrylamide gels corresponding to peaks from profiles I and II. Peaks and corresponding lanes for profile I are labeled 1–5 and for profile II labeled A–D.

(0.35 min⁻¹). This amounts approximately to a 12-fold increase over the intrinsic nucleotide exchange rate of Goi,- (32). Ric-8At, like full-length Ric-8A, binds to Goi,-GDP and, upon release of GDP, forms a stable, gel-filterable nucleotide-free complex. Upon addition of GTPγS, the complex dissociates rapidly to form free Ric-8At and GTPγS-bound Goi,- (data not shown).

The C-terminal fragment of AGS3 (residues 465–650), consisting of four GPR/GoLoco motifs (AGS3-C; Fig. 1A), functions as a GDI toward Goi,-GDP and forms a stable gel-filterable AGS3-C:Goi,-GDP complex (39, 40). To determine whether Ric-8A can induce dissociation of Goi,-GDP from AGS3-C, we incubated Ric-8At with a gel-filtered complex of AGS3-C with His6-tagged Goi,-GDP for 2 h at 20 °C. The reaction mixture was then resolved by Ni2⁺-IMAC. SDS-PAGE analysis of the IMAC eluant showed that Ric-8At displaced AGS3-C from the AGS3-C:His6:Goi,-GDP complex to form a Ric-8At:Goi,- complex (Fig. 1B, lane 1). To test whether the converse is true, that AGS3-C causes dissociation of Goi,- from Ric-8At, we incubated AGS3-C with Ric-8At:His6:Goi,- complex and treated the mixture as described above. Intact Ric-8At: His6:Goi,- complex was retained on the Ni2⁺-IMAC column showing that nucleotide-free Ric-8At:His6:Goi,- complex does not dissociate in the presence of AGS3-C, even at 50-fold molar excess to Ric-8At (Fig. 1B, lane 2). Gel filtration chromatography of mixtures, containing either AGS3-C:Goi,-GDP and Ric-8At or Ric-8At:Goi,- and AGS3-C, showed that Ric-8At can displace Goi,- from the complex with AGS3-C. AGS3-C, on the other hand, does not form a stable complex with Goi,- bound to Ric-8At:Goi,- (Fig. 1C).

Isothermal titration calorimetry (ITC) was performed to rule out the possibility of weak interactions between AGS3-C and Ric-8At:Goi,-. ITC measurements showed that binding of Goi,-GDP to AGS3-C is exothermic and indicated that four molecules of Goi,-GDP bind to one molecule of AGS3-C, in agreement with previously published results (40) (Fig. 2). In contrast, isothermal titration of AGS3-C into a solution containing Ric-8At:Goi,- complex generated no significant heat of formation (Fig. 2).

Binding of GTPγS to wild type Goi,- is accompanied by an increase in tryptophan fluorescence at 340 nm (45). The change in fluorescence upon addition of GTPγS binding to Goi,- in the presence of equimolar amounts of Ric-8At is 10–12-fold faster than that observed in the absence of Ric-8At (Fig. 3). This rate enhancement is consistent with data published earlier using radioactive [35S]GTPγS filter binding assays to monitor Ric-8A-assisted GTPγS uptake by Goi,- (32). This increase in fluorescence is because of structural changes at the nucleotide-binding site of Goi,-, because no fluorescence enhancement was observed for Ric-8At-catalyzed GTPγS binding for (W211A)Goi,- in which Trp-211 was replaced by alanine (Fig. 3A, blue trace). However, filter binding experiments using radiolabeled [35S]GTPγS showed that Ric-8At catalyzes GTPγS binding to (W211A)Goi,- at a rate similar to that for wild type Goi,- (supplemental Fig. 1). The rate of [35S]GTPγS binding to (W211A)Goi,- determined by this method is 0.031 min⁻¹, and the Ric-8At-stimulated rate is 0.38 min⁻¹. In comparison, the rate of [35S]GTPγS binding to wild type Goi,- is 0.038 min⁻¹, and the Ric-8At-assisted rate is 0.43 min⁻¹.

Experiments conducted in the presence of varying concentrations of Ric-8At showed that the fluorescence-based method...
can be used to quantify the catalytic activity of Ric-8At as a GEF toward \( \alpha_{1i} \) subunits (Fig. 3). We assayed the rate of exchange of GDP for GTP\( \gamma \)S at various concentrations of \( \alpha_{1i} \)GDP in the presence of a fixed concentration of Ric-8At. Lineweaver-Burk analysis of these rate profiles yielded a \( V_{\text{max}} \) of 0.37 \( \mu \text{M} \) min\(^{-1} \) and a \( K_m \) of 1 \( \mu \text{M} \) for the binding of GTP\( \gamma \)S to \( \alpha_{1i} \) in the presence of Ric-8At (Fig. 4).

The observation that Ric-8At is able to displace AGS3-C from an AGS3-C:G\( \alpha_{1i} \)-GDP complex suggests that AGS3-C, as a GDI, may function as an inhibitor of Ric-8At-catalyzed G\( \alpha_{1i} \) guanine nucleotide exchange activity. Earlier studies demonstrated that high concentrations of AGS3-C and the paralogous protein LGN-C indeed act as inhibitors of Ric-8A GEF activity (42). To test the activity of AGS3-C as an inhibitor of Ric-8At GEF action, and to determine the kinetic mechanism of inhibition, we assayed the rate of Ric-8At-catalyzed exchange of GDP for GTP\( \gamma \)S at different concentrations of AGS3-C. In the presence of AGS3-C, the \( V_{\text{max}} \) for Ric-8At-catalyzed nucleotide exchange was reduced, but the \( K_m \) value was not affected significantly (Fig. 4). Therefore, AGS3-C acts as a noncompetitive inhibitor of Ric-8At, suggesting that AGS3-C and Ric-8At may bind to different sites on G\( \alpha_{1i} \)-GDP.

The noncompetitive mode by which AGS3-C inhibits Ric-8At permits the speculation that Ric-8At might bind to the AGS3-C:G\( \alpha_{1i} \)-GDP complex and form a transient multimeric Ric-8At:AGS3-C:G\( \alpha_{1i} \) complex. To test this hypothesis, we reasoned that the association between an N-terminally YFP-tagged Ric-8At and a complex of G\( \alpha_{1i} \)-GDP with CFP-tagged AGS3-C would result in production of a transient FRET signal upon irradiation at the fluorescence excitation wavelength of CFP, CFP- and YFP-tagged proteins were expressed in \textit{E. coli}, purified to near-homogeneity, and used in subsequent binding assays. By the use of gel filtration and \([^{35}\text{S}]\)GTP\( \gamma \)S binding and fluorescence assays, we determined that YFP-Ric-8A and CFP-AGS3-C exhibit GEF and GDI activities, respectively, toward G\( \alpha_{1i} \)-GDP (supplemental Fig. 2).

A rapid mixing experiment was designed to monitor the interaction of YFP-Ric-8A with CFP-AGS3-C:G\( \alpha_{1i} \)-GDP. Equal volumes of these two proteins in identical buffers were rapidly mixed in the cell of a pneumatically driven stopped-flow apparatus, and the evolution of FRET at 527 nm upon excitation of CFP at 415 nm was measured. The evolution of FRET after mixing exhibited two distinct phases. The initial phase, an additive value of the FRET and basal YFP fluorescence lasting \( \sim 15-20 \) s, consisted of a sharp rise in fluorescence to a maximal value, which was followed by a second phase characterized by a monotonic decay in fluorescence back to the basal (415 nm wavelength excited) YFP levels (Fig. 5, top panel, green trace). The magnitude and rate of the initial portion of the FRET signal were found to be dependent on the concentration of the two reacting species. Analysis of the evolution of FRET intensity over a range of YFP-Ric-8A and CFP-AGS3-C concentrations yielded a second-order association constant for the two species of \( 6.3 \times 10^5 \) min\(^{-1} \) M\(^{-1} \). The monotonic decay phase was concentration-independent and proceeded at a rate of 1.2 min\(^{-1} \). We attribute this phase to dissociation of the YFP-Ric-8A:CFP-AGS3-C:G\( \alpha_{1i} \) complex following the release of AGS3 and GDP from G\( \alpha_{1i} \). A control experiment wherein equal volumes of YFP-Ric-8A and CFP-AGS3-C were rapidly mixed under identical conditions produced no fluorescence fluctuations at the YFP emission wavelength, corresponding only to the basal YFP (415 nm wavelength excited) fluorescence (Fig. 5, top panel, black trace). Similarly, the rapid mixing of YFP-Ric-8A-G\( \alpha_{1i} \) complex with CFP-AGS3-C produced no significant fluores-
attribute the rise and fall of FRET intensity shown in this latter...GTP binding, with the release of Ric-8At, which binds exclusively to a GDP-bound Gαi$_{11}$ as a function of Trp-211 fluorescence loss. Earlier experiments indicated that binding of AGS3-C to Gαi$_{11}$ does not induce a change in fluorescence at 340 nm (data not shown), because AGS3-C stabilizes the GDP-bound, low fluorescence state of Gαi$_{11}$, and AGS3-C itself lacks tryptophan residues. We also established that, upon formation of a nucleotide-free complex with Ric-8At, there is a decrease in the intrinsic fluorescence of Gαi$_{11}$ because of Trp-211 (this effect is not observed upon complexation with (W211A)Gαi$_{11}$). By utilizing this effect, we monitored the rate of GDP release from a preformed complex of AGS3-C:Gαi$_{11}$GDP upon addition of stoichiometric amounts of Ric-8At, and we found that it proceeded at a rate of 0.38 min$^{-1}$ (Fig. 5, top panel, red trace). Addition of a 5-fold molar excess of Ric-8At to the complex increased the rate of fluorescence quenching to 0.49 min$^{-1}$ (Fig. 5, top panel, blue trace). The processes subsumed in these rates include association of Ric-8At with AGS3-C:Gαi$_{11}$GDP and possibly dissociation of AGS3 from the transient complex with Ric-8At and Gαi$_{11}$ as well as GDP dissociation from the latter.

**DISCUSSION**

Recent biochemical studies demonstrated that Ric-8A, a mammalian GEF for G$_i$ and G$_q$ class G-protein α subunits, is able to catalyze exchange of GTP for GDP from Gαi$_{11}$GDP bound to LGN or the LGN:NuMA complex (42). LGN, like its paralog AGS3, contains four GPR/GoLoco repeats, each of which binds exclusively to a GDP-bound Gα subunit and inhibits nucleotide release (34, 46). LGN also weakly inhibits the GEF activity of Ric-8A toward Gαi$_{11}$GDP (42). Experiments conducted in our laboratory showed that AGS3-C can also inhibit Ric-8A GEF activity. These observations prompted us to ask...
whether Ric-8A competes directly with AGS3-C in binding to
Gα11-GDP or, as suggested by Tall et al. (42), is able to bind
directly to the AGS3-Gα11-GDP complex.

The experiments described here illustrate a key point regarding
the physiological function of Ric-8A action upon AGS3:
Gα11-GDP. The effects of Ric-8A cannot be reversed by AGS3 or
LGN within the same round of the G-protein catalytic cycle.
Protein pulldown and gel filtration experiments show that the
AGS3-C:Gα11-GDP complex dissociates in the presence of Ric-
8At, whereas the Ric-8At:Gα11 complex does not dissociate in
the presence of AGS3-C. Gel filtration experiments confirm the
pulldown assay results and further indicate that incubation of
Ric-8At with AGS3-C:Gα11-GDP yields GDP and nucleotide-
free Ric-8At:Gα11 complex. The high affinity of Ric-8At for
nucleotide-free Gα11 and the low affinity of AGS3-C toward the
same are probably the underlying causes of this irreversibility.
The difficulty in isolating active Gα11 in the nucleotide-free
state prohibits direct testing of this hypothesis. However, the
absence of heat evolution or absorption upon isothermal titra-
tion of Ric-8At:Gα11 with AGS3-C strongly suggests that
AGS3-C does not bind to nucleotide-free Ric-8A-Gα11 near
physiological temperatures.

Ric-8A acts catalytically to promote the exchange of GDP for
GTP (or that of GTP analogs) upon its substrate, Gα11-GDP
(32). We have used nonmyristoylated Gα11 for experiments
described here. The rate of Ric-8A-stimulated nucleotide exchange is
reported to be 2-fold greater for myristoylated versus unmodified
Gα11, and the reaction is less sensitive to inhibition by GPR/GoLoco
proteins (42). Thus, quantitative but not qualitative differences from the
results reported here would be expected for similar experiments
conducted with myristoylated Gα11.

The Ric-8At:Gα11 complex is a stable and readily isolatable inter-
mediate of this reaction that dissociates rapidly in the presence of
GTP to yield Ric-8At and Gα11-GTP. The data presented here show that
Ric-8At catalyzes GTPγS binding to
Gα11 with a Km for Gα11-GDP of ~1
μM and a turnover number (kcat)
near 8 min⁻¹. AGS3-C is a noncom-
petitive inhibitor of Ric-8At and
reduces Vmax, but it does not affect the
Km value for Gα11-GDP. The Kj
value for AGS3-C appears to be less
than 1 μM, but it was not accurately
determined in our experiments.
That AGS3-C is a noncompetitive
inhibitor of Ric-8A with respect to
Gα11 suggests the possibility that
Ric-8A and AGS3 bind to distinct
sites on Gα11.

We have conducted time-resolved FRET experiments that
conclusively demonstrate that CFP-AGS3-C:Gα11-GDP
interacts with YFP-Ric-8A to form a ternary complex with a second-
order rate constant of 6.3 × 10⁵ M⁻¹ min⁻¹. The FRET
signal subsequently decays (at the rate of 1.2 min⁻¹) back to the basal
YFP fluorescence levels. The strong transient FRET emission
seen in our experiments indicates that at least one YFP domain is
present within a Förster radius <50 Å of the CFP tag of
AGS3-C. Because Ric-8At does not bind to AGS3-C, FRET can
arise only from binding of YFP-Ric-8A to Gα11-GDP present in a
CFP-AGS3-C:Gα11-GDP complex. Control experiments rule out
the possibility that FRET arises from nonspecific interactions
between YFP-Ric-8A and CFP-AGS3-C.

We note that it was not possible to determine the stoichiom-
etry of the transient ternary complex composed of Gα11-GDP,
AGS3-C, and Ric-8At. At the protein concentrations used in
these experiments, three to four molecules of Gα11 may be
expected to bind to the four available GPR/GoLoco motifs in
N-terminally CFP-tagged AGS3-C. However, the magnitude of
FRET emission that arises from binding of YFP-Ric-8A to each of
the four possible Gα11 molecules arrayed on CFP-AGS3-C
may differ, and the four Gα11 molecules may not be equally
accessible for interaction with YFP-Ric-8A. Binding of even a
single YFP-Ric-8A molecule to one of four interaction sites in
RIC-8A Catalyzes GEF on Ga\(_i_1\), Bound to AGS3

![Image](image_url)

**FIGURE 5.** FRET detection of transient ternary complex formation by YFP-Ric-8A with CFP-AGS3-C:GDP. Top panel, evolution and decay of FRET emission resulting from the interaction of YFP-Ric-8A with CFP-AGS3-C:GDP (12 \(\mu\)M) in a 20-\(\mu\)l cell of a pneumatically driven stopped-flow apparatus. FRET emission fluorescence at 527 nm upon excitation at 415 nm was measured for 300 s (green trace). A control experiment was performed by rapidly mixing equal volumes (120 \(\mu\)l) of YFP-Ric-8A (55 \(\mu\)M) with CFP-AGS3-C (12 \(\mu\)M) under the same reaction conditions (black trace). Traces shown are averages of 12–15 independent experiments performed under identical conditions. GDP release from AGS3-C:GDP (50 \(\mu\)l, 12 \(\mu\)M) was monitored by tryptophan fluorescence emission (340 nm) after rapid mixing with 50 \(\mu\)l of Ric-8At (55 \(\mu\)M, red trace, or 250 \(\mu\)l, blue trace). Bottom panel, evolution of FRET because of addition of GTP to a reaction mixture identical to that shown in the top panel at the 150-s time point. Equal volumes of YFP-Ric-8A (110 \(\mu\)l, 55 \(\mu\)M) and CFP-AGS3-C:GDP (12 \(\mu\)M complex) were rapidly mixed into an aging loop. After 150 s, GTP (110 \(\mu\)l, 150 \(\mu\)M) was then mixed with 110 \(\mu\)l of the aging loop contents into the optical cell of the stopped-flow apparatus (note that, because of the 1:4 stoichiometry of the CFP-AGS3-C:GDP complex, the concentration of GTP injected into the optical cell is in ~3-fold excess to GDP). FRET emission at 527 nm was measured for 1300 s. The green trace represents the reaction sequence depicted in top panel, and the black trace shows the evolution of FRET emission because of association of CFP-AGS3-C:GDP with YFP-Ric-8A upon GTP binding and hydrolysis by Ga\(_i_1\). Arrow indicates the time point at which GTP was added to the aged reaction mixture. The FRET emission base line for first reaction sequence at 150 s (green trace) was aligned with that for the second phase of the reaction (black trace).

**FIGURE 6.** A cycle of nonreceptor-mediated guanine nucleotide binding and hydrolysis by Ga\(_i_1\). The cycle includes Ga\(_i_1\), (Ga\(_i_1\)), in the presence of Ric-8At (R), AGS3-C (A), and a hypothetical GAP protein (Gp) (reactions involving the latter are shown in gray, because GAP proteins were not included in the present work). Kinetically reversible reactions are shown with double-headed arrows. The outer arc (labeled 1) describes the reaction, initiated at the point indicated by the perpendicular bar, corresponding to the green FRET traces shown in Fig. 5. The inner circle (labeled 2) describes the cyclic course of the reaction initiated at the point indicated by the perpendicular bar, corresponding to the black FRET trace in Fig. 5, bottom panel.

CFP-AGS3-C:Ga\(_i_1\):GDP could potentially generate FRET emission. Likewise, subsequent decay of FRET emission to the basal state could be due largely to dissociation of a single YFP-Ric-8A from the CFP-AGS3-C complex.

Dissociation (or loss of FRET from) of the YFP-Ric-8A:CFP-AGS3-C complex must arise through a spontaneous change in state of the complex subsequent to its formation. Ric-8At-catalyzed release of GDP from one or more Ga\(_i_1\) molecules bound to CFP-AGS3-C might induce such a change. Formation of nucleotide-free Ric-8At:Ga\(_i_1\) is associated with quenching of Ga\(_i_1\) tryptophan fluorescence (Fig. 5, red and blue traces). Quenching upon addition of Ric-8At to the AGS-3C:Ga\(_i_1\):GDP complex occurs with a rate constant (0.38 min\(^{-1}\)) that is only slightly slower than the quenching rate when Ric-8At binds to and releases nucleotide from free Ga\(_i_1\):GDP (~0.50 min\(^{-1}\)). Hence, disassembly of the Ga\(_i_1\):AGS-3C by Ric-8At apparently occurs two to three times more rapidly than the rate at which Ric-8A stimulates dissociation of GDP from Ga\(_i_1\), as illustrated by Scheme 1 (in which unitary stoichiometry among components is assumed for simplicity).

\[
\text{AGS3:Ga}i_1\text{:GDP} + \text{Ric8A} \leftrightarrow \text{AGS3:Ga}i_1\text{:GDP}:\text{Ric8A} \rightarrow \\
\text{AGS3} + \text{Ric8A}:\text{Ga}i_1\text{:GDP} \rightarrow \text{Ric8A}:\text{Ga} + \text{GDP}
\]

**SCHEME 1**

Either of two mechanisms would be consistent with the observed kinetics of FRET emission and GDP release described above. In one scheme, the YFP-Ric8-At:Ga\(_i_1\):GDP:CFP-AGS3-C complex undergoes a spontaneous rearrangement...
that results in the release of AGS3-C from the complex and subsequent dissociation of GDP from Ric-8At:Gαi1-GDP. Alternatively, dissociation of Gαi1 molecules from the four AGS3-C GPR/GoLoco motifs could occur cooperatively, triggered by Ric-8At-catalyzed GDP release from one or two Gαi1 molecules in the complex. Support for the latter model comes from the observation that assembly of AGS3-C-Gαi1-GDP complexes may be cooperative (40).

Our sequential mixing experiments indicate that the nucleotide-free Gαi1 in YFP-Ric-8At:Gαi1 complex is competent to undergo additional nucleotide binding/hydrolysis cycling reactions when liberated by Ric-8At from an initial AGS3-C-bound state as shown schematically in Fig. 6. This cycling process is akin to the classical receptor-mediated G-protein cycling event, but it should be stressed that, unlike the receptor that recognizes only the G-protein heterotrimers, Ric-8A can bind to both free Gαi1-GDP and AGS3-bound Gαi1-GDP.

The results presented here have implications for the molecular mechanism of Ric-8A action. That Ric-8A and AGS3-C are capable of forming a transient complex with Gαi1 implies that the two must occupy distinct or at least partially nonoverlapping binding sites. The crystal structure of the GPR/GoLoco peptide of RGS14 bound to Gαi1-GDP revealed interactions between the peptide and the Switch I and Switch II segments of Gαi1, extending across the catalytic site and into the helical domain (41). Because Ric-8A and AGS3-C form a transient complex with Gαi1, Ric-8A is likely to interact with at least one site that lies outside of this region. Because the K_m value of Ric-8A for Gαi1 is not affected by AGS3-C, it is probable that the binding sites for the two proteins on Gαi1 do not overlap. The Gα-binding site for G-protein-coupled receptors, which also catalyze release of GDP from G-proteins, include C-terminal residues of Gα that are distinct from the switch regions (47–49). GPCRα interact most productively with G-protein αβγ heterotrimers. AGS3 and Gβγ both bind to the switch segments of the GDP-bound form of Gα to inhibit nucleotide release (6, 41). It is reasonable to propose that the Gα-binding sites for Ric-8A and GPCRs are the same or share similar structural elements.

Ric-8A is a GEF capable of acting on either free and or GPR/GoLoco-bound Gaii1-GDP subunits (32). Because Ric-8A is a cell matrix or peri-centriolar protein that cannot catalyze nucleotide exchange of Gβγ-bound Gαi1 (32), it does not compete directly with receptor-mediated signaling. Ric-8A may serve as the upstream regulator of Gαi1 subunits in a pathway that directs dynamic aster-microtubule events during cell division. In this pathway, Ric-8A may activate receptor-independent Gαi1 catalysis by catalyzing GDP → GTP exchange. Catalysis would be completed by the concerted action of GAP proteins, such as C. elegans RGS7 that deactivate Gαi through acceleration of GTP hydrolysis (Fig. 6) (27). In this context, Gαi activation and deactivation may be coupled, respectively, to the release and sequestration of the microtubule-binding protein NuMA from the AGS3 homolog LGN. When bound to Gαi1-GDP, LGN recruits NuMA to the cell periphery while preventing its association with microtubules (43). Ric-8A has been shown, in vitro, to liberate NuMA from LGN through control of allosteric Gαi1-GDP binding. Liberated NuMA potentially participates in interactions with microtubules. We have speculated that this functional cycle might be responsible for generation of the pulsatile aster microtubule pulling forces that act upon the mitotic spindle. That Ric-8A is able to act on Gaii1-GDP subunits that are cooperatively assembled on LGN or AGS3 suggests that Ric-8A-assisted G-protein cycling evolved primarily for multiple and rapid activation events. The unidirectional nature of the reaction is ensured by the fact that AGS3 cannot interact with Ric-8A:Gαi1 complex following GDP release, or with Gαi-GTP that is generated subsequently.

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