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# Ric-8B Is a GTP-dependent G Protein $\alpha_s$ Guanine Nucleotide Exchange Factor*[S]

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***ric-8*** **(resistance to inhibitors of cholinesterase 8) genes have positive roles in variegated G protein signaling pathways, including G$\alpha_q$ and G$\alpha_s$ regulation of neurotransmission, G$\alpha_i$-dependent mitotic spindle positioning during (asymmetric) cell division, and G$\alpha_{olf}$-dependent odorant receptor signaling. Mammalian Ric-8 activities are partitioned between two genes,** ***ric-8A*** **and** ***ric-8B*****. Ric-8A is a guanine nucleotide exchange factor (GEF) for G$\alpha_i$/$\alpha_q$/$\alpha_{12/13}$ subunits. Ric-8B potentiated $G_s$ signaling presumably as a G$\alpha_s$-class GEF activator, but no demonstration has shown Ric-8B GEF activity. Here, two Ric-8B isoforms were purified and found to be G$\alpha$ subunit GDP release factor/GEFs. In HeLa cells, full-length Ric-8B (Ric-8BFL) bound endogenously expressed G$\alpha_s$ and lesser amounts of G$\alpha_q$ and G$\alpha_{13}$. Ric-8BFL stimulated guanosine 5′-3-*O*-(thio)triphosphate (GTP$\gamma$S) binding to these subunits and G$\alpha_{olf}$, whereas the Ric-8B$\Delta$9 isoform stimulated G$\alpha_{s\ short}$ GTP$\gamma$S binding only. Michaelis-Menten experiments showed that Ric-8BFL elevated the $V_{max}$ of G$\alpha_s$ steady state GTP hydrolysis and the apparent $K_m$ values of GTP binding to G$\alpha_s$ from ~385 nM to an estimated value of ~42 $\mu$M. Directionality of the Ric-8BFL-catalyzed G$\alpha_s$ exchange reaction was GTP-dependent. At sub-$K_m$ GTP, Ric-BFL was inhibitory to exchange despite being a rapid GDP release accelerator. Ric-8BFL binds nucleotide-free G$\alpha_s$ tightly, and near-$K_m$ GTP levels were required to dissociate the Ric-8B·G$\alpha$ nucleotide-free intermediate to release free Ric-8B and G$\alpha$-GTP. Ric-8BFL-catalyzed nucleotide exchange probably proceeds in the forward direction to produce G$\alpha$-GTP in cells.**

Heterotrimeric G proteins transduce signals received from ligand-bound G protein-coupled receptors (GPCRs)[2] to intracellular effector enzymes. Agonist-bound GPCRs activate coupled G protein heterotrimers by accelerating the rate of GDP for GTP exchange on the G$\alpha$ subunit (1). The understanding of G protein signaling pathway complexity expanded beyond this traditional paradigm when modulatory proteins that regulate G protein activation apart from receptors were uncovered and characterized (2–7).

One well characterized non-receptor G protein activator is Ric-8 (resistance to inhibitors of cholinesterase 8A). *ric-8* was first identified in a genetic screen devised to find mutants of genes that positively regulated *Caenorhabditis elegans* neurotransmission (8). Through genetic epistasis analyses, it was predicted that *ric-8* action was elicited upstream of G$\alpha_q$ and/or G$\alpha_s$ to regulate divergent G protein signaling outputs (8–10). Ric-8 was first linked physically to G proteins when two homologous mammalian Ric-8 proteins (so-named Ric-8A and Ric-8B) were isolated in yeast two hybrid screens using G$\alpha_o$ and G$\alpha_s$ baits, respectively. Ric-8A interacted with G$\alpha_{i/o}$, G$\alpha_q$, and G$\alpha_{13}$ subunits (7). Ric-8B interacted with G$\alpha_s$ and G$\alpha_q$ (7, 11). Evidence of a preferred interaction of Ric-8A with G$\alpha_i$-GDP led to experimentation showing that Ric-8A was a guanine nucleotide exchange factor for the monomeric G$\alpha$ subunits it bound *in vitro*. Purified Ric-8A stimulated intrinsic G$\alpha$ GDP release, leading to accelerated GTP binding kinetics and steady state GTPase activity (7, 12).

Technical issues of Ric-8B protein purification have prevented an examination of its putative G$\alpha$ GEF activity. Based on its yeast two-hybrid G$\alpha$ binding preferences, we hypothesized that Ric-8B was a GEF for G$\alpha_s$- and/or G$\alpha_q$-class subunits. Evidence in support of this has since been provided by demonstration that full-length Ric-8B (Ric-8BFL) positively influenced $G_s$-class signaling in cells. Ric-8BFL overexpression potentiated ligand-dependent GPCR activation of $G_{olf}$- and $G_s$-dependent cAMP production (13, 14). A shorter expressed isoform of Ric-8B that lacks the entirety of the region encoded by exon 9 of Ric-8BFL (termed Ric-8B$\Delta$9) did not enhance $G_{olf}$ signaling and actually appeared to be a modest inhibitor. Interestingly, Ric-8BFL is one of the long sought components required to reconstitute odorant receptor signaling in heterologous systems (14–17). Ric-8BFL overexpression in HEK cells with odorant receptors and receptor co-factors promoted odorant- and $G_{olf}$-dependent cAMP accumulation. Despite these findings, no direct demonstration that Ric-8B is a G$\alpha$ GEF has been made, and the role that Ric-8B might have in positively regulating $G_s$-class signaling has not been elucidated.

An idea not intuitively consistent with Ric-8B-GEF-mediated support of G$\alpha_s$-class signaling outputs was provided from studies showing that *ric-8* may control G protein expression. Genetic ablation of the single *C. elegans* or *Drosophila melanogaster ric-8* gene reduced levels of plasma membrane-associated G$\alpha_i$ and G$\beta$ subunits (18–21). RNAi disruption of *ric-8B* in

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[2] The abbreviations used are: GPCR, G protein-coupled receptor; G$\alpha_{s\ short}$, G protein $\alpha$s short isoform; G$\alpha_{s\ long}$, G protein $\alpha$s long isoform; GRF, GDP release factor; GEF, guanine nucleotide exchange factor; GTP$\gamma$S, guanosine 5′-3-*O*-(thio)triphosphate; SA, specific activity; TAP, tandem affinity purification; TEV, tobacco etch virus; C12E10, deionized polyoxyethylene 10 lauryl ether; GEF, guanine nucleotide exchange factor; m/v, mass/volume.

NIH-3T3 cells reduced $G\alpha_s$ steady state expression, and some of the remaining $G\alpha_s$ was marked for ubiquitin-mediated degradation (22). Ric-8A and Ric-8B greatly potentiated co-expressed recombinant $G\alpha$ subunit levels in insect cells (23). These findings raise the possibility that Ric-8B may not potentiate adenylyl cyclase signaling as a direct $G_s/G_{olf}$ GEF signaling activator but may do so by supporting $G\alpha_{olf}$ (or enhancing $G\alpha_s$) plasma membrane expression in systems, such as HEK cells, where $G\alpha_{olf}$ is not normally expressed. These ideas necessitated experimentation to address directly whether Ric-8B is a GEF activator of $G\alpha$ subunits and to determine its mechanism of action.

Here we show by direct biochemical demonstration that Ric-8BFL is a $G\alpha$ GDP release factor (GRF) and GEF for $G\alpha_s$ and $G\alpha_{olf}$, $G\alpha_q$, and, $G\alpha_{13}$. Ric-8BΔ9 is a $G\alpha_s$-specific GRF/GEF but was actually a modest inhibitor of $G\alpha_{olf}$ GTPγS binding. A stringent correlation was observed between Ric-8BFL and Ric-8A binding to endogenously expressed $G\alpha$ subunits in cells with respective Ric-8 protein capacity to support $G\alpha$ nucleotide release and exchange *in vitro*. GTP titration experiments indicate that Ric-8B would act as a directional GEF in cells to promote formation of $G\alpha_s$-GTP.

## EXPERIMENTAL PROCEDURES

*Plasmids, Antibodies, and Reagents*—To create *ric-8B* baculovirus donor constructs, mouse Ric-8BFL (Invitrogen, LLAM collection, clone 6490136) and rat Ric-8BΔ9 splice form cDNAs were subcloned by PCR into pFastBacGSTTEV (7). To create tandem affinity purification (TAP)-tagged *ric-8* constructs, a triple FLAG tag was inserted by PCR between the TEV-protease cleavage site and the Ric-8 coding sequences in the pFASTBacGSTTEV *ric-8* vectors (A and BFL). TAP-tagged *ric-8* cDNAs were excised with SalI and NotI restriction enzymes and subcloned into those sites in pFB Hygro (a gift from the Alliance for Cell Signaling). G protein subunit-specific antisera were used to detect $G\alpha_{i1/2}$ (BO84) (24), $G\alpha_q$ (WO82) (25), $G\alpha_{q/11}$ (C19) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), $G\alpha_{13}$ (A20) (Santa Cruz), $G\alpha_s$ (584) (26), $G\beta_{1/2}$ (U49) (24), and $G\beta_{1-4}$ (B600) (24). [$^{35}$S]GTPγS, [α-$^{32}$P]GTP, and [γ-$^{32}$P]GTP were purchased from PerkinElmer Life Sciences.

*Purification of Recombinant Proteins*—$G\alpha_{s\ short}$ and $G\beta_1\gamma_2$ were purified as described (27–29). $G\alpha_{olf}$, $G\alpha_q$, and $G\alpha_{13}$ were purified from insect cell detergent lysates using a Ric-8 association technique as described (23). Ric-8A was purified as described (7, 30). GST-tagged *ric-8B* baculoviruses were produced and amplified according to the manufacturer's instructions using the Bac-to-Bac *Sf*9 cell expression system (Invitrogen). Hi5 insect cells were grown in Sf900II medium to a density of $2 \times 10^6$ cells/ml and infected with amplified GST-*ric-8B* baculoviruses for 48 h. Cells were collected and lysed in lysis buffer (20 mM Hepes, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, protease inhibitor mixture (23 μg/ml phenylmethylsulfonyl fluoride, 21 μg/ml *Nα-p*-tosyl-L-lysine-chloromethyl ketone, 21 μg/ml L-1-*p*-tosylamino-2-phenylethyl-chloromethyl ketone, 3.3 μg/ml leupeptin, and 3.3 μg/ml lima bean trypsin inhibitor)) (Sigma-Aldrich) by nitrogen cavitation using a Parr Bomb (Parr Instrument Co., Moline, IL). Lysates were centrifuged sequentially at 3000 × *g* and at 100,000 × *g* for 45 min. The final supernatant was adsorbed to glutathione-Sepharose 4B resin (GE Healthcare). The resin was washed with lysis buffer and incubated with TEV protease for 16 h at 4 °C. Released Ric-8B proteins were bound to a 5-ml Hi-trap Q column (GE Healthcare) and eluted with a linear salt gradient from 100 to 500 mM NaCl. Monomeric Ric-8B proteins were separated from Ric-8B multimers by Superdex 200 10/300 GL gel filtration chromatography (GE Healthcare). Intact GST-TEV-Ric-8 proteins were eluted from glutathione-Sepharose 4B resin with lysis buffer containing 20 mM reduced glutathione. GST-TEV-Ric-8B fusion proteins were purified using successive Hi-trap Q and Superdex gel filtration chromatographies.

*Protein Interaction Assays*—GST-Ric-8 fusion proteins (500 nM) were incubated with purified $G\alpha$ (1 μM) or $G\alpha$ (1 μM) and $G\beta_1\gamma_2$ (1 μM) for 30 min at 22 °C in incubation buffer (20 mM Hepes, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% (m/v) deionized polyoxyethylene 10 lauryl ether (C12E10), and 1 μM GDP). Protein mixtures were then incubated with 20 μl of glutathione-Sepharose 4B for 2 h at 4 °C. The beads were washed with incubation buffer, and Ric-8 or Ric-8-bound proteins were released by digestion with AcTEV protease (Invitrogen) for 16 h at 4 °C, processed in reducing SDS-PAGE sample buffer, resolved by SDS-PAGE, and visualized by Coomassie Blue staining.

GST-Ric-8 interactions with G proteins extracted from brain membranes with detergents were performed as described previously with more sensitive Western blotting conditions (7). Rat brain membrane extracts were prepared by homogenizing whole rat brains in 10 mM Tris-HCl, pH 8.0, 11% sucrose, and protease inhibitor mixture using a Dounce homogenizer. The homogenate was centrifuged at 100,000 × *g*, and membrane pellets were pooled and homogenized in extraction buffer (20 mM Hepes, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT) before the addition of 1% (m/v) C12E10 and 10 μM GDP to solubilize membranes for 1 h at 4 °C. The samples were centrifuged at 100,000 × *g*, and the detergent-protein extract supernatant was collected. GST-Ric-8BFL, GST-Ric-8BΔ9, GST-Ric-8A, or GST protein (100 μg of each) was adsorbed to a 60-μl bed volume of glutathione-Sepharose 4B pre-equilibrated in equilibration buffer (20 mM Hepes, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and protease inhibitor mixture) for 2 h at 4 °C. The resins were collected by centrifugation at 500 × *g* and washed twice with 1 ml of equilibration buffer. Detergent protein extract (11.4 mg) was then incubated with affinity and control resins for 2 h at 4 °C. The resins were collected by centrifugation, washed five times with extraction buffer, and incubated with 45 μl of extraction buffer containing 5 μl of AcTEV protease (Invitrogen) for 16 h at 4 °C. The resins were pelleted, and the 50-μl supernatants were combined with a 70-μl subsequent resin wash. Eluted proteins were resolved by SDS-PAGE, and the gels were transferred to nitrocellulose and Western blotted with G protein subunit-specific antisera.

*Ric-8 Tandem Affinity Purification (TAP) of G Proteins*—Phoenix 293T cells were transfected with the TAP-tagged *ric-8* pFB Hygro constructs, and recombinant retroviruses were produced according to the manufacturer's instructions (Orbigen, Inc., San Diego, CA). HeLa S3 cells (CCL-2.2, ATCC, Manassas, VA) were infected with the viruses, and stable expression of

TAP *ric-8A* or *ric-8BFL* was selected with 400 μg/ml hygromycin B for 7 days. Stable TAP Ric-8 HeLa S3 cell lines were expanded and grown in suspension paddle culture in $Ca^{2+}$-free minimum essential medium containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, 200 μg/ml hygromycin B, and 0.1% pluronic acid. Suspension cells ($3 \times 10^9$) were recovered and lysed in 20 mM Hepes, pH 8.0, 150 mM NaCl, 1 mM DTT, 2 mM EDTA, and protease inhibitor mixture by Parr Bomb nitrogen cavitation. Lysates were cleared by centrifugation at 100,000 × *g* for 40 min, and the supernatants were applied to glutathione-Sepharose 4B. The Sepharose was washed with lysis buffer and incubated with TEV protease for 18 h at 4 °C. Proteins released by TEV digestion were diluted with 7 ml of PBS containing 1 mM EDTA and protease inhibitor mixture and batch-bound to 250 μl of anti-FLAG M2 affinity gel (Sigma-Aldrich) for 16 h at 4 °C. The FLAG resin was washed with PBS, suspended in reducing SDS-PAGE sample buffer, and boiled for 5 min. The FLAG resin eluates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for G protein subunits.

*GTPγS Binding and Release Assays*—Gα GTPγS binding assays were described previously (7, 31). Briefly, Gα (100 nM) was mixed with Ric-8 proteins (200 nM or as indicated otherwise) at 25 or 30 °C in GTPγS binding buffer (20 mM Hepes, pH 8.0, 100 mM NaCl, 4 mM DTT, 1 mM EDTA, 10 mM $MgCl_2$, 0.05% (m/v) C12E10 ($G\alpha_{i1}$, $G\alpha_{s\ short}$, $G\alpha_{13}$) or 0.05% Genapol C-100 ($G\alpha_q$, $G\alpha_{olf}$)), and 10 μM [$^{35}$S]GTPγS (SA 10,000 cpm/pmol). Triplicate aliquots were taken from the reactions at the indicated time points, quenched in GTP quench buffer (20 mM Tris, pH 7.7, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM GTP, and 0.08% (m/v) C12E10), and filtered onto BA-85 nitrocellulose filters. The filters were washed (20 mM Tris, pH 7.7, 100 mM NaCl, 2 mM $MgCl_2$), dried, and subjected to scintillation counting. GTPγS release measurements were performed using the aforementioned nitrocellulose filter binding method, but $G\alpha_{s\ short}$ or $G\alpha_q$ was first preloaded to completion with [$^{35}$S]GTPγS. $G\alpha_{s\ short}$ was simply incubated in GTPγS binding buffer for 30 min at 25 °C with 10 μM [$^{35}$S]GTPγS (SA 10,000 cpm/pmol). $G\alpha_q$ (10 μM) was preloaded with 100 μM [$^{35}$S]GTPγS (SA 5,000 cpm/pmol) in gel filtration buffer containing Ric-8A catalyst (5 μM) for 18 h at 4 °C and 10 min at 25 °C. $G\alpha_q$-GTPγS was then separated from Ric-8A by gel filtration as described (7, 30). GTPγS release from $G\alpha_{s\ short}$ or $G\alpha_q$ (100 nM) was initiated at 25 °C or 30 °C, respectively, by the addition of Ric-8 protein (500 nM) and challenge with 100 μM non-radioactive GTPγS. Free $Mg^{2+}$ at 1 mM was used in the $G\alpha_q$ experiments to accelerate the observed rate of GTPγS release.

*GDP Release Assay*—Gα GDP release assays were described previously (12). Gα (100 nM) was loaded with 10 μM [α-$^{32}$P]GDP (SA 50,000 cpm/pmol) for 1 h at 30 °C in 50 mM Hepes, pH 8.0, 0.5 mM DTT, 5 mM EDTA, 0.8 mM $MgCl_2$, 4% glycerol, and 0.05% (m/v) C12E10. GDP release was initiated at 25 °C upon the addition of Ric-8 proteins (0 or 200 nM) in reaction buffer (20 mM Hepes, pH 8.0, 1 mM DTT, 2 mM $MgCl_2$, 100 mM NaCl, and 100 μM GTPγS). Duplicate aliquots were taken from the reactions at the indicated time points; quenched in 20 mM Tris, pH 7.7, 100 mM NaCl, 30 mM $MgCl_2$, 30 μM $AlCl_3$, 5 mM NaF, 50 μM GDP; and filtered onto BA-85 nitrocellulose filters. The filters were washed with $AlF_4^-$-containing quench buffer, dried, and subjected to scintillation counting.

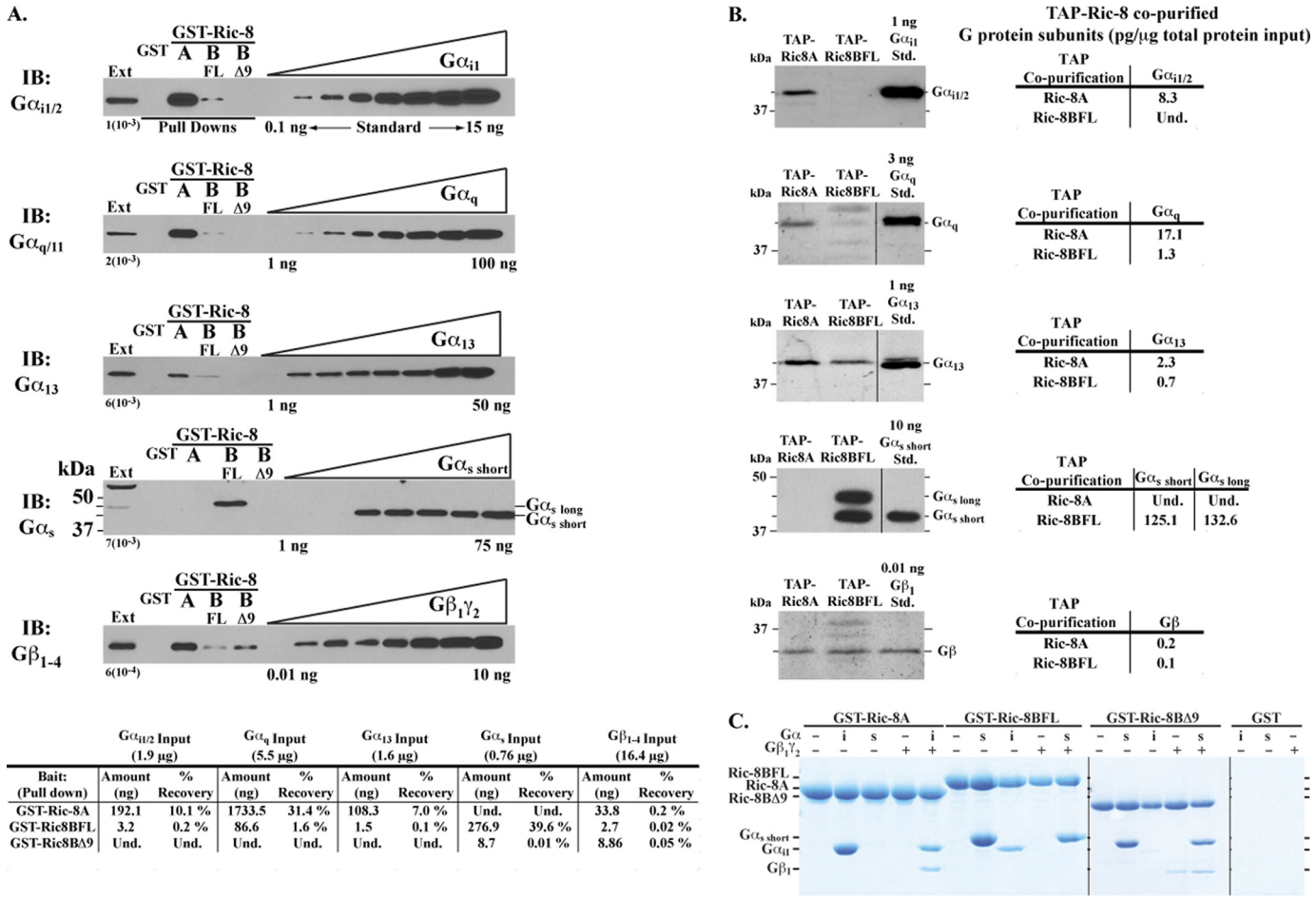
*Steady State GTP Hydrolysis (GTPase)*—Ric-8 proteins (indicated concentrations) and Gα (50 nM) were mixed in buffer containing 20 mM Hepes, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 or 10 mM $MgCl_2$, and 0.05% Genapol C100 ($G\alpha_q$) or 0.05% (m/v) C12E10 ($G\alpha_{s\ short}$). Triplicate reactions were started by the addition of 0.5–50 μM [γ-$^{32}$P]GTP (SA ≥10,000 cpm/pmol) at 25 °C. Reactions were quenched with 5% Norit charcoal in 50 mM $NaH_2PO_4$, pH 3.0, and processed as described previously (32). The amount of hydrolyzed $P_i$ was calculated after scintillation counting. In assays where 500 nM $G\beta_1\gamma_2$ was included, Gα plus $G\beta_1\gamma_2$ or Gα alone were preincubated for 15 min at 22 °C. Reactions were initiated by the addition of Ric-8 (500 nM) and 0.5 μM [γ-$^{32}$P]GTP in reaction buffer.

*Single Turnover GTPase*—$G\alpha_{s\ short}$-[γ-$^{32}$P]GTP was prepared by limited modification of the method of Ross (32). $G\alpha_{s\ short}$ (5 μM) was incubated with 10 μM [γ-$^{32}$P]GTP (SA 30,000 cpm/pmol) in Buffer C (50 mM Hepes, pH 8.0, 100 mM NaCl, 1 mM DTT, 5 μg/ml BSA, 0.05% (m/v) C12E10) containing 10 mM EDTA for 15 min at 25 °C. $G\alpha_s$-[γ-$^{32}$P]GTP was separated from free [γ-$^{32}$P]GTP over a G25-Sephadex column (fine grade). Fractions containing Gα-[γ-$^{32}$P]GTP were pooled and diluted to ~50 nM in Buffer C containing 10 mM EDTA, 3 μg/ml BSA, and 1 μM GTP. Single-turnover GTPase reactions were started by the addition of 88 mM $MgCl_2$ and Ric-8 (0 or 500 nM) at 4 °C. Aliquots from the reactions were taken at the indicated times and processed as described above for steady state GTPase.

*Gel Filtration Assays*—Ric-8B proteins (5 μM) were incubated with $G\alpha_{s\ short}$ (10 μM) or $G\alpha_q$ (10 μM) in gel filtration buffer (20 mM Hepes, pH 8.0, 150 mM NaCl, 3 mM DTT, 2 mM $MgCl_2$, 1 mM EDTA) and 100 μM GDP or [$^{35}$S]GTPγS (SA 35,000 cpm/pmol) for 15 min at 25 °C. The reactions were centrifuged at 21,000 × *g* for 10 min, and the supernatants were resolved over Superdex 75 and 200 10/300 GL columns arranged in series (GE Healthcare). Column eluates were fractionated, and fractions were subjected to Coomassie-stained SDS-PAGE and scintillation counting to measure GTPγS.

## RESULTS

*Ric-8 and G Protein Interactions*—Individual interactions between G protein subunits and Ric-8B or Ric-8A have been described (7, 11, 13, 22, 33). However, a complete and comparative profile of the interactions between Ric-8BFL, Ric-8BΔ9, and Ric-8A with representatives of all four classes of Gα subunits or Gβγ has not been made. We tested Ric-8B and Ric-8A binding to G proteins expressed endogenously in cells and *in vitro* using membrane detergent extracts and purified components. Purified GST-TEV-Ric-8 fusion proteins or GST were adsorbed to glutathione-Sepharose and incubated with detergent extracts of rat brain membranes. The resins were washed, and G proteins bound specifically were released by TEV protease digestion and identified by quantitative Western blot (Fig. 1*A*). Purified G protein subunit standards were used to calibrate the Western blot signals (Fig. 1 and supplemental Fig. S2). No G proteins were recovered with control GST resin. Ric-8BFL bound members of all four Gα classes, but significantly more




| Bait: (Pull down) | $G\alpha_{i1/2}$ Input (1.9 μg) Amount (ng) | % Recovery | $G\alpha_q$ Input (5.5 μg) Amount (ng) | % Recovery | $G\alpha_{13}$ Input (1.6 μg) Amount (ng) | % Recovery | $G\alpha_s$ Input (0.76 μg) Amount (ng) | % Recovery | $G\beta_{1-4}$ Input (16.4 μg) Amount (ng) | % Recovery |
|---|---|---|---|---|---|---|---|---|---|---|
| GST-Ric-8A | 192.1 | 10.1 % | 1733.5 | 31.4 % | 108.3 | 7.0 % | Und. | Und. | 33.8 | 0.2 % |
| GST-Ric8BFL | 3.2 | 0.2 % | 86.6 | 1.6 % | 1.5 | 0.1 % | 276.9 | 39.6 % | 2.7 | 0.02 % |
| GST-Ric8BΔ9 | Und. | Und. | Und. | Und. | Und. | Und. | 8.7 | 0.01 % | 8.86 | 0.05 % |

FIGURE 1. **Ric-8A and Ric-8B bind different sets of G protein subunits.** *A*, whole rat brain membrane detergent extracts (11.4 mg) were incubated with 100 μg of purified GST-TEV, GST-TEV-Ric-8A, GST-TEV-Ric-8BFL, or GST-TEV-Ric-8BΔ9 proteins and applied to glutathione-Sepharose 4B resin. The resins were washed, and bound proteins were released by TEV protease digestion. Detergent extract input material (*Ext.*), the proteins released by TEV digestion, and increasing concentrations of purified G protein subunit standards ($G\alpha_{i1}$, $G\alpha_q$, $G\alpha_{13}$, $G\alpha_{s\ short}$, $G\beta_1\gamma_2$) were resolved by SDS-PAGE. The gels were transferred to nitrocellulose and Western blotted with G protein subunit-specific antisera as indicated. Immunoblot (*IB*) signals were calibrated by densitometry analysis in supplemental Fig. S2. The total amount of G protein isolated with each GST-Ric-8 bait is reported in ng and as percentage recovery of input. *B*, TAP-tagged Ric-8A or Ric-8BFL were stably expressed in HeLa S3 cells and purified from soluble (detergent-free) cell lysates by tandem affinity chromatography (glutathione-Sepharose 4B and FLAG affinity resins). Eluates from the FLAG affinity column and the indicated amounts of purified G protein subunit standards were resolved by SDS-PAGE and Western blotted with G protein subunit-specific antisera. The amount of each G protein subunit (pg/μg of input) that co-purified with TAP-tagged Ric-8A or Ric-8BFL was measured by quantitative densitometry analysis of the immunoblots. *C*, purified GST-TEV-Ric-8 proteins (500 nM) were incubated with purified $G\alpha_{i1}$ or $G\alpha_{s\ short}$, with or without $G\beta_1\gamma_2$ (1 μM). The protein mixtures were adsorbed to glutathione-Sepharose 4B resin. The resins were washed, and proteins bound specifically were released by TEV protease digestion. The released proteins were processed in reducing SDS sample buffer, resolved by SDS-PAGE, and visualized by Coomassie Blue staining.

$G\alpha_s$ was recovered (39.6% of the total $G\alpha_s$ input *versus* 1.6% or less for other $G\alpha$ subunits). Ric-8BΔ9 bound very low levels of $G\alpha_s$ exclusively (Fig. 1*A* and supplemental Fig. S3). Ric-8A bound appreciable $G\alpha_{q/11}$, $G\alpha_{i1/2}$, and $G\alpha_{13}$ (31.4, 10.1, and 7.0% of the input, respectively) but did not bind $G\alpha_s$. Ric-8B was reported to interact with Gγ subunits by a yeast two-hybrid assay and overexpression/co-immunoprecipitation (33). Sensitive immunoblotting with an anti-$G\beta_{1-4}$ common antibody revealed that Gβs (and presumably Gγs) were recovered at very low levels, albeit specifically by all three GST-Ric-8 proteins from the membrane detergent extracts (≤0.2% of input).

A TAP strategy was used to investigate the complete profile of interactions between TAP-tagged Ric-8 proteins and endogenously expressed cytosolic G protein subunits using a single approach (Fig. 1*B*). HeLa S3 cell lines were created that stably expressed TAP-tagged Ric-8A or TAP-tagged Ric-8BFL. The TAP-tagged Ric-8 versions were expressed ~6–8-fold higher than endogenous Ric-8A or Ric-8BFL (data not shown). Soluble (cytosolic) fractions of native lysates were prepared from these cell lines and purified successively by the two TAP affinity steps (glutathione-Sepharose and anti-FLAG affinity chromatography). Gα subunits recovered from the FLAG affinity resin were identified by quantitative Western blot using G protein subunit-specific immunoreagents (Fig. 1*B*). Ric-8BFL bound the long and short isoforms of $G\alpha_s$ selectively. No $G\alpha_s$ was bound to Ric-8A in cells. Ric-8A bound $G\alpha_{i1/2}$ selectively. No $G\alpha_{i1/2}$ was bound to Ric-8BFL in cells. 17-Fold more $G\alpha_q$ and 3-fold more $G\alpha_{13}$ were co-purified with Ric-8A compared with the amounts co-purified with Ric-8BFL. Very low amounts of total Gβ co-purified with Ric-8A or Ric-8BFL from the soluble lysates. These results define the subsets of G protein subunits that Ric-8A and Ric-8BFL interact with in the cell and demonstrate that one subcellular site of these interactions is the cytosol.

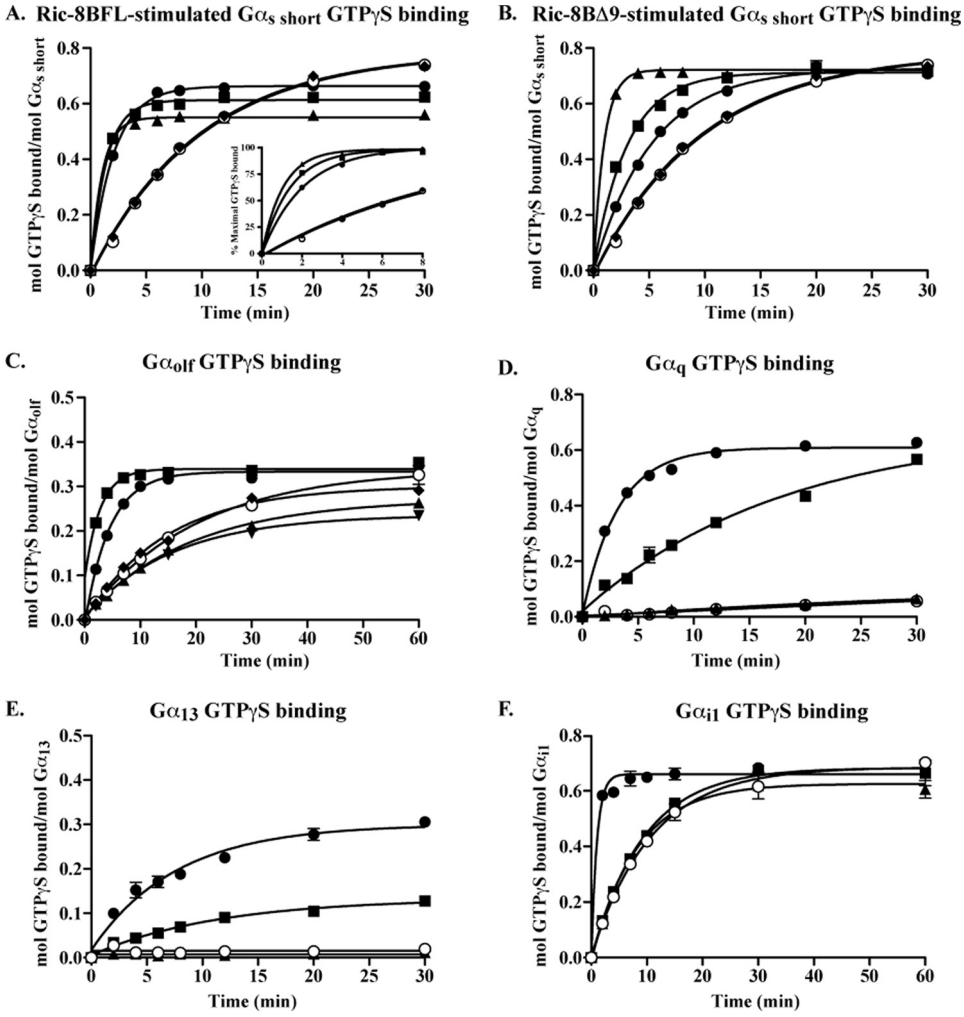



FIGURE 2. **Ric-8BFL is a $G\alpha_q$, $G\alpha_{13}$, and $G\alpha_s$/$G\alpha_{olf}$ GEF, and Ric-8BΔ9 is a $G\alpha_s$ GEF.** The kinetics of GTPγS binding to $G\alpha_{s\ short}$ (*A* and *B*), $G\alpha_{olf}$ (*C*), $G\alpha_q$ (*D*), $G\alpha_{13}$ (*E*), and $G\alpha_{i1}$ (*F*) were measured in the absence (○) or presence of Ric-8 proteins (*closed symbols*). Purified G proteins (100 nM each) were added to reactions containing 10 μM [$^{35}$S]GTPγS (SA 10,000 cpm/pmol) and the following purified Ric-8 proteins: 200 nM (●), 500 nM (■), and 2 μM (▲) Ric-8BFL or Ric-8BΔ9 or 200 nM Ric-8A (♦) (*A* and *B*); 200 nM Ric-8BFL (●), Ric-8BΔ9 (▲), or Ric-8A (♦) or 1 μM Ric-8BFL (■) or Ric-8BΔ9 (▼) (*C*); 200 nM Ric-8BFL (■), Ric-8BΔ9 (▲), or Ric-8A (●) (*D–F*). The reactions were incubated at 25 °C (30 °C for $G\alpha_{i1}$) for the indicated times. Triplicate aliquots were withdrawn from the reactions, quenched, and filtered through nitrocellulose filters. The filters were washed, dried, and subjected to scintillation counting to quantify the amount of G protein-bound GTPγS at each time point. The data were fit to exponential one-phase association functions or linear regression using GraphPad Prism version 5.0. Results are presented as the mean ± S.E. (*error bars*) of three experiments. *A* (*inset*), each data set was plotted as the percentage of maximal GTPγS bound to show that Ric-8BFL stimulated the rate of observed $G\alpha_{s\ short}$ GTPγS binding with increasing Ric-8BFL concentration. Notes that most error bars are smaller than actual *plotted symbols*. Intrinsic Gα rates (○) were measured in each experiment, although these points were often hidden by other data.

To examine the observed specificity of the Ric-8B and $G\alpha_s$ or the Ric-8A and $G\alpha_i$ interaction and to discriminate whether Ric-8 proteins bind $G\beta\gamma$ and/or G protein trimers directly, GST-Ric-8 pull-down experiments were conducted using purified components with conditions that were probably well above the $K_d$ values for relevant Ric-8-Gα subunit interactions (500 nM Ric-8A and 1 μM G protein subunits) (Fig. 1*C*). Ric-8A bound $G\alpha_{i1}$ and $G_i$ trimer but did not bind $G\alpha_{s\ short}$ or $G\beta_1\gamma_2$ alone. Ric-8BFL bound $G\alpha_{s\ short}$ and substoichiometric $G\alpha_{i1}$ but did not show appreciable binding to the $G_{s\ short}$ trimer or $G\beta_1\gamma_2$ alone. In contrast, Ric-8BΔ9 bound $G\alpha_{s\ short}$, $G\beta_1\gamma_2$ alone, and perhaps $G_{s\ short}$ trimer but did not bind $G\alpha_{i1}$.

*Ric-8B Is a G Protein α Subunit GEF*—Ric-8B was proposed to be a GEF for $G\alpha_s$- and $G\alpha_q$-class subunits because of its homology to Ric-8A and abilities to bind these subunits and positively regulate $G_s$/$G_{olf}$-induced cAMP accumulation in cells (Fig. 1) (7, 11, 13, 33). However, the mechanism of Ric-8B regulation of G protein signaling is not clear, and no positive result demonstrating Ric-8B GEF activity has been observed. A procedure was developed to purify active Ric-8BFL and Ric-8BΔ9 from insect cells for the purpose of measuring putative Ric-8B GEF activities. Representatives of all four Gα subunit families ($G\alpha_{s\ short}$ and $G\alpha_{olf}$, $G\alpha_q$, $G\alpha_{13}$, and $G\alpha_{i1}$, 100 nM each) were incubated in timed GTPγS binding reactions alone or with purified Ric-8BFL, Ric-8BΔ9, or Ric-8A (200 nM each or doses as indicated). The amount of [$^{35}$S]GTPγS bound to Gα over time was measured using a nitrocellulose filter-binding assay (7, 31). The purity and use of proteins in these and subsequent studies are shown and denoted in supplemental Fig. S1. $G\alpha_{s\ short}$ or $G\alpha_{s\ short}$ in the presence of Ric-8A bound GTPγS at a rate of 0.1 $min^{-1}$, consistent with a previous report (34). Ric-8BFL and Ric-8BΔ9 (200 nM each) increased this rate to 0.48 and 0.19 $min^{-1}$, respectively (Fig. 2, *A* and *B*). Inclusion of increasing doses of Ric-8BFL and Ric-8BΔ9 (200 nM to 2 μM

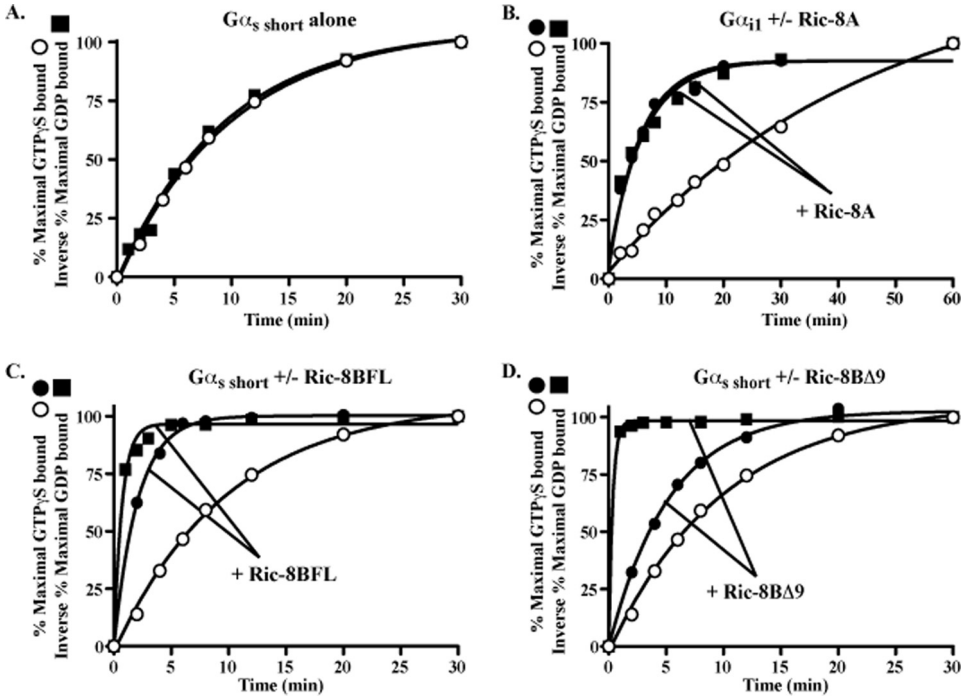



FIGURE 3. **Ric-8B delayed GTPγS binding to nucleotide-free $G\alpha_s$ after stimulating rapid GDP release.** Purified $G\alpha_{s\ short}$ or myristoylated $G\alpha_{i1}$ (100 nM each) was loaded to completion with 10 μM [α-$^{32}$P]GDP (SA 50,000 cpm/pmol) and then added to reactions with or without purified Ric-8 proteins as indicated. Gα GDP release was measured at 25 °C by quenching aliquots of each reaction in $AlF_4^-$-containing buffer and filtering them onto nitrocellulose filters. The filters were washed, dried, and subjected to scintillation counting to quantify the amount of GDP that remained bound to Gα at each time point. The inverse of the percentage of maximal GDP release (■) was co-plotted with the percentage of maximal Ric-8-stimulated (●) or intrinsic (○) GTPγS binding (at 25 °C) over time for $G\alpha_{s\ short}$ alone (*A*), $G\alpha_{i1}$ and Ric-8A (*B*), $G\alpha_{s\ short}$ and Ric-8BFL (*C*), or $G\alpha_{s\ short}$ and Ric-8BΔ9 (*D*). The data were fit to exponential one-phase association functions using GraphPad Prism version 5.0. All assay results are representative of at least three independent experiments that contained 2–3 replicates/assay.

each) in the $G\alpha_{s\ short}$ GTPγS binding reactions resulted in distinct effects. Both Ric-8B isoforms increased the $G\alpha_{s\ short}$ GTPγS binding rate in a dose-dependent manner (0.48–0.96 $min^{-1}$ (Ric-8BFL) and 0.19–1.1 $min^{-1}$ (Ric-8BΔ9)). However, Ric-8BFL uniquely lowered the $Y_{max}$ of GTPγS binding (end point stoichiometry) from 0.66 to 0.56 mol of GTPγS/mol of $G\alpha_{s\ short}$ (at 200 nM to 2 μM Ric-8BFL).

The inhibitory effects of Ric-8BFL on $G\alpha_s$ end point GTPγS binding were unusual. End point ($Y_{max}$) $G\alpha_{s\ short}$ and $G\alpha_q$ GTPγS binding were compared directly over a wide range of Ric-8 protein concentrations (supplemental Fig. S4). Ric-8BFL dose-dependently inhibited end point $G\alpha_{s\ short}$ GTPγS binding but increased $G\alpha_q$ GTPγS binding. Ric-8BΔ9 did not affect either G protein, and lower doses of Ric-8A (200 nM) resulted in saturated $G\alpha_q$ GTPγS binding. One possible explanation for the observed Ric-8BFL-dependent loss of end point $G\alpha_{s\ short}$ GTPγS binding was that Ric-8BFL caused irreversible denaturation of a portion of $G\alpha_{s\ short}$ over the course of the assay. To test this possibility, Ric-8BFL or Ric-8BΔ9 (5 μM each) was incubated with $G\alpha_{s\ short}$-GTPγS (10 μM) for 30 min at 25 °C. The protein mixtures were gel-filtered to separate monomeric G proteins from G protein·Ric-8B dimeric complexes and higher ordered aggregates (supplemental Fig. S5). Virtually all of the $G\alpha_{s\ short}$-GTPγS was recovered as active monomer or in complex with Ric-8BFL. Ric-8BFL did not cause $G\alpha_{s\ short}$ denaturation and aggregation. Ric-8BΔ9 itself was prone to aggregation, but the released $G\alpha_{s\ short}$-GTPγS present in this reaction was not.

We recently reported the primary GTP binding characteristics and adenylyl cyclase activating activities of purified olfactory/brain-specific $G\alpha_s$ homolog, $G\alpha_{olf}$ (23). Ric-8BFL stimulated the $G\alpha_{olf}$ GTPγS binding rate in a dose-dependent manner, whereas Ric-8BΔ9 was actually a modest inhibitor of both end point $G\alpha_{olf}$ GTPγS binding stoichiometry and the GTPγS binding rate (Fig. 2*C*). Ric-8A did not affect GTPγS binding characteristics of $G\alpha_{olf}$.

$G\alpha_q$ and $G\alpha_{13}$ bind GTPγS negligibly in detergent solution (Fig. 2, *D* and *E*) (35, 36). Ric-8BFL and Ric-8A dramatically increased the $G\alpha_q$ GTPγS binding rate from negligible values to 0.06 $min^{-1}$ and 0.31 $min^{-1}$, respectively (Fig. 2*D*), and increased the negligible $G\alpha_{13}$ GTPγS binding rate to 0.09 $min^{-1}$ and 0.13 $min^{-1}$, respectively (Fig. 2*E*). Ric-8BΔ9 did not affect the kinetics of $G\alpha_q$ or $G\alpha_{13}$ GTPγS binding, consistent with the observation that Ric-8BΔ9 did not bind either Gα subunit. A small amount of $G\alpha_{i1}$ (0.2% of input) was recovered by Ric-8BFL from the membrane extracts (Fig. 1*A*), but Ric-8BFL did not stimulate $G\alpha_{i1}$ GTPγS binding. Conversely, Ric-8A bound substantial $G\alpha_{i1/2}$ and stimulated $G\alpha_{i1}$ GTPγS binding dramatically (Fig. 2*F*), as shown previously (7).

*Ric-8B Is a GRF*—G protein nucleotide exchange is limited by the slow GDP release step and followed by rapid GTP binding to the open form of Gα (37). GEFs stimulate GDP release. Ric-8-stimulated Gα GDP release measurements were made and compared directly to the corresponding rates of observed Gα GTPγS binding at equivalent Ric-8 concentrations. Intrinsic $G\alpha_{s\ short}$ GDP release (plotted as the inverse) and GTPγS binding rates were nearly equivalent (0.1 $min^{-1}$ each; Fig. 3*A*), as were the Ric-8A-stimulated $G\alpha_{i1}$ GDP release and GTPγS binding rates (0.13 and 0.14 $min^{-1}$, respectively; Fig. 3*B*). How-

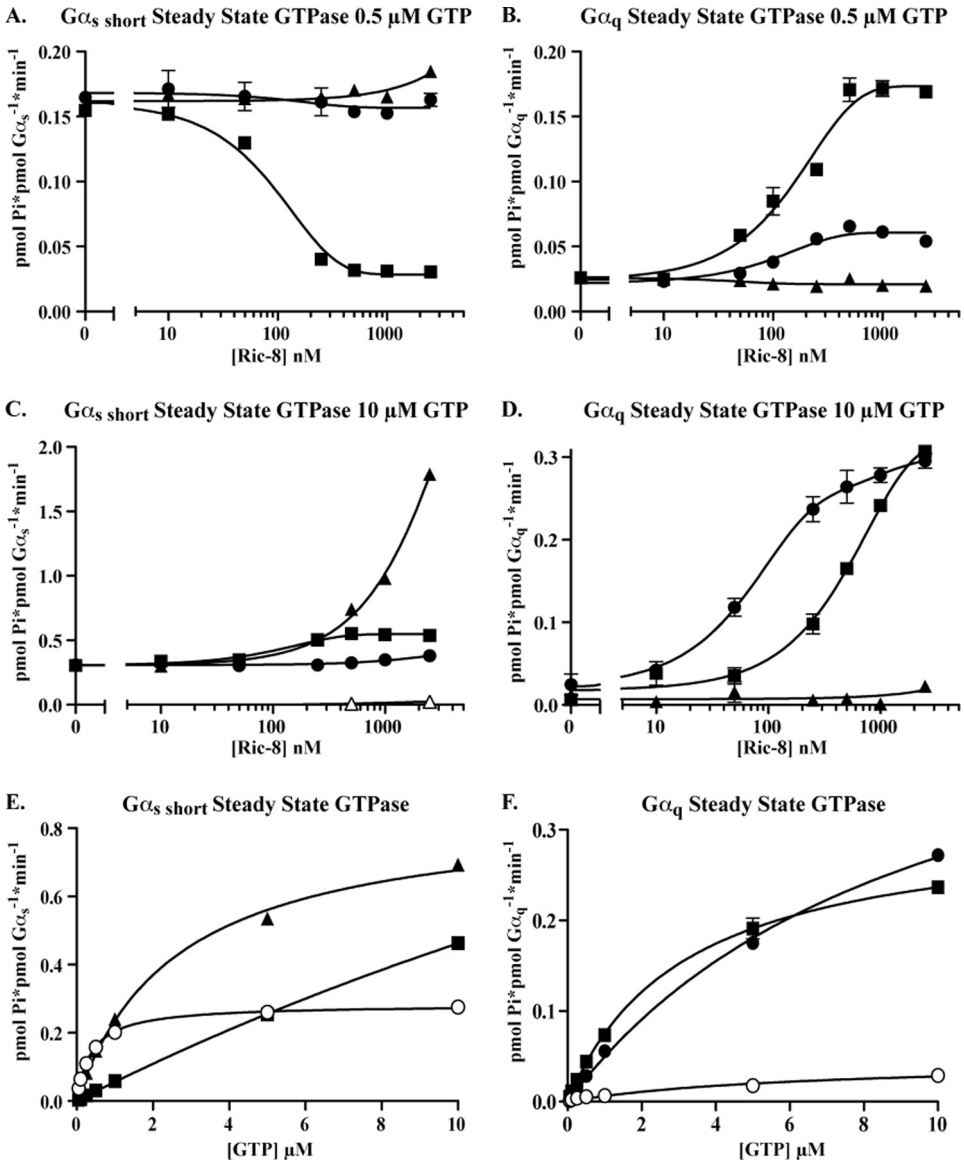



FIGURE 4. **Ric-8B regulation of $G\alpha_{s\ short}$ and $G\alpha_q$ steady state GTP hydrolytic activities are GTP-dependent.** $G\alpha_{s\ short}$ (*A*, *C*, and *E*) or $G\alpha_q$ (*B*, *D*, and *F*) (50 nM each, total of 1 pmol/assay) was mixed in triplicate with Ric-8BFL (■), Ric-8BΔ9 (▲), or Ric-8A (●) (0–2.5 μM) and the indicated concentrations of [γ-$^{32}$P]GTP (SA 10,000–70,000 cpm/pmol) to initiate steady state GTPase reactions at 25 °C. The reactions were quenched after 5–7 min in acidic charcoal suspension and processed as described. *C*, the Ric-8BΔ9 (△) preparation did not contain a contaminating GTPase. In the GTP titration experiments (*E* and *F*), Gα alone (○) or Gα and the indicated Ric-8 proteins (500 nM each, closed symbols) were used. All assay results are representative of at least three independent experiments that contained 3 replicates/assay. *Error bars*, S.E.

ever, Ric-8BFL- or Ric-8BΔ9-stimulated $G\alpha_{s\ short}$ GDP release rates (1.41 and 2.98 $min^{-1}$, respectively) were markedly faster than the corresponding stimulated $G\alpha_{s\ short}$ GTPγS binding rates (0.48 and 0.19 $min^{-1}$, respectively; Fig. 3, *C* and *D*). At the concentration of GTPγS used in these assays (10 μM), Ric-8B proteins were more effective $G\alpha_s$ GRFs than GEFs.

*Ric-8B-influenced G Protein Steady State GTP Hydrolysis (GTPase) Activity*—GTPγS binding to nucleotide-free Ric-8B·$G\alpha_s$ complexes was apparently slower than binding to nucleotide-free $G\alpha_{s\ short}$. Steady state GTPase measurements were conducted to explore the mechanism of this kinetic delay. Because steady state GTPase is limited by GDP release, a GEF should enhance this rate (38). Surprisingly, at low GTP concentrations typically used in these assays (500 nM), Ric-8BFL potently inhibited $G\alpha_{s\ short}$ GTPase activity ($IC_{50}$ ~35 nM) (Fig. 4*A*). High concentrations of Ric-8BΔ9 increased $G\alpha_{s\ short}$ activity marginally, and Ric-8A had no effect. Conversely, at low GTP concentration, Ric-8BFL and Ric-8A stimulated the intrinsically low $G\alpha_q$ steady state GTPase activity to maximal velocities of 0.17 and 0.07 $min^{-1}$, with estimated $EC_{50}$ values of ~150 and ~110 nM, respectively (Fig. 4*B*). Ric-8BΔ9 did not affect $G\alpha_q$ steady state GTPase activity. A higher level of GTP (10 μM) was tested next to determine whether Ric-8 effects were dependent on GTP concentration. Interestingly, Ric-8BFL was no longer inhibitory but activated $G\alpha_{s\ short}$ GTPase activity weakly (Fig. 4*C*). High concentrations of Ric-8BΔ9 (250 nM to 2.5 μM) activated $G\alpha_{s\ short}$ with increasing efficacy and did not appear to be saturating even at the highest concentration tested. Ric-8-dependent $G\alpha_q$ activation was increased modestly at high GTP concentration, but a potency difference

between Ric-8A and Ric-8BFL was revealed. Ric-8A and Ric-8BFL activated $G\alpha_q$ to similar maximal rates with $EC_{50}$ values of ~130 and ~750 nM, respectively (Fig. 4*D*). Ric-8BΔ9 did not influence $G\alpha_q$ GTPase activity at any GTP concentration.

Quantitative analyses of $G\alpha_{s\ short}$ and $G\alpha_q$ steady state GTPase activities were performed by conducting GTP titrations. The data were plotted using Michaelis-Menten models to estimate the $K_m$ and $V_{max}$ values with or without Ric-8 proteins present. The calculated $K_m$ of $G\alpha_{s\ short}$ for GTP was 385.1 ± 10 nM, which was consistent with previous reports for Gα subunits (39, 40). Ric-8BFL and Ric-8BΔ9 increased the $K_m$ dramatically to ~42.4 ± 8.1 and ~2.6 ± 0.2 μM, respectively. $V_{max}$ was elevated from ~0.28 $min^{-1}$ to 2.4 ± 0.4 and 0.85 ± 0.02 $min^{-1}$ when Ric-8BFL or Ric-8BΔ9 proteins were assayed, respectively (Fig. 4*E*). The $K_m$ of $G\alpha_q$ for GTP (alone) could not be estimated reliably because $G\alpha_q$ has such low intrinsic GTPase activity. Ric-8A and Ric-8BFL increased $G\alpha_q$ activity in a substrate-dependent manner to estimated $V_{max}$ values of 0.53 ± 0.02 and 0.31 ± 0.01, respectively. The estimated $K_m$ values of $G\alpha_q$ for GTP in the presence of Ric-8A and Ric-8BFL were 9.8 ± 0.7 and 3.1 ± 0.3 μM, respectively. Due to technical limitations of the assay (high $P_i$ product background with increasing substrate concentration), the $K_m$ values of GTP for $G\alpha_{s\ short}$ in the presence of Ric-8BFL (~42 μM) and GTP for $G\alpha_q$ in the presence of Ric-8A (~9.8 μM) can only be considered estimates because measurements could not be made using GTP concentrations above the estimated $K_m$ values. Nonetheless, Ric-8BFL and Ric-8A dramatically increased the apparent $K_m$ values of $G\alpha_{s\ short}$ and $G\alpha_q$ for GTP, respectively. These elevated $K_m$ values provide a partial explanation for the observation that GTP binding to open Ric-8B·$G\alpha_{s\ short}$ complexes was inhibited and for why Ric-8BFL was inhibitory to $G\alpha_{s\ short}$ GTP binding at low GTP concentrations. At physiological GTP concentrations (250–700 μM) (41), Ric-8BFL would act as a GEF activator (and not an inhibitor) of $G\alpha_{s\ short}$.

Gβγ is obligatory for GPCR GEF activity but inhibitory to Ric-8A activation of $G\alpha_q$ (7). Because Gβγ was found to bind Ric-8 isoforms weakly (Fig. 1*C*) (33), it was tested for its capacity to regulate Ric-8B-influenced $G\alpha_{s\ short}$ and $G\alpha_q$ steady state GTPase activities at low GTP concentration and with reduced $Mg^{+2}$ levels (both reagents inhibit Gβγ binding to Gα). Gβγ markedly inhibited intrinsic and all Ric-8 isoform-influenced $G\alpha_{s\ short}$ and $G\alpha_q$ steady state GTPase activities (supplemental Fig. S6). Ric-8 and Gβγ regulate Gα activity independent of each other *in vitro*.

*Guanine Nucleotide Content of Ric-8B·Gα Complexes*—To clarify findings from the kinetic assays and better define the functional characteristics of Ric-8B interactions with G protein subunits, the guanine nucleotide states of $G\alpha_{s\ short}$ and $G\alpha_q$ when bound to Ric-8 proteins were determined using a gel filtration-based assay in which Ric-8A was shown to bind nucleotide-free $G\alpha_{i1}$ (7). A molar excess of $G\alpha_{s\ short}$ or $G\alpha_q$ (10 μM) was incubated with Ric-8 protein (5 μM) in the presence of 100 μM [$^{35}$S]GTPγS or GDP. The protein mixtures were resolved over Superdex 75 and 200 size exclusion columns arranged in tandem. The column eluates were fractionated. Proteins and GTPγS present in the fractions were identified by Coomassie-stained SDS-PAGE and $UV_{280}$ absorbance or scintillation

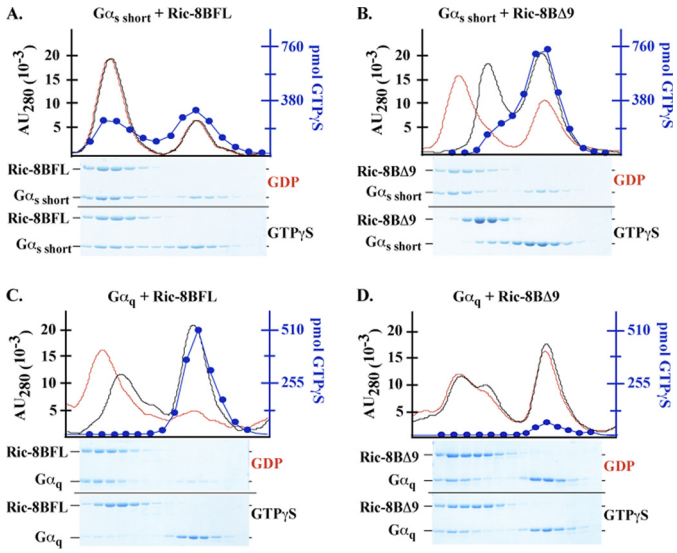



FIGURE 5. **Ric-8BFL and Ric-8BΔ9 bind differentially to GDP- and GTPγS-bound $G\alpha_{s\ short}$ and $G\alpha_q$.** Ric-8B proteins (5 μM) were mixed with $G\alpha_{s\ short}$ (*A* and *B*) or $G\alpha_q$ (10 μM each) (*C* and *D*) in the presence of 100 μM GDP or [$^{35}$S]GTPγS (SA 35,000 cpm/pmol) and incubated for 15 min at 22 °C. The protein/nucleotide mixtures were centrifuged to remove particulate and gel-filtered over Superdex 75 and Superdex 200 columns arranged in tandem. The column eluates were fractionated, and protein-containing fractions were analyzed by Coomassie-stained SDS-PAGE and scintillation counting to quantify the amount of GTPγS contained in each fraction (*blue traces*, [$^{35}$S]GTPγS experiments only). UV absorbance traces ($AU_{280}$) of the column eluates for the GDP (*red traces*) and GTPγS (*black traces*) experiments were co-plotted with the GTPγS measurements (*blue traces*) on double-labeled *y* axis plots. *Left* to *right*, species eluted in decreasing molecular weight from the columns.

counting. Ric-8BFL and Ric-8BΔ9 formed stoichiometric complexes with $G\alpha_{s\ short}$ in the presence of GDP (Fig. 5, *A* and *B*). Given that both Ric-8B isoforms were efficacious $G\alpha_{s\ short}$ GDP release factors (Fig. 3), and by analogy to the nucleotide-free Ric-8A·$G\alpha_{i1}$ complex, it was concluded that Ric-8B·$G\alpha_{s\ short}$ complexes formed in the presence of GDP were nucleotide-free (7, 30). Interestingly, Ric-8BFL, but not Ric-8BΔ9, formed substantial stable complex with $G\alpha_{s\ short}$-GTPγS. Based on a protein complex stoichiometry of 1:1, ~60.8% of the Ric-8BFL was bound to $G\alpha_{s\ short}$-GTPγS (Fig. 5*A*). The measured fraction of GTPγS bound to $G\alpha_{s\ short}$ in complex with Ric-8BFL did not differ from that bound to monomeric $G\alpha_{s\ short}$ (~38%). The experiments with $G\alpha_q$ confirmed the capacity of Ric-8BFL and Ric-8A, but not Ric-8BΔ9, to stimulate $G\alpha_q$ nucleotide exchange. Ric-8BFL bound $G\alpha_q$ with 1:1 stoichiometry in the presence of GDP but was bound to very little detectable $G\alpha_q$ in the presence of GTPγS (Fig. 5*C*). The monomeric $G\alpha_q$ pool had a substantial fraction of bound GTPγS (~27%), showing that Ric-8BFL promoted $G\alpha_q$ GTPγS binding. Ric-8A acted similarly to Ric-8BFL in promoting $G\alpha_q$ GTPγS binding, although a substantial portion of Ric-8A remained bound to $G\alpha_q$-GTPγS (~50%) (supplemental Fig. S7). At the high protein concentrations used in these experiments, a nucleotide preference of Ric-8BΔ9 $G\alpha_q$ binding was not observed. Ric-8BΔ9 bound ~35% of the $G\alpha_q$, whether GDP or GTPγS was present, but the Ric-8BΔ9·$G\alpha_q$ complex did not contain GTPγS (Fig. 5*D*). Notably, the monomeric $G\alpha_q$ pool in the Ric-8BΔ9 experiment had very little bound GTPγS, confirming that Ric-8BΔ9 is not a $G\alpha_q$ GEF.

*Ric-8 Interactions with Gα-GTP*—Isolation of stable Ric-8BFL·$G\alpha_{s\ short}$·GTP and Ric-8A·$G\alpha_q$·GTP complexes may reflect high affinities that Ric-8BFL and Ric-8A have for the respective G protein subtypes. Ric-8 proteins might influence Gα steady state GTPase activity as a consequence of interaction with Gα-GTP by altering the rate of single turnover GTP hydrolytic activity or by promoting GTP release from Gα prior to hydrolysis. The latter possibility would explain the ability of Ric-8BFL to greatly increase the apparent estimated $K_m$ for GTP binding to $G\alpha_{s\ short}$ (Fig. 4*E*) and/or to reduce $G\alpha_{s\ short}$ GTPγS end point binding stoichiometry (Fig. 2 and supplemental Fig. S4). Measurements of Ric-8 influence on $G\alpha_{s\ short}$ single turnover GTP hydrolytic activity were conducted. $G\alpha_{s\ short}$ was loaded with [γ-$^{32}$P]GTP in the absence of $Mg^{2+}$, gel-filtered to remove excess nucleotide, and incubated in timed, single turnover GTPase reactions containing $MgCl_2$ and/or excess Ric-8 proteins (500 nM) at 4 °C. The rates of $G\alpha_{s\ short}$ GTP hydrolysis were nearly equivalent (range of 1.07–1.16 $min^{-1}$) in each experiment (Fig. 6). All Ric-8 proteins (and notably Ric-8BFL) did not affect $G\alpha_{s\ short}$ single turnover GTPase activity.

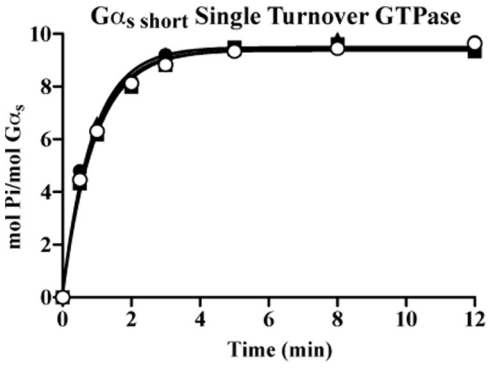



FIGURE 6. **Ric-8 proteins do not affect $G\alpha_{s\ short}$ single turnover GTPase activity.** $G\alpha_{s\ short}$ was loaded with [γ-$^{32}$P]GTP at 25 °C in buffer lacking $Mg^{+2}$ and separated from free GTP by rapid gel filtration. $G\alpha_{s\ short}$-[γ-$^{32}$P]GTP (60 nM, actual concentration) single turnover GTPase reactions were initiated at 4 °C by the addition of buffer containing $MgCl_2$ (○), $MgCl_2$ and Ric-8BFL (■), Ric-8BΔ9 (▲), or Ric-8A (●) (500 nM each). Duplicate reactions were quenched in acidic charcoal suspension at the indicated times and processed as described. Data are representative of three or more independent experiments. The mol of phosphate (*Pi*) released/mol of $G\alpha_{s\ short}$ over time were plotted using GraphPad Prism version 5.0 and one-phase association functions. Note that some points were hidden by other data.

Ric-8-influenced GTPγS release measurements from prepared Gα-[$^{35}$S]GTPγS substrates were conducted in the face of excess GTPγS or GDP challenge. These measurements allowed determination of Ric-8 promotion of Gα GTP release, GTP for GTP futile nucleotide exchange, and/or GDP for GTP reverse nucleotide exchange. With GTPγS challenge, Ric-8BFL stimulated rapid and complete $G\alpha_{s\ short}$ GTPγS release. $G\alpha_{s\ short}$ alone or $G\alpha_{s\ short}$ in the presence of Ric-8BΔ9 or Ric-8A retained prebound GTPγS (Fig. 7*A*). With GDP challenge, Ric-8BFL stimulated $G\alpha_{s\ short}$ GTPγS release with similar kinetics, but the extent of the release did not go to completion (~45%) (supplemental Fig. S8). As a consequence, Ric-8BFL catalyzes $G\alpha_{s\ short}$ GTP for GTP futile nucleotide exchange but probably does not induce GDP for GTP exchange. Stimulation of $G\alpha_q$ GTPγS release by Ric-8 proteins was far less dramatic than that observed for the Ric-8BFL and $G\alpha_{s\ short}$ pair and did not go to completion (Fig. 7*B*). Only Ric-8A stimulated measurable $G\alpha_q$ GTPγS release. Ric-8BFL did not. This was consistent with the finding that a stable complex of Ric-8A·$G\alpha_q$·GTPγS (supplemental Fig. S7) but not Ric-8BFL·$G\alpha_q$·GTPγS (Fig. 5*C*) could be isolated and reflects the higher affinity that Ric-8A probably has over Ric-8BFL for $G\alpha_q$.

## DISCUSSION

We report that Ric-8BFL binds natively expressed $G\alpha_s$ and, to lesser degrees, $G\alpha_q$ and $G\alpha_{13}$. Ric-8BFL is a guanine nucleotide exchange factor for these G proteins and $G\alpha_{olf}$. GTP concentration was an essential parameter that influenced Ric-8B exchange-stimulatory activity for $G\alpha_s$. At higher GTP substrate concentrations (≥10 μM), both Ric-8B isoforms were efficacious $G\alpha_s$ GEF activators of steady state GTPase activity and GTPγS binding. At lower GTP levels (≤1 μM), Ric-8BΔ9 marginally activated $G\alpha_s$ steady state GTPase activity, and Ric-8BFL was a potent inhibitor. These observations were reconciled by the idea that Ric-8 proteins interact with highest affinity with the nucleotide-free form of the Gα subunits that each bind. As a consequence, Ric-8 proteins dramatically raised the apparent $K_m$ values of GTP binding to Gα. After rapid Ric-8B stimulation of GDP release from $G\alpha_s$, cellular levels of GTP (~500 ± 200 μM) would displace Ric-8B from the nucleotide-free Ric-8B·$G\alpha_\sigma$ complex and drive the exchange reac-

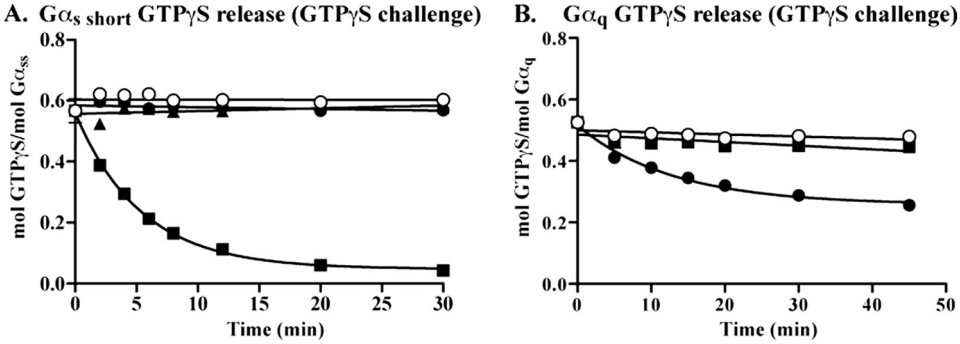



FIGURE 7. **Ric-8 proteins catalyze GTPγS/GTPγS futile nucleotide exchange.** $G\alpha_{s\ short}$ (*A*) and $G\alpha_q$ (*B*) were loaded to completion with [$^{35}$S]GTPγS as described. GTPγS release from Gα (100 nM) was measured over the indicated time courses at 25 °C ($G\alpha_{s\ short}$) or 30 °C ($G\alpha_q$) after the addition of 100 μM non-radioactive GTPγS (○) and/or Ric-8BFL (■), Ric-8A (●), and/or Ric-8BΔ9 (▲) (for $G\alpha_{s\ short}$ only) (500 nM each) using the GTPγS binding assay nitrocellulose filter binding method. The data were fit to one-phase exponential dissociation functions (Ric-8BFL/$G\alpha_{s\ short}$ and Ric-8A/$G\alpha_q$) or otherwise plotted by linear regression using GraphPad Prism. Results are the mean ± S.E. of three independent experiments. Note that most error bars are smaller than the actual *plotted symbols*, and some points were hidden by other data.

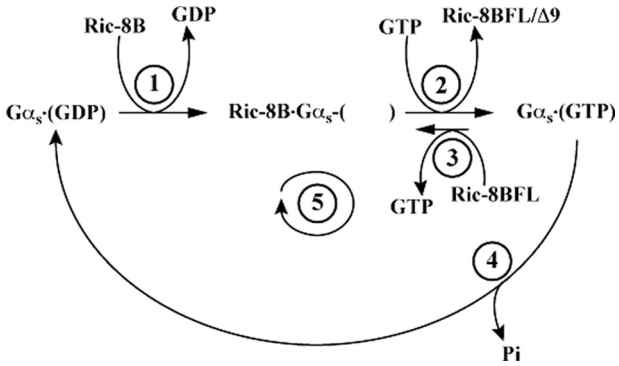



FIGURE 8. **Ric-8B regulation of $G\alpha_s$ catalysis.** Ric-8B is a $G\alpha_s$ GRF with GTP-dependent GEF activity. At low GTP (<10 $\mu$M), Ric-8B-stimulated $G\alpha_{s\ short}$ GDP release (*step 1*) was significantly faster than observed Ric-8B-stimulated GTP($\gamma$S) binding (*step 1* plus *step 2*), whereas intrinsic $G\alpha_{s\ short}$ GDP release and observed GTP$\gamma$S binding rates were equivalent. Ric-8B-FL potently inhibited $G\alpha_{s\ short}$ steady state GTPase activity (*step 5*) at low GTP, due to its capacity to dramatically increase the $K_m$ of GTP for $G\alpha_{s\ short}$ (~385 nM to an estimated value of ~42 $\mu$M) and to stimulate futile GTP for GTP exchange (*step 3*). Ric-8BΔ9 did not increase the $K_m$ of GTP for $G\alpha_{s\ short}$ nearly as much (~2.6 $\mu$M) and did not stimulate futile GTP/GTP exchange. These results probably reflect a higher affinity that Ric-8BFL has over Ric-8BΔ9 for $G\alpha_s$. Neither Ric-8B isoform had any effect on $G\alpha_s$ single turnover GTPase activity (*step 4*). At higher GTP (>10 $\mu$M), Ric-8BFL and Ric-8BΔ9 stimulated $G\alpha_{s\ short}$ nucleotide exchange and steady state GTPase activities. At physiological GTP, Ric-8B-catalyzed exchange is predicted to proceed in the forward direction to produce activated $G\alpha_s$-(GTP).

tion to completion in the forward direction to produce dissociated Ric-8B and activated $G\alpha_s$-GTP (Fig. 8). Ric-8BFL and Ric-8A also activated Gαq with GTP dependence, but both were activators at all concentrations of GTP tested.

Comparison of the results from the quantitative Ric-8·G protein binding studies (Fig. 1) with the guanine nucleotide kinetic assays (Figs. 2–7) allowed prediction of the relevant cellular interactions between the individual Ric-8 proteins (A, BFL, and BΔ9) and G protein subtypes of all four classes. With little exception, the amount of G protein recovered from cells or membrane extracts using particular Ric-8 baits correlated with the ability and degree to which that Ric-8 protein stimulated Gα subunit nucleotide exchange. The relevant Gα interactions with Ric-8A probably include the $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ classes. Ric-8A recovered the highest percentages of these G proteins in the pull-down experiments and activated the GTP$\gamma$S binding rates of each infinitely faster ($G\alpha_{i1}$), 5 times faster ($G\alpha_q$), or 2.3 times faster ($G\alpha_{13}$) than an equivalent concentration of Ric-8BFL. Ric-8A also increased the apparent $K_m$ of $G\alpha_q$ for GTP more so than Ric-8BFL.

Relevant Ric-8BFL cellular interactions certainly include and may be restricted to the $G\alpha_s$ class, although low amounts (<1.6% of input) of $G\alpha_q$ and $G\alpha_{13}$ were recovered in Ric-8BFL pull-down experiments (Fig. 1*A*). Ric-8BΔ9 appeared to have a quite low albeit exclusive affinity for $G\alpha_s$-class subunits. The dramatic enhancement of the estimated $K_m$ of GTP for $G\alpha_{s\ short}$ in the presence of Ric-8BFL (~110 times higher than $G\alpha_s$ alone) further supported the idea that $G\alpha_{s\ short}$ is the relevant Ric-8BFL-interacting Gα subunit in cells. Ric-8BΔ9 enhanced the apparent $K_m$ of GTP for $G\alpha_{s\ short}$ to a lesser degree (~6.7 times higher than $G\alpha_s$ alone). This was consistent with the fact that little $G\alpha_s$ was isolated by Ric-8BΔ9 in membrane extract pull-down experiments despite Ric-8BΔ9 activating $G\alpha_{s\ short}$ GDP release and GTP$\gamma$S binding activities efficaciously. The measured concentration of $G\alpha_{s\ short}$ in the extract pull-down input material was ~5–10 nM (Fig. 1*A*). $G\alpha_{s\ short}$ concentrations used in the GEF assays (Figs. 2 and 3) and purified component pull-down experiments (Fig. 1*C*) were 100 nM and 1 $\mu$M, respectively. If the $K_d$ of $G\alpha_s$·Ric-8BΔ9 binding lies between 10 and 100 nM, this could explain the apparent discrepancy in these results. Because Ric-8BΔ9 only activated $G\alpha_{s\ short}$ and did not activate $G\alpha_{olf}$ or any other tested class of Gα subunit, structural features of the 40-amino acid region within Ric-8BFL that are absent in the Ric-8BΔ9 isoform (by alternative splicing of the entirety of exon 9) may allow Ric-8BFL to bind Gα subunits with higher affinity and/or to possess a better ability to act as a GEF.

The biochemical data here show that Ric-8B should act as a directional $G\alpha_s$ GEF at physiological GTP levels. This seemingly corroborates propositions that overexpressed Ric-8B potentiated $G_s/G_{olf}$ signaling in cells by acting as a G protein activator (GEF) (13, 16, 17, 33). Although plausible, we must consider an alternate interpretation to this model, given that many independent studies have shown that Ric-8 proteins regulate G protein steady state and plasma membrane expression (18–23). Ric-8B might not facilitate $G_s/G_{olf}$ signaling as a direct G protein activator but may do so as a facilitator or enhancer of $G\alpha_{s\ short}/G\alpha_{olf}$ protein expression. In this capacity, Ric-8 might promote G protein biosynthesis and/or prevent G protein turnover by acting analogously toward Gα subunits as PhLP1 (phosducin-like protein 1) acts upon Gβ and Drip78 (dopamine receptor-interacting protein 78) acts on Gγ prior to Gβγ dimer formation (42–47). In this putative role, perturbation of Ric-8 expression would result in Gα protein chains that do not fold efficiently and/or be passed off from Ric-8 to Gβγ for initial G protein trimer assembly on intracellular membranes.

Another possible means of action of predominantly cytosolic Ric-8 proteins is as an escort-like component for G proteins that shuttle among membranes. The time required for $G\alpha_q$ to transit in retrograde fashion from the plasma membrane to the Golgi during a proposed palmitoylation/depalmitoylation cycling process occurred much faster than expected if the trafficking was a vesicle-mediated transport event (48–50). This implied that Gα transits rapidly through the cytosol to reach the outer face of the Golgi. One could easily envision that an escort protein, such as Ric-8 or Gβγ, is required to aid Gα during this transit lest it signal inappropriately. In conditions of reduced Ric-8 expression, Gα subunits not escorted to the proper cellular compartment(s) might be expected to be more sensitive to turnover.

How can these models of Ric-8 control of G protein expression be reconciled with our biochemical results that clearly show that Ric-8B and Ric-8A are GEFs? We envision a model in which so-called GEF activity may not necessarily or always be a means to control G protein activation status to directly evoke a signaling output. Rather, GEF activity could be a means to simply dissociate Ric-8 from Gα. With the exception of a few specific low affinity interactions of Ric-8 isoforms and Gα-GTP (Figs. 5 and 7 and supplemental Fig. S7), Ric-8 proteins dissociate from Gα when Gα adopts the GTP-bound conformation. It stands to reason that whatever Ric-8 proteins do to promote

or preserve Gα expression, they must bind Gα at one point and become dissociated at another. Use of the G protein GDP/GTP conformational switch could be the mechanism by which Ric-8 is dissociated from Gα at the proper temporal/spatial location. The question of what activates or regulates Ric-8 GEF activity in this regard also becomes pertinent. If Ric-8 is an escort factor for Gα during folding or a particular trafficking step, then a third component in addition to GTP may be necessary when the Ric-8·Gα complex reaches its destination to "activate" exchange and dissociate Ric-8 and Gα. This would release Gα to perform non-Ric-8 functions.

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