# Novel Activity of RGS14 on Goα and Giα Nucleotide Binding and Hydrolysis Distinct from Its RGS Domain and GDI Activity<sup>†</sup>

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ABSTRACT: The bifunctional protein RGS14 is both a GTPase activating protein (GAP) for Gia and Goa and a guanine nucleotide dissociation inhibitor (GDI) for Gi $\alpha$ . This GDI activity is isolated to a region of the protein distinct from the RGS domain that contains an additional G protein-binding domain (RBD/ GL). Here, we report that RGS14 missing its RGS domain (R14-RBD/GL) binds directly to Go and Gi to modulate nucleotide binding and hydrolysis by mechanisms distinct from its defined GDI activity. In brain pull-down assays, full-length RGS14 and R14-RBD/GL (but not the isolated RGS domain of RGS14) bind Goa-GDP, Gia-GDP, and also  $G\beta\gamma$ . When reconstituted with M2 muscarinic receptors (M2) plus either Gi or Go, RGS4 (which has no RBD/GL domain) and full-length RGS14 each markedly stimulates the steady-state GTPase activities of both G proteins, whereas R14-RBD/GL has little or no effect. R14-RBD/GL potentiates RGS4 GAP activity in membrane-based assays by increasing the apparent affinity of RGS4 for Gia and Goa, suggesting a cooperative interaction between the RBD/GL domain, RGS4, and  $G\alpha$ . This activity of R14-RBD/GL on RGS4 is not apparent in single-turnover solution GAP assays with purified Gia or Goa, suggesting that membranes and/or receptors are required for this activity. When these findings are taken together, they indicate that regions of RGS14 outside of the RGS domain can bind inactive forms of Go and Gi to confer previously unappreciated activities that influence  $G\alpha$ nucleotide binding and/or hydrolysis by mechanisms distinct from its RGS domain and established GDI activity.

Heterotrimeric G proteins  $(G\alpha\beta\gamma)$  transduce signals from cell-surface receptors to intracellular signaling pathways to mediate the actions of many neurotransmitters and hormones at target cells (1-4). After agonist binding and receptor activation, G proteins are activated such that G $\alpha$ -guanosine triphosphate (GTP)<sup>1</sup> and a  $G\beta\gamma$  complex act alone or in concert to regulate the action of target effector proteins. G $\alpha$ subunits are GTPases that act as molecular switches, and the lifetime of G $\alpha$ -GTP dictates the lifetime of the signaling event. The protein regulators of G protein signaling (RGS proteins) act as  $G\alpha$  GTPase activating proteins (GAPs) to limit the duration of the linked  $G\alpha$  signaling event (5). Mammalian RGS and RGS-like proteins comprise a family of more than 30 distinct proteins, which serve to modulate and/or integrate receptor and linked G protein signaling (6, 7). All family members share a conserved 130 amino acid RGS domain that binds to activated  $G\alpha$  subunits and confers GAP activity. In addition, RGS proteins can directly interfere with effector binding to  $G\alpha$  to serve as effector antagonists (8, 9). A hallmark property of smaller simple RGS protein family members and isolated RGS domains in particular is their preferential binding to activated  $G\alpha$ subunits, whereas inactive GDP-bound forms of  $G\alpha$  have much lower affinity and bind RGS proteins poorly or not at all (5).

While many RGS family members are small simple proteins with few features outside of the conserved RGS domain, other RGS proteins are larger and more complex with additional domains that confer other signaling functions and/or bind to other protein partners (7). RGS14 is a multifunctional 61-kDa protein that contains a defined RGS domain, a tandem Rap1/2 binding domain (RBD), a GPR/GoLoco (GL) motif, and other regions with no known function (10, 11). RGS14 interacts selectively with members of the Gi/o $\alpha$  subfamily of G proteins to regulate their guanine nucleotide binding/hydrolysis activity and signaling functions. The RGS domain of RGS14 binds directly to activated

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<sup>&</sup>lt;sup>1</sup> Abbreviations: RGS, regulators of G protein signaling; AGS3, activator of G protein signaling 3; GPR, LGN, and GL (synonymous terms for the same domain), GPR, G protein regulatory domain; LGN, human mosaic protein containing Leu-Gly-Asn repeats; GL, Gi/oα–Loco interaction or GoLoco domain; Pcp2, Purkinje cell protein 2; RapIGAPII, GTPase activating protein for rap1, type II isoform; GAP, GTPase activating protein; RBD, Rap-binding domain; GDI, guanine nucleotide dissociation inhibitor; AMF, AIF<sub>4</sub> and Mg<sup>2+</sup>; ATP, adenosine trisphosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; Ni-NTA, nickel-nitrilotriacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SF9, *Spodoptera frugiperda*; Tx, thioredoxin; H6, hexahistidine.

Gia/o and confers nonselective GAP activity toward Gia and Goa (11-13). RGS14 also inhibits the guanine nucleotide exchange activity of free Gia but not Goa to serve as a selective guanine nucleotide exchange inhibitor (GDI) (13, 14, 26). This GDI activity has been isolated to regions C-terminal relative to the RGS domain containing the RBD and the GL domains, suggesting this region binds Gia but not Goa to selectively modulate Gia but not Goa function. Indeed, a peptide encoding the GL motif binds directly and selectively to inactive Gia1-GDP but not Goa-GDP and is sufficient to mimic the GDI activity of RGS14 (14, 15). When these findings are taken together, they predict that RGS14 selectively binds to and acts on Gia-GDP but not Goa-GDP.

Previous studies demonstrate that certain other proteins containing GPR/GL motifs can interact with Goa (16-21)and, in several cases, modulate its function (17, 18), which raises the possibility that RGS14/Ga interactions may be more complex than previously expected. To investigate this possibility, we further examined biochemical and functional interactions between RGS14, isolated domains of RGS14, and Goa and Gia. We report that RGS14 and in particular regions of RGS14 contained within the RBD/GL domains and outside of the RGS domain can bind inactive forms of Go and Gi. Interaction of RBD/GL with Go and Gi in membranes expressing M2 muscarinic receptors confers previously unappreciated activities that influence Ga nucleotide binding and hydrolysis by mechanisms distinct from its established GAP and GDI activities.

## MATERIALS AND METHODS

Materials. Sources of baculoviruses encoding M2 muscarinic acetylcholine receptor, Gi $\alpha$ , Go $\alpha$ , G $\beta$ 1, and G $\gamma$ 2 were as described previously (22, 23). cDNA encoding TxH<sub>6</sub>-RGS14 was a generous gift of D. P. Siderovski (University of North Carolina, Chapel Hill, NC). cDNA encoding H<sub>6</sub>-R14-RGS and TxH<sub>6</sub>-R14-RBD/GL was generated by PCR using primers to the base pairs corresponding to amino acids 1-205 and 299-544, respectively, and cloned into the bacterial expression vectors pQE60 (H<sub>6</sub>-R14-RGS) and pET20b (TxH<sub>6</sub>-R14-RBD/GL) as described (13). Carbachol, tropicamide, and nonradioactive nucleotides were purchased from Sigma. Lubrol (undefined MW) was obtained from MP Biomedicals, Inc. Polyclonal antisera that specifically recognizes Goa and Gia, respectively, was purchased from Santa Cruz Biotechnologies. Rabbit polyclonal antisera that recognizes  $G\beta$  (common  $G\beta$  sera) was kindly provided by Dr. Susanne Mumby (UT Southwestern Medical Center, Dallas, TX). [<sup>3</sup>H]GDP was purchased from New England Nuclear, and  $[\gamma^{-32}P]$ GTP was purchased from ICN or Perkin-Elmer.

Protein Expression. Hexahistidine (H6)-tagged thioredoxin (Tx), Tx- and H6-tagged RGS14 (TxH<sub>6</sub>-RGS14), H6-tagged RGS14 encoding amino acids 1-205 of RGS14 including the RGS domain (H<sub>6</sub>-RSG14), and a Tx- and H6-tagged amino-truncated protein encoding amino acids 299–544 of RGS14 including the RBD and GL domains (TxH<sub>6</sub>-R14-RBD/GL) were constructed and expressed in BL21DE3 bacterial cells as described (*13*). Where indicated, fusion of RGS protein with Tx was necessary to generate an intact, stable protein. The cells were grown to mid-log phase, and

protein production was induced with 1 mM IPTG for 2 h. Cells were lysed using the French Press method, and the supernatant was recovered, loaded to a Ni<sup>2+</sup> HiTrap affinity column (Amersham Pharmacia, NJ), and purified by FPLC. Proteins were eluted with an imidazole gradient from 20 to 200 mM imidazole in 50 mM HEPES at pH 7.4 and 150 mM NaCl. For TxH<sub>6</sub>-R14, the cell supernatant was loaded to Ni-NTA agarose beads, washed and eluted using 200 mM imidazole, and further purified by FPLC using a superdex-200 column (Pharmacia-Biotech). Histidine-tagged RGS4, Gi1 $\alpha$ , and Go $\alpha$  were grown in *Escherichia coli* and purified as described previously (*13, 22, 23*).

Pull-Down Assays. Pull-down assays were performed as described (24). Rat brains were lysed in hypotonic buffer [50 mM Hepes at pH 8 and phenylmethyl-sulfonyl fluoride (PMSF)] and separated into cytosolic and membrane fractions by centrifugation at 100,000g. Brain membranes (500  $\mu$ g) were incubated with 10  $\mu$ g of Trx-H<sub>6</sub> (control protein), TxH<sub>6</sub>-RGS14, H<sub>6</sub>-R14-RGS, or TxH<sub>6</sub>-R14-RBD/GL in 20 mM Hepes, 150 mM NaCl, 1 mM DTT, 20 mM imidazole, and either GDP or GDP +  $AlF_4/Mg^{2+}$  and then extracted with 1% cholate. The extract was incubated with 100  $\mu$ L of Ni-NTA for 30 min. The beads were pelleted by centrifugation and then washed 5 times with 500  $\mu$ L of 20 mM Hepes, 150 mM NaCl, 0.1% Lubrol, 3 mM DTT, 40 mM imidazole, and GDP  $\pm$  AMF, as appropriate. Bound proteins were eluted into 120  $\mu$ L of 1× Laemmli buffer. Samples were denatured, subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted using anstisera specific for either Goa, Gia, or  $G\beta$ .

Receptor-Mediated Steady-State GTPase Assays. Sf9 cells were infected for 48 h with baculoviruses encoding the M2 muscarinic acetylcholine receptor,  $G\beta 1$  and  $G\gamma 2$ , plus either Gi $2\alpha$  or Go $\alpha$ , and membranes from these cells were prepared as described previously (22, 23) and stored at -80 °C. For GTPase assays, reaction mixtures containing 20 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 1  $\mu$ g/mL leupeptin, 10  $\mu$ g/mL aprotinin, 10 mM NaCl, and 2 mM MgCl<sub>2</sub> (calculated free Mg<sup>2+</sup> = 0.5 mM) plus membranes (6 µg), purified proteins, and muscarinic drugs, were preincubated on ice for 60 min; at that time, nucleotides were added [1  $\mu$ M GTP, 500  $\mu$ M ATP, [ $\gamma$ -<sup>32</sup>P]GTP (1 ×  $10^6$  cpm/assay)], and tubes were transferred to a 30 °C shaking water bath for 5 min. The assay was stopped by adding 950 µL of ice cold 5% (w/v) Norit in 0.05 M NaH<sub>2</sub>- $PO_4$  (pH 3) and centrifuging. Radioactivity of  $[^{32}P]P_i$  in the resulting supernatant was determined by liquid scintillation counting. The nonspecific membrane GTPase signal was estimated by adding 1 mM unlabeled GTP to the above assay mix. Agonist- and RGS-dependent GTPase activity was calculated as described (22).

Single-Turnover Solution-Based GTPase Assays. The GTPase activity of Gi $\alpha$  and Go $\alpha$  were measured as described (13). Purified recombinant His6-tagged G $\alpha$  subunits (0.5 uM) were loaded with 1  $\mu$ M [ $\gamma$ -<sup>32</sup>P]GTP at room temperature for either 20 min (Go $\alpha$ ) or 1 h (Gi<sub>1</sub> $\alpha$ ). Proteins were cooled to 4 °C and then added to a reaction tube containing an excess of GTP (100  $\mu$ M final) and MgCl<sub>2</sub> (5 mM final), as well as either buffer or buffer and RGS proteins (R14-RBD/GL, RGS4, and R14-RGS or a mixture of proteins as described). At 1 min, samples were quenched with ice-cold-activated charcoal and then centrifuged, and hydrolyzed [<sup>32</sup>P]P<sub>1</sub> was



FIGURE 1: Purified recombinant RGS14 and truncated forms of RGS14 proteins. (Left) Schematic diagram illustrating Tx- and H6tagged (TrxH<sub>6</sub>)-RGS14, H<sub>6</sub>-R14-RGS, TrxH<sub>6</sub>-R14-R/GL, and TrxH6 alone. (Right) Coomassie-blue-stained gel of purified TrxH<sub>6</sub>-RGS14 (RGS14), H<sub>6</sub>-R14-RGS, TrxH<sub>6</sub>-R14-RBD/GL, and TrxH6 control protein. Proteins were expressed in bacteria, purified as described in the Materials and Methods, and then resolved using SDS–PAGE.



FIGURE 2: RGS14 binds inactive Go $\alpha$ -GDP, Gi $\alpha$ -GDP, and G $\beta\gamma$ from brain membranes. (A) Pull-down assays were performed as described in the Materials and Methods. Brain membranes were incubated with RGS14 or TrxH6 control protein in the presence of GDP with or without AlF<sub>4</sub> and Mg<sup>2+</sup> (ÅMF). Membrane-bound proteins were extracted with 1% cholate, and the lysate was incubated with 100  $\mu$ L Ni<sup>2+</sup>-NTA beads, which were pelleted and washed with buffer with or without AlF<sub>4</sub>. Proteins were eluted into 1× laemmli sample buffer, resolved by SDS-PAGE, and immunoblotted using antisera that specifically recognize either Gia or Goa. Results are representative of five (RGS14) and two (Trx-H6) different experiments. (B) Brain membranes were incubated with either RGS14, R14-RGS, or R14-RBD/GL in the presence of GDP. Otherwise, membranes were treated and extracted, and pulldown assays were performed as described above. Samples were resolved by SDS-PAGE and immunoblotted using antisera that specifically recognize either Gia, Goa, or G $\beta$ . Results are representative of four (Go $\alpha$  and Gi $\alpha$ ) and two (G $\beta$ ) different experiments.

recovered from the supernatant and quantitated by liquid scintillation counting.

### RESULTS

A Truncated Form of RGS14 Lacking the RGS Domain Binds to Inactive Go $\alpha$ -GDP and Gi $\alpha$ -GDP (Figures 1 and 2). Our previous findings (13) and those of others (14, 15, 26) have demonstrated that RGS14 is a selective GDI for Gi $\alpha$  but not Go $\alpha$ . This suggests that RGS14 directly and selectively interacts with Gi $\alpha$ -GDP but not Go $\alpha$ -GDP. In contrast, several studies have shown that GL motif-containing proteins can modulate Go $\alpha$  activity (17, 18), suggesting that RGS14/G $\alpha$  interactions may be more complex than previously thought. To investigate this possibility, we examined RGS14 interactions with Gia and Goa present in brain extracts. Histidine-tagged RGS14 was expressed as a fulllength protein and in two truncated forms and purified by nickel chromatography (shown in Figure 1). One mutant, R14-RGS, includes the conserved RGS domain but lacks the carboxy-terminal 245 amino acid residues that contain the GL motif and the tandem RBD domains. The other truncated form, R14-RBD/GL, includes the GL and RBD domains but lacks the RGS domain. Active, full-length RGS14 is difficult to obtain from bacterial expression systems, and thus, as before (13), it was purified as a Tx-RGS14 fusion protein. Similarly, the yield of R14-RBD/GL when expressed as a Tx fusion protein was greatly enhanced compared to previous purification efforts (13). Additionally, we also purified recombinant His-tagged Tx to test in parallel as a control.

Because RGS14 is expressed in the brain (10, 11, 13), we tested whether these proteins bound Gia or Goa from extracted brain membranes in "pull-down" assays (Figure 2). When brain extracts were incubated with GDP,  $Mg^{2+}$ , and aluminum fluoride (AMF) to activate G proteins, RGS14 bound both Gia and Goa as expected (Figure 2A). However, when brain extracts were incubated with GDP alone to stabilize inactive G $\alpha$ . RGS14 also bound both Gi $\alpha$ -GDP and  $Go\alpha$ -GDP, which was unexpected. We investigated this further by determining whether  $G\alpha$ -GDP bound to either R14-RGS or R14RBD/GL. When brain extracts were incubated with GDP alone, RGS14 and R14-RBD/GL bound Goa, Gia, and G $\beta$  (and presumably G $\gamma$ ), whereas R14-RGS did not bind any of the G protein subunits under these assay conditions (Figure 2B). These findings suggest a novel functional interaction between RGS14 and inactive Go and Gi. However, we should note that Go is more abundant than Gi in the brain, and our findings do not address whether R14-RBD/GL binds Go and Gi with different relative affinities. As noted above, both full-length RGS14 and R14-RBD/GL were purified as Trx-H6 fusion proteins; however, this does not appear to affect their interactions with G proteins because a Trx-H6 control protein did not bind to either Gi $\alpha$  or Go $\alpha$  under either activating or nonactivating conditions.

Our unexpected observation that R14-RBD/GL binds inactive Go $\alpha$ -GDP indicates that a region distinct from the RGS domain of RGS14 can modulate the function of this G $\alpha$ . To investigate this, we examined whether R14-RBD/ GL influences the guanine nucleotide binding/exchange properties of target G $\alpha$ . We and others have shown that RGS14 acts as a GDI for Gi $\alpha$ 1 but not Go $\alpha$ , effectively slowing the exchange of GDP for GTP (13–15). Consistent with previous studies (13–15), R14-RBD/GL impeded the binding of [<sup>3</sup>H]GDP in exchange for bound GDP with Gi $\alpha$ at 30 °C but not with Go $\alpha$  at either 20 or 30 °C; Go $\alpha$ -GDP was labile at the higher temperature, as reported previously (25), but notably this thermal effect was decreased in the presence of R14-RBD/GL (data not shown), suggesting that the latter binds to free Go $\alpha$ .

The Isolated RBD/GL Domain of RGS14 Markedly Potentiates the Stimulatory Effects of RGS4 on Gia and Goa Steady-State GTPase Activities (Figures 3 and 4). We next examined whether R14-RBD/GL affected the stimulatory effects of RGS on Gia and Goa GTPase activity. For these studies, we first established an experimental system for measuring receptor-directed steady-state GTPase activity of



FIGURE 3: RGS4 and RGS14 stimulate M2-activated Gi and Go steady-state GTPase activity in membranes. Membranes derived from Sf9 cells coexpressing the M2 muscarinic acetylcholine receptor plus either heterotrimeric Gi (black bars) or heterotrimeric Go (white bars) were assayed with the agonist carbachol (100  $\mu$ M) either alone or in the presence of RGS4 or full-length RGS14 at the concentrations indicated. Nonspecific signal with each membrane was defined as that observed in the absence of RGS protein and in the presence of the antagonist atropine (10  $\mu$ M), and this was subtracted out to yield the values indicated. This experiment was carried out 3 times and representative results are shown. Further details are described in the Materials and Methods.



FIGURE 4: R14-RBD/GL potentiates RGS4 effects on Gi or Go steady-state GTPase activity in membranes. The GTPase activities of membranes derived from Sf9 cells expressing the M2 muscarinic acetylcholine receptor together with either heterotrimeric Gi (A) or heterotrimeric Go (B) were measured as described in the Materials and Methods with either no added RGS protein, 50 nM RGS4, 1  $\mu$ M R14-RBD/GL, or both RGS4 and R14-RBD/GL, plus either no drug, 100  $\mu$ M carbachol, or 10  $\mu$ M tropicamide. This experiment was carried out 5 times with Gi and 3 times with Go, and representative results are shown.

Gia and Goa (23). Sf9 insect cells were multiply infected with baculoviruses encoding the M2 muscarinic cholinergic (M2) receptor plus either Go (Goa + G $\beta$ 1 + G $\gamma$ 2) or Gi (Gia + G $\beta$ 1 + G $\gamma$ 2). Plasma membranes were isolated from these cells and used to measure receptor-directed GTPase activity. When purified full-length recombinant RGS4 or RGS14 were reconstituted with these membranes, we found that each markedly potentiated carbachol-stimulated steadystate GTPase activity of Go $\alpha$  and Gi $\alpha$  (Figure 3). The isolated RGS domain of RGS14, R14-RGS, exhibited barely detectable GAP activity in these steady-state GTPase assays with either G protein (data not shown). The reasons for this are unclear, but this observation is consistent with our previous finding that R14-RGS is a relatively weak GAP for purified free Gi $\alpha$  and Go $\alpha$  when compared with fulllength RGS14, even though R14-RGS readily binds activated Gi/o $\alpha$  from brain membranes in "pull-down" assays (13).

We next examined the effects of reconstituting purified R14-RBD/GL on receptor and RGS-stimulated GTPase activity of Gia and Goa (Figure 4). Because we had difficulty obtaining a measurable GAP signal for the isolated RGS domain of RGS14 (R14-RGS) in this steady-state assay, we examined the effects of R14-RBD/GL on RGS4. Addition of R14-RBD/GL had little or no effect on the steady-state GTPase activity of Gia in the presence of M2 agonist carbachol, the M2 inverse agonist tropicamide, or no receptor ligand (Figure 4). RGS4 at a subsaturating concentration of 50 nM marginally stimulated the GTPase activity of Giα in the absence of drug, and this activity was enhanced with carbachol but completely blocked by tropicamide. On the basis of the observed effects of R14-RBD/GL in solutionbased nucleotide-binding assays, we expected to see a diminution of RGS4-dependent GTPase activity, at least with M2 plus Gi membranes. Surprisingly, addition of R14-RBD/ GL greatly potentiated the stimulatory effect of RGS4 on Gia GTPase activity either alone or in the presence of carbachol, although this activity again was completely blocked by the inverse agonist tropicamide (Figure 4). The stimulatory effects of RGS4 and potentiation of those effects by R14-RBD/GL also were observed for Goa (Figure 4).

The Isolated RBD/GL Domain of RGS14 Does Not Alter the GAP Effects of RGS on Purified Gia and Goa GTPase Activity in Single-Turnover, Solution-Based Assays (Figure 5). The observed stimulatory effect of R14-RBD/GL on RGS4 in membranes was entirely unexpected. Therefore, to understand underlying mechanisms, we next examined whether these effects of R14-RBD/GL on RGS4 or R14-RGS are preserved in solution-based, single-turnover GTPase assays of pure Gia or Goa (Figure 5). Goa or Gia were preloaded with [32P]GTP, and the binding reaction was quenched by the addition of excess unlabeled GTP, while GTPase activity was initiated with Mg<sup>2+</sup>. G $\alpha$ -directed nucleotide hydrolysis was measured by the simultaneous addition of 0, 30, or 3000 nM R14-RBD/GL plus either no additional protein, 50 nM RGS4, or 300 nM R14-RGS. Gia and Goa exhibited very low basal GTPase activities after 1 min at 4 °C, and as expected, both RGS4 and R14-RGS stimulated this activity. Consistent with our observations in membranes, RGS4 at a relatively low concentration stimulates GTPase activity of free  $G\alpha$  much more robustly than does R14-RGS at a relatively high concentration. Somewhat surprisingly, R14-RBD/GL alone also exhibited a small stimulatory effect on  $G\alpha$  GTPase activity. This was clearly mediated via Ga, because R14-RBD/GL alone had no measurable intrinsic GTPase activity (data not shown). When  $G\alpha$  GTPase activity was measured in the presence of both R14-RBD/GL and either R14-RGS or RGS4, the effects were either additive or subadditive. Thus, R14-RBD/GL appears neither to stimulate nor to inhibit RGS domain-dependent increases in GTP hydrolysis rates (Figure 5). This differs



FIGURE 5: R14-RBD/GL has no effect on RGS-directed stimulation of purified Gia or Goa GTPase activity in single-turnover, solutionbased assays. Purified recombinant His6-tagged Gia1 (500 nM) (A) or Goa (500 nM) (B) were preloaded with [ $\gamma$ -<sup>32</sup>P]GTP and then incubated alone or with the indicated concentrations of R14-RBD/GL in the absence or presence of RGS4 (50 nM) or R14-RGS (300 nM) at 4 °C. After 1 min, reactions were stopped and hydrolyzed GTP (accumulated [<sup>32</sup>P]P<sub>i</sub>) was measured. Further details are described in the Materials and Methods. Results are pooled averaged data (mean ± standard deviation) from three separate experiments.

from its marked stimulatory effects on RGS4-dependent steady-state GTPase activity seen in membrane-based assays (Figure 4).

R14-RBD/GL Potentiates Receptor-Dependent RGS4 GAP Activity by Increasing the Apparent Affinity of RGS4 for Gia and Goa (Figures 6 and 7). R14-RBD/GL did not alter the RGS stimulatory effects on purified free Goa or Gia GTPase activity in solution-based assays, suggesting that it does not affect GTP dissociation from  $G\alpha$  nor does it increase the affinity of RGS4 or R14-RGS for free Gα-GTP. Therefore, we next turned our attention back to membranes where R14-RBD/GL markedly potentiated RGS4 actions. We first examined the effects of varying amounts of R14-RBD/GL on a single concentration of RGS4 mixed with M2 membranes expressing either Go or Gi (Figure 6). We found that the potentiation of the effects of RGS4 on Gia and Goa GTPase activities by R14-RBD/GL was most evident at higher concentrations of R14-RBD/GL and excess molar ratios (R14-RBD/GL > G $\alpha$ ). We next tested the effects of a single high concentration of R14-RBD/GL on varying amounts of RGS4. We found that R14-RBD/GL markedly increased the potency of RGS4 for stimulation of M2/Goor M2/Gi-directed steady-state GTPase activity in membranes (Figure 7), suggesting that R14-RBD/GL acts to increase RGS4 affinity for Go and Gi in membranes when M2 receptors and  $G\beta\gamma$  are present.



FIGURE 6: Concentration dependence of the R14-RBD/GL effect on agonist- and RGS4-stimulated G protein steady-state GTPase activity in membranes. GTPase activities of membranes derived from Sf9 cells expressing the M2 muscarinic acetylcholine receptor together with either heterotrimeric Gi (A) or heterotrimeric Go (B) were measured as described in the Materials and Methods in the presence of the agonist carbachol (100  $\mu$ M) plus RGS4 (50 nM), together with R14-RBD/GL at the concentrations indicated on the abscissa. This experiment was carried out 7 times with Gi and 2 times with Go, and representative results are shown.

#### DISCUSSION

A Novel RGS14-Go Interaction. RGS14 is a complex, multifunctional protein that contains two identified binding sites for Gi/o $\alpha$  family members, each with distinct activities on target Ga. Previous work has indicated that the RGS domain of RGS14 binds activated Gia and Goa to confer GAP activity, whereas the GPR/GoLoco motif binds inactive, GDP-bound Gia to confer GDI activity (13-15). The present results confirm and extend these findings to show that binding of R14-RBD/GL, which lacks the N-terminal 60% of the protein including the RGS domain, binds not only to inactive Gia but also to inactive Goa and  $G\beta\gamma$  from brain membranes. Functionally, the binding of R14-RBD/GL appears to decrease the susceptibility of free  $Go\alpha$  to thermal denaturation and also to promote its ability to hydrolyze bound GTP, although there is no apparent effect on nucleotide exchange.

Previous studies have reported the interaction of the RGS14 GPR/GoLoco (GL) domain with Goa (14, 15, 26). The apparent failure of a 35 amino acid peptide corresponding to the RGS14 GPR/GoLoco (GL) domain to bind Goa (14) suggests that R14-RBD/GL binding here involves additional G protein contact points within this truncation mutant. Notably, residues in the RGS14 GPR/GoLoco peptide located C-terminally to the GoLoco motif of RGS14



FIGURE 7: R14-RDB/GL increases the apparent affinity of RGS4 for Gi and Go in steady-state GTPase activity in membranes. GTPase activities of membranes derived from Sf9 cells expressing the M2 muscarinic acetylcholine receptor together with either heterotrimeric Gi (A) or heterotrimeric Go (B) were measured as described in the Materials and Methods in the presence of the agonist carbachol (100  $\mu$ M) plus R14-RBD/GL (1  $\mu$ M), together with RGS4 at the concentrations indicated on the abscissa. This experiment was carried out 3 times, and representative results are shown.

conferred specificity for Gia interaction (15). Given this, our findings suggest that residues within RGS14 located N-terminal relative to the GPR/GoLoco motif may confer Goa binding capacity. Located between the RGS domain and the GPR/GoLoco motif are the tandem RBD motifs and a 50–60 amino acid stretch with no homologous identity or described function. Further studies will be required to define this putative Gi/oa binding domain of RGS14.

The observed binding of Goa to R14-RBD/GL recalls the behavior of other mammalian proteins containing GPR/ GoLoco motifs that bind to and interact with Goa including AGS3, LGN, Rap1GAPII, and Pcp2 (16-21, 27). Pcp2 contains two nonidentical tandem GPR/GoLoco motifs, and the second of these has been shown to bind inactive Goa-GDP and regulate its guanine nucleotide exchange activity (15). Surprisingly, while the isolated GRP/GoLoco motifs of Pcp2 inhibit nucleotide exchange, the action of full-length Pcp2 on Go $\alpha$  is to stimulate guanine nucleotide release (17). Whereas a peptide encoding the isolated GPR/GoLoco motif of RGS14 does not bind Go $\alpha$ , a chimeric peptide encoding the RGS14 GPR/GoLoco motif fused with residues derived from Pcp2 that are located C-terminal to its second GPR/ GoLoco motif does bind Goa and inhibits nucleotide exchange (15). Together with the present data, these findings suggest that regions outside of the GPR/GoLoco motif are required for RGS14 binding to Goa and its effects on Goa activity.

RGS14 and specifically RBD/GL also bound G $\beta$  (and presumably G $\gamma$ ) in brain membranes. This was not entirely unexpected because RGS14 also bound inactive G $\alpha$ -GDP, which is presumed to be complexed with G $\beta\gamma$ . Our findings

do not distinguish whether RGS14 binds heterotrimer or free subunits. One previous study reports that RGS4 binds  $G\beta\gamma$ and also G $\alpha$  and PLC $\beta$  (28), suggesting that some RGS proteins may participate in scaffolding complexes with multiple related signaling proteins. Our findings suggest that RGS14 effects on RGS4 are dependent on membranes and membrane components. It is possible that  $G\beta\gamma$  and/or receptors (e.g., M2 muscarinic) may help to facilitate RGS14 interactions with RGS4/G $\alpha$  within the membrane, although further studies will be required to test this idea.

Effects of RGS14 on Steady-State GTPase Activity. Receptors promote guanine nucleotide exchange on  $G\alpha$ ; thus, agonist-activated receptors and RGS proteins together act synergistically to increase steady-state hydrolysis of GTP by G proteins (29). Effects of GPR/GoLoco containing proteins on steady-state Ga GTPase activity have not been investigated previously. The observed GDI function of RGS14 would be expected to blunt the effects of its RGS domain on steady-state GTPase activity (with Gi), although the present results seem to contradict this idea. Full-length RGS14 increased steady-state GTPase activity similarly to RGS4 and did not differ drastically between Gi and Go. While lower concentrations of R14-RBD/GL may have produced a modest (~10%) decrease in agonist- and RGS4dependent GTPase activity with Gi (Figure 6), higher concentrations produced, surprisingly, a marked potentiation of RGS4-directed increases in  $G\alpha$  GTP hydrolysis for each G protein, both in the absence or in the presence of muscarinic agonist carbachol. These effects are difficult to reconcile with current interpretations of RGS14 activity, and the seeming discrepancy underscores the difficulties in extrapolating ideas based on rudimentary biochemical assays to complex multiprotein systems.

Mechanism(s) underlying this novel R14-RBD/GL stimulatory function are unknown, although they appear distinct from established GAP activity of the RGS domain. Because R14-RBD/GL augments the effect of RGS4 and full-length RGS14 has a greater effect on GTPase activity than R14-RGS, one possibility is that the GPR/GoLoco domain itself has GAP activity. R14-RBD/GL exhibits some, albeit modest, intrinsic GAP activity toward purified Gia and Goa in solution-based assays (Figure 5). Another possibility is that binding of R14-RBD/GL to G proteins promotes GDP dissociation thereby facilitating RGS GAP activity under steady-state conditions. This idea directly contradicts accepted notions that GPR/GoLoco proteins inhibit GDP dissociation. We also found that R14-RBD/GL has no effect on RGS GAP activity in single-turnover assays, whereas we would expect a decrease in hydrolysis if dissociation occurs.

One interpretation of the present results is that the effects of R14-RBD/GL on steady-state G $\alpha$  GTPase activity reflect its modulation by other signaling components present in membranes, including the receptor, the RGS protein, or G $\beta\gamma$ . In a related example, the GPR/GoLoco domains of AGS3 were found to have profound effects on the Gi- and Goregulated binding of serotonin to the 5HT1A receptor, possibly reflecting the GPR/GoLoco-dependent dissociation of G $\beta\gamma$  from G $\alpha$  (20). Our results suggest that M2 receptors remain coupled to G $\alpha$  under such conditions, raising the possibility that R14-RBD/GL can substitute for G $\beta\gamma$  in facilitating receptor–G $\alpha$  interactions (1). We found that RGS14 is capable of binding G $\beta\gamma$  and G $\alpha$ -GDP from brain membranes (Figure 2), and regardless of whether  $G\beta\gamma$  fully dissociates, R14-RBD/GL could conceivably promote the effects of either the receptor or RGS4 (or both) on the G protein, thereby increasing steady-state GTPase activity.

Another possibility to consider is that R14-RBD/GL may increase the affinity of RGS4 for target  $G\alpha$ . We tested this idea and found that R14-RBD/GL does indeed markedly enhance the potency of RGS4 for stimulating Ga steadystate GTPase activity in membranes (Figure 7), although such an effect was not evident in solution-based assays (Figure 5). This suggests the possibility that R14-RBD/GL and RGS4 may bind independently to distinct sites on target  $G\alpha$  (or to multiple  $G\alpha$  proteins associated with a receptor oligomer) and act cooperatively to modulate agonist and receptordirected Ga GTPase activity. R14-RBD/GL itself may also promote GTP hydrolysis (Figure 5), and this tendency could be facilitated by RGS4 (Figures 4, 6, and 7); however, R14-RBD/GL does not seem to have a direct effect on nucleotide exchange, because its synergistic effect with RGS4 was not observed in the presence of the inverse agonist tropicamide, which blocks receptor-mediated exchange (Figure 4). The observed lack of synergy in solution-based experiments suggests that components present in the membrane-based assays serve as organizers of RGS protein-G protein interactions, although the details of this remain unclear.

The foregoing discussion suggests that the separate domains within RGS14 may function in concert with one another, and indeed, one recent report suggests that the RGS domain and GoLoco domain of RGS14 act cooperatively to regulate M2 receptor and Gαi-mediated signaling (Erk phosphorylation) in HEK cells (*30*). This might explain why the isolated RGS domain of RGS14 did not produce a reliable signal in our receptor-based GTPase assays, although we were unable to detect synergy between R14-RGS and R14-RBD/GL. Reasons for this are unclear, although the simplest explanation is that truncated proteins expressed independently require the remainder of the protein for full activity. Still, the observed effects of R14-RBD/GL on RGS4 activity suggest that such interactions can occur and point to a need for further study of interdomain activities of RGS14.

In summary, we demonstrate for the first time that RGS14 binds inactive Go $\alpha$ -GDP and G $\beta\gamma$  and that RGS14 binding confers to both Go $\alpha$  and Gi $\alpha$  novel activities that are distinct from established GAP and GDI activities. These studies extend our understanding of RGS14 interactions with established binding partners Gi $\alpha$  and Go $\alpha$  and raise new questions regarding cooperative intermolecular interactions between known functional domains (RGS, RBD, and GPR/GoLoco) and possible new domains involved in RGS14 modulation of receptor and G protein signaling.

### REFERENCES

- Neer, E. J. (1995) Heterotrimeric G proteins: Organizers of transmembrane signals, *Cell 80*, 249–257.
- 2. Hamm, H. E. (1998) The many faces of G protein signaling, *J. Biol. Chem.* 273, 669–672.
- Bourne, H. R. (1997) How receptors talk to trimeric G proteins, Curr. Opin. Cell Biol. 9, 134–142.
- Hepler, J. R., and Gilman, A. G. (1992) G proteins, *Trends Biochem. Sci.* 17, 383–387.
- Ross, E. M., and Wilkie, T. M. (2000) GTPase-activating proteins for heterotrimeric G proteins: Regulators of G protein signaling

(RGS) and RGS-like proteins, Annu. Rev. Biochem. 69, 795-827.

- De Vries, L., Zheng, B., Fischer, T., Elenko, E., and Farquhar, M. G. (2000) The regulator of G protein signaling family, *Annu. Rev. Pharmacol. Toxicol.* 40, 235–271.
- 7. Hollinger, S., and Hepler, J. R. (2002) Cellular regulation of RGS proteins: Modulators and integrators of G protein signaling, *Pharmacol. Rev.* 54, 527–559.
- Carman, C. V., Parent, J. L., Day, P. W., Pronin, A. N., Sternweis, P. M., Wedegaertner, P. B., Gilman, A. G., Benovic, J. L., and Kozasa, T. (1999) Selective regulation of Gα(q/11) by an RGS domain in the G protein-coupled receptor kinase, GRK2, *J. Biol. Chem.* 274, 34483–34492.
- 9. Hepler, J. R., Berman, D. M., Gilman, A. G., and Kozasa, T. (1997) RGS4 and GAIP are GTPase-activating proteins for Gq α and block activation of phospholipase C β by γ-thio-GTP-Gq α, *Proc. Natl. Acad. Sci. U.S.A.* 94, 428–432.
- Snow, B. E., Antonio, L., Suggs, S., Gutstein, H. B., and Siderovski, D. P. (1997) Molecular cloning and expression analysis of rat Rgs12 and Rgs14, *Biochem. Biophys. Res. Commun. 233*, 770–777.
- Traver, S., Bidot, C., Spassky, N., Baltauss, T., De Tand, M. F., Thomas, J. L., Zalc, B., Janoueix-Lerosey, I., and Gunzburg, J. D. (2000) RGS14 is a novel Rap effector that preferentially regulates the GTPase activity of Gαo *Biochem. J.* 350 (Part 1), 19–29.
- Cho, H., Kozasa, T., Takekoshi, K., De Gunzburg, J., and Kehrl, J. H. (2000) RGS14, a GTPase-activating protein for Giα, attenuates Giα- and G13α-mediated signaling pathways, *Mol. Pharmacol.* 58, 569–576.
- Hollinger, S., Taylor, J. B., Goldman, E. H., and Hepler, J. R. (2001) RGS14 is a bifunctional regulator of Gαi/o activity that exists in multiple populations in brain, *J. Neurochem.* 79, 941– 949.
- 14. Kimple, R. J., De Vries, L., Tronchere, H., Behe, C. I., Morris, R. A., Gist Farquhar, M., and Siderovski, D. P. (2001) RGS12 and RGS14 GoLoco motifs are Gα(i) interaction sites with guanine nucleotide dissociation inhibitor activity, *J. Biol. Chem.* 276, 29275–29281.
- Kimple, R. J., Kimple, M. E., Betts, L., Sondek, J., and Siderovski, D. P. (2002) Structural determinants for GoLoco-induced inhibition of nucleotide release by Gα subunits, *Nature 416*, 878–881.
- Bernard, M. L., Peterson, Y. K., Chung, P., Jourdan, J., and Lanier, S. M. (2001) Selective interaction of AGS3 with G-proteins and the influence of AGS3 on the activation state of G-proteins, *J. Biol. Chem.* 276, 1585–1593.
- Luo, Y., and Denker, B. M. (1999) Interaction of heterotrimeric G protein Gαo with Purkinje cell protein-2. Evidence for a novel nucleotide exchange factor, J. Biol. Chem. 274, 10685–10688.
- Natochin, M., Gasimov, K. G., and Artemyev, N. O. (2001) Inhibition of GDP/GTP exchange on G α subunits by proteins containing G-protein regulatory motifs, *Biochemistry* 40, 5322– 5328.
- Redd, K. J., Oberdick, J., McCoy, J., Denker, B. M., and Luo, Y. (2002) Association and colocalization of G protein α subunits and Purkinje cell protein 2 (Pcp2) in mammalian cerebellum, *J. Neurosci. Res.* 70, 631–637.
- Ma, H., Peterson, Y. K., Bernard, M. L., Lanier, S. M., and Graber, S. G. (2003) Influence of cytosolic AGS3 on receptor-G protein coupling, *Biochemistry* 42, 8085–8093.
- Mochizuki, N., Ohba, Y., Kiyokawa, E., Kurata, T., Murakami, T., Ozaki, T., Kitabatake, A., Nagashima, K., and Matsuda, M. (1999) Activation of the ERK/MAPK pathway by an isoform of rap1GAP associated with Gα(i), *Nature 400*, 891–894.
- Cladman, W., and Chidiac, P. (2002) Characterization and comparison of RGS2 and RGS4 as GTPase-activating proteins for m2 muscarinic receptor-stimulated G(i), *Mol. Pharmacol.* 62, 654–659.
- Mao, H., Zhao, Q., Daigle, M., Ghahremani, M. H., Chidiac, P., and Albert, P. R. (2004) RGS17/RGSZ2, a novel regulator of Gi/o, Gz, and Gq signaling, J. Biol. Chem. 279, 26314–26322.
- 24. Saitoh, O., Kubo, Y., Odagiri, M., Ichikawa, M., Yamagata, K., and Sekine, T. (1999) RGS7 and RGS8 differentially accelerate G protein-mediated modulation of K+ currents, *J. Biol. Chem.* 274, 9899–9904.
- Ferguson, K. M., Higashijima, T., Smigel, M. D., and Gilman, A. G. (1986) The influence of bound GDP on the kinetics of guanine nucleotide binding to G proteins, *J. Biol. Chem.* 261, 7393–7399.

- Mittal, V., and Linder, M. E. (2004) The RGS14 GoLoco domain discriminates among Gαi isoforms, J. Biol. Chem. 279, 46772– 46778.
- 27. Jordan, J. D., Carey, K. D., Stork, P. J., and Iyengar, R. (1999) Modulation of rap activity by direct interaction of  $G\alpha(o)$  with Rap1 GTPase-activating protein, *J. Biol. Chem.* 274, 21507– 21510.
- 28. Dowal, L., Elliott, J., Popov, S., Wilkie, T. M., and Scarlata, S. (2001) Determination of the contact energies between a regulator of G protein signaling and G protein subunits and phospholipase C β 1, *Biochemistry 40*, 414–421.

- 29. Roy, A. A., Lemberg, K. E., and Chidiac, P. (2003) Recruitment of RGS2 and RGS4 to the plasma membrane by G proteins and receptors reflects functional interactions, *Mol. Pharmacol.* 64, 587–593.
- Traver, S., Splingard, A., Gaudriault, G., and De Gunzburg, J. (2004) The RGS (regulator of G-protein signaling) and GoLoco domains of RGS14 cooperate to regulate Gi-mediated signaling, *Biochem. J.* 379, 627–632.

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