Novel Activity of RGS14 on Goα and Giα Nucleotide Binding and Hydrolysis Distinct from Its RGS Domain and GDI Activity†

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ABSTRACT: The bifunctional protein RGS14 is both a GTPase activating protein (GAP) for Giα and Goα and a guanine nucleotide dissociation inhibitor (GDI) for Giα. 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 Goα-GDP, Giα-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 Giα and Goα, suggesting a cooperative interaction between the RBD/GL domain, RGS4, and Gα. This activity of R14-RBD/GL on RGS4 is not apparent in single-turnover solution GAP assays with purified Giα or Goα, 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 Gα 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 Gα-guanosine triphosphate (GTP)[1] and a G$\beta\gamma$ complex act alone or in concert to regulate the action of target effector proteins. Gα subunits are GTPases that act as molecular switches, and the lifetime of Gα-GTP dictates the lifetime of the signaling event. The protein regulators of G protein signaling (RGS proteins) act as Gα GTPase activating proteins (GAPs) to limit the duration of the linked Gα 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 Gα subunits and confers GAP activity. In addition, RGS proteins can directly interfere with effector binding to Gα 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 Gα subunits, whereas inactive GDP-bound forms of Gα 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/oα 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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[1] 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; Rap1GAPII, GTPase activating protein for rap1, type II isoform; GAP, GTPase activating protein; RBD, Rap-binding domain; GDI, guanine nucleotide dissociation inhibitor; AMF, AlF_4 and Mg^{2+}; 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.

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Giα/o and confers nonselective GAP activity toward Giα and Goα (*11*−*13*). RGS14 also inhibits the guanine nucleotide exchange activity of free Giα but not Goα 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 Giα but not Goα to selectively modulate Giα but not Goα function. Indeed, a peptide encoding the GL motif binds directly and selectively to inactive Giα1-GDP but not Goα-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 Giα-GDP but not Goα-GDP.

Previous studies demonstrate that certain other proteins containing GPR/GL motifs can interact with Goα (*16*−*21*) and, in several cases, modulate its function (*17*, *18*), which raises the possibility that RGS14/Gα 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 Goα and Giα. 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 Gα 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, Giα, Goα, Gβ1, and Gγ2 were as described previously (*22*, *23*). cDNA encoding TxH_6-RGS14 was a generous gift of D. P. Siderovski (University of North Carolina, Chapel Hill, NC). cDNA encoding H_6-R14-RGS and TxH_6-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_6-R14-RGS) and pET20b (TxH_6-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 Goα and Giα, respectively, was purchased from Santa Cruz Biotechnologies. Rabbit polyclonal antisera that recognizes Gβ (common Gβ sera) was kindly provided by Dr. Susanne Mumby (UT Southwestern Medical Center, Dallas, TX). [^{3}H]GDP was purchased from New England Nuclear, and [γ-^{32}P]GTP was purchased from ICN or Perkin−Elmer.

Protein Expression. Hexahistidine (H6)-tagged thioredoxin (Tx), Tx- and H6-tagged RGS14 (TxH_6-RGS14), H6-tagged RGS14 encoding amino acids 1−205 of RGS14 including the RGS domain (H_6-RSG14), and a Tx- and H6-tagged amino-truncated protein encoding amino acids 299−544 of RGS14 including the RBD and GL domains (TxH_6-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^{2+} 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_6-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, Gi1α, and Goα 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 100,000*g*. Brain membranes (500 μg) were incubated with 10 μg of Trx-H_6 (control protein), TxH_6-RGS14, H_6-R14-RGS, or TxH_6-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 μL of Ni-NTA for 30 min. The beads were pelleted by centrifugation and then washed 5 times with 500 μL of 20 mM Hepes, 150 mM NaCl, 0.1% Lubrol, 3 mM DTT, 40 mM imidazole, and GDP ± AMF, as appropriate. Bound proteins were eluted into 120 μL of 1× Laemmli buffer. Samples were denatured, subjected to SDS−PAGE, transferred to nitrocellulose, and immunoblotted using anstisera specific for either Goα, Giα, or Gβ.

Receptor-Mediated Steady-State GTPase Assays. Sf9 cells were infected for 48 h with baculoviruses encoding the M2 muscarinic acetylcholine receptor, Gβ1 and Gγ2, plus either Gi2α or Goα, 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 μg/mL leupeptin, 10 μg/mL aprotinin, 10 mM NaCl, and 2 mM $MgCl_2$ (calculated free Mg^{2+} = 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 μM GTP, 500 μM ATP, [γ-^{32}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_2PO_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 Giα and Goα were measured as described (*13*). Purified recombinant His6-tagged Gα subunits (0.5 uM) were loaded with 1 μM [γ-^{32}P]GTP at room temperature for either 20 min (Goα) or 1 h ($Gi_1\alpha$). Proteins were cooled to 4 °C and then added to a reaction tube containing an excess of GTP (100 μM final) and $MgCl_2$ (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 [^{32}P]P_i was

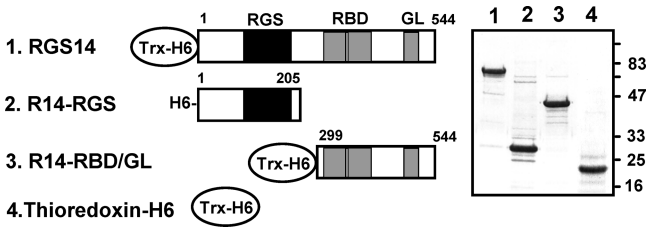

FIGURE 1: Purified recombinant RGS14 and truncated forms of RGS14 proteins. (Left) Schematic diagram illustrating Tx- and H6-tagged ($TrxH_6$)-RGS14, H_6-R14-RGS, $TrxH_6$-R14-R/GL, and TrxH6 alone. (Right) Coomassie-blue-stained gel of purified $TrxH_6$-RGS14 (RGS14), H_6-R14-RGS, $TrxH_6$-R14-RBD/GL, and TrxH6 control protein. Proteins were expressed in bacteria, purified as described in the Materials and Methods, and then resolved using SDS−PAGE.

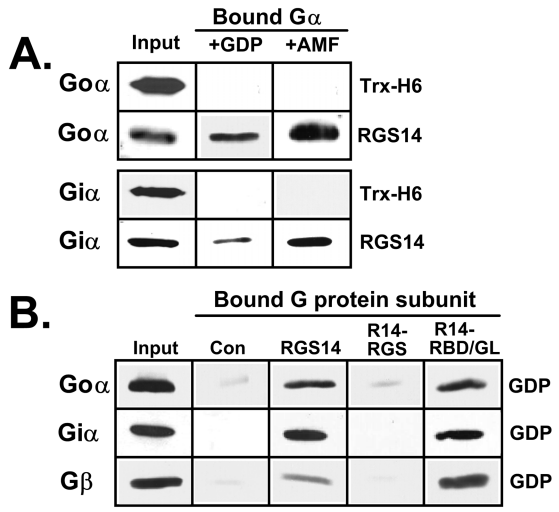

FIGURE 2: RGS14 binds inactive Goα-GDP, Giα-GDP, and G$\beta\gamma$ from brain membranes. (A) Pull-down assays were performed as described in the Materials and Methods. Brain membranes were incubated with RGS14 or TrxH6 control protein in the presence of GDP with or without AlF_4 and Mg^{2+} (AMF). Membrane-bound proteins were extracted with 1% cholate, and the lysate was incubated with 100 μL Ni^{2+}-NTA beads, which were pelleted and washed with buffer with or without AlF_4. Proteins were eluted into 1× laemmli sample buffer, resolved by SDS−PAGE, and immunoblotted using antisera that specifically recognize either Giα or Goα. 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 pull-down assays were performed as described above. Samples were resolved by SDS−PAGE and immunoblotted using antisera that specifically recognize either Giα, Goα, or Gβ. Results are representative of four (Goα and Giα) and two (Gβ) 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 Goα-GDP and Giα-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 Giα but not Goα. This suggests that RGS14 directly and selectively interacts with Giα-GDP but not Goα-GDP. In contrast, several studies have shown that GL motif-containing proteins can modulate Goα activity (*17*, *18*), suggesting that RGS14/Gα interactions may be more complex than previously thought. To investigate this possibility, we examined RGS14 interactions with Giα and Goα present in brain extracts. Histidine-tagged RGS14 was expressed as a full-length 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 Giα or Goα 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 Giα and Goα as expected (Figure 2A). However, when brain extracts were incubated with GDP alone to stabilize inactive Gα, RGS14 also bound both Giα-GDP and Goα-GDP, which was unexpected. We investigated this further by determining whether Gα-GDP bound to either R14-RGS or R14RBD/GL. When brain extracts were incubated with GDP alone, RGS14 and R14-RBD/GL bound Goα, Giα, and Gβ (and presumably Gγ), 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 Giα or Goα under either activating or nonactivating conditions.

Our unexpected observation that R14-RBD/GL binds inactive Goα-GDP indicates that a region distinct from the RGS domain of RGS14 can modulate the function of this Gα. To investigate this, we examined whether R14-RBD/GL influences the guanine nucleotide binding/exchange properties of target Gα. We and others have shown that RGS14 acts as a GDI for Giα1 but not Goα, effectively slowing the exchange of GDP for GTP (*13*−*15*). Consistent with previous studies (*13*−*15*), R14-RBD/GL impeded the binding of [^{3}H]GDP in exchange for bound GDP with Giα at 30 °C but not with Goα at either 20 or 30 °C; Goα-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 Goα.

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

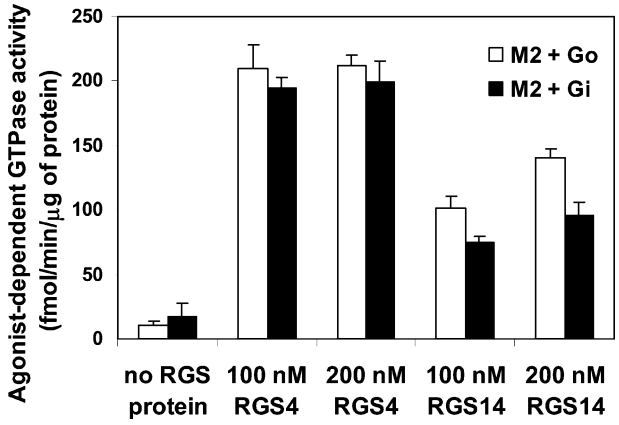

FIGURE 3: RGS4 and RGS14 stimulate M2-activated Gi and Go steady-state GTPase activity in membranes. Membranes derived from Sf9 cells coexpressing the M2 muscarinic acetylcholine receptor plus either heterotrimeric Gi (black bars) or heterotrimeric Go (white bars) were assayed with the agonist carbachol (100 μM) 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 μM), 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.

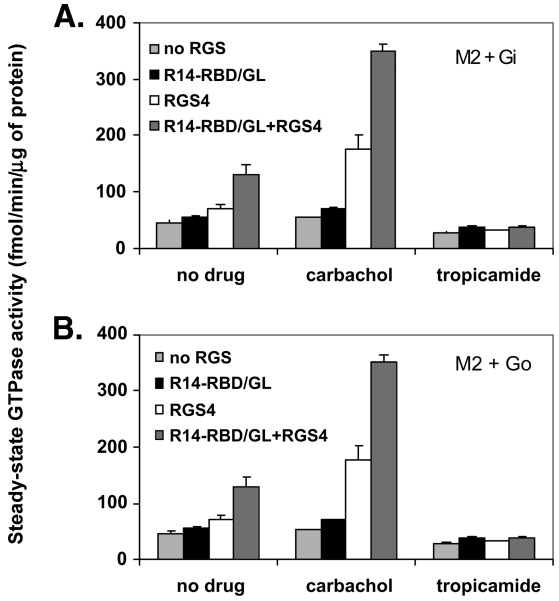

FIGURE 4: R14-RBD/GL potentiates RGS4 effects on Gi or Go steady-state GTPase activity in membranes. The GTPase activities of membranes derived from Sf9 cells expressing the M2 muscarinic acetylcholine receptor together with either heterotrimeric Gi (A) or heterotrimeric Go (B) were measured as described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

Giα and Goα (*23*). Sf9 insect cells were multiply infected with baculoviruses encoding the M2 muscarinic cholinergic (M2) receptor plus either Go (Goα + Gβ1 + Gγ2) or Gi (Giα + Gβ1 + Gγ2). 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 steady-state GTPase activity of Goα and Giα (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 Giα and Goα when compared with full-length RGS14, even though R14-RGS readily binds activated Gi/oα 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 Giα and Goα (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 Giα 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 solution-based 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 Giα 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 Goα (Figure 4).

The Isolated RBD/GL Domain of RGS14 Does Not Alter the GAP Effects of RGS on Purified Giα and Goα 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 Giα or Goα (Figure 5). Goα or Giα were preloaded with [^{32}P]GTP, and the binding reaction was quenched by the addition of excess unlabeled GTP, while GTPase activity was initiated with Mg^{2+}. Gα-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. Giα and Goα 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 Gα 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 Gα GTPase activity. This was clearly mediated via Gα, because R14-RBD/GL alone had no measurable intrinsic GTPase activity (data not shown). When Gα 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

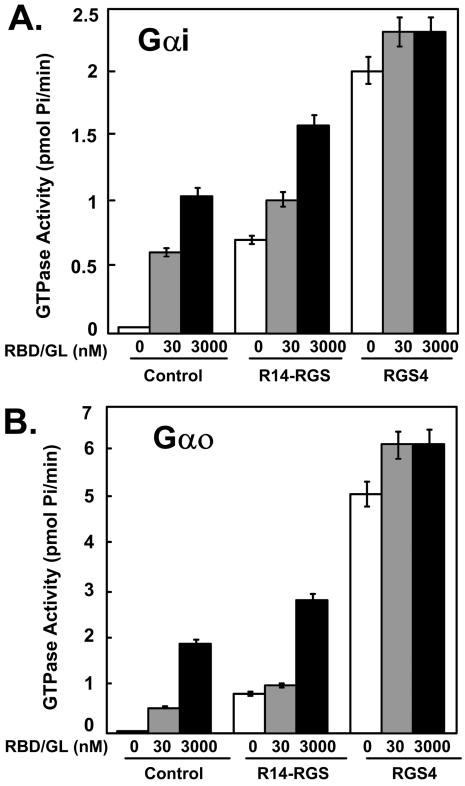

FIGURE 5: R14-RBD/GL has no effect on RGS-directed stimulation of purified Giα or Goα GTPase activity in single-turnover, solution-based assays. Purified recombinant His6-tagged Giα1 (500 nM) (A) or Goα (500 nM) (B) were preloaded with [γ-^{32}P]GTP and then incubated alone or with the indicated concentrations 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 [^{32}P]P_i) was measured. Further details are described in the Materials and Methods. Results are pooled averaged data (mean ± standard deviation) from three separate experiments.

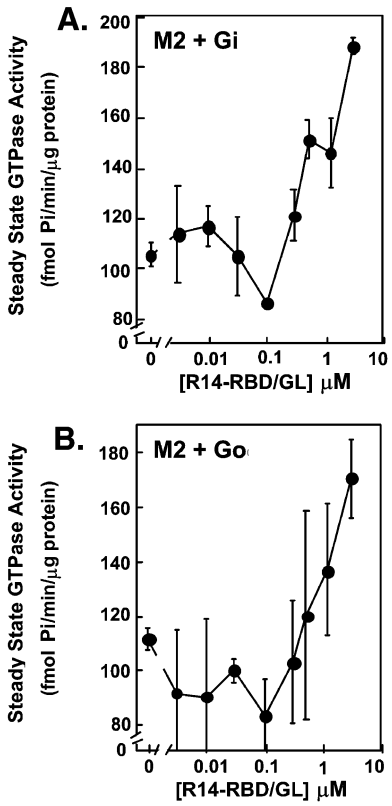

FIGURE 6: Concentration dependence of the R14-RBD/GL effect on agonist- and RGS4-stimulated G protein steady-state GTPase activity in membranes. GTPase activities of membranes derived from Sf9 cells expressing the M2 muscarinic acetylcholine receptor together with either heterotrimeric Gi (A) or heterotrimeric Go (B) were measured as described in the Materials and Methods in the presence of the agonist carbachol (100 μM) 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.

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 Giα and Goα (Figures 6 and 7). R14-RBD/GL did not alter the RGS stimulatory effects on purified free Goα or Giα GTPase activity in solution-based assays, suggesting that it does not affect GTP dissociation from Gα 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 Giα and Goα GTPase activities by R14-RBD/GL was most evident at higher concentrations of R14-RBD/GL and excess molar ratios (R14-RBD/GL > Gα). 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/Go- or 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.

DISCUSSION

A Novel RGS14-Go Interaction. RGS14 is a complex, multifunctional protein that contains two identified binding sites for Gi/oα family members, each with distinct activities on target Gα. Previous work has indicated that the RGS domain of RGS14 binds activated Giα and Goα to confer GAP activity, whereas the GPR/GoLoco motif binds inactive, GDP-bound Giα 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 Giα but also to inactive Goα and G$\beta\gamma$ from brain membranes. Functionally, the binding of R14-RBD/GL appears to decrease the susceptibility of free Goα 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 Goα (*14*, *15*, *26*). The apparent failure of a 35 amino acid peptide corresponding to the RGS14 GPR/GoLoco (GL) domain to bind Goα (*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

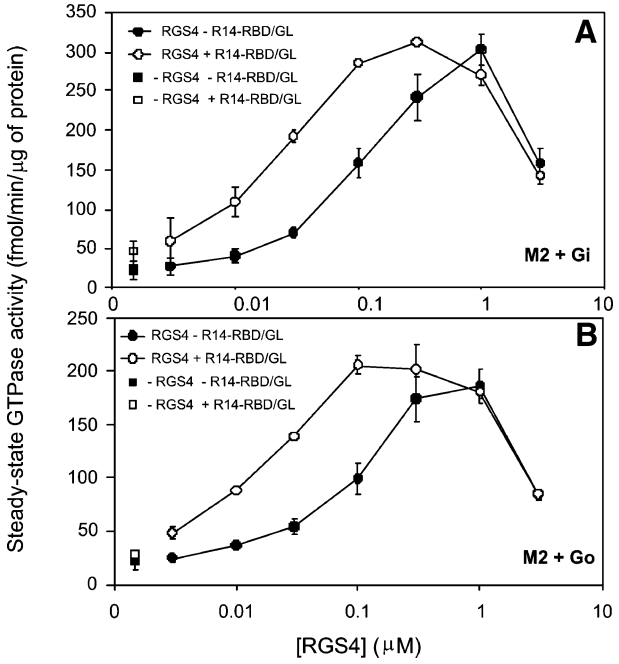

FIGURE 7: R14-RDB/GL increases the apparent affinity of RGS4 for Gi and Go in steady-state GTPase activity in membranes. GTPase activities of membranes derived from Sf9 cells expressing the M2 muscarinic acetylcholine receptor together with either heterotrimeric Gi (A) or heterotrimeric Go (B) were measured as described in the Materials and Methods in the presence of the agonist carbachol (100 μM) plus R14-RBD/GL (1 μM), 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 Giα interaction (*15*). Given this, our findings suggest that residues within RGS14 located N-terminal relative to the GPR/GoLoco motif may confer Goα 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/oα binding domain of RGS14.

The observed binding of Goα to R14-RBD/GL recalls the behavior of other mammalian proteins containing GPR/GoLoco motifs that bind to and interact with Goα 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 Goα-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 Goα is to stimulate guanine nucleotide release (*17*). Whereas a peptide encoding the isolated GPR/GoLoco motif of RGS14 does not bind Goα, 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 Goα 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 Goα and its effects on Goα activity.

RGS14 and specifically RBD/GL also bound Gβ (and presumably Gγ) in brain membranes. This was not entirely unexpected because RGS14 also bound inactive Gα-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 Gα and PLCβ (*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/Gα 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 Gα; 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 Gα 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 RGS4-dependent GTPase activity with Gi (Figure 6), higher concentrations produced, surprisingly, a marked potentiation of RGS4-directed increases in Gα 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 Giα and G*o*α 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 Gα 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 Go-regulated binding of serotonin to the 5HT1A receptor, possibly reflecting the GPR/GoLoco-dependent dissociation of G$\beta\gamma$ from Gα (*20*). Our results suggest that M2 receptors remain coupled to Gα under such conditions, raising the possibility that R14-RBD/GL can substitute for G$\beta\gamma$ in facilitating receptor−Gα interactions (*1*). We found that RGS14 is capable of binding G$\beta\gamma$ and Gα-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 Gα. We tested this idea and found that R14-RBD/GL does indeed markedly enhance the potency of RGS4 for stimulating Gα steady-state 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 Gα (or to multiple Gα proteins associated with a receptor oligomer) and act cooperatively to modulate agonist and receptor-directed Gα 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 Goα-GDP and G$\beta\gamma$ and that RGS14 binding confers to both Goα and Giα novel activities that are distinct from established GAP and GDI activities. These studies extend our understanding of RGS14 interactions with established binding partners Giα and Goα 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.

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