RGS17/RGSZ2, a Novel Regulator of $G_{i/o}$, $G_{z}$, and $G_{q}$ Signaling*

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To identify novel regulators of $G_{o}$, the most abundant G-protein in brain, we used yeast two-hybrid screening with constitutively active $G_{o}$ as bait and identified a new regulator of G-protein signaling (RGS) protein, RGS17 (RGSZ2), as a novel human member of the RZ (or A) subfamily of G-proteins. RGS17 contains an amino-terminal cysteine-rich motif and a carboxyl-terminal RGS domain with highest homology to hRGSZ1- and hRGS-Gα-interacting protein. RGS17 RNA was strongly expressed as multiple species in cerebellum and other brain regions. The interactions between hRGS17 and active forms of $G_{a_{1-3}}, G_{o}, G_{z}$, or $G_{q}$, but not $G_{q}$, were detected by yeast two-hybrid assay, in vitro pull-down assay, and co-immunoprecipitation studies. Recombinant RGS17 acted as a GTPase-activating protein (GAP) on free $G_{o}$ and $G_{q}$, under pre-steady-state conditions, and on M2-muscarinic receptor-activated $G_{o_{11}}, G_{o_{2}}, G_{o_{3}}, G_{o_{4}}, G_{z}$, and $G_{q}$ in steady-state GTPase assays in vitro. Unlike RGSZ1, which is highly selective for $G_{o}$, RGS17 exhibited limited selectivity for $G_{o}$, among $G_{o}/G_{z}$ proteins. All RZ family members reduced dopamine-D2 $G_{o}$-mediated inhibition of cAMP formation and abolished thyrotropin-releasing hormone receptor-$G_{o_{m}}$-mediated calcium mobilization. RGS17 is a new RZ member that preferentially inhibits receptor signaling via $G_{i/o}$, $G_{z}$, and $G_{q}$ over $G_{o}$ to enhance cAMP-dependent signaling and inhibit calcium signaling. Differences observed between in vitro GAP assays and whole-cell signaling suggest additional determinants of the G-protein specificity of RGS GAP effects that could include receptors and effectors.

Regulators of G-protein signaling (RGS) proteins accelerate the intrinsic GTPase activity of heterotrimeric G-protein $G_{o}$ subunits. All RGS proteins contain a conserved RGS core domain, which is an interaction site for the $G_{o}$ subunits (1–3).

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There are more than 30 human RGS or RGS-like proteins that are classified into six subfamilies based upon their sequence homology and that have conserved functional and targeting domains outside of the RGS domain. For instance, a membrane-targeting domain immediately proximal to the RGS core domain directs small G-proteins such as RGS1–5 and -16 to the cell membrane (4). Putative nuclear localization signals have been found within (5) and also outside of the RGS core domain (6), and this may direct certain RGS subtypes to the nucleus (7, 8). RZ family members, such as RGSZ1 and RGS-Gα-interacting protein (GAIP), contain a cysteine-rich motif that may serve as a palmitoylation site for membrane association (9, 10). A more recent study showed that RZ family members also serve as adapter proteins for $G_{o}$ subunit degradation (11). The cysteine-rich motif interacts with the leucine-rich region of GAIP-interacting protein N terminus, an E3 ubiquitin ligase responsible for $G_{o_{m}}$ degradation. Recently RGSZ1 and RGS6 have been shown to associate with SCG-10, a protein involved in neuronal differentiation (12, 13), and the $G_{z}$ GTPase-activating protein (GAP) effects of RGSZ1 (and other RGS proteins) were found to be negatively regulated by synapsin-1a (14). Thus, RZ proteins may play diverse and important roles in the regulation of signaling and cytoskeletal events in the brain.

G-protein specificity of RGS proteins has been investigated in various studies. Some RGS proteins display a preference for $G_{o}$ subfamilies (15), with the majority regulating $G_{o_{11}}$ and a subset of these also acting on $G_{o_{6}}$ (16–18, 25). Other RGS members are more selective regulators of specific $G_{o}$ subunits. For instance, p115RhoGEF has a preference for $G_{o_{12/13}}$ subunits (19). RGSZ1 seems to be selective for $G_{o_{1}}$ (20, 21), although at higher concentrations it has been shown to interact with and negatively regulate $G_{o_{1}}$ subunits (22). Similarly, a series of R7 family RGS proteins displayed a relative hierarchical preference in the regulation of $G_{o}$ proteins rather than an absolute specificity for one subtype (23).

The most abundant G-protein expressed in the brain is $G_{o}$. However, many actions of $G_{o}$ are mediated by means of $G_{o}G_{y}$ subunits, and little is known of the effectors for the $G_{o_{6}}$ subunit. To identify $G_{o}$-dependent regulators or effectors, we screened human brain cDNA library for interacting proteins using a yeast two-hybrid system with constitutively active $G_{o_{6}}$ as bait. RGS17 was identified among a number of clones. RGS17 contains a cysteine-rich string at its N terminus followed by the highly conserved RGS domain, and is most homologous to RGSZ1 and other RZ family members. We have addressed the G-protein specificity of RGS17 by GAP interaction and functional assays. Our results indicate that RGS17, as well as RGSZ1, negatively regulates the functions of $G_{o_{11}}, G_{z}$, and $G_{o_{6}}$ subunits.
**Materials and Methods**

**Plasmid Constructs**—Constitutively active rat Gαo179C (24) cDNA was subcloned into EcoRI-cut pAS2-1 (Clontech) for two hybrid-screening. GST-RGS17, GST-RGSZ1, His-Gαo17, and His-Gαo4 subunits were cloned into pGEX-4T-1 vectors. The gly-→ ser (GTS) mutants of all Gα subunit cDNAs were constructed using the Stratagene GeneMiner site-directed mutagenesis protocol. All constructs were confirmed by DNA sequencing.

**Expression of Proteins**—For expression in mammalian cells were constructed in pCDNA3. For the purification of proteins from mammalian cells, GST-RGS17, GST-RGSZ1, His-RGS17, and His-Gαo4 were kindly provided by Dr. John Hepler (Emory University). Baculoviruses for Gαo17 and Gαo4 were kindly provided by Dr. Armen H. Tashjian, Jr. (Harvard University, Boston, MA). Dr. John Hepler (Emory University) and Dr. Pat Casey (Duke University) were supplied by Dr. Armen H. Tashjian, Jr. (Harvard University, Boston, MA). Baculoviruses for GST-RGSZ1 were kindly provided by Dr. John Hepler (Emory University). Baculoviruses for GST-RGSZ1 were kindly provided by Dr. John Hepler (Emory University) and Dr. Pat Casey (Duke University). Other baculoviruses were obtained as noted previously (25). Rat thyrotrphin-releasing hormone receptor (TRH-R1) cDNA was kindly provided by Dr. Armen H. Tashjian, Jr. (Harvard University, Boston, MA). Rat Tyr-oR179C was used as a bait to screen 5 × 10^10 colonies from a human brain cDNA library (HL4004AH, Clontech) in AH109 yeast strain using the LiAc method (27). The yeast colonies were grown at 30 °C. Transformants (1.4 × 10^4) were selected on S.D.-Trp-His plates with 40 mM 3-amino-1,2,4-triazole and screened by X-galactosidase assay. DNA from positive clones, re-transformed to MH6 cells, and selected on M9-Leu plates for sequencing.

For purification of histidine-tagged RGS4, Gαo17, and Gαo4, were kindly provided by Dr. John Hepler (Emory University). Baculoviruses for Gαo17 and Gαo4, respectively, were gifts from Dr. John Hepler (Emory University) and Dr. Pat Casey (Duke University). Other baculoviruses were obtained as noted previously (25). Rat thyrotrphin-releasing hormone receptor (TRH-R1) cDNA was kindly provided by Dr. Armen H. Tashjian, Jr. (Harvard University, Boston, MA). Rat Tyr-oR179C was used as a bait to screen 5 × 10^10 colonies from a human brain cDNA library (HL4004AH, Clontech) in AH109 yeast strain using the LiAc method (27). The yeast colonies were grown at 30 °C. Transformants (1.4 × 10^4) were selected on S.D.-Trp-His plates with 40 mM 3-amino-1,2,4-triazole and screened by X-galactosidase assay. DNA from positive clones, re-transformed to MH6 cells, and selected on M9-Leu plates for sequencing.

For purification of β-galactosidase, the plasmids pAS2-Gαo17 were subcultured in S.D.-Leu medium (30 °C) overnight and were diluted to 10^6 for the yeast cells grown for 3 days. For liquid induction was commenced by adding 30 μM ATP, [ 32P]GTP (1 μCi/mM, 30 μM MgCl2, 20 μM β-ME, 0.5 μM PMSF, and either 2 μM (first dialysis) or 0.5 μM (second dialysis) urea. The dialysis was centrifuged at 27,000 × g for 60 min at 4 °C, and the resultant supernatant was equilibrated with 5% Ni-NTA affinity resin (Qiagen) for 90 min in the cold room. The resin was packed into a column and then washed with ~10 volumes of a buffer containing 50 mM HEPES (pH 7.5), 500 mM NaCl, 20 μM β-ME, 10% glycerol, 6 μM guanidine HCl, and protease inhibitors and dialyzed twice against a buffer containing 50 mM HEPES (pH 7.5), 500 mM NaCl, 20 μM β-ME, 10% glycerol, 6 μM guanidine HCl, and protease inhibitors, plus 20 mM imidazole. This first washing was followed by a second wash using the same buffer but with Triton X-100 and with imidazole increased to 35 mM. The protein was eluted by further increasing the imidazole concentration to 500 mM. The chromatographic removal of imidazole and urea (Superdex 75 column) resulted in precipitation of the purified protein; therefore, RGS17 was stored in the elution buffer and added directly into assays (residual imidazole concentration in assays ranged from 2–6 mM, which was held equal for all conditions within a given experiment). N-terminally histidine-tagged RGS4 was purified as described previously (25). GST-RGSZ1 was purified essentially as described previously (20) and assayed in the uncleaved form, which exhibited GAP activity comparable with that of untagged RGSZ1.

For purification of histidine-tagged Gαo4 and Gαo17 proteins, transformed E. coli BL21(DE3) cells were stored in buffer A (50 mM HEPES, pH 8.0, 100 mM NaCl, 20 μM β-ME, 1% Triton X-100, 5 μM GDP, and 5 μM PMSF) and protease inhibitors were thawed, 0.2 mg/ml lysozyme was added, and the cells were incubated on ice for 30 min. The mixture was then centrifuged at 140,000 × g for 30 min at 4 °C, and the supernatant was collected and washed 25 μM dialyzed into 25 mM imidazole, and then washed with 30 ml of buffer A without Triton X-100 and supplemented with 5% glycerol and 20 mM imidazole. G-protein was eluted with the final wash buffer plus 200 mM imidazole. The elution buffer was removed by dialysis or size-exclusion chromatography using a buffer of 50 mM HEPES, pH 8.0, 100 mM NaCl, 0.1 mM PMSF, 5% glycerol, 2 μg/ml GTP, and 1 mM EDTA, 0.5 mM diethiothreitol. The purified protein was collected and stored with addition of 0.5 M (RGS17) or 0.1 M (RGSZ1) PMSF at −20 °C.

**Steady-state GTPase Assay—**Sf9 insect cells at a density of 2 × 10^6/ml were infected with baculoviruses encodiing the following: Gαo17, Gαo4, Gαo4, or Gαo4, plus N-terminally cmyc-tagged M2 muscarinic receptors, Gβ, and Gγ. After 48 h of infection, cell membranes were prepared as described previously (25) and stored at −80 °C in a buffer containing 20 mM Tris, pH 8.0, 0.5 mM PMSF, 1 mM MgCl2, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 μM ATP, 10–50 mM NaCl, and 0.5 mM GTP, calculated as 7.5 mM. Membranes were incubated at 30 °C for 5 min with 1 μM GTP, 500 μM ATP, [ 32P]GTP (1 × 10^5 cpm/assay), either 100 μM carbachol or 10 μM atropine, and cell mem-

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2 J. Wang, personal communication.
branes (2 µg/assay). The assay was stopped by adding 950 ml of ice cold 5% (w/v) Norit in 0.05 m NaH2PO4 (pH 5) and centrifuging. Radioactivity of [32P]Pi in the supernatant was determined by liquid scintillation counting. The nonspecific membrane GTPase signal was estimated by adding 1 mM of unlabeled GTP to the above assay mix. agonist- and RGS-dependent GTPase activity was calculated as described (25).

Pre-steady-state GTPase Assay—Measurement of pre-steady-state, single-turnover GTP hydrolysis by Ga2, and Ga, was conducted as described (29) with slight modifications. Purified Ga2 or Ga (1 µM) was incubated with 2 x 106 cpm [32P]GTP at 20 °C (Ga2) or 30 °C (Ga) for 15 min and then kept on ice. After 5 min, a single round of GTP hydrolysis was initiated by the addition of MgCl2 (10 mM), GTP (500 µM), to quench the binding reaction, and varying amounts of RGS protein. Reactions were terminated at various time points by mixing aliquots of sample with activated charcoal. The charcoal was pelleted, and the released 32Pi in the supernatant was measured by scintillation counting. GTP hydrolysis rates were determined by fitting to a single exponential function (Origin 6).

In Vitro Pull-down Assay and Western Blotting—E. coli strain BL21DE3 was transformed with pET-Gea179c, pGEX-4T-1, or pGEX-RGS17, induced with 0.5 mM isopropylthio-B-D-galactoside (37 °C, 4 h), harvested, and resuspended in lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM MgCl2, 1 mM dithiothreitol, 0.1% Triton X-100). The bacteria were sonicated, centrifuged at 14,000 rpm for 15 min, and the supernatants were incubated with Ni-NTA resin on ice for 3 h. After washing three times with lysis buffer, the resin was washed 3 x with wash buffer containing 40 mM imidazole, then 200 mM imidazole, and resolved on an SDS-12% polyacrylamide gel. The gel was transferred onto a polyvinylidene difluoride membrane and blocked overnight at 4 °C in 5% skimmed milk in TTBS buffer (0.1% Tween 20, 50 mM Tris, pH 8, 1 mM NaCl). The membrane was blocked with anti-His (Covance Inc., Princeton, NJ) or anti-GST (Santa Cruz Biotechnology), and then horseradish peroxidase–linked secondary antibodies. The membrane was incubated with chemiluminescence substrate (peroxidase (POD), Roche Applied Science) and exposed to Kodak X-OMAT film.

Immunoprecipitation—The pcDNA3-FLAG-RGS17 construct was transiently co-transfected with individual pcDNA3myc-Ga mutants into HEK293 cells by the CaPO4 precipitation method (30). Forty-eight hours after transfection, the cells were scraped in Nonidet P-40 lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl; 1% Nonidet P-40, protease inhibitor mixture, Roche Applied Science) and left on ice for 30 min to lyse. The cell lysate was centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatants of FLAG-RGS17 and mycGa subunits were incubated with ~5 µl of anti-FLAG M2-agarose affinity gel (Sigma) at 4 °C overnight. The gel was washed three times with Nonidet P-40 lysis buffer, resuspended in 1 x glycine buffer (pH 3.3) at room temperature for 5 min to elute the proteins. The eluted supernatants were resolved on an SDS-12% polyacrylamide gel and analyzed by Western blotting.

cAMP Assay—HEK293 cells were co-transfected with dopamine-D1 (Ga-coupled) and -D2 (Ga-coupled) receptor expression plasmids with or without pcDNA3FLAG-RGS17, pcDNA3FLAG-GaI, pcDNA3FLAG-RGSZ1, or PTX-sensitive rat Ga subunits as described (30). Plasmid pcDNA3-FLAG was added to equalize the total DNA content for each transfection. After 24 h, cells were plated into six-well dishes. Forty-eight hours after transfection, the cells were pre-treated with 100 ng/ml PTX (Sigma) for four hours and assayed in serum-free Dulbecco’s modified Eagle’s medium/10 µM isobutylmethylxanthine (IBMX) with compounds indicated in the figure legends (37 °C, 20 min). The medium was collected, floating cells were pelleted (10,000 × g for 30 s), and the supernatant was collected for cAMP radioimmunoassay (30).

Calcium Mobilization—Ltk+ cells in 15-cm plates were transiently co-transfected with 5 µg of THRHR1 cDNA (28) and 10 µg of vector pcDNA3FLAG, pcDNA3FLAG-RGS17, pcDNA3FLAG-GaI, or pcDNA3FLAG-RGSZ1 by DEAE-dextran method (31, 32). Forty-eight hours after transfection, the cells were trypsinized and washed in HBBS buffer (20 mM HEPES, 0.1 mM NaCl, 4.6 mM KCl, 10 mM glucose, 1 mM CaCl2) and incubated with 2 µM Fura-2 AM in HBBS at 37 °C for 30 min with shaking (100 rpm). The cells were washed twice with HBBS, resuspended in 2 ml of HBBS, and subjected to fluorometric measurement. Experimental compounds were added directly to the cuvette from 100-fold concentrated solutions.
RESULTS

RGS17, a Novel RZ-RGS—To identify novel Gαo-interacting proteins, we screened a human brain cDNA library with constitutively active GαoR179C for interacting proteins using a yeast two-hybrid system. Among eight positive clones, human RGS17 (GenBank accession no. AF202257.3) was identified. Its cDNA sequence encodes a protein of 210 amino acids, with a predicted molecular mass of 24.4 kDa (Fig. 1). RGS17 contains a core RGS domain (Fig. 1, underlined) and a cysteine-repeat motif (Fig. 1, dotted underlined) at the N-terminal region. There are six putative casein kinase 2 sites (Fig. 1, black arrowheads) and three potential PKC sites (Fig. 1, white arrowheads). hRGS17 shares 93% amino acid identity to the rat RGS17 (GenBank accession no. XM/H14061217837), 92% identity with the Gallus gallus homolog (GenBank accession no. AF151968), and 91% identity to murine RGSZ2 (GenBank accession no. AF191555), indicating that these are species homologues of RGS17. Human RGS17 has the highest homology to RZ subfamily RGS proteins with amino acid identity of 61 and 57% compared with hRGSZ1 and hRGS-GAIP, respectively, suggesting that RGS17 is a new member of the RZ subfamily.

Expression of RGS17—To verify that RGS17 is expressed in vivo, we examined the distribution of RGS17 mRNA in human tissues by Northern blotting. At least five RGS17 transcripts were found in various brain regions, with three dominant bands at 8, 3, and 2 kb (Fig. 1B). Throughout the central nervous system, the 8-kb RGS17 transcript was expressed most abundantly in the cerebellum (Fig. 1B, lane 1), whereas the 2-kb species was more abundant in cortex and medulla (lanes 2 and 3). The multiple RGS17 transcripts in brain tissues may represent alternately spliced mRNA products. In contrast, in a variety of human peripheral tissues, only the single 2-kb RGS17 mRNA transcript was weakly detected (data not shown). The size of this transcript is similar to the 1.8-kb RGSZ1 (20, 21) and the 1.6-kb RGS-GAIP (33). Thus, multiple RGS17 transcripts are predominantly expressed in the cerebellum and other brain regions.

Because we detected several large RGS17 RNA species in brain, we performed 5'-RACE studies of a human brain cDNA...
library (Marathon, Clontech) to search for alternate translational start sites (Fig. 1C). We obtained a single major band which, when subcloned and sequenced, was identical to and no longer than the cDNA sequence. Thus, the human RGS17 RNA variants in brain appear to have the same coding sequence, and variations in RNA size may be due to different 3'-untranslated regions.

*RGS Specificity of RGS17 Interaction*—To demonstrate direct interactions between RGS17 and Gao17C, in vitro pull-down assays were done using recombinant proteins (Fig. 2). In the absence of His-Gao1, neither GST nor GST-RGS17 bound to the Ni-NTA resin. GST-RGS17, but not GST, was pulled down with His-Gao1, indicating a direct interaction between RGS17 and Gao1 (Fig. 2A). Similar results were obtained from recombinant proteins expressed in HEK293 cells transiently transfected with expression constructs demonstrating a specific interaction of His-Gao17C with FLAG-RGS17 (Fig. 2B).

To further assess its G-protein specificity, interactions between RGS17 and several mammalian Gα subtypes and their constitutively active mutants were examined in yeast. In multiple experiments, RGS17 interacted more strongly with the Gα Gln → Leu mutant (QL) and Gao-QL than with their wild-type counterparts, with the exception of Gao1 and Gao3 (Fig. 3A). No interaction was detected between RGS17 and Gao-QL or Gao-QL in yeast-mating assays (data not shown). Thus, RGS17 is selective for Gao10 and Gao3 proteins and preferentially binds the GTP-activated form.

A specific GS point mutation of the Gao11 and Gao13 subunits disrupts their interaction with RGS proteins (34). The role of this site in RGS17 interaction with G-proteins was assessed using the yeast interaction assay (Fig. 3B). The interaction between RGS17 and constitutively active Gao1 Arg → Cys mutant (RC), Gao2QL, and Gao3QL proteins was abolished upon incorporation of the GS mutation. Similar results were observed for the interaction of RGS-GAP with these Gα-proteins. By contrast, Gao23-33 (a non-RGS Gao3-interacting protein) showed no difference in interaction with single- or double-Gα mutants, indicating the selectivity of the GS mutation for RGS interactions. The interaction specificity was further confirmed by co-immunoprecipitation (Fig. 3C). FLAG-RGS17 and c-myc-Gao12, c-myc-Gao32, or c-myc-Gao3 (single- or double-mutants) were co-expressed transiently in transfected HEK293 cells. The QL mutants of Gao12, Gao32, and Gao3 all co-immunoprecipitated with FLAG-RGS17, but the double QL/GO mutants did not. These data provide further evidence that the interaction with RGS17 requires specific structural determinants of Gα subunits.

*G-protein Specificity of RGS17 GAP Function*—The function of RGS17 was assessed using steady-state and single-turnover GAP assays. Purified recombinant RGS17 increased the rate at which GTP was hydrolyzed by members of the Gα family under all conditions tested. In pre-steady-state, single-turnover GTPase assays, observed basal rates of hydrolytic activity were consistently greater with Gα2 (0.18 ± 0.03 min⁻¹) than with Gα1 (0.06 ± 0.01 min⁻¹). RGS17 at 10 nM or greater increased the rate of GTP hydrolysis of these free G-proteins (Fig. 4). Similarly, RGS17 even at low nanomolar concentrations produced an appreciable GAP effect on membranes from SF9 cells expressing these G-proteins in heterotrimeric form together with M2 muscarinic receptor in steady-state, agonist-driven assays (Fig. 5). RGS17 GAP activity reached a plateau between 300 and 1200 nM in these assays, implying a saturating effect. The GAP activity of RGS17 was also observed in membranes from SF9 cells co-expressing the M2 muscarinic receptor plus Gα2, Gα11, or Gα3 (Fig. 6). Although RGS17 and RGSZ1 GAP effects on Gα11 are suggested by functional data in eukaryotic cells (see below), such changes in receptor-dependent Gα11 GTPase activity could not be reliably demonstrated in these *in vitro* biochemical assays (data not shown).

Relative to RGS4, RGSZ1 produced somewhat greater GTPase activation of Gα2 and Gα3 than other Gα proteins, whereas RGS17 was active on all five Gα subunits over a similar concentration range, with a slight preference for Gα1 and Gα3 (Fig. 6C). In all cases, the maximal GAP activity of RGS17 in steady-state assays was found to be greater than RGSZ1 activity but lower than that of RGS4. The reason for
Fig. 6. Comparison of RGS17 and RGSZ1 GAP activities. The hydrolysis of pre-bound \[^{32}P\]GTP by purified Go12 (A) and Go13 (B) was measured as in Fig. 4 in the absence of RGS protein (control) or in the presence of the indicated concentrations of either RGS17 or RGSZ1. C, steady-state GTPase activity was measured in the presence of 100 \(\mu\)M carbachol plus RGS proteins in membranes derived from Sf9 cells expressing the M2 muscarinic receptor plus heterotrimeric Ga12, Go12, Go13, or Go14 (as in Fig. 5). RGS GAP activity was taken as the amount of GTP hydrolysed over that observed with the agonist alone, and values obtained with 300 or 1200 nM RGS17 or RGSZ1 were normalized, taking as 100% the level of hydrolysis observed in the presence of pre-bound \[^{32}P\]GTP in the absence of RGS protein (as in Fig. 5). The hydrolysis of Go12 was roughly double the effect of its homologue (Fig. 6, A–B). For example, with Go12 300 nM RGS17 increased the rate of GTP hydrolysis to 1.7 min \(^{-1}\), while the same concentration of RGSZ1 increased it to 2.0 \(\pm\) 1.1 min \(^{-1}\). The difference between RGS17 and RGSZ1 was more pronounced in receptor-dependent steady-state assays (Fig. 6C). Unlike the near-saturating (i.e. 60–80% of maximal) GAP activity of 300 nM RGS17 on M2 muscarinic receptor-stimulated Go12, Go13, Go1, and Go12, an

**Fig. 7.** Concentration-dependent attenuation of Go12-mediated inhibition of cAMP by RGS17. A, HEK293 cells were transiently co-transfected with D1R and D2R along with or without increasing amounts of FLAG-RGS17. Forty-eight hours after transfection, the cells were treated for 20 min at 37 \(^\circ\)C with 10 \(\mu\)M IBMX (basal), 10 \(\mu\)M IBMX + 1 \(\mu\)M apomorphine (Apo), or 10 \(\mu\)M IBMX + 1 \(\mu\)M apomorphine + 0.1 \(\mu\)M spiperone (Apo+Spi), and cAMP levels were measured by radioimmunoassay. B, percentage inhibition of D1R-mediated cAMP formation was calculated from the cAMP data in panel A. Statistical analysis was calculated using Prism software. **, \(p < 0.01\); ***, \(p < 0.001\). Inset, Western blot of HEK293 cell extracts from one experiment stained with anti-FLAG antibody showing the level of transduced RGS17 expression.

**Fig. 8.** Specificity of RZ-RGS proteins for Go12 or Go13-mediated inhibition of cAMP. Constructs of D1R/D2R, Go1-PTX-insensitive subunits with pcDNA3 (empty vector), RGS17, GAIP, or RGSZ1 constructs were co-transfected into HEK293 cells. The cells were pre-treated with 100 ng/ml PTX for 4 h and then treated with drugs, and medium was then collected for cAMP assay. Percentage of inhibition was calculated as in Fig. 5; n = 4–7, **, \(p < 0.05\); ***, \(p < 0.001\). Inset, PTX-sensitive inhibition of Go13-induced cAMP in HEK293 cells; n = 5.

this partial effect compared with RGS4 is unclear. Although RGS17 is a less potent GAP than RGS4 under pre-steady-state conditions, we found no indication that the two differ in efficacy with respect to their interactions with free G-proteins in pre-steady-state GAP assays (data not shown). Thus, unknown factors seem to be limiting the maximal effect of RGS17 in the membrane-based assay system relative to the effect of RGS4.

Using both steady-state and pre-steady-state GAP assays,
equivalent concentration of RGSZ1 had little or no effect. In contrast, 300 nM RGSZ1 increased the rate of GTP hydrolysis by free Gᵢ₁, Gᵢ₂, Gᵢ₃, and Gₒ, by an order of magnitude. Quadrupling the concentration of RGSZ1 to 1200 nM allowed us to consistently observe an increase over background (i.e. agonist-stimulated) GTPase activity; however, maximal RGSZ1 activity could not be determined because of limitations in purified protein stock concentrations. Relative to RGS4 activity, RGSZ1 produced its greatest GAP effects in membranes from cells expressing the receptor plus Gₒ₃. Also, whereas the effect of 1200 nM RGSZ1 was only half that of 300 nM RGS17 with Gᵢ₁, Gᵢ₂, Gᵢ₃, and Gₒ, it was roughly equivalent with Gₒ₃, indicating the selectivity of RGSZ1 for Gₒ₃ in the presence of muscarinic receptor.

Actions of RGS17 on Effector Activities—To examine the functional activity and specificity of RGS17 in living cells, its activity to inhibit Gᵢ₁-mediated inhibition of cAMP formation was tested. HEK293 cells, which lack dopamine receptors, were co-transfected with dopamine D₁ receptors (to stimulate cAMP formation) and dopamine D₂ receptors (to inhibit cAMP) (30), in the absence or presence of RGS17. Cells were treated with 1 mM apomorphine to activate both receptors or with apomorphine/spiperone to block D₂ but permit D₁ receptor signaling. The difference in cAMP production between the two treatments measures the inhibitory effect mediated by D₂ receptor-Gᵢ₁ activation (Fig. 7A). In vector-transfected cells, D₁-stimulated cAMP formation was reduced by 80% upon activation of D₂ receptors (Fig. 7B). The inhibitory effect of D₂-Gᵢ₁ was progressively reduced by cotransfection of increasing amounts of FLAG-RGS17, which was detected with anti-FLAG antibody (Fig. 7, inset). By contrast, there was no alteration of D₁-mediated cAMP induction (1 µg of RGS17, 21.2 ± 5.1 nM; 2 µg of RGS17, 24.8 ± 2.1 nM; 4 µg of RGS17, 22.9 ± 3.3 nM), which is consistent with the observed lack of physical interaction between RGS17 and Gᵢ₁.

The G-protein specificity of RGS17 and other RZ family members on individual Gᵢ₇ or Gᵢ₉-mediated inhibition of cAMP was assessed by transfecting HEK293 cells with Gᵢ₇ or PTX-insensitive Gᵢ₉ subunits (Fig. 8). In the absence of PTX-insensitive Gᵢ₇ subunits (Fig. 8, inset), PTX pretreatment greatly diminished the inhibitory effect mediated by endogenous Gᵢ₉ (79 versus 14% inhibition, n = 5). Co-transfection of RGS17, RGS-GAIP, or RGSZ1 with Gᵢ₇ or PTX-insensitive Gᵢ₉ subunit, significantly reduced the inhibitory effect mediated by those Gᵢ₉ subunits, with the exception of RGS17 on Gᵢ₉, upon which the effect was not statistically significant. Although this assay was not sensitive enough to detect a preference of RGSZ1 for Gᵢ₉, RGSZ1 selectively targeted Gᵢ₉, as shown by the in vitro GAP assay (Fig. 6; Ref. 21).

To address the physiological relevance of the interaction between RGS17 and GaqQL (Fig. 3C), we tested the effect of RZ family members on calcium mobilization in Ltk cells. We transiently transfected Ltk cells with TRHR1 and vector RGS17, GAIP, or RGSZ1 and assayed TRHR1-Gq/₁₁-mediated calcium signaling. Upon addition of 1 µM thyrotropin-releasing hormone (TRH), an immediate increase in [Ca²⁺]ᵢ was observed because of Gq₁₁-induced phospholipase C activation (35). Calcium mobilization mediated by TRHR1 was completely blocked by each RZ member (Fig. 9). Importantly, the level of specific [³H]methyl-TRH binding in whole cells was similar in all conditions (data not shown), indicating that RGS proteins did not alter the TRHR1 receptor number. These data indicate that RZ family members function as GAPs for Gα₁₁ signaling or, alternatively, may serve as effector antagonists in this pathway.

Fig. 9. RZ family members reduce Gᵢ₉-mediated calcium mobilization. Ltk⁻ cells were transiently co-transfected with TRHR1 and pcDNA3, RGS17, GAIP, or RGSZ1 using the DEAE-dextran method. Forty-eight hours after transfection, the cells were collected and calcium mobilization-tested by treating cells with 1 µM TRH and 1 µM ionomycin as indicated. This experiment was conducted three times with similar results.
G-protein Specificity of RGS17

DISCUSSION

RGS17, a Novel RZ Member—Based on its interaction with Gαo, we have identified RGS17, a novel RGS protein with homology to RZ or class A subfamily members (2, 3). Northern blot analysis indicated that RGS17 is transcribed as multiple, tissue-specific mRNA species, with multiple species enriched in brain areas, particularly the cerebellum. Similarly, RGSZ1 is expressed primarily in the brain (20, 21, 36). Thus its distribution is different from that of the related protein RGS-GAIP, which is highly expressed in peripheral tissues and weakly expressed in brain (33).

The human RGS17 gene spans more than 33 kb and contains at least four exons (37), and thus could be subject to alternate splicing to generate multiple RNA species. In the RGS20 gene, upstream exons are spliced in to generate Ret-RGS, a large retinal-specific form of RGS17 (36). RGS17 homologues have now been identified in several vertebrate species, including human (GenBank accession no. AF202257.3 and NM.012419), rat (GenBank accession no. XM.217837), mouse (GenBank accession no. AF191555), and chicken (GenBank accession no. AF151988). All mammalian homologues have the same translation initiation site, with a short (24 bp) in-frame open reading frame located 93 or 99 nucleotides upstream of the translation initiation codon. Upstream in-frame open reading frames seem to cause mis-priming of the translational initiation complex, resulting in decreased protein translation (38). The presence of the upstream stop codon suggests that longer RGS17 RNA variants do not encode upstream start sites, and we found no evidence for alternate splicing between the stop codon and the initiation ATG site. Hence, it seems that unlike RGS20, RGS17 does not contain an alternately spliced amino-terminal.

RGS17 Structure—Except for RET-RGS, a splice variant of RGSZ1 with an extended N-terminal domain that is expressed in retina (36), the RZ subfamily consists of small, simple RGS proteins with short N- and C-terminal sequences. The N-terminal cysteine-string motif is highly conserved and serves as a protein interaction site (11). The cysteine-string motif, once palmitoylated, is believed to direct localization of RGS-GAIP and RGSZ1 to cytoplasmic structures such as the trans-Golgi apparatus and post-Golgi secretory vesicles (20, 39). Deletion of the N-terminal domain of RGSZ1 enhanced its nuclear localization, suggesting that the N-terminal domain of RGS17 may direct its localization (5), perhaps by means of specific protein interactions. Recently, the cysteine string of RZ members, including RGS17/RGSZ2, were shown to interact with GAIP-interacting protein N terminus, a novel protein that seems to enhance the degradation of RZ proteins (11). In addition, there is a putative leucine-rich nuclear export sequence (5) embedded in the RGS domain of RGS17 (LFWLACEDL), which suggests that export of RGS17 to the cytoplasm may be regulated. Further studies will be required to identify the roles of these domains in RGS17 function.

G-protein Specificity of RZ Proteins—RGS17 interacted with members of the Gαo subfamily (Gαq and Gαo) and Gαi/o in yeast and in vitro studies (pull-down and co-immunoprecipitation assays), but lacked interaction with Gαo and Goe. The observed physical interactions were reflected in functional effects wherein RGS17 exhibited GAP activity toward Gα1i, Gα2q, Gα3i, Gαo and Gαi and inhibited downstream signaling via Gα2q, Gα3i, and Gαi. Results from yeast β-gal assays suggest possible RGS17 selectivity toward Gαo. We were unable to test actions of RGS17 on Gαq-mediated inhibition of cAMP because Gαq failed to couple to inhibition of cAMP levels in HEK293 cells (results not shown). Gαq selectively inhibits adenyl cyclase V that is lacking in HEK293 cells (40), which might explain the lack of detectable Gαq signaling in these cells. However, greater increases in GTP hydrolysis of receptor-activated Gαq were observed compared with Gαq, although the concentration-dependence of RGS17 GAP activity was the same for both. RGS17 also exhibited a relatively strong GAP effect on receptor-activated Gαq; however, it caused only a minor effect on the regulation of intracellular cAMP levels by this G-protein. In general, RGS effects were more difficult to detect with Gαq, which is consistent with its low GTP turnover relative to other Gα proteins (20) and its relatively weak ability to mediate receptor-dependent effects on cAMP (Fig. 7). Taken together, our results suggest that RGS17 is a GAP for members of the Gαq protein subfamily of heterotrimeric G-proteins, with potential selectivity among these, and that this GAP activity attenuates their intracellular signaling. In contrast, we were unable to demonstrate RGS17 GAP activity on Gα1i2, although it did bind to Gαi2 and inhibit its downstream signaling. This discrepancy may reflect signal-to-noise ratio differences between the assays in question or, alternatively, may point to a different mechanism of signaling inhibition. The latter possibility, that RGS17 may act as an effector antagonist, is suggested by recent reports showing that the inhibition by RGS proteins of Gαi-dependent phospholipase Cβ activation (and hence intracellular calcium release) does not necessarily require RGS GAP activity (41, 42).

RGS17 is most homologous to Gαq-selective RGSZ1 (20, 21). In contrast to RGS17, RGSZ1 and RGS-GAIP both produced a statistically significant attenuation of Gαq-cAMP signaling (Fig. 8). Relative to RGS17, RGSZ1 thus appeared to be selective for Gαq, which is consistent with receptor-dependent GTPase assays in the present study. In accord with previous findings (20), the potency of free RGSZ1 on receptor-activated heterotrimeric G-proteins was low compared with its effects on purified Gαo proteins. RGSZ1 is considered to be a selective GAP for Gαo, as demonstrated previously by GAP assays on free Gαo (20, 21). However, RGSZ1 in the present study also showed an equal or greater effect on two Gαq subunits compared with Gαq in regulating D2S receptor-mediated inhibition of cAMP. Wang et al. (22) recently found that RGSZ1 interacts with Gα1i, and enhances its single-turnover GTPase activity, whereas previous studies had reported minimal GAP effects on Gαq and Gαi (20, 21). The present results suggest that the differences noted between previous studies may have been overstated. The more recent study (22) found that 100 nM RGSZ1 caused a 10-fold increase in the rate of GTP hydrolysis by free Gα1i, with 20 nM RGSZ1 producing roughly a 3-fold (i.e. 200%) stimulation. Prior studies using similar low concentrations of RGSZ1 showed increases ranging from 30–50% (probably Gα1i, Ref. 20) to 150% (Gαo and Gαq, Ref. 21). In our study, 30 nM RGSZ1 produced increases of ~40 and 110% on free Gαq and Gαo, respectively, with a higher concentration producing effects consistent with those described on free Gα1i (22). In steady-state assays, RGSZ1 produced comparable GAP effects on receptor-coupled Gα1i, Gαq, Gαo, and Gαq. Taken together, the foregoing observations suggest that RGSZ1 is a selective Gαq GAP and a general inhibitor of signals mediated via members of the Gαo subfamily. Apparent inconsistencies between effects of RGS17 and RGSZ1 on GTP hydrolysis and second messenger levels suggest the possibility that the specificity of RGS proteins to enhance Gαq.o GAP activity in vitro may not correspond exactly to their abilities to regulate receptor-mediated signaling in vivo.

RGS17 as an Integrator of Cell Signaling—As described above, RZ family members are known to regulate Gαo/Gαq-mediated inhibition of cAMP formation. Our novel finding that RGS17 also interacts with Gαo and that RGS17 and other RZ members inhibited TRH receptor/Gαo-mediated calcium mobilization suggests a role for these proteins as inte-
G-protein Specificity of RGS17

Markers of cellular signaling. Thus, RGS17 negatively regulates both inhibitory coupling of the dopamine-D2 receptor and stimulatory TRHR1/Gαs signaling. In the pituitary and in GH4Zr7 cells, D2 receptor signaling generally inhibits TRH action, although the acute phase of TRH-induced calcium mobilization is spared (43, 44). Expression of RGS17 in GH4Zr7 cells suggests a role to shift signaling, attenuating cAMP-mediated increases in cAMP. The physiological outcome of RGS17 action remains unclear, but one possibility is that cAMP-mediated actions would play a more important role in cells expressing high levels of RZ family members.

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