G Protein Selectivity Is a Determinant of RGS2 Function*

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RGS (regulator of G protein signaling) proteins are GTPase-activating proteins that attenuate signaling by heterotrimeric G proteins. Whether the biological functions of RGS proteins are governed by quantitative differences in GTPase-activating protein activity toward various classes of Gα subunits and how G protein selectivity is achieved by differences in RGS protein structure are largely unknown. Here we provide evidence indicating that the function of RGS2 is determined in part by differences in potency toward Gαq versus Gαi family members. RGS2 was 5-fold more potent than RGS4 as an inhibitor of Gαq-stimulated phosphoinositide hydrolysis in vivo. In contrast, RGS4 was 8-fold more potent than RGS2 as an inhibitor of Gαi-mediated signaling. RGS2 mutants were identified that display increased potency toward Gαi family members without affecting potency toward Gαq. These mutations and the structure of RGS4-Gαi complexes suggest that RGS2-Gαi interaction is unfavorable in part because of the geometry of the switch I binding pocket of RGS2 and a potential interaction between the α8-α9 loop of RGS2 and αi of Gαi class α subunits. The results suggest that the function of RGS2 relative to other RGS family members is governed in part by quantitative differences in activity toward different classes of Ga subunits.

Many hormones, neurotransmitters, and sensory stimuli exert their effect on target tissues by activating receptors that are coupled to heterotrimeric G proteins1 (1, 2). Receptor activation results in exchange of GTP for GDP on Gα subunits, dissociation of GTP-bound Gα subunits from the Gβγ heterodimers, and activation of downstream effector pathways. Signals are terminated following Ga-catalyzed hydrolysis of GTP and reformation of G protein heterotrimers. Thus, G proteins are molecular switches that coordinate physiological responses elicited by a variety of stimuli.

RGS (regulator of G protein signaling) proteins are a family of more than 20 members that regulate G protein signaling in part by acting as GTPase-activating proteins (GAPs) for several classes of G protein α subunits (3–6). The GAP activity of RGS proteins decreases the lifetime of active, GTP-bound Ga subunits, thereby attenuating responses or accelerating the kinetics of signal termination (7, 8). Binding of RGS proteins to active Ga subunits can also antagonize effector activation, thereby blocking signal propagation (9). These activities are mediated by the conserved RGS domain of ~120 amino acids that is characteristic of this protein family.

Higher eukaryotes express several types of RGS proteins, potentially to provide selective regulation of distinct types of G protein signaling pathways. Consistent with this hypothesis, RGS proteins are structurally diverse, distinguished by various domains that are likely to confer specific functions. For example, the N terminus of RGS4 confers receptor-selective regulation of Gαq-coupled responses (10, 11), the PDZ domain of RGS12 binds peptides from the C termini of certain G protein coupled receptors (12), and the GGL domain of RGS7 selectively binds Gβδ (13, 14). Differences in expression pattern (15, 16), subcellular localization (17–19), and interaction with other signaling or regulatory proteins are also likely to give RGS family members distinct biological functions.

It is less clear to what extent differences in Ga subunit selectivity govern the biological function of various RGS proteins. Whereas a few RGS proteins, such as RGSZ1 (20) and the RGS domain of p115Rho-GEF (6), are highly specific for certain Ga subunits, many RGS proteins are promiscuous in vitro. For example, RGS1, RGS2, RGS4, and RGS-GAIP stimulate the GTPase activity of Gα family members and Gαiα in vitro. Less selective members of the RGS family do display quantitative differences in GAP activity toward various classes of Ga subunits in vitro (21, 22), but the significance of this in vivo is unknown. Transfection studies have shown that RGS proteins can be more selective for certain types of signaling pathways in vivo than they are as GAPs in vitro (23–25). Whether the regulatory specificity of RGS proteins observed in intact cells is achieved by differences in G protein selectivity or other mechanisms remains unknown. Accordingly, the mechanisms that govern the intrinsic Ga subunit selectivity of RGS proteins remain poorly understood.

To examine the importance of Ga selectivity as a determinant of the biological functions of RGS proteins, we have focused on RGS2 and RGS4. RGS2 or RGS4 can act in vivo and in vitro on Gα or Gαi class α subunits, although RGS2 is much less potent than RGS4 as a GAP for Gαi subunits in vitro. Here we have examined whether RGS2 and RGS4 differ in potency as inhibitors of Gαq versus Gαi signaling in intact cells and used mutagenesis to determine whether differences in intrinsic G protein selectivity govern the function of RGS2. The results...
suggest that RGS2 preferentially regulates Gα, an effect that is mediated by unique structural features of its G protein-binding interface. They further implicate G protein selectivity as an important determinant of RGS2 function.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cytomegalovirus promoter-driven plasmids used to express RGS4 (pCR3) and RGS2 (pEGFP-C1) were from Invitrogen (Carlsbad, CA) and CLONTECH (Palo Alto, CA), respectively. cDNAs were fused to sequences encoding three tandem copies of a Myc epitope by polymerase chain reaction as described previously (26). The pVT102U-GFP and YCp50 expression plasmids have been described (27). Bacterial expression plasmids containing RGS2 and RGS4 cDNAs were generated in previous studies (26, 28). Expression constructs for RGS2/RGS4 chimeras and point mutants were made using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) and verified by DNA sequencing of the entire protein coding region. Unless otherwise stated, all other reagents and chemicals were from Sigma.

**Transfections**—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, 2 mm glutamine, 10 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a humidified atmosphere with 5% CO2. To generate stably transfected cell lines, HEK293 cells (7 × 106 cells) in 10-cm plates were transfected with a plasmid containing the neomycin (G418) resistance gene and plasmids (10 μg) that direct expression of RGS2 or RGS4 and tagged at their C termini with three copies of the c-Myc epitope. Stable clones were selected for growth in 0.5 mg/ml geneticin. Subclones were screened for RGS expression levels by Western blotting and for clonality by immunofluorescence using the mouse 9E10 monoclonal antibody. To determine the relative expression levels of RGS proteins in stably transfected cell lines, cells from tetrypsinized plates were counted, pelleted, and lysed (2 × 106 cells/ml) in Laemmli sample buffer. Following sonication and boiling, samples were resolved by SDS-polyacrylamide gel electrophoresis. Protein expression levels were determined by immunoblotting using the 9E10 antibody, ECL (Amersham Pharmacia Biotech), and densitometry.

**Phosphoinositide Hydrolysis Assays**—Inositol trisphosphate production was measured essentially as described by Venkatakrishnan and Exton (29). Briefly, 0.3–0.5 × 106 cells/well in a 12-well dish were labeled in complete Dulbecco’s modified Eagle’s medium (without inositol) containing 4 μCi/ml [3H]inositol (NEN Life Science Products) for 17–24 h. In cases where pertussis toxin (List Biologicals, Campbell, CA) was used, it was added (100 ng/ml) 2–4 h prior to completion of the labeling period. Cells were washed once with Dulbecco’s modified Eagle’s medium (without bicarbonate) containing 25 mM Hepes, pH 8, and incubated for 10 min in the same medium containing 5 mM LiCl. Following agonist or vehicle addition to each well, reactions were allowed to continue for 30–60 min and then stopped with 750 μl of ice-cold formic acid. Samples were incubated for 30 min at 4 °C. The supernatant (700 μl) was mixed with 34254 μl of 0.7 M NH4OH, and soluble inositol-containing material was separated by ion exchange chromatography as described previously (29). Portions of the total inositol-containing material and IP3 fraction from each sample were added to 10 ml of Ready-Size scintillant (Beckman, Fullerton, CA) and analyzed by liquid scintillation spectrometry. IP3 levels were expressed as the fraction of the total soluble inositol-labeled material.

**Purification of Recombinant Proteins**—N-terminally histidine-tagged forms of RGS2, RGS4, and Gα were expressed in Escherichia coli (BL21(DE3)) and purified by Ni2+-nitrilotriacetic acid chromatography as described previously (26, 28). The sources of G proteins used for GAP assays have been described previously (4). Recombinant His-tagged Gα was prepared and purified as described (30). Barvuloviruses encoding untagged Gα, Gβ, and His-tagged Gγ subunits and methods used to express and purify untagged Gα from Sf9 cells have been described previously (31). Purified phospholipase Cβ1 was a generous gift of Drs. R. Ball and P. Sternweis (University of Texas Southwestern Medical Center, Dallas, TX).

**G Protein Recycling, G Protein Binding, and Gα-mediated PLCβ Activation**—The rate of GTP hydrolysis by Gα during a single catalytic turnover was measured as described previously (4). RGS protein binding to purified Gα and Gβ and regulation of Gα-mediated activation of reconstituted PLCβ1 were determined as described previously (21).

**Assays of Yeast Pheromone Response**—RGS2 and RGS4 cDNAs were expressed in yeast from the constitutive ADH promoter. Various portions of the coding sequences of RGS2 and RGS4 were generated by polymerase chain reaction as BamHI/XhoI fragments and inserted in frame upstream of the GFP or GFF-Ras2 (C-terminal amino acids of Ras2p; CAAAX box) coding sequences in the high copy plasmid pVT102U (27). To generate single-copy versions of selected constructs, an SpH1 fragment containing the ADH promoter, GFP coding sequences, and ADH transcriptional terminator was subcloned into YCp50. The yeast strain used was BC180 (MATα leu2-3, 112, ura3-52, his3A1, ade2-1, sst2-34254). Yeast cells were grown at 30 °C in synthetic dextrose medium lacking uracil (SD-Ura) medium. Phorone responsiveness was determined by performing holo assays as described previously (27). RGS protein expression from various GFP fusion constructs was assessed by Western blotting and quantified by fluorescence activated cell sorting of yeast cells (2 × 104) in a FACScan flow cytometer (Becton Dickinson).

**Confocal Microscopy**—As described previously (27) the yeast strain SWYS15 ADE2 (MATα ura3Δ1, his3-11, 15 leu2-3, 115 trpl-1, can1-100) was used for microscopy. Cells bearing the various constructs were grown to log phase at 30 °C in SD-Ura liquid medium. Confocal microscopic observation was performed on live cells using a Zeiss Axioskop microscope coupled to an MRC-1000 laser scanning confocal microscope (Bio-Rad). Images represent single equatorial planes obtained with a 63× objective. Confocal images were processed with Adobe Photoshop 4.0.

**RESULTS**

RGS2 Is More Potent than RGS4 as an Inhibitor of Gα Signaling in Vivo—To compare the inhibitory potencies of RGS2 and RGS4, we first established an assay system by identifying receptors expressed endogenously by HEK293 cells that stimulate phosphoinositide hydrolysis via Gαi (Fig. 1A). By stimulating cells with various agonists, we could show that HEK293 cells respond to carbachol, resulting a 4-fold increase in IP3 production relative to untreated controls. This response was insensitive to pertussis toxin, indicating that PLCβ activation probably occurs through Gαi-coupled m1 or m3 muscarinic acetylcholine receptors. In contrast, lysophosphatic acid, somatostatin (Fig. 1A), bradykinin, and angiotensin II (data not shown) did not elicit detectable stimulation of IP3 production.

To examine the relative potencies of RGS2 and RGS4 as inhibitors of Gα, signaling, we appended three copies of the c-Myc epitope to the C termini of RGS2 and RGS4 and generated HEK293 cell lines that stably express either of these tagged proteins. Tagging was necessary to directly compare expression levels of RGS2 and RGS4; previous studies have shown that C-terminally tagged and untagged RGS4 have equivalent activity (26). Two stable lines were obtained that...
express equivalent levels of RGS2-Myc (RGS2-1 and RGS2-2), and four were obtained that express various levels of RGS4-Myc (RGS4-1 through RGS4-4). Initially we analyzed the RGS2-1 and RGS4-1 lines by comparing the levels of IP₃ produced in response to increasing concentrations of carbachol. At each concentration of carbachol, IP₃ accumulation was inhibited to a similar extent in RGS2-1 and RGS4-1 cells relative to controls (Fig. 1B). However, immunoblotting showed that RGS4-Myc expression was approximately 5-fold higher than RGS2-Myc in these cell lines (Fig. 1B, inset), indicating that RGS2 may be more potent than RGS4 as an inhibitor of Gq signaling in these cells. The observed low expression levels of RGS2-Myc are likely due to inefficient translation initiation because inclusion of an optimal Kozak consensus initiation sequence (32) significantly increased RGS2-Myc expression.² Nevertheless, neither RGS2-Myc nor RGS4-Myc was expressed at high levels in these stably transfect lines.

As a further means of determining whether RGS2 is more potent than RGS4 as an inhibitor of Gᵦₐ signaling, we compared carbachol-induced IP₃ production in the RGS2-1 line with that in cell lines expressing various levels of RGS4-Myc (Fig. 1C). The results indicated that the extent of inhibition of IP₃ production correlated directly with RGS4-Myc expression levels. More importantly, IP₃ production was inhibited strongly only when RGS4-Myc expression was severalfold higher than that of RGS2-Myc (Fig. 1, B and C, compare RGS2-1 with RGS4-1 and RGS4-2). Furthermore, expression of RGS4-Myc at a level similar to that of RGS2-Myc had little inhibitory effect (Fig. 1, B and C, compare RGS2-1 with RGS4-2 and RGS4-3). The results of these experiments suggested that RGS2 is approximately 5-fold more potent than RGS4 as an inhibitor of Gᵦₐ-mediated signaling in HEK293 cells. This difference in inhibitory potency in vitro is likely to be mediated at the G protein and/or effector level, because recombinant RGS2 is 5–10-fold more potent than RGS4 as an inhibitor of Gᵦₐ stimulation of purified PLCβ1 in vitro (21). Differences in potency of RGS2 and RGS4 could also be due in part to distinct patterns of subcellular localization. Accordingly, subsequent experiments were aimed at determining the role of G protein selectivity on the function of RGS2 and RGS4.

**RGS4 Is More Potent than RGS2 as an Inhibitor of G, Class α Subunits—**Several observations prompted us to investigate whether RGS2 and RGS4 differ in their potencies as inhibitors of G class α subunits. First, although recombinant RGS2 appears to be 1000-fold less potent than RGS4 as a GAP for Gᵦₐ subunits (21), either RGS protein is capable of stimulating GTP hydrolysis when reconstituted with receptors and Gᵦ in lipid vesicles (5). Second, we and others have shown that RGS4 is much more potent than RGS2 as an inhibitor of the yeast mating pheromone response pathway (16), which uses a G class α subunit. However, in these studies it had not been determined whether the relative potencies of RGS2 and RGS4 are due to differences in G protein selectivity, expression level, subcellular localization, or other factors. Answering this question was the objective of the following experiments.

To explore the mechanisms that account for the different potencies of RGS2 and RGS4 as inhibitors of G class α proteins, we tagged RGS2 and RGS4 at their C termini with GFP as a means of examining their expression level and subcellular localization when expressed in yeast. We also generated versions of the RGS-GFP fusions that contain a C-terminal prenylation sequence (CAAX) from the yeast Ras2 protein to target the chimeric proteins efficiently and specifically to the plasma membrane, as we have done previously with RGS4 (27).

² S. P. Heximer and K. J. Blumer, unpublished results.
TABLE I

Comparison of the phenrome-resistant phenotypes of cells expressing wild type and triple mutant derivatives of RGS2 and RGS4

| Protein expressed | Phenome resistance | Protein level | Normalized phenome resistance |
|-------------------|--------------------|---------------|------------------------------|
| GFP               | 1 ND                | 0.01          | NA                           |
| (A–1/63)RGS2-GFP-CAAX | 3 14                | 0.2 (1)       | NA                           |
| (A–1/63)RGS2(triple)-GFP-CAAX | 60 17               | 3.5 (18)      | NA                           |
| (A–1/33)RGS4-GFP-CAAX | 200 130             | 1.5 (8)       | NA                           |
| (A–3/33)RGS4(triple)-GFP-CAAX | 60 150              | 0.4 (2)       | NA                           |

a Proteins are expressed from the ADH promoter in the 2-μm plasmid pVIT102U.

b GFP fluorescence determined by quantitation of 20,000 events using FACS. Reported values (arbitrary units) have been corrected for background fluorescence of the host strain.

c ND, not determined.

d NA, not applicable.

Features of RGS2 That Limit Its Ability to Act on G, Class G Proteins—Based on the preceding results, it appeared that RGS2 and RGS4 differ considerably in their relative activities toward Gq and Gi family members. To test this hypothesis further and to investigate the mechanisms responsible for achieving different G protein selectivities, we sought to define structural features of RGS2 that may attenuate its ability to act on G, class α subunits. One possibility was that differences in Ga selectivity could be determined by the structures of the G protein-binding surfaces of RGS2 and RGS4. To test this idea, we used sequence alignments and structural data for the G protein-binding surface of RGS4 (33) to identify features of RGS2 that may account for its G protein selectivity. We assumed that the structures of the RGS domains of RGS2 and RGS4 are similar. This seemed likely because the hydrophobic residues that appear to stabilize the RGS fold are conserved even in more highly diverged members of the RGS family such as GRK3 and p115Rho-GEF.

Features of RGS2 that may govern its G protein selectivity were identified by analyzing the properties of RGS2/4 chimeras and point mutants (Fig. 3); none of the chimeras or point mutants were insoluble, indicating that they folded relatively normally. Three regions of the RGS core domain of RGS4 (overlined) contact Gα1. With the exception of a highly conserved asparagine residue, the middle region lacked a strong consensus among the RGS proteins studied and, therefore, seemed unlikely to account for RGS2-specific Ga selectivity. In contrast, the N- and C-terminal subregions (labeled A and B, respectively, in Fig. 3) were highly conserved among other RGS proteins but contained a significant number of RGS2-specific sequences. These regions were studied further.

To determine whether regions A and B of the RGS core domain influence Ga selectivity, we analyzed a series of chimeric proteins in which the RGS2 and RGS4 were transplanted. Region A of RGS2 (residues 99–109) was replaced with the equivalent region of RGS4 to produce the RGS2(4A) chimera. Similarly, the RGS2(4B) chimera was made by replacing domain B (residues 181–191) of RGS2 with the corresponding region of RGS4. A third chimera, RGS2(4AB), was generated replacing both the A and B regions of RGS2 with those of RGS4. Each protein was expressed as a His-tagged molecule in E. coli, purified, and analyzed for GAP activity using Gα1 as a substrate (Fig. 4A). Introducing either the A or B domain of RGS4 into RGS2 did not result in detectable GAP activity. However, replacing both the A and B domains of RGS2 with those of RGS4 was sufficient to obtain GAP activity. Titration experiments indicated that the activity of RGS2(4AB) is at least 100-fold greater than wild type RGS2 and 5–10-fold less potent than wild type RGS4 (Fig. 4B).

As a means of defining the specific sequence features of regions A and B of RGS4 that confer G,α GAP activity when introduced into RGS2, we constructed and analyzed a series of point mutants. Region A of RGS2 and RGS4 differ at only two sites (Phe105 and Cys186 of RGS2 correspond, respectively, to Tyr264 and Ser265 of RGS4). Accordingly, we determined whether the remaining two residues (Asn183 and Glu191) in region B of RGS2 affect G protein selectivity. These residues in region B were changed singly or in combination to their RGS4 equivalents in the context of the RGS2 chimera containing only the B region of RGS4. This yielded RGS2(4B)/F105Y, RGS2(4B)/C106S, and RGS2(4B)/F105Y,C106S. A similar approach was used to identify which of three amino acid differences in region A affect the G protein selectivity of RGS2. One of these, Asn183, seemed less likely to be involved because changing its equivalent in RGS4 (Lys163) to alanine does not affect function (26). Accordingly, we determined whether the remaining two residues (Asn184 and Glu191) in region B of RGS2 affect G protein selectivity. These residues in region B were changed singly or in combination to their RGS4 equivalents in the context of the RGS2 chimera containing the A region of RGS4. This yielded RGS2(4A)/N184D, RGS2(4A)/E191K, and RGS2(4A)/N184D,E191K.

Results shown in Fig. 5A indicated that one residue in region A and two in region B are likely to influence the G protein selectivity of RGS2. In region A, the C106S substitution increased the G,α GAP activity of the RGS2(4B) chimera,
that influence selectivity toward G protein α subunits in vitro. A, GAP activities of RGS2, RGS4, RGS2/4 chimeras, and RGS2/4 chimeras containing point mutations. Single turnover GTPase assays used Gα and the indicated RGS proteins (100 nM). Average values obtained from three assays are expressed as the amount of phosphate released after a 45-s reaction; S.E. are indicated by error bars. B, GAP activities of wild type and mutant forms of RGS2 and RGS4. Single turnover GTPase assays used Gα and the indicated RGS proteins at various concentrations. The RGS proteins tested were: RGS4 (closed circles), RGS4-triple mutant (S85C,D163N,K170E; open circles), RGS2 (closed squares), and RGS2-triple mutant (C106S,N184D,E191K; open squares). Data are expressed as the amount of phosphate released after a 45-s reaction. Data shown are the mean of three assays; S.E. are indicated by error bars.

whereas the F105Y substitution had little effect. In region B, the N184D or E191K substitution increased Gα GAP activity of the RGS2(4A) chimera to intermediate levels, whereas the two together yielded activity similar to the RGS2(4AB) chimera.

To determine whether the three residues implicated by the preceding results are primary determinants of the G protein selectivity of RGS2, we introduced the C106S, N184D, and E191K substitutions into otherwise wild type RGS2, yielding the RGS2-triple mutant. Furthermore, we introduced the reciprocal substitutions into RGS4 (S85C,D163N,K170E = RGS4-triple), reasoning that this might attenuate its GAP activity toward Gα. The GAP activities of recombinant the RGS2-triple and RGS4-triple mutants were compared with their wild type counterparts in titration experiments (Fig. 5B). As expected, the RGS2-triple mutant functioned as a GAP for Gα; like the RGS2(4AB) chimera it was 5–10-fold less potent than wild type RGS4. By contrast, even at a high concentration (1 μM) the RGS4-triple mutant was ineffective as a GAP for Gα, further suggesting that the sequence features we have defined are involved in governing G protein selectivity.

To determine whether the RGS-triple mutants have altered potencies toward Gα class G proteins in intact cells, we examined their ability to inhibit yeast mating pheromone response. This approach was chosen for several reasons. First, the sensitivity and reproducibility of pheromone response assays allows detection of quantitative differences in RGS potency. Second, inhibitory potency in yeast is determined mainly by G protein selectivity because the activities of wild type RGS proteins and their prenylated RGS core domain counterparts are similar. Third, our previous studies indicate that RGS mutants have similar activities toward yeast and mammalian Gα class α subunits (26).

Accordingly, we removed the N-terminal membrane targeting domains of wild type and triple mutant versions of RGS2 and RGS4 and appended GFP containing a CAAK motif to their C termini to quantify protein expression and obtain efficient plasma membrane targeting. As indicated by the results of pheromone response assays, the RGS2-triple mutant was 17-fold more active than the equivalent wild type protein, even though protein expression levels and membrane localization efficiency were similar (Table I and data not shown). Conversely, the RGS4-triple mutant was 4-fold less potent than the equivalent wild type protein, without significant differences in protein expression or membrane localization. These results are probably a more accurate indicator of the effects of these mutations on the activity of RGS2 and RGS4, because single turnover GTPase assays underestimate the activity of RGS2 toward Gα class α subunits. This difference between in vitro and in vivo activity toward Gα family members may indicate that the membrane, Gβγ, or the receptor modulates RGS2 activity. Indeed, this is also suggested by the recent demonstration that RGS2 can mediate the receptor-stimulated steady-state GTPase activity of Gα in a reconstituted system (5).

Although the preceding results suggest that G protein selectivity of RGS2 and RGS4 is affected by the point mutations, they could instead suggest that potency toward any G protein is affected. If so, the RGS2-triple mutant should be more potent than wild type RGS2 as an inhibitor of Gα than wild type RGS4 as an inhibitor of Gα, and the RGS4-triple mutant should be less potent than wild type RGS4 as an inhibitor of Gα. Contrary to these expectations, titration experiments indicated that the point mutations did not dramatically affect the potencies of RGS2 or RGS4 as inhibitors of Gα-mediated activation of purified PLCβ1 (Fig. 6). Therefore, we suggest that these residues in RGS2 are not general inhibitors of Gα interaction but rather act as determinants of G protein selectivity.

DISCUSSION

In this study we have provided three lines of evidence indicating that RGS2 inhibits signaling by Gα in a more potent manner than it inhibits Gα class G proteins. RGS2 is 5-fold more potent than RGS4 as an inhibitor of Gα function in vivo and in vitro. In contrast, RGS2 is 8-fold less potent than RGS4 as an inhibitor of yeast mating pheromone signaling, which is mediated by a Gα class α subunit. Finally, RGS2 mutants have been identified that are more potent toward Gα class α subunits without affecting potency toward Gα. Thus, RGS2 and RGS4 differ considerably in terms of their relative intrinsic potencies as inhibitors of Gα versus Gα class G proteins.

Mechanism of G Protein Discrimination by RGS2—A mecha-
The second structural feature of RGS2 that appears to attenuate activity toward G\(i\) class \(\alpha\) subunits is at the edge of the interaction footprint defined by the RGS4-G\(i,\alpha\) crystal structure. This interfering interaction is proposed to involve the putative a\(\delta\)-a\(\theta\) loop of RGS2 and a\(\alpha\)A of G\(i\) class \(\alpha\) subunits, because we have found that a E191K substitution in the a\(\delta\)-a\(\theta\) loop of RGS2 is required to increase activity toward G\(i\) class subunits. One interpretation is that charge repulsion occurs between Glu\(^{191}\) of RGS2 and a conserved glutamic acid residue (Glu\(^{65}\)) in a\(\alpha\) of G\(i,\alpha\). However, the closest approach between the equivalent residue in the a\(\delta\)-a\(\theta\) loop of RGS4 (Lys\(^{170}\)) and Glu\(^{65}\) of a\(\alpha\) in G\(i,\alpha\) is 6.5 Å, a distance over which charge repulsion may be negligible. Nevertheless, this distance could be shorter earlier in the reaction mechanism, disfavoring a high affinity interaction. Alternatively, the structure of RGS2 may differ from that of RGS4, placing the a\(\delta\)-a\(\theta\) loop closer to a\(\alpha\)A of the Go subunit. G\(i,\alpha\) may bind tightly to wild type RGS2 because the equivalent of E65 in a\(\alpha\) (D71) is positioned farther away from Glu\(^{191}\) in the a\(\delta\)-a\(\theta\) loop, either because of its shorter side chain or conformational differences of G\(i,\alpha\) relative to G\(i\) class \(\alpha\) subunits. Consistent with these hypotheses, recent studies suggest that sequences in the helical domain (which includes a\(\alpha\)) of transducin \(\alpha\) subunits mediate preferential interaction with the RGS9 core domain (34). Indeed, it would be interesting to determine whether such interactions generally affect the selectivity of RGS-Go interaction.

Two findings suggest that the relative inability of RGS2 to interact with G\(i\) class \(\alpha\) subunits could also involve other sequence or structural features. None of the RGS2 mutants we have analyzed is as active toward yeast or mammalian G\(i\) class \(\alpha\) subunits as RGS4. Furthermore, the RGS4-triple mutant, which is identical to RGS2 at the three residues proposed to attenuate G\(i\) interaction, is 2-fold more potent than RGS2 as an inhibitor of yeast mating pheromone response. Potentially, sequences flanking the RGS domain could influence the G\(\alpha\) selectivity of RGS2.

G protein selection by other RGS family members may use somewhat different mechanisms. We suggest this because the RGS domain of p115Rho-GEF, which is specific for G\(12/13\), is highly diverged from other RGS family members (6), suggesting that its Go interaction surface is quite different. Furthermore, RGSZ1 (which acts on G\(z\) but not other G\(i\) family members or G\(q\)) is identical to RGS4 (which acts on all G\(i\) family members including G\(i,\alpha\)) at the two of the three positions we have identified as G protein selectivity determinants in RGS2 (20, 35). The selectivity of RGSZ1 may be mediated by other features of its RGS domain or by flanking sequences.

Biological Implications of the G Protein Selectivity of RGS2—G protein selectivity is likely to be one of several factors that govern the function of RGS2. G\(i\) selectivity may be important because RGS2 does not yet appear to have preference for certain types of G protein coupled receptors (10). This is in contrast to RGS1, RGS4, and RGS16, which display pronounced selectivity toward different types of G\(_i\)-coupled receptors (10). Expression level is also likely to determine which types of G protein signaling pathways are regulated by RGS2. Lower level expression of RGS2 may be sufficient to attenuate G\(_i\) signaling without affecting G\(_q\)-mediated responses, whereas higher level expression of RGS2 could attenuate both types of signaling pathway. Thus, regulated expression of RGS2, as occurs in response to rises in cAMP or changes in neuronal activity (5, 10, 36), may provide a mechanism whereby various types of stimuli regulate G\(_i\) and G\(_q\) signaling pathways separately or in a coordinated fashion, depending on the expression level of RGS2 that is achieved. Other processes such as Go modification (e.g. palmitoylation or phosphorylation) or associ-
ation with other signaling or regulatory molecules could further augment the ability of RGS2 to effect various regulatory outcomes.

In conclusion, it appears that quantitative differences in GAP activity toward G_{i} and G_{q} class α subunits is one factor that governs the biological function of RGS2 and presumably other RGS isoforms as well. Expression of mutants having altered G protein selectivity should provide a means of determining to what extent intrinsic selectivity for various classes of G proteins, receptors, or effectors governs the function of RGS2 mammalian cells.

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