Structural Determinants of G-protein α Subunit Selectivity by Regulator of G-protein Signaling 2 (RGS2)∗§

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“Regulator of G-protein signaling” (RGS) proteins facilitate the termination of G protein-coupled receptor (GPCR) signaling via their ability to increase the intrinsic GTP hydrolysis rate of Gα subunits (known as GTPase-accelerating protein or “GAP” activity). RGS2 is unique in its in vitro potency and selectivity as a GAP for Gαq subunits. As many vasoconstrictive hormones signal via Gq heterotrimer-coupled receptors, it is perhaps not surprising that RGS2-deficient mice exhibit constitutive hypertension. However, to date the particular structural features within RGS2 determining its selectivity for Gαq over Gαi/o substrates have not been completely characterized. Here, we examine a trio of point mutations to RGS2 that elicits Gαq-directed binding and GAP activities without perturbing its association with Gαq. Using x-ray crystallography, we determined a model of the triple mutant RGS2 in complex with a transition state mimetic form of Gαq at 2.8-Å resolution. Structural comparison with unliganded, wild type RGS2 and of other RGS domain/Gα complexes highlighted the roles of these residues in wild type RGS2 that weaken Gαi subunit association. Moreover, these three amino acids are seen to be evolutionarily conserved among organisms with modern cardiovascular systems, suggesting that RGS2 arose from the R4-subfamily of RGS proteins to have specialized activity as a potent and selective Gαq GAP that modulates cardiovascular function.

G protein-coupled receptors (GPCRs)§ form an interface between extracellular and intracellular physiology, as they convert hormonal signals into changes in intracellular metabolism and ultimately cell phenotype and function (1–3). GPCRs are coupled to their underlying second messenger systems by heterotrimeric guanine nucleotide-binding protein (“G-proteins”) composed of three subunits: Gα, Gβ, and Gγ. Four general classes of Gα subunits have been defined based on functional couplings (in the GTP-bound state) to various effector proteins. Gq subfamily Gα subunits are stimulatory to membrane-bound adenylyl cyclases that generate the second messenger 3′,5′-cyclic adenosine monophosphate (cAMP); conversely, Gi subfamily Gα subunits are generally inhibitory to adenylyl cyclases (4). G12/13 subfamily Gα subunits activate the small G-protein RhoA through stimulation of the GEF subfamily of RGS proteins, namely p115-RhoGEF, LARG, and PDZ-RhoGEF (5). Gq subfamily Gα subunits are potent activators of phospholipase-Cβ enzymes that generate the second messengers diacylglycerol and inositol triphosphate (6); more recently, two additional Gαq effector proteins have been described: the receptor kinase GRK2 and the RhoA nucleotide exchange factor p63RhoGEF (7, 8).

The duration of GPCR signaling is controlled by the time Gα remains bound to GTP before its hydrolysis to GDP. Gα proteins are key modulators of GPCR signaling by virtue of their ability to accelerate the intrinsic GTP hydrolysis activity of Gα subunits (reviewed in Refs. 9 and 10). RGS2/G058, one of the first mammalian RGS proteins identified (11) and member of the R4-subfamily (10), has a critical role in the maintenance of normostatic blood pressure both in mouse models (12, 13) and in humans (14, 15); additionally, Rgs2-deficient mice exhibit impaired aggression and increased anxiety (16, 17), behavioral phenotypes with potential human clinical correlates (18, 19).

Although many RGS proteins are promiscuous and thus act on multiple Gα substrates in vitro (e.g. Ref. 20), RGS2 exhibits exquisite specificity for Gαq in biochemical binding assays and single turnover GTPase acceleration assays (20, 21). Consistent with this in vitro selectivity,§ mice deficient in RGS2 uniquely exhibit constitutive hypertension and prolonged responses to

§ Independent reports (e.g., Refs. 57–59) have demonstrated that, in membrane-reconstitution systems containing GPCRs and G-protein heterotrimeric RGS2 can affect the agonist-dependent GTPase activity of Gq-coupled signaling systems. The basis for this discrepancy between RGS2 selectivity for Gαq in binary, solution-based assays and apparent RGS2 activity on Gαq in reconstituted systems has not yet been resolved, but it is important to note that RGS2 (like other RGS proteins) is known to interact with other components of GPCR signal transduction beyond Gα subunits (60), including isoforms of the Gαi effector target, adenylyl cyclase (37).
vasoconstrictors, as would be expected upon loss of a potent negative regulator of G\(_{\alpha_q}\) that mediates signaling from various vasoconstrictive hormones such as angiotensin II, endothelin, thrombin, norepinephrine, and vasopressin (22). In addition, RGS2-deficient mice respond to sustained pressure overload with an accelerated time course of maladaptive cardiac remodeling (23), a pathophysiological response that evokes myocardial hypertrophy known to be critically dependent on G\(_{\alpha_q}\) signaling (24, 25).

To gain insight into the structural basis of the unique G\(_{\alpha}\) substrate selectivity exhibited by RGS2, a series of point mutants in RGS2 were evaluated that enable this protein to bind and accelerate GTP hydrolysis by G\(_{\alpha}\); we subsequently delineated the structural determinants of the G\(_{\alpha}/\)mutant RGS2 interaction using x-ray crystallography. Three key positions, first identified by Heximer and colleagues (21) and highlighted in our structural studies as key determinants of RGS2 substrate selection, were also found to be conserved throughout the evolution of the RGS2 protein in a manner suggestive of specialization toward cardiovascular signaling modulation.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Assay Materials**—Unless otherwise noted, all chemicals were the highest grade available from Sigma or Fisher Scientific (Pittsburgh, PA).

**Protein Expression and Purification**—Using ligation-independent cloning, DNA encoding human RGS2 (Lys\(^{71}\)–His\(^{209}\)), fused to either hexahistidine alone (His\(_{6}\)) or to His\(_{6}\)-tagged enhanced yellow fluorescent protein (YFP), was hybridized into a Novagen (San Diego, CA) pET vector-based prokaryotic expression construct as previously described (26, 27). Point mutations corresponding to Cys106 to serine (C106S), Asn184 to aspartate (N184D), Arg188 to glutamate (R188E), and Glu191 to lysine (E191K) were made using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA). For expression of hexahistidine- and His\(_{6}\)-YFP fusion RGS2 constructs, BL21(DE3) Escherichia coli were grown to an 600 nm of 0.7–0.8 at 37 °C and pelleted after culturing for 14–16 h at 20 °C, cells were pelleted and centrifuged before 600–D-thiogalactopyranoside. After cryoprotection in a solution of 2M ammonium sulfate, Ni\(^{2+}\)-chelating fast protein liquid chromatography column (FF HisTrap; GE Healthcare) using an excitation wavelength of 433 nm (455 nm cutoff) and emission scans from 470 to 535 nm at 2-nm intervals.

**Surface Plasmon Resonance**—Optical detection of protein-protein interactions by surface plasmon resonance (SPR) was performed using a Biacore 3000 (GE Healthcare) exactly as previously described (20, 29, 30).

** Förster Resonance Energy Transfer (FRET)-based Binding Assays**—Förster resonance energy transfer was used to measure binding between G\(_{\alpha_i}\) and the triple point mutant RGS2 (C106S,N184D,E191K) as previously described (26, 28). In brief, FRET between recombinant G\(_{\alpha_i}\)-CFP and YFP-RGS2(C106S,N184D,E191K) proteins was measured using a SpectraMax Gemini fluorescence reader (Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 433 nm (455 nm cutoff) and emission scans from 470 to 535 nm at 2-nm intervals.

**Structure Determination**—Purified G\(_{\alpha_i}\) and RGS2(C106S,N184D,E191K) proteins were mixed at a molar ratio of 1:1.5 and incubated at 4 °C for 20 min. The sample was passed through an S200 gel filtration column pre-equilibrated with 25 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM dithiothreitol, 2 mM dithiothreitol, 100 mM AlCl\(_3\), 20 mM sodium fluoride, and 100 mM GDP. Protein fractions that eluted as a complex were identified using SDS-PAGE and the fractions were pooled and concentrated to 23 mg/ml prior to crystallization condition screens using a 150-nl drop volume with an TTP Labtech Mosquito nanoliter liquid-handling system. The crystal of the RGS2(C106S,N184D,E191K)-G\(_{\alpha_i}\) complex used for data collection was crystallized by vapor diffusion in sitting drops of 400 nl of protein and 200 nl of reservoir solution containing 0.1 M HEPES, pH 7.5, and 2 M ammonium sulfate (TTP Labtech Mosquito).

After cryoprotection in a solution of 2 M ammonium sulfate, 0.1 M HEPES, pH 7.5, and 20% (w/v) D-glucose, crystals were flash cooled in liquid nitrogen. A complete data set was col-
lected at 100 K on a Rigaku/MSC FR-E rotating anode x-ray generator equipped with an R-AXIS HTC image plate detector. Diffraction images were evaluated with MOSFLM (31), and data were scaled using SCALA (32). The crystal belonged to the space group P3221 with unit cell dimensions $a = 114.54$ Å, $b = 114.54$ Å, and $c = 99.33$ Å. A molecular replacement solution was found in this space group using PHASER (33) with the RGS10/G$i3$ complex (PDB code 2IHB) as the search model. The RGS2 coordinates from PDB code 2AF0 were superimposed onto the RGS10 coordinates of the RGS10/G$i3$ positioned complex and rigid body refinement into the electron density was performed using REFMAC5 (34). Difference density in the GDP binding site was modeled using the higher resolution structure of G$i3$ in the RGS8/G$i3$ complex (PDB code 2ODE) with one molecule of GDP, a tetrafluoroaluminate ion, and a magnesium ion coordinated by two additional water molecules. Several rounds of manual rebuilding in COOT (35) and restrained refinement with REFMAC5 (34), using Translation/Libration/Screw (TLS) groups calculated with TLSMD (36), resulted in the final structural model described in Table S1. Coordinates of the RGS2(C106S,N184D,E191K)-G$i3$ complex were deposited in the Protein Data Bank with entry code of 2V4Z.

Cellular cAMP Signaling Assays—HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen) in 6-well dishes with 4 µg of total DNA including pGloSensor™-20F cAMP biosensor plasmid (Promega Corp., Madison WI), dopamine D2 receptor, and empty vector, HA-RGS2(WT), or HA-RGS2(C106S,N184D). Twenty-four hours post-transfection, cells were re-plated on poly-D-lysine-treated, clear-bottom, white 96-well plates at a density of 60,000 cells/well. Forty-eight hours post-transfection, culture medium was aspirated and cells were washed once with assay medium (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (without phenol), 15 mM HEPES, pH 7.4) before being incubated for 2 h with 40 µl/well of equilibration medium (assay medium with 4% GloSensor TM substrate). After 2 h, 6.6 µl of 6× final concentration of quinpirole (diluted in 10 µM forskolin-containing assay medium) was added to...
RGS2/Gα Complex Reveals Key Features of Selectivity

Evaluating Point Mutations to RGS2 That Facilitate Interaction with Gαi1—RGS2 is the only member of the R4-subfamily known to bind specifically to Gαq and not to Gαi/o heterotrimeric G-protein subunits in vitro (20, 21). Three amino acids within RGS2 were identified by Heximer and colleagues (21) as potential selectivity determinants in studies of Gαq-directed GAP activity by RGS domain chimaera derived from RGS2 and RGS4 sequences: namely, cysteine 106, asparagine 184, and glutamate 191. In the present study, we mutated these three amino acids to the highly conserved corresponding amino acids in R4-subfamily members (Cys106 to serine, Asn184 to aspartate, and Glu191 to lysine; supplementary Fig. S1) to identify their respective contributions to Gαq substrate specificity.

RGS2 proteins containing these point mutations, either singly, in tandem, or all three together, were expressed in E. coli and purified to homogeneity (Fig. S2). SPR spectroscopy was used (e.g. Fig. 1) to assess if any individual mutation, or combination of point mutations, was capable of changing the selectivity of RGS2. All mutants retained wild type binding toward Gαq1 (e.g. Fig. 1B). Single mutations to RGS2 (C106S, N184D, or E191K) did not enhance binding to Gαi1 and only minimal enhancements to binding were observed with the C106S, N184D, C106S,N191D, and E191K, N184D double mutants (e.g. Fig. 1A); in contrast, the triple mutant RGS2 exhibited a dramatic increase in Gαi1 binding versus wild type.

RESULTS AND DISCUSSION

Evaluating Point Mutations to RGS2 That Facilitate Interaction with Gαq1—RGS2 is the only member of the R4-subfamily known to bind specifically to Gαq and not to Gαi/o heterotrimeric G-protein subunits in vitro (20, 21). Three amino acids within RGS2 were identified by Heximer and colleagues (21) as potential selectivity determinants in studies of Gαq-directed GAP activity by RGS domain chimera derived from RGS2 and RGS4 sequences: namely, cysteine 106, asparagine 184, and glutamate 191. In the present study, we mutated these three amino acids to the highly conserved corresponding amino acids in R4-subfamily members (Cys106 to serine, Asn184 to aspartate, and Glu191 to lysine; supplementary Fig. S1) to identify their respective contributions to Gαq substrate specificity.

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FIGURE 2. Quantitation of RGS2 binding to Gαq1. SPR was performed as described in the legend to Fig. 1, with the concentration of the RGS2 analyte titrated from 1 nM to 50 μM. Sensorgrams were subsequently used in equilibrium saturation binding analyses to determine RGS2/Gαq1 interaction binding affinities. Dissociation constants (Kd values) were estimated to be =21.1 (95% CI, 11.6 – 30.7 μM), =5.3 (95% CI, 3.1 – 7.5 μM), and =8.6 (95% CI, 5.4 – 11.9 μM) for the double mutants RGS2(C106S,E191K), RGS2(C106S,N184D), RGS2(N184D,E191K), respectively, and determined to be 1.25 (95% CI, 1.0 – 1.6 μM) for the triple mutant RGS2(C106S,N184D,E191K). A Kd value for the wild type RGS2/Gαq1 interaction could not be estimated because saturation was not obtained at concentrations tested.
RGS2. Although the magnitude of binding of the RGS2 double mutants was significantly less than that observed with the triple mutant, binding isotherms were nonetheless generated for all double mutants along with the triple mutant by increasing concentrations of RGS2 protein over the $G_{\alpha_i}$·GDP·AlF$_4^-$ surface. Using equilibrium binding analyses (Fig. 2), dissociation constants ($K_D$ values) for the RGS2/ $G_{\alpha_i}$·GDP·AlF$_4^-$ interaction were estimated to be $\geq$5.3, $\geq$8.6, and $\geq$21.1 μM for C106S,N184D,E191K, N184D, and C106S,E191K, respectively, whereas the $K_D$ value was determined to be 1.25 μM for the RGS2(C106S,N184D,E191K) triple mutant. Dissociation constants derived for the RGS2 double mutants are likely underestimated given an inability to attain saturating concentrations of these particular RGS2 analytes and thereby attain maximal binding ($B_{\text{max}}$).

To determine whether the enhanced affinity of the RGS2 triple mutant was the result of improvements to a canonical RGS domain/$G_{\alpha}$ interaction interface, a highly conserved, surface-exposed arginine within this canonical interface (Arg$^{188}$ in the $\alpha$VIII helix; Fig. S1) was mutated to glutamic acid. As has been shown for other RGS proteins (38), this single charge-reversal point mutation (R188E) on the $G_{\alpha}$-binding surface of the RGS2 triple mutant abolished binding to $G_{\alpha_i}$·GDP·AlF$_4^-$ (Fig. 2B, bottom panel).

To quantify any difference in the ability of the RGS2(C106S,N184D,E191K) triple mutant to bind $G_{\alpha_i}$, increasing concentrations of wild type RGS2 and RGS2 triple mutant proteins were separately injected over an immobilized $G_{\alpha_i}$·GDP·AlF$_4^-$ surface (Fig. 3). Dissociation constants were determined to be 55 nM (95% confidence interval (CI) of 23–87 nM) and 17 nM (95% CI, 9–27 nM) for wild type RGS2- and RGS2(C106S,N184D,E191K)-bound $G_{\alpha_i}$, respectively.

To confirm these SPR-derived results with an orthogonal technique of assessing the RGS domain/$G_{\alpha}$ interaction, FRET measurements were performed using a YFP-RGS2 (C106S, N184D,E191K)/$G_{\alpha_i}$-CFP pair, similar to the RGS4/$G_{\alpha_i}$ interaction FRET assay we have previously described (28). In the presence of GDP, aluminum tetrafluoride, and Mg$^{2+}$ ("AMF"), binding between RGS protein and the Go subunit is observed as an increase in YFP emission and decrease in CFP emission; in the presence of GDP alone, no binding is observed as expected (28, 39) and so the ratio of YFP to CFP emission remains low. The relative affinities of wild type RGS2, RGS16, and RGS2 triple mutant were assessed by using this FRET binding assay in a competitive manner: unlabeled RGS protein was added in increasing amounts to a fixed concentration of YFP-RGS2(C106S,N184D,E191K) and $G_{\alpha_i}$-CFP proteins. As expected, only unlabeled RGS2(C106S,N184D, E191K) and RGS16 proteins were able to inhibit the binding of the RGS2(C106S,N184D,E191K)/$G_{\alpha_i}$ FRET pair (Fig. 4), with observed IC$_{50}$ values of 526 nM (95% CI, 236–1171 nM) and 115 nM (78–168 nM), respectively. At no concentration tested was wild type RGS2 able to inhibit binding of the RGS2(C106S,N184D,E191K)/$G_{\alpha_i}$ FRET pair (Fig. 4B), consistent with the lack of affinity between wild type RGS2 and $G_{\alpha_i}$ subunits seen in our present SPR analyses and previously published studies (20, 21).

**Determinants of RGS2 GAP Activity on $G_{\alpha_i}$ in Vitro**—Using SPR and FRET, we demonstrated that all three point mutations were required to facilitate high affinity binding of RGS2 to $G_{\alpha_i}$. To determine whether this enhanced binding affected the ability of RGS2 to accelerate GTP hydrolysis by $G_{\alpha_i}$, we performed single turnover GTPase assays with both wild type and triple mutant RGS2 proteins (Fig. 5). At no concentration tested was wild type RGS2 capable of increasing GTP hydrolysis over the intrinsic GTP hydrolysis rate of $G_{\alpha_i}$ (Fig. 5A). In contrast, a stoichiometric amount of RGS16 (a known $G_{\alpha_i}$ GAP; Ref. 40) was able to accelerate $G_{\alpha_i}$ GTPase activity; complete hydrolysis of bound GTP was observed in less than 15 s at 0 °C. Unlike wild type RGS2, the RGS2(C106S,N184D,E191K) triple mutant was able to increase the rate of $G_{\alpha_i}$ GTP hydrolysis in a dose-dependent manner (Fig. 5B); however, adding the R188E mutation to
the triple mutant resulted in a complete loss in GAP activity, consistent with the loss of Goi1 binding observed in SPR and FRET assays. To further confirm that the mechanism of action of the RGS2(C106S, N184D, E191K) triple mutant in increasing GTP hydrolysis by Goi1 was related to a canonical RGS domain/Go interaction and not the inadvertant addition of a contaminating GTPase, we assessed the effects of both RGS2(C106S, N184D, E191K) and RGS16 proteins on an RGS-insensitive Goi1 point mutant: specifically, G183S in the Go switch I region (41). Neither RGS2(C106S, N184D, E191K) nor RGS16 proteins were able to increase the intrinsic rate of GTP hydrolysis exhibited by this RGS-insensitive Goi1 (Fig. 5, C and D).

**Determinants of RGS2 Activity on G coupled GPCR Signaling in Cells**—To validate in a cellular context the change in Go specificity exhibited in vitro by the RGS2(C106S, N184D, E191K) triple mutant, we used an intracellular cAMP biosensor to measure Gi heterotrimer-mediated inhibition of forskolin-stimulated cAMP production in HEK293T expressing the G1-coupled D2 dopamine receptor along with either wild type RGS2 or the RGS2(C106S, N184D, E191K) mutant. Upon treatment of transfected cells with forskolin, a robust increase in luminescence was observed from the cAMP sensor, reflecting direct activation of adenyl cyclase by forskolin (4); upon administration of the dopamine D2/D3-receptor selective agonist, quinpirole, dose-dependent inhibition of this cAMP production was observed. Wild type RGS2 had no effect on the IC50 of quinpirole (Fig. 6). However, cellular expression of the RGS2(C106S, N184D, E191K) triple mutant resulted in a significantly higher IC50 for quinpirole (762 versus 18 nM for empty vector; Fig. 6), indicating that the gain of Goi1-directed activity is readily apparent in a cellular context as well as in vitro for the RGS2 triple mutant.

**Structural Determinants of RGS2 Interaction with Ga Subunits**—To determine the structural basis for the Go selectivity of RGS2, we used x-ray crystallography to obtain a structural model of the RGS2 triple mutant bound to a Goi1 subunit. A diffraction pattern data set was collected on a single crystal containing a complex between the RGS2(C106S, N184D, E191K) triple mutant and Goi1 GDP-ALF4 and was refined to 2.8-Å resolution (supplemental Table S1). The resultant structural model revealed canonical RGS domain/Go interactions

**FIGURE 5.** The triple mutant RGS2(C106S,N184D,E191K), but not wild type RGS2, accelerates the GTP hydrolysis rate of Goi1. A, increasing concentrations of wild type RGS2 (as indicated) are unable to accelerate the GTP hydrolysis of 200 nM Goi1. Intrinsic GTP hydrolysis by isolated Goi1 (kobs) was measured at 0.0075 s−1, whereas kobs values of 0.0076 (0.0055–0.0097), 0.0066 (0.0054–0.0078), and 0.0086 (0.0069–0.010) s−1 were observed upon the addition of 50, 2500, or 5000 nM wild type RGS2, respectively. Higher concentrations of RGS2(triple) protein (200, 500, 1000, and 5000 nM) led to GTPase rates of at least 0.1–0.2 s−1 (again underestimated due to sampling time constraints). The triple mutant also containing a fourth, loss-of-function point mutation (namely, RGS2(C106S,N184D,E191K,R188E)) was unable to accelerate GTP hydrolysis by Goi1 with a kobs value of 0.0076 (0.0066–0.0086) s−1. C, the single point mutation to Goi1 glycine 183 to serine, Goi1(G183S) (41) renders Goi1 insensitive to the GAP activity of RGS proteins. The intrinsic hydrolysis rate of the Goi1(G183S) mutant was determined to be 0.0053 (0.0037–0.0069) s−1. Upon addition of 200, 3000, or 5000 nM of the RGS2(C106S,N184D,E191K) triple mutant, the kobs was found to be 0.0036 (0.0026–0.0046), 0.0042 (0.0060–0.0078), and 0.0025 (0.00017–0.00048) s−1, respectively; the kobs for GTP hydrolysis after addition of 200 nM RGS16 was observed to be 0.0064 (0.0052–0.0076) s−1. D, the kobs values are plotted versus concentration of RGS protein to demonstrate the dose-dependent increase in GAP activity upon the addition of RGS2(C106S,N184D,E191K) protein to wild type Goi1, but not the RGS-insensitive Goi1(G183S) mutant.

**FIGURE 6.** The triple mutant RGS2(C106S,N184D,E191K), but not wild type RGS2, inhibits dopamine D2-receptor influence on forskolin-stimulated cAMP production. HEK293T cells were transiently co-transfected with expression vectors for the GloSensor cAMP biosensor and the Gi-coupled dopamine D2-receptor with empty vector, wild type RGS2, or the RGS2(triple) mutant. Inhibition of forskolin-stimulated cAMP production was determined after activation of the D2 receptor with various concentrations of quinpirole as indicated. The IC50 (95% CI) for quinpirole was determined to be 18 (12–26), 14 (9–22), and 762 (498–1170) nM in the presence of empty vector; wild type RGS2; and the triple mutant, respectively. Inset, post-transfection cell lysates were immunoblotted with anti-HA epitope tag antibody to confirm the equivalent overexpression of HA-RGS2 and HA-RGS2(C106S,N184D,E191K) proteins.
One of the three mutation sites within the RGS2 triple mutant, aspartate 184, is observed to form a double salt bridge (Fig. 8A and Fig. S3) with the neighboring arginine 188, the latter being an αVIII residue completely conserved among all other R4-subfamily RGS domains (Fig. S1). Asparagine 184 of wild type RGS2, located between αVII and αVIII, is an aspartic acid in all other R4-subfamily RGS domains (Fig. S1). The additional terminal oxygen present in the aspartate side chain (and missing in asparagine) normally allows two salt bridges to be formed (Fig. 8A) with the conserved αVIII helix arginine residue (e.g. Arg170 of RGS16, Arg188 of RGS2). These salt bridges are not consistently observed in all unliganded RGS domain structures (20); however, this double salt bridge is present in all R4-subfamily RGS domains complexed with Gαi/o subunits (Table S2), suggesting that their formation is important for making the RGS domain competent to bind Gαi/o subunits. The importance of this Arg-Asn side chain interaction is supported by the loss of Gαi/o binding and Gαi/o-directed GAP activity when this αVIII helix arginine is mutated to glutamate (Figs. 2 and 5). The significance of this intramolecular interaction is further supported by observations that mutating the analogous αVIII helix arginine in RGS4 (Arg167) and RGS12 (Arg821) results in loss of Gαi/o binding and Gαi/o-directed GAP activity (38, 43, 44). Although Arg188 of RGS2 does not make any critical contacts with Gαi3 per se, it has a critical role in orienting Asp184 (Fig. 8B) to form a conserved hydrogen bond with the main chain amide of a threonine residue in the Gα switch I region (Thr182 of Gαi3 (20, 42); Thr183 of Gαo (45)).

FIGURE 7. Overall structural features of the RGS2(C106S,N184D,E191K)-Gαi3-GDP-AlF4− complex. A, the tertiary structure of Gαi3 is composed of a Ras-like domain (red) and an all α-helical domain (blue) and is present in a transition-state mimetic form bound to a molecule of GDP (magenta) and tetrafluoroaluminate (AlF4−) ion (gray/blue sticks). The three critical switch regions of Gα (numbered Sw I to Sw III) are colored cyan. All three switch regions are engaged by the RGS2 RGS domain (yellow-green). Panel B represents the same structural model as in panel A, but rotated to highlight contacts made by residues serine 106, aspartate 184, and lysine 191 of the RGS2(C106S,N184D,E191K) triple mutant. This same orientation of the complex is presented in Fig. 8B.
forms only a single hydrogen bond with terminal amine of Arg^{188} and, rotated in this manner, the side chain cannot at the same time form a hydrogen bond with the Thr^{182} backbone (Fig. 8A and Table S2).

The aspartate substitution at position Asn^{184} is critical to allow binding of RGS2 to Go\(_q\); however, this single substitution alone is not sufficient to engender robust Go\(_q\) binding (Fig. 1). Ser^{106} is completely conserved among all R4-subfamily RGS domains except RGS2, in which this position is a cysteine residue (Fig. S1). Mutating Cys^{106} to serine was also necessary to obtain high affinity binding to Go\(_q\) subunits (Figs. 1 and 2); whereas the Ser^{106} side chain was not observed in the structural model to make any critical contacts with Go\(_{13}\), this residue is tightly packed among other residues (Fig. 8B). The structure of the RGS2(C106S,N184D,E191K)-Go\(_{13}\) complex reveals that the \(\beta\)-carbon of Ser^{106} is closely juxtaposed with the backbone carbonyl and \(\gamma\)-hydroxyl of Thr^{182} within switch I of Go\(_{13}\); additionally, the \(\alpha\)-carbon of Ser^{106} is 3.8 Å from the \(\beta\)-carbon of Ser^{106}. In conjunction with the SPR binding data, the observed tight packing of Ser^{106} within the RGS2(C106S,N184D,E191K)-Go\(_{13}\) complex suggests that the Cys^{106} residue of wild type RGS2 prevents high affinity binding to Go\(_{13}\) subunits by steric blockade of interactions with switch I and switch II of the Go\(_q\) subunit.

Although amino acid positions 106 and 184 are completely conserved among all R4-subfamily RGS domains except RGS2, the specific amino acid at position 191 is conserved only in its basic character, being either a lysine or an arginine in all R4-subfamily RGS domains (Fig. S1). In wild type RGS2, this position is instead an acidic residue (glutamate 191). In the structural data derived from the RGS2(C106S, N184D,E191K)-Go\(_{13}\) complex, electron density was present only for the \(\alpha\)-, \(\beta\)-, and \(\gamma\)-carbons of the mutated Lys^{191}; however, the final ordered carbon atom was found to be only 5.1 Å from the hydroxyl oxygen of Glu^{65} in the \(\alpha\)A helix of the Go\(_{13}\) all-helical domain. Electron density was present to fit the Ca, C\(\beta\), C\(\gamma\), and C\(\delta\) atoms of the Lys^{191} residue (Fig. S3). The C\(\epsilon\) and terminal amine were modeled by superimposing a Lys over those parts of the carbon atom chain that could be placed with electron density, revealing that this basic side chain would be less than 3.0 Å from the hydroxyl oxygen of Go\(_{13}\) Glu^{65} and thus within hydrogen bonding distance. It is possible that the high salt concentration necessary for crystallization screened the electrostatic contribution of this interaction away, resulting in a partially disordered side chain. In wild type RGS2, this salt bridge would be lacking and this position instead would create electrostatic repulsion between RGS2 Glu^{191} and the all-helical domain of Go\(_{13}\). The importance of all-helical domain contacts to RGS protein selectivity for Go substrates has been previously speculated for the retinal-specific proteins RGS9-1 and Go\(_{q}\)-transducin (46); our present finding with RGS2 provides one of the first structural insights into these interactions. These RGS domain/all-helical domain interactions, whereas typically underestimated when considering the structural determinants of the RGS protein/Go interaction interface (e.g. Refs. 42 and cf. 20), may provide a unique point of interdiction to exploit with selective RGS protein inhibitors.

**Unique Determinants of RGS2 Go\(_q\) Selectivity Are Conserved among Species with Cardiovascular Systems**—Current knowledge of Go selectivity suggests that R4-subfamily members, as well as proteins from the more ancestral RZ-subfamily (e.g. RGS17, -19, and -20), can act as GAPs for both Go\(_q\) and Go\(_{13}\) subunits (20, 47), with the R4-protein RGS2 particularly attuned to Go\(_{13}\) over Go\(_{q}\). Given its unique Go selectivity and its specialized role in cardiovascular signal transduction, RGS2 is likely to have arisen from the R4-subfamily in response to the development of cardiovascular structures and function.

In evolutionary terms, Go\(_{13}\) emerged as the harbinger of a distinct and recognizable Go subfamily in fungi, and Go\(_{13}\) subunits are present in all metazoans including sponges (48, 49). Although RZ-subfamily RGS proteins are represented within the genomes of nematodes and arthropods (50), a distinct R4-subfamily does not appear until the evolution of urochor-
As chordates evolved into the Gnathostomata (jawed vertebrates), the cardiovascular system rapidly developed coronary vessels, inhibitory vagal innervation, excitatory adrenergic innervation, and responses to prostaglandins, nitric oxide, and endothelin (56). This advance is marked in *Danio rerio* by the addition of multiple R4 proteins, specifically including a *Gαq*-specific RGS2 protein (Fig. 9). This unique member of the R4-subfamily, with cysteine, aspartagine, and aspartate at the three key specificity positions, is highly conserved in the extant representatives of all subsequent evolutionary steps: amphibians (e.g. *Xenopus laevis* and *Xenopus tropicalis*), avians (e.g. *Gallus gallus*) and mammals (Fig. 9); the three defining residues are seen to be unique among all R4-subfamily members within a given species (e.g. human R4 paralogs aligned in Fig. S1). Only amphibians (*X. laevis* and *X. tropicalis*) do not contain all three RGS2-defining amino acids (Fig. 9): whereas the RGS2 signature residue aspartagine is present at position 184, serine (not cysteine) is present at position 106, and a neutral glutamine (not glutamate) is present at position 191. (Note that the latter glutamine is not seen in RGS2, RGS4, nor RGS20 paralogs.) Even though the conservation is not absolute in the amphibians, we have shown that asparagine in position 184 is sufficient on its own to significantly reduce Gαq affinity (i.e. ~20-fold; compare $K_d$ of >21 μM for the C106S,E191K RGS2 double mutant versus $K_d$ of 1.25 μM for the C106S,N184D,E191K triple mutant in Fig. 2). In conclusion, the conservation of these three key residue positions suggests that RGS2 has indeed evolved from the R4-subfamily to be a specialized Gαq GAP for the modern cardiovascular system by acquiring particular residues at one or more of these key positions that have been highlighted in our mutagenesis/crystallography studies.

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