Interplay between negative and positive design elements in G\textalpha\ helical domains of G proteins determines interaction specificity toward RGS2

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Regulators of G protein signaling (RGS) proteins inactivate G\textalpha\ subunits, thereby controlling G protein-coupled signaling networks. Among all RGS proteins, RGS2 is unique in interacting only with the G\textalpha\textsubscript{q} but not with the G\textalpha\textsubscript{i} subfamily. Previous studies suggested that this specificity is determined by the RGS domain and, in particular, by three RGS2-specific residues that lead to a unique mode of interaction with G\textalpha\textsubscript{q}. This interaction was further proposed to act through contacts with the G\textalpha\ GTPase domain. Here, we combined energy calculations and GTPase activity measurements to determine which G\textalpha\ residues dictate specificity toward RGS2. We identified putative specificity-determining residues in the G\textalpha\ helical domain, which among G proteins is found only in G\textalpha\ subunits. Replacing these helical domain residues in G\textalpha\ with their G\textalpha\textsubscript{q} counterparts resulted in a dramatic specificity switch toward RGS2. We further show that G\textalpha\–RGS2 specificity is set by G\textalpha\ residues that perturb interactions with RGS2, and by G\textalpha\textsubscript{q} residues that enhance these interactions. These results show, for the first time, that the G\textalpha\ helical domain is central to dictating specificity toward RGS2, suggesting that this domain plays a general role in governing G\textalpha\–RGS specificity. Our insights provide new options for manipulating RGS–G protein interactions in vivo, for better understanding of their ‘wiring’ into signaling networks, and for devising novel drugs targeting such interactions.

Introduction

Heterotrimeric (\alpha\beta\gamma) G proteins are molecular switches that mediate signaling initiated by G protein-coupled receptors (GPCRs) and play central roles in numerous cellular signaling cascades [1–3]. G proteins are activated by the exchange of bound GDP nucleotide within the G\textalpha\ subunit for GTP, enabling the G\textalpha\ subunit to stimulate downstream effectors. The activity of G\textalpha\ subunits is terminated by interaction with regulators of G protein signaling (RGS) proteins. The RGS domain in these proteins accelerates GTP hydrolysis by the G\textalpha\ subunit, thereby acting as a GTPase-activating protein (GAP) [4–8]. Based on their sequence similarities, G\textalpha\ subunits are divided into the G\textalpha\textsubscript{i}, G\textalpha\textsubscript{q}, and G\textalpha\textsubscript{12/13} subfamilies. The 20 ‘canonical’ RGS proteins, which are organized into four subfamilies (R4, R7, R12, and RZ), can potentially recognize and inactivate G\textalpha\ subunits from the G\textalpha\textsubscript{i} and G\textalpha\textsubscript{q} subfamilies [9]. However, the molecular determinants of G\textalpha–RGS specificity remain to be fully defined [10].

In all G\textalpha–RGS complex structures that were solved experimentally to date, the RGS domain binds to the GTPase domain of the G\textalpha\ subunit [11–17]. Previous structural and biochemical studies have further shown that three substructures within the GTPase domain (termed switches I, II, and III) play central roles in mediating interactions with RGS domains [2,10,11,14–20]. On the other hand, the G\textalpha\ subunit contains a second domain, the G\textalpha\ helical domain, which can also interact with RGS domains [10,13,14,16,17]. For years, the biological role of the G\textalpha\ helical domain, which among all G proteins is unique to G\textalpha\ subunits, was unclear [21], although it was previously implicated in increasing affinity for GTP [22], acting as a tethered intrinsic GAP [23], or participating in effector recognition [24].
More recently, it was shown that the Gα helical domain mediates binding to inhibitory proteins, such as guanine-nucleotide dissociation inhibitors [25], and plays a central role in Gα activation by GPCRs and nucleotide exchange [26]. Previous structural studies observed that RGS contacts with the Gα helical domain can be heterogenic, suggesting that they might contribute to interaction specificity [13–17]. However, previous studies did not show a functional role for the Gα helical domain in interactions with RGS proteins, and particularly, in determining the specificity of these interactions.

Among the 20 canonical RGS proteins, RGS2 is unique in its extreme specificity for members of the Gq subfamily, showing no measurable GAP activity or affinity toward any member of the Gi subfamily [27]. RGS2 is involved in many physiological functions, such as blood pressure homeostasis [28,29], cardiac function [30–33], airway contraction [34,35], immunity [36], and synaptic function [36–39]. RGS2 was shown to play crucial roles in mental disorders, such as anxiety, depression, and stress [36,40–42], and has been implicated in numerous pathological conditions, such as hypertension [43–46], cardiac hypertrophy [47], bacterial infection [48], and various cancers [49–54]. Presumably, these varied biological roles are dependent on the interaction specificity of RGS2. To identify the basis for RGS2 selectivity, Heximer et al. [55] compared the sequences of RGS2 and other RGS proteins and identified three crucial residues that are highly conserved among the RGS family, yet differ in RGS2. These RGS2 residues (C106, N184, and E191) are located at the interface with the Gα subunit and were suggested to prevent RGS2 activity toward the Gi subfamily [15,55]. Exchanging these three residues for their RGS4 counterparts yielded a gain-of-function phenotype that enabled the triple-mutant RGS2 to bind and down-regulate Gα [15,55]. More recently, the structure of the Gαq–RGS2 complex showed that RGS2 adopts a unique pose believed to enable interaction with the Gαq switch regions [16]. A subsequent structure of Gαq–RGS8 [17] and comparison with previous Gα–RGS structures [11,14,15] revealed the manner with which RGS2 complexed with Gαq was indeed unique; all other RGS domains bind their Gα partners similarly. Taken together, these studies concluded that the unique sequence of RGS2 leads to a distinct mode of interaction with Gαq which, via interactions with the Gα switch regions in the GTPase domain, determines RGS2 specificity and its inability to inactivate members of the Gi subfamily [14–17]. Yet, it was not shown experimentally whether interaction specificity with RGS2 is indeed determined by the GTPase domain of Gα subunits.

We have previously shown that several Gα-helical domain residues substantially contribute to interactions with RGS domains [10]. Here, we extended our energy calculations to compare RGS complexes with Gα and Gαq. Our analysis identified helical domain residues that can dictate specificity toward RGS proteins in general and toward RGS2 in particular. Analyzing these residues at the 3D structure level allowed us to predict Gα residues that would perturb interactions with RGS2, as well as Gαq residues that would favorably contribute to such interactions. These computational predictions were validated by Gα-to-Gαq mutagenesis, showing that substitutions in the Gα helical domain were sufficient for an RGS2 specificity switch toward Gαq.

Materials and methods
Protein structures and sequences
We used the following 3D structures in our analysis and visualization of Gα–RGS complexes (with PDB codes for each structure): Gαi1–RGS4 (1AGR) [11], Gαi1–RGS16 (2IK8) [14], Gαi1–RGS1 (2GTP) [14], Gαq–RGS2 (4EKD) [16], and Gαq–RGS8 (5DO9) [17]. Missing short segments in PDB entry 2IK8 (Gαi1 residues 112–118) were modeled on the basis of the Gαi1–RGS4 structure (PDB 1AGR) using the program Nest [56], with partial or missing side chains being modeled using Scap [57]. 3D structural visualization and superimposition were carried out with PyMol (http:// pymol.org).

Energy calculations for identifying Gαi/q residues determining specificity toward RGS2
We followed the methodology described previously [10,58,59] to analyze the per-residue contributions of Gα residues to their RGS partners in the crystal structures mentioned above. We used the finite difference Poisson–Boltzmann (FDPB) method to calculate the net electrostatic and polar contributions (∆∆GElec) of each residue within 15 Å of the dimer interface. Residues that substantially contribute to the interaction were defined as those contributing ∆∆GElec ≥ 1 kcal/mol to the interactions (i.e. twice the numerical error of the electrostatic calculations) [60]. Non-polar energy contributions (∆∆Gnp) were calculated as a surface–area proportional term, by multiplying the per-residue surface area buried upon complex formation using surfv [61] by a
surface tension constant of $-0.05 \text{kcal/mol}/\AA^2$ [60]. Residues that substantially contribute to binding were defined as those contributing $\Delta G_{\text{on}} \geq 0.5 \text{kcal/mol}$ to the interactions (namely, bury more than 10 Å$^2$ of each protein surface upon complex formation).

Protein expression, purification, and mutagenesis
The human RGS2 domain (residues 70–211) [15,55] and Go$_{i1}$ (residues 31–354) [25] were expressed using the pNIC-SGC1 (Addgene) and pProEXHTb (Invitrogen) vectors, respectively. Go$_{i1}$ mutants were produced using a QuickChange site-directed mutagenesis kit (Invitrogen). Proteins were expressed in Escherichia coli BL21 (DE3) cells and grown in 0.5 or 1 l of LB broth at 37°C for RGS or Go protein expression, respectively, until an $A_{600 \text{ nm}} \geq 1.4$ was reached. The temperature was then reduced to 15°C and protein expression was induced by the addition of 500 or 100 μM isopropyl-$\beta$-thiogalactopyranoside for RGS or Go proteins, respectively. After 16–18 h, cells were harvested by centrifugation at 6000 $g$ for 30 min at 4°C, followed by freezing the pellets at $-80^\circ$C. Bacterial pellets were suspended in lysis buffer [50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 5 mM MgCl$_2$, 5 mM $\beta$-mercaptoethanol, protease inhibitor cocktail (Roche), and 0.5 mM PMSF (for Go proteins only)], and the cells were lysed using a Sonics Vibra-Cell sonicator, followed by centrifugation at 24 000 $g$ for 30 min at 4°C. The supernatants were equilibrated to 500 mM NaCl and 20 mM imidazole and loaded onto 1 ml HisTrapFF columns (GE Healthcare Life Sciences). The columns were washed with >20 volumes of wash buffer [20 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 20 mM imidazole] at 4°C and the tagged proteins were eluted with elution buffer [20 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 100 mM imidazole]. The elute was loaded onto a HiLoad 16/600 Superdex 75 PG gel filtration column (GE Healthcare Life Sciences) at 4°C with ≥1.5 volumes of GF elution buffer [50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 5 mM $\beta$-mercaptoethanol, and 1 mM MgCl$_2$ (for Go subunits only)]. The elute was dialyzed against dialysis buffer [GF elution buffer containing 40% glycerol (v/v)]. All purified proteins were estimated to be $>$95% pure, as assessed by SDS–PAGE electrophoresis and Coomassie staining. Protein concentrations were determined by measuring absorbance at $A_{280 \text{ nm}}$, using predicted extinction coefficients (ProtParam, Swiss Institute for Bioinformatics) based on the sequence of each expressed protein.

RGS GAP activity dose–response analysis
RGS activity measurements using dose–response analysis were performed as in previous studies [16,62]. Wild-type and mutant Go$_{i1}$ proteins were loaded with 1 μM [$\gamma$-$^{32}$P]-GTP for 15 min at 30°C in reaction buffer [50 mM HEPES (pH 7.5), 0.05% polyoxyethylene (v/v), 5 mM EDTA, 5 μg/ml BSA, and 1 mM dithiothreitol] and then cooled on ice for 5 min. Each assay was initiated by adding RGS protein at different concentrations in assay buffer (5 mM MgCl$_2$ and 100 μM cold GTP) to a tube containing Go$_{i1}$ (500 nM) on ice. Each reaction was terminated after 30 s by adding 100 μl of 5% perchloric acid and quenched with 700 μl of 10% (w/v) charcoal slurry in 50 mM phosphate buffer (pH 7.5), followed by centrifugation at 12 000 $g$ for 5 min at room temperature. Aliquots (200 μl) of the supernatants were transferred to 3 ml scintillation fluid and analyzed using a Tri-Carb 2810 TR scintillation counter (PerkinElmer). EC$_{50}$ values were determined from a three-parameter sigmoidal curve using SigmaPlot 10.0.

Results
Go$_{i/q}$ residues that determine specificity toward RGS2 are located in the Go helical domain
To identify Go residues that underlie specific RGS recognition by the $G_i$ and $G_q$ subfamilies, we applied energy-based calculations to compare which Go$_{i1}$ and Go$_{q}$ residues substantially contribute to interactions with RGS proteins in the relevant X-ray structures of Go–RGS complexes. Using the computational methodology we developed [10,58,59], the three available X-ray structures of Go$_{i1}$ bound to the high-activity RGS domains RGS1, 4, 16 [11,14] were compared with the two available structures of Go$_{q}$ bound to RGS2 and RGS8 [16,17]. We used the FDPB method to calculate the net electrostatic and polar contributions ($\Delta G_{\text{elec}}$) of each Go residue that is within 15 Å of the Go–RGS interface in each complex. Non-polar energy contributions ($\Delta G_{\text{on}}$) were calculated as a surface-area proportional term by multiplying the per-residue surface area buried upon complex formation by a surface tension constant of $-0.05 \text{kcal/mol}/\AA^2$.

Our calculations showed that the majority of Go$_{i1}$ and Go$_{q}$ residues contributing to interactions with RGS domains do so electrostatically (Figure 1A–E; Supplementary Figures S1 and S2). About two-thirds of the
contributing residues are found in the Gα GTPase domain, located exclusively in the three Gα switch regions; however, a third of the Gα residues that contribute significantly to interactions with RGS domains are located in the Gα helical domain (Figure 1A,B, cf. C). We next classified Gα-contributing residues into two major groups. The first group contains ‘Significant & Conserved (S&C) residues’, whose energy contributions are conserved across both G1i and G3q structures. Note that the conservation required in this group is of energy contributions, rather than sequence identity. However, we do observe that these residues not only contribute similarly to interactions with RGS domains across all G1i and G3q structures, as shown in red spheres. G1i/q specificity-determining residues, which contribute to specific interactions with RGS domains only in G1i or in G3q structures, are shown as purple spheres. All five structures shown in A and B were superimposed and are visualized as above.

Figure 1. Gα S&C residues are restricted to the GTPase domain, while putative G1i/q specificity-determining residues are mainly located in the helical domain.
(A) G1i1 residues that substantially contribute to interactions with high-activity RGS proteins, shown as spheres and colored according to the type of energy contribution, as in the caption (sc or mc elec, side chain or main chain polar/electrostatic contributions, respectively; np, non-polar contributions). The following three crystal structures of G1i1–RGS complexes (with PDB codes) were superimposed: G1i1–RGS1 (2GTP), G1i1–RGS4 (1AGR), and G1i1–RGS16 (2IK8). Gα subunits are shown as ribbon diagrams, colored according to Gα domains: teal (GTPase domain) and olive (helical domain). A representative RGS domain (RGS16) is shown as a transparent orange molecular surface. (B) G1i1 residues that substantially contribute to interactions with RGS2 and RGS8, shown as spheres and colored as in (A). The crystal structures of G1q–RGS2 (4EKD) and G3q–RGS8 (5DO9) were superimposed and are shown as ribbon diagrams, colored according to Gα domains: blue (GTPase domain) and pink (helical domain). A representative RGS domain (RGS2) is shown as a transparent gray molecular surface. (C) A ribbon diagram of a representative complex (G1i1–RGS16) showing the location of the Gα GTPase and helical domains, and the three ‘switch regions’ that regulate interactions with Gα partners such as RGS domains. (D) 3D visualization as in A, rotated 90° about the y-axis. (E) 3D visualization as in B, rotated 90° about the y-axis. (F) Gα S&C residues, which contribute similarly to interactions with RGS domains across all G1i and G3q structures, are shown as red spheres. G1i/q specificity-determining residues, which contribute to specific interactions with RGS domains only in G1i or in G3q structures, are shown as purple spheres. All five structures shown in A and B were superimposed and are visualized as above.
loop in the helical domain (Supplementary Figure S2). Comparing these putative specificity-determining positions across the paralogs of Goα1 and Goαq, we saw that the subfamily-specific residues in these positions are conserved among the Gi homologs and among the Gq homologs that do interact, or do not interact with RGS2, respectively. Moreover, the residues in these positions diverge in the homologs of the visual G protein transducin (Goα1–3), which are down-regulated by RGS9 and its homologs (Supplementary Figure S3). Therefore, the prevalence of Goαq specificity-determining residues in the Ga helical domain suggested that these helical domain residues might determine Ga specificity toward RGS proteins and thus play a possible role in the unique specificity of RGS2 toward Goαq and its homologs. We next compared these Goα1 and Goαq structures to identify which of these helical domain residues combine together into functional structural motifs that can discriminate between RGS domains.

Replacing the αB–αC motif in the Goα1 helical domain with the corresponding Goαq residues increased RGS2 activity

Some of the putative Gi/q specificity-determining residues we identified are located in the αB–αC loop of the Ga helical domain. This loop was previously shown to adopt different conformations in Goα1 and Goαq when bound to RGS8 and was thus suggested as being a potential selectivity determinant [17]. Therefore, we compared the αB–αC loop (residues 112–120 in Goα1 and residues 115–125 in Goαq) conformations and adjacent residues in the three Goα1–RGS and two Goαq–RGS complexes. We observed that the region between Goα residues F108–F118 and Goαq residues R114–A123, which includes the C-terminus of the αB helix and most of the αB–αC loop, adopts distinct conformations in each set of structures (Figure 2). We termed this structurally dissimilar region ‘the αB–αC motif’. Interestingly, we note that this region has higher thermal B-factors in the three crystal structures of Goα1–RGS complexes than in the two Goαq–RGS complexes (Supplementary Figure S4), suggesting that the αB–αC motif also has a different dynamic behavior in the two Ga subfamilies. In contrast, the regions surrounding this segment were structurally aligned in all five structures and adopted very similar 3D conformations.

To test whether the αB–αC motif plays a role in Ga specificity toward RGS2, we substituted this segment in Goα1 with the corresponding residues from Goαq. When the GAP activities of the RGS2 domain toward wild-type Goα1 (Goα1-wt) and this chimera (Goα-i/q-1) were compared (Figure 3), we saw an increase in RGS2 activity, with the EC50 decreasing more than three-fold, from >10 μM (Goα1-wt) to 3.2 μM (Goα-i/q-1). We note that the EC50 of RGS2 toward Goα1-wt is probably much higher than 10 μM, given our inability to reach saturating concentrations of RGS2. However, the increased GAP activity of RGS2 toward the Goα-i/q-1 chimera was still much lower than the high GAP activity of RGS domains from the R4 family toward Goα1, which is in the tens of nM range [55]. Therefore, we next considered the individual Gi density-determining positions identified above and their potential roles in RGS2 interactions.

Figure 2. The helical domains of Goα1 and Goαq contain a structurally dissimilar region.

(A) Superimposition of five crystal structures of Goα1 and Goαq complexed with RGS proteins, as in Figure 1F. The structurally dissimilar region, termed here the αB–αC motif, consisting of residues F108–F118 in Goα1 and R114–A123 in Goαq, which includes the αB–αC loop and some adjacent residues, is colored green (Goα1) or magenta (Goαq). (B) Same as (A), rotated 90° about the y-axis (180° about the y-axis, relative to Figure 1A,B).
Residues in the Gα helical domain αA helix co-operate with the αB–αC motif to interact with RGS2

One of the substantial contributions identified in the αB–αC motif was that of Gαi1–E116, which forms an electrostatic and hydrogen bond network with a glutamate–lysine motif in the cognate RGS domain (E161 and K162 in RGS4; Figure 4A). This interaction is also conserved in the complexes of Gαi1 with RGS1 and RGS16 (Figure 4A). However, our calculations identified substantial contributions from three additional residues (A71, V72, and S75), all found in a different part of the Gαi1 helical domain, the αA helix. These three residues

Figure 3. Substitution of the αB–αC motif in the Gαi1 helical domain with the corresponding residues from Gαq increases RGS2 activity.

Dose–response analysis of RGS2 activity toward wild-type Gαi1 (black circles) and the Gαi/q-1 mutant (where the Gαi1 αB–αC motif, as defined in Figure 2, was replaced with the corresponding residues from Gαq; open triangles). EC50 values were calculated using three-parameter sigmoidal curves in SigmaPlot 10.0. EC50 of Gαi1-wt >10 000 nM; EC50 of Gαi/q-1 = 3200 ± 250 nM. Data presented are means ± s.e.m. of experiments performed in triplicate and are representative of three (Gαi1-wt) and four (Gαi/q-1) independent biological replicates each.

Figure 4. The αB–αC motif co-operates with Gα αA helix residues to interact with RGS domains.

(A) Gαi1 helical domain residues that, together with the Gαi1 αB–αC motif, interact with a glutamate–lysine motif in RGS1, 4, and 16. Interacting residues are shown as sticks, with favorable electrostatic interactions/hydrogen bonds marked with dashed black lines. The Gαi1–RGS1 (2GTP), Gαi1–RGS4 (1AGR), and Gαi1–RGS16 (2IK8) complexes are superimposed and shown as ribbon diagrams, colored olive (Gαi1 helical domain from 1AGR), dark-yellow (RGS1), yellow (RGS4), or orange (RGS16). Residues are numbered according to the Gαi1–RGS4 structure. (B) Potential interactions of the Gαi1 helical domain residues shown in A with RGS2. RGS2 (from 4EKD, colored gray) was superimposed onto the RGS4–Gαi1 complex and visualized as in A. Potential unfavorable interactions are marked with dashed red lines. (C) The corresponding Gαq–RGS2 interactions in the Gαq–RGS2 complex, visualized as in A, and colored pink (Gαq helical domain) and gray (RGS2).
make a non-polar intermolecular contribution to interactions with the RGS residues mentioned above (Supplementary Figure S2) and to the RGS4-unique residue R166 (not shown). Importantly, Goαi1–S75 also participates in the hydrogen bond network with the RGS glutamate–lysine motif (Figure 4A). These three Goαi1 αA helix residues also make intramolecular non-polar interactions with the αB–αC loop (data not shown) and can thereby stabilize the loop in a particular conformation. As such, they also affect αB–αC loop interactions with the RGS domain indirectly.

Further analysis by modeling the interactions of Goαi1 residues with RGS2 predicted that RGS2–E182 interacts unfavorably with Goαi1–E116 (Figure 4B), and that the favorable interactions seen in Figure 4A would be lost in Goαi1 interactions with RGS2. In contrast, visualizing the comparable residues in Goqi revealed a different interaction network across the interface with RGS2. Here, the Goqi αB–αC motif adopted a distinct conformation from that seen in Goαi1, with Goqi–E119 forming an intermolecular salt bridge with RGS2–K175 (Figure 4C). This RGS2 residue is too far to interact favorably with Goαi1–E116 (Figure 4B). The residues in the Goqi αA helix also interact differently both across the interface and with the Goqi αB–αC motif than do the corresponding Goαi1 residues (Figure 4C). Goqi–K77 forms an intermolecular salt bridge with RGS2–E182. Goqi–L78, which corresponds to Goαi1–V72, makes a favorable non-polar intermolecular contribution to interactions with RGS2–N183 (not shown). Goqi–Q81 forms a hydrogen bond with RGS2–N183 and also contributes an intramolecular non-polar interaction with the Goqi αB–αC loop (not shown). We, therefore, predict that Goqi αA helix residues K77, L78, and Q81 work together as a structural motif that co-operates with the αB–αC motif in forming favorable interactions with RGS2. Accordingly, we added the corresponding Goαi1-to-Goqi mutations (i.e. A71K, V72L, and S75Q) to the Goα–q-1 mutant to generate the Goα–i/q-2 mutant. Indeed, these additional changes increased RGS2 GAP activity toward this mutant, with the EC50 decreasing more than four-fold to 760 nM (Figure 5).

Goαi1 helical domain residues E65 and Q68 perturb interactions with RGS2

Two additional residues that can affect interactions with RGS2, Goαi1–E65 and Q68, are located in the N-terminal portion of the Goα αA helix. These two residues form a structural motif that interact favorably with a lysine residue in high-activity RGS domains (K170 in RGS4, Figure 6A) via a salt bridge (with Goαi1–E65) and a hydrogen bond (to Goαi1–Q68). On the other hand, this position in RGS2 is occupied by a glutamate (E191), which is predicted to form unfavorable interactions with Goαi1–E65 and might interact less favorably with Goαi1–Q68 (Figure 6B). In Goqi, the side chain of D71, which corresponds to Goαi1–E65, is shorter and, therefore, likely to interact less unfavorably with RGS2–E191 (Figure 6C). Goqi–G74, which corresponds to Goαi1–Q68, does not interact at all with RGS residues (Figure 6C). Therefore, we predicted that Goαi1–E65 and Goαi1–Q68 perturb interactions with RGS2 more than do their Goqi counterparts.

Figure 5. Substitution of three additional αA helix residues in the Goαi1 helical domain with the corresponding residues from Goqi further increases RGS2 activity.

Dose–response analysis of RGS2 activity toward the Goα–i/q–2 mutant (Goα–i/q–1 + A71K, V72L, S75Q; open circles). EC50 of Goα–i/q–2 = 760 ± 60 nM. RGS2 activity toward wild-type Goαi1 (as in Figure 3) is shown for reference (black circles). EC50 values were calculated as in Figure 3. The data presented are means ± s.e.m. of experiments performed in triplicate and are representative of three independent biological replicates each.
To test these predictions, the appropriate mutants were generated. As hypothesized, replacing this motif (G\text{\(\alpha\)i1–E65 and Q68}) with its G\text{\(\alpha\)}q counterpart (G\text{\(\alpha\)}q–D71 and G74) on the G\text{\(\alpha\)-i/q-2} background (generating G\text{\(\alpha\)-i/q-3}) substantially decreased the EC\text{50} to 330 nM (Figure 7). Further structural analysis identified an additional G\text{\(\alpha\)i1} residue (K54) that can also affect the G\text{\(\alpha\)i1–RGS2} intermolecular interaction via intramolecular interactions with G\text{\(\alpha\)i1–E65}. We hypothesized that, in G\text{\(\alpha\)}q, the corresponding arginine residue might reduce the unfavorable interactions of D71 with RGS2–E191, when compared with G\text{\(\alpha\)i1–K54}, because of the longer side chain and different geometry of the arginine residue (Supplementary Figure S5). Indeed, adding the K54R substitution to the G\text{\(\alpha\)-i/q-3} mutant further decreased the EC\text{50} of RGS2 toward the resulting G\text{\(\alpha\)-i/q-4} mutant to 110 nM (Figure 7). In summary, substituting the a\(B\)–\(\alpha\)C motif and the additional G\text{\(\alpha\)i1} helical domain residues identified here with their G\text{\(\alpha\)}q counterparts increased the EC\text{50} of RGS2 by more than two orders of magnitude (Figure 7).

Figure 6. Residues in the N-terminal portion of the G\text{\(\alpha\)i1} \(\alpha\)A helix may interact unfavorably with RGS2–E191. (A) G\text{\(\alpha\)i1} helical domain residues that interact favorably with a lysine conserved in RGS1, 4, and 16. Interacting residues are shown as sticks, with favorable electrostatic interactions/hydrogen bonds marked with dashed black lines. G\text{\(\alpha\)} helical domains and RGS proteins are shown as in Figure 4. (B) Potential interactions of the G\text{\(\alpha\)i1} helical domain residues shown in A with RGS2, as in Figure 4B. Potential unfavorable interactions are marked with dashed red lines. (C) The corresponding G\text{\(\alpha\)q–RGS2} interactions in the G\text{\(\alpha\)q–RGS2} complex, as in Figure 4C. Unfavorable interactions, which we predict are weaker than the unfavorable interactions modeled in Figure 6B, are marked with an orange dotted line.

Figure 7. Replacement of G\text{\(\alpha\)i1} helical domain residues with their G\text{\(\alpha\)}q counterparts further increases RGS2 activity. Dose–response analysis of RGS2 activity toward the G\text{\(\alpha\)-i/q-3} mutant (G\text{\(\alpha\)-i/q-2 + E65D + Q68G; open circles) and toward the G\text{\(\alpha\)-i/q-4} mutant (G\text{\(\alpha\)-i/q-3 + K54R; black triangles). EC\text{50} of G\text{\(\alpha\)-i/q-3} = 330 ± 50 nM and G\text{\(\alpha\)-i/q-4} = 110 ± 20 nM. RGS2 activity toward wild-type G\text{\(\alpha\)}i1 (as in Figure 3) is shown for reference (black circles). EC\text{50} values were calculated as in Figure 3. The data presented are means ± s.e.m. of experiments performed in triplicate, representative of three (G\text{\(\alpha\)-i/q-3}) and four (G\text{\(\alpha\)-i/q-4}) independent biological replicates each.
Discussion

Our results show that residues in the G\(\alpha\) helical domain play a major role in determining interaction specificity toward RGS2. While the G\(\alpha\)_q–RGS2 X-ray structure showed some RGS2 interaction with the G\(\alpha\)_q helical domain, mutation of selected individual G\(\alpha\)_q helical domain residues had only minor effects on the ability of RGS2 to interact with G\(\alpha\)_q [16]. Similarly, although G\(\alpha\)_q helical domain residues also interact with RGS8 in the G\(\alpha\)_q–RGS8 structure [17], mutagenesis experiments performed in this study, which had only minor effects on RGS interactions, also led to the conclusion that the helical domain is not a major selectivity determinant for such interaction. Rather, previous studies suggested that the unique sequence of RGS2 is the sole determinant of its dramatic selectivity for the G\(\alpha\) rather than the G\(\alpha\)_q G\(\alpha\) subfamily [14–17,55], and that interactions with the G\(\alpha\) switch regions are critical mediators of this specificity [14–17,20]. However, we have shown here that G\(\alpha\)\(\alpha\)_1-to-G\(\alpha\)_q substitutions within the \(\alpha\)B-\(\alpha\)C motif and the additional helical domain residues we identified are sufficient to produce a dramatic gain of function toward RGS2. The quantitative effect of changing these helical domain residues is similar to the gain-of-function effect of the RGS2 triple mutant (Supplementary Figure S6), which was characterized previously [15,55]. Since both the RGS2 triple mutant and the G\(\alpha\)-i/q-4 mutant increased GAP activity by more than two orders of magnitude (Supplementary Figures S6 and S7, respectively), and since the former was also shown to increase affinity by more than two orders of magnitude [15], it is likely that, in both cases, the increase in activity is due to a corresponding increase in affinity. Our results therefore suggest that the G\(\alpha\) helical domain plays a crucial role in determining specificity toward RGS proteins, and particularly in recognizing the unique RGS2 sequence/structure.

Structural moieties that determine specific interactions are often termed positive- and negative-design elements [63,64]. In the context of our work, G\(\alpha\) residues that contribute favorably to interactions with RGS2 act as positive-design elements, while G\(\alpha\) residues that perturb favorable interactions with RGS2 can be considered as negative-design elements. Accordingly, our results define the G\(\alpha\)\(\alpha\)_1 \(\alpha\)B-\(\alpha\)C motif as a positive-design element toward RGS1, 4, and 16, yet as a negative-design element toward RGS2. In addition, our computational analysis predicted that, in G\(\alpha\)_q, this motif is a positive-design element that interacts favorably with the unique residues in RGS2, a prediction that was validated by the increased activity of RGS2 toward the G\(\alpha\)-i/q-1 mutant. Furthermore, we suggest that three residues in the middle of the G\(\alpha\)\(\alpha\)_1 \(\alpha\)A helix (A71, V72, and S75) form a structural motif that, together with the \(\alpha\)B-\(\alpha\)C motif, functions as a positive-design element toward RGS1, 4, and 16. Conversely, the corresponding elements in G\(\alpha\)_q function together as positive-design elements toward RGS2. Indeed, substituting these G\(\alpha\)\(\alpha\)_1 residues with their G\(\alpha\)_q counterparts further increased RGS2 activity toward the G\(\alpha\)-i/q-2 mutant. Lastly, the two N-terminal G\(\alpha\)\(\alpha\)_1 \(\alpha\)A helix residues E65 and Q68 function as a negative-design element toward RGS2, and substituting these residues with their G\(\alpha\)_q counterparts increased RGS2 activity even further.

The predicted combinations of G\(\alpha\)\(\alpha\)_1-negative-design elements and G\(\alpha\)\(\alpha\)_q-positive-design elements that are missing in G\(\alpha\)_1 as explaining the differential recognition of RGS2 are supported by an additional experiment. When we mutated all of the G\(\alpha\)\(\alpha\)_1 residues mentioned above to alanines, the activity of RGS2 toward this mutant (G\(\alpha\)\(\alpha\)_1-HD-Ala\(_{\alpha\}) increased to a value similar to that measured with the G\(\alpha\)-i/q-1 mutant (Supplementary Figure S7). This suggests that negative design plays only a partial role in preventing RGS2 from interacting with G\(\alpha\) and that the positive-design elements in the G\(\alpha\)_q helical domain are required for achieving substantial RGS2 activity. We note that we did not investigate the potential role of water molecules or the dynamic properties of these proteins in mediating additional specificity-determining interactions, and further studies using complementary methods such as molecular dynamic simulations will probably expand our understanding of such determinants. Nevertheless, it is evident that the combination of the positive- and negative-design elements we identified in the G\(\alpha\) helical domain is sufficient to produce the extreme selectivity of the G\(\alpha\) versus the G\(\alpha\)_q subfamily in terms of RGS2 recognition.

In a broader context, previous work assigned specific and central roles for the G\(\alpha\) helical domain in nucleotide exchange [26] and in recognizing nucleotide exchange inhibitors [25]. Here, we identified a new and crucial role of the G\(\alpha\) helical domain, namely dictating specificity toward RGS2 in particular and RGS proteins in general. Our results provide a detailed mechanistic basis for further investigations of RGS–G protein interactions. In doing so, these findings raise the possibility of specifically manipulating RGS–G protein interactions in vivo so as to understand their ‘wiring’ into signaling networks, and for the development of drugs that target such interactions. The particular involvement of RGS2 in pathologies such as mental disorders, hypertension, cardiac hypertrophy, and cancer and the unique determinants of its specificity with the G\(\alpha\)_q subfamily further underscores how our findings can be used toward therapeutics that target these interactions.
Abbreviations
FDPB, finite difference Poisson–Boltzmann; GAP, GTPase-activating protein; GPCRs, G protein-coupled receptors; RGS, regulators of G protein signaling; S&C, Significant & Conserved.

Author Contribution
M.K. designed the research, conducted most of the experiments and structural analysis, analyzed results, and wrote the paper. S.G. conducted structural analysis and experiments, and analyzed results. I.S. conducted structural analysis and some experiments, and analyzed results. M.A-S. conducted experiments, supervised laboratory work, and analyzed results. M.K. designed and supervised the research, and wrote the paper. All the authors were involved in the writing of the paper and approved the final version.

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Competing Interests
The Authors declare that there are no competing interests associated with the manuscript.

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Supplementary Information

Figure S1: The physicochemical classification of Gαi and Gαq residues that contribute significantly to interactions with RGS domains. A. Gαi residues that contribute significantly to the interaction with RGS subunits (as in Fig. 1D), shown as spheres and colored as follows: basic residues, blue; acidic residues, red; polar residues, orange; all other residues, green. The three representative structures of Gαi subunits and the RGS domain are visualized as in Fig. 1D. B. Gαq residues that contribute significantly to the interaction with RGS subunits (as in Fig. 1E), shown as spheres and colored as in A. The two representative structures of the Gαq subunits and the RGS domain are visualized as in Fig. 1E.

Figure S2: Significantly contributing residues in the Gαi and Gαq helical domains. A residue-level sequence map summarizing our structure-based energy calculations of the representative Gαi and Gαq complexes with RGS proteins (as in Fig. 1). Colored boxes mark Gα residues that contribute substantially to interactions with RGS proteins in each structure (with PDB IDs on the left) – magenta (side-chain polar/electrostatic and non-polar contributions), red (side-chain polar/electrostatic contribution only), cyan (main-chain polar/electrostatic and nonpolar contributions), or green (non-polar contribution only). Relevant Gα secondary structure elements are marked above the alignment.
Fig S3: Comparison of helical domain residues that contribute to interactions with RGS domains in \( \alpha_{i1} \), \( \alpha_{q} \), and their related paralogs. The related human homologs of \( \alpha_{i1} \) and \( \alpha_{q} \) (UNIPROT sequences: \( \alpha_{i1}, P63096; \alpha_{i2}, P04899; \alpha_{i3}, P08754; \alpha_{o}, P09471; \alpha_{t1}, P11488; \alpha_{t2}, P19087; \alpha_{t3}, A8MTJ3; \alpha_{q}, P50148; \alpha_{11}, P29992; \alpha_{14}, O95837 \) were aligned using the MAFFT server (https://mafft.cbrc.jp/alignment/server/) and visualized using the BoxShade server (https://embnet.vital-it.ch/software/BOX_form.html). Identical residues in the majority of sequences are shaded black, and conserved residues are shaded gray. Residues that make direct contributions to interactions with RGS proteins (as in Fig. S2) are marked above the alignment as follows: non-polar contributions (green plus signs), side chain contributions (red cross), side chain contributions and non-polar contributions (magenta double crosses).

Fig S4: Normalized thermal B-factors for the helical domain of \( \alpha_{i1} \) and \( \alpha_{q} \) in complex with RGS domains. B-factors were taken from the same five crystal structures analyzed in Figs. 1, S1, and S2 (with PDB codes): \( \alpha_{i1}\)-RGS1 (2GTP), \( \alpha_{i1}\)-RGS4 (1AGR), \( \alpha_{i1}\)-RGS16 (2IK8), \( \alpha_{q}\)-RGS2 (4EKD), and \( \alpha_{q}\)-RGS8 (5ODE).
Figure S5: $\text{G}_{\alpha_q}$-R60 can affect the unfavorable interaction of $\text{G}_{\alpha_q}$-D71 with RGS2-E191. $\text{G}_{\alpha_q}$-R60 forms an electrostatic inter- and intra-molecular network with $\text{G}_{\alpha_q}$-D71 and RGS2-E191, visualized as in Fig. 4C, with the $\text{G}_{\alpha_q}$ GTPase domain colored blue.

Figure S6: The RGS2-SDK triple mutant increased GAP activity. Dose-response analysis of the RGS2-SDK (C106S, N184D, E191K) triple mutant (white triangles); EC$_{50}$ = 120 ± 20 nM. RGS2 activity towards wild type $\text{G}_{\alpha_l}$ (black circles), as in Fig. 3, is shown for reference. EC$_{50}$ values were calculated as in Fig. 3. Data presented are means ± s.e.m. of experiments performed in triplicate, and are representative of three independent biological replicates each.
Figure S7: Substituting $\alpha_{i1}$ helical domain residues that interact with RGS domains with alanines increase RGS2 activity. Dose-response analysis of RGS2 activity towards the $\alpha_{i1}$-HD-Ala$_6$ mutant, in which all six $\alpha_{i1}$ residues that contribute to RGS interactions, as in Fig. 1A, are replaced with alanines (black triangles). EC$_{50}$ of $\alpha_{i1}$-HD-Ala$_6 = 3100 \pm 550$ nM. RGS2 activity towards wild type $\alpha_{i1}$ (black circles) and $\alpha_{i1}$-i/q-4 (open circles), as in Fig. 7, is shown for reference. EC$_{50}$ values were calculated as in Fig. 3. Data presented are means ± s.e.m. of experiments performed in triplicate, and are representative of three ($\alpha_{i1}$-HD-Ala$_6$) independent biological replicates each.