Structural motifs in the RGS RZ subfamily combine to attenuate interactions with Gα subunits

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1. Introduction

Heterotrimeric (αβγ) G proteins function as ubiquitous molecular switches in signal transduction pathways. Activated Gα subunits are turned “off” by Regulators of G-protein Signaling (RGS) proteins, which mediate numerous physiological functions and human pathologies — mostly by functioning as GTPase Activating Proteins (GAPs) towards the Gα subfamily. Yet, which RZ subfamily members mediate particular functions and how their GAP activity and specificity are governed at the amino acid level is not well understood. Here, we show that all RZ subfamily members have similar and relatively low GAP activity towards Gαs. We characterized four RZ-specific structural motifs that mediate this low activity, and suggest they perturb optimal interactions with the Gα subunit. Indeed, inserting these RZ-specific motifs into the representative high-activity RGS16 impaired GAP activity in a non-additive manner. Our results provide residue-level insights into the specificity determinants of the RZ subfamily, and enable to study their interactions in signaling cascades by using redesigned mutants such as those presented in this work.

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ABSTRACT

Regulators of G-protein Signaling (RGS) proteins inactivate heterotrimeric G proteins, thereby setting the duration of active signaling. In particular, the RGS RZ subfamily, which consists of RGS17, RGS19, and RGS20, mediates numerous physiological functions and human pathologies — mostly by functioning as GTPase Activating Proteins (GAPs) towards the Gα subfamily. Yet, which RZ subfamily members mediate particular functions and how their GAP activity and specificity are governed at the amino acid level is not well understood. Here, we show that all RZ subfamily members have similar and relatively low GAP activity towards Gαs. We characterized four RZ-specific structural motifs that mediate this low activity, and suggest they perturb optimal interactions with the Gα subunit. Indeed, inserting these RZ-specific motifs into the representative high-activity RGS16 impaired GAP activity in a non-additive manner. Our results provide residue-level insights into the specificity determinants of the RZ subfamily, and enable to study their interactions in signaling cascades by using redesigned mutants such as those presented in this work.

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RGS19 and RGS20. We found that all three RZ subfamily members attenuate interactions with Gt12-GTPase and helical domains, suggesting they combine to modulate specific interactions with Gα subunits.

Here, we characterized the structural role of the seven specificity-determining residues of RGS17 and compared RGS17 to RGS19 and RGS20. We found that all three RZ subfamily members have similar activity towards Gαo, governed by these seven “RZ-specificity determining” residues. We characterized these structural motifs using structure-based modeling, suggesting they attenuate interactions with Gα subunits by a combined interaction with residues from both the Gα GT-Pase and helical domains. Indeed, insertion of these RZ-specificity determining residues into the high-activity RGS16 substantially reduced RGS GAP activity. This residue-level understanding of the functional specificity determinants of the RGS RZ subfamily can guide the development of RGS-directed therapeutics aimed at this subfamily.

2. Materials and methods

2.1. 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): Gαo•human RGS16 (2IK8) and RGS17 (1ZV4) [22]. Missing residues in 2IK8 (Gαo residues 112–118) and 1ZV4 (S145) were predicted using Nest [23], and partial or missing side chains in 1ZV4 (L144, R184) were predicted using Scap [24].

2.2. Protein expression, purification, and activity analysis

RGS19 and RGS20 were obtained from the cDNA Resource Center (www.cdna.org), while RGS16 and RGS17 were obtained from Addgene. Rat Gαo was a gift from Vadim Arshavsky (Duke University). All GRS domains were expressed in the pLIC-SGC1 vector (Addgene). All proteins were expressed as N-terminally His6-tagged fusion proteins and purified from transformed Escherichia coli BL21 (DE3) cells as described previously [21]. Dose-response analyses of RGS GAP activity were performed as in Ref. [21], using 500 nM Gαo pre-loaded with 1 μM [γ-32P]-GTP and RGS domains in concentrations ranging from 0.5 nM to 3 μM at 4°C.

3. Results

3.1. RZ subfamily members show lower GAP activity towards Gαo than the high-activity RGS16

We measured the GAP activities of the three RZ subfamily members (RGS17, RGS19, and RGS20) towards the representative Gαo subfamily member Gαo, and compared it to that of RGS16, a representative R4 high-activity RGS domain [20,21]. We used dose response analysis to quantify and compare the GAP activity of these RGS domains, as this analysis provides a more accurate measurement of GAP activity [21]. This comparison showed that all three RZ family members have similarly low GAP activities compared to RGS16. As expected from previous studies [20], replacing all seven RGS17 specificity-determining residues with their corresponding RGS16 residues (the RGS17 > 16 mutant) increased the GAP activity of this mutant to that of RGS16, confirming that these seven residues are sufficient to determine the lower GAP activity of RGS17.

3.2. The RZ subfamily contains four structural motifs that are conserved across this subfamily but diverge from high-activity RGS domains

To characterize the functional role of the seven RGS17 specificity-determining positions, we compared these amino acid positions in the RZ subfamily and across representative high-activity members from the R4 subfamily (Fig. 2). We found that all seven residues are essentially conserved across all RZ subfamily members, and can be assigned into four distinct motifs (Fig. 2A). Three of these (the “ILS”, “S”, and “HR” motifs) are identical across all three RZ members, while the “N” motif, which is an asparagine in RGS17 and RGS20, is a serine in RGS19 (Fig. 2A). As shown previously [20,21], residues in the high-activity R4 RGS domains that correspond to these four motifs contribute favorably to the interactions of these RGS domains with Gαo and Gαo (Fig. 2B). Supporting the functional importance of these positions, mutations in R4 residues located in these four motifs were shown to impair GAP activity [20,21,25–27]. Two of these positions (RGS16 A126 and N131) were previously classified as S&C residues that contribute to interactions with Gα subunits in all high-activity RGSs, while four positions were classified as Modulatory residues that are usually non-conserved and can contribute to interactions with Gα subunits only in some RGS domains (Fig. 2B) [20,21]. Moreover, the HR motif in the RZ subfamily corresponds to a Disruptor motif that was identified in the R12 RGS subfamily; a lysine-tyrosine or a lysine-phenylalanine motif in the corresponding positions in the R12 subfamily members RGS10 and RGS14 led to significantly impaired GAP activity [21].

We modeled the RGS17-Gαo complex by superimposing the RGS17 monomer, as a structural representative of the RZ subfamily, onto the RGS16 coordinates in the RGS16-Gαo complex. We see that the four RZ-specific motifs are spaced along the RGS domains with no apparent intramolecular interactions between them (Fig. 2C). The ILS and S motifs interact only with the Gα GT-Pase domains, with the former in the periphery of the interface, and the latter
buried in the middle of the interface (Fig. 2D). On the other hand, the HR and N motifs are closer together and interact with the Gα helical domain (Fig. 2D).

3.3. RZ-specificity determining motifs are predicted to attenuate interactions with Gα subunits

To investigate the mechanistic basis for how the seven RZ-specificity determining residues attenuate Gα recognition, we compared the interactions of RGS17 with Gα to those of RGS16, a representative of the high-activity R4 subfamily, with Gα (Fig. 3). Note that Gαi and Gαs interact similarly with high-activity Gα domains, and that Gαi is better characterized among the available Gα complexes with RGS domains [21]; Gαi is therefore a more reliable choice for such structural comparisons [28].

Analyzing the model of the RGS17-Gα complex, we see that the RGS17 ILS motif consists of two large hydrophobic residues (RGS17 S145 and L144; Fig. 3A, upper). These residues cannot form the electrostatic or polar interactions with Gαi. This hypothesis is supported by analysis of the NMR structure of RGS19, which shows enhanced flexibility in the α5-α6 loop that contains the ILS motif (Supp. Fig. S1) and by B-factor analysis of the monomer x-ray structure of RGS17, which also suggests enhanced flexibility of the same loop (Supp. Fig. S2).

The RGS17 HR motif corresponds to a glutamate-lysine motif in high activity Gα domains that forms an electrostatic and hydrogen bond network with multiple residues on both sides of the interface [21]. As detailed above, the corresponding residues in RGS10 (lysine-tyrosine) and RGS14 (lysine-phenylalanine) were shown to perturb these interactions and attenuate GAP activity [21]. When we compared RGS17 and RGS16, we saw that while RGS17 R184 can potentially interact with Gαi S75 and E116 similarly to the corresponding RGS16 K165, the RGS17 H183 residue cannot substitute for the intra-molecular salt bridge formed by RGS16 E164 (Fig. 3B). This suggests that the RGS17 HR motif may partially perturb interactions with Gαi, but less so than the RGS10 and RGS14 Disruptor residues. Notably, the RGS17 HR motif is adjacent to the N motif (N192), and this asparagine residue is too short to interact favorably with Gαi E65 (not shown). Therefore, despite this difference in amino acids, the N motif of all members of RZ subfamily is predicted to have a similar effect on interactions with the Gα subunit. This analysis also suggests that due to the proximity of the HR and N motifs, their effect on interactions with Gα subunits is not mutually exclusive, and should be regarded as a joint motif, which we call the HR + N motif.

The RGS17 S* motif (S150) stands out in its pivotal location at the center of the interface with Gαi (Fig. 2D) and its multiple interactions with critical Gα residues (Fig. 3C). The RGS16 residue in this position is an asparagine (termed here Asnα46C) that is conserved across all RGS domains except for the RZ subfamily. This
Fig. 3. Predicted interactions of the RGS17 specificity-determining motifs with Gαi, compared to the corresponding interactions in RGS16 bound to Gαs. The RGS17-Gαs complex was modeled as in Fig. 2. RGS17 (colored green, upper panels), RGS16 (colored orange, lower panels), and the Gα subunit (colored grey) are shown as ribbon diagrams. Salt bridges or hydrogen-bonds in the crystal structure are marked with dashed lines, while predicted salt bridges between RGS17 and Gα are marked with dotted lines. (A) The RGS17 ILS motif (I143, L144, and S145, upper) cannot form the favorable interactions of the corresponding RGS16 residues (S124, E125 and A126, lower) with Gα. (B) The RGS17 HR and N motifs interact with adjacent residues in the Gα helical domain. The RGS17 HR motif (H183 and R184) can only partially form the favorable interactions of the corresponding RGS16 E164 and K165 residues, which form a network of intra- and inter-molecular interactions with Gα S75 and E116 (lower). The RGS17 N motif (N192, upper) cannot form the favorable interaction made by the corresponding RGS16 K173 with Gα E65 (lower). (C) The RGS17 S* motif cannot form the intricate network of interactions made by the RGS16 AsnSac residue with Gα E207, the catalytic residue Q204, and K180 (lower). The Gα catalytic residue R178 is also shown, as the nearby K180 likely affects its orientation. The guanine nucleotide (from PDB ID 2IK8) is shown in ball and stick representation, colored by element. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Inserting the RZ-specifcity determining motifs into the high-activity RGS16 impairs GAP activity

To examine the mechanistic effect of the RZ-specificity determining motifs characterized above, we inserted them into the high-activity RGS16 and measured the effect using dose response analysis (Fig. 4). Inserting the individual ILS motif into RGS16 had a minor effect on GAP activity (Fig. 4A). The combined HR + N motif had a more substantial effect, increasing the EC50 by about two-fold (Fig. 4B). The majority of this effect comes from the HR residues in this combined motif (Fig. 4B). Combing all three motifs together into the RGS16 ILS + HR + N mutant increased the EC50 further, from 7 nM (RGS16 wild-type) to 17 nM (Fig. 4C). Surprisingly, when we mutated the RGS16 AsnSac to a serine (the RGS16-S* mutant), the GAP activity of this mutant decreased substantially, with an EC50 an order of magnitude lower (380 nM) than that of RGS16 (Fig. 4D).

4. Discussion

Our results show that compared to the high-activity RGS16, RZ subfamily members RGS17, RGS19, and RGS20 have lower GAP activity towards Gαo. We posit that this low activity is the result of four RZ-specific motifs (Fig. 2), which function in an identical way across the RZ subfamily. Substitution of the corresponding residues in RGS16 with the RGS17 ILS, HR and N motifs reduced the GAP activity of RGS16, validating the suggested disruptive nature of these motifs. The disruptive effect of the ILS motif correlates with high flexibility in this region. The HR and N motifs function jointly by partially disrupting a polar/electrostatic network with the helical domain of the Gα subunit — a Gα domain that was recently shown to play an important role in interactions with other RGS subfamilies [21,28]. The HR and N motifs reduce GAP activity compared to their RGS16 counterparts, but to a lesser extent than the corresponding KY/KF Disruptor motifs that were previously characterized in the R12 subfamily [21]. Furthermore, the combination of the ILS, HR, and N motifs reduced RGS16 GAP activity more substantially than each motif separately. Importantly, substitution of the AsnSac residue in RGS16 with the S* motif had a more dramatic effect on GAP activity than all other three motifs combined. This quantitative effect is supported by previous studies that mutated the AsnSac residue in RGS4 and RGS16 and showed a substantial impairment of GAP activity towards Gαo and Gαi [25,26,30]. Nevertheless, the GAP activity of wild type RGS17 is higher than the RGS16 N131S mutant (Fig. 4D), suggesting all of the RZ-specificity determining residues combine in a non-additive way to produce this difference in activity.

More generally, because the RGS17 specificity-determining motifs are essentially identical to those of RGS19 and RGS20, we suggest that the four motifs we characterized here function similarly across the entire RZ subfamily. Therefore, these residue-level insights into the specificity determinants of the RZ subfamily enable to study the interactions of individual RZ subfamily...
members with specific Gα subunits by inserting redesigned mutants, such as those presented in this work, into relevant cells and tissues.

**Author contributions**

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

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.08.033.

**Transparency document**

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.08.033.

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Figure S1: The RZ subfamily representative RGS19 shows enhanced flexibility in the $\alpha_{5-6}$ loop compared to the R4 representative RGS4. (A) The 20 NMR models of the RGS19 monomer structure (PDB ID 1CMZ, green ribbon) show enhanced conformational variability in the $\alpha_{5-6}$ loop, which contains the ILS motif. (B) The 30 NMR models of monomeric RGS4 (PDB ID 1EZY, blue ribbon) show that the $\alpha_{5-6}$ loop has a fixed rigid structure among all models. The structures are shown as ribbon diagram with the $\alpha_{5-6}$ loop colored red.
Figure S2: The RGS17 ILS motif has higher thermal B-factors than the corresponding residues in representative R4 subfamily crystal structures. Plotted are normalized thermal B-factors for the RGS domains from the following structures (with PDB IDs): RGS16-Gαi1 (2IK8), RGS16-Gαo (3C7K), RGS4-Gαi1 (1AGR), RGS17 (1ZV4), and RGS16 (3C7L).