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

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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 $G\alpha_i$ 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 $G\alpha_o$. 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.

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 [1–4], and are therefore considered promising therapeutic targets [5–7]. RGS proteins inactivate Gα subunits by allosterically accelerating their intrinsic GTPase activity. In particular, the ~120 residue "RGS domain", which is present in all RGS proteins, underlies their function as GTPase Activating Proteins (GAPs) [1]. A notable RGS subfamily is the RZ subfamily, whose members RGS17, RGS19, and RGS20 were identified as GAPs for the $G\alpha_i$ subfamily [8–11]. This subfamily has been implicated in central processes such as cell proliferation, neuronal regulation, and tumorigenesis [12–15]. However, which RZ subfamily members mediate particular signaling cascades and what are the molecular determinants of their specific interactions with Gα subunits are not well understood.

Previous reports of the RZ subfamily GAP activity towards members of the $G\alpha_i$ subfamily vary [11]. Earlier studies showed that RGS20 is selective for $G\alpha_z$ and suggested it has minimal GAP activity towards other $G\alpha_i$ subfamily members such as $G\alpha_{i2}$ and $G\alpha_o$ [16,17]. In contrast, Wang et al. showed that RGS19 and RGS20 had similarly high GAP activity towards $G\alpha_{i1}$ as compared to RGS4 [18], a representative high-activity RGS from the R4 subfamily [19]. On the other hand, Mao et al. measured higher GAP activities of RGS17 than RGS20 towards various members of the $G\alpha_i$ subfamily, while both RZ subfamily members had lower GAP activities than RGS4 [10]. More recently, RGS17 was shown to exhibit low GAP activity towards $G\alpha_o$, compared to the high-activity RGS4 and RGS16 [20]. It is therefore unclear what is the relative GAP activity of each RZ subfamily member, and how these activities are governed at the amino acid level.

In previous studies, we classified RGS residues that determine interactions with Gα subunits into three groups, based on their mechanistic role in interactions with Gα subunits. The first group, "Significant & Conserved" (S&C) residues, contains residues that contribute favorably and similarly to interactions with Gα subunits across all high-activity RGS domains [20,21]. The second group, "Modulatory" residues, contains residues that contribute to interactions with Gα subunits only in some high-activity RGS domains and were proposed to fine-tune G protein recognition [20]. The third group, "Disruptor" residues, was recently identified in the

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RGS R12 subfamily and shown to function as negative design elements; namely these residues attenuate RGS activity for particular Gα subunits by reducing GAP activity in a specific fashion [21]. With regard to RGS17, previous work identified seven residues that determine its interaction with $G\alpha_o$, and showed that substitution of all seven residues led to a gain of function, increasing the activity of RGS17 to that of the high-activity RGS16 [20]. However, the mechanistic role of these putative RGS17 "specificity-determining" residues was not investigated, nor how 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 $G\alpha_o$, 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 GTPase 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): $G\alpha_{i1}$—human RGS16 (2IK8) and RGS17 (1ZV4) [22]. Missing residues in 2IK8 ($G\alpha_{i1}$ 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 $G\alpha_o$ was a gift from Vadim Arshavsky (Duke University). All RGS domains were expressed in the pLIC-SGC1 vector (Addgene). All proteins were expressed as N-terminally His_6-tagged fusion proteins and purified from transformed *Escherichia coli* BL-21 (DE3) cells as described previously [21]. Dose-response analyses of RGS GAP activity were performed as in Ref. [21], using 500 nM $G\alpha_o$ pre-loaded with 1 μM [γ-^{32}P]-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 $G\alpha_o$ than the high-activity RGS16

We measured the GAP activities of the three RZ subfamily members (RGS17, RGS19, and RGS20) towards the representative $G\alpha_i$ subfamily member $G\alpha_o$, 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 RGS 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 $G\alpha_i$ and $G\alpha_o$ (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-$G\alpha_i$ complex by superimposing the RGS17 monomer, as a structural representative of the RZ subfamily, onto the RGS16 coordinates in the RGS16-$G\alpha_i$ 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 GTPase domains, with the former in the periphery of the interface, and the latter

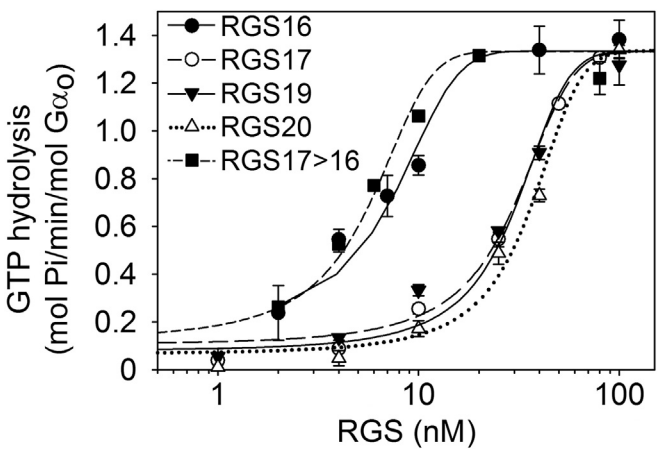

Fig. 1. RGS RZ subfamily members RGS17, 19, and 20 show similarly low GAP activity towards $G\alpha_o$, compared to the high-activity RGS16. Dose-response analysis of the GAP activity of the following RGS domains toward $G\alpha_o$: RGS16, RGS17, RGS19, RGS20, and the RGS17 > 16 mutant (where all seven previously-identified RGS17 specificity-determining residues were substituted with the corresponding RGS16 residues: I143S + L144E + S145A + S150N + H183E + R184K + N192K). EC_{50} values are: RGS16 = 7 ± 1 nM, RGS17 = 30 ± 2 nM, RGS19 = 29 ± 3 nM, RGS20 = 36 ± 2 nM, RGS17 > 16 = 5 ± 1 nM, and were calculated using three-parameter sigmoidal curves in SigmaPlot 10.0. Data are means ± SEM of experiments performed in triplicate, representative of three or more independent biological replicates for each RGS.

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