High-resolution structure of RGS17 suggests a role for Ca\(^{2+}\) in promoting the GTPase-activating protein activity by RZ subfamily members

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Regulator of G protein signaling (RGS) proteins are negative regulators of G protein–coupled receptor (GPCR) signaling through their ability to act as GTPase-activating proteins (GAPs) for activated G\(\alpha\) subunits. Members of the RZ subfamily of RGS proteins bind to activated G\(\alpha_i\), G\(\alpha_o\), and G\(\alpha_{13}\) proteins in the nervous system and thereby inhibit downstream pathways, including those involved in Ca\(^{2+}\)-dependent signaling. In contrast to other RGS proteins, little is known about RZ subfamily structure and regulation. Herein, we present the 1.5-Å crystal structure of RGS17, the most complete and highest-resolution structure of an RZ subfamily member to date. RGS17 co-crystallized with Ca\(^{2+}\) bound to conserved positions on the predicted G\(\alpha\)-binding surface of the protein. Using NMR chemical shift perturbations, we confirmed that Ca\(^{2+}\) binds in solution to the same site. Furthermore, RGS17 had greater than 55-fold higher affinity for Ca\(^{2+}\) than for Mg\(^{2+}\). Finally, we found that Ca\(^{2+}\) promotes interactions between RGS17 and activated G\(\alpha\) and decreases the \(K_{m,G}\) for GTP hydrolysis, potentially by altering the binding mechanism between these proteins. Taken together, these findings suggest that Ca\(^{2+}\) positively regulates RGS17, which may represent a general mechanism by which increased Ca\(^{2+}\) concentration promotes the GAP activity of the RZ subfamily, leading to RZ-mediated inhibition of Ca\(^{2+}\) signaling.

G protein–coupled receptors (GPCRs)\(^{3}\) regulate many physiological processes in response to the binding of an extracellular ligand, leading to the activation of diverse pathways, including vision and hormonal signaling. The intracellular response to ligand binding is mediated by heterotrimeric G proteins, which consist of G\(\alpha\) and G\(\beta\gamma\) subunits. In the inactive state, G\(\alpha\) is bound to GDP and stably associated with G\(\beta\gamma\). The activated GPCR is a guanine nucleotide exchange factor for G\(\alpha\), catalyzing the exchange of GDP for GTP. G\(\alpha\)-GTP and G\(\beta\gamma\) dissociate from one another and bind downstream effector enzymes to stimulate second messenger production (1). G\(\alpha\) subunits are deactivated upon GTP hydrolysis and reassociate with G\(\beta\gamma\), terminating downstream signaling. However, the intrinsic rate of GTP hydrolysis for many G\(\alpha\) subunits is too slow to be physiologically relevant. This discrepancy led to the discovery of the regulator of G protein signaling (RGS) proteins, which are GTPase-activating proteins (GAPs) for some classes of G\(\alpha\) subunits (2–5). RGS proteins increase the rate of GTP hydrolysis by binding to the switch regions of G\(\alpha\)-GTP and stabilizing the transition-state conformation (6).

Over 20 RGS proteins have been identified and are subdivided into four families (R4, R7, R12, and RZ) based on sequence conservation and G\(\alpha\) subunit preference. All RGS proteins share the highly conserved RGS homology (RH) domain that is required for G\(\alpha\)-GTP binding and hydrolysis (7, 8). This domain is composed of terminal and bundle subdomains, which consist of the N and C termini and a four-helix bundle, respectively. Additional subfamily-specific domains or regions flanking the RH domain contribute to the subcellular localization of the RGS protein, the specificity and affinity for the G\(\alpha\)-GTP subunit, and/or participate in protein–protein interactions (9). The majority of RGS proteins act as GAPs for G\(\alpha_{16}\), G\(\alpha_{20}\), and G\(\alpha_{i3}\) subunits. However, some RGS proteins have narrower substrate specificity, such as RGS2, which preferentially binds G\(\alpha_{q}\)-GTP, or broader substrate specificity, like the RZ subfamily, which can also bind G\(\alpha_{i3}\)-GTP (9, 10).

The RZ subfamily, comprised of RGS17 (RGSZ2), RGS19 (GAIP, G\(\alpha_{13}\)-interacting protein), and RGS20 (RGSZ1 or RetRGS) are among the simplest RGS proteins. They consist of the RH domain flanked by short N and C termini. The N terminus quantum coherence; CSP, chemical shift perturbation; CI, confidence interval; GST, glutathione S-transferase; ITC, isothermal titration calorimetry; \(K_{d}\), dissociation constant; GTP\(\gamma\)S, guanosine 5’-3-O-(thio)triphosphate.
contains a highly conserved cysteine string that can be palmitoylated, as has been reported for RGS19, potentially allowing these proteins to be localized to the plasma membrane (11, 12). The defining feature of the RZ subfamily is the residue implicated in GAP activity. The R4, R7, and R12 subfamilies use a highly conserved asparagine residue (e.g., Asn-128 in RGS4) to engage the switch regions of Gα with over 50-fold higher affinity than Mg2+. Whether or how this serine recapitulates the interactions of the canonical asparagine residue in promoting GAP activity is not understood.

RGS17 is a potent regulator of cAMP and Ca2+ signaling and is expressed at highest levels in the cerebellum (10, 15, 16). It was first identified as a GAP for Gαo and subsequently also found to interact with Gαd and Gαt–t. Thus, RGS17 increases cAMP accumulation by inhibition of GαoGDP. RGS17 has also been reported to negatively regulate Ca2+ through a Gαq-dependent mechanism (15). The preferred substrate of RGS17 is therefore likely dependent upon the cellular context (12). More recently, RGS17 has emerged as a promising therapeutic target in several cancers. RGS17 expression in nonneuronal tissues is linked to lung (17, 18) and breast cancer (19) as well as hepatocellular carcinoma (20). The mechanistic role of RGS17 in these diseases is attributed to the increased cAMP driven by the inhibition of Gαi signaling pathways (17). However, how RGS17 itself is regulated is unclear.

Herein, we present the 1.53-Å structure of RGS17, the most complete structure of an RZ family member and the highest-resolution RGS structure reported to date. RGS17 crystallized as a dimer with strong electron density observed for two Ca2+ atoms in each chain. One site is formed by the side chain of Glu-109 and the backbone of Tyr-106, which are situated on the face of RGS17 that is predicted to bind the switch regions of GoGTP. This places the site in close proximity to Ser-150, the RZ subfamily residue thought to be responsible for GAP activity and which is analogous to RGS4 residue Asn-128 (13, 14). We confirmed that RGS17 binds Ca2+ at the Tyr-106–Glu-109 site in solution and at a concentration significantly less than that used to obtain the crystal structure. Furthermore, we found that Ca2+ binding to RGS17 with over 50-fold higher affinity than Mg2+. Finally, we found that although Ca2+ has no effect on the stability of RGS17 itself, Ca2+ enhances the ability of RGS17 to bind GαqGTP. These findings suggest that Ca2+ is a novel potentiator of RGS17 activity. As RGS17 is known to regulate Ca2+ signaling (10, 15, 16), this could represent a mechanism of feedback inhibition wherein elevated Ca2+ promotes RGS17–Gα interactions to terminate Gα signaling.

**Results**

**Crystal structure of RGS17 bound to calcium**

RGS17 (residues 70–206) crystallized as an asymmetric dimer with the final structure refined to 1.53-Å spacings (Table 1 and Fig. 1). The dimer is a crystallographic dimer as RGS17 is monomeric in solution as determined by both size-exclusion chromatography and nuclear magnetic resonance (NMR). Continuous electron density was observed for residues 72–203 in Chain A and 72–206 in Chain X, but the two chains were otherwise essentially identical (r.m.s.d. of 0.11 Å for Ca atoms in residues 72–203). Overall, the RGS17 RH domain is similar to that of other RGS proteins (7) and a previously determined

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**Table 1**

**RGS17 data collection and refinement statistics**

Values in parentheses correspond to the highest resolution data shell. CC1/2 is the Pearson correlation coefficient.

| Data collection       |                       |
|-----------------------|-----------------------|
| X-ray source          | LS-CAT 21-ID-D        |
| Wavelength (Å)        | 1.068                 |
| Dmin (Å)              | 30.0–1.53 (1.56–1.53) |
| Space group           | P12_1                 |
| Cell dimensions       |                       |
| a, b, c (Å)           | 39.6, 59.3, 65.2      |
| α, β, γ (°)           | 90, 100.6, 90         |
| Total reflections     | 190,012               |
| Unique reflections    | 39,255                |
| Rfree (%)             | 13.2 (66.5)           |
| Completeness (%)      | 87.5 (41.4)           |
| I/σ                  | 10.52 (6.95)          |
| Redundancy            | 4.8 (1.7)             |
| CC1/2                 | (0.58)                |

**Refinement**

| Refinement resolution (Å) | 30.0–1.53 (1.56–1.53) |
| Total reflections used    | 37,292                |
| r.m.s.d. bond lengths (Å) | 0.009                 |
| r.m.s.d. bond angles (°)  | 1.31                  |
| Estimated coordinate error (Å) | 0.103               |
| Ramachandran plot         |                       |
| Favor (%)                 | 98.5                  |
| Outliers (%)              | 0.0                   |
| Rwork/Rfree (%)           | 20.3/22.5             |
| Protein atoms             | 2,275                 |
| Ligand atoms              | 39                    |
| Solvent molecules         | 293                   |
| Average B-factor (Å2)     | 18.0                  |
| Protein                  | 16.7                  |
| Ligand                    | 37.2                  |
| Solvent                   | 28.2                  |
| Wilson B factor (Å2)      | 12.9                  |
| PDB code                  | 6AM3                  |

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**Figure 1. Crystal structure of RGS17 in complex with Ca2+.** RGS17 crystallized as a dimer but is monomeric in solution as determined by size-exclusion chromatography and NMR. Chain X is shown color-ramped from blue at the N terminus to red at the C terminus. Chain A is shown in gray, and the r.m.s.d. between chains is 0.6 Å. Strong electron density was observed for four Ca2+ ions (black spheres). The 10σ [Fo − Fc]omit maps for the Ca2+ ions are shown as blue cages, and electron density for these ions persists beyond 20σ.
structure of RGS17 (Protein Data Bank (PDB) code 1ZV4 (21)). However, the two RGS17 structures differ from one another by 1.08 Å (Cα atoms of residues 72–204), which is unexpected given their identical sequence. This arises primarily due to differences within the terminal subdomain and the orientation between the terminal and bundle subdomains of the RH domain (Fig. 2). Superimposing the bundle subdomains from this structure and 1ZV4 confirms that the bundle subdomains are highly similar, with an r.m.s.d. of 0.52 Å for the Cα atoms of residues 106–186. In contrast, superimposing the RGS17 structures over their terminal subdomains results in an r.m.s.d. of 0.86 Å for the Cα atoms of residues 72–105 and 187–206. In addition, the terminal subdomain of our RGS17 structure is rotated by ~17° with respect to its orientation in the 1ZV4 structure (Fig. 2) (22).

Each chain of RGS17 in the crystal structure also contained strong electron density consistent with bound Ca2+, which was present in the crystallization conditions as 200 mM CaCl2. Each RGS17 chain coordinated two Ca2+ atoms, but only one site is conserved between the two chains. This site, located in the loop connecting helices α3 and α4, coordinates Ca2+ through the side chain of Glu-109 and the backbone carbonyl oxygen of Tyr-106 with the rest of the Ca2+ coordination sites occupied by five water molecules (Fig. 3, A and B). These Ca2+ are tightly bound as electron density is still observed when the |$F_{o}$| − |$F_{c}$| omit map is contoured to 20 σ (Fig. 1). This Ca2+ ion is on the same face of RGS17 that is predicted to interact with the switch regions of GoGTP and is ~11 Å from Ser-150 (3, 23, 24). Thus, the Ca2+ ion is positioned to potentially modulate the interactions between RGS17 and its cognate GoGTP.

Each RGS17 chain in the crystal structure also contains strong electron density for a second Ca2+ ion. However, these secondary sites are not conserved between the two chains. In Chain A, a Ca2+ ion is coordinated by the side chain of Glu-148 and the carbonyl oxygen of Ile-143 with the remaining coordination sites occupied by three water molecules (Fig. 3B). This site is located in the loop connecting helices α5 and α6, on the predicted Gα-binding surface, and in close proximity to both Ser-150 (~10 Å) and the Ca2+ site formed by Tyr-106 and Glu-109 (Fig. 3B). In Chain X, a Ca2+ is coordinated by the backbone carbonyl oxygen of Gln-124, located in the loop connecting helices α4 and α5, along with three water molecules that complete the coordination of Ca2+ (Fig. 3C). Although this Ca2+ is near the dimer interface and the N and C termini of Chain A, it does not contribute to the dimer interface or interact with any residues in Chain A. For both of these sites (Ile-143–Glu-148 and Gln124), the Ca2+ ions are tightly bound as electron density is visible when the |$F_{o}$| − |$F_{c}$| omit map is contoured to 20 σ.

**RGS17 binds Ca2+ and Mg2+ in solution**

RGS17 and other RGS proteins have not previously been reported to directly bind Ca2+ or other divalent cations. One possible explanation for the presence of bound Ca2+ in our RGS17 structure is that it is an artifact due to the presence of 200 mM CaCl2 in the crystallization conditions. To establish whether RGS17 binds Ca2+ in solution, we used NMR to monitor changes in the 1H–15N 2D HSQC spectrum of RGS17 in the presence of 15 mM CaCl2. In this spectrum, protons that are
directly bound to $^{15}$N are detected, providing a “fingerprint” of the amide backbone of the protein. To assign each peak in the $^1$H-$^1$H HSQC spectra, the protein backbone and Cβ carbons were first assigned using $^{13}$C- and $^{15}$N-labeled RGS17 and standard triple-resonance experiments.

If RGS17 binds Ca$^{2+}$ in solution, Ca$^{2+}$ will alter the local chemical environment, causing perturbations in the chemical shifts of the amide proton and/or nitrogen of residues in close proximity to the bound ion. In contrast, residues that are distant from the site of binding and/or that are unaffected by Ca$^{2+}$ addition will not have significantly perturbed chemical shifts relative to the $^1$H-$^1$H NMR spectrum of the protein alone. Addition of 15 mM CaCl$_2$ to $^{15}$N-labeled RGS17 caused significant chemical shift perturbations (CSPs) for residues Ser-107 (0.178 ppm), Glu-108 (0.160 ppm), Glu-109 (0.065 ppm), and Asn-110 (0.096 ppm) ($>2$ S.D. from average; 0.017 ppm) for Ser-108 (0.024 ppm) (Fig. 5), similar to the CSP observed in the presence of Ca$^{2+}$. Ser-107 (0.022 ppm) and Glu-109 (0.072 ppm) also displayed substantial shifts, consistent with Mg$^{2+}$ binding to this site in RGS17. 15 mM MgCl$_2$ addition induced CSPs in a second group of residues, including Val-149 (0.031 ppm), Tyr-140 (0.024 ppm), and Ser-150 (>0.023 ppm), located in helix α5 and the α5–α6 loop (Fig. 5). These residues are adjacent to or in close proximity to Ile-143 and Glu-148, which coordinate a second Ca$^{2+}$ site in Chain A of the crystal structure (Figs. 1 and 3) and which display altered chemical shifts in the presence of Ca$^{2+}$ (Fig. 4).

These results demonstrate that both Ca$^{2+}$ and Mg$^{2+}$ bind directly to RGS17 in solution through a site formed by the backbone carbonyl of Tyr-106 and the side chain of Glu-109. This site is also the only Ca$^{2+}$-binding site observed in both chains in the crystal structure. Although secondary Ca$^{2+}$-binding sites are observed within each chain of the crystal structure, the only other site in which significant CSPs could be reliably observed was the site formed by the backbone carbonyl of Ile-143 and the side chain of Glu-148. This second site may be less favorable for cation binding given that only one RGS17 chain has Ca$^{2+}$ bound in this site in the crystal structure. Finally, we also found no evidence that the binding of divalent cations impacts the thermal stability of RGS17 as determined by differential scanning fluorimetry (Fig. S1 and Table S1).

**RGS17 has higher affinity for Ca$^{2+}$ than Mg$^{2+}$**

Having demonstrated that RGS17 can bind both Ca$^{2+}$ and Mg$^{2+}$ at multiple sites, we next determined the affinity of RGS17 for these ions. Using NMR, we observed concentration-dependent changes in CSP for both ions, and the magnitude of CSPs induced by Ca$^{2+}$ binding was greater than that observed upon Mg$^{2+}$ binding (Fig. 6). We determined the dissociation constant ($K_{d}$) for Ca$^{2+}$ and Mg$^{2+}$ for each residue that displayed a significant CSP upon the addition of the divalent cat-

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**Figure 4. RGS17 binds Ca$^{2+}$ in solution.** A, $^1$H-$^1$H 2D HSQC spectra of RGS17 alone (black) or upon addition of 15 mM CaCl$_2$ (red). B, structure of RGS17 where residues that display chemical shift perturbations greater than 0.15 ppm are shown as ball-and-stick. Ca$^{2+}$ is shown as a black sphere. C, graph of CSPs for all the residues that could be assigned in the $^1$H-$^1$N 2D HSQC spectra for RGS17.
The CSP was fit as a function of ion concentration to a one-site binding model. For the binding site created by Tyr-106 and Glu-109, the dissociation constants of Ca\textsuperscript{2+} ranged from 98 μM (35–197, 95% CI) for Glu-109 to 181 μM (98–296, 95% CI) for Glu-108. Averaging the K\textsubscript{D,avg} values obtained for residues 107–110 in the presence of Ca\textsuperscript{2+} yielded a K\textsubscript{D,avg} of 132 μM. A similar K\textsubscript{D,avg} of 91 μM was observed for the binding site near Ile-143 and Glu-148 through the analysis of Ser-145, which had a K\textsubscript{D} of 86 μM (31–172, 95% CI), and Val-149, which had a K\textsubscript{D} of 95 μM (37–182, 95% CI).

Titration of RGS17 with increasing concentrations of Mg\textsuperscript{2+} revealed that RGS17 had much lower affinity for this metal at both sites examined. For the binding site formed by Tyr-106 and Glu-109, the K\textsubscript{D,avg} was 34 ± 23 mM with individual residues exhibiting K\textsubscript{D} values ranging from 21 mM (13–38, 95% CI) for Asn-110 to 68 mM (45–104, 95% CI) for Glu-108. The binding site formed by Ile-143 and Glu-148 also displayed lower affinity for Mg\textsuperscript{2+} where the K\textsubscript{D,avg} was 20 ± 4 mM. Ser-145 alone had a K\textsubscript{D} of 17 mM (12–25, 95% CI), and Val-149 had a K\textsubscript{D} of 22 mM (20–25, 95% CI). Thus, RGS17 has 257- and 219-fold higher affinity for Ca\textsuperscript{2+} than Mg\textsuperscript{2+} at the sites formed by Tyr-106 and Glu-109 and Ile-143 and Glu-148, respectively.

RGS17 binds cations with higher affinity than RGS4 or RGS2

The RH domain is highly conserved across the RGS family, including the residues we have shown to bind cations in RGS17. Additionally, as some of the interactions are mediated by the peptide backbone, it is possible that all RGS proteins bind cations in solution. We used NMR spectroscopy to determine whether RGS4 and RGS2 bind Ca\textsuperscript{2+} and/or Mg\textsuperscript{2+} in solution. RGS4 and RGS2 share 40 and 38% identity with RGS17 across the RH domain and are well-characterized with respect to their structure and regulation (7) (Fig. 7 and Fig. S2).

The spectrum of RGS4 has been fully assigned (25), allowing us to identify amino acids that show CSPs upon the addition of divalent cation. Two cation-binding sites were identified on RGS17, one formed by Tyr-106 and Glu-109 and a secondary site formed by Ile-143 and Glu-148. Residues adjacent to these binding sites that displayed CSPs > 2 S.D. greater than the average CSP were used to calculate the K\textsubscript{D,avg} for each site by fitting the CSP as a function of ion concentration to a one-site binding model. A, CSP as a function of increasing Ca\textsuperscript{2+} concentration. The K\textsubscript{D,avg} for residues Ser-107, Glu-108, Glu-109, and Asn-110 is 132 ± 35 μM, and the K\textsubscript{D,avg} for residues Ser-145 and Val-149 is 91 ± 6 μM. B, CSP as a function of increasing Mg\textsuperscript{2+} concentration. The K\textsubscript{D,avg} for residues Ser-107, Glu-108, Glu-109, and Asn-110 is 34 ± 23 mM, and the K\textsubscript{D,avg} for residues Ser-145 and Val-149 is 20 ± 4 mM.

**Figure 5.** RGS17 binds Mg\textsuperscript{2+} in solution. A, ¹H-¹⁵N 2D HSQC spectra of RGS17 alone (black) or upon addition of 15 mM MgCl\textsubscript{2} (red). B, structure of RGS17 where residues with CSPs greater than 0.15 ppm are shown in ball-and-stick in red. In contrast to spectra obtained in the presence of CaCl\textsubscript{2}, MgCl\textsubscript{2} induces CSPs in two regions of RGS17. The locations of the Mg\textsuperscript{2+} ions (black spheres) are modeled based on the location of Ca\textsuperscript{2+} atoms observed in the RGS17–Ca\textsuperscript{2+} crystal structure. C, graph of CSPs for all the residues that could be assigned in the ¹H-¹⁵N 2D HSQC spectra for RGS17.

**Figure 6.** RGS17 binds Ca\textsuperscript{2+} with higher affinity than Mg\textsuperscript{2+}. The K\textsubscript{D,avg} for Ca\textsuperscript{2+} and Mg\textsuperscript{2+} binding to RGS17 was determined for each amino acid that displayed a significant CSP upon the addition of divalent cation. Two cation-binding sites were identified on RGS17, one formed by Tyr-106 and Glu-109 and a secondary site formed by Ile-143 and Glu-148. Residues adjacent to these binding sites that displayed CSPs > 2 S.D. greater than the average CSP were used to calculate the K\textsubscript{D,avg} for each site by fitting the CSP as a function of ion concentration to a one-site binding model. A, CSP as a function of increasing Ca\textsuperscript{2+} concentration. The K\textsubscript{D,avg} for residues Ser-107, Glu-108, Glu-109, and Asn-110 is 132 ± 35 μM, and the K\textsubscript{D,avg} for residues Ser-145 and Val-149 is 91 ± 6 μM. B, CSP as a function of increasing Mg\textsuperscript{2+} concentration. The K\textsubscript{D,avg} for residues Ser-107, Glu-108, Glu-109, and Asn-110 is 34 ± 23 mM, and the K\textsubscript{D,avg} for residues Ser-145 and Val-149 is 20 ± 4 mM.
RGS17, the 1H-15N HSQC spectrum of RGS4 was obtained alone and in the presence of a 40-fold molar excess of CaCl$_2$ or MgCl$_2$. Similar to RGS17, two regions of RGS4 showed significant CSPs (>2 S.D. from average; 0.019 ppm in the presence of Ca$^{2+}$ or 0.006 ppm in the presence of Mg$^{2+}$). The first region corresponds to the cation-binding site formed by Tyr-106 and Glu-108 in RGS17 (Tyr-84 and Glu-86 in RGS4). In the presence of Ca$^{2+}$, RGS4 residues Tyr-84 (0.056 ppm), Ser-85 (0.206 ppm), Glu-86 (0.291 ppm), Glu-87 (0.043 ppm), Asn-88 (0.081 ppm), and Ile-89 (0.115 ppm) all show significant CSPs. The second site is equivalent to the site formed by RGS17 Ile-143 and Glu-148 (RGS4 Val-121 and Glu-126) with significant CSPs observed for residues Ala-123 (0.019 ppm), Lys-125 (0.066 ppm), and Val-127 (0.056 ppm). The $K_{D_{av},avg}$ for these Ca$^{2+}$ binding sites was calculated as a function of ion concentration using a one-site binding model. For the site encompassing residues Tyr-84, Ser-85, Glu-86, Glu-87, Asn-88, and Ile-89, the calculated $K_{D_{av},avg}$ was 9.6 ± 3 mM, whereas the $K_{D_{av},avg}$ for the site associated with residues Ala-123, Lys-125, and Val-127 was 6.1 ± 1.6 mM. Thus, RGS4 has ~10–15-fold lower affinity for Mg$^{2+}$ relative to Ca$^{2+}$ and binds both cations more weakly than RGS17.

The ability of RGS2 to bind Ca$^{2+}$ and Mg$^{2+}$ in solution was then tested. The NMR spectrum of RGS2 has not been assigned, and thus the 1H-15N HSQC spectra of RGS2 were compared with spectra obtained with increasing concentrations of CaCl$_2$ or MgCl$_2$ (Fig. S2). Addition of a 20- or 250-fold molar excess of CaCl$_2$ to 15N-labeled RGS2 caused few changes in the 1H-15N HSQC spectra (Fig. S2A). This is consistent with RGS2 binding weakly to Ca$^{2+}$ in solution. Similarity, addition of up to a 500-fold molar excess of MgCl$_2$ to $^{15}$N-labeled RGS2 had essentially no impact on the 1H-15N HSQC spectra (Fig. S2B), demonstrating that RGS2 binds Mg$^{2+}$ very weakly under these experimental conditions.

**Calcium enhances interactions between RGS17 and activated G$\alpha$**

The binding sites for Ca$^{2+}$ and Mg$^{2+}$ on RGS17 are located on the same face of the protein predicted to interact with activated G$\alpha$ subunits (6, 14, 15) and in close proximity...
to the putative GAP residue, Ser-150. To determine whether Ca$^{2+}$ impacts the ability of RGS17 to bind activated G$\alpha_\text{o}$, an AlphaScreen protein interaction assay (26) was used to quantify association. Briefly, RGS17 was biotinylated and immobilized on streptavidin-coated donor beads, whereas activated GST-tagged G$\alpha_\text{o}$, GDP was immobilized on anti-GST acceptor beads. RGS17–G$\alpha_\text{o}$ binding was quantified as an increase in bead-based fluorescence. RGS17 binds activated G$\alpha_\text{o}$ in a concentration-dependent manner, with saturation occurring at $\sim 5$ nM G$\alpha_\text{o}$-GDP-AlF$_4$ (Fig. 8). Addition of 5 mM CaCl$_2$ had no impact on the apparent affinity of the interaction but did cause a significant increase ($p < 0.01$) in the amount of bead-based fluorescence relative to the control, suggesting increased binding between RGS17 and G$\alpha_\text{o}$-GDP-AlF$_4$ (Fig. 8, A and B). In contrast, addition of 10 mM EDTA, which preferentially chelates free Ca$^{2+}$, had no significant effect on the amount of bead-based fluorescence, suggesting that the increased signal depends upon the presence of Ca$^{2+}$. This increase in binding in the presence of Ca$^{2+}$ was only observed between RGS17 and G$\alpha_\text{o}$-GDP-AlF$_4$ as Ca$^{2+}$ had no impact on the interaction between G$\alpha_\text{o}$-GDP-AlF$_4$ and the closely related RGS4 protein (Fig. 8, C and D).

Mg$^{2+}$ binds the same sites on RGS17 as Ca$^{2+}$ in solution and thus could also impact the RGS17–G$\alpha_\text{o}$-GDP-AlF$_4$ interaction. However, because Mg$^{2+}$ is required to stabilize the transition state of G$\alpha_\text{o}$-GDP-AlF$_4$, its role in the protein–protein interaction cannot be directly assessed. To indirectly probe the role of Mg$^{2+}$ in binding, 10 mM EDTA was used to chelate free Mg$^{2+}$ in the binding reaction. Under these conditions, bead-based fluorescence decreased relative to the control. However, it is not possible to determine how much of the decrease is due to perturbation of the RGS17–G$\alpha_\text{o}$-GDP-AlF$_4$ interaction versus destabilization of activated G$\alpha_\text{o}$. It is possible that Ca$^{2+}$ alters the affinity between RGS17 and G$\alpha_\text{o}$-GDP; however, the affinity of RGS17 for inactive G subunits is too low to accurately determine.

### Ca$^{2+}$ increases the GTPase activity of RGS17

Ca$^{2+}$ selectively binds to RGS17 and increases its interactions with G$\alpha_\text{o}$-GDP-AlF$_4$. Therefore, Ca$^{2+}$ may alter the abil-
Table 2

|           | $k_{cat}$ | $K_m$ | $V_{max}$ | $k_{cat}/K_m$ |
|-----------|-----------|-------|-----------|--------------|
| RGS17 + Go | 57.71 ± 2.94 | 1.49 ± 0.26 | 57.1 ± 2.94 | 38.7 ± 6.89 |
| RGS17 + Go + CaCl2 | 51.93 ± 2.18 | 0.56 ± 0.14 | 51.9 ± 2.12 | 92.9 ± 23.2 |
| RGS4 + Go | 61.78 ± 2.18 | 0.39 ± 0.08 | 61.8 ± 2.18 | 160.4 ± 34 |
| RGS4 + Go + CaCl2 | 60.38 ± 2.06 | 0.34 ± 0.07 | 60.4 ± 2.06 | 176 ± 38.1 |

Steady-state kinetic parameters for RGS17 and RGS4 GTPase activity

The GT-Pase activity of RGS17 and RGS4 was measured using a rate-altered Go, variant (26, 45) in the presence or absence of saturating CaCl2 (100 μM). Data represents the mean of four independent experiments ± S.E. (*p = 0.018).

Calcium alters the binding mechanism between RGS17 and activated Go

Isothermal titration calorimetry (ITC) was utilized to further characterize the RGS17–Go, binding interaction in the presence and absence of saturating concentrations of Ca2+ (Fig. 10). We observed no significant difference in the $K_d$ of the RGS17–Go, interaction in the presence (596 ± 257 nM) or absence (611 ± 128.5 nM) of Ca2+. The stoichiometry of the Go,–RGS17 complex was also found to be unchanged in the presence or absence of Ca2+. However, the binding enthalpy for the interaction in the presence and absence of Ca2+ was found to be significantly different at −2.76 ± 0.74 and −7.33 ± 0.72 kcal/mol respectively. The presence of Ca2+ in the experiment increased the enthalpy by 4.57 kcal/mol, which is consistent with Ca2+ changing the binding mechanism between RGS17 and Go,.

Discussion

RGS proteins are critical negative regulators of GPCR signaling through their ability to act as GAPs for activated Go subunits. The RZ subfamily inhibits GPCR signaling in the nervous system where they inactivate Go, Go, and Go, thereby preventing G-dependent inhibition of adenylyl cyclase (9, 10). RGS17, a member of the RZ family, has also been reported to negatively regulate Ca2+–signaling, suggesting that it also contributes to the regulation of Go,–dependent processes (15). RGS17 has emerged as adriver in cancer, in particular lung and breast cancers (17–19), where its overexpression results in increased inhibition of Go,–dependent signaling, thereby increasing CAMP and PKA activity (17). However, how RGS17 itself is regulated is not well characterized. In this study, we report a high-resolution crystal structure of RGS17 revealing that this protein binds Ca2+ and provide support for a mechanism wherein Ca2+–binding to RGS17 enhances its interactions with activated Go.

RGS17 crystallized as a dimer, and both chains in the asymmetric unit preserve the canonical RH fold (Fig. 1) (7). Despite being identical in sequence to a previously published RGS17 structure (PDB code 1ZV4 (21)), our RGS17 structure differs from 1ZV4 in two major ways. First, the terminal subdomain is rotated with respect to the bundle subdomain by ~17° relative to their orientation in the 1ZV4 structure (Fig. 2) (21, 22). This suggests that the terminal and bundle subdomains may be flexible in their relative orientation, which could potentially facilitate binding to activated Go subunits. Second, our structure of RGS17 shows strong electron density for four well-resolved Ca2+ ions, with each chain in the asymmetric unit binding two Ca2+ ions (Fig. 1). One Ca2+ site, formed by the backbone carbonyl of Tyr-106 and the side chain of Glu-109, is observed in both chains of the crystal structure (Fig. 3, A and B). We confirmed that RGS17 binds Ca2+ or Mg2+ in solution by monitoring C3P in the NMR spectra of 15N-labeled RGS17 in the presence or absence of CaCl2 or MgCl2 (Figs. 4 and 5). Each RGS17 chain in the crystal structure also bound a second Ca2+...
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Ca\(^{2+}\) and Mg\(^{2+}\) bind the same face of RGS17 that is predicted to interact with the switch regions of activated G\(\alpha\) sub-units to promote GTP hydrolysis (Fig. S3). Comparison of the RGS17–Ca\(^{2+}\) structure with RGS–G\(\alpha\) complexes (6, 30–33) would suggest that Ca\(^{2+}\) could inhibit G\(\alpha\) binding. To test this hypothesis, an AlphaScreen protein interaction assay was used to measure binding between RGS17 and G\(\alpha\). Addition of Ca\(^{2+}\) increased the observed binding between RGS17 and G\(\alpha\), whereas addition of EGTA had no effect on the interaction (Fig. 8). The increased interactions between RGS17 and G\(\alpha\) may also contribute to changes in GTP hydrolysis. We found that Ca\(^{2+}\) decreased the \(K_{D,\text{avg}}\) for RGS17-stimulated GTP hydrolysis on G\(\alpha_o\) but had no impact on RGS4-stimulated GTP hydrolysis (Fig. 9 and Table 2). Finally, isothermal titration calorimetry revealed a significant difference in the binding enthalpy for the RGS17–G\(\alpha_o\) interaction brought about by the presence of Ca\(^{2+}\) (Fig. 10). Ca\(^{2+}\) was found to increase the binding enthalpy by 4.57 kcal/mol, demonstrating that Ca\(^{2+}\) changes the binding mechanism for RGS17–G\(\alpha_o\). Polar interactions and/or conformational changes are typically reflected in the enthalpy of binding (28, 29). Thus, Ca\(^{2+}\) may alter binding by decreasing the polar contacts between RGS17 and G\(\alpha_o\) and/or altering the local structure at the protein interfaces.

Taken together, our data suggest Ca\(^{2+}\) regulates RGS17 activity. Although Ca\(^{2+}\) binding directly to other RGS proteins has not been reported previously, there is precedent for Ca\(^{2+}\)-mediated regulation of RGS activity. For example, Ca\(^{2+}\)/calmodulin is a known regulator of some RZ and R4 subfamily members. These RGS proteins are inhibited when bound to phosphatidylinositol-3,4,5-triphosphate at the cell membrane, and this inhibition is relieved upon binding Ca\(^{2+}\)/calmodulin.
Thus, these proteins are activated following G_{q}-dependent \( \text{Ca}^{2+} \) release, providing a feedback mechanism to inhibit further G_{q} signaling (34, 35). Additional studies will be required to determine whether \( \text{Ca}^{2+} \), alone or in combination with calmodulin, may be a general regulator of RZ RGS function.

**Experimental procedures**

**RGS17 expression and purification**

A construct for the RGS17 protein, encoding residues 70–206, was obtained from Addgene (catalog number 39141) and was a gift from Nicola Burgess-Brown (The Structural Genomics Consortium). RGS17 was purified largely as described previously (21). Briefly, RGS17 was expressed in BL21 CodonPlus(DE3)-RIPL cells and grown at 37 °C and 275–300 rpm until an OD_{600} of 2.0 was reached. Protein production was induced with 0.5–1 mM isopropyl-1-thio-\( \beta \)-D-galactopyranoside, and the culture was incubated for an additional 16 h at 18 °C while shaking at 275–300 rpm. Bacterial cells were then pelleted and resuspended in 50 mM HEPES, 500 mM NaCl, 1 mM \( \beta \)-mercaptoethanol, 10 mM imidazole at pH 8 (Buffer A) at 4 °C. Cells were lysed with lysozyme (1 mg ml\(^{-1}\) cell pellet), and DNase I (~2 mg) was added. Lysate was then subjected to multiple freeze–thaw cycles in liquid N\(_{2}\), and the soluble lysate fraction was separated by centrifugation at 100,000 \( \times \) g. His-tagged RGS17 was then separated from the supernatant using an \( \text{AKTA FPLC} \) (GE Healthcare) equipped with an immobilized metal affinity chromatography column (Ni-Sepharose 6 Fast Flow, GE Healthcare). Eluted fractions containing RGS were then treated with His-tagged tobacco etch virus protease at a molar ratio of 1:20 tobacco etch virus:RGS and dialyzed overnight at 4 °C against 5 liters of Buffer A to cleave the His_{6} tag. Samples were again subjected to the immobilized metal affinity chromatography column, and the flow-through was collected. Size-exclusion chromatography (10 mM borate, 500 mM NaCl, and 1 mM DTT at pH 7.0) was then used to obtain 99+\% pure RGS17 as determined by SDS-PAGE.

Isotope-labeled (\( ^{15}\text{N} \) and \( ^{13}\text{C},^{15}\text{N} \)) RGS17 was purified largely as above with the exception that when culture OD_{600} reached 1.5, cells were pelleted at 3,500 \( \times \) g at 4 °C for 15 min and resuspended in an equal volume of M9 minimal medium supplemented with 2 g liter\(^{-1}\) D-[\( ^{13}\text{C}_{6}\)]glucose and 1 g liter\(^{-1}\) \( ^{15}\text{NH}_{4}\)Cl for \( ^{13}\text{C},^{15}\text{N} \)-labeled sample or 1 g liter\(^{-1}\) \( ^{15}\text{NH}_{4}\)Cl for \( ^{15}\text{N} \)-labeled sample. Isotope-labeled samples were concentrated to >1 mM in 20 mM K\(_{2}\)HPO\(_{4}\) buffer with 100 mM NaCl, 0.5 mM \( \beta \)-mercaptoethanol, and 2 mM Na_{2}SO\(_{4}\) at pH 7.6. Prior to all NMR experiments, RGS17 was exhaustively dialyzed against 55 mM HEPES, 110 mM NaCl, and 0.55 mM \( \beta \)-mercaptoethanol at pH 7.6 to remove phosphate buffer.

**Crystallization of RGS17**

Initial crystallization conditions were determined using commercially available screens. Hanging-drop vapor-diffusion experiments were set up in 96-well polystyrene microplates (Greiner Bio-one) using a Mosquito LCP crystallization robot (TTP Labtech) at 25 °C. The drops contained an equal volume (200 nl) of RGS17 (20.6 mg/ml in 10 mM borate, pH 7.0, 500 mM NaCl, and 1 mM DTT) and reservoir solution (0.2 m CaCl\(_{2}\) and 20% PEG 3350, pH 5.1) of the PEG/Ion Screen ( Hampton Research) suspended over 50 μl of reservoir. Conditions were optimized in-house using 24-well SuperClear Pregereated plates (Crystalgen) with drops containing equal volumes (0.5 μl) of RGS17 (16 mg/ml) and precipitant solution. Final crystals were obtained from reservoirs containing 0.2 m CaCl\(_{2}\), 22% (w/v) PEG 3350, and 0.1 m MES, pH 6.0, at 12 °C using streak seeding. Crystals were harvested in 0.2 m calcium chloride dihydrate, 40% PEG 3350, and 0.1 m MES, pH 6.0, and frozen on nylon loops in liquid N\(_{2}\).

**Data collection, processing, and refinement**

Diffraction data were collected at 100 K using an Eiger detector at the Advanced Photon Source at LS-CAT 21-ID-D. HKL2000 was used to integrate and scale the data, and Phaser in CCP4 (37) was used to solve the structure by molecular replacement with the prior structure of RGS17 (PDB code 1ZV4 (21)) as a starting model. The structure was built by manual model building in Coot (38) alternating with translation/libration/screw (TLS) refinement in REFMAC5 (39). The correctness of the structure was assessed using MolProbity (40). Structure figures were generated using PyMOL 1.8.6.2 (Schrodinger, LLC).

**Nuclear magnetic resonance**

The following triple-resonance experiments were performed at 25 °C using a 600-MHz Varian INOVA NMR spectrometer equipped with a triple-resonance gradient probe to assign RGS17-RH backbone (and Cβ) chemical shifts: HNCACB, CBCA(CO)NH, HNCO, and HN(CA)CO. Data were processed and analyzed using NMRPipe (41) and CCPNAnalysis (42), respectively. 260 μM RGS17 in 50 mM HEPES, 100 mM NaCl, and 0.5 mM \( \beta \)-mercaptoethanol, pH 7.6, in 10% D_{2}O was incubated with or without the indicated concentration of CaCl\(_{2}\) or MgCl\(_{2}\). \( ^{1}H\)-\( ^{15}N \) HSQC spectra were then acquired at 25 °C using either a 500-MHz Bruker Avance II or a 600-MHz Varian INOVA NMR spectrometer, each equipped with a triple-resonance gradient probe.

NMR experiments with RGS4 and RGS2 were carried out as described for RSG17. \( ^{1}H\)-\( ^{15}N \) HSQC spectra of 375 μM RGS4 in 50 mM HEPES, 100 mM NaCl, and 10 mM \( \beta \)-mercaptoethanol at pH 7.6 and 10% D_{2}O or 300 μM RGS2 in 50 mM HEPES, 100 mM NaCl, and 11 mM \( \beta \)-mercaptoethanol at pH 7.6 in 10% D_{2}O were acquired followed by addition of increasing concentrations of CaCl\(_{2}\) or MgCl\(_{2}\). Chemical shift assignments for RGS4 were confirmed using prior assignments (25), whereas the spectrum of RGS2 has not been assigned.

CSPs between control and metal-treated samples were calculated by measuring the distance between the centers of the peaks using the following equation,

\[
\text{CSP} = \sqrt{(\Delta \delta_{H})^2 + (0.101 \times \Delta \delta_{N})^2}
\]

where \( \Delta \delta_{H} \) and \( \Delta \delta_{N} \) are the difference in chemical shift in the absence and presence of cation in the indicated dimension.

\( K_{D} \) values for individual residues with CSP ≥2 S.D. from the mean were obtained using GraphPad Prism 7 by fitting CSP titration data to a one-site binding model with correction for ligand depletion as follows,
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$$\text{CSP} = \text{CSP}_{\text{max}}(\left\{[M^{2+}] + 2[P] + K_{D}\right\} - \sqrt{\left\{[M^{2+}] + 2[P] + K_{D}\right\}^2 - 4 \times 2[P][M^{2+}]/(2 \times 2[P])} \quad \text{(Eq. 2)}$$

where CSP$_{\text{max}}$ is the maximum CSP value observed, [M$^{2+}$] is the concentration of metal ion, [P] is the concentration of protein, and $K_{D}$ is the dissociation constant. Note that 2 × [P] was used to account for the two metal-binding sites on each molecule of RGS17. $K_{D}$ values obtained for residues were averaged to determine the $K_{D}$avg of metal-binding sites. Identical residues were used for Mg$^{2+}$ $K_{D}$ determinations.

**Differential scanning fluorimetry**

The impact of Ca$^{2+}$ or Mg$^{2+}$ on the thermal stability of RGS17 was determined by measuring the change in fluorescence of SYPRO Orange (Molecular Probes, Eugene, OR) due to protein denaturation as a function of temperature (43). RGS17 was exchanged into buffer containing 10 mM borate, pH 9.0, 500 mM NaCl, and 1 mM DTT. RGS17 at a final concentration of 0.9 mg/ml was incubated with SYPRO Orange dye and increasing concentrations of CaCl$_2$ or MgCl$_2$ (0.2 mM–200 mM) for 30 min on ice in a final volume of 20 μl. Samples were assayed in triplicate in a MicroAmp Optical 96-well plate, sealed with MicroAmp Optical Adhesive Film (Applied Biosystems), and centrifuged for 1 min. Differential scanning fluorimetry assays were carried out on a ViiA7 qPCR instrument (Thermo Fisher). The change in fluorescence was measured at 0.2 °C intervals from 25 to 95 °C. The $T_m$ was calculated by fitting the increase in fluorescence as a function of temperature to a Boltzmann sigmoid (GraphPad Prism 7.0). Data represent the mean of at least three experiments performed in triplicate ± S.E.

**AlphaScreen method for the RGS17–Gαo interaction**

RGS17–Gαo binding was assessed as described previously (44). Biotinylated RGS17 was conjugated to streptavidin donor beads in ALPHA Buffer (20 mM HEPES, 100 mM NaCl, 1% BSA, and 1% Lubrol, pH 8). GST–Gαo purified as described previously (27), was conjugated to anti-GST acceptor beads in ALPHA Buffer. The Gαo mixture was supplemented with 5 μM AlCl$_3$, 5 μM MgCl$_2$, and 5 mM NaF (AMP) and 2.5 mM GDP. Final concentrations were 100 nM RGS17, the indicated concentration of Gαo, and 15 ng/μl for each bead. The assay was incubated for 1 h at ambient temperature, and then fluorescence was measured using a PerkinElmer Life Sciences Envision plate reader. Wells lacking AMF represented negative control and were normalized to 0%, and wells containing RGS17 in ALPHA Buffer alone were normalized to 100%. Data analysis was performed using GraphPad Prism 7.

**GTP hydrolysis assays**

RGS-stimulated GTP hydrolysis was measured using a rate-altered Gαi variant, Gαi1 R178M/A326S, which was expressed and purified as described previously (27, 45). GTase activity was measured using the GTase-Glo assay (Promega, Madison, WI) as described (46) but with some modifications. Briefly, 1 μM Gαi1, R178M/A326S and 1 μM RGS17 or RGS4 were incubated in GTase/GAP reaction buffer (50 mM Tris-HCl, pH 7.5, 50 μM NaCl, and 10 μM MgCl$_2$) in the presence or absence of 100 μM CaCl$_2$ for 30 min. The reaction was initiated by the addition of 2.5 μM GTP (10-μl final volume) and allowed to proceed for increasing time points before the addition of an equal volume of GTase-Glo reagent and a 30-min incubation. The GTase-Glo reagent uses a nucleoside-diphosphate kinase and ADP to convert remaining GTP to ATP and GDP. The GTase-Glo activity inversely correlates with ATP production and is measured with a detection reagent containing a luciferase/luciferin mixture. 20 μl of this detection reagent was added to 20 μl of the reaction mixture and incubated in the dark for 10 min. Assay plates (Corning, 3572; 384-well) were read on a Synergy 2 plate reader (BioTek, Winoski, VT) in luminescence mode. Time points at 0.5, 1, 2, 3, 4, 5, and 10 min post-GTP addition were taken. Wells without GTP were used in normalizations of values for data analysis to represent 100% GTP hydrolysis, whereas wells without Gαi1 R178M/A326S represented 0% GTP hydrolysis. Data analysis was performed using GraphPad Prism 7.

**Isothermal titration calorimetry**

Gαo was concentrated in ITC sample buffer (50 mM HEPES, pH 8.0, 200 mM NaCl, 2 mM β-mercaptoethanol, and 50 μM GTPγS) to 3 μM. RGS17 was concentrated in ITC sample buffer to 60 and 53.4 μM for samples without and with Ca$^{2+}$, respectively. 45 mM Ca$^{2+}$ was included in Gαo and RGS17 samples to achieve saturation as determined by 1H-15N 2D HSQC NMR. RGS17 injections of 14 μl over 20 total injections were added to the ITC cell containing 1.4 ml of Gαo to reach a molar ratio (RGS17:Gαo) of 4.5. An injection duration time of 14 s and a spacing of 240 s were set for each injection. All experiments were conducted on a GE MicroCal VP-ITC System (GE Healthcare) at 25 °C. Heats of dilution were determined by averaging the heat evolved by the last five injections and subtracted from the raw data. The values for affinity, stoichiometry, and change in enthalpy were then determined using the Origin software provided by the manufacturer. Replicates for each run were further analyzed using GraphPad Prism 7.

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