Selective Inhibition of α₁A-Adrenergic Receptor Signaling by RGS2 Association with the Receptor Third Intracellular Loop*

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Regulators of G-protein signaling (RGS) proteins act directly on Go subunits to increase the rate of GTP hydrolysis and to terminate signaling. However, the mechanisms involved in determining their specificities of action in cells remain unclear. Recent evidence has raised the possibility that RGS proteins may interact directly with G-protein-coupled receptors to modulate their activity. By using biochemical, fluorescent imaging, and functional approaches, we found that RGS2 binds directly and selectively to the third intracellular loop of the α₁A-adrenergic receptor (AR) in vitro, and is recruited by the unstimulated α₁A-AR to the plasma membrane in cells to inhibit receptor and Gαq/11 signaling. This interaction was specific, because RGS2 did not interact with the highly homologous α₁B- or α₁D-ARs, and the closely related RGS16 did not interact with any α₁-ARs. The N terminus of RGS2 was required for association with α₁-ARs and inhibition of signaling, and amino acids Lys219, Ser220, and Arg238 within the α₁A-AR i3 loop were found to be essential for this interaction. These findings demonstrate that certain RGS proteins can directly interact with preferred G-protein-coupled receptors to modulate their signaling with a high degree of specificity.

Signaling through G-protein-coupled receptors (GPCRs) must be tightly regulated in cells to maintain functional specificity. Recent studies have identified a large family of proteins, the Regulators of G-protein Signaling (RGS), which bind GαGTP to increase the rate of GTP hydrolysis and rapidly terminate responses (1–4). Because more than 30 RGS proteins have been identified and divided into six subfamilies (5–7), it was initially postulated that the specificity of RGS function would be controlled by formation of individual RGS/Gα pairs. However, subsequent studies showed that many RGS proteins, in particular most members of the B/R4 subfamily (RGS1–5, -8, -13, -16, and -18), could nonselectively bind and inhibit Gαq and Gα11 function in reconstituted systems (5, 7), suggesting other factors may regulate their functional specificity.

Recent studies suggest that RGS proteins may interact with GPCRs to regulate their function (8–14). In fact, a plant GPCR containing an RGS domain embedded within its C-terminal tail has been identified (15), indicating that RGS proteins and GPCRs are physically and functionally coupled in plants, and suggests that they may have evolved as separate genes in higher organisms allowing for the formation of specific paired complexes. Consistent with this idea, we showed recently (16) that purified RGS2 binds directly to the third intracellular (i3) loops of the Gαq-coupled M1, M3, and M5 muscarinic acetylcholine receptors but not the Gαq-coupled M2 and M4. Binding of RGS2 to the M1 receptor was shown to recruit recombinant RGS2 and to regulate the activity of endogenous Gαq/11 in cell membranes (16). Taken together, these data support the idea that RGS proteins form direct functional complexes with preferred GPCRs in order to modulate the signaling properties of these receptors and their linked G-proteins (17, 18). However, previous work has not yet demonstrated direct RGS interactions with full-length receptors in intact systems, the specific amino acids within a receptor that bind the RGS protein, and the general applicability of this phenomenon across GPCRs.

α₁-Adrenergic receptors (ARs) mediate responses to norepinephrine (NE) and epinephrine (19). There are three α₁-AR subtypes (α₁A, α₁B, and α₁D) which, like M1 receptors, also activate Gαq and release intracellular Ca²⁺. α₁-ARs play an essential role in the regulation of vascular tone and blood pressure and are an important target for treatment of hypertension (19, 20). Most interestingly, a recent report showed that RGS2 knock-out mice have a strongly hypertensive phenotype, with increased mean arterial pressure, renovascular abnormalities, and persistent constriction of the peripheral vasculature (21, 22). Thus, we examined the possibility that RGS2 might interact specifically with α₁-AR subtypes to modulate their signaling. Unexpectedly, we found that RGS2 binds specifically to the i3 loop of the α₁A-AR but not the closely related α₁B- or α₁D-AR. This interaction requires specific amino acids in the α₁A-AR i3 loop and results in inhibition of agonist-stimulated responses in intact cells. The findings of this study demonstrate that specific RGS proteins interact directly with preferred GPCRs and that this interaction is essential for controlling RGS specificity and function.

EXPERIMENTAL PROCEDURES

Constructs-GST-α₁A-i3, Constructs—α₁A-i3, α₁B-i3, and α₁D-i3 loop constructs were originally cloned into the pET41b vector to encode a fusion protein with an N-terminal GST tag and a C-terminal His tag. Each receptor i3 loop was amplified from corresponding regions of the human full-length receptor as follows: α₁A, amino acids 213–260; α₁B, amino acids 233–280; α₁D, amino acids 283–334. α₁A and α₁B were amplified as BamHI/XhoI fragments, and α₁B was amplified as an EcoRI/XhoI frag-
ment. In order to perform pull-down assays with purified His-tagged RGS proteins, fragments encoding the i3 loops of α1A- and α1B-ARs were subcloned further into the pGEX4T vector to eliminate the His tag. α1A was amplified as a BamHI/BglII fragment, and α1B was amplified as an EcoRI/HindIII fragment and cloned in-frame with an N-terminal GST tag. α1A/i3 Loop Chimeras—α1A/i3 loop chimeras were created by using a nested primer PCR strategy (23) and inserted into the pGEX4T-1 vector. The α1A/i3 chimera encodes amino acids 214–239 of α1A fused to amino acids 259–280 of α1B. The α1A/i3 chimera encodes amino acids 234–258 of α1B fused to amino acids 239–260 of α1A. The junction of these chimeras occurs between conserved lysine-asparagine residues.

The α1A/i3-1 constructs encode single, double, or triple amino acid substitutions of the α1A/i3 sequence with α1B placed into the GST–α1A/i3 chimeric template. Substitutions were made as denoted below, where the amino acid number corresponds to position of the residue in the context of the full-length α1A–AR receptor sequence: α1A/i3-1, E214T/S215T; α1A/i3-2, R216K/G217N; α1A/i3-3, R219E/S220A; α1A/i3-4, L222V/K223M; α1A/i3-5, T224K/D225E/K226M; α1A/i3-6, D228N; α1A/i3-7, E230K/Q231E/V232L; α1A/i3-8, R235S. The mutations demonstrating the largest loss in RGS2 binding capacity were then inserted into the full-length α1A–AR in the pDT vector using the Quikchange site-directed mutagenesis kit (Strategene) for use in functional assays examining RGS2 inhibition of [3H]InsP formation.

RGS Constructs—RGS2-HA, RGS6-HA, N2/RGS6-HA, RGS2-His, RGS6-His, RGS2-GFP, and RGS6-GFP were created as described previously (16). α1-AR Constructs—Full-length α1A–AR i3 loop mutant constructs were created using the Quikchange site-directed mutagenesis kit (Strategene), using HA-α1A-AR in pDoubleTrouble (pDT) as a template. N-terminally HA epitope-tagged human α1A–ARs were produced as described previously (24).

Induction and Purification of GST Fusion Proteins—GST–α1A–AR i3 loop fusion proteins (GST–α1A/i3, GST–α1B/i3, GST–α1A/i3–α1B, GST–α1A/i3–α1B–i3, GST–α1A/i3–1A/i3 chimeras and GST–α1A/i3 mutants) were transformed into BL21(DE3) Escherichia coli and purified as described previously (16).

Cell Cultures and Transfections—Human embryonic kidney (HEK293) cells were propagated in Dulbecco’s modified Eagle’s medium with sodium pyruvate supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a humidified atmosphere with 5% CO2. Confluent plates were subcultured at a ratio of 1:5 for transfection. HEK293 cells were transfected with 3 μg of DNA of each construct for 24 h using Lipofectamine 2000 transfection reagent, and cell lysates were harvested as described previously (16).

Radioligand Binding—Confluent 150-mm plates were washed with PBS and harvested by scraping. Cells were collected by centrifugation, resuspended in PBS, and sonicated for 10 s and centrifuged at 30,000 × g for 5 min at 4 °C. Samples were sonicated for 10 s and centrifuged for 5 min at 10,000 × g. Samples were subjected to anion exchange chromatography to isolate [3H]InsP, which were quantified by scintillation counting. Percent hydrolysis of total myo-[3H]inositol incorporated into [3H]InsPs was counted and expressed as mean ± S.E. Mean values were compared using the one-sample t test, with a p value less than 0.01 considered significant.

RESULTS

RGS2 Selectively Associates with the i3 Loop of the α1A-AR—To determine whether selected B/E4 RGS proteins directly associate with α1A–ARs, we examined the capacity of closely related RGS2 and RGS16 to associate with the i3 loops of all three subtypes (α1A, α1B, and α1D) by using pull-down assays. As shown in Fig. 1, RGS2 was capable of binding to the α1A/i3, but not to the α1B/i3 or α1D/i3, whereas RGS16 did not associate with any of the three α1-AR subtype i3 loops. To identify a specific RGS2 binding domain within the α1A/i3, we created α1A/i3 chimeras encoding amino acids 214–239 of α1A fused to amino acids 259–280 of α1B and α1A/i3 chimeras encoding amino acids 234–258 of α1B fused to amino acids 239–260 of α1A (Fig. 1B). Chimeras were then fused to GST and tested for their capacity to associate with RGS2-His by using pull-down assays. Most interestingly, we found that RGS2 robustly associated with the GST–α1A/i3 chimera (Fig. 1C), indicating that the N-terminal half of the α1A/i3 contains an RGS2-binding motif. In contrast, we found RGS2 interacted very weakly or not at all with the GST–α1B/i3 chimera, suggesting the first 26 amino acids of the i3 loop is predominantly responsible for RGS2 interaction. Therefore, these data indicate that an RGS2-binding motif may be contained within the proximal domain of the α1A/i3.

RGS2 Co-localizes at the Plasma Membrane with α1A/ARs in HEK293 Cells—To determine whether RGS2 associates with α1A–ARs in a cellular context, we co-transfected GFP-tagged RGS proteins with HA-tagged α1A–ARs into HEK293 cells, and we examined their cellular localization by using confocal microscopy. However, to ensure that these effects are not a result of receptor and/or RGS overexpression, we performed radioligand binding experiments using 125I-BE2254 on cells tran-
FIG. 1. RGS2 binds selectively to the \( \alpha_{1A/-3} \)-i3 loop. A, cell lysates from Chinese hamster ovary cells transiently transfected with RGS2-HA or RGS16-HA were incubated with equal amounts (6 \( \mu \)g) of GST-\( \alpha_{1A/-3} \), GST-\( \alpha_{1B/-3} \), GST-\( \alpha_{1A/-3} \), or GST alone bound to glutathione-Sepharose beads. Following centrifugation, recovered beads were washed three times and subjected to Western blot analysis using anti-HA antibody. Results are representative of three individual experiments. B, schematic of GST-tagged \( \alpha_{1A/-3} \), \( \alpha_{1B/-3} \), \( \alpha_{1A/-3} \), and \( \alpha_{1B/-3} \) chimeras. Chimeras were created and isolated as described under “Experimental Procedures.” C, association of \( \alpha_{1A/-3} \)-i3 chimeras with RGS2. Purified RGS2-His (0.5 \( \mu \)g) was incubated with equal amounts of GST-\( \alpha_{1A/-3} \), GST-\( \alpha_{1B/-3} \), GST-\( \alpha_{1A/-3} \), or GST alone bound to glutathione-Sepharose. Western blot analysis was performed using anti-His antibody. Results are representative of three individual experiments.

FIG. 2. RGS2 translocates and co-localizes with \( \alpha_{1A/-3} \)Rs at the plasma membrane in HEK293 cells. A, cellular localization of RGS proteins and \( \alpha_{1A/-3} \) subtypes. HEK293 cells were transiently transfected with plasmid cDNA encoding GFP-tagged RGS2 (upper left), GFP-tagged RGS16 (upper right), HA-tagged \( \alpha_{1A/-3} \)Rs (lower left), or HA-tagged \( \alpha_{1A/-3} \)Rs (lower right). B, HEK293 cells were transiently transfected with plasmid cDNA encoding RGS2-GFP and either HA-tagged \( \alpha_{1A/-3} \)Rs (upper panel) or HA-tagged \( \alpha_{1A/-3} \)Rs (middle panel). HA-tagged \( \alpha_{1A/-3} \)Rs co-expressed with GFP-tagged RGS16 are shown in the lower panel. C, HEK293 cells transiently transfected with RGS2-GFP and HA-\( \alpha_{1A/-3} \)Rs were incubated for 30 min with either 100 \( \mu \)M nifedipine (upper panel), 100 \( \mu \)M prazosin (middle panel), or 10 \( \mu \)M NE (lower panel). Green GFP fluorescence at 488 nm (left), anti-HA rhodamine red fluorescence at 522 nm (middle), and merged images (right) are depicted. Yellow in the merged images indicated overlapping localization. Each image is a representative picture of many cells observed in 2–3 individual transfections.
after the plasma membrane localization of α1A-AR/RGS, although the proteins appeared to be clustered and nonoverlapping in their distribution (Fig. 2C, middle panel). However, stimulation of cells with 10 μM NE for 30 min resulted in marked α1A-AR internalization with an apparent disruption of the α1A-AR-RGS2 complex (Fig. 2C, lower panel). This was also observed following 5 and 15 min of stimulation with 10 μM NE and with 5, 15, and 30 min of stimulation with 1 μM NE (data not shown). Therefore, these data suggest that agonist stimulation results in α1A-AR internalization, which is followed by subsequent RGS2 dissociation from the receptor and redistribution to the cytosol.

RGS2 Selectively Inhibits α1A-AR Functional Responses in HEK293 Cells—We next examined if RGS2 can selectively regulate α1A-AR functional responses, by transiently transfecting α1A-ARs into HEK293 alone and in combination with RGS2 proteins and assaying for NE-stimulated [3H]InsP formation. Both HA- and GFP-tagged RGS2 significantly inhibited [3H]InsP formation by α1A-ARs in response to 100 nM NE by 60.6 ± 3.3 and 61.4 ± 5.3%, respectively (Fig. 3, left). Transfection with pEGFP or pcDNA3.1 vector alone had no effect on α1A-AR signaling. However, HA- and GFP-tagged RGS2 had no significant effect on α1B-AR-stimulated [3H]InsP formation (Fig. 3, right). Combined with our previous findings, these data indicate that RGS2 selectively associates with α1A-ARs at the plasma membrane to facilitate uncoupling of the receptor with G-protein-mediated functional responses.

Effect of RGS2/α1A-AR Association on Agonist Binding Affinity—It is generally accepted that agonists display multiple affinity states for GPCRs, as a result of G-protein binding to GPCR i3 loops. Typically, binding of the G-protein induces low affinity agonist binding interactions with the GPCR, and uncoupling of the G-protein by GTP decreases agonist affinity by >10-fold. In our studies, we find that RGS2 can directly associate with the α1A-i3. We therefore tested whether RGS2 affected agonist affinity for binding to the α1A-AR. To examine this, we harvested membranes from HEK293 cells stably expressing wild-type α1A-ARs, which were then preincubated for 30 min at 4 °C with concentrations of purified RGS2-HA reported previously to be sufficient for maximal association with M1 receptors in isolated Chinese hamster ovary cell membranes (16). After 30 min, the affinity of the nonselective adrenergic receptor agonist NE was determined using 125I-BE2254 competition radioligand binding. As reported in Table I, NE bound with low affinity to α1A-ARs when expressed alone. However, preincubation with 10, 30, or 100 nM RGS2 caused no significant change in affinity, suggesting that RGS2 binding to the α1A-AR does not affect ligand-receptor interactions.

The N Terminus of RGS2 Determines Selectivity of Receptor Association—Previously, we and others have shown the N-terminal domains of RGS proteins are required for promoting association with the Go subunits and with GPCRs (16, 31–33). However, the functional significance of this interaction was not examined in intact cells. Therefore, we examined the capacity of a chimera containing the N-terminal portion of RGS2 fused to the RGS domain and C-terminal portion of RGS16 (N2/RGS16-HA) to associate with and inhibit the signaling of α1A-ARs (Fig. 4A). In pull-down assays, we found RGS2-HA and N2/RGS16-HA selectively associated with α1A-i3 GST-fusion proteins, whereas RGS16-HA did not (Fig. 4A). In HEK293 cells transiently co-transfected with HA-α1A-ARs alone or with RGS constructs, co-expression with RGS2-HA resulted in significant inhibition of α1A-AR maximal response (Fig. 4B). However, co-expression with N2/RGS16-HA caused a decrease in NE-stimulated [3H]InsP formation, whereas co-expression with RGS16-HA caused no significant decrease in the α1A-AR maximal response (Fig. 4B). Thus far, we have determined that RGS2 associates with the α1A-AR through a direct association with the i3 loop.

Identification of Amino Acids within the α1A-i3 Responsible for Binding of RGS2—Thus far, we have determined that RGS2 associates with the α1A-AR through a direct association with the proximal half of the i3 loop. Next, we initiated studies to identify specific amino acids within the α1A-i3 that are responsible for promoting this interaction. In Fig. 1, we showed RGS2 binds the α1AAR but not the α1B-i3 chimera. By comparing the sequence homology between the α1A-i3 and α1B-i3 (Fig. 5A), nonhomologous amino acids in the proximal half were identified between the receptors to target for substitution mutation, and used to create a series of α1A-i3 mutants in which specific amino acids in the α1A-i3 were replaced with the corresponding amino acids in the α1B-i3, using the α1AAR as a template. A total of eight mutant α1A-i3 constructs were created using PCR, each involving between 1 and 3 amino acid substitutions (Fig. 5A). Constructs were then expressed as GST fusion proteins and were used for pull-down assays with purified RGS2-His. Of the eight α1A-i3 mutants examined, the constructs containing a Lys212-Arg238 to Glu-Ala (A/B3), Leu222-Lys233 to Val-Met (A/B4), and single Arg238 to Ser (A/B8)
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**Fig. 4.** The N terminus of RGS2 confers selectivity for interactions with α1A-ARs. A, schematics of RGS2-HA, RGS16-HA, and chimeric N2/RGS16-HA (left) and their corresponding associations with α1A-i3 (right). N2/RGS16-HA was created by fusing the RGS2 N terminus with the RGS domain and C-terminal tail of RGS16. Purified RGS-HA proteins (0.5 μg) were incubated with equal amounts of GST-α1A-i3 bound to glutathione-Sepharose. Immunoblot analysis was performed using anti-HA antibody. Results are representative of three individual experiments. B, HEK293 cells were transiently transfected with HA-α1A-ARs alone or HA-α1A-ARs co-transfected with RGS2-HA, RGS16-HA, or N2/RGS16-HA. After 24 h post-transfection, cells were preincubated with 1 μCi of myo-[3H]inositol for 24 h and stimulated with 100 μM NE for 1 h. The values for each experiment are represented as percent hydrolysis, with 100% hydrolysis equal to the level of NE-stimulated hydrolysis in HEK293 cells expressing HA-tagged α1A-ARs alone. Mean basal [3H]InsPs and maximal stimulation levels in counts/min were 1149.1 ± 147 and 6801 ± 330, respectively. The data are expressed as mean ± S.E. of data from four separate experiments performed in duplicate or triplicate and were statistically compared using a one-sample t test (*p < 0.01). Immunoblot analysis of HEK293 cell lysates transfected with HA-tagged RGS proteins alone are shown below. Immunoblotting was performed using anti-HA antibody as described under “Experimental Procedures.” This figure is representative of three independent experiments.

mutation demonstrated the most severe loss in RGS2 binding (Fig. 5B).

To determine whether the loss of binding correlated with a decrease in RGS2 inhibition of α1A-AR functional responses, the Lys-Ser/Glu-Ala, Leu-Lys/Val-Met, and Arg/Ser mutations were introduced into full-length α1A-ARs via site-directed mutagenesis, using the full-length α1A-AR in pDT vector as a template. Full-length α1A-ARs carrying the Lys-Ser/Glu-Ala, Leu-Lys/Val-Met, and Arg/Ser mutations were then transiently transfected into HEK293 alone or in combination with RGS2-HA and assayed for NE-stimulated [3H]InsP formation. In comparison to full-length α1A-ARs (B_max = 321 ± 32 fmol/mg protein), each of the three α1A-AR mutants demonstrated relatively equal (B_max values, Lys-Ser/Glu-Ala = 401 ± 15, Arg/Ser = 300 ± 24 fmol/mg protein) or higher (B_max, Leu-Lys/Val-Met = 710 ± 45 fmol/mg protein) binding site densities. As shown in Fig. 6, RGS2 caused a significant decrease in the efficacy of NE for stimulating α1A-AR-mediated phosphatidylinositol hydrolysis (Fig. 6A). However, α1A-ARs containing either the Lys-Ser/Glu-Ala (Fig. 6B) or Arg/Ser mutations (Fig. 6C) were rendered insensitive to RGS2, indicating that these mutations abrogated the direct association between RGS2 and α1A-ARs. However, α1A-ARs containing the Leu-Lys/Val-Met mutation remained susceptible to RGS2 inhibition of functional responses (Fig. 6D) indicating that in the context of the full-length receptor in a cellular environment, these amino acids alone are not required for RGS2 association with receptor. Taken together, these data suggest that RGS2 associates with the α1A-AR through a direct interaction within the proximal half of the α1A-i3 and that three amino acids within this domain (Lys219, Ser220, and Arg238) appear to be critical for this interaction.

**DISCUSSION**

Many RGS proteins, particularly those of the B/R4 family, show little selectivity in inhibiting G-protein signaling when assayed as purified proteins in reconstituted systems, but they appear to have a high degree of specificity in intact cells (5). Recent reports (8–11,16) suggest this may be due to the capacity of GPCRs to recruit RGS proteins to the plasma membrane and thus regulate the specificity of RGS function, although these studies provide only indirect evidence in support of this hypothesis. Our recent work (16) demonstrates that RGS2 binds directly and selectively to the intracellular third loop of the M1 muscarinic receptor to modulate receptor and Gq/11 signaling in cell membranes. However, this and other previous work has not demonstrated direct RGS interactions with full-length receptors in intact systems, the specific amino acids within a receptor that binds RGS proteins, or the general applicability of this phenomenon across GPCRs. In this study, we demonstrate that RGS2 directly associates with α1A-ARs through an interaction between the N-terminal domain of RGS2 and three specific amino acids in the proximal half of the α1A-i3. This, and other previous work, indicates that in the context of the full-length receptor in a cellular environment, these amino acids alone are not required for RGS2 association with receptor.
Our findings show that RGS2 is selectively recruited to the plasma membrane by the unstimulated α1A-AR to regulate receptor and G-protein signaling. These findings are consistent with our previous findings with M1 muscarinic receptors (16) and those of others (10) showing specific membrane recruitment of certain G-proteins by unstimulated receptors. Taken together, these findings suggest that receptors and RGS proteins can form preferred functional pairs and predict a model where GPCR and RGS are prebound prior to signal initiation. By forming a complex with a specific GPCR at the plasma membrane, the RGS is positioned to modulate the linked G-protein upon agonist activation. In this way, the receptor determines RGS protein/G-protein coupling and functional specificity. Consistent with this model, we found that RGS16 is capable of blocking receptor-mediated Gq/11 signaling only when it contained the N terminus of RGS2 and is capable of binding α1A-AR. Further studies will be needed to confirm these models.

Based on a number of recent studies, it is now apparent that RGS proteins play a prominent role in regulating GPCR function in the cardiovascular system (37). Both protein and mRNA for GRS members are detectable in a variety of cardiac (38–42) and vascular tissues (43, 44), where they appear to modulate GPCR functional responses (42, 43, 45, 46). In a recent report, RGS2 knock-out mice demonstrate a profound hypertensive phenotype and significant alterations in cardiovascular function and remodeling (21, 22). Preliminary evidence also implicates other RGS proteins in the development of cardiovascular disease, including stroke (47), cardiac hypertrophy (48), and congestive heart failure (42, 49). The α1A-AR subtype is known to contribute strongly to the regulation of blood pressure (50) and vascular remodeling (51, 52). Therefore, the hypertensive phenotype observed in RGS2 knock-out mice may be due to an increased activity of the α1A-AR in the peripheral vasculature. Thus, based on the results of this and previous studies, RGS proteins appear to be attractive new therapeutic targets for development of novel pharmaceutical agents (37, 53).

This study presents evidence that RGS2 binds directly to α1A-ARs to modulate their function. This interaction occurs with a high degree of specificity through identified domains within the receptor and the RGS protein. Thus, we propose that RGS proteins form stable, functional complexes with preferred GPCRs to selectively modulate the signaling functions of those receptors and linked G-proteins. Such direct interactions are likely to play significant roles in the still poorly understood specificity of RGS actions in vivo.

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