Identification of RGS2 and Type V Adenyllyl Cyclase Interaction Sites*

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The production of cAMP is controlled on many levels, notably at the level of cAMP synthesis by the enzyme adenyllyl cyclase. We have recently identified a new regulator of adenyllyl cyclase activity, RGS2, which decreases cAMP accumulation when overexpressed in HEK293 cells and inhibits the in vitro activity of types III, V, and VI adenyllyl cyclase. In addition, RGS2 blocks adenylyl cyclase-stimulating antibodies that lead to elevated cAMP levels in olfactory neurons. Here we examine the nature of the interaction between RGS2 and type V adenyl cyclase. In HEK293 cells expressing type V adenyl cyclase, RGS2 inhibited the activity of recombinant type V adenyl cyclase, and to bind adenyl cyclase. Further mutational analysis indicated that the C terminus of RGS2, not the RGS domain is required for inhibition of adenyl cyclase. Alanine scanning of the N-terminal amino acids of RGS2 identified three residues responsible for the inhibitory function of RGS2. Furthermore, we show that RGS2 interacts directly with the C3 domain but not the C2 domain of type V adenyl cyclase and that the inhibition by RGS2 is independent of inhibition by Gaq. These results provide clear evidence for functional effects of RGS2 on adenyl cyclase activity that adds a new dimension to an intricate signaling network.

Heterotrimeric G protein-mediated receptor signaling pathways are pivotal parts of the intricate and diverse biological processes dictating cellular function. G proteins transduce signals to a variety of effectors including adenyl cyclase (AC) (1). The hormone-sensitive AC system is a typical archtype of G protein-mediated signal transduction. Appropriate agonist-bound, heptahelical receptors activate Ga in catalyzing the exchange of GDP for GTP. The GTP-bound subunit of Ga in turn activates AC, increasing the rate of synthesis of cyclic AMP from ATP (1, 2). All isoforms of mammalian AC are

stimulated by the heterotrimeric G protein Ga. Many other regulatory influences including RGS (regulators of G protein signaling) proteins have crucial effects on various AC isofoms (3). These enzymes thus serve critical roles as integrators of diverse inputs.

RGS2 is a 211-amino acid protein that like other members of this family mediates its GAP activity via its core domain (4–6). Experiments using recombinant RGS2 and membranes prepared from insect cells (SF9) expressing different AC isofoms indicate that RGS2 suppresses the activity of AC III, the predominant isotype in the olfactory system, and the cardiac isoforms, V and VI (3). RGS2 has also been shown to decrease CAMP accumulation in bTC3 insulinoma cells (7). When added to purified recombinant type V AC cytoplasmic domains, recombinant RGS2 decreases CAMP production stimulated by either Gaq or forskolin. The structural basis for the inhibitory effect of RGS2 remains unknown, although it is likely a direct effect. In vivo, odorant-elicited CAMP stimulation resulted in the opening of cyclic nucleotide gated channels. The microinjection of RGS2 antibody into olfactory neurons dramatically augments the currents observed in whole cell voltage clamp recordings of odorant-stimulated olfactory neurons. These results indicate that the level of RGS2 in an olfactory neuron may regulate the responsiveness of that neuron to olfactory stimulants (3). This study provided evidence for a novel role of RGS proteins as direct regulators of AC activity.

The mechanisms for RGS2 regulation are as yet unclear (8). Phosphorylation of RGS2 by protein kinase C decreases its capacity to negatively regulate phospholipase C activation (9). Signal-induced redistribution of RGS2 function (10) and have important effects on cellular signaling. RGS2 shares with RGS4 and RGS16 a conserved N-terminal domain that is necessary and sufficient for plasma membrane targeting (10). Until recently, most reports of the intracellular localization of members of the RGS family of proteins have found them to be associated with the membrane fraction or distributed between the membrane and cytosolic fractions (11, 12); however, it becomes self-evident from the present study that the localization of RGS2 proteins has found to be associated with the membrane fraction. Thus, the RGS2 localization of RGS2 may be in fact controlled in ways not yet appreciated.

The list of non-G protein-binding partners for RGS proteins continues to grow (8, 16). It remains to be determined whether interactions of RGS2 with Gaq, Gaq, AC, and coat protein (β-COP) can explain the putative roles for RGS2 in control of behavior, T cell function, and synaptic development in hippocampal CA1 neurons as revealed by RGS2-deficient mice.

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‡ The abbreviations used are: AC, adenyl cyclase; β2AR, β2-adrenergic receptor; GTPγS, guanosine 5'-O-(β-thiotriphosphate); HA, hemagglutinin; Ni-NTA, nickel-nitrilotriacetic acid; GST, glutathione S-transferase.
(17). We describe herein a direct interaction between RGS2 and the C1 domain of type V AC. In addition, we report the nature of the AC-binding site on RGS2.

EXPERIMENTAL PROCEDURES

Materials—The anti-HA (hemagglutinin A) and Ni-NTA-horseradish peroxidase conjugate antibodies were purchased from Roche Molecular Biochemicals and Qiagen, respectively. Anti-Gαs, anti-GαQ, and anti-GST antibodies were purchased from Santa Cruz Biotechnology. The anti-RGS2 antiserum was generated in rabbits against a C-terminal peptide (PQITTEFPHAT) coupled to keyhole limpet hemocyanin followed by affinity purification using immunizing peptide. Goat anti-rabbit IgG horseradish peroxidase conjugate was obtained from Bio-Rad, and sheep anti-mouse IgG horseradish peroxidase was from Amersham Biosciences. FuGENE 6 transfection reagent was purchased from Roche Molecular Biochemicals and Qiagen, respectively. Anti-Gαs antibodies were purchased from Santa Cruz Biotechnology. The expression vector for Gβ′/γ′-HCCT (22) into the mammalian expression vector, pcDNA3, using the restriction enzymes

FIG. 1. Representation of truncated RGS2 constructs. Wt, wild type.

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transfected using FuGENE 6 transfection reagent following the manufacturer’s protocol with constructs expressing either β3-adrenergic receptor (0.5 μg/well) or GαQL227L (0.5 μg/well) along with RGS2 (1.0 μg/well) and type V AC (0.2 μg/well). The vector pcDNA3 (Invitrogen) was used to normalize all of the DNA concentrations to 1.7 μg/well. After 36 h of transfection, the cells were starved overnight in medium containing 1% fetal bovine serum, followed by starvation in medium devoid of serum for 2 h.

To measure cAMP accumulation in cells transfected with GαQL227L or corresponding control DNA constructs, the cells were treated with 1 mm 3-isobutyl-1-methylxanthine for 15 min and subsequently harvested in 250 μl of hypotonic lysis buffer (50 mm Tris, pH 7.5, 4 mm EDTA, plus protease inhibitors). A portion of the cell lysate (50 μl) was used for Western blotting to monitor protein expression, and the remaining lysate was boiled at 100 °C for 10 min to remove cellular debris. The supernatant was used to measure the total cAMP accumulation by cAMP enzyme immunoassay detection. For β3-adrenergic receptor activation and corresponding controls, the cells were treated with isoproterenol (0.1 μM) for 15 min before subsequent harvesting, lysis, and cAMP detection. The data are represented as the means ± S.E., and statistical significance is determined by Student’s test (p < 0.05 is considered significant).

Western Blotting—The cell lysates were boiled for 5 min in Laemmli sample buffer and sonicated. The proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The protein expression levels were determined by immunoblotting using anti-HA antibody. Immunoreactive protein bands were detected by a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection (Amersham Biosciences, Arlington Heights, IL). All GST fusion proteins were efficiently expressed in E. coli. The N-terminal (VC1) or C-terminal (VC2) hexahistidine tag and associated GST tag on each fusion protein was efficiently recognized by affinity chromatography.

Purification of Recombinant Proteins—RGS2 polypeptide chain fragments digested with BspHI and BamHI were inserted in-frame with an N-terminal hexahistidine tag in pQE60 using NolI and BamHI. The purified protein fractions were dialyzed in buffer containing 10 mm Hepes, pH 7.4, 10 mm β-mercaptoethanol, 10% glycerol, 200 mm NaCl, pooled, concentrated, and stored at −70 °C. Type V AC domains were expressed with an N-terminal (VC3) or C-terminal (VC1) hexahistidine tag and purified as previously described (25). GST-VC1 was purified using glutathione affinity resin. BL21(DE3) cells containing the GST-VC1 expression plasmid were grown at 30 °C in T7 medium until the A600 reached 1.0. Synthesis of GST-VC3 was induced with 0.5 mm isopropyl-β-D-thiogalactoside, and incubation continued for 4 h at 30 °C. The cells were harvested and resuspended in lysis buffer containing 50 mm Tris, pH 7.7, 1 mm EDTA, 2 mm dithiothreitol, 120 mm NaCl, and protease inhibitors prior to incubation with 0.2 mg/ml lysozyme for 30 min at 4 °C and then subsequent incubation with 5 mm MgCl2 and DNase (0.01 mg/ml) for 30 min. Cellular debris was removed by filtration (100,000 × g for 30 min). The supernatant was applied to a glutathione column, which was first washed with lysis buffer containing 400 mm NaCl (25 column volumes) followed by lysis buffer without NaCl (5 column volumes). The protein was eluted with 20 mm Hepes, 1 mm EDTA, 2 mm dithiothreitol, 100 mm NaCl, 5% glycerol, and 12 mm glutathione. The protein was then concentrated and dialyzed overnight in elution buffer lacking glutathione.

All G protein α subunits were synthesized in E. coli and purified as described (26, 27). Purified α subunits were activated by incubation with [35S]GTPγS at 30 °C for 30 min (Gαα), or 2 h (Gαγ). Free GTPγS was removed by gel filtration.

Interaction of Adenovirus Recombinants—Adenoviruses for RGS2 and RGS4 were generated as described (28, 29). RGS2 and RGS4 were cloned into the adenoviral shuttle vector pCAsk2.cmv using the restriction enzymes KpnI and XhoI. Both constructs contained C-terminal HA tags. To obtain recombinant viruses, HEK293 cells were co-transfected with the recombinant shuttle vector and pJM17. Viral plaques were isolated and propagated, and viral DNA was sequenced. HEK293 cells (10–15 × 106) were infected with HA-tagged RGS2 and RGS4 adenoviruses (multiplicity of infection = 2). The cells were washed once in ice-cold phosphate-buffered saline and harvested 24 h post-infection in 1 ml of hypotonic lysis buffer (50 mm Tris, pH 7.5, 4 mm EDTA plus protease inhibitors). The cell lysates were subjected to 50 strokes of Dounce homogenization on ice, followed by low speed centrifugation for 5 min. To eliminate cellular debris, and subsequent centrifugation at 100,000 × g at 4 °C for 20 min. The supernatant was checked for RGS protein expression using anti-HA antibody and used for AC binding assays as described below. Note that the high overexpression resulting from adenoviral expression often leads to a doublet for both RGS2 and RGS4. This is most likely due to proteolytic cleavage of the triple HA tag.

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Fig. 2. Interaction of RGS2 and Type V AC. A, extracts containing HA-tagged RGS4 (R4) and RGS2 (R2) were obtained from adenovirus-infected HEK293 cells (Load). Equal amounts of cell lysates were added in the reactions and incubated with His-tagged C2 (H6-VC2) and C1 domains (VC1-H6) of type V AC at 4 °C for 30 min. Ni-NTA alone was incubated with RGS2 cell extract as a negative control (Ni-NTA Beads). Nontagged GTPyS-Gαi and nontagged GTPyS-Gαq were incubated with the C2 and C1 domain, respectively, as positive controls in the absence of cell extracts. Ni-NTA resin was then added to all of the reactions, mixed for 2 h at 4 °C and washed as described under “Experimental Procedures.” The proteins were eluted with SDS sample buffer, run on SDS-PAGE, and blotted with anti-HA antibody (Ab). The dots were then stripped and reprobed for Gαi and Gαq. The load represents 30% of the total protein present in these reactions, whereas 100% of the elution is run on SDS-PAGE. RGS2 and RGS4 were run as a doublet because of proteolytic clipping of the HA tag in adenovirus-infected cell extracts (see “Experimental Procedures”). B, equal amounts of purified His-tagged RGS4 and RGS2 (Load) were used in additional binding reactions. His-tagged RGS proteins were incubated with glutathione beads alone (Glut. Beads) or with glutathione beads bound with GST (GST-alone) or GST-fused to the C1 domain of type V AC (GST-VC1). The proteins were incubated for 1 h at 4 °C, washed, eluted with SDS sample buffer, run on SDS-PAGE, and blotted with Ni-NTA-horseradish peroxidase antibody to detect both RGS2 and RGS4. The load represents 20% of the total protein used in the binding reactions, whereas 100% of the eluted RGS protein is shown.

Fig. 3. Inhibition of cAMP accumulation by RGS2 and N-terminal truncations in the presence of Gαs-Q227L or the β2-adrenergic receptor. A, production of cAMP was measured in HEK293 cells transfected as indicated with type V AC, Gαs-Q227L, and wild type, mutant (NT49A), and truncated (N-terminal deletions NT1 (Δ1–19), NT2 (Δ1–39), and NT3 (Δ1–62)) RGS2. Protein expression levels were determined by immunoblotting using anti-HA antibody. The data are expressed as the means ± S.E. from three different experiments each performed in duplicate. B, cAMP production was measured as in A, except isoproterenol stimulation of the β2AR was used instead of Gαs-Q227L for activation of type V AC. HEK293 cells transfected with control constructs or β2AR were stimulated with 0.1 μM isoproterenol for 15 min prior to lysis, cAMP detection, and Western blotting. The data are expressed as the means ± S.E. from three different experiments each performed in duplicate. Wt, wild type.
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RESULTS AND DISCUSSION

RGS2 but Not RGS4 Directly Binds to the C1 Domain of Type V Adenylyl Cyclase—Mammalian AC consists of a short N terminus, a set of six transmembrane spans, a cytoplasmic domain (designated C1), followed by a second set of six transmembrane spans, and a C-terminal cytoplasmic domain (C2) (2). Inhibition of the activities of types III, V, and VI AC reported earlier (3) could be attributed to the direct binding of RGS2 with either cytoplasmic domain of the enzyme. To demonstrate that RGS2 directly binds to AC, we performed binding assays using hexahistidine-tagged C1 and C2 domains of type V AC. These cytoplasmic domains are fully active when reconstituted with each other and retain most of the properties of the native enzyme (21, 31–33). An excess of His-tagged C1 and C2 domains from type V AC were incubated with HEK293 extracts containing C-terminal HA-tagged RGS2 or RGS4 (Fig. 2A). Nontagged GTPγS-Gqα or GTPγS-Gsα were used as positive controls for the C1 and C2 domains, respectively. Ni-NTA resin was used to pull down the interacting proteins that were eluted with SDS sample buffer, followed by immunoblotting for the respective proteins. Approximately 30% of the HA-tagged RGS2 present in the HEK293 extracts was bound to the C1 domain of type V AC. No binding of RGS4 to the C1 domain was observed, although the C1 domain tightly bound Gqα. Neither RGS2 nor RGS4 bound to Ni-NTA beads alone or to the C2 domain, although this protein was capable of binding Gsα (22).

Because the above experiment utilized HEK293 extracts, it was still possible that a second protein was required to mediate this binding event. Therefore, we tested the direct interaction of E. coli purified His-tagged RGS proteins with the C1 domain fused to GST versus GST alone. Glutathione resin was used to pull down the interacting proteins that were detected by immunoblotting with the Ni-NTA-horseradish peroxidase antibody or an antibody directed against GST. Because of the limited expression of GST-VC1, an excess of purified RGS proteins was used in these reactions. RGS2 associated with GST-VC1 but not with GST alone (Fig. 2B). No interaction was observed with RGS4. These results further support our hypothesis of a direct interaction between RGS2 with type V AC.

RGS2 Inhibits Type V AC in Vivo Independent of RGS2 GAP Activity—Previous studies have shown that RGS2 inhibits Gaq- or Gaiα-induced type III AC activation in vivo (3). To verify that RGS2 inhibits Gaqα-induced type V activation in vivo, we transfected HEK293 cells with the expression vectors for type V AC and a GAPα-deficient form of Gaqα (Gaα-Q227L), in the presence or absence of RGS2. Co-expression of Gaqα-Q227L and type V AC increased cAMP accumulation 3-fold (Fig. 3A). Additional expression of RGS2 reduced Gaqα-Q227L-stimulated cAMP accumulation to near basal levels. A similar result was obtained with non-tagged RGS2, ruling out any possible unwarranted effects of the HA tag (data not shown). The inhibition by RGS2 is unusual because many RGS proteins including RGS1, RGS4, and GAIP have no effect or show increased cAMP accumulation (34–36). However, RGS-PX1, RGS3, and RGS13 have also been reported to have inhibitory effects on cAMP accumulation by either Gαq GAP activity or other unknown mechanisms (36–38).

To test the role of RGS GAP activity, we have utilized a mutation within the highly conserved GAP domain. Replacement of Asn129 with Ala in RGS4 reduces GAP activity and binding to G proteins by over 3 orders of magnitude (39, 40). Structural data also indicates that Asn129 in RGS4 plays a crucial role in the interaction of RGS and G proteins (41). We made the corresponding mutation in RGS2, Asn49 to Ala, and examined its effect on cAMP production. This mutant form of RGS2 also suppressed cAMP accumulation to the same level as wild type RGS2 (Fig. 3A). Hence, a mutation created at the Gaα-RGS binding interface in RGS2 that disrupts GAP activity does not limit its ability to inhibit cAMP. However, deletion of the N-terminal amino acids of RGS2 (∆NT1, ∆NT2, and ∆NT3) abolished its ability to inhibit cAMP generated by type V AC. Even the shortest deletion of 19 amino acids (∆NT1) was sufficient to eliminate RGS inhibitory activity (Fig. 3A). Wild type, mutant, and truncated forms of RGS2 were all expressed at similar levels as detected by immunoblotting using anti-HA antibodies.

Similar results were observed in HEK292 cells, transfected with type V AC and the β2-adrenergic receptor (β2AR). Wild type and mutant (N149A) RGS2 inhibited isoproterenol-stimulated cAMP accumulation to a similar extent (Fig. 3B), whereas N-terminal truncations of RGS2 displayed no significant inhibition.

RGS2 and RGS2-N149A but Not ∆NT1 Bind to the C1 Domain of AC—Using binding assays as described in Fig. 2A, we assayed whether mutant and truncated RGS2 proteins were deficient in binding the C1 domain from type V AC. Cell extracts from HEK293 cells expressing HA-tagged proteins were incubated with His-tagged C1 domain. Strong interactions were observed with the C1 domain for both wild type and mutant RGS2-N149A but not the ∆NT1 truncated protein (Fig. 4). Incubation with the C1 protein is sufficient to completely clear the cytosol of wild type and N149A-RGS2 protein, whereas almost all of the ∆NT1 truncated protein remains. Hence, deletion of the N-terminal 19 amino acids is sufficient to eliminate detectable interactions between RGS2 and AC.

The N-terminal 19 Amino Acids of RGS2 Are Sufficient for Inhibition of AC—The RGS2 box domain is located from amino acids 72–199, as defined by structural homology to the RGS4 domain to near basal levels. A similar result was obtained with non-tagged RGS2, ruling out any possible unwarranted effects of the HA tag (data not shown). The inhibition by RGS2 was still possible that a second protein was required to mediate this binding event. Therefore, we tested the direct interaction of E. coli purified His-tagged RGS proteins with the C1 domain fused to GST versus GST alone. Glutathione resin was used to pull down the interacting proteins that were detected by immunoblotting with the Ni-NTA-horseradish peroxidase antibody or an antibody directed against GST. Because of the limited expression of GST-VC1, an excess of purified RGS proteins was used in these reactions. RGS2 associated with GST-VC1 but not with GST alone (Fig. 2B). No interaction was observed with RGS4. These results further support our hypothesis of a direct interaction between RGS2 with type V AC.

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Zeng et al. (42) have shown that the N-terminal residues (1–33) of RGS4 confer high affinity and receptor selective signaling via Gq. Deletion of the N-terminal domain decreased the potency of RGS4 inhibition of Gq signaling by 104-fold. RGS2 and RGS4 share a limited homology outside the RGS box from amino acid 42–72, but the extreme N terminus of RGS2 is 20 amino acids longer and displays no homology with the N terminus of RGS4. Although the N-terminal domain of RGS2 appeared to be a likely candidate to interact with AC, we could not rule out additional sites of interaction.

RGS2 contains 12 amino acids at the C terminus that are unique to this RGS protein. In addition, residues within the RGS core domain could also play a role. This domain shares many hydrophobic amino acids among all RGS family mem-

**Fig. 5.** Inhibition of cAMP accumulation by deletions of the box domain and C terminus of RGS2 with activation by either Go-Q227L or β2AR. Production of cAMP was measured in HEK293 cells transfected as indicated with type V AC, wild type (Wt), mutant (N149A), and deletions of box (Δ72–211) and C-terminal (Δ199–211) domains of RGS2 constructs in the presence of Go-Q227L (A) or β2AR (B) as described in the legend to Fig. 3 and under “Experimental Procedures.” The protein expression levels were determined by immunoblotting using anti-HA antibody. Because of the small size of RGS2-Δbox (Δ72–211), it was run on a 20% gel and is shown separately. The data are expressed as the means ± S.E. from a single experiment (n = 2) and are representative of three different experiments each performed in duplicate.

**Fig. 6.** Alanine scanning mutations of the N-terminal 19 amino acids of RGS2: cAMP accumulation, localization, and binding assays. A, a series of mutations were constructed within the N-terminal 19 amino acids of RGS2. Groups of three amino acids were mutated to alanine as shown. B, cAMP production was measured in HEK293 cells transfected as indicated with type V AC, Go-Q227L, and wild type (Wt) and mutant RGS2 constructs (RGS2-MFL, RGS2-VQH, RGS2-DCR, and RGS2-PMD). The protein expression levels were determined by immunoblotting using anti-HA antibody. The data are expressed as the means ± S.E. from two different experiments each performed in duplicate. C, HA-tagged wild type and two mutants of RGS2 (N149A and VQH) were obtained from transfected HEK293 cells. Equal amounts of each protein was loaded in the reactions (LOAD). His-tagged C1 domain of type V AC was incubated with all three protein extracts at 4 °C for 30 min. Unbound proteins (SUPERNATANT) and the bound eluted proteins (VC1-H6 Elution) were run on SDS-PAGE and blotted with HA-antibody. The load and supernatant represent 60% of the protein present in the reaction, whereas 100% of the VC1-H6 eluted protein is shown. D, the subcellular localization of wild type, mutant (RGS2-VQH), and truncated (ΔNT1) RGS2 was determined as described under “Experimental Procedures.” Equal amounts of protein from whole cell lysate (LOAD), cytosol, and membrane fractions were loaded on SDS-PAGE and detected by immunoblotting.
 bers; however, significant differences between RGS2 and RGS4 lie within α helices 3 and 6 of the RGS fold (41). To determine the precise regions of RGS2 critical for inhibition of type V AC, we created two additional truncations of RGS2. RGS2-Δbox is devoid of the RGS core domain and contains only the N-terminal 71 amino acids. RGS2-ΔC lacks the 12-amino acid overhang at the C terminus. Type V AC and Goα-Q227L were co-expressed in the presence or absence of constructs directing the expression of full-length RGS2 and the Δbox and ΔC truncations in HEK293 cells (Fig. 5A). Both truncations inhibited cAMP production to the same extent as wild type RGS2, indicating that the N terminus is sufficient for RGS2-mediated inhibition with no significant contributions from the RGS box or C terminus. Similar results were obtained by co-transfection with the β2AR and stimulation with isoproterenol (Fig. 5B).

**Fig. 7.** Mutations in the Goα-binding site of type V AC prevent inhibition by carbachol but have no effect on inhibition by RGS2. A, production of cAMP was measured in HEK293 cells transfected as indicated with Goα-Q227L and wild type and mutant type V AC (E411A and L472A) constructs in the presence or absence of carbachol (1 μM) for 15 min. B, cAMP production was measured in HEK293 cells transfected with constructs directing the expression of wild type and mutant type V AC (E411A and L472A) and Goα-Q227L in the presence and absence of wild type RGS2 (1 μg/well). The protein expression levels were determined by immunoblotting using anti-HA antibody (data not shown). The data are expressed as the means ± S.E. from three different experiments each performed in duplicate. C, cAMP production was measured in HEK293 cells transfected with constructs as described for B, except that a variable amount of RGS2 DNA was used (25 ng, 50 ng, 1 μg, and 2 μg). The data are shown as percentages of the fold activation in the absence of RGS2 for each type V AC construct, where the fold activation is simply the level of cAMP of type V AC plus Goα-Q227L as compared with Goα-Q227L alone. The data are expressed as the means ± S.E. from a single experiment (n = 2) and are representative of two different experiments each performed in duplicate. D, wild type (●, 60 nM), L472A (△, 80 nM), and E411A (■, 225 nM) mutant VC1(670) was reconstituted with 0.5 μM VC2 and assayed with 400 nM activated Goα in the presence of the indicated concentrations of purified RGS2. The activities are expressed as percentages of control values (1630, 1950, and 240 nmol/min/mg for wild type and L472A and E411A mutant VC1(670), respectively) and are representative of two different experiments performed in duplicate. wt, wild type.

**Mutational Analysis of RGS2 Identifies a Series of Residues Important for Inhibition of Type V AC—**The N-terminal 19 amino acids of RGS2 clearly play a pivotal role in inhibiting type V AC-generated cAMP production. We conducted a limited alanine scanning analysis of the first 19 amino acids in the N-terminal region of RGS2 to identify residues important for RGS2 function. Sets of three residues along the 19-amino acid stretch were mutated to alanine as shown in Fig. 6A. Both truncations inhibited cAMP production to the same extent as wild type RGS2, indicating that the N terminus is sufficient for RGS2-mediated inhibition with no significant contributions from the RGS box or C terminus. Similar results were obtained by co-transfection with the β2AR and stimulation with isoproterenol (Fig. 5B).

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of cAMP accumulation as wild type RGS2 (Fig. 6B). The mutation VQH displayed no inhibition of cAMP production by type V AC, although it expressed as well as if not better than any of the other mutant proteins. In addition to blocking inhibition of cAMP production by RGS2, mutation of the residues VQH to alanine also prevented binding to the C1 domain of type V AC (Fig. 6C). The N-terminal 19-amino acid stretch of RGS2 displays no homology with RGS3 or RGS13, which have been reported to impair Gi signaling (36, 37), nor with any other mammalian protein sequence in the GenBank™ data base.

Membrane Localization of Wild Type, ΔNT1 (1–19) RGS2, and RGS2-VQH—Localization can be a key determinant of RGS2 function (10). One trivial explanation for the loss of inhibition by RGS2-ΔNT1 and the mutation of residues VQH would be a possible mislocalization of the truncated or mutated protein to regions away from the plasma membrane. Earlier reports identified amino acid residues 33–67 as an amphipathic α-helical domain that was sufficient for plasma membrane targeting (10). To rule out possible additional effects from the N-terminal 19 amino acids, we fractionated HEK293 cells expressing wild type, ΔNT1 truncated RGS2, and the mutant RGS2-VQH. All three forms of RGS2 were present in the plasma membrane and in the cytosol of HEK293 cells (Fig. 6D). Therefore, it is unlikely that gross mislocalization of the truncated or mutant RGS2 proteins results in a loss of activity.

RGS2 Does Not Interact with the Gαi-binding Site on Type V AC and Is Independent of Inhibition by Gαi—Because RGS2 bound to the C1 domain of type V AC, we tested whether RGS2 may utilize the Gαi inhibitory binding site. We made use of two mutations in type V AC, ACV-E411A and ACV-L472A, that showed a 60-fold reduction in the IC50 for Gαi-mediated inhibition of type V AC (21). The Gαi-binding site was defined as a cleft formed by the α2 and α3 helices of the C1 domain. The amino acids Glu411 and Leu472 reside on the α2 and α3 helices, respectively. Mutation of these residues had no effect on the activation of type V AC by Gαi, nor with any other mammalian protein sequence in the GenBank™ data base.

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