Unique Hydrophobic Extension of the RGS2 Amphipathic Helix Domain Imparts Increased Plasma Membrane Binding and Function Relative to Other RGS R4/B Subfamily Members

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RGS2 and RGS5 are inhibitors of G-protein signaling belonging to the R4/B subfamily of RGS proteins. We here show that RGS2 is a much more potent attenuator of M1 muscarinic receptor signaling than RGS5. We hypothesize that this difference is mediated by variation in their ability to constitutively associate with the plasma membrane (PM). Compared with full-length RGS2, the RGS-box domains of RGS2 and RGS5 both show reduced PM association and activity. Prenylation of both RGS-box domains increases activity to RGS2 levels, demonstrating that lipid bilayer targeting increases RGS domain function. Amino-terminal domain swaps confirm that key determinants of localization and function are found within this important regulatory domain. An RGS2 amphipathic helix domain mutant deficient for phospholipid binding (L45D) shows reduced PM association and activity despite normal binding to the M1 muscarinic receptor third intracellular loop and activated G\(_\alpha_q\). Replacement of a unique dileucine motif adjacent to the RGS2 helix with corresponding RGS5 residues disrupts both PM localization and function. These data suggest that RGS2 contains a hydrophobic extension of its helical domain that imparts high efficiency binding to the inner leaflet of the lipid bilayer. In support of this model, disruption of membrane phospholipid composition with \(N\)-ethylmaleimide reduces PM association of RGS2, without affecting localization of the M1 receptor or G\(_\alpha_q\). Together, these data indicate that novel features within the RGS2 amphipathic \(\alpha\) helix facilitate constitutive PM targeting and more efficient inhibition of M1 muscarinic receptor signaling than RGS5 and other members of the R4/B subfamily.

Heterotrimeric G-protein-coupled receptors mediate cellular responses to a variety of extracellular ligands, including hormones, neurotransmitters, and sensory stimuli. Proper coordination of G-protein-coupled receptor signaling at the cellular and tissue level is required to ensure appropriate physiologic responses to rapidly changing environmental conditions. An important component of G-protein-coupled receptors signal coordination in human cells is the RGS (regulator of G-protein signaling) superfamily of proteins. RGS proteins inhibit G-protein signaling via their activities as GTPase-activating proteins for G-protein \(\alpha\) subunits (1–4).

The human genome contains 37 RGS proteins (5) characterized by a ~120-amino acid domain called the “RGS-box.” The RGS protein superfamily can be further subclassified based on the architectural organization and RGS-box function of its members (6). Many subfamilies are made up of larger RGS-box-containing proteins that include the RGS7-like, RGS12-like, RhoGEF-containing, and G-protein-coupled receptor kinases. These subfamilies are mainly composed of members with multiple modular signaling domains. By contrast, the RGSZ-like and RGS4-like (R4/B) subfamilies are made up of smaller proteins containing a RGS-box domain flanked by short amino-terminal and carboxyl-terminal extensions. These two families are distinguished by the presence of a polycysteine domain in the amino terminus of the RGSZ-like members (7, 8) that is believed to be reversibly palmitoylated (9). Members of the mammalian R4/B subfamily include RGS1, RGS2, RGS3, RGS4, RGS5, RGS8, RGS16, RGS18, and RGS21. Each R4/B subfamily member contains a domain in its short amino terminus that may form an amphipathic \(\alpha\) helix upon association with a lipid bilayer (10). It is believed that this domain is important for proper plasma membrane association and function of mammalian RGS proteins (11–13).

The R4/B subgroup members are important regulators of G\(_\alpha_q\) function in the cardiovascular system. Our laboratory studies RGS proteins as regulators of vascular function.
Because RGS2 and RGS5 mRNAs are both highly expressed in vascular smooth muscle cells, we set out to characterize the determinants that are important for their relative function. Preliminary data from our laboratory showed that RGS2 localizes to the plasma membrane more efficiently and behaves as a more potent inhibitor of muscarinic receptor-dependent Goq-mediated signaling than RGS5. This observation was at odds with a recent report that showed that RGS2 and RGS5 were similarly effective as inhibitors of muscarinic receptors (14). To help rationalize this discrepancy, we investigated the mechanisms regulating differential localization and function of the R4/B subfamily members.

It is known that RGS2 localizes to the mammalian cell plasma membrane more efficiently than other R4/B subfamily members and also functions as a more potent inhibitor of muscarinic receptor Goq signaling (15–17). However, the relative contribution of three previously reported membrane targeting mechanisms to its localization and receptor inhibition has not been determined. First, the amino terminus of RGS2 can bind to the third intracellular loop of the M1 muscarinic receptor, whereas other R4/B subfamily members cannot (17). Second, RGS2 contains three unique residues in its RGS-box that confer Goq selectivity (15) compared with other R4/B subfamily members. Third, the amino-terminal amphipathic helix of RGS2 is sufficient to confer lipid binding and plasma membrane localization in mammalian cells, whereas the same domain in RGS4 localizes evenly throughout the cell (13). Moreover, this domain is necessary for increased recruitment of RGS2 to the plasma membrane by constitutively active Goq, an effect that is not seen for any of the other R4/B subfamily members tested except RGS3 (18). Here, we exploited the functional differences between RGS2 and RGS5 to dissect the relative contribution of these molecular determinants to membrane targeting and M1 muscarinic receptor inhibition. The data show that unique hydrophobic residues adjacent to the core amphipathic α helix domain in RGS2 are necessary to mediate its tonic plasma membrane association and increased function relative to RGS5. Since changing these residues does not interfere with receptor or Goq binding, and interfering with the anionic lipid composition on the inner leaflet of the plasma membrane results in RGS2 dissociation, these data point to differences in steady state association with the lipid bilayer in mammalian cells as a key explanation for the functional difference between RGS2 and RGS5.

EXPERIMENTAL PROCEDURES

Materials—The cytomegalovirus promoter of the pEYFP-C1 plasmid (Clontech/BD Biosciences) was used to drive expression of all RGS protein constructs used in this study. The polyclonal anti-green fluorescent protein antibody (catalog number 632376) was also from Clontech/BD Biosciences. Fura-2/AM and all tissue culture media and transfection reagents were from Invitrogen. HEK293 cells stably expressing the M1 muscarinic receptor (M1-HEK) were a kind gift from P. Burgon and E. Peralta (Harvard University, Cambridge, MA). The green fluorescent protein-tagged Goq expression construct was a kind gift from C. Berlot (Weis Center for Research, Danville, PA).

Unless otherwise stated, all other reagents and chemicals were from Sigma.

Cell Culture—HEK cells were grown in maintenance medium (Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (1:1), supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 10 µg/ml streptomycin, and 100 units/ml penicillin) at 37 °C in a humidified atmosphere with 5% CO2. M1-HEK cells were grown as above with 0.5 mg/ml Geneticin added to the medium.

cDNA Constructs—All RGS protein cDNA constructs were prepared by PCR cloning using Pfu polymerase (Fermentas, Burlington, Canada). The indicated RGS protein chimeras were generated by PCR-mediated ligation of cDNA sequences encoding amino-terminal (RGS2, aa1–37; RGS5, aa1–51) and carboxy-terminal (RGS2, aa72–211; RGS5, aa52–184) residues. All RGS proteins were made as carboxyl-terminal YFP fusions by insertion into unique Nhel and AgeI restriction sites ahead of YFP in pEYFP-C1. Robust expression was ensured by inclusion of an optimized translation initiation signal (19) in the context of the first methionine codon (“Kozak consensus,” GCCACATGGCGG). Point mutations were introduced by the QuickChange site-directed mutagenesis kit (Qiagen, Missis- sauga, Canada), and primer sequences are available upon request. All constructs were purified using the Endofree Maxi kit (Qiagen) and verified by sequencing of the complete protein-coding region.

Phosphoinositide Hydrolysis Assays—COS-7 cells (~0.2 × 106 cells in 12-well plates) were transiently transfected with 250 ng of M1 muscarinic receptor and 250 ng of either control vector or RGS protein expression constructs. Inositol phosphate production was measured 48 h after transfection according to the method of Ventkatakrishnan and Exton (20). Briefly, ~0.5 × 106 cells/well in a 12-well dish were labeled in complete Dulbecco’s modified Eagle’s medium (without inositol) containing 4 µCi/ml myo-[3H]inositol for 17–24 h, washed twice, and treated with 200 µM carbachol in the presence of 5 mM LiCl. Incubations were carried out for exactly 45 min before stopping them by media aspiration and the immediate addition of 750 µl of ice-cold 20 mM formic acid. The entire contents of the well were collected and spun at 13,000 × g for 15 min in a micro-centrifuge. The supernatant fraction (700 µl) was neutralized with 214 µl of 0.7 M NH4OH before proceeding to the ion exchange chromatography steps. For each well to be measured, a separate 3-ml Dowex resin (AG 1-X8, 200–400-mesh, formate form) column was prepared. The entire sample was added to the column, and unbound 3H-labeled material consisting of the total inositol-containing fraction was collected after the addition of 4 ml of water. Following a wash with 4 ml of 40 mM ammonium formate, the inositol phosphate-containing fraction was eluted into collection tubes with 5 ml of 1.2 M ammonium formate. 0.5 ml of each sample from total inositol-containing and inositol phosphate-containing fractions was added to 10 ml of scintillation fluid and counted. Inositol phosphate levels were expressed as the fraction of the total soluble 3H-la-
beled inositol material (inositol phosphate + total inositol-containing fraction) for each sample.

**Intracellular Calcium Imaging—** M1-HEK cells were seeded at 50% confluence on polylysine-coated #1 glass coverslips in 6-well plates prior to transfection with 2 μg of plasmid DNA in Lipofectamine (Invitrogen) according to the manufacturer’s instructions. After 24 h of transfection, coverslips were washed and incubated in calcium imaging buffer (11 mM glucose, 130 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl₂, 17 mM HEPES, and 1 mM CaCl₂, pH 7.3) containing 5 μM Fura-2/AM and 0.05% pluronic acid for 40 min at 37 °C. Fura-2-loaded cells were washed again and incubated for at least 10 min in calcium imaging buffer to allow hydrolysis of the acetoxyethyl ester. Coverslips were mounted in a TC1-SL25 open bath chamber (BioScience Tools, San Diego, CA) and imaged on an Olympus BX51WI upright microscope using a ×10 water-dipping objective. Excitation light was provided by a DeltaRam V monochrometer (PTI, Lawrenceville, NJ). Fluorescence imaging was performed with ImageMaster imaging software (PTI). Images were acquired with a Photometrics Cascade 512B cooled charge-coupled device camera (Roper Scientific, Tucson, AZ). YFP-expressing cells were identified using 505 ± 3 nm excitation light in conjunction with a YFP filter set (Chroma Technology Corp., Brattleboro, VT) and selected as regions of interest within the ImageMaster software. Relative YFP fluorescence (RGS expression) and Fura-2 ratiometric (intracellular calcium) was determined for each region of interest and was calculated as mean pixel fluorescence value following 50 and 100 ms of exposure, respectively. For Fura-2 imaging, alternating excitation wavelengths (355 ± 5/396 ± 5 nm) were provided at ~1 excitation pair/s in conjunction with a 495-nm dichroic mirror and a 510 ± 20-nm emission filter (Chroma Technology Corp.), and paired images were collected after a 100-ms exposure. Fluorescent ratio values for the image pairs were determined for regions of interest previously selected on the basis of their YFP expression. Base-line fluorescence ratios (FRs) of nonstimulated cells were collected for 30 frames prior to the addition of 100 μM carbachol. The percentage increase from base-line FR levels to the peak stimulated FR was calculated only for cells with a relative YFP fluorescence of <12,000. Identical excitation/emission conditions and data collection parameters were maintained for all individual experiments performed in this study. For determination of absolute calcium levels, FR data were converted to intracellular calcium concentration ([Ca²⁺]) using a look-up table generated using a mixture of two Molecular Probes™ Ca²⁺ calibration kits (catalog numbers C3009 and C3722; Invitrogen) to a final [Mg²⁺] of 375 nM.

**Confocal Microscopy—** Polylysine-coated 25-mm circular #1 glass coverslips containing transfected cells were mounted in a modified Leyden chamber containing calcium imaging buffer. Confocal microscopy was performed on live cells at 37 °C using an Olympus Fluoview 1000 laser-scanning confocal microscope. Images represent single planes on the basal side of the cell obtained with a ×60 oil objective. Confocal images were processed with Adobe Photoshop 7.0.

**Measurement of RGS Protein Binding to M1 Muscarinic Receptor Third Intracellular Loop—** HEK293T cells at 80% confluence in 10-cm tissue culture dishes were transiently transfected with the indicated constructs using Lipofectamine (40 ml of Lipofectamine to 8 ml of total plasmid DNA). 24 h post-transfection, cells were lysed in hypotonic lysis buffer (10 mM HEPES, pH 8.0, 50 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100) supplemented with protease inhibitors (Complete Mini mixture; Roche Applied Science) and incubated on ice for 30 min. Total cell lysates were then spun at 100,000 × g for 30 min to remove cellular debris. The supernatant was snap-frozen in liquid nitrogen until use. The RGS pull-down assay using recombinant glutathione S-transferase-tagged M1 muscarinic receptor third intracellular loop protein (GST-M1i3) was performed as we previously described (17). Briefly, cell lysates were mixed with GST-M1i3 prebound to glutathione-Sepharose beads (10 ml) and incubated by rotating at 4 °C overnight. Glutathione-Sepharose beads were collected by centrifugation at 500 × g for 5 min at 4 °C and washed twice in harvest buffer, the second time in the absence of Triton X-100. Protein was eluted from the beads by the addition of 2 × SDS sample buffer. Both load and bound RGS proteins were detected by subsequent immunoblotting for YFP as described above.

**Fluorescent Labeling of RGS2 Proteins—** Recombinant histidine-tagged wild type and mutant RGS2 proteins were expressed in bacteria and purified as previously described (21) before dialysis in storage buffer (50 mM HEPES, pH 8.0, 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol). RGS2 proteins in storage buffer were fluorescently labeled with Alexa Fluor 532 carboxylic acid succinimidyl ester (Invitrogen) at a 3:1 fluorophore/protein ratio. The reaction was incubated at 4 °C in the dark for 1 h and then quenched with a 10-fold excess of glycine for 15 min. Excess fluorophore was removed by filtration through a Sephadex G25 spin column.

**Biotinylation of Goq —** Purified Goq by was chemically biotinylated using biotinamidohexanoic acid N-hydroxysuccinimide ester (Sigma) at a 5:1 biotin/Goq ratio for 1 h at room temperature. The reaction was quenched with a 10-fold excess of glycine for 15 min at room temperature. Goq by was then activated by a 10-fold dilution into activation buffer (20 mM HEPES, pH 8.0, 100 mM NaCl, 50 mM MgCl₂, 10 mM β-mercaptoethanol, 50 mM (GDP, 30 mM AlCl₃, 10 mM NaF, and 10 mM CHAPS) and allowed to incubate for 20 min at room temperature. AMF (50 mM GDP, 30 mM AlCl₃, and 10 mM NaF)-activated Goq by was then loaded onto a 1-mL Ni²⁺-nitrilotriacetic acid (Qiagen) column. Biotinylated-Goq by was eluted with elution buffer (20 mM HEPES, pH 8, 100 mM NaCl, 5 mM MgCl₂, 10 mM β-mercaptoethanol, 50 mM GDP, 30 mM AlCl₃, 10 mM NaF, and 20 mM imidazole).

**Flow Cytometry Protein Interaction Assay—** Equilibrium binding for AF532-RGS2 proteins and biotin-Goq by was determined using the flow cytometry protein interaction assay method, as described previously (22). Briefly, Luminex LumAvidin beads were washed with bead-coupling buffer (phosphate-buffered saline, 1% bovine serum albumin, and 1 mM dithiothreitol) and incubated for 30 min in the dark with biotin-Goq by at a 5:n final concentration. Beads were then washed with a flow buffer (50 mM HEPES, pH 8.0, 100 mM NaCl, 0.1% Lubrol, and 1 mM dithiothreitol), and AMF was added for AMF-activated biotin-Goq by samples. Fluorescently labeled RGS2 proteins were diluted into flow buffer (with or without AMF) and were dispensed into a
96-well plate according to their concentration. 50 ml of biotin-Gq (with or without AMF)-labeled beads were dispensed into each well and allowed to equilibrate at room temperature for at least 30 min in the dark. The plate was then read on a Luminex 100IS 96-well plate-reading flow cytometer. Median fluorescence intensity values, measured in each experiment as duplicates, were plotted against the RGS2 concentration and fit to a single binding hyperbola (MF1 = Bmax × [AF532-RGS2]/(Kd × [AF532-RGS2]) using GraphPad Prism 4.

Statistical Analysis—For calcium imaging studies, shown are the averages of >50 cells on one experimental day that is representative of at least three independent experiments carried out on separate days. Confocal microscopy pictures show representative images from at least 50 cells. One-way analysis of variance was used to indicate statistical differences between the multiple groups studied in each experiment. Pairwise comparisons between groups were made using the unpaired Student’s t test. A p value of <0.05 was deemed significant.

RESULTS

RGS2 Is a More Potent Inhibitor of M1 Muscarinic Receptor-mediated Phosphatidylinositol Hydrolysis Compared with RGS5—Data from several groups, including our own, have shown that RGS2 is a more potent inhibitor of Gq,-coupled muscarinic receptor signaling than other members of the R4/B subfamily of RGS proteins. Anger et al. (14), however, reported that RGS5 inhibited Gq,-dependent muscarinic receptor-coupled signaling with similar efficiency to that of RGS2. To investigate this apparent discrepancy, we compared the ability of RGS2 and RGS5 to inhibit muscarinic receptor-mediated stimulation of phosphatidylinositol hydrolysis. Carbachol-mediated increases in inositol phosphate accumulation were measured in COS-7 cells cotransfected with M1 muscarinic receptor and either control vector (pEYFP-C1), RGS2-YFP, or RGS5-YFP (Fig. 1A). Immunoblotting confirmed that these constructs were expressed in similar amounts as single protein bands of the expected size (Fig. 1A, inset). At odds with the report of Anger et al. (14), RGS2 was found to be a more potent inhibitor of M1 muscarinic receptor-mediated inositol phosphate accumulation in this system.

RGS2 Is a More Potent Inhibitor of M1 Muscarinic Receptor-mediated Intracellular Calcium Signaling Compared with RGS5—We next used intracellular calcium measurements to determine whether RGS2 and RGS5 were also different in their ability to regulate rapid physiologic responses (Fig. 1, B and C). pEYFP control plasmid or a RGS-YFP fusion construct was transfected into HEK293 cells containing stably expressed M1 muscarinic receptor. Fura-2-loaded cells were stimulated with carbachol, and intracellular calcium was measured using microscopy with ratiometric imaging software. RGS function is measured in single cells, preselected on the basis of their RGS-YFP expression levels, to afford a means for standardizing RGS function with protein expression in individual cells.

Cells expressing low to intermediate levels (3500 < relative YFP fluorescence < 12,000) of YFP fluorescence were selected for study. This YFP expression range corresponded to RGS5 protein expression levels that are on the linear portion of its expression versus function curve (data not shown). In 13 separate experiments, the intracellular calcium data for >750 individual YFP-expressing (control) cells showed resting and peak carbachol-stimulated intracellular [Ca2+]i of 105 ± 2 and 274 ± 12 nM, respectively. Fig. 1B shows the typical kinetic response following the addition of carbachol to YFP-, RGS2-YFP-, and RGS5-YFP-expressing cells (n > 50). Notably, the Fura-2 ratio response is reproducibly blunted in RGS2-expressing compared with YFP-expressing cells (55 ± 3%), whereas RGS5 typically showed much lower levels of inhibition (21 ± 2%). Fig. 1C shows the average percentage increase from base line of the fluorescent ratios for the traces in Fig. 1B. When all 13 experiments were analyzed in this manner, peak stimulated ratio increases were significantly lower for RGS2-YFP and RGS5-YFP compared with YFP controls in 13 of 13 experiments and 11 of 13 experiments, respectively. Moreover, peak stimulated Fura-2 ratios were significantly lower for RGS2-YFP compared with RGS5-YFP in 12 of 13 experiments. Taken together, these data indicate that RGS2 is more effective than RGS5 as an inhibitor of rapid intracellular calcium responses to M1 muscarinic receptor activation.

Plasma Membrane Targeting Is a More Important Functional Determinant than Intrinsic RGS-box Function—Previous data from our laboratory showed that RGS2 contained three unique amino acid residues in its RGS-box domain that mediate its selective inhibition of Gq,- versus Gq,-mediated signaling (15). To determine whether Gq,-selectivity could explain the enhanced relative function of RGS2, we compared the ability of the RGS-box domains of RGS2 and RGS5 to act as inhibitors of M1 muscarinic receptor signaling. YFP fusions of the carboxyl-terminal RGS-box domains or RGS-box domains tagged with the K-Ras plasma membrane targeting sequence (CAAX motif) were constructed according to the schematic in Fig. 2A. Immunoblotting for YFP confirmed that all of the constructs expressed as protein bands of the expected sizes (Fig. 2B). Confocal microscopy was used to follow the localization of these clones in HEK cells (Fig. 2C). RGS2 localized to the nucleus and plasma membrane, whereas RGS5 was distributed uniformly through the cell. Notably, RGS2 stably associated with the plasma membrane under normal culture conditions (no carbachol) and after treatment with the muscarinic antagonist scopolamine (supplemental materials). Moreover, the stable RGS2 association with the plasma membrane was not decreased by serum starvation or increased by agonist addition. In dramatic contrast, RGS5 did not stably associate with the plasma membrane under any of the experimental conditions tested. We used the isolated RGS-box domains to study the relative functional contribution of membrane association and intrinsic RGS-box function for RGS2 and RGS5.

The carboxyl-terminal RGS-box domains of RGS2 and RGS5 showed no measurable activity in two of three and one of three experiments, respectively. In cases where RGS-box activity of RGS5 could be detected compared with no RGS controls, its function was similar to that of full-length RGS5. By contrast, the function of the RGS-box of RGS2 was markedly impaired relative to the full-length protein (Fig. 3A). Moreover, appending a carboxyl-terminal prenylation (CAAX-box) membrane-targeting sequence increased the inhibitory function of the RGS-box domains for both RGS2 and RGS5 to levels similar to
that of the wild type RGS2 (Fig. 3B). These data argue strongly against a role for differences in the RGS-box domains as the main functional difference between RGS2 and RGS5. Instead, these studies suggested that promoting constitutive plasma membrane association of the RGS-box domain of RGS2 and RGS5 is crucial for their respective functions.

**FIGURE 1.** Comparison of RGS2 and RGS5 function as inhibitors of M1 muscarinic receptor-mediated inositol phosphate accumulation and peak intracellular calcium elevation. A, inhibition of agonist-stimulated production of inositol phosphate in COS-7 cells expressing RGS2 or RGS5. COS-7 cells were transiently co-transfected with M1 muscarinic receptor and the indicated RGS or control construct. After 24 h, wells containing the transfected cells were labeled with [3H]inositol and treated with the indicated concentrations of carbachol in the presence of 10 mM LiCl for 45 min before determination of inositol phosphate accumulation values as described under “Experimental Procedures.” Inositol phosphate values represent the average of duplicate or triplicate samples (shown in parentheses) and were expressed as the percentages of soluble IPx relative to total soluble inositol-labeled material; error bars indicate the range of experimental values for each condition. RGS-YFP protein expression (inset) was determined by immunoblotting, as described under “Experimental Procedures.” B, M1-HEK cells on coverslips were transiently transfected with the indicated construct and loaded with Fura-2/AM. Transfected cells identified as low to medium fluorescence intensity (3500 < relative YFP fluorescence units < 12,000) were selected for analysis of their intracellular calcium responsiveness to carbachol. Changes in intracellular calcium levels were recorded as changes in fluorescence ratio (FR = (emission at 510 nm upon excitation at 355 nm)/(emission at 510 nm upon excitation at 396 nm)). Shown are mean FR trace values (n > 50 kinetic cells) expressing YFP control, RGS2, and RGS5 in a typical experiment showing base-line and relative FR change following the addition of 100 μM carbachol (arrow). C, peak calcium response to carbachol in M1-HEK cells expressing RGS2 and RGS5. Transfected M1-HEK cells were processed and selected for calcium imaging as described in B. Peak relative increases in intracellular calcium levels for each cell were calculated as follows: percentage of FR increase above base line = (peak stimulated FR/unstimulated base-line FR) − 1) × 100%. All experiments show mean percentage FR increase above base line ± S.E. for n > 50 cells. All data are representative of three independent experiments. *, p < 0.005.

**FIGURE 2.** Expression and subcellular localization of YFP-tagged full-length and carboxyl-terminal RGS-box domains of RGS2 and RGS5 in HEK cells. A, shown is the schematic representation for construction of the full-length, carboxyl-terminal RGS-box domain and CAAX-tagged RGS-box domains of RGS2 (dark shading) and RGS5 (light shading). All RGS constructs are tagged with YFP at the carboxyl terminus. B, HEK293 cells were transfected with the indicated RGS- YFP constructs, and expression was examined by immunoblotting as described under “Experimental Procedures.” C, HEK cells were transfected with the indicated constructs and seeded onto #1 circular glass coverslips, and subcellular localization of YFP-tagged proteins was analyzed in live untreated cells by laser-scanning confocal microscopy as described under “Experimental Procedures.” Pictures are of cells with low to intermediate fluorescence intensity and are representative of at least 50 cells transfected with the same construct.
RGS5 is a critical determinant of their function as inhibitors of M1 muscarinic receptor signaling. Unique Sequences in the RGS2 Amino Terminus Impart Stable Plasma Membrane Localization and Increased Function Compared with RGS5—Chimeric RGS proteins were created such that the amino-terminal domain of one protein was fused to the RGS-box domain of the other. The resulting constructs were named RGS2/5 and RGS5/2, and a schematic diagram showing their domain architecture is shown in Fig. 4A. Immunoblotting for YFP confirmed that both chimeric-YFP fusion proteins were expressed as a single protein species in HEK cells (Fig. 4B). Plasma membrane localization of the chimeric clones was completely dependent on their respective amino-terminal sequences (Fig. 4D). Specifically, RGS2/5 and RGS5 were evenly distributed and noticeably absent from the plasma membrane. Functional assays supported our model that RGS-box function is dependent on their stable association with the plasma membrane prior to agonist stimulation. Specifically, RGS5/2 and RGS5 showed similarly modest levels of inhibitory function, whereas RGS2/5 and RGS2 showed efficient receptor inhibition. Similar results were obtained in inositol phosphate accumulation.
RGS2 Amino Terminus Directs Increased Membrane Targeting

**FIGURE 5.** Testing whether mutation of the conserved hydrophobic residues in amphipathic α helix in RGS2 and RGS2/5 alters tonic plasma membrane localization and function. A negatively charged aspartate residue was introduced into the conserved hydrophobic face of the amphipathic helix in RGS2 and RGS2/5. Immunoblotting (A), live cell confocal microscopy (B), and peak relative FR increases from baseline (C) were used to describe the expression, subcellular localization, and inhibitory function of the indicated RGS protein constructs, respectively, as described for Fig. 4. *, p < 0.001.

Membrane Association and Regulatory Function—Previously, we showed that increased plasma membrane targeting of RGS2 by activated Goαq was prevented by mutation of hydrophobic or basic residues in the amphipathic helix (13). Thus, the amino-terminal helix domain may mediate plasma membrane association with phospholipid bilayers containing physiologic levels of negatively charged phospholipids (13). To study the functional consequence of disrupting the amphipathic helix, the L45D mutation was introduced into the RGS2 and RGS2/5. Immunoblotting for YFP confirmed the expression of a single protein species for both mutant constructs (Fig. 5A). Consistent with the increased plasma membrane association observed for the L45D variants (Fig. 5B) compared with wild type RGS2, in transfected M1-HEK cells expressing L45D variants of RGS2 and RGS2/5, the relative extent of M1 muscarinic receptor signal inhibition was reduced to a similar level as that of wild type RGS5 (Fig. 5D), indicating that the functional advantage conferred by the RGS2 amino terminus was ablated by disruption of the hydrophobic interface of this amphipathic helix domain. However, it remained to be determined whether the amphipathic helix domain mediate stable plasma membrane localization via lipid binding, receptor binding, or association with other signaling components, such as Goαq.

**Unique Dileucine Motif in RGS2 Mediates Tonic Plasma Membrane Association and Regulatory Function**—Previously, we showed that increased plasma membrane targeting of RGS2 by activated Goαq was prevented by mutation of hydrophobic or basic residues in the amphipathic helix (13). Thus, the amino-terminal helix domain may mediate plasma membrane associ-
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Ation by binding to the phospholipid bilayer and/or enhancing RGS2 association with activated Gαq. Enigmatically, RGS5 also contains an amino-terminal amphipathic α helix domain but does not stably associate with the plasma membrane under any condition examined thus far. Thus, we asked what were the unique determinants within the RGS2 amphipathic helix that promote its increased plasma membrane targeting efficiency in mammalian cells. Sequence alignment of the core amphipathic helix of RGS2 with RGS5 and other R4/B family members revealed no difference in the organization of aliphatic or basic amino acid residues that could obviously explain the functional differences between these domains (Fig. 7A). However, in the region flanking the core helix domain, RGS2 contained two unique aliphatic leucine residues at amino acid positions 37 and 38 and some additional basic residues. We hypothesized that these residues extended the hydrophobic face of the RGS2 amphipathic helix by one extra turn compared with other R4/B family members (Fig. 7B) and, therefore, provided a more hydrophobic interaction surface for phospholipids, Gαq, or other membrane-bound effectors.

First, to determine whether the dileucine motif in RGS2 was required for increased plasma membrane localization and function, we replaced it with the corresponding pair of less hydrophobic residues in RGS5 (arginine, alanine) to produce the RGS2(LL-RA) expression construct. Immunoblotting (A), live cell confocal microscopy in the absence of carbachol (B), and peak relative FR increases from base-line levels following the addition of 100 μM carbachol (C) were used to describe the expression, subcellular localization, and inhibitory function of the indicated RGS protein constructs, respectively, as described in detail above. *, p < 0.001.

FIGURE 7. Primary sequence alignment and helical net comparisons of RGS2, RGS5, and other R4/B subfamily members. The sequences for the conserved amphipathic α helical domain in RGS2 were aligned to the corresponding sequences of RGS5, RGS4, RGS16, and RGS3 by the Clustal method (A). Shading indicates the position of hydrophobic residues that are located on the lipid-binding face of the helix. The same region was represented by a helical net (B), with the shaded region indicating the length of the hydrophobic surface along the lipid-binding face of the helix. Aliphatic and nonpolar hydrophobic residues within this surface are shown in black.

FIGURE 8. Determining the functional contribution of residues in RGS2 and RGS5 that flank the conserved amphipathic α helical domain. The dileucine motif in RGS2 was replaced with the corresponding pair of less hydrophobic residues in RGS5 (arginine, alanine) to produce the RGS2(LL-RA) expression construct. Immunoblotting (A), live cell confocal microscopy in the absence of carbachol (B), and peak relative FR increases from base-line levels following the addition of 100 μM carbachol (C) were used to describe the expression, subcellular localization, and inhibitory function of the indicated RGS protein constructs, respectively, as described in detail above. *, p < 0.001.

severely impaired with respect to its ability to inhibit M1 muscarinic receptor signaling in transfected M1-HEK cells (Fig. 8C). Evidence that the dileucine motif itself was sufficient to impart membrane-targeting function to another amphipathic helix domain came from the reciprocal experiment where it was incorporated instead of Arg15/Ala16 in RGS5. Inclusion of the dileucine motif directed RGS5 to the plasma membrane as well as to other intracellular membranes, including the endoplasmic reticulum and nuclear envelope (supplemental materials). Together, these data suggested that sequences in the region flanking the core amphipathic helix regulate the tonic localiza-
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**FIGURE 9.** Binding of AF532-labeled RGS2, RGS2(L45D), RGS2(LL-RA), and RGS2(EN-AA) to biotin-labeled G<sub>q</sub> using the flow cytometry protein interaction assay method. A, binding of AF532-RGS2 proteins is dependent on AMF activation of G<sub>q</sub>. The curves shown are representative of four different experiments with n = 2 for each data point. MFI, mean fluorescence intensity. B, specific binding of AF532-RGS2 to biotin-G<sub>q</sub>, RGS2, RGS2(L45D), and RGS2(LL-RA) exhibit similar K<sub>d</sub> values, whereas RGS2(EN-AA), which has a disrupted RGS-box, does not bind biotin-G<sub>q</sub>. The fitted K<sub>d</sub> values are given in Table 1.

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**TABLE 1.** Affinity of AF532-RGS2 proteins for biotin-G<sub>q</sub>

| Protein           | K<sub>d</sub> ± S.E. |<br>nm |
|-------------------|----------------------|------|
| Wild type RGS2    | 17.6 ± 2             |
| L45D              | 8.6 ± 3              |
| LL-RA             | 9.1 ± 2              |
| EN-AA             | NB<sup>a</sup>       |

<sup>a</sup> No binding.

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The amino terminus of RGS2 directs plasma membrane localization by facilitating the amphipathic nature of the helical domain. Notably, disruption of the hydrophobic surface of the helical domain is known to prevent RGS2 interaction with the phospholipid bilayer, which could not exclude the possibility that the extended hydrophobic surface acted as a supplemental interaction site for G<sub>q</sub>. In this case, the increased function and plasma membrane targeting efficiency of RGS2 could be a consequence of its increased affinity for G<sub>q</sub>. The contribution of the RGS2 amphipathic helix domain toward G<sub>q</sub> binding was studied using the flow cytometry protein interaction assay as previously described by Neubig and co-workers (22). First, the affinity of histidine-tagged wild type and mutant RGS2 proteins for GDP-bound (inactive) and AIF<sub>5</sub>-activated G<sub>q</sub> was measured. RGS2 wild type, L45D, and L37R/L38A proteins all bound much more efficaciously to activated than to inactive G<sub>q</sub>, whereas the G-protein-binding defective mutant E108A/N109A did not bind to G<sub>q</sub> in either activation state (Fig. 9A). These data suggested that the RGS-box domain of RGS2 was necessary to promote G<sub>q</sub> binding, whereas the amino terminus could not direct G<sub>q</sub> binding in the context of a full-length RGS2 containing a nonfunctional RGS-box. We next used dissociation constant measurements to test whether the RGS2 amino terminus played an important ancillary role during G<sub>q</sub> binding. The K<sub>d</sub> of wild type RGS2 for activated G<sub>q</sub> was similar to that of L45D and L37R/L38A (Fig. 9B, summarized in Table 1), indicating that the amphipathic helix and flanking dileucine motif did not contribute significantly to G<sub>q</sub>-binding.

Interfering with Plasma Membrane Lipid Composition Disrupts Localization of RGS2 without Affecting M1 Muscarinic Receptor or G<sub>q</sub>—Last, we examined the role of anionic phospholipid head groups in targeting the unique RGS2 helical domain to the lipid bilayer. Specifically, we determined whether chemical disruption of the anionic lipid composition on the inner leaflet of the plasma membrane affected RGS2 localization. Mammalian cells continuously shuttle phosphatidylserine (PS) and phosphatidylethanolamine from the outer to the inner leaflet of the plasma membrane to maintain a higher concentration of anionic lipids on the cytoplasmic face. N-Ethylmaleimide (NEM) has been shown to rapidly inhibit PS shuttling, resulting in shuffling of phospholipid pools and increased exposure of PS on the extracellular surface. Treatment of HEK cells with NEM resulted in the expected level of lipid shuffling and extracellular exposure of PS, as indicated by fluorescein isothiocyanate-labeled annexin V-binding using fluorescence-activated cell-sorting analysis (Fig. 10A). Notably, as levels of PS on the inner leaflet decreased, RGS2 rapidly (<2 min) delocalized from this site. By contrast, M1 muscarinic receptor and G<sub>q</sub>, two other signaling proteins that are known to affect RGS2 localization, remained tightly associated with the lipid bilayer in NEM-treated cells (Fig. 10B). Together, these data suggest that the highly anionic lipid interface of the inner leaflet of the plasma membrane is required for stable RGS2 targeting. Moreover, it confirms our biochemical data showing that the amino terminus of RGS2 directs plasma membrane localization by factors that are independent from its G<sub>q</sub> or M1 muscarinic receptor binding. Thus, the differential affinity of RGS amphipathic helices for plasma membrane phospholipids appears to be a key determinant of RGS protein function that can explain the observed functional differences between RGS2 and RGS5.

**DISCUSSION**

To understand the biologic function of RGS proteins, we must first determine the spectrum of signaling pathways they regulate in living organisms. These efforts are complicated by the fact that most tissues express several RGS proteins with overlapping functions. Thus, a critical step is to define the key functional determinants that specify differences in regulation of G-protein signaling by different RGS proteins.

**RGS2 Is a More Potent Inhibitor of M1 Muscarinic Receptor-mediated Signaling Compared with RGS5**—When the activities of RGS2 and RGS5 as inhibitors of M1 muscarinic receptor signaling were compared in assays measuring phosphatidylinositol and intracellular calcium, RGS2 was found to be a more efficacious inhibitor of this receptor. These data appeared to
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Increased function of the isolated RGS-box domains, even in the absence of amino-terminal regulatory sequences.

Consistent with this notion, RGS2/5 chimeras showed that the RGS2 amino terminus is necessary to promote stable plasma membrane localization and increased RGS-box function in mammalian cells, whereas the RGS5 amino terminus lacks this key function. This was the first demonstration that functional amino/carboxyl-terminal chimeras can be used to study the relative function of different R4/B subfamily members. Although the RGS2 amino terminus is known to be sufficient to promote plasma membrane targeting, the relative functional contribution of phospholipid binding via the amphipathic helix and receptor binding via an unknown domain is unknown. Attempts to study the role of phospholipid binding using constitutive Goq (13) suggested that amphipathic helix-mediated lipid bilayer targeting of RGS2 was not critical for its function. Specifically, the L45D mutant did not bind to the plasma membrane but showed wild type inhibitory activity toward constitutively active Goq. The inconsistency between those and our current data may be the result of limitations in the previous experimental protocols. Our current studies allow us to select cells based on their level of RGS protein expression. Thus, we are able to compare individual cells with similar RGS protein expression. In previous experiments, overexpression may have masked any potential functional differences between wild type RGS2 and the L45D mutant. Furthermore, Mende and co-workers (14) have recently shown that in the presence of constitutive Goq, RGS2 is dramatically up-regulated, leading to its even higher overexpression. Our current experimental protocol allowed us to titrate expression levels and examine the more subtle differences between RGS mutants; therefore, we felt it was important to characterize the functional contribution of the amphipathic helix in this assay.

Extension of the Hydrophilic Face of the RGS2 Amphipathic α Helix Promotes Plasma Membrane Association—The molecular characteristics of amphipathic helix function in the R4/B subfamily have not been rigorously studied. Chimera studies showed that the amino-terminal domain of RGS2 was able to direct the RGS-box domains of both RGS2 and RGS5 to the plasma membrane, whereas the amino-terminal domain of RGS5 lacked this important function. By mutating the RGS2 amphipathic α helix domain (L45D), we demonstrated the importance of this feature in mediating both plasma membrane localization and increased relative function of RGS2 and RGS5. Although RGS2 and RGS5 both contain amphipathic helix domains in the same position relative to their RGS-box, sequence alignment revealed two unique leucine residues and two other basic residues that we predicted should extend the length of its amphipathic nature by one additional turn of the helix. We thus hypothesized that the extended helix in RGS2 contributes to its more efficient association with the lipid bilayer component of the plasma membrane. In support of this model, replacement of the two leucine residues in RGS2 with their less hydrophobic counterparts in RGS5 (arginine and alanine) was sufficient to disrupt the majority of plasma membrane localization and function. Thus, for the first time, our studies prove that the length of the amphipathic surface of the amino-terminal helical domain is a critical functional determin-

contradict those of Anger et al. (14), who reported no difference in the function of RGS2 and RGS5 as inhibitors of M3 muscarinic receptor signaling. There are two potential differences in the experimental design of the two studies that may explain this discrepancy. First, we used M1 muscarinic receptor compared with M3 muscarinic receptor used in the study by Anger et al. (14). Although it is possible that receptor-selective mechanisms explain this discrepancy, RGS2 has been shown to inhibit both M1 muscarinic receptor and M3 muscarinic receptor signaling with higher relative efficiency relative to other R4/B class proteins (15, 16). Second, our study measured RGS activity under conditions of similar RGS protein expression as determined by either Western blotting or relative green fluorescent protein fluorescence. By contrast, Anger et al. (14) measured RGS activities relative to the amount of plasmid used per transfection, a method that would not account for potential differences in the rates of synthesis or degradation of the different RGS proteins.

Constitutive Plasma Membrane Targeting Mediates Increased RGS Function in Mammalian Cells—We set out to determine whether the increased activity of RGS2 compared with RGS5 reflected its stronger constitutive association with plasma membrane. Support for this idea came initially from the observation that CAAX-box targeting to the lipid bilayer greatly

![Figure 10. Effect of NEM-mediated phospholipid shuffling on tonic plasma membrane localization of RGS2, M1 muscarinic receptor, and Goq. A, HEK cells were treated with medium with or without 10 mM NEM for 5 min before incubation with fluorescein isothiocyanate-conjugated annexin V. Phosphatidylserine exposure on the extracellular surface of the lipid bilayer was determined by the extent of annexin V binding using cell sorting analysis as previously described (30). B, HEK cells were transiently transfected with green fluorescent protein-tagged RGS2, RGS2-CAAX, M1 muscarinic receptor, or Goq (cotransfected with untagged Gq2 and Gq1). NEM was added to a final concentration of 10 mM for 5 min. Shown are representative confocal images of at least 50 low expressing cells captured before and after the addition of NEM.](image-url)
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nant of the function of the R4/B subgroup of RGS proteins. Furthermore, the only other R4/B subfamily member that has a similar amphipathic extension, RGS3, is also a more efficient inhibitor of muscarinic receptor signaling than RGS4 (16) and, like RGS2, can be more efficiently recruited to the plasma membrane by constitutive Goq expression (13, 18, 25). Recent studies have suggested that the RGS2 amino terminus can interact with several other proteins that impact its function. We specifically examined whether the amphipathic helix domain in RGS2 directed plasma membrane association via interactions with the M1 muscarinic receptor or Goq.

Mutations That Prevent RGS2 Plasma Membrane Localization Do Not Affect M1 Muscarinic Receptor Binding—Data from our group and others have shown that the amino terminus of RGS2 interacts with the intracellular loops of M1 muscarinic and other G-protein-coupled receptors (17, 23, 24). Although the direct mechanism mediating this interaction is still not known, it has been proposed that this model can account for the increased membrane targeting and function of RGS2 toward specific receptors (26). Our current studies show that mutations within the amino-terminal amphipathic α-helix, while disrupting both function and plasma membrane localization, do not affect receptor interaction.

Furthermore, NEM treatment causes the dissociation of RGS2 from the plasma membrane without affecting the M1 muscarinic receptor localization. Thus, although it is possible that receptor association is one component required for plasma membrane targeting, it is probably not the primary determinant. One potential possibility that would incorporate both of these models is that once RGS2 is stably associated with the plasma membrane via its extended amphipathic helix, its ability to interact with receptor imparts an ability to select specific targets from within the receptorome. Future studies in this area could also be used to elucidate the mechanisms that explain the reported selectivity of RGS5 for the angiotensin II receptor AT1 (27).

Mutations That Prevent RGS2 Plasma Membrane Localization Do Not Affect Goq Binding—Interaction between the RG5-box domain and Goq is critical for proper RGS2 function. The G-protein binding-defective E108A/N109A mutation (EN-AA) of RGS2 does not have measurable affinity for activated Goq and does not inhibit calcium signaling in our assay. Our mutations resulting in both total disruption of helix function (L45D) or removal of the dileucine motif (LL-RA) both resulted in large changes in localization and function without changing RGS2 affinity for Goq. Finally, NEM treatment resulted in the disassociation of RGS2 from the plasma membrane without any change in Goq localization. Together, these data suggest that, in the case of the M1 receptor interaction, Goq-RGS2 interactions are not sufficient to promote plasma membrane localization and increased relative function.

Extended Hydrophobic Helix of RGS2 Binds Anionic Phospholipids on the Plasmid Membrane—Taken together, our data show that disruption of RGS2 plasma membrane localization and function occur independently from both receptor and Goq interaction. The fact that NEM, a compound that alters anionic phospholipid concentrations on the plasma membrane, is able to decrease RGS2 localization provides mechanistic support for our hypothesis that it is the lipid bilayer that binds to the elongated amphipathic helix of RGS2. Although the possibility does remain that interaction between RGS2 and another NEM-sensitive factor recruits RGS2 to the plasma membrane, no such factor has yet been identified. Indeed, all of our present data point to the phospholipid bilayer as the most likely target of this specialized helix domain.

Can the Length of the Amphipathic Helix be Regulated by Palmitoylation in Mammalian Cells?—If the lipid bilayer is indeed the primary target of the RGS helices, it raises the possibility that palmitoylation of a conserved cysteine at position 12 in RGS5, RGS4, and RGS16 could extend the hydrophobic face of their respective amphipathic helices (see Fig. 7) and thus provide a potential regulatory site for membrane targeting and function. Consistent with this notion, alanine mutations at this position have been shown to inhibit the localization and function of RGS16 (28, 29). It remains to be demonstrated whether RGS5 is similarly modified and, if so, whether constitutively palmitoylated RGS4, RGS5, or RGS16 proteins would show increased efficiency of membrane association and function in mammalian cells.

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