RGS proteins serve as GTPase-activating proteins and/or effector antagonists to modulate Gα signaling events. In live cells, members of the B/R4 subfamily of RGS proteins selectively modulate G protein signaling depending on the associated receptor (GPCR). Here we examine whether GPCRs selectively recruit RGS proteins to modulate linked G protein signaling. We report the novel finding that RGS2 binds directly to the third intracellular (i3) loop of the Gq/11-coupled M1 muscarinic cholinergic receptor (M1 mAChR; M1i3). This interaction is selective because closely related RGS16 does not bind M1i3, and neither RGS2 nor RGS16 binds to the Gqα-coupled M2 muscarinic receptor (M2 mAChR; M2i3). This interaction is therefore blocked by M2i3 membranes. These findings predict a model where the i3 loops of GPCRs selectively recruit specific RGS protein(s) via their N termini to regulate the linked G protein. Consistent with this model, we find that the i3 loops of the mAChR subtypes (M1–M5) exhibit differential profiles for binding distinct B/R4 RGS family members, indicating that this novel mechanism for GPCR modulation of RGS signaling may generally extend to other receptors and RGS proteins.

Cells rely upon G protein-coupled receptors (GPCRs)1 to convey signals from extracellular hormones and neurotransmitters to intracellular effectors and linked signaling pathways. Agonist occupancy of the GPCR activates a heterotrimeric G protein (Gαβγ) by catalyzing the exchange of GDP for GTP on the Gα subunit (1). This initiates dissociation of the trimer into free Ga and Gβγ, which independently or in coordinated fashion activate downstream effectors and linked signaling pathways. Members of the regulators of G protein signaling (RGS) family of proteins are direct modulators of G protein activity. RGS proteins are best understood as GTPase-activating proteins (GAPs), which bind to the activated form of Gα and accelerate its GTPase activity thereby promoting the termination of G protein signaling (2–5). By virtue of their interactions with activated Ga, RGS proteins also serve as effector antagonists to block activation of downstream effector molecules (6, 7).

All RGS proteins share a conserved RGS core domain of ~130 amino acids that contains binding sites for Gα and is responsible for their GAP activity (5, 8, 9). Outside of the RGS domain, however, the more than 30 family members are widely divergent. Some RGS proteins are quite complex and contain multiple domains for binding a variety of signaling proteins. Other RGS proteins are simple, with relatively short, featureless N- and C-terminal regions flanking the RGS domain. RGS proteins are classified into at least six subfamilies based on RGS domain identities, and related subfamily members also share conserved regions outside of their RGS domains (5, 9). The B/R4 subfamily contains the simplest of RGS proteins. Members of this subfamily (RGS1, 2, 3, 4, 5, 6, 13, and 16) share a conserved amphipathic helix near their N termini but have no other identifiable features outside of their RGS domains. These family members are often considered prototypical because they do not appear to have functions outside of modulating G protein activity.

Although the biochemical mechanism of GAP activity and structure of RGS proteins has been well characterized, much less is understood about the regulation of RGS proteins in cells (8). Of particular interest is how RGS proteins can function in cells to selectively control the activity of specific G proteins. In single-turnover GTP-hydrolysis assays and G protein binding experiments using purified proteins, many studies have demonstrated that most B/R4 RGS proteins share overlapping pro-thione S-transferase; CHO, Chinese hamster ovary cells; PMSF, phenylmethylsulfonyl fluoride; PiPγ, phosphatidylinositol 4,5-bisphosphate; PE, phosphatidylethanolamine; AMF, AlCl3 + MgCl2 + NaF; WT, wild type; BSA, bovine serum albumin; PLC, phospholipase C; DTT, di-thiothreitol; PBS, phosphate-buffered saline; HA, hemagglutinin; AppNH2p, adenylyl-5′-yl β,y-imidodiphosphate; GDPβS, guanyl-5′-yl thiotriphosphate; GTPγS, guanyl-5′-yl thiotriphosphate.

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files of G protein interactions and nonselectively bind to and/or inhibit signaling by \( G_{\alpha_i}, G_{\alpha_o}, G_{\alpha} \) and \( G_{\alpha} \) (Refs. 5 and 9 and references therein). However, more recent studies have suggested that B/R4 RGS proteins can distinguish among receptor signaling pathways that are coupled to the same G protein when examined in the context of a living cell. One study demonstrated that recombinant RGS1, -2, -4, and -16 displayed different relative potencies of inhibiting signaling through \( G_{\alpha_i} \)-linked muscarinic cholinergic, bombesin, or cholecystokinin receptors, suggesting that the particular GPCR could dictate RGS actions (10). Similarly, selective disruption of endogenous expression of RGS3 or RGS5 mRNA and protein in rat vascular smooth muscle cells differentially enhanced cellular responses to carbachol or angiotensin, respectively (11). Consistent with these findings, a separate study showed that RGS3, but not RGS1, -2, or -4, specifically inhibited gonadotropin-releasing hormone receptor-mediated PI turnover in transfected COS cells (12). The idea thatGPCRs determine the specificity of RGS signaling in cells is also supported by a recent finding that RGS proteins selectively translocated from the cytoplasm or nucleus to the plasma membrane of a cell when co-transfected with a functionally coupled receptor (13). Taken together, these results suggest that GPCRs, either alone or in coordination with their linked G protein, can selectively recruit certain RGS proteins to the plasma membrane to determine their signaling functions, and point to a model in which GPCRs possess intrinsic differential affinities for RGS proteins. Based on their differing affinities for RGS protein recognition, GPCRs may recruit a specific RGS protein to act on its downstream G protein signaling pathway(14, 15).

Molecular mechanisms underlying functional coupling between certain GPCR and RGS proteins have remained obscure. One complex RGS protein (RGS12) contains a PDZ domain that may bind one GPCR (16). However, no other RGS proteins contain PDZ domains and there has not yet been any demonstration of a direct GPCR-RGS interaction. In this study, we begin to examine whether there is a direct physical interaction between GPCRs and GPCRs that could dictate selectivity. For these studies, we used members of the B/4 subfamily of RGS proteins and muscarinic acetylcholine receptor (mAChR) subtypes as prototype protein binding partners. The mAChRs contain PDZ domains and there has not yet been any demonstration of RGS interactions with them. Based on their differing affinities for RGS protein binding, the mAChRs possess intrinsic differential affinities for RGS proteins. For these studies, we used members of the B/4 subfamily of RGS proteins and muscarinic acetylcholine receptor (mAChR) subtypes as prototype protein binding partners. The mAChRs contain PDZ domains and there has not yet been any demonstration of RGS interactions with them.

**Induction and Purification of GST Fusion Proteins**

GST-mACHR i3 loop fusion proteins (GST-M13, GST-M23, GST-M33, GST-M43, and GST-M53) have been described previously (22). GSF-Tagged RGS Protein Constructs—All constructs were verified by sequencing.

**GST-His pET20b Constructs—** RGS2-HA, RGS5-HA, and \( \Delta \text{NRGS2}-\text{HA} \) were cloned into the pET20b vector (Novagen) for bacterial expression with a C-terminal hexahistidine tag. Each insert was amplified as a NdeI-NotI fragment from the corresponding pCDNA3.1 construct for insertion into pET20b. For RGS2 the primers were 5'-CAGGTACCATCGTGGTCAGAAGCATTTGG-3' (sense) and 5'-CTTAGGTTTGAAGGCTCAGC-3' (antisense). For \( \Delta \text{NRGS2}-\text{HA} \) the primers were 5'-GGGTTTAAAATCAGTGTTACCA-3' (sense) and 5'-CTAGAAGCCTTGATGAAAGCCTGCGAC-3' (antisense). For these constructs were verified by sequencing.

**Glutathione S-Transferase (GST)-mACHR i3 Constructs—**

GST-His pET20b—RGS2-HA, RGS5-HA, and \( \Delta \text{NRGS2}-\text{HA} \) were cloned into the pET20b vector (Novagen) for bacterial expression with a C-terminal hexahistidine tag. Each insert was amplified as a NdeI-NotI fragment from the corresponding pCDNA3.1 construct for insertion into pET20b. For RGS2 the primers were 5'-CAGGTACCATCGTGGTCAGAAGCATTTGG-3' (sense) and 5'-CTTAGGTTTGAAGGCTCAGC-3' (antisense). For \( \Delta \text{NRGS2}-\text{HA} \) the primers were 5'-GGGTTTAAAATCAGTGTTACCA-3' (sense) and 5'-CTAGAAGCCTTGATGAAAGCCTGCGAC-3' (antisense). For \( \Delta \text{NRGS2}-\text{HA} \) the primers were 5'-GGGTTTAAAATCAGTGTTACCA-3' (sense) and 5'-CTAGAAGCCTTGATGAAAGCCTGCGAC-3' (antisense). For these constructs were verified by sequencing.

**GST-His Constructs—** The construct for expression of C-terminally His-tagged G\( \alpha \) (G\( \alpha \)-CH6) was described in Ref. 23.
side for 2 h at 37 °C. Cells were pelleted, and the cell pellets were suspended in 25 ml of harvest buffer (10 mM HEPES, pH 8.0, 50 mM NaCl, 5 mM EDTA, 0.5% Triton X-100) supplemented with Complete Mini protease inhibitor mixture (Roche Diagnostics GmbH) and lysozyme. The pellets were then frozen at −80 °C overnight. Samples were thawed and sonicated, followed by centrifugation at 15,000 × g, 4 °C to collect soluble material. Streptomycin sulfate (30 mg) was added to precipitate remaining nucleic acid material, and the samples were centrifuged a third time to produce the soluble bacterial lysate. The lysates were incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) for 1 h, 4 °C, rotating end-over-end to bind the fusion proteins. Protein-bead complexes were washed three times with harvest buffer and then stored as a slurry in harvest buffer at −80 °C. In some cases, the volume of GST protein used for each protein varied among the GST fusion proteins. For each protein used, a single common batch of protein/beads were generated and aliquoted for freezing and storage at −80 °C. Direct quantitation of total bound protein by Bradford assay was unsuccessful because of nonspecific interaction of the resin with Bradford reagent. Therefore, the concentration of protein bound was compared by comparison of Coomassie staining intensity of GST fusion proteins with a known amount of protein standard (BSA). For experiments, the volume of beads used for each protein was adjusted to ensure that the same amount of total protein was used for binding interactions.

**Cell Culture, Transfection, and Preparation of CHO Cell Lysates**

Wild-type CHO-K1 cells (WT-CHO) were grown and maintained in Dulbecco’s modified Eagle’s medium (CellGro) containing 10% fetal bovine serum (Atlanta Biologicals), 1× nonessential amino acids (CellGro), and penicillin/streptomycin. To produce cell lysates containing RGS-HA proteins, WT-CHO were grown to 80–90% confluence in 10-cm dishes and transfected with LipofectAMINE reagent (Invitrogen) using the protocol from the manufacturer. 24–36 h after transfection, cells were washed once with PBS and then lysed in harvest buffer containing 1% Triton X-100 containing a protease inhibitor mixture (Roche Diagnostics GmbH) (200 μl/plate). Lysates were scraped off the dishes using a razor blade and incubated at 4 °C, rotating end-over-end for 30–60 min to solubilize the cells. Lysates were subjected to centrifugation at 300,000 × g (75,000 rpm) for 15 min in a TLA100.3 rotor to separate the insoluble material. The soluble lysate was diluted to 0.5% Triton X-100 in harvest buffer, then aliquoted and snap-frozen for use in GST pull-down assays. Lysates expressing each RGS-HA protein were assessed by Western blot using the HA antibody to determine the relative concentrations of RGS proteins.

**Laser Confocal Microscopy**

CHO cells transiently transfected with cDNA constructs (RGS2-GFP alone, RGS16-GFP alone, or these constructs co-transfected with cDNA encoding HA-M1 mACHr) were grown on sterile coverslips, fixed for 2 h at room temperature, then 20 min on ice. GST or GST-M1i3 bound to glutathione-Sepharose beads (10–50 μl) with harvest buffer and protease inhibitors. Equal amounts of GST fusion proteins were added as determined by Coomassie staining. The total reaction volume was 250 μl. Reactions were incubated by rotating overnight at 4 °C. Beads were collected by centrifugation at 500 × g for 5 min at 4 °C and washed one time with harvest buffer, then one time with harvest buffer without Triton X-100. Bound proteins were eluted from the beads by the addition of 2× SDS sample buffer. Bound RGS proteins were detected by Western blot. For experiments in which Gαi-His was included, RGS2-His and Gαi-His were pre-incubated in harvest buffer along with 5 mM NaF, 5 mM MgCl2, 30 μM α-Ct, and 1 mM GFP for 10 min at room temperature, then 20 min on ice. GST or GST-M1 bound to glutathione-Sepharose beads was then added to the reaction and incubated and washed as above. Samples were boiled for 1 min, centrifuged briefly to pellet the beads, resolved on 13.5% SDS-PAGE, and transferred to nitrocellulose for Western blot analysis.

**Immunoblots**

Nitrocellulose membranes were incubated in blocking buffer (Tris-buffered saline with 5% milk, 0.5% Tween 20, 0.02% sodium azide) 1 h at room temperature or overnight at 4 °C. Membranes were then probed with either mouse anti-HA antibody (Covance) or mouse anti-His (Qiagen) antibodies diluted 1:1000 in blocking buffer for 1–2 h at room temperature. Membranes were washed three times with Tris-buffered saline + 0.1% Tween 20, then probed with horseradish peroxidase-conjugated goat anti-mouse (Rockland) diluted 1:2000 in Tris-buffered saline + 0.1% Tween 20. The protein bands were visualized using chemiluminescence and exposed to film.

**Measurement of PLCβ Activity in Cell Membranes**

Assays were performed as previously described (6) with the following modifications: CHO cells stably expressing the M1 mACHr (M1-CHO cells) were grown to confluence in 150-mm dishes (3–5 dishes/experiment). Cells were washed once with phosphate-buffered saline and then scraped from the dish in phosphate-buffered saline (5 ml/plate) using a razor blade. Cells were pelleted for 10 min in a clinical centrifuge. The pellet was resuspended in hypotonic buffer (1 ml/dish; 10 mM HEPES, pH 7.2, 1 mM EDTA) and Complete Mini protease inhibitor mixture (Roche Diagnostics GmbH). Cells were passed 20 times through a ball-bearing cell cracker (0.0012-inch clearance), and 250 μm sucrose in hypotonic buffer was added to the homogenate. The lysate was centrifuged for 10 min, 4 °C at 500 × g to pellet the cell nuclei and unbroken cells. The supernatant was then subjected to centrifugation at 100,000 × g in a TLA100.3 rotor for 30 min at 4 °C to separate cell membranes. The resulting membrane pellet was suspended in membrane buffer (250 μl/plate; 50 mM HEPES, pH 7.2, 1 mM EDTA, 3 mM EGTA, 5 mM MgCl2, 2 mM DTT, 100 mM NaCl, and PMSF) by 10 strokes with a Dounce homogenizer. M1-CHO membranes (10 μl/reaction) were pre-incubated on ice with purified RGS-His in assay buffer (50 mM HEPES, pH 7.2, 3 mM EGTA, 1 mM DTT, 80 mM KCl, and PMSF) for 30–90 min on ice to allow interaction of the RGS and M1 mACHRs. Next, 100 μM carbachol, 3 μM GTPγS, 10 mM GDPβS were added along with [3H]IP/PE vesicles.
Reactions were initiated by the addition of 1.3 mM CaCl₂ for a total volume of 70 μl and incubated at 30 °C for 40 min. Reactions were terminated by precipitation with 250 μl of 10% trichloroacetic acid and 100 μl of 10 mg/ml BSA. Samples were centrifuged at 200 × g for 10 min. The soluble supernatants containing [3H]inositol phosphates were counted in a scintillation counter.

**Measurement of Steady State GTPase Activity in Sf9 Insect Cell Membranes**

Sf9 insect cells grown to a density of 2 × 10⁶ cells/ml were triply infected with baculoviruses encoding M1-G₁,α fusion protein, G₂β, and G₃γ. The baculovirus encoding M1-G₁,α fusion protein was kindly provided by Dr. Tatsuya Haga (Gakushuin University, Tokyo, Japan) and baculoviruses encoding G₂β and G₃γ were a gift from Dr. Terry Hebert (Montreal Heart Institute, Montreal, Quebec, Canada) (25). Following a 48-h infection at 27 °C, membranes were prepared essentially as described (25). The cells were harvested by centrifugation (228 × g), washed with PBS, and finally resuspended in lysis buffer (20 mM Tris, pH 8.0, 0.1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin). After a 10-min wait on ice, the cells were disrupted by Polytron and centrifuged at 500 × g to remove any whole cells, and the membrane fraction pelleted at 48,000 × g for 30 min, resuspended in 0.1 volume of the lysis buffer, aliquoted, and stored at ~80 °C. The membranes were assessed for muscarinic receptor density using [3H]quinuclidinyl benzilate and found to contain 2.3 fmol of specific binding sites/assessed for muscarinic receptor density using [3H]quinuclidinyl benzilate and found to contain 2.3 fmol of specific binding sites/assessed for muscarinic receptor density using [3H]quinuclidinyl benzilate and found to contain 2.3 fmol of specific binding sites.

Steady state GTPase assays were adapted from a previously described method (25). Briefly, membranes (6 μg of protein/assay) from Sf9 cells expressing M1 mACHR-G₁α fusion protein plus G₂β and G₃γ were preincubated with drugs and purified RGS proteins on ice for 1 h, at which time nucleotides were added to yield 40-μl reaction mixtures containing 20 mM HEPES, pH 7.5, 1 mM EDTA, 2 mM MgCl₂, 10 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 0.3 μM GTP, 1 mM ATP, 0.1 mM App[NH]₃p, and 1 × 10⁶ cpm/assay [³²P]GTP. The assay tubes were incubated at 30 °C for exactly 5 min and the reaction terminated by the addition of 960 μl of ice-cold 5% Norit in 50 mM NaH₂PO₄, pH 3. The mix was centrifuged (2000 g) at 4 °C, membranes were prepared essentially as described (25). The scaled value was subtracted from experimental measurement. The nonspecific signal was estimated as described (25). Membrane GTPase signal was estimated by adding 1 mM unlabeled GTP to the above assay mix and this value subtracted off the total cpm. In each experiment, separate controls were carried out to determine the GTPase activity attributable to the purified RGS protein preparations. This was taken as the difference in [³²P], between samples with and without maximal levels of RGS proteins that lacking membranes, and then scaled according to the GRS protein concentration at each assay point. The scaled value was subtracted from experimental measurements of membrane-dependent GTPase activity as appropriate. Agonist-dependent GTPase activity was taken as that observed in the presence of the muscarinic receptor agonist carbachol (100 μM) minus that in the presence of the inverse agonist tropicamide (10 μM). Of the materials used for these assays, [³²P]GTP was purchased from ICN and PerkinElmer Life Sciences. GTP, ATP, App[NH]₃p, carbachol, tropicamide, and PMSF were purchased from Sigma. Leupeptin and aprotinin were purchased from Roche. Cell culture reagents were purchased from Invitrogen.

**RESULTS**

**Interaction of RGS2 with the Third Intracellular Loop of M1 mACHR**—Based on recent observations that B/R4 RGS proteins can selectively inhibit signaling through different GPCRs (10–12), we hypothesized that these RGS proteins interact selectively with functionally preferred receptors. We used the mACHRs as model GPCRs to test this idea. To determine whether RGS proteins form a stable complex with mACHRs, we took advantage of an affinity binding “pull-down” assay that has been used previously to identify binding partners for other GPCRs (19–21, 26). Fusion proteins consisting of GST with the intracellular third loop of the M1 and M2 mACHRs (GST-M13 and GST-M23) or GST alone were expressed in bacteria and recovered by binding to glutathione-Sepharose beads. RGS proteins with C-terminal HA tags (RGS-HA) were transiently transfected in CHO cells. Total cell lysates of these cells (Fig. 1A) were incubated with equal protein amounts of the immobilized GST fusion proteins (Fig. 1B). After the beads were collected and washed, the bound proteins were eluted by the addition of SDS sample buffer. Samples were subjected to SDS-PAGE and immunoblotting with the HA antibody to detect bound RGS proteins. A, left lane (input), samples represent the total amount of RGS-HA in the cell lysates that were used for each reaction. Results are representative of at least three independent experiments. IB, immunoblotting. B, Coomassie stain of GST, GST-M13, and GST-M23 proteins (1 μg each) used in A.

![Fig. 1. The M13 loop selectively interacts with RGS2-HA from CHO cell lysates.](http://www.jbc.org/)

**Direct RGS2-M1 mAChR Interactions**

In Intact Cells—Thus far, our studies indicate that RGS2 and M1 mAChR form a direct physical complex, which supports the idea that these proteins are functionally paired. However, for functional pairing to occur in a cell, these proteins also must co-localize in an intact cellular environment. We therefore tested whether RGS2 and M1 mAChR are present at the same subcellular location when expressed together in the same cell (Fig. 3). When CHO cells were transfected with plasmid encoding RGS2-GFP alone, RGS2 was found predominantly at the plasma membrane and also in the nucleus in all cells observed (Fig. 3A). When co-expressed with M1 mAChR containing an HA epitope tag (HA-M1), RGS2-GFP and HA-M1 were both found together at the plasma membrane (Fig. 3B). This observed distribution pattern for RGS2 is consistent with previ...
purified RGS2-His or RGS16-His (2 μg each) were incubated with equal amounts (6.5 μg) of GST-M1i3 or GST alone bound to glutathione-Sepharose, and reactions were processed as in Fig. 1. Western blot analysis was performed using an anti-His antibody. Results are representative of at least two independent experiments. A, RGS2-His, but not RGS16-His bound to the M1i3 loop. Left lane (input), samples represent the total amount of purified protein used for each reaction. B, increasing concentrations of purified RGS2-His or RGS16-His from E. coli were incubated with GST or GST-M1i3 (0.5 μg each) as indicated. Immunoblot of actual total RGS protein inputs are shown in the bottom row.

FIG. 2. RGS2 binding to the M1i3 loop is direct. RGS2-His and RGS16-His were expressed and purified from E. coli using nickel-nitrilotriacetic acid affinity chromatography (see “Experimental Procedures”). Purified RGS2-His or RGS16-His (2 μg each) were incubated in cell membranes (Fig. 5). Purified RGS2-His and RGS16-His were expressed and purified from E. coli using nickel-nitrilotriacetic acid affinity chromatography (see “Experimental Procedures”). Purified RGS2-His or RGS16-His (2 μg each) were incubated with equal amounts (6.5 μg) of GST-M1i3 or GST alone bound to glutathione-Sepharose, and reactions were processed as in Fig. 1. Western blot analysis was performed using an anti-His antibody. Results are representative of at least two independent experiments. A, RGS2-His, but not RGS16-His bound to the M1i3 loop. Left lane (input), samples represent the total amount of purified protein used for each reaction. B, increasing concentrations of purified RGS2-His or RGS16-His from E. coli were incubated with GST or GST-M1i3 (0.5 μg each) as indicated. Immunoblot of actual total RGS protein inputs are shown in the bottom row.

FIG. 3. RGS2 and M1 mAChR co-localize at the plasma membrane of CHO cells. CHO cells were transiently transfected with plasmid eDNA encoding GFP-tagged RGS2 (RGS2-GFP) (A), HA-tagged full-length human M1 mAChR (HA-M1) plus RGS2-GFP (B), RGS16-GFP (C), or HA-M1 plus RGS16-GFP (D). After 36 h of transfection, cells were fixed and visualized using laser confocal microscopy as detailed under “Experimental Procedures.” Green GFP fluorescence at 510 nm (left), anti-HA rhodamine red fluorescence at 522 nm (middle: rhod), and merged images (right) are depicted. Yellow in the merged images indicates overlapping localization. Each image is representative of at least 24 cells examined for each condition derived from three independent experiments.

These data suggest that membrane-targeting factors intrinsic to RGS2 are sufficient for its localization at the plasma membrane, as is the case for RGS4-terminus (28). In contrast, RGS16-GFP localized predominantly to the cytosol and nucleus when expressed alone in CHO cells (Fig. 3C). This localization pattern was observed for RGS16 in all cells and also when it was co-expressed with HA-M1, which localized to the plasma membrane (Fig. 3D). When localization patterns of RGS2 were examined in HEK293 cells, RGS2-GFP was visualized by laser confocal microscopy at the plasma membrane in 31% of cells heterologously expressing that protein only (29 cells), and in 73% of cells additionally expressing the M1 receptor (26 cells).2 Taken together, these findings indicate that RGS2 and M1 mAChR are both present at the plasma membrane when co-expressed in the same cell, and support the idea that these two proteins are targeted to the same subcellular location necessary for functional coupling.

The M1i3 Loop Interacts with the N Terminus of RGS2—Previous studies have demonstrated that the N terminus of RGS4 is important for its functional selectivity among receptor pathways (29). Therefore, we tested whether the N terminus of RGS2 is responsible for its interactions with M1i3. Full-length RGS2-HA, RGS16-HA, and a truncation mutant of RGS2 in which the first 78 amino acids of the protein were deleted (ΔNRGS2-HA) were each expressed in CHO cells. Lysates from these cells were incubated with GST-M1i3 or GST alone. We found that, whereas full-length RGS2-HA bound as before, neither RGS16-HA nor ΔNRGS2-HA bound to GST-M1i3 (Fig. 4). This result indicates that sites within the N-terminal region upstream of the RGS domain of RGS2 are necessary for receptor binding. To determine whether the RGS2 N terminus is sufficient for receptor binding, we designed a chimeric protein in which the first 71 amino acids of RGS2 were fused to the RGS domain and C terminus of RGS16 (N2/RGS16-HA). When lysates from CHO cells expressing N2/RGS16-HA were incubated with GST-M1i3, we found that, unlike WT RGS16-HA, the chimera did interact with the receptor (Fig. 4). Thus, the N terminus is both necessary and sufficient for RGS2 binding to the M1i3 loop.

The N Terminus Is Required for RGS2 Modulation of M1 mAChR Signaling—In an attempt to examine the functional significance of the interaction between RGS2 and M1i3, we tested the relative capacities of RGS2, RGS16, and a truncated form of RGS2 missing its first 78 N-terminal amino acids (ΔNRGS2) to inhibit M1 mAChR- and Gq/11-mediated activation of phospholipase Cβ in cell membranes (Fig. 5). Purified

2 L. Baert and P. Chidiac, unpublished observation.
then incubated with PIP2/PE vesicles containing \([3H]\)PIP2 tracer in a receptor contribution to the signaling event (see Ref. 6). Membranes were interactions. Experimental conditions were designed to optimize the re-
mixture of \(3/100\)M carbachol (100 \(\mu\)M GTP \(\gamma\S\) and 10 \(\mu\)M GDP\(\beta\S\), in the presence or absence of carbachol (100 \(\mu\)M). The excess GDP\(\beta\S\) inhibits receptor-independent activation of the membrane G protein, but the GTP\(\beta\S\)/GDP\(\beta\S\) mixture permits agonist/receptor-stimulated nucleotide loading of G\(\alpha\). CaCl\(_2\) was added to start the reactions. The reactions were incubated at 30 °C for 30 min, then terminated by the addition of trichloroacetic acid and BSA to precipitate membranes and unhydrolyzed lipids. The superna-
tant containing \(\Delta\text{HIP}_{2}\) (inositol (1,4,5) triphosphate) was collected and radiolabel measured. At each concentration, average cpn in the absence of carbachol was subtracted from the carbachol-stimulated values to give the carbachol-dependent activity. This was expressed as a percentage of total PIP\(_2\) hydrolysis in the absence of RGS. Maximal activity resulting from guanine nucleotides in the absence of carbachol was 39.9% of the total activity in the presence of carbachol. The data are pooled results from two individual experiments, each performed in duplicate, and are representative of results from two to four total experiments for each protein. C, Sf9 insect cell membranes containing baculovirally expressed M1 mAChR-G\(\alpha\) fusion protein and G\(\beta\gamma\) were incubated with increasing concentrations of purified RGS2-His (circles), RGS16-His (squares), or \(\Delta\text{NRGS2}-\text{His}\) (triangles) to promote receptor-RGS in-
teractions. Experimental conditions were designed to optimize the re-
ceptor contribution to the signaling event (see Ref. 6). Membranes were therefore incubated with 1 mM GDP in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of AMF (5 mM NaF, 5 mM MgCl\(_2\), 30 \(\mu\)M AlCl\(_3\)) for 10 min at room temperature, then for 20 min on ice. GST-M1i3 bound to glutathione-Sepharose beads were added to the reactions and incubated overnight at 4 °C with rotation. Beads were collected by centrifugation and washed as described. Bound proteins were eluted by suspension of the beads in SDS sample buffer. Proteins were subjected to SDS-PAGE, transferred, and detected by Western blot using an anti-His antibody. Leftmost two lanes (input), samples represent total amount of G\(\alpha\)-His (50 pmol) and RGS2-His (40 pmol) added to the reactions. Results are representative of three independent experiments.

RGS-His proteins (Fig. 5A) were incubated with membranes from CHO cells stably expressing M1 mAChRs. Experimental conditions were modified to favor measurement of receptor-
directed phosphoinositide signaling (see Ref. 6). To the reaction, \(\Delta\text{HIP}_{2}/\text{PE vesicles with GTP}\gamma\S\) and GDP\(\beta\S\) were added in the presence or absence of 100 \(\mu\)M carbachol as described under “Experimental Procedures.” The presence of a nonhydrolyzable guanine nucleotide analog such as GTPyS is required for G\(11\)\(\alpha\) activity in mammalian cell membranes (6). By includ-
ing GDP\(\beta\S\), PLC\(\beta\) activity resulting from receptor uncoupled G\(11\)\(\alpha\) is suppressed, whereas receptor-catalyzed G\(11\)\(\alpha\) activity is enhanced. Although G\(11\)\(\alpha\) cannot hydrolyze GTPyS, RGS inhibition of PLC\(\beta\) activity is readily measured under these conditions because of the effector antagonist effects of RGS (6). We found that RGS2 could inhibit carbachol-stimu-
lated PIP\(_2\) hydrolysis and inositol 1,4,5-triphosphate accumulation at concentrations much lower than was observed with RGS16 (Fig. 5B). Furthermore, deletion of the N terminus of RGS2 severely reduced its capacity to inhibit PLC\(\beta\) activity in this assay (Fig. 5B). These findings are consistent with the biochemical selectivity of M1i3 binding affinity for RGS2 over RGS16 (Figs. 1 and 2) and the importance of the N terminus for RGS binding (Fig. 4). Although full-length RGS2 inhibited activity by almost 90% at a concentration of 10 \(\mu\)M, \(\Delta\text{NRGS2}\) inhibited activity by only 5–10% at 100 \(\mu\)M concentration. At a concentration of 1 \(\mu\)M, \(\Delta\text{NRGS2}\) inhibited M1 mAChR-mediated membrane PLC\(\beta\) activity in membranes by 53% (data not shown), indicating that the protein retains some RGS effector antagonist activity, but is much less potent than full-length RGS2. A similar marked reduction in activity upon deletion of the N terminus has been observed for RGS4 (29). In separate

\[\text{G}_\alpha \text{His} \quad \Delta\text{NRGS2}\text{-His} \quad \text{RGS2}\text{-His}\]
have demonstrated here that RGS2 binds the receptor through its N terminus. It therefore seems reasonable that RGS2 could interact with both M1 mAChR and with Gq,α at the same time to effectively modulate the pathway. To test this idea, we examined whether RGS2 can form a stable complex with both the M13 loop and Gq,α (Fig. 6). We pre-incubated RGS2-His and Gq,α-His with GDP in the absence or presence of AlF4− and Mg2+/AMF. AMF activation places Gq,α in a conformation that is most favored for RGS2 binding (31). GST-M13 bound to glutathione-Sepharose beads was then added to the samples for pull-down assays. We found that, whereas RGS2 alone bound to M13 (Fig. 6, lanes 1 and 2), Gq,α alone did not associate with the M13 loop (Fig. 6, lanes 3 and 4). When RGS2 and Gq,α were preincubated together in the presence of GDP, very little or no Gq,α associated with the RGS2 that bound to M13 (Fig. 6, lane 5). However, in the presence of AMF, RGS2 and Gq,α formed a stable heterotrimERIC complex with the M13 loop (Fig. 6, lane 6). These findings indicate that RGS2 can bind both activated Gq,α and the M13 loop simultaneously through distinct domains.

Differential Binding Profiles of Various RGS Proteins with the i3 Loops of the Five mAChR Subtypes—Our studies suggest a model for selective recruitment by M1 mAChR of target RGS proteins. If the interaction of RGS proteins with receptor i3 loops is a means for selective coupling in cells, then we would expect differential profiles of RGS binding among different receptor i3 loops. To test whether the selectivity of RGS2/M1 can be extended to the other B/R4 family members and mAChRs, we examined the interactions of RGS1, RGS2, RGS4, and RGS16 with each of the five mAChR i3 loops (M1–M5) (Fig. 7). Each of the HA-tagged RGS proteins was expressed in CHO cells (Fig. 7A). Pull-down assays were performed on lysates from these cells with the GST fusion proteins (Fig. 7B). In addition to its interactions with M1, we found that RGS2 bound strongly to GST-M5i3 and weakly to GST-M3i3. RGS4 also bound M13 and M5i3 loops. RGS16 bound GST-M3i3 weakly, whereas RGS1 did not appear to interact with any of the i3 loops. None of the RGS proteins tested bound to the M2i3 or M4i3 loops. These findings demonstrate that each RGS protein interacts with a distinct subset of GPCRs.

**DISCUSSION**

A newly emerging model for RGS protein regulation of G protein signaling suggests that RGS proteins are selectively recruited by GPCRs, which, in turn, direct RGS activity toward the linked G protein signaling cascade (11, 13–15). Consistent with this hypothesis, we provide here the first evidence for direct RGS protein-GPCR interactions and a possible mechanism for functional coupling. We found that RGS2, but not RGS16, interacts directly and selectively with the M1 mAChR, and that this interaction involves the third intracellular loop of the M1 mAChR and the N terminus of RGS2. The N terminus has been implicated in determining the functional selectivity of B/R4 RGS proteins in cells. Deletion of the N terminus of RGS4 abrogated its capacity to discriminate between mAChR and CCK-mediated signaling pathways (29). In addition, the isolated N terminus of RGS4 could partially modulate carbachol-mediated signaling in this system (29). Similarly, the isolated RGS8 N terminus in the absence of the rest of the protein altered α2-adrenergic receptor-stimulated GIRK channel kinetics (32). It therefore seemed likely that the N terminus contains the contact sites for RGS2 binding to the M1 mAChR. Indeed, we found that the N terminus of RGS2 is both necessary and sufficient to mediate its binding to GST-M1i3. Deletion of this region from RGS2 resulted in a corresponding decrease in potency of the protein to inhibit M1-mediated signaling. Our findings support the hypothesis that the affinity of the N terminus for particular receptors is critical in controlling the specificity of RGS/G protein interactions in cells.

Our data do not address whether the receptor and RGS are “prebound” prior to agonist stimulation of the receptor or whether receptor activation promotes RGS binding. A recent study suggests that receptor activation is not necessary for recruitment of RGS proteins because the expression of preferred receptors was sufficient to promote translocation of RGS proteins to the membrane without agonist stimulation (13). This would predict that RGS proteins and receptors are complexed in a resting cell. In addition to containing receptor-binding sites, the N termini of RGS2 and its subfamily members also contain an amphipathic helix that serves as a membrane-targeting domain (28). Thus, the recruitment of RGS proteins to the plasma membrane by GPCRs (13) likely involves a combination of membrane targeting and receptor binding factors. Consistent with this idea, we observed that RGS2 is localized to the plasma membrane when expressed alone in CHO cells, indicating the involvement of membrane targeting features intrinsic to RGS2. When co-expressed with M1 mAChR in the same cell, expression patterns of RGS2 and M1 mAChR are overlapping at the plasma membrane, indicating that RGS2 is positioned nearby for recruitment by functional coupling with M1 mAChR and Gq/11. It remains unclear exactly how receptor binding and N-terminal features of RGS each contribute to influence the signaling capacity of RGS proteins in cells. It will be an important next step to identify the specific contact sites on both the RGS protein and receptor required for this interaction, and to determine whether these sites overlap with or are distinct from sites that are critical for RGS protein membrane targeting.

If recruitment of RGS2 by M1 mAChR allows RGS2 to selectively regulate the downstream pathway, then its RGS domain needs to be in a conformation that allows it to interact with the associated Gq,α. M1 mAChR is functionally linked to Gq/11, and we have shown previously that RGS2 selectively interacts with Gq,α (31, 33, 34). Initially our studies did not distinguish whether the RGS-Ga interaction could occur while RGS2 is bound to M1i3, or whether the RGS protein was released prior to its actions on Ga. We found that, although Gq,α alone did not
bind the M1i3 loop, activated Gaα prebound with RGS2 could form a stable heterotrimeric complex with the M1i3 loop (Fig. 6). Thus, RGS2 is capable of binding the receptor through its N terminus at the same time it interacts with activated Gα11α through its RGS domain.

Based on our findings with RGS2 and M1 mAChR, we propose a model for the signaling selectivity of RGS proteins in a cell. First, the receptor recruits and directly binds a specific RGS protein based on its affinity for the i3 loop. With RGS bound, hormone binding to receptor then activates the linked G protein heterotrimer, which is bound to specific sites within the i2 and i3 loops of the receptor (35). The RGS protein remains bound to the i3 loop, in close proximity to the Ga. Once the Ga is activated, RGS is in position to act on the G protein, inhibiting the downstream signaling pathway. In this manner, the RGS protein is directed to a specific G protein(s), depending on the identity of the receptor. Such a model would help to explain why RGS proteins are nonselective with regard to their Ga interactions in in vitro assays using purified proteins, but are selective regulators in the context of a cell or cell membrane. In solution, purified RGS proteins would be free to interact with any available Ga. However in a cellular context, GPCR recruitment at the plasma membrane would restrict RGS protein activity to the linked Ga.

RGS proteins can negatively regulate Ga signaling by serving both as GAPs and/or effector antagonists (6, 7), and a recent report shows differential contributions of GTPase activation and effector antagonism to explain the inhibitory actions of RGS2 and RGS16 on receptor and Gα11α signaling (30). Specifically, RGS2 appeared to inhibit the stimulation of phospholipase Cβ activity by acting both as a GAP and as an effector antagonist for Gα, whereas RGS16 appeared to act as a GAP but not as an effector antagonist. Consistent with these findings, we observed that, although RGS2 and RGS16 each can act as an effector antagonist and a GAP for M1 mAChR-directed Gα11α signaling in membranes, RGS2 was more potent than RGS16 as an effector antagonist and RGS16 was more potent than RGS2 as a GAP. Surprisingly, the isolated RGS domain of RGS2 missing the N terminus (∆NRGS2) was a fully functional (albeit slightly less potent than RGS2) GAP for Ga, but could not bind the M1i3 loop and exhibited little or no activity as an effector antagonist. Taken together, these findings suggest that RGS2 modulation of receptor signaling may reflect in large part its effector antagonist activity, which is greatly facilitated by the binding of RGS2 to the i3 loop. In support of this idea, RGS2 simultaneously bound the M1i3 loop by its N terminus and AlF4 activated Gαi by its RGS domain. Independent of receptor i3 loop binding, ∆NRGS2 also can modulate Ga signaling by its GAP activity, although completely dissociating a receptor contribution was not possible because a receptor-Gα fusion protein was required to load Ga with GTP for GAP measurements. One possible explanation for this apparent segregation of function may be that RGS GAP and effector antagonist activities exist under distinct conformational states. In this scenario, binding to the i3 loop would confer to RGS2 a conformation that favors high affinity Ga binding and effector antagonist activity. Whereas RGS GAP activity may play a transient role in modulating the strength of Ga/effector signals independent of receptor, RGS effector antagonism may play a more important role in long term receptor desensitization by completely uncoupling Ga from effector signaling. Nonetheless, the significance of segregating GAP and effector antagonist activity on RGS2 cellular functions is at present unclear (30), and a full understanding will require further study.

We chose to focus on muscarinic receptors because of previous evidence indicating that B/R4 RGS proteins inhibited signaling through carbachol- and mAChR-mediated pathways (10). However, there was no precedent to indicate whether RGS selectivity would extend to other subtypes of the same receptor family. When we compared the interactions of the closely related RGS1, -2, -4, and -16 to the i3 loops of each of the mAChR subtypes, we found distinct profiles of binding selectivity (Fig. 7). We found that RGS2 and RGS4 bound M1i3 and M5i3; RGS2 also bound M3i3 with lower affinity. In a subset of experiments, we observed RGS16 binding weakly to M3i3. RGS1 did not interact with any of the i3 loops, and none of the RGS proteins bound to either M2i3 or M4i3. These results suggest that GPCR recruitment of RGS proteins is likely to be a general mechanism for GPCR selectivity among some subfamilies of RGS proteins.

We did not observe significant binding of any of the RGS proteins tested with Gβγ-coupled M2i3 or M4i3 loops. RGS2 stands out among the B/R4 RGS subfamily as the only member shown to be a selective GAP for Gαi (31, 33, 34). Thus, it seems reasonable that RGS2 would not be recruited by receptors that activate Gαi pathways. However, RGS4 is an efficient GAP in vitro for both Gαi and Gαi (6) and has been shown to modulate M2i3-mediated signaling in many cases (25, 34, 36), yet we did not detect RGS4 binding to the M2i3 or M4i3 loop. One explanation for this discrepancy is that the relative expression levels of receptor, RGS and Gaβγ in these assay systems may allow the RGS-Ga coupling to predominate such that RGS can inhibit Ga despite a lack of RGS-M2 interaction. Fluorescence resonance energy transfer studies of RGS4 binding to Gαi, Gβγ, and PLCβ1 indicated that the strongest interactions occur between the RGS and activated Ga protein, and that the interactions with other components of the signaling pathway are significantly weaker (37). Thus, it is likely that the relative ratios of G protein subunits and receptor could influence which interaction dictates the response. Consistent with this idea, overexpression of Ga subunits is sufficient to recruit RGS2 and RGS4 to the plasma membrane (13, 27, 38). Alternatively, the mechanism for RGS recruitment among Gβγ-linked receptors may differ from that of Gaα-linked receptors such that different regions of the receptor bind to the RGS.

We initially chose to focus on the i3 region of the mAChRs as potential binding domains for RGS proteins because the mAChR subtypes are most diverse within this domain and thus were the most likely regions to confer RGS selectivity. Other proteins critical for downstream signaling and regulation of the receptor also bind to this region of receptors. The extreme ends of the i3 loop, at the junctions with transmembrane spans 5 and, in particular, span 6 (which are not contained within our construct boundaries) contain important sites for Ga coupling (35). The i3 loop of mAChRs also contains sites for interactions with G protein-coupled receptor kinases and β-arrestins, proteins that mediate receptor desensitization and internalization (19). G protein-coupled receptor kinase 2 (GRK2) phosphorylates agonist-occupied M3 mAChR at specific serine residues with the assistance of Gβγ, which binds sites in the i3 loop (20, 39). The interactions of these proteins indicates that regions critical for receptor trafficking are contained within the i3 loop and raises the possibility that RGS proteins could have undiscovered roles in this process. Further studies will be needed to define the contact sites for RGS2 and M1i3 interactions, and whether RGS binding sites overlap with or are distinct from the interaction sites of other regulatory proteins that bind to the i3 loop.

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