The Gβ_5−RGS7 Complex Selectively Inhibits Muscarinic M3 Receptor Signaling via the Interaction between the Third Intracellular Loop of the Receptor and the DEP Domain of RGS7[†]

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ABSTRACT: Regulators of G protein signaling (RGS) make up a diverse family primarily known as GTPase-activating proteins (GAPs) for heterotrimeric G proteins. In addition to the RGS domain, which is responsible for GAP activity, most RGS proteins contain other distinct structural motifs. For example, members of the R7 family of RGS proteins contain a DEP, GGL, and novel DHEX domain and are obligatory dimers with G protein β subunit Gβ_5. Here we show that the Gβ_5−RGS7 complex can inhibit Ca^{2+} mobilization elicited by muscarinic acetylcholine receptor type 3 (M3R), but not by other Gq-coupled receptors such as M1, M5, histamine H1, and GNRH receptors. The isolated DEP domain of RGS7 is sufficient for the inhibition of M3R signaling, whereas the deletion of the DEP domain renders the Gβ_5−RGS7 complex ineffective. Deletion of a portion of the third intracellular loop allowed the receptor (M3R-short) to signal but rendered it insensitive to the effect of the Gβ_5−RGS7 complex. Accordingly, the recombinant DEP domain bound in vitro to the GST-fused i3 loop of the M3R. These results identify a novel molecular mechanism that can impart receptor subtype selectivity on signal transduction via Gq-coupled muscarinic receptors.

G protein-coupled receptors (GPCRs)[1] regulate numerous physiological functions in eukaryotes. Agonist-bound GPCRs catalyze the exchange of GDP bound to the G protein α subunits for GTP, which allows the G proteins to modulate the activity of their effector enzymes and ion channels. For example, heterotrimeric G proteins that belong to the Gq class stimulate phospholipase C, which leads to inositol triphosphate-mediated release of Ca^{2+} from intracellular stores. The duration and amplitude of the activated state of a G protein cascade depend largely on the lifetime of the GTP-bound form of the G protein. For most G proteins, the rate of GTP hydrolysis is increased by a distinct class of approximately 30 diverse proteins known as regulators of G protein signaling (RGS). Their interaction with the G proteins is mediated by an ∼120-amino acid RGS domain, which serves as a GTPase activating protein (GAP) for Gα subunits (*1, 2*). Most RGS proteins also contain other structural motifs that are implicated in a variety of functions (*3, 4*).

The R7 subfamily of RGS proteins is comprised of four gene products, RGS6, RGS7, RGS9, and RGS11 (*5*−*7*). In addition to the RGS domain, they have three other domains, GGL, DEP, and DHEX. The function of the DHEX (DEP helical extension) domain, which was recently identified by crystallography (*8*), has not been determined. The GGL (G γ like) domain is responsible for the interaction with the unique neuro-specific G protein β subunit, Gβ_5 (*9, 10*). It was shown that Gβ_5 and the R7 family of RGS proteins form obligatory dimers in vivo (*6, 7*). The DEP domain (first identified in Disheveled, EGL-10, and pleckstrin) was found in a variety of signaling proteins (*11*). The function of the DEP domains in the R7 family remained unknown until it was demonstrated that they could bind to R9AP and R7BP, novel proteins that anchor R7 family proteins to the membranes (*12*−*15*). It is interesting to note that a large pool of the Gβ_5−RGS7 complex in the native tissue is present in the cytosol apart from the membrane-bound R7BP (*16*). Furthermore, the knockout of R7BP produced no apparent phenotype in mice and only slightly affected membrane association of the Gβ_5−RGS7 complex (*17*). Thus, it appears that the Gβ_5−RGS7 complex in the native tissues can exist as the dimer or trimer with R7BP.

Certain functions of RGS proteins cannot be explained solely by their GAP activity. For example, RGS4 inhibited muscarinic acetylcholine M3 receptor (M3R) with a much higher potency than the cholecystokinin receptor, another Gq-coupled GPCR (*18*). This selectivity was dependent on the presence of the N-terminal region of RGS4, but not on the RGS domain. Likewise, another study showed that RGS8 was more potent toward M1R compared to M3R (*19*). One of the suggested explanations for the receptor selectivity of

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[1] Abbreviations: RGS, regulator of G protein signaling; GPCR, G protein-coupled receptor; R7BP, R7 family RGS binding protein; R9AP, RGS9 anchoring protein; GTP, guanosine triphosphate; GST, glutathione *S*-transferase; CHO, Chinese hamster ovary; FRET, fluorescence resonance energy transfer; SD, standard deviation.

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RGS action was their direct interaction with GPCRs. Indeed, it was later shown that RGS8 could directly bind to M1R (*20*).

All GPCRs share the same overall architecture with seven transmembrane domains, but the difference in their intracellular loops and the C-termini allows them to couple to distinct G proteins and other signaling molecules. For example, muscarinic receptor subtypes M1, M3, and M5 couple to members of the Gq family of G proteins, whereas M2 and M4 receptors couple to Gi. The intracellular regions of GPCRs also contain sites for phosphorylation and arrestin binding, the processes involved in GPCR desensitization (*21*). The sites for the interaction of the G protein subunits and arrestins were mapped to the third intracellular (i3) loops of M3 and M2 receptors (*22*−*24*). Studies have also shown that the i3 loops directly bind to proteins such as calmodulin (*25*), RGS2 (*26*), casein kinase α (*27*), and SET, a putative oncogene and protein phosphatase 2A inhibitor (*28*).

In this paper, we show that the DEP domain of RGS7 can directly bind to the third intracellular loop of the M3R and attenuate receptor-induced Ca^{2+} mobilization in a M3 subtype-selective manner.

MATERIALS AND METHODS

Reagents and Antibodies. The cDNA clones for human muscarinic M3, M1, and M5, histamine H1, gonadotropin-releasing hormone (GNRH), and serotonin 2c (5HT2c) receptors were obtained from the Missouri S&T cDNA Resource Center (www.cdna.org). The GFP antibody was from Clontech. RGS7, Gβ_5, and Gβ_1 antibodies have been described previously (*29*). The rabbit polyclonal antibody against R7BP was provided by K. Blumer (Washington University, St. Louis, MO). Carbamoylcholine chloride (carbachol), histamine dihydrochloride, serotonin hydrochloride, and human luteinizing hormone releasing hormone acetate were all obtained from Sigma.

GST−*MR i3 Constructs.* The GST fusion constructs encoding the third intracellular loops (i3) of M1−5 muscarinic receptors were kindly provided by J. Hepler (Emory University, Atlanta, GA) and have been previously described (*26, 30*). The DNA fragment encoding the M3R i3 loop region (Asn^{304}−Gln^{390}) was amplified from the full-length human M3R using the forward primer 5′-TCCGGATCCAACAGGAGGAAGTAT-3′ and the reverse primer 5′-CACGAATTCCTGCAGGTTGTCCGA-3′. The shorter fragment encoding the Ser^{345}−Gln^{390} part of the i3 loop was amplified using the forward primer 5′-GCCGGTCCTCCCTGGAGAACTCC-3′ and the reverse primer used for Asn^{304}−Gln^{390}. The fragments were cloned into the pGEX-2T vector at the BamHI and EcoRI sites. The GST fusion constructs encoding rat M3R i3 Arg^{252}−Gln^{490} and Val^{390}−Gln^{490} (*22, 31*) were kindly provided by S. Lanier (Medical University of South Carolina, Charleston, SC).

Preparation of Brain Homogenates. Mouse brains were homogenized in lysis buffer [20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, and 2 mM β-mercaptoethanol] and centrifuged at 150000*g* for 1.5 h at 4 °C. The supernatant fraction was collected (cytosolic extract), and the pellet containing the membranes was washed twice and resuspended in the same buffer containing 1% sodium cholate. This suspension was left on ice for 15 min then centrifuged at 150000*g* for 45 min at 4 °C, and the supernatant was retained as the membrane extract.

Purification of GST Fusion Proteins. The purification of the GST fusion proteins was done as previously described (*32*). Briefly, bacterial cultures were grown at 37 °C, and protein expression was induced with the addition of 0.4 mM IPTG for 1−1.5 h at 37 °C. The cultures were harvested and centrifuged, and pellets were resuspended in STE [150 mM NaCl, 100 mM Tris (pH 8.0), and 1 mM EDTA] buffer containing lysozyme, 5 mM DTT, and protease inhibitors. The cell suspension was briefly sonicated on ice, and Sarkosyl (final concentration of 1.5%) and Triton X-100 (final concentration of 2%) were added to the lysate. After gentle rotation at 4 °C for 1 h, the lysate was centrifuged at 19000 rpm at 4 °C for 30 min. The clarified lysate was batch processed using GST−Sepharose 4B beads (GE) (0.5 mL of packed beads per 10 mL of lysate, which contained 2−5 mg/mL total protein) overnight at 4 °C. The beads were washed and eluted with 20 mM glutathione. The purified GST fusion protein was desalted on Sephadex G-25 pre-equilibrated with buffer containing 100 mM Tris (pH 8.0), 150 mM NaCl, and 15% glycerol and stored frozen in aliquots at −80 °C.

Constructs for Expression in Mammalian Cells. The plasmid harboring the M3R-short receptor, which has 196 amino acids deleted from the i3 loop (amino acids Ala^{274}−Lys^{469}), was previously described (*33*) and was kindly provided by J. Wess (National Institutes of Health). To generate the construct corresponding to amino acids 1−124 of full-length bovine RGS7 (YFP-DEP), nucleotides 1−372 were amplified using the forward primer 5′-TCCGGACTCAGATCTATGGCCCAGGGG-3′ and the reverse primer 5′-GTCTGTGTTAAGCTTTTCCGGCTCCCA-3′. The RGS7 construct that lacks the RGS domain and the C-terminus (ΔRGS) was generated by PCR amplification of nucleotides 1−963 corresponding to amino acids 1−321 using the forward primer 5′-TCCGGACTCAGATCTATGGCCCAGGGGG-3′ and the reverse primer 5′-CCCAAGCTTTTCTTTGCTTGC-3′. These fragments were cloned into the pEYFP-C1 vector at BglII and HindIII sites. The constructs encoding the C-terminal part of RGS7 ($RGS7^{249-469}$), YFP-RGS7, and CFP-fused Gβ_5 were described previously (*32, 34*).

Cell Culture and Transfection. CHO-K1 cells were cultured in F-12K Nutrient Mixture (Kaighn's modification, Gibco) with 10% fetal bovine serum and penicillin/streptomycin. CHO-R7BP cells (*32*) were cultured like the CHO-K1 cells with the addition of 400 μg/mL Geneticin. Twenty-four hours prior to transfection, the cells were plated to achieve a density of $0.8-1.0 \times 10^6$ cells per 100 mm plate. Transfection was carried out using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. The DNA ratio of RGS7 to Gβ_5 was maintained at 5:1, with a total of 8.0 μg of DNA per plate. Lac Z DNA was used as a control to ensure that the total DNA per plate used in the CHO-K1 cotransfection assays remained constant. Forty-eight hours after transfection, cells were washed with HBSS, harvested, lysed in hypotonic buffer, and used for pull-down assays.

GST Pull Down. Glutathione Sepharose 4B beads were prewashed with PBS and 0.1% CHAPS, incubated at 4 °C with purified recombinant GST or the GST fusion proteins for 1−2 h, and then washed three times with PBS and 0.1% CHAPS to remove excess protein. The slurry was incubated

overnight at 4 °C on a rotary mixer with the investigated CHO cell lysates, as determined by the experiment. At the end of the incubation, the beads were settled by gravity and the supernatant was collected as the unbound fraction. In a typical assay, the packed volume of the resin was 30 μL, and the volume of the protein lysate was 300 μL. The beads were washed three times with 600 μL of PBS and 0.1% CHAPS buffer and eluted with 30 μL of 2× SDS sample loading buffer. The unbound and eluted fractions were resolved by gel electrophoresis and analyzed by Western blotting.

Ca^{2+} Mobilization Assay. CHO-K1 cells were transiently transfected with cDNAs for M1, M3, M5, GNRH, H1, and 5HT2c receptors, RGS7 and Gβ_5, or Lac Z, as required by the experiment. Transfected cells were grown on 12 mm glass coverslips (Electron Microscopy Sciences). Forty-eight hours after transfection, they were washed with 2% FBS in HBSS and then incubated in 2% FBS in HBSS containing 1 μM fura-2AM for 45 min at ambient temperature in the dark. This was followed by a 30 min incubation in Locke's buffer to permit de-esterification of fura-2AM. The coverslips were then secured in a flow chamber and mounted on the stage of a Nikon TE2000 inverted fluorescence microscope. The cells were continuously perfused with Locke's buffer and stimulated with varying agonist concentrations in the same buffer as required by the experiment. The images were collected in real time every 2 s using a 20× UV objective lens and recorded using Metafluor. The excitation wavelengths were 340 and 380 nm, and the emission was set at 510 nm. The free Ca^{2+} concentration was determined from the fluorescence measurements using the fura-2 Ca^{2+} imaging calibration kit (Molecular Probes) according to the manufacturer's instructions.

[^{3}H]NMS Binding Assay. Muscarinic receptor density was determined by the ligand binding assay using the muscarinic antagonist *N*-methyl scopolamine chloride ([^{3}H]NMS, 70 Ci/mmol, Perkin-Elmer) essentially as previously described in ref *35*. Briefly, CHO-K1 cells were transfected in 24-well plates with the M1, M3, or M5 muscarinic receptor alone or together with the Gβ_5−RGS7 complex, as required by the experiment. Twenty-four hours after transfection, cells were washed and incubated with 1 mL of [^{3}H]NMS in a buffer also containing 10 mM HEPES (pH 7.4), 4.2 mM $NaHCO_3$, 11.7 mM glucose, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.7 mM KCl, 118 mM NaCl, and 1.3 mM $CaCl_2$. The [^{3}H]NMS concentrations ranged from 20 pM to 14 nM, with seven points used for the saturation curve and Scatchard analysis. Each [^{3}H]NMS concentration was used in duplicate (two wells of cells). Nonspecific binding was assessed in the presence of 20 μM atropine. Following incubation for 1 h at 37 °C, cells were rapidly washed twice with 1 mL of ice-cold buffer and then lysed with 0.5 mL of 0.1 M NaOH added to the wells. This lysate was neutralized with 0.5 mL of 0.1 M HCl, and the mixture was transferred to the vials for liquid scintillation counting.

RESULTS

The Gβ_5−RGS7 Complex Inhibits M3R-Mediated Ca^{2+} Mobilization. Previous studies showed that the Gβ_5−RGS7 complex attenuated Ca^{2+} mobilization elicited by the muscarinic acetylcholine M3 receptor (M3R) by approximately

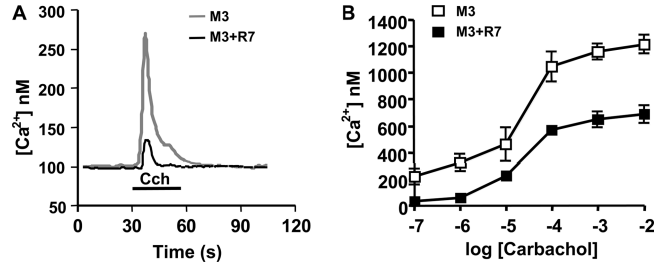

FIGURE 1: Gβ_5−RGS7 inhibits Ca^{2+} mobilization elicited by the muscarinic M3 receptor. CHO-K1 cells were transiently transfected with the M3R and Lac Z or the M3R, Gβ_5, and RGS7. Cells were plated on glass coverslips and loaded with fura-2AM, and the changes in free intracellular Ca^{2+} concentrations in response to the application of carbachol were recorded in real time as described in Materials and Methods. (A) Representative traces from cells transfected with plasmids encoding M3R and Lac Z (gray) and cells expressing M3R together with Gβ_5−RGS7 (black). The application of 100 nM carbachol (Cch) is denoted with the black bar. (B) Dose−response curve obtained by treating cells expressing M3R alone (□) or M3R and the Gβ_5−RGS7 complex (■) with increasing concentrations of carbachol. The peak Ca^{2+} response was recorded from the entire visual field that included 30−60 individual cells. The data represent the mean ± the SD of six independent experiments.

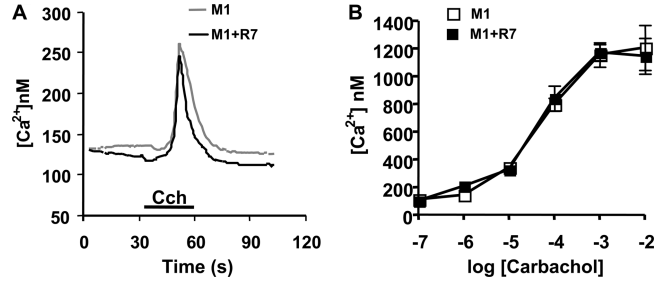

FIGURE 2: M1R-mediated Ca^{2+} release is not affected by the Gβ_5−RGS7 complex. CHO-K1 cells were transfected with the M1R and Lac Z or the M1R, Gβ_5, and RGS7, and the calcium responses were recorded and analyzed as described in the legend of Figure 1. (A) Representative traces from single cells. The gray trace represents data from cells with M1R alone, and the black trace shows data from cells expressing M1R together with Gβ_5−RGS7. (B) Dose−response plot from increasing carbachol concentrations: (□) M1R alone and (■) M1R with Gβ_5−RGS7. The data represent the mean ± the SD of three independent experiments from measurements of the amplitude of free Ca^{2+}.

50% (*29, 32, 34*). Here, we tested if this inhibition could occur to a greater extent at a lower agonist concentration. We reasoned that given the high expression level of the receptor in the transiently transfected cells, the amount of Gβ_5−RGS7 complex was insufficient to quench the receptor-mediated activation of Gq, particularly at saturating agonist concentrations. To reduce the amount of activated receptor, we stimulated cells with a range of carbachol concentrations. We found that at carbachol concentrations of 1 and 0.1 μM (below the EC_{50}), the Gβ_5−RGS7 complex-mediated inhibition of Ca^{2+} responses was nearly complete (Figure 1).

Receptor Selectivity of Gβ_5−RGS7. While Gβ_5−RGS7 displayed a robust inhibitory effect on M3R, it had no effect on other tested Gq-coupled receptors. For example, Figure 2 shows that M1R was insensitive to Gβ_5−RGS7 over the wide range of tested agonist concentrations. Likewise, the receptors for histamine (H1), serotonin (5HT2c), GNRH, and muscarinic acetylcholine receptor M5R were also not affected by Gβ_5−RGS7 (Figure 3). We determined the number of binding sites on live CHO-K1 cells expressing M1, M3, and

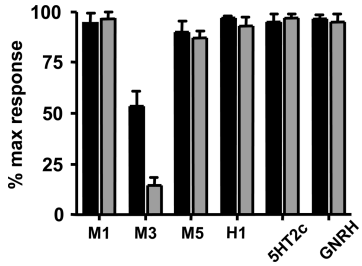

FIGURE 3: Gβ_5−RGS7 complex inhibits Gq signaling in a receptor selective manner. CHO-K1 cells were transiently transfected with cDNAs encoding Gq-coupled receptors: M1, M3, and M5 muscarinic, 5HT2c serotonin, H1 histamine, and GNRH. Lac Z or the Gβ_5 plus RGS7 plasmids were cotransfected with the receptor-encoding plasmids. In each experiment, changes in free intracellular Ca^{2+} concentration were measured upon treatment of the cells with two agonist concentrations. The highest agonist concentration (black bars) used for the M1, M3, and M5 muscarinic receptors was 10 mM, and the lowest concentration (gray bars) was 100 nM. The highest and lowest concentrations of 5HT2c, histamine, and GNRH were 10 μM and 10 nM, respectively. The determined response in the presence of Gβ_5−RGS7 was expressed as the percent of the signal recorded from cells transfected only with the indicated receptor. Shown is the mean amplitude ± the SD of the Ca^{2+} response from at least three independent experiments.

M5 receptors using the nonselective muscarinic antagonist [^{3}H]NMS. The binding of [^{3}H]NMS was saturable and dose-dependent, and the B_{max} values in M1R, M3R, and M5R transfected cells were as follows: 856 ± 181.4 (n = 3), 1066 ± 187.9 (n = 4), and 1024 ± 81.5 (n = 2) fmol/mg of total protein, respectively. The determined K_d values for [^{3}H]NMS were 0.310 ± 0.06, 0.228 ± 0.09, and 0.401 ± 0.02 nM, respectively. We found that the coexpression of Gβ_5−RGS7 slightly increased the B_{max} of all three tested receptors [985 ± 118.0 (n = 3), 1310 ± 135.2 (n = 4), and 1304 ± 153.0 (n = 2) fmol/mg, respectively], which can likely be attributed to a minor positive effect on the transfection efficiency of the CHO cells. The K_d values in the presence of Gβ_5−RGS7 were 0.277 ± 0.08, 0.216 ± 0.04, and 0.392 ± 0.06 nM, respectively, not appreciably different from the values in the absence of Gβ_5−RGS7. Thus, all three muscarinic receptors were expressed at approximately the same level with or without Gβ_5−RGS7, however, Gβ_5−RGS7 inhibited only signaling elicited by the M3 receptor subtype.

Since all tested receptors act through Gq, such receptor selectivity indicated that inhibition of M3R signal transduction occurs upstream of the G protein. Therefore, we hypothesized that the Gβ_5−RGS7 complex inhibited M3R signal transduction via a mechanism that does not utilize its GAP activity.

The DEP Domain of RGS7 Is Responsible for M3R Inhibition. To determine the mechanism by which Gβ_5−RGS7 inhibits M3R signaling, we investigated which domain of RGS7 was responsible for this effect. We prepared the following three constructs (Figure 4A): (1) the N-terminal portion of RGS7, which lacked the RGS domain and the C-terminus, termed ΔRGS, (2) the C-terminal portion that lacked the N-terminus and the DEP and DHEX domains, termed RGS7$^{249-469}$, and (3) the N-terminal portion (amino acids 1−124) termed the DEP domain. The ΔRGS and the DEP domain constructs were fused to the C-terminus of YFP to enhance their expression and detection. The ΔRGS and RGS7$^{249-469}$ truncations of RGS7 were coexpressed in CHO cells together with Gβ_5. Our results showed that the ΔRGS

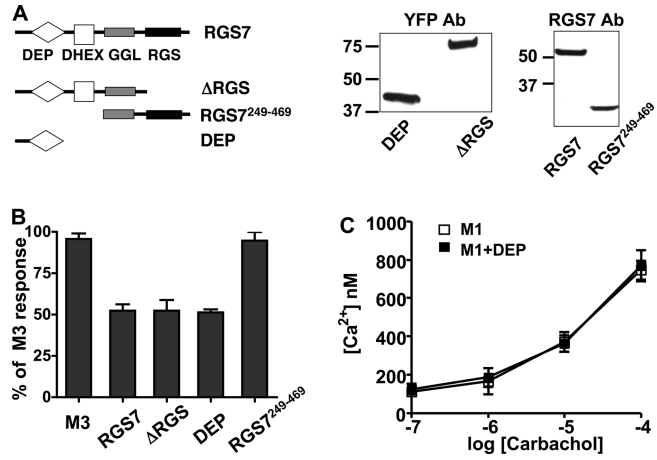

FIGURE 4: DEP domain of RGS7 is sufficient for the inhibition of M3R signaling. (A) CHO-K1 cells were cotransfected with the M3R and RGS7 constructs encoding full-length RGS7, ΔRGS, RGS7$^{249-469}$, or the DEP domain. The schematic illustrates the location of the DEP (diamond), DHEX (white square), GGL (gray rectangle), and RGS (black rectangle) domains within the RGS7 molecule. The RGS7 constructs containing the GGL domain were cotransfected together with Gβ_5 cDNA. The Western blot panels show the expression of these constructs in CHO-K1 lysates (12 μg of total protein) that were used in these experiments. ΔRGS and DEP−YFP fusion proteins were probed with the YFP antibody. Full-length RGS7 and RGS7$^{249-469}$ were probed with the RGS7 antibody. (B) Effects of the constructs described in panel A on the M3R-mediated Ca^{2+} response. The average response in the presence of the constructs was expressed as the percent of the signal from cells transfected with M3R alone. Data show the mean amplitude ± the SD from at least three independent experiments. (C) RGS7 DEP does not affect the M1 receptor. Ca^{2+} responses in the M1R-transfected cells were recorded in the absence (□) or presence of the DEP domain (■), over the range of indicated carbachol concentrations. The data represent the mean ± the SD for two independent experiments.

and DEP constructs inhibited M3R-induced Ca^{2+} mobilization in a manner similar to that of the full-length RGS7 protein (Figure 4B). In contrast, RGS7$^{249-469}$, which lacked the DEP domain, had no effect on M3R signaling. These results indicated that the RGS domain is not essential for this inhibition, supporting the hypothesis that Gβ_5−RGS7 inhibits M3R-mediated signal transduction via a non-GAP mechanism. Instead, the Gβ_5−RGS7 complex inhibits M3R signaling via the DEP domain. Similar to full-length Gβ_5−RGS7 (Figure 2), the DEP domain did not inhibit signaling from the M1R, showing the selectivity of the DEP domain of RGS7 toward the M3 receptor subtype (Figure 4C).

The DEP Domain of RGS7 Directly Binds to the Third Loop of M3R. The selectivity of Gβ_5−RGS7 toward M3R indicated that it acts upstream of the G protein, suggesting that it may directly bind to the receptor. The i1 and i2 loops are very short and well-conserved among all five muscarinic receptors. In contrast, the i3 loops of muscarinic receptors are longer, are much more diverse, and were previously shown to interact with multiple proteins such as Gαq (*24*), G$\beta\gamma$ (*31*), β-arrestin (*23*), RGS2 (*26*), calmodulin (*25*), and SET (*28*). Therefore, we reasoned that the likely binding site for Gβ_5−RGS7 could be located within the third intracellular loop. To test this hypothesis, we first investigated if Gβ_5−RGS7 had an effect on M3R-short, the deletion mutant of M3R that lacked a large portion of the i3 loop (Figure 5A). Our results showed that Gβ_5−RGS7 had no effect on

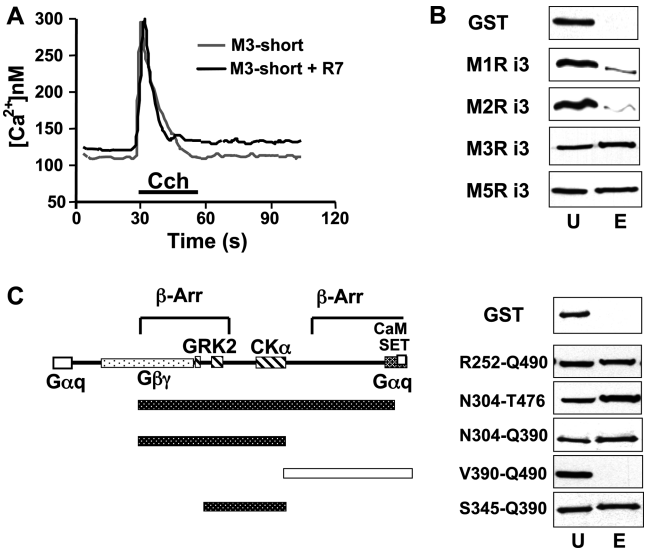

FIGURE 5: Direct protein−protein interaction between the third intracellular loop of M3R and the DEP domain of RGS7. (A) The M3 receptor deletion mutant lacking the third intracellular loop (M3R-short) was transfected into CHO-K1 cells together with Lac Z or Gβ_5-RGS7. Changes in Ca^{2+} were recorded upon the application of 100 nM Cch (black bar). The gray line represents a Ca^{2+} transient recorded from cells transfected with M3R-short alone. The black line represents a transient for M3R-short transfected together with Gβ_5−RGS7. (B) GST fusions of the i3 loops of the M1, M2, M3, and M5 muscarinic receptors or GST were immobilized on glutathione−agarose beads. The beads were incubated with the extract from CHO-K1 cells transfected with the YFP−DEP fusion protein, as described in Materials and Methods. After the slurry was spun down and unbound material was collected, the resin was washed and eluted with SDS sample buffer. The unbound (U) and eluted (E) material was analyzed by Western blot using the anti-GFP antibody. (C) Schematic representation of the sequence of the entire M3R i3 loop. The approximate location of the binding sites for Gαq, G$\beta\gamma$, SET, regions phosphorylated by GRK2 and casein kinase α (CKα), calmodulin (CaM), and β-arrestin (β-Arr) are indicated according to the literature (see the text). The truncated GST-fused M3R i3 loop regions are shown under the full-length M3R i3 loop. Filled boxes designate constructs that bound the RGS7 DEP domain, and the empty box shows the construct that did not bind to the DEP domain. The representative results of the pull-down assays obtained with these GST fusion proteins are shown at the right.

Ca^{2+} mobilization elicited by this M3R mutant. We further tested the hypothesis that the DEP binding site is localized within the i3 loop using a pull-down assay with the i3 loop of M3R fused to the C-terminus of GST (Figure 5B). We found that the DEP domain of RGS7 bound to the M3R i3 loop, but not to GST. The DEP domain also bound to the i3 loop of M5R and also exhibited very weak binding to the loops of M1 and M2 receptors.

To locate the putative binding site for the DEP domain, we tested its interaction with shorter fragments of the M3R i3 loop. Our results showed that the DEP domain binds to the region encompassing amino acids 345−390 in human M3R, corresponding to the central portion of the i3 loop (Figure 5C). Thus, it appears that RGS7 DEP binds to the region that is most distant from the membrane, whereas the reported binding sites for the G protein are located at the ends of the third loop (*36*), presumably near the membrane surface.

Effects of Gβ_5 and R7BP on the Interaction of RGS7 with M3R. We found that full-length monomeric RGS7 bound to the i3 loop of M3R, but the Gβ_5−RGS7 dimer did not

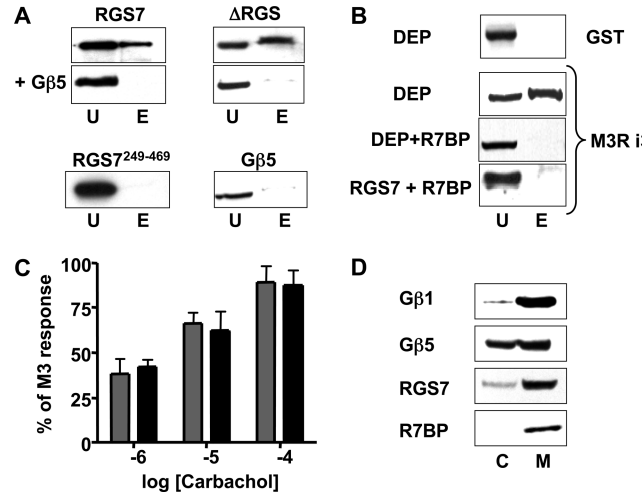

FIGURE 6: Gβ_5 and R7BP inhibit the interaction of the DEP domain of RGS7 with the third intracellular loop of M3R. (A). The N304−I375 region of the i3 loop of M3R fused to GST was used in a series of pull-down assays with the lysates of CHO cells transfected with full-length monomeric RGS7, ΔRGS (with or without Gβ_5), Gβ_5 alone, or RGS7$^{249-469}$ with Gβ_5. The unbound (U) and eluted (E) material was analyzed by Western blot using the anti-RGS7 (for the full-length RGS7 and the RGS7$^{249-469}$ construct) or anti-GFP (for ΔRGS or CFP−Gβ_5 constructs) antibodies. (B) Wild-type CHO cells or CHO cells stably expressing R7BP were transiently cotransfected with DEP or full-length RGS7. The total cell lysate from the wild-type cells and the membrane extracts from CHO-R7BP cells were compared in the pull-down assay using the GST fusion of the M3R i3 (N304−I375) loop. (C) CHO-R7BP cells were transiently transfected with M3R (gray bars) or M3R with Gβ_5−RGS7 (black bars). The Ca^{2+} responses at three indicated carbachol concentrations were recorded. The data represent the mean amplitude ± the SD for two independent experiments. (D) Membrane association of R7BP and RGS7 in the brain tissue. Mouse brain was fractionated to obtain cytosolic (C) and membrane (M) fractions as described in Materials and Methods. These fractions were analyzed by Western blot for the presence of Gβ_1, Gβ_5, RGS7, and R7BP. Results are representative of at least three experiments.

(Figure 6A). Likewise, Gβ_5 blocked the interaction of the ΔRGS construct with the M3R i3 loop. Neither the Gβ_5−RGS7$^{249-469}$ complex nor monomeric Gβ_5 bound to the i3 loop.

We also investigated the effects of R7BP on the interaction of Gβ_5−RGS7 with M3R (Figure 6B−D). Our results showed that R7BP prevented the interaction of the DEP domain or the full-length monomeric RGS7 with the i3 loop of M3R (Figure 6B). This result is consistent with the observation that in CHO cells stably expressing R7BP, Gβ_5−RGS7 did not influence M3R-mediated Ca^{2+} mobilization (Figure 6C and ref *32*). We confirmed that Gβ_5−RGS7 in the native tissue was present in both membranes and cytosol, whereas R7BP was found only in the membranes (Figure 6D). These results indicate that binding of DEP to the i3 loop and binding to R7BP are mutually exclusive and suggest that the regulation of M3R is carried out by the cytosolic form of Gβ_5−RGS7.

DISCUSSION

In this work, we investigated the function of a neuronal regulator of G protein signaling, the Gβ_5−RGS7 complex. Our study highlights two novel aspects: the remarkable selectivity of Gβ_5−RGS7 toward muscarinic M3 receptors

(M3R) and the direct interaction of the DEP domain of RGS7 with the receptor.

Receptor Selectivity. Our experiments showed that $G\beta_5$−RGS7 robustly inhibited signaling from M3R, but under the same experimental conditions, it did not influence other Gq-coupled receptors, including M1R (Figures 1−3). This selectivity toward M3R indicated that $G\beta_5$−RGS7 inhibits M3R signaling not via the GAP activity toward Gq, but upstream of the G protein. We hypothesized that $G\beta_5$−RGS7 interacts directly with the receptor. Since it was shown that M3R contains the binding site for $G\beta\gamma$ subunits (*22*), we initially thought that the $G\beta_5$/GGL moiety was responsible for this interaction. However, our results showed that the effect of $G\beta_5$−RGS7 was mediated by the DEP domain, whereas neither $G\beta_5$/GGL nor the RGS domain was needed for the inhibition of M3R (Figure 4). Previous studies showed that $G\beta_5$−RGS7 has GAP activity toward Gi but not Gq family G proteins (*37, 38*). Therefore, it was not clear why $G\beta_5$−RGS7 inhibited Ca^{2+} mobilization elicited by the Gq-coupled M3R (*29, 32, 34*). Our current findings showed that this inhibition is mediated by the interaction between the receptor and the DEP domain of RGS7. The identification of this novel mechanism resolved the controversy between the lack of GAP activity toward Gαq and the functional effect of $G\beta_5$−RGS7 on the M3R in cells.

The M3R is expressed in a variety of peripheral tissues as well as in the central nervous system. According to the Allen Brain Atlas (www.brain-map.org), M3R and RGS7 mRNAs colocalize, especially in the cerebral cortex and hippocampus. Interestingly, muscarinic receptors of different subtypes can be found in the same neurons (*39, 40*). It is not clear why two Gq-coupled receptors of acetylcholine are needed in one cell. It is reasonable to hypothesize that the interaction with $G\beta_5$−RGS7 differentiates the neuronal M3R from the M1R and from the M3R expressed in peripheral cells. Mice lacking the neuronal M3R have a distinct lean phenotype (*41*−*43*), which is different from the phenotypes of other muscarinic receptor knockouts (*44*). The $G\beta_5$ knockout mice are born runty (*45*) and remain lean throughout their lifetime, even on a high-fat diet (V. Z. Slepak laboratory, unpublished data). The similarity in the phenotypes of $G\beta_5$ and neuronal M3R knockouts may indicate that they participate in the same pathway that is unique for neuronal signaling.

New Role of the DEP Domain. We found that the isolated DEP domain of RGS7 mimicked the functional effect of the entire RGS7 complex, supporting the concept that RGS proteins can regulate signal transduction not only via their GAP activity (*4*). Our results show that the DEP domain of RGS7 can directly bind to the i3 loop of M3R, which contains binding sites for several other proteins, including Gαq, $G\beta\gamma$, arrestin, calmodulin, and SET (*22, 28*). In contrast to Gαq, RGS7 binds to the middle of the loop, which can potentially protrude far into the cytosol. We speculate that the distance of approximately 100 amino acids between the RGS7 site and the juxtamembrane site for Gq could allow the cytosolic $G\beta_5$−RGS7 dimer to bind to the receptor at the same time with Gαq. This would be consistent with previous studies that detected FRET between the fluorescently tagged $G\beta_5$−RGS7 complex and Gαq in cells (*34, 46*). The RGS7 binding site partially encompasses the region phosphorylated by GRK2 and casein kinase and a binding site for β-arrestin, which suggests that $G\beta_5$−RGS7 could have a role in the processes of M3R desensitization and β-arrestin-mediated signaling.

Our results showed that binding of the RGS7 DEP domain to the isolated i3 loops of muscarinic receptors (Figure 5B) had lower subtype selectivity than the inhibitory effect on the full-length receptors (Figures 1−3). Binding to the M3R i3 loop was the most robust, but the DEP domain also bound well to the M5R i3 loop and exhibited much weaker interaction with the i3 loops of M1 and M2 receptors (Figure 5B). The reason for the reduced selectivity in the GST pull-down assay compared to the Ca^{2+} mobilization experiments is not clear at this point. One can speculate that the size of the M3R i3 loop allows the RGS7 complex to remain associated once the G protein binds to the juxtamembrane regions and thus be more effective in inhibiting Gq activation. It is also possible that there is an additional site in the full-length M3R that stabilizes the interaction with the DEP domain and which is absent in other receptors.

It was recently shown that the DEP domain of the yeast RGS protein Sst2 could directly bind to the G protein-coupled receptor, Ste2 (*47*). That interaction occurred at the C-terminal tail of Ste2, which is different from the RGS7−M3R interaction. There is no obvious homology between the C-tail of Ste2 and the i3 loop of M3R; however, both these regions contain the sites for phosphorylation and play a role in desensitization. Another study showed that the dopamine D2 receptor facilitated the membrane localization of RGS9-2, a member of the R7 family of RGS proteins (*48*). Like the interaction of M3R with RGS7 reported in this paper, the effect of the dopamine receptor was selective for the D2 subtype and was mediated by the DEP domain of RGS9-2. However, it did not require the third loop or the C-terminus of the D2 receptor, indicating that structural elements involved in the interaction with DEP domains can be different for specific receptor subtypes. It is worth noting that another DEP domain-containing signaling protein, Disheveled, also binds to its cognate seven-pass transmembrane receptor, Frizzled. However, this interaction is mediated by its PDZ domain rather than the DEP domain of Disheveled (*49*). It will be interesting to determine whether the DEP domains found in RGS proteins are unique in their ability to interact with GPCRs.

Potential Roles of $G\beta_5$ and R7BP. The fact that the G protein β subunit $G\beta_5$ interacts with RGS proteins of the R7 family instead of Gγ subunits was established a decade ago (*9, 10, 50, 51*). $G\beta_5$ and the associated RGS protein stabilize each other against rapid proteolysis (*29*). This mutual stabilization explained why R7 proteins and $G\beta_5$ have not been found apart from each other in the native tissues (*50, 51*) and why R7 proteins are absent in $G\beta_5$ knockout animals (*45, 52*). The knockout of $G\beta_5$ also causes the disappearance of R7BP (*16, 53*). However, it is not clear why such basic function as stabilization of the RGS protein would require association with a G protein β subunit. It appears that there must also be a functional role for $G\beta_5$ within the R7 complex. Early studies suggested that $G\beta_5$ attenuated the interaction of RGS7 with Gαo (*10*). This notion was supported by the analyses of RGS9-1, which showed that its GAP activity toward transducin is reduced in the presence of $G\beta_5$ (*54, 55*). In this paper, we show that $G\beta_5$ can prevent the protein−protein interaction between the

DEP domain and the i3 loop of the M3 receptor (Figure 6). Thus, it appears that Gβ_5 can serve as the negative regulator of both the DEP and RGS domains of RGS7.

We found that like Gβ_5, R7BP prevents the DEP domain from binding to the i3 loop of M3R. R7BP also blocks the effect of the Gβ_5−RGS7 complex on M3R signaling (*32*). Therefore, R7BP and Gβ_5 differ in their effects on the interaction of RGS7 with the full-length M3R in intact cells. The Gβ_5−RGS7 complex is as effective as the isolated DEP domain of RGS7 in its ability to block M3R signaling under the same experimental conditions (Figure 4). This indicates that Gβ_5 can allow the DEP domain to interact with the i3 loop protruding from the full-length receptor in intact cells. In our previous study, we suggested a model in which the Gβ_5−RGS7 molecule can exist in (at least) two conformations: "closed", when Gβ_5 associates with the DEP domain, and "open", when they do not (*32*). On the basis of the results presented in this paper, we now hypothesize that the agonist-bound M3R can open the cytosolic Gβ_5−RGS7 dimer so that the DEP domain binds to the i3 loop, which then inhibits M3R−Gq coupling. The interaction with R7BP at the membrane restricts the action of Gβ_5−RGS7 to Gi-coupled receptors, such as muscarinic M2 (*13*), which occurs via the canonical GAP mechanism that involves the RGS domain of RGS7.

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