The Membrane Anchor R7BP Controls the Proteolytic Stability of the Striatal Specific RGS Protein, RGS9-2**

Garret R. Anderson, Arthur Semenov, Joseph H. Song, and Kirill A. Martemyanov

From the Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455

A member of the RGS (regulators of G protein signaling) family, RGS9-2 is a critical regulator of G protein signaling pathways that control locomotion and reward signaling in the brain. RGS9-2 is specifically expressed in striatal neurons where it forms complexes with its newly discovered partner, R7BP (R7 family binding protein). Interaction with R7BP is important for the subcellular targeting of RGS9-2, which in native neurons is found in plasma membrane and its specializations, postsynaptic densities. Here we report that R7BP plays an additional important role in determining proteolytic stability of RGS9-2. We have found that co-expression with R7BP dramatically elevates the levels of RGS9-2 and its constitutive subunit, Gβ5. Measurement of the RGS9-2 degradation kinetics in cells indicates that R7BP markedly reduces the rate of RGS9-2-Gβ5 proteolysis. Lentivirus-mediated RNA interference knockdown of the R7BP expression in native striatal neurons results in the corresponding decrease in RGS9-2 protein levels. Analysis of the molecular determinants that mediate R7BP/RGS9-2 binding to result in proteolytic protection have identified that the binding site for R7BP in RGS9 proteins is formed by pairing of the DEP (Disheveled, EGL-10, Pleckstrin) domain with the R7H (R7 homology), a domain of previously unknown function that interacts with four putative α-helices of the R7β core. These findings provide a mechanism for the regulation of the RGS9 protein stability in the striatal neurons.

RGS (regulators of G protein signaling) proteins comprise a large family of proteins that control the duration of signal transduction through G protein-coupled receptors (GPCR)1 (1, 2). RGS proteins act to accelerate the rate of GTP hydrolysis on G protein α subunits, thus facilitating G protein inactivation and the subsequent termination of signaling through GPCRs (reviewed in Ref. 3). A mounting body of evidence from clinical studies and genetic animal models indicate that the action of RGS proteins is essential for the normal functioning of almost all systems in the organism (4–6). In the nervous system, many critical neuronal processes appear to be regulated by the R7 RGS proteins, a subfamily conserved in a variety of animals from Caenorhabditis elegans to humans (2, 7). In mammals, the R7 subfamily consists of four highly homologous proteins: RGS6, RGS7, RGS9, and RGS11, all of which are expressed predominantly in the nervous system (8).

Arguably the best studied member of this group is RGS9. It exists in two splice isoforms, RGS9-1 and RGS9-2, which regulate vision and reward behavior, respectively (9). Although the role of RGS9-1 in vertebrate phototransduction has been well established (reviewed in Ref. 10), much remains to be learned about the molecular mechanisms that regulate RGS9-2 function. Previous studies have found that RGS9-2 in the striatum is involved in the modulation of μ-opioid (11, 12) and D2 dopamine (13–15) receptor responses. Studies of RGS9-deficient mice revealed increased locomotor responses, elevated rewarding effects and increased physical dependence in response to the administration of abused drugs such as morphine and cocaine (12, 13). Interestingly, drug administration has been shown to modulate the protein expression levels of RGS9-2, suggesting a possible mechanism for the adaptive changes in G protein signaling observed in addiction and tolerance (12, 13).

RGS9, as well as other members of the R7 subfamily, is a multidomain modular protein that exists in vivo as a constitutive heterodimer with the type 5 G protein β subunit (Gβ5) (16). This association is critical because genetic ablation of Gβ5 results in almost complete elimination of RGS9 protein, as well as all other R7 RGS proteins, presumably because of their proteolytic destabilization (17). In photoreceptors, the stability of the RGS9-1/Gβ5 complex is further dependent upon its association with R9AP (RGS9 anchor protein) (18). Knockout of R9AP leads to a profound reduction in both RGS9-1 and Gβ5 protein levels (19), whereas hyperexpressing R9AP leads to an elevation in the RGS9-1 and Gβ5 protein levels (20). In mammals, R9AP is expressed only in photoreceptors, but we have recently found that in striatum, RGS9-2 forms a complex with a novel R9AP homolog that we named R7BP (R7-binding protein) (21). Unlike R9AP, which is available for binding only to RGS9-1, R7BP interacts with all four members of the R7 RGS protein family (21, 22). Studies by us and others indicate that, depending on its palmitoylation status, R7BP can target R7 RGS proteins to the plasma membrane, nucleus, and postsynaptic densities (22, 23). Furthermore, R7BP binding to RGS7 can potentiate its ability to terminate G protein signaling (22, 24).

In this study we report that an additional role for R7BP is to regulate the proteolytic stability of the RGS9-2-Gβ5 complex. We have found that co-transfection of RGS9-2-Gβ5 with R7BP...
increases the expression level of the complex and increases the half-time of its degradation in neuronal cell lines. Using lentivirus-mediated RNAi knockdown of R7BP expression in primary striatal cultures, we demonstrate that decreases in R7BP levels lead to corresponding reductions in the levels of RGS9-2. We have further employed site-directed mutagenesis and chimeric approaches to dissect the molecular determinants that mediate the binding of R7 RGS proteins to R7BP to result in the observed stabilization of the complex.

**EXPERIMENTAL PROCEDURES**

**Recombinant Proteins, Antibodies, and Reagents—**Generation of sheep anti-R7BP (N-terminal epitope) (21), anti-R9AP (against full-length mouse R9AP) (25), and sheep anti-RGS9-2CT (C-terminal epitope) (21) has been described previously. Antibodies were affinity-purified and stored in PBS buffer containing 50% glycerol. Rabbit anti-RGS7 (7RC1) (26) and anti-Gβ5 (SGS) were generous gifts from Dr. William Simonds (NIDDK, National Institutes of Health). Rabbit anti-DARPP-32 antibodies were from Chemicon (Temecula, CA). Mouse monoclonal anti-β-actin (clone AC-15) antibodies were purchased from Sigma. pcDNA3.1 TOPO cloning systems were obtained from Invitrogen. Recombinant GST-tagged R7BP and R9AP proteins were expressed in *Escherichia coli* and purified as described previously (21). His-tagged recombinant R7 RGS complexes with Gβ5 were obtained in Sf9/baculovirus system and purified by nickel-nitrilotriacetic acid affinity chromatography (21, 27). Protein quantification was performed using Bradford reagent (Sigma) according to the manufacturer’s specifications. RGS7/RGS9 chimeric constructs were generated to create fusion proteins of the following amino acid compositions: R7/9-1 (RGS9 1–115, RGS7 123–469); R7/9-2 (RGS7 1–122, RGS9 116–484); R7/9-3 (RGS9 1–209, RGS9 218–469); R7/9-4 (RGS7 1–297, RGS9 332–484); R7/9-5 (RGS9 1–297, RGS7 332–469); and R7/9-6 (RGS7 1–331, RGS9 298–484). All of the constructs were propagated into *E. coli* Top-10 strain (Invitrogen), isolated using Maxiprep kits (Qiagen), and sequenced.

**Cell Culture and Transfections—**NG108-15 cells were purchased from ATCC and maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 0.1 mM sodium hypoxanthine, 0.4 μM aminopterin, 16 μg/ml thymidine, 100 units of penicillin, and 100 μg of streptomycin. 293FT cells were obtained from Invitrogen and cultured at 37 °C and 5% CO₂ in DMEM supplemented with antibiotics, 10% fetal bovine serum, and 4 mM L-glutamine.

NG108-15 and 293 FT cells were transfected at ~70% confluence, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The ratio of Lipofectamine to DNA used was 4 μl/2.5 μg/cm² of cell surface. The cells were grown for 24–48 h post-transfection.

Primary cultures of striatal neurons were essentially prepared as described by Ivkovic and Ehrlich (29). Briefly, the striata were dissected from Swiss Webster mice at postnatal day 1. After dissection, the tissues were treated by papain (Worthington, Lakewood, NJ), triturated, and plated on 12- or 6-well tissue culture plates (Nunc, Denmark) coated with poly-d-lysine (20 μg/ml; BD Bioscience, Bedford, MA). The cultures were maintained in Neurobasal-A medium supplemented with B27 (both from Invitrogen) and 0.5 mM L-glutamine. The cells were plated at a density of 2000 viable cells (e.g. excluded trypan blue)/1 mm² of well square for Western blot analysis and at 500–700 cells/mm² for immunostaining. The cultures were incubated at 37 °C in a humidified 5% CO₂ incubator. One-half of the medium was replaced with the fresh medium every 72 h. From days 4 to 7, the cultures were transduced by lentiviral constructs, incubated for 7–10 days, washed with PBS, and lysed in SDS sample buffer.

**RNA Interference and Generation of Lentiviruses—**R7BP expression was down-regulated by short interfering RNA duplexes. Target regions in R7BP were identified by BLOCK-iT RNAi Designer Program (Invitrogen). Two sequences were used to generate RNAi molecules that target either 248–268 coding region of R7BP gene (RNAi 248, sequence CTCTGCGTCAAGCAGCA) or to the 483–583 region (RNAi 483, sequence AGCGAAGAATTTGGACAGCAA). These sequences were synthesized as DNA oligonucleotides and in addition contained complementary sequences joined by the GTTTTGGC—
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CACTGACTGAC loop. Synthetic duplexes were cloned into the pcDNA6.2GW/EmGFP vector in the middle of the micro RNA 155 (miR155) sequence supplied as a part of the BLOCK-iT Lentiviral Pol II miR RNAi expression system kit (Invitrogen). In the pcDNA6.2GW/EmGFP vector the chimeric miR155-R7BP sequence is located under the control of the cytomegalovirus promoter co-cistronically with EmGFP. Upon processing in the cells by the endogenous machinery, the construct is used to produce anti-R7BP RNA duplex (miRNA-αR7BP). The control construct (miRNA-CTR) was created by cloning a scrambled sequence AAATGACTGGCCTGAGAC into the micr155 environment identically as described for miRNA-αR7BP. The expression cassette was transferred to the lentiviral shuttle vector pLent6/V5-DEST vector (Invitrogen) by Gateway recombination following the kit instructions.

For the generation of infectious lentiviral particles, pLent6/V5-DEST vectors containing miRNA-αR7BP or miRNA-CTR cassettes were co-transfected with ViraPower™ packaging plasmid mixture: pLP1, pLP2, and pLP/VSV-G (Invitrogen) into 293FT cells using Lipofectamine 2000 (Invitrogen). Ten T75 flasks were used to produce each batch of lentiviruses. Virus containing media was collected 60–65 h after transfection, centrifuged at 20,000 x g for 6 min and filtered through a 0.45-µm filter (Millipore), and viral particles were concentrated as described by Coleman et al. (30) with some modifications. Virus-containing supernatants were carefully loaded on conical-bottomed centrifuge tubes (Beckman) and centrifuged at 50,000 x g and 30 min of centrifugation at 20,000 x g. The supernatant was carefully loaded on 50,000 x g for 2.5 h at 4 °C using a swinging bucket rotor SW-28 (Beckman). The medium just above the media/ OptiPrep interface was carefully removed and discarded. The residual medium containing OptiPrep and viruses (~500 µl in each tube) were mixed gently by shaking and pooled into 3 ml of 10% dialyzed fetal bovine serum (Invitrogen), DMEM without l-Methionine or l-Cysteine (Gibco) and incubated at 37 °C and 5% CO2 for 30 min. After this step, the cells were washed with D-MEM buffer and incubated with 1 pmol of purified RGS9-2 CT antibodies for 10 min, followed by three washes. The proteins were eluted in SDS sample buffer, and RGS proteins retained by the beads were detected by Western blotting with specific antibodies.

Pulse-Chase Degradation Experiments—NG108-15 cells grown in T-25 flasks were co-transfected with RGS9 and Gβ5 constructs with or without R7BP or R9AP plasmids. Twenty-four hours after transfection, the cells were rinsed twice with PBS and placed into 5 ml of starvation medium (10% dialyzed fetal bovine serum (Invitrogen), DMEM without l-Methionine or l-Cysteine (21013-024, Invitrogen) and incubated at 37 °C and 5% CO2 for 30 min. After this step, the cells were rinsed twice with 2 ml of D-MEM and incubated with 5 ml of fully supplemented medium supplemented by additional 2 mM l-methionine and 2 mM L-cysteine (Sigma-Aldrich) at 37 °C for the indicated incubation times. At the end of the incubation, the cells were scraped into 5 ml of ice-chilled PBS pellet by centrifugation at 3,200 x g for 1 min and resuspended in 900 µl of radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.8, 300 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate) supplemented with protease inhibitors (Complete; Roche Applied Science). The cells were allowed to lyse by incubating them at 4 °C for 20 min with gentle shaking. The suspension was centrifuged at 4 °C and 14,000 x g for 30 min, and the supernatant was incubated with 10 µl of protein G beads and 3 µg of RGS9-2 CT antibody for 1 h at 4 °C. The beads were then washed three times with 500 µl of radioimmune precipitation assay buffer, and immunoprecipitated RGS9 proteins were eluted from the beads by 50 µl of SDS sample buffer. 25 µl of the samples were run on the SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was air-dried and incubated on a phosphorimaging screen overnight. This screen was then scanned using a STORM phosphor-imager (Molecular Dynamics), and the bands were quantified.
this protein appears to be more stable than RGS9-1 and could be detected even when RGS9-1 was completely eliminated in photoreceptors of R9AP knockout mice (19). Control experiments utilizing RGS9-2 mutant deficient in its ability to interact with both R9AP and R7BP, RGS9-2ΔDEP reveal that its expression level is not modulated by co-expression with membrane anchors, confirming that the R7BP/R9AP elicit their effects through the complex formation with RGS9-Gß5 complexes. We have found that the effects of the membrane anchors on the expression of R7 Gß proteins did not depend on the cell line, and similar results were obtained using cultured NG108-15 cells (data not shown).

**Association with R7BP Reduces the Rate of RGS9-2 Degradation**—The observed enhancement of the RGS9-Gß5 protein levels by R7BP may be a result of either increased protein synthesis or decreased degradation. Several studies of RGS7 and RGS9 complexes with Gß5 strongly indicate that the major regulation of their expression occurs at the post-translational level (17, 19, 31). We therefore studied the mechanism of RGS9-2 expression modulation by analyzing the effect of R7BP on the degradation kinetics of RGS9-2. We utilized a pulse-chase strategy for the metabolic labeling of proteins expressed in 293 cells. In these experiments a small fraction of newly synthesized RGS9-2 was radioactively labeled, and the rate of its degradation was measured by analyzing the decay of radioactivity present in the full-length RGS9-2 protein following its immunoprecipitation from cellular lysates. The data presented in Fig. 2 demonstrate that the RGS9-2-Gß5 complex degrades rapidly, such that by 6 h after synthesis the proteins are nearly undetectable. However, co-expression with R7BP decreases its degradation rate by ~5-fold from 59.3 ± 5.8 to 287.1 ± 41.3 min (Fig. 2C). At the same time, co-transfection with R7BP did not appreciably change the extent of the RGS9-2 labeling, indicating that R7BP did not increase the rate of RGS9-2 protein synthesis. Western blot analysis of RGS9-2 present in the samples served as a loading control because the total amount of RGS9-2 expressed in the cell remains constant during the time of the experiment. These results indicate that binding to R7BP greatly stabilizes the RGS9-2-Gß5 complex by protecting it from proteolytic degradation.

**The Protective Effect of R7BP Requires Protein Binding but Not Membrane Association**—The R7BP homolog R9AP has been shown to regulate RGS9-1 levels in vivo (19, 20). Because RGS9-1/Gß5 and RGS9-2/Gß5 bind to both R9AP and R7BP with approximately equal efficiency (18, 21) and result in similar modulation of the expression levels upon co-transfection (Fig. 1), we asked whether R9AP and R7BP were also similar in their ability to protect RGS9-2-Gß5 complexes from degradation in the pulse-chase degradation assays. As shown in Fig. 3, co-transfection of RGS9-2-Gß5 with R9AP also results in an ~5-fold reduction in the rate of RGS9-2 proteolysis, demonstrating that R7BP and R9AP are equal in their ability to protect RGS9-2-Gß5 complexes.

Because association of RGS9-2-Gß5 with either R9AP or R7BP results in a translocation of the complex to the membrane (22, 23), one can imagine two potential mechanisms that can contribute to the protective effects of the anchors: targeting of RGS9-2 away from the site of proteolysis and/or stabilization using ImageQuant Software (Molecular Dynamics). Each experiment was repeated at least twice.

**RESULTS**

**Co-expression with R7BP Increases RGS9 Levels**—It is well established in the literature that R7 Gß proteins are susceptible to degradation and require association with Gß5 for their increased stability and high expression levels (17, 31). Previous studies by us and others indicate that R7 Gß proteins bound to Gß5 can readily form complexes with their partner R7BP when co-transfected into cultured cell lines (22, 23). Here, we investigated how expression levels of R7 Gß-Gß5 complexes are affected by their co-expression with R7BP. For these experiments we chose to use complexes of Gß5 with RGS9 as model R7 Gß protein because of its documented instability in vivo (19). The use of RGS9 also allowed us to compare potential effect of R7BP to the effects produced by R9AP, a protein known to stabilize the RGS9-1-Gß5 complex in photoreceptors (18, 33). As illustrated in Fig. 1, co-expression with R7BP in 293 cells substantially elevates the levels of both the RGS9-1-Gß5 and RGS9-2-Gß5 complexes. Quantification of protein expression levels indicated that R7BP increases the levels of RGS9 by ~3.5-fold and Gß5 by 2-fold. Interestingly, the extent of the effect of R7BP on the RGS9-2-Gß5 expression was similar to the one observed with R9AP. The weaker effects of R7BP and R9AP on the expression of Gß5 is expected because
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It has been previously shown that the interaction with R7BP and R9AP is mediated by the DEP domain of RGS9 (21, 33). Our earlier studies indicated that although full-length RGS9-1 could not be expressed without interacting with R9AP (19, 20), we were able to transgenically express a deletion variant of RGS9-1 lacking the DEP domain in photoreceptors (33). This result suggested that the DEP domain of RGS9-1/RGS9-2 might contain elements that destabilize RGS9 when it is not in complex with R9AP or R7BP (33). We used the pulse-chase degradation assay to directly determine whether the deletion of the DEP domain would result in the stabilization of RGS9-2-Gβ5 in the absence of its interaction with R7BP. The data presented in Fig. 3 show that RGS9-2 lacking the DEP domain exhibits the same rate of degradation as full-length RGS9-2 arguing either that the elements that destabilize RGS9 are contained in other regions of the molecule or that the stabilizing effect of R7BP is brought about by conformational rearrangement of the RGS9-2 molecule.

Knockdown of R7BP Expression Reduces RGS9-2 Levels in Primary Cultures of Striatal Neurons—The observation that R7BP reduces the degradation rate of RGS9-2 led us to ask whether binding to R7BP is critical for the expression of RGS9-2 in native neurons. RGS9-2 was reported to be predominantly expressed in the striatum where it is found in most subtypes of medium spiny neurons as well as the cholinergic interneurons (8, 14). We therefore used primary cultures of mouse striatal neurons as a model for studying the effects of R7BP on RGS9-2 expression. To knockdown R7BP expression we employed an RNAi approach in which we used short RNA duplexes containing sequences complementary to R7BP mRNA to induce specific inhibition of its expression (34). Our screen of chemically synthesized siRNA duplexes in transfected 293 cells identified two sequences that were able to induce almost complete knockdown of R7BP expression when co-delivered with an R7BP
expression construct into the cells (data not shown). Nucleotide sequences of these effective RNAs (shRNA) were incorporated into the micro RNA-155 environment in the lentiviral transfer vector pLenti6.2 (Fig. 4A). The vector was used to produce lentiviral particles pseudotyped with the VSV-G envelope protein, which upon infection delivers the construct to the cells. Our control lentiviral construct contained scrambled shRNA placed in the same position of miRNA155. The resulting lentiviral-mediated knockdown of R7BP expression in primary cultures of striatal neurons. A, genetic construct for the inhibition of R7BP expression with lentiviruses. Short hairpin duplex with either the sequences of R7BP gene or scrambled nucleotides (shRNA) were cloned as a part of microRNA155 (miRNA155) and placed under the control of the cytomegalovirus promoter. The construct also allows for the co-cistronic expression of emerald GFP with miRNA155. The expression cassette is flanked by the elements necessary for packaging into lentiviral particles (5’ and 3’ long terminal repeats (LTR), ψ, and RRE). B, representative striatal neurons in culture following lentiviral infection. Cultured cells were infected with equal amounts of anti-R7BP (miR-urR7BP) or control (miR-CTR) lentiviruses. The infection results in the expression of the GFP reporter as monitored by the appearance of the green fluorescence. The images were obtained using fluorescent microscopy. C, Western blot analysis of protein expression in the striatal neurons infected with control or anti-R7BP viruses. When indicated, recombinant lentiviruses were added to cultured cells on day 7 in vitro, and the cultures were maintained for a week after the infection. The cells were lysed in SDS sample buffer, and equal amounts of lysates were loaded on the same gel. Expressed proteins were detected with specific antibodies (see "Experimental Procedures"). D, quantification of changes in protein levels. The intensity of the protein bands was determined by densitometry using ImageJ software (NIH). The resulting values are reflected as percentages of the band density in the uninfected control cultures. Two lentiviruses targeting different regions of R7BP gene showed similar results and were combined in the analysis. The results were averaged from three experiments conducted on independently isolated primary cultures. The error bars reflect S.E. The asterisks indicate statistically significant difference in protein levels in cultures infected by miR-CTR versus miR-urR7BP viruses (p < 0.05, t test).

viruses were able to effectively infect both cultured cells and primary neurons as evidenced by the expression of the GFP reporter in the cells (Fig. 4B). The results presented in Fig. 4C reveal that infection of the striatal neurons with lentiviruses carrying miRNA targeting R7BP but not lentiviruses containing scrambled control miRNA result in the effective knockdown of R7BP expression. We found that our two lentiviral vectors targeting different regions of R7BP mRNA (248 and 483, see "Experimental Procedures" for details) were equal in their ability to bring down R7BP expression level in striatal neurons. Notably, concomitant with decreases in R7BP protein, RGS9-2 protein also showed a comparable reduction in its expression levels. At the same time, the expression of DARPP-32, a signaling protein exclusively expressed in striatal neurons (35), remained unchanged, verifying the specificity of the R7BP and subsequent RGS9-2 knockdown. Together, these data demonstrate that the knockdown of R7BP expression in native striatal neurons specifically destabilizes RGS9-2, resulting in a marked reduction in its expression levels.

The RGS-binding Site in R7BP Is Formed by Four Putative α-Helices—To gain insight into the stabilizing properties of the R7BP/RGS9-2-Gβ5 complex, we sought to define the molecular determinants that mediate the interaction of R7 RGS proteins with R7BP. As we previously reported (21), the core of R7BP secondary structure is predicted to be formed by four α helices, designated H1–H4 with the weakly structured regions at both N and C termini (Fig. 5). In addition to its propensity to form an α helix, the H4 region also contains heptad repeats (21, 22) and is identified by the COILS algorithm (36) to have more than 90% probability to form a coiled-coil fold. Interestingly, analysis of R9AP organization, the closest R7BP homolog, also predicts the same overall structural organization (33, 37).

We have used this model to perform a deletion mutagenesis of R7BP. Full-length R7BP and its deletion mutants were obtained as C-terminal fusions with GST. Recombinant proteins were expressed in E. coli, affinity-purified to homogeneity, and assessed for their ability to bind recombinant R7 RGS-Gβ5 complexes (Fig. 5). Among the four R7 RGS proteins RGS9 is highly homologous to RGS11, and RGS7 shares closest homology with RGS6, separating the subfamily into two groups. Therefore, to account for the potential differences assayed in the binding properties of R7BP mutants, we analyzed their binding to both RGS9 and RGS7. As evident from Fig. 5, the interaction pattern of both RGS proteins was similar across all deletion mutants used. Both N- and C-terminal elements of R7BP were found to be dispensable for high affinity binding of R7BP to R7 RGS proteins. However, all four helices were required for its ability to form complexes with RGS proteins. Any truncations of the H1–H4 resulted in the complete loss of R7BP binding to RGS proteins, even though all of the generated constructs were soluble and retained their ability to bind to glutathione. This indicates that the minimal RGS-binding site in R7BP is formed by helices H1–H4, which are likely to be organized in a single structural unit. This organization of the binding site is different from R9AP where only three helices, H1–H3, were shown to constitute the minimal binding site for RGS proteins (37).
R7BP and R9AP Have Competitive but Distinct Modes of Binding to RGS9-2—In addition to the observed difference in the minimal RGS-binding site between R7BP (H1–H4 helices) and R9AP (H1–H3 helices), these proteins also differ in their binding specificity for R7 RGS proteins. Although R7BP can bind all four R7 RGS proteins, R9AP forms complexes only with RGS9 and its closest homolog RGS11. To explore the molecular basis of this specificity of binding, we generated a series of R7BP/R9AP chimeric molecules in which we interchanged one or more helices and used pull down assays to determine

FIGURE 5. Determination of the RGS-binding site in R7BP. A, schematic diagram of the R7BP deletion mutants. Cylinders (H1–H4) indicate regions predicted to form α helices, blocks (NT and CT) correspond to poorly structured regions. The numbers at the bar indicate the amino acid position number where the truncations were made. B, results of the GST pulldown assay. Deletion mutants were expressed as GST fusions, purified and bound to the beads. The beads were incubated with R7 RGS5 complexes and washed, and bound proteins were eluted with SDS sample buffer. The retention of the RGS proteins was analyzed by Western blotting. GST-R7BP mutants (baits) were detected by Ponceau S staining of the polyvinylidene difluoride membrane to ensure their equal binding to the beads. C, schematic diagram of the R7BP mutants with additional truncation in the H1–H4 region. D, Western blotting analysis of the RGS protein retention by the additional R7BP deletion mutants. RGS9 and RGS7 were detected using specific antibodies.

FIGURE 6. RGS binding properties of R7BP/R9AP chimeras. A, schematic representation of the generated R7BP/R9AP chimeras. Boxes indicate predicted α helical regions that are shared between R7BP and R9AP. Shaded boxes designate elements in R7BP, and white boxes designate elements in R9AP. B, results of the pulldown binding assay between R7BP/R9AP chimeras bound to beads and RGS proteins present in the solution. RGS7/G85 and RGS9/G85 proteins retained by the beads were detected by Western blotting with specific anti-RGS antibodies.
whether the ability to bind to RGS9 or to both RGS9 and RGS7 was altered (Fig. 6). The data presented in Fig. 6 indicate that no region from R9AP can functionally substitute for the RGS-binding determinants in R7BP unless most of the protein is substituted by the R9AP sequences that alone can form an RGS-binding site (chimera 4). At the same time, both R7BP and R9AP sites of interaction with RGS9 overlap, as indicated by the binding competition assays (Fig. 7). These results indicate that despite structural and functional similarity of these two proteins, R7BP and R9AP use different modes of binding to RGS proteins.

R7BP/R9AP-binding Site in R7 RGS Proteins Is Formed by Both DEP and R7H Domains—Continuing our search of the molecular determinants of R7BP-RGS9-Gβ5 complex formation, we have next focused on the identification of the R7BP-binding site in RGS proteins. Previous studies by us and others have identified the DEP domain as a critical element that was required for RGS9 binding to both R7BP and R9AP (21, 33, 38, 39). However, when expressed alone the DEP domain failed to bind R9AP, indicating that by itself it is not sufficient for mediating interactions with the membrane anchors (38). Our attempts to determine the minimal binding site for R9AP and R7BP in RGS9 by deletion mutagenesis were not successful because of the high instability of resulting mutants.³

In the experiments presented in Fig. 8, we employed chimeric replacement strategy to identify the elements in R7 RGS proteins that form the minimal binding site for its membrane anchors. We took advantage of the fact that RGS7 and RGS9 have clearly different binding specificities for R7BP and R9AP. RGS9 forms complexes with both R7BP and R9AP, whereas RGS7 can bind only to R7BP (21). Therefore, the minimal segment shared between the RGS proteins, the replacement of which would completely and reciprocally reverse their respective binding specificities, should reveal a minimal binding site for the membrane anchors R9AP/R7BP.

The data presented in Fig. 8 reveal that the replacement of the DEP domain of RGS9 for the DEP domain of RGS7 did not result in the acquisition of the ability of the chimera to bind R9AP. The reciprocal substitutions have dramatically reduced the ability of the chimeric protein to bind to R7BP and eliminated its binding to R9AP. Consistent with our earlier observations, this result indicates that the DEP domain, although necessary, is not sufficient for mediating the binding of R7 RGS protein with their membrane anchors R7BP and R9AP. Strikingly, the replacement of both DEP and R7H domains in the next set of chimeras completely switched the binding specificities of the RGS proteins. RGS7 containing the DEP and R7H domains of RGS9 gained an ability to interact with R9AP, whereas the ability of RGS9 to bind to R9AP was lost. Including the GGL (G gamma like)-Gβ5 module to the parts replaced between RGS7 and RGS9 did not change this altered binding specificity pattern. Overall, these results indicate that the binding site for membrane anchors R7BP and R9AP is formed by pairing DEP and R7H domains of R7 RGS proteins.

DISCUSSION

The main finding of our study is that RGS9-2-Gβ5 is inherently susceptible to rapid degradation and requires the association with its binding partner, R7BP, to maintain its stability.

³ S. Baker and K. Martemyanov, unpublished observations.
and high expression levels. Regulation of protein stability is an emerging mechanism for the control of R7 RGS protein function at the cellular level. Previous studies have found that the stability of RGS9, as well as other members of R7 RGS family, depends on complex formation with their binding partner, Gβ5 (31, 32, 40). Studies of RGS9 and Gβ5 knockout animals firmly establish these proteins as obligate heterodimers (17, 19, 41). The results obtained in our study introduce R7BP as an additional player that in turn determines the stability of the RGS9/Gβ5 at the post-translational level. This result parallels our earlier observation that the photoreceptor specific splice isofrom of RGS9, RGS9-1, is also highly unstable when present in complex only with Gβ5. The levels of the RGS9-1/Gβ5 complex in photoreceptors is controlled by its association with R7BP-like protein R9AP because the genetic ablation of the R9AP gene photoreceptors is controlled by its association with R7BP-like protein R9AP (44). These observations argue that activity-dependent modulation of RGS9-2-Gβ5 expression levels is a possible compensatory mechanism that regulates altered GPCR activity.

We propose that the regulation of RGS9-2-Gβ5 stability by the association with its membrane anchor R7BP may serve as a potent mechanism for the rapid modulation of the RGS9-2-Gβ5 protein levels in the striatal neurons. Dissociation of RGS9-2-Gβ5 from R7BP would destabilize the protein complex resulting in rapid degradation, which would in turn prolong the duration of the GPCR responses. Conversely, formation of the ternary complex would stabilize the RGS9-2-Gβ5, resulting in shorter responses. Mechanisms that regulate the RGS9-2-Gβ5 association with R7BP and mediate rapid proteolytic degradation of RGS9-2-Gβ5 heterodimer will be a timely goal for future investigations.

Acknowledgment—We are grateful to Dr. Sheila Baker for critical comments on the manuscript.

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