Subcellular Targeting of RGS9-2 Is Controlled by Multiple Molecular Determinants on Its Membrane Anchor, R7BP*

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RGSG9-2, a member of the R7 regulators of G protein signaling (RGS) protein family of neuronal RGS, is a critical regulator of G protein signaling. In striatal neurons, RGS9-2 is tightly associated with a novel palmitoylated protein, R7BP (R7 family binding protein). Here we report that R7BP acts to target the localization of RGS9-2 to the plasma membrane. Examination of the subcellular distribution in native striatal neurons revealed that both R7BP and RGS9-2 are almost entirely associated with the neuronal membranes. In addition to the plasma membrane, a large portion of RGS9-2 was found in the neuronal specializations, the postsynaptic densities, where it forms complexes with R7BP and its constitutive partner Gβ5. Using site-directed mutagenesis we found that the molecular determinants that specify the subcellular targeting of RGS9-2-Gβ5-R7BP complex are contained within the 21 C-terminal amino acids of R7BP. This function of the C terminus was found to require the synergistic contributions of its two distinct elements, a polybasic motif and palmitoylated cysteines, which when combined are sufficient for directing the intracellular localization of the constituent protein. In differentiated neurons, the C-terminal targeting motif of R7BP was found to be essential for mediating its postsynaptic localization. In addition to the plasma membrane targeting elements, we identified two functional nuclear localization sequences that can mediate the import of R7BP into the nucleus upon depalmitoylation. These findings provide a mechanism for the subcellular targeting of RGS9-2 in neurons.

G protein signaling pathways mediate a wide range of critical neuronal processes (1). The normal functioning of these pathways is dependent on the tight control of signal duration mediated by regulators of G protein signaling (RGS) proteins (2, 3). RGS proteins act to control the inactivation of heterotrimeric G proteins by dramatically accelerating the rate of their GTP hydrolysis (4, 5). Much recent attention has been focused on the R7 subfamily of RGS proteins due to their emerging roles in the regulation of a variety of critical neuronal functions (6, 7). The R7 subfamily contains four highly homologous proteins, RGS6, RGS7, RGS9, and RGS11, which are expressed exclusively in the nervous system (8) as constitutive complexes with a type 5 G protein β subunit (Gβ5) (9–11).

RGSG9, with its two splice isoforms, is one of the best-studied members of the R7 subfamily. The short isoform RGS9-1 is expressed in the photoreceptor neurons where it regulates the visual signal transduction cascade (12, 13). The long splice isoform RGS9-2 is predominantly expressed in the striatum and the central nervous system structures, mediating nociceptive responses where it was shown to regulate dopamine and μ-opioid pathways (14–17). Disruption of RGS9-2 in mice resulted in the stimulation of locomotor and reward behavior, increased physical dependence and analgesic effects of morphine, and movement disorders (15–17). In photoreceptors, the function of RGS9-1 is controlled by its interaction with the transmembrane protein R9AP (RGS9 anchor protein) (18), which determines its subcellular targeting (19), catalytic activity (19, 20), and expression (21). We and others have found that, in the brain, RGS9-2 as well as other R7 RGS proteins also exist in complexes with the novel membrane protein R7BP (R7 binding protein), a close homolog of R9AP (22, 23). Membrane localization of R7BP has been shown to be mediated by its palmitoylation, without which it accumulates in the nucleus (23). Examination of the role of R7BP in R7 RGS protein function revealed that it can potently stimulate RGS-mediated inactivation of the GIRK (G protein-gated inwardly rectifying potassium) channel responses elicited by the activation of m2 muscarinic receptors in live cells (23).

In this study, we analyzed the mechanisms that regulate the intracellular targeting of R7BP and its partner, RGS9-2, in neurons. Using primary neuronal cultures and established neuronal cell lines, we have found that the major targeting determinants that control the intracellular localization of RGS9-2-R7BP complexes are contained within the short C-terminal domain of R7BP. We identified that the C terminus of R7BP carries two distinct types of signals, a functional nuclear localization sequence and a plasma membrane retention sequence. We have further demonstrated that, in striatal neurons, the presence of the C-terminal domain is required for the targeting of RGS9-2-R7BP complex to the functional specializations of the neuronal plasma membranes, the postsynaptic densities.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Generation of sheep anti-R7BP (N-terminal epitope) and anti-RGS9-2CT (C-terminal epitope) has been described previously (22). Affinity-purified antibodies were stored in PBS buffer containing 50% glycerol at a concentration of 3 mg/ml (RGS9-2CT) and 12 mg/ml (R7BP) and used at a 1:10,000 dilution in Western blot analysis. Goat antibodies against PSD-95 and rabbit antibodies against lamin A were purchased from ABCam and used at 1:1,000 dilutions. Sheep antibodies against the C terminus of Gβ5 were a gift from Dr. Vadim Arshavsky (Duke University). pcDNA3.1 TOPO cloning systems were obtained from Invitrogen. All general chemicals were purchased from Sigma Aldrich.

Cell Culture, Immunostaining, and Microscopy—NG108-15 cells were provided by Dr. Ping-Yee Law (University of Minnesota). Cells were grown on poly-l-lysine-coated coverslips (BD Biosciences) in standard Dulbecco’s modified Eagle’s medium (Invitrogen) and supple-

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mented with 10% fetal bovine serum, 0.1 mM sodium pyruvate, 0.4 µM aminopterin, 16 µM thymidine, 100 units of penicillin, and 100 µg of streptomycin. When cells reached ~70% confluency, they were transfected with DNA constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The ratio of Lipofectamine to DNA used was 4 µl/µg DNA. Briefly, on embryonic day 17, fetal rat hippocampi were dissected and maintained as described previously with minor modifications (24).

When neurons were disassociated by trituration with four flame-restricted 5-ml glass pipettes and spun down for 10 min at 1000 g, the supernatant was discarded, and the cells were washed five times with PBS buffer solution for 15 min each wash and then incubated with goat anti-rabbit antibodies conjugated to Alexa Fluor 488 (Invitrogen) and washed five times with PTS buffer and mounted using Fluoromount-G (SouthernBiotech). Following 1 h of incubation with anti-R7BP antibody at a 1:1,000 dilution, the cells were washed five times with PBS solution for 15 min each wash and then incubated with goat anti-rabbit antibodies conjugated to Alexa Fluor 568 (Invitrogen) at a 1:2,000 dilution in blocking solution for an hour. Coverslips were washed five times with PBS buffer and mounted with Fluoromount medium (Biomeda).

Cultures of hippocampal neurons were obtained from neonatal rats and maintained as described previously with minor modifications (24). Briefly, on embryonic day 17, fetal rat hippocampi were dissected and placed into Ca2+/-Mg2+/-free HEPES-buffered Hanks’ salt solution (CMF-HH). HH is composed of the following (in mM): 20 HEPES, 137 NaCl, 1.3 CaCl2, 0.4 MgSO4, 0.5 MgCl2, and 5.0 KCl. Hippocampal cells were dissociated by trituration with four flame-restricted 5-ml glass pipettes and spun down for 10 min at 1000 g. The supernatant was removed and cells were then resuspended in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1 µg/ml penicillin/streptomycin. The cells were triturated for a second time using the same four flame-restricted glass pipettes. The cells were counted and plated at a density of 16,250 cells/well on 25-mm round coverglasses that were embedded in Fluoromount (Biomeda) on glass slides. Images of the cells were captured using a Bio-Rad 1024 multiphoton confocal microscope (Bio-Rad). The confocal is equipped via LaserSharp version 3.1 software (Bio-Rad). GFP was observed by exciting the cells at 488 nm with the krypton-argon laser and monitoring the emission through a central plane of the cells that was then frame-averaged to reduce random noise fluctuations. Images were processed and analyzed using Confocal Assistant version 4.02 software.

DNA Constructs and Site-directed Mutagenesis—The coding region for GβSS was amplified by PCR using the 5’ primer GAAGATGGCACC-CCATGGGGC and the 3’ primer TTATGGCAAAATCTTGGGTTG- GATC, from the Marathon-ready mouse brain cDNA library (Invitrogen) and cloned into the pcDNA3.1/V5-His-TOPO vector. RGS9-2 was cloned into the same vector after PCR amplification of the coding region using a cDNA clone provided by Dr. S. Gold (University of Texas Southwestern). The cloning of R7BP cDNA has been previously described (22). Full-length R7BP cDNA was subcloned into the mammalian expression pcDNA3.1/V5-His-TOPO vector. For the expression of R7BP fused to GFP, the R7BP coding region was placed at the 3’ end of the GFP open reading frame in the vector. The resulting construct encoded the GFP-R7BP fusion protein under the control of the cytomegalovirus promoter. C-terminal mutants of R7BP were generated by PCR amplification using the 5’ flanking primer (5’-GATATGAGTCTG-CACCGAATTGGG-3’) and the following mutagenic primers at the 3’ end: R7BPACT (amino acids 1–126), 5’-CTACGTTAGATTCAGA-GAGCTGCGC-3’; R7BP-RE (R243E,R246E), 5’-CTAGCTTGGAGACAA- GGAACATTAAGCCGAACAACTCTCCCTGCTTCGACCA-TGG-3’; and R7BP-CA (C252A,C253A), 5’-CTAGCTTGGAGACAA-GCAGCTAAGCGGAAGA-3’.

The R7BP-PA mutant lacking amino acids 242–247 was generated by ligating a linker coding for the C-terminal part of R7BP (amino acids 248–257) into the Ncol-treated pcDNA3.1/NT-GFP-TOPO containing wild-type R7BP gene. For the generation of the GFP-CT construct (amino acids 242–257), a synthetic oligonucleotide duplex coding for the C-terminal 16 amino acids of R7BP was cloned at the 3’ end of the GFP sequence in the same open reading frame. The same approach was used for the generation of the GFP-CTRE construct (amino acids 242–257 with R243E,R246E mutations).

Quadruple mutants R7BP-RECA (C252A,C253A,R243E,R246E) and R7BP-CAANL-2S (C252A,C253A,(Δ242–247)) were generated by amplifying the coding regions of R7BP-RE or R7BP-PA, respectively, with the mutagenic primer used for the generation of R7BP-CA. R7BP-CAANL-1 (C252A,C253A,(Δ1–11)) was generated by amplifying the cDNA of R7BP-CA mutant with the 5’ mutagenic primer AGCGGTTCAC-CGGCTCC and the 3’ flanking primer. The same strategy was used to generate the R7BP-CAANL-1+S1NLS-2 mutant (Δ1–11,Δ257–257). All cDNAs coding for R7BP mutants were cloned into the pcDNA3.1/NT-GFP-TOPO vector to produce GFP fusions. Constructs were propagated into Escherichia coli Top10 strain (Invitrogen) isolated using Maxiprep kits (Qiagen) and sequenced.

Fractionation of Brain Extracts—C57/B6 mice (Charles River) were sacrificed by brief CO2 inhalation followed by cervical dislocation. Brains were dissected into ice-cold HEPES-sucrose buffer (4 mM HEPES, pH 7.4, 0.32 M sucrose) supplemented with protease inhibitor cocktails (Sigma). All procedures were performed at 4 °C. For the biopsies of striatal tissues, whole mouse brains were cut into 3-mm-thick coronal sections. The caudate/putamen regions were punched out from the sections using a 2-mm trephine. Tissues (~80 mg) were homogenized in 500 µl of HEPES-sucrose by repeated passes through a series of 18–26-gauge needles. Subcellular fractionation of brain extracts was performed using a variation of the method described by Blackstone et al. (25) and Lau et al. (26). Homogenates were briefly subjected to sedimentation at 1,000 × g for 10 min. The pellet containing the nuclei (P1) was resuspended in 500 µl of HEPES-sucrose and resedimented. The resulting pellet was designated as the nuclear fraction (N). Postnuclear supernatants (S) were combined and sedimented at 10,000 × g for 15 min. The pellet was resuspended in 500 µl of HEPES-sucrose and resedimented yielding a crude synaptosomal fraction. The supernatant was removed and subjected to a 45-min centrifugation at 100,000 × g to prepare the membrane (M; plasma membrane and microsomes) and cytosolic (C) fractions. Synaptosomes were lysed by resuspending in 450 µl of ice-cold H2O followed by prompt adjustment to 4 mM HEPES, pH 7.4.
7.4. The lysate was centrifuged at 25,000 × g for 20 min to yield a crude synaptic vesicle fraction (supernatant) and a lysed synaptosomal membrane fraction (pellet). For the isolation of pure synaptic plasma membrane, synaptosomal membranes were resuspended in 200 μl of HEPES-sucrose and layered on top of a 0.8, 1, and 1.2 M sucrose gradient. Samples were centrifuged at 150,000 × g for 2 h. Synaptic plasma membranes were recovered from the interface between 1.0 and 1.2 M sucrose, diluted to 0.32 M sucrose, and sedimented at 150,000 × g for 30 min. The resulting pellet was designated as the purified synaptic plasma membrane fraction.

Post-synaptic densities were isolated as described previously (27, 28). The synaptosomal membrane pellet was resuspended in 500 μl of 50 mM HEPES, pH 7.4, 2 mM EDTA, 0.5% Triton X-100, and protease inhibitor mixture (Sigma) and incubated with end-over-end mixing for 15 min at +4 °C. The Triton X-100-resistant fraction (T1P) was recovered by centrifuging the sample at 32,000 × g for 20 min. The resulting pellet was resuspended in the same buffer, incubated for an additional 15 min, and sedimented at 100,000 × g for 20 min to pellet the postsynaptic densities (T2P). Protein content in the fractions was quantified by Bradford assay. Samples containing 15 μg of total protein were loaded onto a polyacrylamide gel and subjected to Western blot analysis.

**Immunoprecipitation**—Immunoprecipitation was conducted as described earlier (22). The postsynaptic density fraction T1P prepared from the striatum of one mouse was extracted with PBS buffer containing 1% Triton X-100 (Sigma) and protease inhibitor mixture (Sigma) for 1 h on ice. The extract was centrifuged and the resulting supernatant applied to 10 μl of protein G-Sepharose beads (GE Healthcare) covalently attached to 100 μg of sheep affinity-purified anti-RGS9-2 CT using BS3 cross-linker ( Pierce). In the control immunoprecipitation, equal amounts of non-immune sheep IgGs (Sigma) were used. After 1 h of incubation, the beads were washed three times with 1% Triton X-100-PBS buffer. Bound proteins were eluted with SDS-PAGE sample buffer and analyzed by Western blotting.

**Metabolic Labeling**—NG108-15 cells were grown in T-75 flasks until confluency, at which point they were transfected with the DNA constructs containing R7BP genes using Lipofectamine 2000. 48 h after transfection, the medium was aspirated and replaced with 5 ml of fresh labeling medium composed of Dulbecco’s modified Eagle’s medium supplemented with 5% dialyzed fetal bovine serum (Invitrogen) and 330 μCi/ml [3H]palmitic acid (40 Ci/mmol; PerkinElmer Life Sciences). Cells in the labeling medium were incubated at 37 °C and 5% CO₂ for 3 h. Following incubation, the cells were washed twice with PBS (Invitrogen) and scraped into 15 ml of ice-cold PBS supplemented with 1% Triton X-100 and 0.01% SDS. The cells were lysed by passage through 23-gauge needles, and insoluble material was removed by 30 min of centrifugation at 10,000 × g. The resulting extract was applied to 10 μl of protein G-agarose beads (Amersham Biosciences) coupled to 60 μg of anti-R7BP antibodies. Immunoprecipitated R7BP proteins were eluted from the beads with SDS sample buffer, separated by polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membrane. Membranes were soaked in Amplify (Amersham Biosciences), dried, and exposed to x-ray film (BioMax MR, Kodak) for 1 week. Following fluorographic analysis, polyvinylidene difluoride membranes were rewetted in methanol and used in Western blot analysis with anti-R7BP antibodies.

**RESULTS**

**R7BP Targets RGS9-2 Localization to the Plasma Membrane**—We have recently identified R7BP as the protein that interacts nearly stoichiometrically with RGS9-2 in the membrane fractions of the striatum (22). In this study, we started addressing the functional role(s) of this interaction by analyzing the intracellular localization of RGS9-2 and R7BP upon their co-expression in cell cultures. Because RGS9-2 has been shown to exist as an obligatory complex with the Gβ5 subunit in the brain (10, 22), we co-expressed RGS9-2 with the brain-specific splice isoform of Gβ5, Gβ5S. The expression of RGS9-2, Gβ5, and R7BP in vivo is restricted to neuronal tissues, which suggests that much of their native properties may be dependent upon the specific intracellular environment of neurons. Therefore, we chose to use the NG108-15 mouse neuroblastoma/rat glioma hybrid cells, which share many common properties with native differentiated neurons (29).

As illustrated in Fig. 1, RGS9-2-Gβ5S and R7BP show distinctly different localization patterns when expressed in these cells separately. Although RGS9-2-Gβ5S is localized exclusively in the cytoplasm, R7BP is restricted to the plasma membrane. However, co-expression of GFP-labeled RGS9-2-Gβ5S with R7BP resulted in the co-localization of both proteins to the plasma membrane. This result demonstrates that R7BP acts to target the membrane localization of the otherwise soluble RGS9-2-Gβ5S. This is consistent with the observation that palmitoylated R7BP is capable of interaction with all four members of the R7 GRS protein family in the plasma membrane (23).

**Plasma Membrane Localization of R7BP Is Mediated by Its C Termi-**

**nus**—The R7BP molecule can be roughly divided into two parts, a four-helical N-terminal domain that shares significant homology with a related protein, R9AP, and a unique C terminus occupying the position corresponding to the transmembrane helix in R9AP (22, 23). This 20-amino-acid C-terminal domain of R7BP is structurally similar to the C-terminal sequences of K-Ras proteins and contains two main features, a polybasic stretch of six consecutive arginines/lysines and a conserved cysteine doublet that undergoes post-translational palmitoylation (Fig. 2A) (22, 23).

Fig. 2B indicates that the C-terminal domain plays a crucial role in determining subcellular localization of R7BP. Deletion of this domain resulted in complete abolishment of the membrane attachment of the truncated mutant, which was instead found distributed throughout the cytoplasm and the nucleus of the cells. To test whether the C-terminal domain of R7BP is sufficient in providing plasma membrane localization, we expressed a construct containing the C-terminal domain of R7BP fused to the C terminus of GFP. Unlike soluble GFP, which is evenly distributed throughout the cytoplasm, GFP-CT was localized exclusively to the plasma membrane, mimicking the localization pattern
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A schematic representation of the R7BP constructs used in the experiments. Shaded boxes mark two conserved features at the C terminus (CT) of R7BP, Wavy lines represent palmitoyl moieties. B, localization of GFP-R7BP constructs in transfected NG108-15 cells as monitored by GFP.

of wild-type R7BP. These data indicate that the plasma membrane-targeting signal is located within the C-terminal domain of R7BP and that this signal is sufficient to determine its subcellular localization.

Plasma Membrane Targeting of R7BP Requires Synergistic Contributions of a Polybasic Region and Palmitoylated Cysteines—The sufficiency of the C-terminal domain in directing R7BP plasma membrane localization prompted us to determine the relative contributions of the two structural elements located within this domain, the polybasic region and the palmitoylated cysteines. Polybasic clusters adjacent to sites of lipid modifications have been shown to mediate membrane localization in several members of the Ras superfamily (30), suggesting a similar functional significance in R7BP. To test this possibility, we used site-directed mutagenesis to construct R7BP mutants with disrupted polybasic motifs (Fig. 3A). This was achieved by either deletion of all six basic amino acids (R7BPΔPB) or neutralization of the net charge by replacing positively charged arginines with negatively charged glutamic acids at positions Arg-243 and Arg-246. As shown in Fig. 3B, the disruption of the polybasic region resulted in dramatic mislocalization of R7BP. Only trace amounts of R7BPΔPB or R7BP-RE were associated with the plasma membrane, whereas most were trapped in intracellular vesicular structures. This mislocalization pattern was replicated when a sequence containing the last 21 amino acids of the mutated C-terminal sequence was placed onto a GFP molecule, indicating that this effect is not mediated by the unique composition of the rest of the R7BP molecule (Fig. 3B).

Introduction of the C252A and C253A mutations, which abolished R7BP palmitoylation, also abolished the vesicular trapping of the R7BP-RECA mutant, leading to its diffuse distribution throughout the cytoplasm (Fig. 3B). This result suggests that mislocalization of R7BP to intracellular membrane vesicles in the absence of a functional polybasic motif is palmitoylation-dependent. To further demonstrate that mutations in the polybasic motif affect the intracellular routing of R7BP rather than its ability to undergo palmitoylation, we examined the palmitoylation status of the R7BP-RE mutant by metabolic labeling (Fig. 3C). Our data demonstrate that the palmitoylation level of the R7BP-RE mutant is similar to that of wild-type R7BP. The absence of [3H]palmitic acid incorporation into the R7BP-CA mutant is consistent with palmitoylation occurring specifically at the Cys-252 and Cys-253 residues of R7BP.

In summary, these data indicate that plasma membrane targeting of R7BP is achieved by the synergistic contributions of two elements located at the C terminus. Although cysteine palmitoylation alone provides R7BP with the ability to associate with membranes, the addition of the polybasic region enables R7BP to selectively associate with the plasma membrane.

R7BP Contains Functional Nuclear Localization Sequences—In addition to directing membrane localization, palmitoylation of R7BP has also been shown to be involved in determining its nuclear accumulation. Up to 40% of the wild-type R7BP was found in the nucleus of the transfected cells of non-neuronal origin (23). In most cases, import into the nucleus requires the presence of a functional nuclear localization sequence (NLS) on the transported protein. Although individual NLS motifs differ in their amino acid sequences, they are usually highly enriched in positively charged residues (31). The sequence analysis of R7BP revealed the presence of two basic amino acid clusters that could potentially serve as the NLS, the N-terminal sequence RKKR (amino acids 8–11), designated as NLS-1, and the C-terminal polybasic motif (NLS-2) described above (Fig. 4A). To explore the possibility that the nuclear targeting of R7BP is directed by either of these putative NLS sequences, we constructed R7BP mutants where one or both motifs were deleted. Because R7BP localizes to the nucleus only when it is not palmitoylated, the effects of these mutations were studied on the background of the palmitoylation-deficient mutant (R7BP-CA). As illustrated in Fig. 4B, almost all non-palmitoylated R7BP localized to the nucleus of the NG108-15 cells, a result consistent with earlier findings by Drenan et al. (23). The deletion of either NLS-1 or -2 shifted the distribution of R7BP in cells toward the cytoplasm, whereas a significant portion of it was still localized to the nucleus. Deletion of both NLS sequences resulted in the soluble distribution of R7BP throughout the entire cell cytoplasm, coinciding with the distribution of GFP alone (Fig. 4B). This result demonstrates that non-palmitoylated R7BP is targeted into the nucleus by two functional nuclear localization sequences.
Most of R7BP Is Localized in the Plasma Membrane of Striatal Neurons—The identification of signals in R7BP capable of targeting the protein to either the nucleus or the plasma membrane led us to ask where endogenous R7BP is localized in neurons. We addressed this question by biochemically separating mouse striatal neurons into discrete subcellular fractions.

A schematic representation of the cell fractionation strategy is illustrated in Fig. 5A. The first step involved the separation of cellular lysate into a nuclear pellet and postnuclear supernatant that was subsequently used in the isolation of various membrane fractions and cytosol. To monitor the isolation efficiency of the nuclear and membrane fractions, we used two marker proteins, the nuclear envelope protein Lamin A and the palmitoylated scaffold protein PSD-95 as markers for membrane fractions. In addition to its localization in the plasma membrane, PSD-95 is also found to be dynamically associated with specialized neuronal plasma membrane structures, the postsynaptic densities (32). As evident from the results of a typical fractionation experiment (Fig. 5B), very little R7BP was found in the nuclear fraction, whereas most of it localized to the various plasma membrane compartments. In fact, the nuclear content of R7BP did not exceed the content of membrane-bound PSD-95 in the same fraction and therefore may have resulted from minor contamination of the nuclear fraction by membranes.

In addition to the plasma membranes and microsomes, a large portion of R7BP was found in the synapses. Further fractionation of the synaptosomes revealed that R7BP is localized to synaptic membranes but not synaptic vesicles (Fig. 5B). This pattern of localization at the synapses is also characteristic of the distribution of PSD-95, which is primarily found in the postsynaptic densities. To further test the possibility that R7BP in the synaptic membrane fraction is associated with the postsynaptic densities, we performed a sucrose gradient purification of the synaptic plasma membranes following the isolation of the postsynaptic densities. As evident from Fig. 5C, R7BP is highly enriched in the purified synaptic plasma membrane fraction, which is resistant to treatment with 0.5% Triton X-100, a feature characteristic of postsynaptic density proteins. Accordingly, the partner of R7BP, RGS9-2, showed a similar postsynaptic localization pattern. An immunoprecipitation experiment conducted with extracts from isolated postsynaptic density fractions revealed that R7BP forms complexes with RGS9-2 and its constitutive subunit Gβ5 at these postsynaptic sites (Fig. 5D).

We next examined the role of the C-terminal targeting sequence of R7BP in its postsynaptic localization. This was addressed in primary cultures of hippocampal neurons, a model ubiquitously used to study postsynaptic densities in neurons. GFP-R7BP transfected in hippocam-
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Members of the R7 family of RGS proteins are emerging as critical regulators of neuronal G protein signaling. RGS9-2 is one of the best-studied members of this family and is implicated in the development and progression of visual diseases (33), movement dysfunctions (17), neurodegenerative disorders (34), and drug addiction (16). However, the mechanisms that control RGS9-2 function on the molecular and cellular levels are poorly understood. In this study, we examined one of the most recently discovered properties of RGS9-2, its interaction with the membrane anchor R7BP (22, 23). The major result of this study is the identification of targeting signals in R7BP that specify its subcellular localization and determine the localization of the RGS9-2 complex in neurons. We have found that the major determinant of R7BP targeting is contained within its 21-amino-acid C-terminal domain, which alone is sufficient for directing the intracellular localization of the constituent protein. The presence of the small sorting signal at the C terminus of R7BP provides yet another illustration of

DISCUSSION

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FIGURE 4. R7BP contains two functional nuclear localization sequences (NLS). A, schematic representation of R7BP structure showing potential NLS sequences (shaded boxes). Asterisks indicate the position of the C252A and C253A mutations that abolish R7BP palmitoylation. B, analysis of nuclear/cytoplasmic distribution of R7BP mutants by confocal microscopy. Green, GFP of R7BP constructs. Blue, nuclei stained with DAPI.
the intracellular protein sorting mechanisms that are common to such diverse proteins families as G protein-coupled receptors and Ras, which rely on short C-terminal tags to reach their intracellular destinations (see Refs. 30 and 35 for reviews).

The C-terminal domain of R7BP contains two structural elements that are responsible for its targeting properties. One is a cysteine doublet that undergoes post-translational palmitoylation (23); another is a polybasic cluster composed of five arginines and a lysine residue. We have determined that lipidation of R7BP provides it with the ability to bind to the membranes, whereas the polybasic region carries two types of signals. First, acting together with palmitoylated cysteines, the polybasic region routes R7BP specifically to plasma membrane compartments instead of intracellular vesicular structures. Second, when R7BP is depalmitoylated, this region serves as a functional nuclear localization sequence directing R7BP to the nucleus. Interestingly, a very similar organization of C-terminal targeting signals is also observed in members of the Ras superfamily, K-Ras4B and Rac1 (30, 36). Both of these proteins undergo lipid modification by prenylation at the C-terminal cysteines and contain polybasic regions immediately adjacent to the sites of modification. As in the case of R7BP, the presence of intact polybasic motifs in K-Ras4B was shown to be required for its efficient plasma membrane delivery, and disruption of the positive charge led to its accumulation in intracellular membranous compartments (37). Likewise, the interaction of Rac1 with intracellular membranes diminishes upon the disruption of its polybasic region (38). In addition, the polybasic region of Rac1 has been shown to serve as a nuclear localization sequence (39). These similarities suggest that the mechanisms regulating the sorting and delivery of R7BP and Ras proteins are governed by common principles. This provides an exciting opportunity to apply a wealth of information gained in the studies of Ras protein trafficking to probe the principles governing the intracellular fate of R7BP bound to R7 RGS proteins.

The results presented in our study, as well as in the recent report by Drenan et al. (23), indicate that one function of R7BP is to target RGS9-2/Gb5 complexes and other members of the R7 RGS family to plasma membrane compartments. These findings parallel our earlier observations that intracellular targeting of the short splice isoform of RGS9, RGS9-1, to the place where it functions (the outer segments of photoreceptors) requires its interaction with R9AP, a close homolog of R7BP (19). Taken together, these data suggest the general principle that R7 RGS protein trafficking in the cell is mediated by specialized adapter proteins, such as R7BP and R9AP, which carry intracellular sorting signals allowing the delivery of the RGS proteins to the sites of their function. In this model of assisted delivery, R7 RGS proteins do not contain elements specifying their intracellular targeting per se but must rely on specific protein-protein interactions to reach their destination. This mechanism should provide powerful regulatory points where the localization of RGS proteins, and therefore their G protein inactivating activity, can be controlled by modulating the interactions with trafficking proteins.
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adapters. In this respect, R7BP and R9AP may represent only a fraction of multiple trafficking adapters that could potentially control various intracellular destinations of GRS proteins.

One of the central findings in this study is that, in the brain, nearly all R7BP and its partner RGS9-2 are localized to the plasma membrane and that a significant portion of R7BP-RGS9-2 is associated with postsynaptic densities. Moreover, we found that this localization pattern of the complex is determined by the targeting sequence at the C terminus of R7BP. To our knowledge, this is the first demonstration that GRS proteins are specifically localized to discrete postsynaptic compartments. This finding places R7BP and its complexes with R7 RGS proteins in line with other components of the G protein signal transduction machinery that are enriched at the postsynaptic densities, such as G protein-coupled receptors (40) and G protein effectors (41).

It is interesting to consider this result in view of recent findings on the functional association of RGS9-2 (14, 15, 17) with D2 and μ-opioid receptors (16, 42). The targeting of RGS9-2 to the receptors was found to require the presence of its N-terminal DEP domain, although no direct interactions have been established (14, 17). Because the DEP domain is the module also mediating the interaction of RGS9-2 with R7BP (22), it seems plausible to suggest that functional coupling of RGS9-2 to G protein-coupled receptors is indirect and is mediated by its postsynaptic targeting via the interaction with R7BP rather than by a direct association with a specific receptor subtype. Because R7BP can interact with all four members of the R7 subfamily and not just with RGS9-2, this mode of R7BP function is likely to be universal for the entire R7 RGS subfamily. Indeed, a recent study demonstrated that R7BP potently stimulates RGS7 function by controlling physiological responses to m2 muscarinic receptor stimulation and that this stimulation is dependent on the membrane targeting of R7BP (23). This finding indicates that the targeted regulation of G protein signaling by RGS proteins at postsynaptic sites might constitute a part of the mechanisms that underlie synaptic plasticity.

Another interesting property of R7BP is its ability to accumulate in the nucleus upon depalmitoylation. In this study, we identified two sequences that provide the molecular basis for the ability of R7BP to undergo nuclear translocation. Importantly, one of the NLS sequences is also a part of the plasma membrane targeting sequence acting in conjunction with palmitoyl tails resulting in the mutually exclusive nature of the R7BP localization in the cell. Although palmitoylated R7BP is localized only on the plasma membrane, in the absence of the palmitoylation, the ability of R7BP to localize to the plasma membrane is completely lost and is mostly found in the nucleus. Our results obtained with transfected NG108-15 cells, hippocampal neurons, and biochemical fractionation of the striatum consistently indicate that wild-type R7BP is almost exclusively found in plasma membrane compartments with only trace amounts present in the nuclei. Accordingly, we found that the subcellular localization of RGS9-2 in the brain is identical to the localization pattern of R7BP. These data appear to be in disagreement with results obtained by Drenan et al. (23), who found up to 40% of the wild-type R7BP in the nucleus of human embryonic kidney-293 cells and transfected hippocampal neurons. Because the palmitoylation status of the R7BP in the nucleus was not determined in that study, one possibility is that nuclear R7BP represents non-palmitoylated protein. In that case, it may be the activity level of endogenous palmitoyl acyltransferase enzyme, perhaps different between various cell lines and culturing conditions, that determines how efficiently R7BP is palmitoylated in the cells and is therefore targeted to membranes. Similar reasons might also explain the predominant membrane association of RGS9-2 in our experiments as opposed to its presence in the nucleus and cytoplasm reported previously (43).

The presence of functional NLS signals could trigger nuclear accumulation of R7BP through a change in palmitoylation status, a mechanism that has been described to control the intracellular destination of many signaling proteins (44). Although the physiological conditions at which R7BP undergoes nuclear translocation, as well as the functional roles it may play in the nucleus remain to be elucidated, one important consequence of such translocation is the sensitization of G protein signaling (23) that presumably would occur due to the retargeting of R7 RGS away from the GPCR transduction machinery at the plasma membrane.

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