

## R7BP, a Novel Neuronal Protein Interacting with RGS Proteins of the R7 Family\*

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**The R7 subfamily of the regulators of G protein signaling (RGS) proteins is represented by four members broadly expressed in the mammalian nervous system. Here we report that in the brain all four R7 proteins form tight complexes with a previously unidentified protein, which we call the R7-binding protein or R7BP. We initially identified R7BP as a protein co-precipitating with the R7 protein, RGS9, from extracts obtained from the striatal region of the brain. We further showed that R7BP forms a tight complex with RGS9 *in vitro* and that this binding occurs via the N-terminal DEP domain of RGS9. R7BP is expressed throughout the entire central nervous system but not in any of the tested non-neuronal tissues. All four R7 RGS proteins co-precipitate with R7BP from brain extracts and recombinant R7 proteins bind recombinant R7BP with high efficiency. The closest homolog of R7BP is R9AP which was previously found to interact with RGS9 in photoreceptors. Both R7BP and R9AP are related to the syntaxin subfamily of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins involved in vesicular trafficking and exocytosis. In photoreceptors R9AP regulates several critical properties of RGS9 including its intracellular targeting, stability and catalytic activity. This suggests that R7BP interactions with R7 proteins in the brain may also bear major functional significance.**

RGS<sup>1</sup> proteins regulate the duration of G protein signaling by stimulating the rate of the GTP hydrolysis on G protein  $\alpha$  subunits (reviewed in Refs. 1 and 2). RGS proteins are classified in six to nine subfamilies based on the homology within the

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<sup>1</sup> The abbreviations used are: RGS, regulators of G protein signaling; G $\beta$ 5, type 5 G protein  $\beta$  subunit; R9AP, RGS9 anchor protein; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; DEP, disheveled, EGL10, pleckstrin homology domain; G $\alpha_o$ , the  $\alpha$  subunit of G protein G<sub>o</sub>; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; R7BP, R7-binding protein; GST, glutathione S-transferase.

RGS catalytic domain (1–3). Most RGS proteins contain additional noncatalytic domains that modulate the properties of their catalytic domains and allow them to interact with their partners in a broad range of signaling pathways. The R7 (also called "C") subfamily of RGS proteins contains four members expressed in the mammalian nervous system, RGS6, RGS7, RGS9, and RGS11 (reviewed in Ref. 4). R7 proteins share common domain composition and exist as constitutive complexes with the type 5 G protein  $\beta$  subunit (G $\beta$ 5).

Marked progress in the understanding of the function of the R7 protein came from the studies of the short splice variant of RGS9, RGS9-1, which regulates signal duration in the phototransduction pathway in vertebrate rod and cone photoreceptors. Several functional properties of RGS9-1 in photoreceptors are regulated by its interacting partner, a SNARE-like protein R9AP. R9AP is responsible for targeting RGS9-1 to the photoreceptor outer segment, an intracellular compartment where phototransduction takes place (5). It also determines the RGS9-1 expression level by protecting RGS9-1 from proteolytic degradation in the cell (6). Finally, R9AP enhances the ability of RGS9-1 to stimulate G protein GTPase activity thus allowing the visual signal to be terminated on the physiologically rapid time scale (5, 7, 8). RGS9-1 interaction with R9AP is mediated by the N-terminal domain of RGS9-1 called DEP (7, 8).

The physiological significance of RGS9-1 interaction with R9AP was emphasized in a recent human study of patients bearing defects in either RGS9 or R9AP molecules (9). The mutations of either protein resulted in a clinical phenotype characterized by impaired ability to adjust to bright light and by impaired temporal resolution of the visual signals resulting in reduced ability to see moving objects.

The importance of RGS9-1 interaction with R9AP in the visual system suggests that similar interactions may regulate the function of other R7 proteins, each containing the N-terminal DEP domain. In mammals, all R7 proteins, including RGS6, RGS7, RGS11, and the long splice variant of RGS9, RGS9-2, are expressed in the brain (10, 11), whereas R9AP expression is restricted to the retina (12). In this study we searched for unknown R9AP homolog(s) that could interact with R7 proteins in the brain. We have found that indeed all four R7 proteins interact with a novel neuronal homolog of R9AP, which we call R7-binding protein (R7BP).

### EXPERIMENTAL PROCEDURES

**Cloning, Expression, and Purification of Recombinant Proteins**—The coding region of R7BP was amplified by PCR from Marathon-ready mouse brain cDNA library (BD Biosciences) using the NdeI-containing upstream primer: 5'-GGAATTCCATATGAGTTCTGCACCGAATGGG-C-3' and the EcoRI-containing downstream primer: 5'-TGGCGAATTCCTAGCTTGAGACAAGACAACATAAGC-3'. The PCR product was cloned into the pGEX2T *Escherichia coli* expression vector downstream from the GST coding sequence at BamHI and EcoRI sites via a BamHI-NdeI linker. The R7BP coding region was sequenced and found to be identical to the sequence found in GenBank™ (accession no. AK046733). The resulting vector was transformed into the BL21(DE3) *E. coli* strain, and the cells were induced by 1 mM isopropyl  $\beta$ -thiogalactoside at OD<sub>600</sub> = 0.8. Three hours post-induction cells were collected and disrupted by sonication. R7BP found in the particulate fraction was solubilization by 6 M urea and dialyzed into the Buffer A: 12 mM potassium phosphate (pH 7.4), 3.7 KCl, 447 mM NaCl, and complete protease inhibitor mixture (Roche Applied Science). Renatured protein was purified on the Hi-Trap glutathione-Sepharose column (Amersham Biosciences) following the manufacturer's protocol.

The construction of the pVL1392 plasmids encoding RGS9-1, the

RGS9 $\Delta$ DEP mutant of RGS9-1 (amino acids 112–484), and the short splice variant of G $\beta$ 5 was described previously (13). RGS7 cDNA cloned in pVL1392 was a gift from William Simonds (NIDDK, National Institutes of Health). The cDNAs coding for mouse RGS6 and RGS11 were cloned by reverse transcription-PCR from total RNA isolated from the mouse brain. The RGS6 specific primers used were: BamHI-containing upstream 5'-CGCGGATCCATGGCTCAGGGGTCCGGGGAC-3' and MfeI-containing downstream 5'-GCGCAATTGTCTCAGGAGGACTGCATCAGGCC-3'. The RGS11 specific primers used were: BamHI-containing upstream 5'-CGCGGATCCATGGCCATGGTTACCCGCTGC and MfeI-containing downstream 5'-GCGCAATTGTCTCCTCCATCGGCACCTTC-3'. PCR products were digested with BamHI and MfeI restriction endonucleases and cloned into the modified baculovirus transfer vector pVL1392 treated with BamHI and EcoRI enzymes (13). The resulting constructs encoded RGS6 and RGS11 preceded by an amino acid sequence containing a His<sub>6</sub> tag and a thrombin cleavage site. The coding regions for RGS proteins were sequenced and found to match those published in GenBank™ (NM\_015812 for RGS6 and XM\_128488 for RGS11).

The R7 and G $\beta$ 5 constructs were used to generate recombinant baculoviruses through the custom service of Orbigen (San Diego, CA). Proteins were expressed in Sf-9 cells and purified as described previously (13). Control experiments conducted as described in (14) revealed that all purified R7/G $\beta$ 5 proteins were catalytically active as judged by their ability to stimulate transducin and G $\alpha_o$  GTPase activity.

**Antibodies**—Affinity-purified sheep antibodies derived against a fragment of RGS9-1 between residues 226–484 were described in (15). Specific antisera against the following peptides corresponding to the mouse proteins were obtained from both sheep and rabbit by Elmira Biologicals (Iowa City, IA): the N-terminal R7BP peptide CSAPNGRKRPSRSTRSSI; the C-terminal RGS6 peptide CKKGKSLAGKRLTGLMQSS, the C-terminal RGS7 peptide CRKGKLTLSKSLTSLVQSY, the C-terminal RGS9-2 peptide DSDDPRAGESGDQTTEKEVICPWESELAEGKAG, and the C-terminal RGS11 peptide CSPALQSTPREAATSSPEGADGE. Each peptide was purified by high performance liquid chromatography and coupled to the maleimide-activated keyhole limpet hemocyanin (Pierce); the resulting conjugates were used to immunize sheep and rabbits. Specific antibodies were purified from the immune sera by affinity chromatography on the columns with immobilized peptides used for the immunization.

**Preparation of the Brain Extracts**—All procedures were performed at 4 °C unless stated otherwise. Whole brains were removed from mice and dissected on ice. For the analysis of the R7BP expression, various brain structures were removed, placed in 1.5 ml of Buffer A containing 1% Triton X-100 (Buffer B), and homogenized by sonication. Detergent-insoluble material was removed by centrifugation at 100,000  $\times g$  for 30 min. The resulting detergent extracts were subsequently used for immunoprecipitation by anti-R7BP and RGS9-2 antibodies.

For the preparative immunoprecipitation of RGS9-2, striatums from six mice were placed in 3 ml of Buffer A. The tissue was homogenized by passing it through a series of needles (18–23 gauge), and the resulting homogenate was centrifuged at 100,000  $\times g$  for 30 min to sediment the membrane fraction. The pellet was resuspended in Buffer B and incubated for 1 h on a rocking platform to extract membrane proteins. The non-soluble debris was removed from the detergent-solubilized extract by centrifugation at 100,000  $\times g$  for 30 min.

**Immunoprecipitation**—For protein immunoprecipitation, 1.5 ml of the striatal extract or extracts obtained from other brain regions was incubated on a rocking platform for 1 h at 4 °C with 30  $\mu$ l of protein G-Sepharose (Amersham Biosciences) coupled to either anti-RGS9-2 or IgG antibodies (300  $\mu$ g each). The antibodies were covalently cross-linked to the protein G with BS<sup>3</sup> (Pierce) following the manufacturer's protocol. The beads were then washed three times with 500  $\mu$ l of Buffer B. For preparative experiments, proteins were eluted with 100  $\mu$ l of 5% ammonium hydroxide, lyophilized, solubilized in the SDS-PAGE sample buffer, separated by SDS-PAGE, and visualized with Novex Coomassie-based stain (Invitrogen). For analytical experiments, proteins were eluted by 50  $\mu$ l of the SDS-PAGE sample buffer and analyzed by Western blotting.

**Pull-down Assays**—Pull-down assays were conducted essentially as described previously (14, 16). Briefly, 3 nmol of a GST-tagged protein was coupled to 15  $\mu$ l of glutathione-agarose beads (Amersham Biosciences) equilibrated with the binding buffer: 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 0.25% lauryl sucrose, and 50  $\mu$ g/ml bovine serum albumin. The beads were incubated with 50  $\mu$ l of 0.1  $\mu$ M R7-G $\beta$ 5 complexes for 20 min on ice and then washed three times with 1 ml of the binding buffer. RGS proteins bound to the beads were eluted with 50  $\mu$ l of SDS-PAGE sample buffer. 10- $\mu$ l aliquots of the eluates were subjected

to SDS-PAGE followed by the Western blot detection using specific antibodies against each RGS protein.

**Mass Spectrometry**—Coomassie-stained protein bands were excised from the gel and treated with trypsin using the in-gel digestion kit (Pierce) according to the manufacturer's protocol. Dried peptides were dissolved in 50% acetonitrile, 0.1% trifluoroacetic acid, and 5 mg/ml  $\alpha$ -4-hydroxycinnamic acid as a matrix. 0.6  $\mu$ l of peptide-matrix mix was loaded onto a 192-spot MALDI plate dried at room temperature and subjected to MALDI-TOF MS and MS/MS analyses using the 4700 Proteomics analyzer and GPS software package (Applied Biosystems). A combination of peptide mass fingerprinting and the MS/MS analysis was performed using the Mascot search engine. Fragmentation of the precursors was carried out using the collision-induced dissociation settings. Collision-induced dissociation spectra were submitted for protein identifications with a precursor precision tolerance of 1 Da and MS/MS fragment tolerance of 0.5 Da.

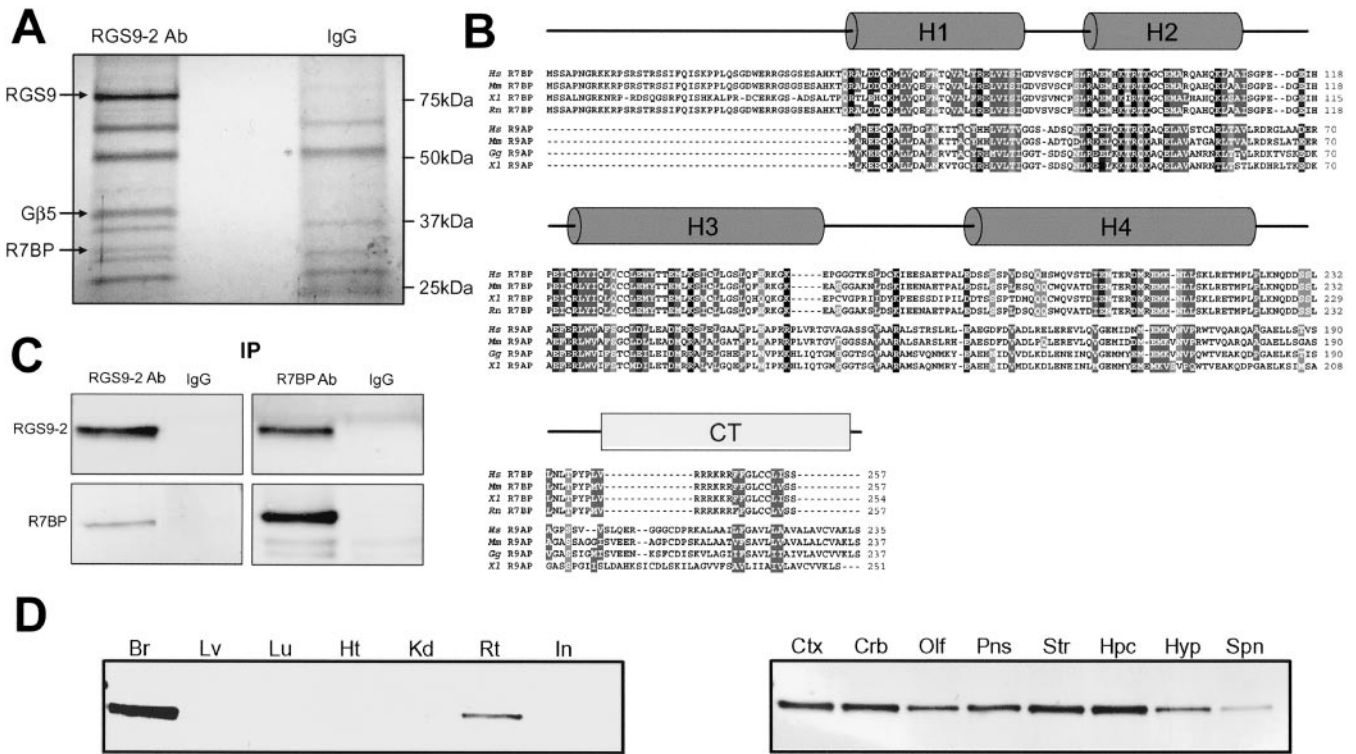
## RESULTS AND DISCUSSION

**R7BP Is a Novel Protein Interacting with RGS9-2 in the Brain**—We searched for proteins interacting with RGS9-2 in the mouse striatum by conducting co-immunoprecipitation studies using affinity purified sheep IgG recognizing the C terminus of RGS9-2. A typical experiment, illustrated in Fig. 1A, revealed the presence of several protein bands including RGS9-2 and its constitutive G $\beta$ 5 subunit. To assess which of the bands present in this gel may be proteins interacting with RGS9-2 specifically we performed a control immunoprecipitation experiment using the total sheep IgG fraction. Only one protein, other than RGS9-2 and G $\beta$ 5, was precipitated by specific antibodies and absent in the control IgG precipitate. The corresponding band of ~30 kDa was excised from the gel, subjected to tryptic digestion, and analyzed by MALDI MS and MS/MS mass spectrometry. Using a combination of peptide mass fingerprinting and MS/MS sequencing, we have identified six peptides corresponding to the sequence of an unnamed protein product with a molecular mass of 29,575 Da (NCBI accession number BAC32849.1; Fig. 1B). Three of these peptides (SICLLGSLQFHR, ELVISIGDVSVSCPSLR, LAAISGPE-DGEIHPEICR) were sequenced by MS/MS with high ion scores that yielded confidence intervals of >99.9% for each peptide, indicating correct identification of the protein. R7BP is also encoded in human, rat, and *Xenopus* genomes (NCBI accession numbers XP\_376386.1, XP\_215473.2, and AAH73094.1, respectively). Based on its ability to interact with all R7 proteins (see below), we named this protein R7-binding protein or R7BP.

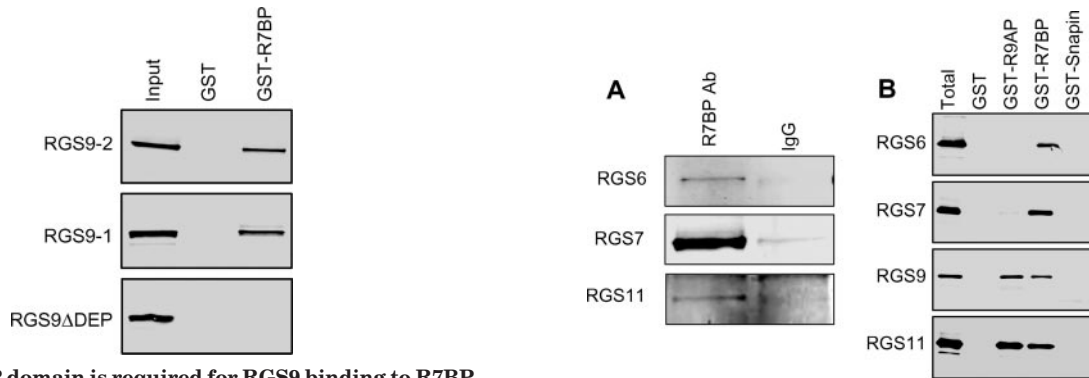
Remarkably, its closest homolog is R9AP, which interacts with RGS9-1 in photoreceptors (Fig. 1B). As in R9AP, the predicted secondary structure of the major N-terminal part of the R7BP molecule contains four  $\alpha$ -helices likely to form coiled coils (12, 17). It also resembles the structure of syntaxin, a SNARE protein participating in membrane fusion (5, 17). However, unlike R9AP and syntaxin, R7BP lacks a C-terminal transmembrane domain. Instead, its C terminus contains a polybasic region followed by two cysteines. This resembles the sequence of the C terminus of Ras where both the polybasic region and lipid modifications of the cysteines were shown to mediate Ras membrane attachment (18, 19). Based on this analogy, R7BP may also be a membrane-associated protein, which is consistent with its presence in brain membrane fractions.

To obtain further evidence that RGS9-2 forms a complex with R7BP in striatum we conducted reciprocal co-precipitation experiments using the sheep anti-R7BP antibodies. As shown in Fig. 1C, RGS9-2 was efficiently precipitated by anti-R7BP antibodies but not by the control, non-immune IgG.

**R7BP Is Specifically Expressed in the Nervous System**—Fig. 1D illustrates our analysis of the R7BP tissue expression profile. Because of a relatively low abundance of R7BP, we first precipitated R7BP from each tissue extract using the sheep



**FIG. 1. The identification and characterization of R7BP.** *A*, Coomassie-stained gel representing proteins immunoprecipitated from striatal extracts by sheep anti-RGS9-2 antibodies and control non-immune IgG. *B*, multiple sequence alignment of R7BP from human (*Hs*), mouse (*Mm*), *Xenopus* (*Xl*), and rat (*Rn*) with R9AP from human, mouse, *Xenopus*, and chicken (*Gg*). The cylinders marked *H1–H4* represent predicted  $\alpha$ -helices. The box marked *CT* represents the C-terminal region, which in R7BP contains the polybasic sequence and two cysteines and in R9AP, the transmembrane domain. *C*, reciprocal co-immunoprecipitation of R7BP and RGS9-2 from striatal extracts. Each protein was precipitated by the corresponding specific antibody from sheep. The presence of RGS9-2 and R7BP in precipitates was analyzed by Western blotting. Non-immune sheep IgG were used in control immunoprecipitation experiments. *D*, tissue specificity of R7BP expression. Extracts from 40 mg of each tissue were prepared as described for the brain extracts under “Experimental Procedures.” R7BP was immunoprecipitated from each extract and detected by Western blotting. The tissues (*left panel*) are: brain (*Br*), liver (*Lv*), lung (*Lu*), heart (*Ht*), kidney (*Kd*), retina (*Rt*), and intestine (*In*). The brain regions (*right panel*) are: cortex (*Ctx*), cerebellum (*Crb*), olfactory bulb (*Olf*), pons (*Pns*), striatum (*Str*), hippocampus (*Hpc*), hypothalamus (*Hyp*), and spinal cord (*Spn*).



**FIG. 2. The DEP domain is required for RGS9 binding to R7BP.** The interaction between recombinant GST-tagged R7BP and RGS9/G $\beta$ 5 constructs was analyzed in pull-down assays as described under “Experimental Procedures.” Proteins bound to R7BP were eluted from the beads and detected by Western blotting using sheep anti-RGS9-2 antibodies (*upper panel*) or anti-RGS9-1 antibodies (*two lower panels*). The nonspecific protein binding to the beads was assessed using recombinant GST instead of the GST-tagged R7BP.

anti R7BP antibody. We then detected the presence of R7BP in each precipitate by Western blotting using the rabbit anti R7BP antibody. These experiments demonstrated that R7BP is ubiquitously expressed throughout the nervous system including the retina but is not detectible in any of the non-neuronal tissues that we tested.

*The Binding of RGS9 to R7BP Is Mediated by the DEP Domain*—Since the binding of RGS9 to R9AP occurs via the

**FIG. 3. The interaction of R7 proteins with R7BP, R9AP, and snapin.** *A*, RGS6, RGS7, and RGS11 interact with R7BP in the brain. R7BP was immunoprecipitated from the whole brain extract by sheep anti-R7BP antibodies. R7 proteins in the precipitate were detected by Western blotting using specific antibodies against each individual protein. Non-immune sheep IgG were used to control nonspecific protein precipitation. *B*, the specificity of the interaction of R7 proteins with R7BP and R9AP. The binding of recombinant GST-tagged R7BP, R9AP, or snapin and recombinant R7-G $\beta$ 5 complexes was studied in pull-down assays as described under “Experimental Procedures.” The nonspecific protein binding to the beads was assessed using recombinant GST protein.

N-terminal domain of RGS9 called DEP, we tested whether the same domain is responsible for RGS9 binding to R7BP. Our experimental approach replicated that used to establish the DEP domain as the R9AP interacting site (7, 8). We used

pull-down assays to monitor the binding of the GST-tagged R7BP to a set of RGS9 constructs. As shown in the two upper panels of Fig. 2, both splice isoforms of RGS9, RGS9-1 and RGS9-2, interact with R7BP with similar efficiency. However, the construct lacking the DEP domain, RGS9 $\Delta$ DEP, does not bind to R7BP at all. These data demonstrate that the DEP domain of RGS9 is required for the R7BP binding, whereas the unique C-terminal sequences of each splice isoform are not required for this interaction. Therefore, the DEP domain is responsible for the RGS9 interaction with both R9AP and R7BP.

**R7BP Interacts with All Four R7 Proteins**—While R7BP is expressed throughout the entire central nervous system (Fig. 1C), the expression of RGS9-2 is restricted to striatum and just a few other brain regions (20–22). This suggests that R7BP may be associated with other members of the R7 RGS family expressed in multiple brain regions (10, 11). We tested this hypothesis by immunoprecipitating R7BP from the mouse brain extract and probing the precipitate for the presence of RGS6, RGS7, and RGS11. The Western blot analysis illustrated in Fig. 3A revealed that all four members of the R7 family were present in the precipitate. No such precipitation was observed when specific anti-R7BP antibodies were substituted with non-immune IgG.

We next studied the interactions between R7BP and R7 proteins using recombinant proteins *in vitro*. Each mouse R7 protein was co-expressed with G $\beta$ 5 in the Sf-9/baculovirus system and purified as described under “Experimental Procedures.” Each of these R7-G $\beta$ 5 complexes was functionally active, as evident by its ability to stimulate the GTPase activities of transducin and G $\alpha_o$  (data not shown). Pull-down assays shown in Fig. 3B demonstrated that each R7-G $\beta$ 5 complex was efficiently retained by R7BP attached to the Sepharose beads via an N-terminal GST tag (Fig. 3B). Control experiments demonstrated that no binding was observed with beads containing the GST tag alone, indicating that the interaction between R7BP and R7 proteins is specific.

Finally, we analyzed the binding of R7 proteins to R9AP and snapin, a protein previously reported as a binding partner of the DEP domain of RGS7 (23). The data shown in Fig. 3B indicate that R9AP interacts with RGS9 and its closest homolog RGS11 but not with RGS6 and RGS7. This indicates that R7BP has a broader range (and perhaps functional role) of interaction with R7 proteins than R9AP. We did not observe the binding of the N-terminally GST-tagged snapin to any of these R7 proteins. At present, we cannot explain this discrepancy with the previous report; it may reflect low binding affinity or unidentified differences in experimental conditions.

**Concluding Remarks**—RGS proteins of the R7 subfamily have been recently shown to play an important role in the normal functioning and survival of mammals. The functional knock-out of all four R7 proteins, which takes place in the G $\beta$ 5 knock-out mice, leads to multiple health problems, low body weight, and a high mortality rate (24). A less severe but also well identifiable phenotype was documented in the RGS9 knock-out mice. In addition to their visual impairment (25), these mice display behavioral abnormalities due to impaired regulation of opioid and dopamine signaling pathways (21, 26).

A milestone in understanding the biology of R7 proteins was the discovery that they all exist as constitutive complexes with G $\beta$ 5 (15, 27–29). G $\beta$ 5 determines the folding and stability of R7

proteins and regulates their catalytic activity (reviewed in Ref. 4).

In this study, we identified another universal partner of all R7 proteins, a novel neuronal protein R7BP. R7BP has a high degree of homology to R9AP from photoreceptors, both being related to the syntaxin subfamily of SNARE proteins. The importance of the R9AP interaction with RGS9-1 for normal vision suggests that the interactions of R7 proteins with R7BP in the brain also bear major functional significance. Our understanding of the R9AP function in photoreceptors provides a strong conceptual framework for studying the function(s) of R7BP in the brain. Future experiments would explore the role of R7BP in maintaining R7 protein stability and expression levels, determining R7 protein intracellular targeting, and regulating R7 protein activity.

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