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Identification of Rat EMAP, a δ-Glutamate Receptor Binding Protein

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While most subtypes of glutamate receptors have been studied extensively, less is known about the δ-glutamate receptors, δ1 and δ2. Although neither forms functional channels when expressed in heterologous cells, genetic analyses have demonstrated the physiological significance of δ2. We used the cytosolic C-terminus of the δ2 glutamate receptor subunit in a yeast two-hybrid screen of a rat brain cDNA library to identify δ-glutamate receptor binding proteins. We isolated rat EMAP, the rat homolog of a microtubule-associated protein initially isolated and characterized in echinoderms. Rat EMAP contains 10 WD-repeats, which are domains important for mediating protein–protein interactions in a wide variety of proteins. Rat EMAP binds to δ-glutamate receptor subunits within a 50-amino-acid segment of the δ C-terminus. It is widely expressed in both brain and peripheral tissues, including high expression in brainstem and enrichment in the postsynaptic density.

Key Words: **EMAP; WD-repeat; glutamate receptor; δ; postsynaptic density.**

Glutamate receptors are the major excitatory neurotransmitter receptors in the central nervous system. The ionotropic glutamate receptors are ligand-gated ion channels that consist of several subtypes including AMPA, NMDA, kainate, and δ receptors. The AMPA, NMDA, and kainate subtypes have been studied extensively, and much is known of their functional characteristics (1). In contrast, very little is known about the δ-glutamate receptors. These receptors do not bind glutamate, nor do they form functional channels when expressed in heterologous cells. Despite this, genetic analyses have confirmed the importance of the δ2 glutamate receptor. For example, mice lacking the δ2 gene display a loss of motor coordination and impaired cerebellar long-term depression (2). In addition, a point mutation in the third transmembrane domain of δ2 results in the mutant phenotype of the Lurcher mouse, which includes severe degeneration of Purkinje neurons (3).

In an attempt to learn more about δ receptor structure and function, we have searched for δ receptor binding proteins using the yeast two-hybrid system. Here we report the identification of rat EMAP, a protein that interacts with the δ1 and δ2 C-termini. Rat EMAP is the rat homolog of EMAP, a microtubule-associated protein initially isolated from echinoderms. EMAPs are highly conserved with homologs reported in echinoderms, *C. elegans*, human, and now rat. All EMAP-related proteins contain a series of WD-repeats, motifs that mediate protein–protein interactions in numerous proteins with functions as diverse as cytoskeletal dynamics and transcriptional regulation. We now present the characterization of rat EMAP, a protein expressed throughout the brain and periphery. Rat EMAP displays a unique distribution in brain, including high expression in brain stem and enrichment in the PSD, consistent with a role in receptor function or localization.

MATERIALS AND METHODS

Screening of the yeast two-hybrid library. The δ2 C-terminus (amino acids 852–1008) was used to screen a rat brain cDNA library in the activation domain vector pGAD 10 (Clontech). Approximately 1.94 million clones were screened, yielding 5 positives. The positive interactors were verified by restreaking and assaying for growth on histidine-deficient plates and β-galactosidase activity.

cDNA library screening and sequencing. The full-length clone of rat EMAP was isolated using standard colony hybridization techniques. In brief, 800,000 clones of a rat cerebellum λZAP cDNA library (Stratagene) were screened using a random-primed [^{32}P]-dCTP-labeled rat EMAP probe under high stringency hybridization conditions. This probe consisted of a 336 bp PCR product amplified from the rat EMAP cDNA isolated in the yeast screen and corresponded to amino acids 358–636 of the deduced rat EMAP protein sequence. Two strongly hybridizing plaques were purified and subjected to automated sequence analysis using an Applied Biosystems 373 DNA sequencer. These plaques corresponded to overlapping

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clones of rat EMAP; however, comparison of these sequences with that of human EMAP revealed the initiation methionine codon and Kozak sequence (4) were missing, suggesting that the 5′ end of our rat EMAP clones was truncated. To isolate the 5′ end of the rat EMAP cDNA, PCR reactions were performed using whole rat brain λZAP cDNA as template and a rat EMAP 5′ antisense primer (5′-ATAGACCCAATCTAGCTT-3′) with either T3 forward primer (5′-AATTACCCTCCACTAAAGGG-3′) or T7 reverse primer (5′-CGGGATATCACTCAGCATAATG-3′). The PCR products were purified by gel electrophoresis, subcloned into pGEM T-Z vector (Promega) and sequenced. The PCR products contained two overlapping fragments of 550 and 700 bp, respectively. These additional sequences encoded the initiation methionine and contained a Kozak consensus sequence. These sequences were appended to the sequences from the original yeast screen and the λZAP screen to generate a full-length rat EMAP cDNA.

Yeast two-hybrid assays. Constructs in GAL 4 DNA binding domain vectors and constructs in GAL 4 activation domain vectors were cotransformed into HF7c yeast cells according to the manufacturer's protocol (Clontech). The yeast were plated onto synthetic dextrose plates lacking tryptophan and leucine and allowed to grow for approximately 3 days. Colonies were then resuspended in 10 mM Tris and 1 mM EDTA, pH 8.0, and restreaked on dextrose plates lacking tryptophan and leucine and also onto plates lacking tryptophan, leucine and histidine (His-deficient plates). Growth on His-deficient plates was scored on a − to +++ scale, comparing cotransformations that yielded equivalent growth on plates lacking tryptophan and leucine, but containing histidine. Colony lifts were performed on His-deficient plates and β-galactosidase activity was assayed using X-gal. Color intensity was scored from − to +++.

In vitro binding assays. The recombinant GST fusion proteins were prepared as previously described (5). The GST–rat EMAP fusion protein included the fragment of rat EMAP isolated in the initial yeast two-hybrid screen, amino acids 358–636. This fusion protein was very sensitive to proteolysis and therefore the yield was low compared to GST alone or other GST fusion proteins. [^{35}S]Methionine-labeled *in vitro* translated δ2 was prepared using the TNT Coupled Reticulocyte Lysate System and Microsomal Membranes (Promega) according to the manufacturer's protocol. The translated δ2 was incubated with 5 μg of either GST or GST-rat EMAP fusion protein attached to glutathione Sepharose beads (Pharmacia) for 2 h at 4°C. The beads were washed two times in binding buffer (PBS containing 0.1% Triton X-100, 1 mg/ml BSA, 2 mM EDTA, 0.1 mM AEBSF, 1 μg/ml leupeptin, 5 mM iodoacetamide), and two final washes in binding buffer without BSA. The beads were resuspended in sample buffer, heated at 100°C for 5 min and resolved by SDS–PAGE on 4–20% gradient gels, and visualized by fluorography.

Northern blot analysis. A nylon membrane containing poly(A)$^+$ RNA from multiple rat tissues (Clontech) was hybridized with a [γ-^{32}P]dCTP-labeled probe in QuickHyb (Stratagene) for 1 h at 68°C. The probe encoded amino acids 429–538 of rat EMAP. The blot was then washed in 6× SSC/0.5% SDS sequentially at 37, 42, and 68°C and then washed twice in 2× SSC/0.5% SDS at 68°C. The blot was then exposed to X-ray film.

Antibodies. The rat EMAP antibodies were generated by immunizing rabbits with the synthetic peptide CTTGGKDTSVLQWRVA, corresponding to the rat EMAP C-terminal 15 amino acids. A cysteine was added at the amino-terminus for coupling to BSA.

Transfections and immunoblotting. HEK-293 cells grown on 10-cm dishes were transfected with empty vector or rat EMAP cDNA (10 μg) using the calcium phosphate coprecipitation method (Clontech). Transfected cells were harvested 36 h following transfection.

Total homogenates of transfected cells or the regions of adult rat brain indicated were resolved by SDS–PAGE on 4–20% gradient gels. The postsynaptic density (PSD) samples were kindly provided by K. Wu, and were prepared from cerebral cortex of adult rat as previously described (6). The proteins were transferred to PVDF membranes, immunoblotted with rat EMAP affinity-purified antibodies (1 μg/ml), and visualized with enhanced chemiluminescence.

In situ hybridization. *In vitro* transcription and labeling of sense and antisense probes were performed according to established procedures using SP6 and T7 RNA polymerases with [^{35}S]dCTP (Amersham Pharmacia Biotech, Piscataway, NJ), using riboprobe *in vitro* transcription systems (Ambion, Austin, Texas). Labeling reactions were performed as suggested by the manufacturers.

Male Sprague–Dawley rats (n = 3; 150–200 g) were deeply anesthetized with a 1:1 mixture of ketamine hydrochloride (Ketaset; 100 mg/ml; Aveco, Fort Dodge, IA) and xylazine (Rompun; 20 mg/ml; Mobay Corp., Shawnee, KS) and perfused through the heart with 100 ml of phosphate-buffered saline (PBS, pH 7.4) at room temperature, followed by 250 ml of ice-cold 4% paraformaldehyde in 1× PBS at pH 7.4. Brains were removed and postfixed in the same fixative for 1 h at 4°C and rinsed in PBS. Brains were then washed overnight in phosphate buffer containing 30% sucrose at 4°C and frozen in isopentane at −60°C. Brains were cut on a cryostat into 10-μm-thick sections and stored at −70°C until use. All animals were handled according to an approved animal protocol.

The *in situ* hybridization method described in this study is a modification of a previously described protocol (7). The slides were dried completely and tissue treated with 0.5% Triton X-100 (w/v) in PBS at room temperature for 30 min and then washed twice with PBS. Sections were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0)/0.9% NaCl at room temperature for 10 min followed by two washes of 2 min each with 2× standard saline citrate (SSC, 0.15 M NaCl, 0.015 M sodium citrate). The sections were dehydrated with a graded ethanol series and air-dried at room temperature for 30 min. The probes were heated at 80°C for 5 min. 10^6 cpm of ^{35}S-labeled riboprobe in 100 μl of hybridization solution [50% formamide, 2× SSC, 1 mM Tris–HCl, pH 7.5, 1× Denhardt's solution, 10% dextran sulfate, 100 mM dithiothreitol (DTT), 100 μg/ml sheared single-stranded salmon sperm DNA and 250 μg/ml yeast transfer RNA] was placed on a slide covered with Parafilm and incubated for 17 h in a humid chamber at 60°C. After hybridization, the sections were cooled down to room temperature and the cover film was carefully removed in 4× SSC and washed four times for 10 min with the same solution containing 1 mM DTT. Slides were washed in a solution of RNase A (20 μg/ml), 10 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 0.25 mM EDTA (pH 8.0) at 37°C for 30 min. The slides were rinsed and gradually desalted with the following solutions: 2× SSC, 5 min, 2 times; 1× SSC, 5 min; 0.5× SSC, 5 min; 0.1× SSC, 65°C, 1 h; finally, slides were washed in 0.1× SSC briefly to cool them to room temperature. 1 mM DTT was added to all solutions. Dehydration was done quickly in ethanol: 50%, 3 min; 70%, 3 min; 90%, 3 min; and 100%, 3 min. We added 1 ml of 20× SSC and 200 μl of 1 M DTT to each 200 ml of 50 and 70% ethanol. The slides were drained and then dried at room temperature for 30 min. The sections either exposed to Kodak Biomax MR film for 2–7 days or coated with NTB2 autoradiographic emulsion (Kodak, Rochester, NY) at 4°C and exposed for 2–3 weeks.

RESULTS AND DISCUSSION

Isolation of rat EMAP as a δ-Glutamate Receptor Binding Protein

In an effort to learn more about δ-glutamate receptors, we have begun identifying δ-glutamate receptor binding proteins. Previously, we reported that δ2 interacts with the PDZ domain-containing protein PSD-93 at parallel fiber synapses of the cerebellum (8). In addition, it has also been shown that δ receptors interact with spectrin (9), and with the protein-

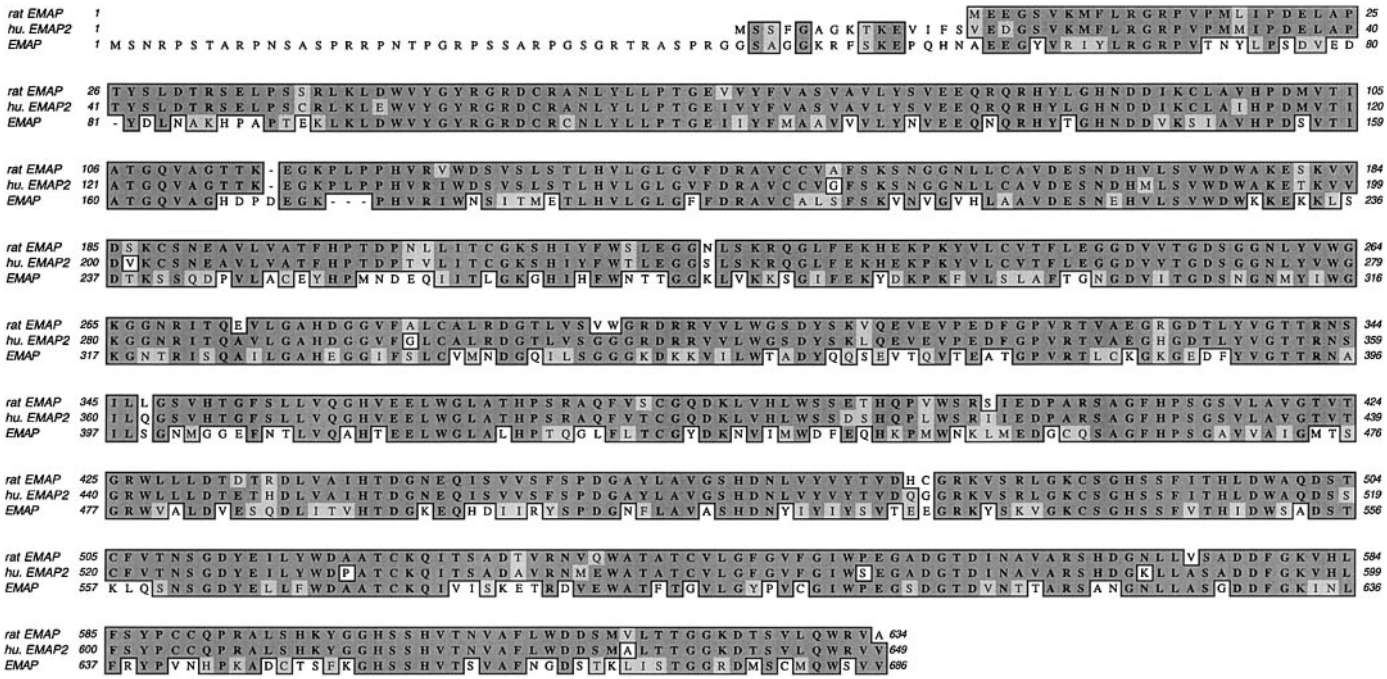

FIG. 1. Primary sequence of rat EMAP. The amino acid sequence of rat EMAP is shown in alignment with human EMAP2 and echinoderm EMAP. Amino acids that are identical to rat EMAP are shaded a dark gray and conserved amino acids are shaded a lighter gray. Rat EMAP is 94% identical/95% similar to human EMAP2 and 58% identical/68% similar to echinoderm EMAP. The rat EMAP sequence has been deposited into GenBank (Accession No. bankit 380047).

tyrosine phosphatase, PTPMEG (10). In this study, we have used the δ2 C-terminus to screen a rat brain yeast two-hybrid library. Of approximately 1.94 million clones screened, 5 δ interactors were isolated. Four of the 5 clones were identified as the rat homolog of the microtubule-associated protein EMAP (11). As in many yeast screens, the cDNA we isolated did not represent a full-length clone. The fragment of rat EMAP that we identified in our initial screen included the entire C-terminus, but lacked the 5′ untranslated region and the initiation methionine. In order to isolate a full-length cDNA, a Lambda ZAP library was screened using a probe derived from the rat EMAP fragment isolated in the yeast screen, and an independent EMAP clone was isolated. However, this clone still lacked the 5′ end, and a PCR-based approach (5′ RACE) was finally used to isolate the 5′ end of the rat EMAP cDNA. The primary sequence of rat EMAP (predicted molecular weight: 69,336 Da) is shown in Fig. 1 in alignment with the echinoderm EMAP (58% identity; 68% similarity), the first EMAP isolated, and with human EMAP2 (94% identity; 95% similarity), the EMAP homolog with the closest identity to rat EMAP (Fig. 1).

Rat EMAP Binds to δ-Glutamate Receptors

The binding of rat EMAP to δ-glutamate receptor subunits was evaluated using the yeast two-hybrid assay. Both the δ1 and δ2 C-termini bind to rat EMAP (aa 357–635) (Fig. 2A). Deletion analysis of the δ2 C-terminus revealed that the middle 50 amino acids of the δ2 C-terminus (aa 904–955) are necessary and sufficient for rat EMAP binding (Figs. 2A and 2B). Thus, the rat EMAP binding site is an internal sequence upstream of the PDZ binding domain that has been characterized previously (8).

To confirm the δ2–rat EMAP interaction we observed in the yeast two-hybrid system using an independent approach, we used a GST fusion protein pull-down assay. We found that *in vitro* translated full-length δ2 binds well to GST-rat EMAP fusion protein, but very poorly to GST alone (Fig. 2C). As with many cytoskeletal proteins, rat EMAP is very insoluble and highly sensitive to proteolysis, precluding any evaluation of binding using traditional immunoprecipitation techniques.

Tissue Distribution of Rat EMAP

Having isolated EMAP from a rat brain cDNA library, we set out to examine if rat EMAP was specifically localized to brain or if it was also expressed in peripheral tissues. We therefore hybridized a blot containing poly(A)$^+$ RNA isolated from a variety of tissues with a rat EMAP probe under high stringency conditions and found that rat EMAP was widely expressed. A 2.6 kb transcript was observed in all peripheral tissues analyzed, with a single larger transcript ob-

A

Gal 4 DNA binding hybrid	Gal 4 activation hybrid	His 3 activation	β Gal activation
δ1 WT C-terminus (851-1009)	rat EMAP (357-635)	+++	+++
δ2 WT C-terminus (852-1008)	rat EMAP (357-635)	+++	+++
δ2 truncated C-terminus (852-1002)	rat EMAP (357-635)	+++	+++
δ2 truncated C-terminus (852-955)	rat EMAP (357-635)	+++	+++
δ2 truncated C-terminus (862-1008)	rat EMAP (357-635)	+++	+++
δ2 truncated C-terminus (904-1008)	rat EMAP (357-635)	+++	+++
δ2 truncated C-terminus (955-1008)	rat EMAP (357-635)	-	-
δ2 truncated C-terminus (904-955)	rat EMAP (357-635)	+++	+++

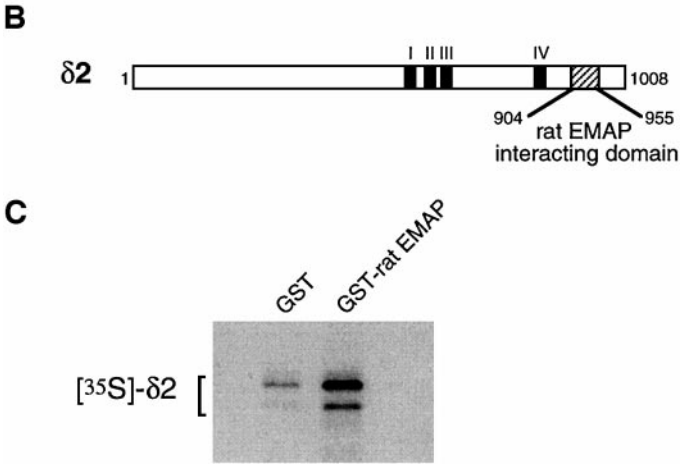

FIG. 2. δ1 and δ2 bind to rat EMAP. (A) The δ1 and δ2 C-termini interact with rat EMAP. Yeast HF7c cells were cotransformed with expression vectors encoding various δ C-terminus-GAL 4 DNA binding domain fusion proteins and the rat EMAP (357–635)-GAL 4 activation domain fusion protein. Each transformation mixture was plated on synthetic dextrose plates lacking tryptophan and leucine. Interaction was measured by the filter assay as described (Clontech), analyzing both extent of growth on histidine deficient media and also activation of the lac Z reporter gene. Data are representative of two independent experiments. (B) Summary of the yeast interaction data indicates that the middle 50 amino acids of the δ2 C-terminus constitute the rat EMAP binding site. (C) δ2 binds to GST-rat EMAP. δ2 was *in vitro* translated using [^{35}S]methionine and incubated with 5 μg of GST or 5 μg of GST-rat EMAP fusion proteins immobilized on glutathione-Sepharose beads as described under Materials and Methods. Following extensive washing, bound δ2 was eluted in sample buffer, resolved by SDS–PAGE, and visualized by fluorography. The exposure shown allows direct comparison of specific binding to GST-rat EMAP and nonspecific binding to GST alone.

served in brain and both large and small transcripts in testes (Fig. 3A). A similar expression pattern has been reported for human EMAP2 (12), including ubiquitous expression and a larger transcript size in brain and spinal cord.

Distribution of Rat EMAP in Brain

We raised an antibody against the extreme C-terminus of rat EMAP (aa 620–634) to characterize the distribution of this protein throughout the brain. The rat EMAP antibody recognized a specific band in HEK-293 cells expressing rat EMAP (Fig. 3B) which migrated at the same apparent molecular weight as rat EMAP from brain supporting both the fidelity of the antibody and the integrity of the full-length rat EMAP cDNA. Immunoblot analysis of tissue homogenates from different brain regions revealed that rat EMAP was expressed throughout the brain and was highly enriched in the superior and inferior colliculi as well as the brainstem (Fig. 3B). In contrast, relatively little rat EMAP was detected in cortex, hippocampus, and cerebellum. We also detected a large amount of the protein in the PSD (Fig. 3B), a Triton X-100 insoluble structure to which many postsynaptic receptors, including δ-glutamate receptors, are localized *in vivo*.

We next used *in situ* hybridization to investigate the expression of rat EMAP in brain in greater detail. We found that rat EMAP mRNA was highly expressed in brainstem (Fig. 4), consistent with our results using immunoblot analysis. Intense hybridization was also observed in many brainstem nuclei including the facial nucleus (shown at higher magnification in Fig. 4C), among others.

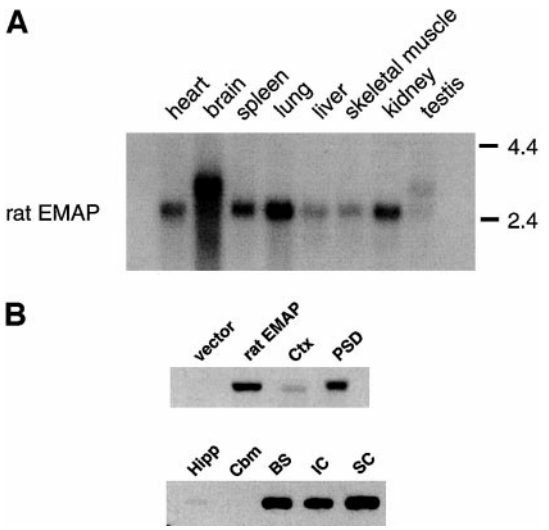

FIG. 3. Distribution of rat EMAP. (A) [γ-^{32}P]dCTP-labeled probe encoding amino acids 429–538 of rat EMAP was hybridized with a multiple tissue Northern blot under high stringency conditions (Clontech). Note that a 2.6 kb transcript is present in all tissues and that an additional transcript is present in brain and testes. (B) Total cell homogenates of HEK-293 cells transiently transfected either with empty vector or with rat EMAP were analyzed together with rat brain cortex (20 μg) and PSD fraction (20 μg; prepared according to Wu *et al.*, 1986). They were resolved by SDS–PAGE, transferred to PVDF membranes, and probed with rat EMAP affinity-purified antibodies (top). In a separate experiment, total homogenates from different brain regions (20 μg each) were resolved by SDS–PAGE, transferred to PVDF membranes, and probed with rat EMAP affinity-purified antibodies (bottom). Ctx, cortex; PSD, postsynaptic density; Hipp, hippocampus; Cbm, cerebellum; BS, brainstem; IC, inferior colliculus; SC, superior colliculus.

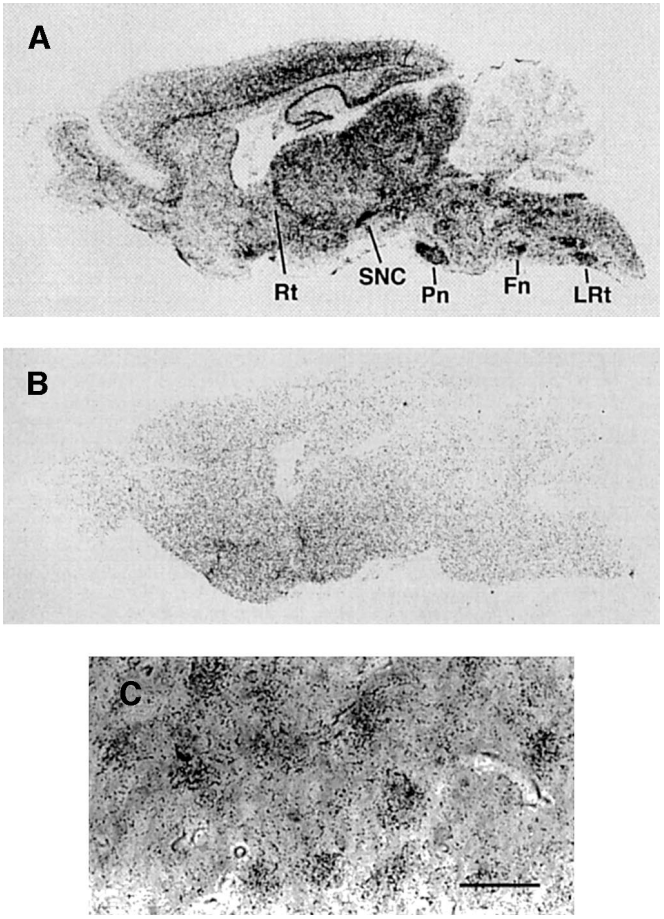

FIG. 4. Rat EMAP displays a unique localization in brain, including high expression in nuclei of the brainstem. (A, B) EMAP mRNA is highly expressed in multiple nuclei of the brainstem, as seen in parasagittal sections at the level of the superior and inferior colliculi. *In situ* hybridization was performed with antisense probes (A) or sense probes (B) of EMAP as described under Materials and Methods. (C) At higher magnification, clusters of silver grains, representing hybridization with ^{35}S-labeled oligonucleotide probes, are detected in cells of the facial nucleus (bar size: 50 μm). Rt, reticular thalamic nucleus; SNC, pars compacta of substantia nigra; Pn, Pontine nucleus; Fn, facial nucleus; LRt, lateral reticular nucleus.

We now report the identification of rat EMAP, the rat homolog of a microtubule-associated protein originally isolated in echinoderms, but later shown to be expressed in *C. elegans* and humans. We have demonstrated a broad distribution for rat EMAP in both brain and peripheral tissues, including enrichment in many discrete neuronal subpopulations. Interestingly, rat EMAP is expressed at relatively low levels in cortex, hippocampus and cerebellum, but is highly expressed in superior and inferior colliculi and nuclei of the brainstem. Since δ2 is almost exclusively expressed in Purkinje cells of the cerebellum where there is very little rat EMAP, it is likely that δ1 is the physiological target for *in vivo* binding to rat EMAP due to its broader distribution throughout the brain (13). Furthermore, our findings that rat EMAP is enriched in the PSD is consistent with a role for this protein in receptor regulation.

Our description of rat EMAP as a novel δ interacting protein expands the list of proteins known to directly bind to δ receptors. δ receptor have been shown to interact directly with PSD-93 (8), PTPMEG (10), spectrin (9), and now rat EMAP, confirming the importance of the δ C-terminus as a protein–protein interacting domain. A 28-amino-acid stretch of the δ2 C-terminus adjacent to the fourth transmembrane domain, termed the juxtamembrane (J) segment, appears to be critical for plasma membrane targeting (14), and it is likely that this region of δ receptors serves as a binding site for an as yet unidentified cytosolic binding protein that facilitates trafficking to the cell surface. The direct binding of rat EMAP and spectrin indicates an important role for the cytoskeleton in regulating δ receptors. The intracellular C-terminus of other glutamate receptors have proven to be essential as has been documented for other glutamate receptors (15). Complete identification and characterization of the variety of δ binding proteins will facilitate study on δ receptors and will help to elucidate the function of this poorly understood class of receptors.

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