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Differential expression of isoforms of PSD-95 binding protein (GKAP/SAPAP1) during rat brain development

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Abstract PSD-95/SAP90, which binds to the C-terminus of NMDA receptor and Shaker-type potassium channel, is one of the major postsynaptic density proteins. Recently, novel classes of proteins interacting with the guanylate kinase domain of PSD-95 have been identified, guanylate kinase-associated protein (GKAP) and SAP90/PSD-95-associated proteins (SAPAPs). Here we report the isolation of new isoforms of PSD-95 binding protein (GKAP/SAPAP1) using the yeast two-hybrid system. The isolated protein directly interacts with the guanylate kinase domain of PSD-95. Northern blot analyses revealed that the expression of these isoforms containing distinct N-terminal sequences is differentially regulated during brain development. The present findings suggest that each isoform of the PSD-95 binding protein is differentially expressed in a development-dependent manner and may be involved in the complex formation of PSD-95 and channel/receptors at the postsynaptic density.

Key words: Postsynaptic density; PSD-95/SAP90

1. Introduction

Postsynaptic density (PSD) is a unique synaptic structure underlying the postsynaptic membrane of excitatory nerve synapses. Morphological analyses revealed a specialization of the PSD during postnatal development [1,2]. Several components have been identified in the PSD: for example, actin, spectrin (calspectin or fodrin), MAP2, tubulin, and Ca^{2+}/calmodulin-dependent protein kinase II [3–6]. However, the overall components and the molecular organization of PSD remain unknown [3–8]. Several PDZ domain-containing proteins such as chapsyn-110, PSD-95/SAP90, SAP97, and SAP102 have been identified in the pre- and postsynaptic sites [9–13]. They have repeating PDZ domains, an Src homology domain 3 (SH3), and/or a domain that has homology with a yeast guanylate kinase (GK) in order from the N-terminus to the C-terminus [14,15]. PSD-95/SAP90 has been identified as one of the abundant proteins in the PSD fraction [10]. It binds to NMDA receptor and Shaker-type potassium channel through the PDZ domain [16–18]. Kim et al. have demonstrated the clustering of NMDA receptor or potassium channel mediated by the PDZ domain of PSD-95 [9,17]. In contrast, there have been few reports regarding the functional role of the C-terminal SH3 or GK domain of PSD-95. To reveal the molecular organization of the PSD, using the yeast two-hybrid system, we searched for proteins interacting with the C-terminal side of PSD-95. During the course of our study, the isolation of new classes of proteins which interact with the GK domain of PSD-95 has been reported by two groups, the so called guanylate kinase-associated protein (GKAP) and SAP90/PSD-95-associated proteins (SAPAPs) [19,20]. We also independently isolated several cDNA clones of PSD-95 binding protein which proved to be isoforms of GKAP/SAPAP1 by sequence analysis. These isoforms have unique sequence variants at the N-terminus. Northern blot analyses using specific probes for each N-terminal sequence revealed that these isoforms show different expressions during development of rat brain. These findings suggest that the expression of these isoforms is differentially regulated at a transcriptional level during brain development.

2. Materials and methods

2.1. Yeast two-hybrid system

The yeast two-hybrid system for the isolation of PSD-95 binding proteins was employed according to the method described previously [21,22]. The C-terminal 340 amino acids (residues 385–724) of rat PSD-95 were obtained by polymerase chain reaction (PCR) and subcloned in frame into yeast expression vector pAS2-1, GAL4 binding domain vector (Clontech, USA) for bait construction. This bait vector was transformed into yeast strain Y-190 harboring the reporter gene HIS3 and lacZ. The bait strain was further transformed with a rat brain cDNA library constructed in pGAD10, GAL4 activation domain vector (Clontech). Positive clones growing on synthetic dropout −tryptophan/−leucine/−histidine plates containing 25 mM of 3-amino-1,2,4-triazole were confirmed by β-galactosidase filter assay, as described previously [21,22].

2.2. 5′ RACE and 3′ RACE

We performed 5′ rapid amplification of cDNA ends (5′ RACE) and 3′ RACE as described elsewhere [23]. We carried out PCR between the 5′ and 3′ sequences obtained by 5′ and 3′ RACE. The amplified fragments and the obtained clone by the yeast two-hybrid system were subcloned into pBluescript SK^- (Stratagene, USA) and sequenced.

2.3. In vitro binding assay

The full-length coding region of the PSD-95 binding protein (clone2-2A, shown in Fig. 1) was subcloned in frame into pQE30, hexahistidine (6×His)-tagged fusion vector (Qiagen, USA). The rat PSD-95 C-terminus (residues 394–724), the SH3 domain with the flanking region (residues 394–533), and the GK domain (residues 534–724) were obtained by PCR and subcloned in frame into pGEX-3X to produce glutathione *S*-transferase (GST)-tagged fusion proteins (Pharmacia, Sweden). The 6×His fusion proteins and the GST fusion proteins were purified by Ni-NTA resin (Qiagen) and glutathione-coupled Sepharose 4B (Pharmacia), respectively. In overlay binding assay, the 6×His-tagged fusion protein of clone2-2A was separated by SDS-PAGE, transferred to nitrocellulose membrane (Schleicher&Schuell, Germany), and probed by GST fusion proteins after blocking with Block Ace (Yukijirushi, Japan). The probes bound to the 6×His-tagged protein were visualized by anti-GST polyclonal

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antibodies (produced in our laboratory) and horseradish peroxidase H-conjugated anti-rabbit IgG (Promega, USA).

2.4. Northern blot analysis

Total RNAs were extracted from adult rat tissues using Isogene RNA extraction kit (Nippon Gene, Japan). To analyze expressional change of the PSD-95 binding protein and PSD-95 mRNAs during brain development, total RNAs were extracted from rat whole brains at indicated developmental stages. 10 μg of total RNAs was separated on 1.0% agarose formaldehyde denaturing gels, and then transferred to nylon membranes (GeneScreen, USA). The fragment which was present in clone2-2A–D and SAPAP1 in common (nucleotide positions 1564–2176 of GenBank AB003594) was ^{32}P-labelled by the random priming method and used as a probe (Fig. 3C, common probe). For Northern blot analysis of PSD-95, PSD-95 cDNA fragment expanding from nucleotide positions 1212–2229 (GenBank M96853) was labelled by the random priming method. As a specific probe for clone2-2A, B, and C, a 145 bp cDNA fragment (nucleotide positions 1–145 of GenBank AB003594) was ^{32}P-labelled using a specific antisense oligonucleotide (5′-CTGTCCATTCATCCTGGGGAAC-3′) (Fig. 3C, probe I). As a specific probe for clone2-2D, a 451 bp cDNA fragment (nucleotide positions 31–481 of GenBank AB005146) was prepared in the same way (Fig. 3E, probe II). For SAPAP1-specific probe [20], a SAPAP1 cDNA fragment (nucleotide positions 272–1270 of GenBank U67137) was obtained by PCR and ^{32}P-labelled by the random priming method (Fig. 3G, probe III). To normalize the applied RNAs, ribosomal RNAs were stained with 0.02% methylene blue.

3. Results and discussion

3.1. Isolation of PSD-95 binding protein

For the isolation of proteins that interact with the C-terminus of PSD-95, the yeast two-hybrid system was introduced to screen a rat brain cDNA library using a bait consisting of the C-terminal 340 amino acids (residues 385–724) of rat PSD-95. 6.0×10^6 independent colonies were screened, and a single clone, clone2-2, carrying a 1260 bp sequence was isolated (Fig. 1). We then applied the 5′ and 3′ RACE methods to obtain the full-length coding sequence of clone2-2. As a result, we obtained four full-length coding sequences, clone2-2A–D, shown in Fig. 1. Sequence analysis of clone2-2A reveals an open reading frame that encodes a sequence of 694 amino acids with a calculated M_r of 77 000. There are presumably three distinct splicing variants at the N-terminus of the isolated clone, clones2-2A and C, clone2-2B, and clone2-2D. A two-amino acid deletion around the N-terminal splicing site was detected in two clones of five clone2-2A by the 5′ RACE (clone2-2B). Since the reading frame is not disrupted, this deleted protein seems to be one of the isoforms of the PSD-95 binding protein. The nucleotide sequence of clone2-2B has been submitted to the GenBank Data Bank with accession number AB003594. In addition to these N-terminal variants, a 10-amino acid deletion was also observed in the middle portion of this molecule (clone2-2C). This deletion most likely represents an alternative splicing based on its in-frame sequence. In comparison with data base entries, the four clones obtained are thought to originate from the same gene with GKAP and SAPAP1 because they contain identical sequences except for the N-terminus and the central region, but clones2-2B and D are novel isoforms that have not yet been reported. The N-terminal sequence of clones2-2A–C is essentially the same with that of GKAP. Clone2-2D is therefore thought to be the rat homologue of human GKAP. Up to this time, four sequence variants have been identified at the N-terminus of the PSD-95 binding protein (Fig. 1).

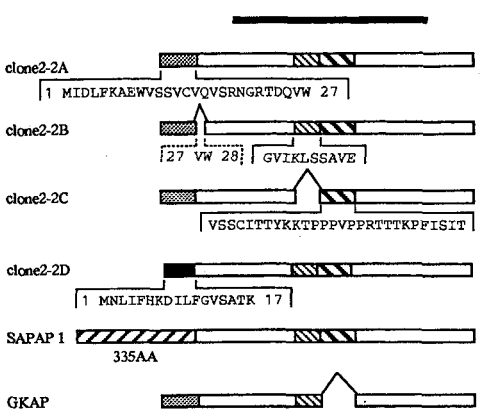

Fig. 1. Schematic structures of isolated clones as compared with GKAP and SAPAP1. The common sequence in all isoforms is depicted as a white bar. The specific N-terminal sequence shared by clones2-2A, B, C and GKAP is indicated as shaded boxes. The black box and the hatched boxes at the N-terminus indicate the specific N-terminal sequences in clone2-2D and SAPAP1, respectively. Hatched boxes in the middle portion refer to the two types of inserted sequence. The amino acid sequences of these boxes are also shown. Clone2-2B has a deletion of two amino acids (amino acid positions 27 and 28 of GenBank U67987) around the N-terminal splicing site. Clone2-2, isolated by the two-hybrid system, is indicated as a black bar at the top in the corresponding position.

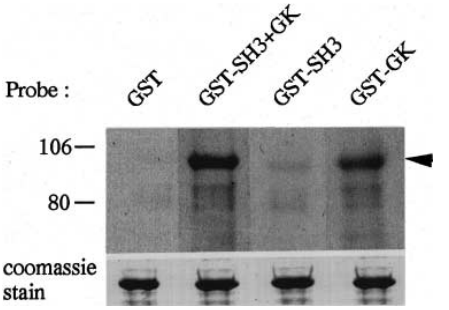

Fig. 2. In vitro interaction between PSD-95 and the PSD-95 binding protein. The direct interaction between PSD-95 and the PSD-95 binding protein was examined using the gel overlay assay. The 6×His-tagged clone2-2A fusion protein separated by SDS-PAGE is probed with GST, GST-SH3+GK, GST-SH3, or GST-GK. Molecular size markers are indicated on the left in kilodaltons. The arrowhead indicates the position of the 6×His-tagged clone2-2A. The SDS gel loading the same sample was stained with Coomassie blue and is shown at the bottom.

3.2. In vitro binding assay

We further examined the interaction between the obtained clone2-2 and PSD-95 using the yeast two-hybrid system. They bound each other even when the inserts were exchanged between the binding domain vector and the activation domain vector. Furthermore, clone2-2 specifically bound to the bait consisting of the GK domain but not to that of the SH3 domain of PSD-95 in the yeast two-hybrid system (data not shown). We performed a gel overlay assay to confirm the direct interaction between the GK domain of PSD-95 and the PSD-95 binding protein in another system. As shown in Fig. 2, the 6×His-tagged clone2-2A fusion protein was separated by SDS-PAGE and transferred to nitrocellulose membrane, followed by overlay assay probed with GST, GST-tagged PSD-95 C-terminus (GST-SH3+GK), GST-tagged SH3 domain with the flanking region (GST-SH3), or GST-tagged GK domain (GST-GK). The 6×His-tagged clone2-2A

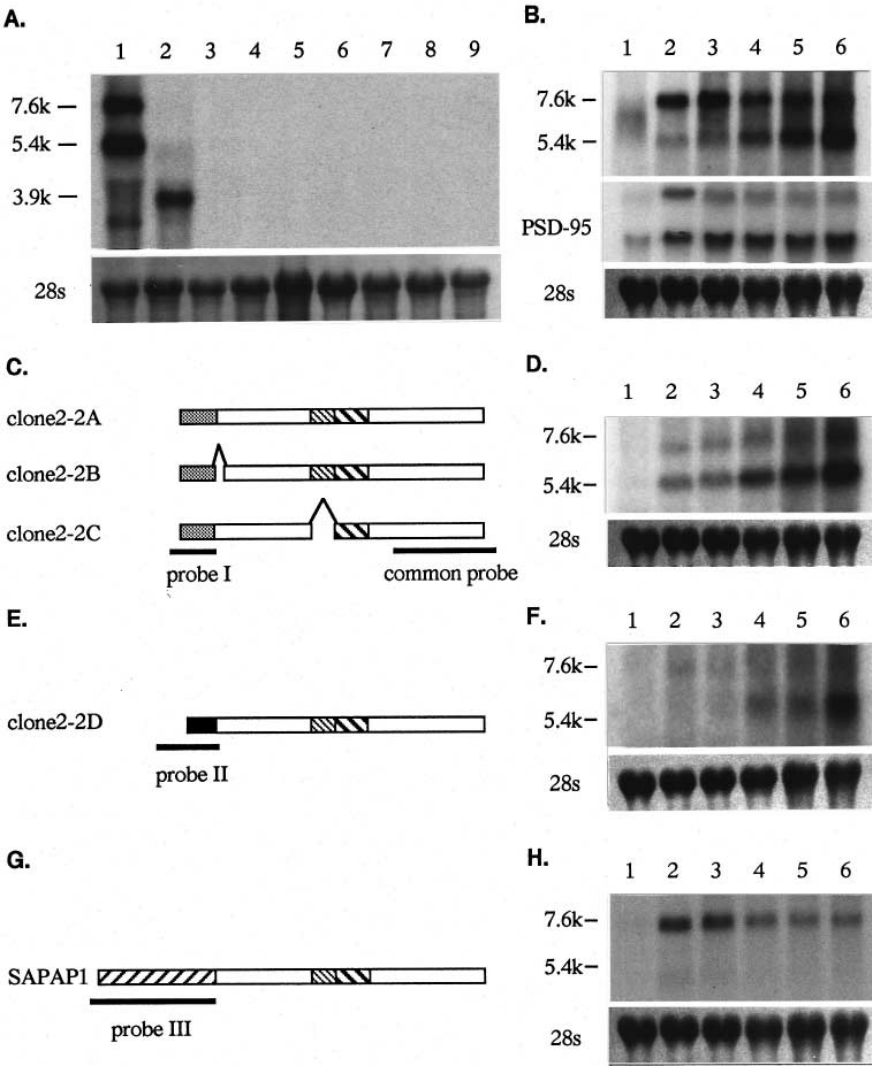

Fig. 3. Expression of the PSD-95 binding protein mRNAs. A: Tissue distribution of the PSD-95 binding protein mRNAs. Total RNAs from adult rat tissues were hybridized with the ^{32}P-labelled common probe shown in C. The lanes indicate: 1, brain; 2, testis; 3, lung; 4, heart; 5, intestine; 6, colon; 7, liver; 8, spleen; 9, kidney. The sizes of the transcripts calculated from the mobility are indicated on the left side in kilobases. B: Expressional changes of the PSD-95 binding protein mRNAs during brain development were revealed by a common probe. Total RNAs from rat whole brains at indicated stages were analyzed. Lane 1, embryonic day 18; 2, postnatal day 0; 3, day 5; 4, day 15; 5, day 30; 6, 6 weeks. To compare the expression pattern of PSD-95, the Northern blot of PSD-95 is shown in the middle panel. 28S RNAs for the standardization are shown at the bottom. C, E, G: Schematic structures are essentially the same as in Fig. 1 and probes used in this figure are shown as bold black lines in the corresponding positions. D: Northern blot analysis using a clone2-2A-, B-, and C-specific sequence at the N-terminus as a probe (probe I shown in C). F: Northern blot analysis using a clone2-2D-specific probe (probe II shown in E). H: Northern blot analysis using an SAPAP1-specific probe (probe III shown in F). Numbers of lanes indicated in D, F, and H are the same developmental days as in B.

protein interacted with GST-SH3+GK and GST-GK, but not with GST or GST-SH3. This result is consistent with that of the two-hybrid system. Kim et al. have performed overlay filter assay and demonstrated the in vitro interaction between PSD-95 and GKAP [19]. We could confirm their results using the same method in spite of some differences in vector constructions and assay conditions.

3.3. Expression of PSD-95 binding protein mRNAs

We performed Northern blots to analyze the expression of the PSD-95 binding protein mRNAs regarding tissue distribution and brain development. Northern blots using a common probe revealed that the PSD-95 binding protein mRNAs were exclusively expressed in brain and testis, but not in any other tissues (Fig. 3A). In adult rat brain, the transcripts were at least composed of two major bands, 7.6 kb and 5.4 kb in size. We consider that both transcripts originate from the same gene encoding PSD-95 binding protein because several fragments containing common sequences, and antisense sequences used as probes, showed the same results under high stringency conditions (data not shown). In the testis, the 3.9 kb transcript was dominant (Fig. 3A). The 7.6 kb and 5.4 kb transcripts in the brain were differentially expressed during development (Fig. 3B). The 7.6 kb transcript was rapidly expressed at birth and increased to reach a plateau by the adult stage. The 5.4 kb transcript was detected at birth and was gradually upregulated thereafter. To determine the expression patterns of various isoforms of the PSD-95 binding protein mRNAs in

the brain, we performed Northern blot analyses using specific probes for each N-terminal sequence variant. When the 145 bp fragment representing clone2-2A, B, and C (probe I, shown in Fig. 3C) was used as a probe, major 5.4 kb and minor 7.6 kb transcripts were detected. Both transcripts appeared at birth and were gradually upregulated during brain development (Fig. 3D). Using the 451 bp fragment representing clone2-2D (probe II, shown in Fig. 3E), only a 5.4 kb transcript was shown. It was first detected at postnatal day 15, and gradually upregulated thereafter (Fig. 3F). The N-terminal-specific sequence of SAPAP1 as a probe (probe III, shown in Fig. 3G) showed only a 7.6 kb mRNA, which was gradually downregulated from birth to the adult stage (Fig. 3H). These results indicate that the 5.4 kb mRNAs using a common probe are composed of transcripts of clones2-2A, B, C and clone2-2D, and the 7.6 kb mRNAs are transcripts combined with SAPAP1, clones2-2A, B, and C. The expression patterns of various isoforms during brain development, therefore, could be divided into two groups: the one is upregulated (clones2-2A, B, C, and D) and the other is downregulated (SAPAP1). On the other hand, PSD-95 mRNAs were composed of two bands, a major 3.5 kb and a minor 6.0 kb one (Fig. 3B). Cho et al. have reported that the former was expressed in both forebrain and cerebellum, and the latter was prominent in cerebellum [10]. The expression patterns of the 5.4 kb mRNAs originating from clones2-2A–D were coordinated with the major 3.5 kb mRNA of PSD-95 during development (Fig. 3B). Consequently, the transcription of the PSD-95 binding protein isoforms containing distinct N-terminal sequences might be differentially regulated in brain at different

toskeleton, and to elucidate the functional and structural mechanisms which regulate their interactions in the PSD.

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