Characterization of the Shank Family of Synaptic Proteins

MULTIPLE GENES, ALTERNATIVE SPlicing, AND DIFFERENTIAL EXPRESSION IN BRAIN 
AND DEVELOPMENT

Shank1, Shank2, and Shank3 constitute a family of proteins that may function as molecular scaffolds in the postsynaptic density (PSD). Shank directly interacts with GKAP and Homer, thus potentially bridging the N-methyl-D-aspartate receptor-PSD-95-GKAP complex and the mGluR-Homer complex in synapses (Naisbitt, S., Kim, E., Tu, J. C., Xiao, B., Sala, S., Valtschanoff, J., Weinberg, R. J., Worley, P. F., and Sheng, M. (1999) Neuron 23, 569–582; Tu, J. C., Xiao, B., Naisbitt, S., Yuan, J. P., Petralia, R. S., Brakeman, P., Doan, A., Aakalu, V. K., Lanahan, A. A., Sheng, M., and Worley, P. F. (1999) Neuron 23, 583–592). Shank contains multiple domains for protein-protein interaction including ankyrin repeats, an SH3 domain, a PDZ domain, an SAM domain, and a proline-rich region. By characterizing Shank cDNA clones and RT-PCR products, we found that there are four sites for alternative splicing in Shank1 and another four sites in Shank2, some of which result in deletion of specific domains of the Shank protein. In addition, the expression of the splice variants is differentially regulated in different regions of rat brain during development. Immunoblot analysis of Shank proteins in rat brain using five different Shank antibodies reveals marked heterogeneity in size (120–240 kDa) and differential spatiotemporal expression. Shank1 immunoreactivity is concentrated at excitatory synaptic sites in adult brain, and the punctate staining of Shank1 is seen in developing rat brains as early as postnatal day 7. These results suggest that alternative splicing in the Shank family may be a mechanism that regulates the molecular structure of Shank and the spectrum of Shank-interacting proteins in the PSDs of adult and developing brain.

The mechanisms underlying the molecular assemblage of molecules at the synapse are not well understood. Recently, a number of novel anchoring/scaffold proteins that are associated with the PSD1 have been isolated (1–5). In particular, PSD-95/SAP90, GRIP/ABP, and Homer/Vesl have been reported to be putative anchoring proteins for NMDA, AMPA, and metabotropic glutamate receptors, respectively (6–14). These anchoring proteins also interact with a variety of signaling and cytoskeletal proteins, thereby organizing a unique multiprotein complex for each glutamate receptor (15–21). One interesting question is whether there is any physical link between these specific glutamate receptor complexes and whether these links are regulated.

Recently, a synaptic protein, termed Shank, that may bridge the NMDA receptor complex and the mGluR receptor complex has been isolated (22, 23). Shank is made of five domain/regions that are likely involved in protein-protein interactions: ankyrin repeats, an SH3 domain, a PDZ domain, an SAM domain, and a proline-rich region. The PDZ domain of Shank directly interacts with the C-terminal QTRL motif of GKAP/SAPAP/DAP-1 (24–26), a protein that binds to the GK domain of the PSD-95 family of proteins (PSD-95/SAP90 (27, 28), SAP97 (29), chapsyn-110/PSD-93 (7, 30), and SAP102 (12, 13)). The proline-rich region of Shank directly interacts with the EVH1 domain of Homer, a putative anchoring protein for mGluR receptor (11). Thus, Shank may bridge two different (NMDA and metabotropic) glutamate receptor complexes.

Interestingly, the characterization of multiple cDNA clones of Shank1 and Shank2 suggests the presence of diverse splice variants in the Shank family. Here, we show that these splice sites are mainly localized at the boundaries between the domains of Shank, suggesting that Shank proteins with diverse domain compositions can be generated by alternative splicing. We investigated the heterogeneity of Shank in the rat brain at both mRNA and protein level and analyzed the differential regulation and localization of Shank proteins during rat brain development.

EXPERIMENTAL PROCEDURES

Northern Blot Analysis—To make specific probes for Northern blot analysis, the inserts of the original yeast two hybrid clones containing part of Shanks (the r8 clone of Shank1, the r9 clone of Shank2, and the h10 clone of Shank3) (22) were isolated and labeled with [ω-32P]CTP by using the High Prime DNA labeling kit (Roche Molecular Biochemicals). Rat multiple tissue Northern membrane (CLONTECH) was sequentially probed with the Shank probes in ExpressHyb solution following the manufacturer’s protocol (CLONTECH).

RT-PCR Analysis—Total RNA from cortex and cerebellum of adult
and developing rat brains were isolated by using RNAgent total RNA isolation system (Promega). RT-PCR were performed using specific primers (Table I) and the Access RT-PCR system (Promega). RT-PCR conditions were as follows; 40 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 1 min, and elongation at 68 °C for 1 min. Relevant cDNAs and water were used as positive and negative controls, respectively. RT-PCR products were resolved on 3% agarose gels. Calculation of the ratio of band signals with and without an insert was done by scanning positive photographs followed by densitometric reading with the Image-Pro Plus software (Media Cybernetics).

**Expression Constructs, COS Cell Transfection, and Immunohistochemistry**—HA-tagged Shank expression constructs were made as follows. The r8 clone of Shank1 and the r8 clone of Shank2 were amplified by PCR, digested with XhoI, and subcloned into pCDNA3 HA (Invitrogen). The h10 clone of Shank3 was isolated from pGAD10 by EcoRI digestion and subcloned into pCDNA3 HA (Invitrogen). GW1 mammalian expression construct containing the full-length Shank1a has been described elsewhere (22) (GW1, British Biotechnology). GW1 Shank1b construct lacking the C-terminal 1356 amino acid residues long) was made by replacing the C-terminal AvrII-SalI fragment (nucleotides 4340–7040) of Shank1a (GenBank accession number AF131951) with the AvrII-SalI fragment from the Shank1 clone r11 (22), digested with BamHI and CIP. Glutathione S-transferase and thioredoxin fusion protein were purified using glutathione-Sepharose 4B resin (Amersham Pharmacia Biotech) and Probond resin (Promega), respectively. Glutathione S-transferase fusion proteins were used to immunize rabbits, and thiorexin fusion proteins were used to make affinity columns for the purification of specific antibodies (Sulfolink, Pierce). To raise a monoclonal antibody, thiorexin fusion protein containing the SH3 + PDZ domains of Shank2 was made as immunogen. Guinea pig PSD-95 antibody was described previously (7). The monoclonal antibodies specific for PSD-95 (K2858/8.5) and glutamic acid decarboxylase were purchased from Upstate Biotechnologies, Inc. and Sigma, respectively.

**Immunoblot Analysis**—Crude synaptosomal membrane proteins (22) from different regions of adult rat and developing rats and corticobasal ganglia were isolated and used in immunoblot analysis. PSD fractions were isolated as described (27). Protein samples resolved by SDS-PAGE gels were transferred to nitrocellulose membrane, incubated with primary antibodies followed by peroxidase-conjugated secondary antibodies and ECL reagent (Amersham Pharmacia Biotech). Working concentrations for the primary antibody incubation were as follows: Shank polyclonal antibodies, 1–2 μg/ml; Shank monoclonal antibody, 1:1000 dilution; PSD-95 monoclonal and HA antibody, 1 μg/ml.

**Immunohistochemistry on Rat Brain Sections**—Adult and developing rats were perfused with 4% parafomaldehyde, and brain sections were cut by using vibratome. Brain sections were permeabilized by incubating the sections in TE buffer (10 mM Tris-HCl (pH 8.0) + 1 mM EDTA) containing 0.1% Triton X-100 at 37 °C for 10 min, or by incubating in phosphate-buffered saline containing 1% Triton X-100 at room temp for 10 min. For DAB staining, sections were incubated with Shank1 antibody (1356) at 1 μg/ml at room temperature overnight followed by biotinylated anti-rabbit secondary antibody in the Elite Vectastain ABC kit (Vector). For immunofluorescence staining, brain sections were incubated with Shank1 (1356), PSD-95 (guinea pig), or glutamic acid decarboxylase (Sigma) antibody at 1 μg/ml or 1:1000 dilution followed by Cy3- or fluorescein isothiocyanate-conjugated secondary antibody at 1:1000 and 1:200 dilutions, respectively (Jackson Immunoresearch). The images were captured by using LSM510 confocal laser scanning microscope (Zeiss).

**RESULTS**

**Comparison of the Members of the Shank Family**—To extend the protein interaction network extending from the NMDA receptor-PSD-95 complex, we have searched binding partners of GKAP, an abundant protein that binds to the GK domain of PSD-95 (24–26). In a yeast two-hybrid screen using the GKAP C terminus as bait, we isolated partial cDNAs of Shank1, Shank2, and Shank3. Sequences of full-length cDNAs of Shank1 and Shank3 are described by Naisbitt et al. (22). In an effort to get more information on Shank2, we characterized a human EST clone (clone 191111) of Shank2. Alignment of the amino acid sequences of EST19111 and the r9 clone (original yeast two hybrid rat Shank2 clone) reveals 99% of amino acid sequence identity in the region of high homology including the PDZ domain, whereas the N-terminal region of EST19111 is completely different from the r9 clone, suggesting the presence of another splice variation in this region (Fig. 1A). In addition, EST19111 showed 99% identity with the second EST CortBP1, a recently published rat protein enriched in the growth cone (31), except for its C terminus, suggesting the presence of another splice variation in this region (Fig. 1A). These results suggest that the r9 clone, EST191111, and CortBP1 are splice variants of the same gene, termed Shank2/CortBP1.
Alternative Splicing and Differential Expression of Shank

The SH3 domains of Shank1, Shank2, and Shank3 share 63–74% amino acid sequence identity among themselves, but only 20–24% with the SH3 domain of Src (Fig. 1B). The PDZ domains of Shanks share 82–88% amino acid sequence identity, but 24–25% with the second PDZ domain of PSD-95 (Fig. 1C). The similar domain structure and amino acid sequences of Shank1, Shank2, and Shank3 suggest that they are the members of a protein family.

Tissue distribution of mRNAs of Shank1, Shank2, and Shank3 was examined by Northern blotting of poly(A) RNA (Fig. 2). Shank1 mRNA (10 kilobases) is exclusively expressed in rat brain; Shank2 mRNA (9 kilobases) is strongly expressed in brain and at lower levels in liver and kidney, whereas Shank3 mRNA (7–7.5 kilobases) is expressed abundantly in heart and moderately in brain and spleen.

Alternative Splicing in Shank1 and Shank2—Presumptive splice variants of Shank1 and Shank2 that were initially identified by sequence analysis of independent cDNA clones were confirmed by RT-PCR analysis (Figs. 3–5). We found four splice sites in Shank1 and another four in Shank2 (Fig. 3A). Actual nucleotide or amino acid sequences of alternative insertion(s) at each splice site are shown (Fig. 3B). Interestingly, some insertions contain alternative translational start codons (Shank1 Ins 2b and Shank2 Ins 1) or in frame stop codons (Shank1 Ins 4 and Shank2 Ins 4). In addition, most of the splice sites are located between the recognizable domains, except for the first splice site of Shank1 and the fourth splice site of Shank2 (Fig. 3A). These results suggest that alternative splicing in Shank can not only introduce small insertions but also generate Shank splice variants with differential domain compositions. For instance, the N-terminal ankyrin repeats + SH3 domain or the C-terminal SAM domain can be deleted in Shank1. Similarly, the N-terminal ankyrin repeats + SH3 domain, or the proline-rich region + SAM domain can be deleted in Shank2. We cannot exclude the possibility that there are additional alternative splice sites in Shank, for instance, at sites in Shank2 that are equivalent to sites 1 and 4 in Shank1. Systematic characterization of alternative splicing in Shank3...
Alternative Splicing and Differential Expression of Shank

Differential Expression of the Splice Variants of Shank1 and Shank2 in Developing Brain—To test in vivo existence and differential expression of Shank splice variants, we performed RT-PCR analysis on total RNA from cortex and cerebellum of adult and developing rat brains (Fig. 4). At splice site 1 of Shank1, the expression level of Ins 1a (combination of the “a” and “r” primers, Fig. 3A) did not change significantly during development of both cortex and cerebellum (Fig. 4A, A, and B). At splice site 4, the expression of Ins 4 (containing an in frame stop codon that deletes the C-terminal SAM domain) showed a downward trend that did not reach statistical significance (Fig. 4, A and B).

RT-PCR results for Shank2 revealed similar differential expression (Fig. 5). In particular, at splice sites 2 & 3 of Shank2, the bottom band (Fig. 5, arrowhead) is clearly seen in cortex (lanes 2–4), but very faintly in cerebellum (lanes 5–7). Interestingly, the upper diffuse band at Ins 2 & 3 (Fig. 5, arrow) is decreased during the development of cortex (lanes 2–4), whereas they are slightly increased during the development of cerebellum (lanes 5–7). Because the upper band contains probably two bands (containing the insertions (Ins 2 & 3) of similar size, 12 and 21 base pairs, respectively) that could not be separated on agarose gel, calculation of the band ratios and statistical analysis could not be performed. For Ins 1a, Ins 1b, and Ins 4a, we could not detect any significant changes in their expression (Fig. 5). Ins 4b is an interesting insertion in that it contains an in-frame stop codon that would delete the proline-rich region + SAM domain (Fig. 3B, Ins 4 of Shank2). Unfortunately, Ins 4b was found from the human EST1911111 clone. Thus, differential expression of Ins 4b could not be tested with rat RNA. Instead, only the in vivo presence of Ins 4b in human brain was performed confirmed by trying normal PCR, not RT-PCR, on the first strand cDNA from human brain (Marathon-ready cDNA, CLONTECH) (Fig. 5, bottom right panel, P).

Expression of Diverse Shank Proteins in Rat Brain—To study Shank proteins in vivo, we generated five independent antibodies for Shanks: two anti-peptide rabbit polyclonal antibodies, two anti-fusion protein rabbit polyclonal antibodies, and an anti-fusion protein mouse monoclonal antibody. The regions of Shank1 and Shank2 used as immunogens are shown along with the nature of the antibodies in Fig. 6A.

To check the specificity of these antibodies for different Shanks in Western blotting, we immunoblotted COS cells transfected with HA-tagged Shank1, Shank2, and Shank3 proteins to cDNAs or untransfected COS cells as negative controls. Immunoblot with HA-antibody reveals the size and relative amount of HA-tagged Shank1, Shank2, and Shank3 proteins (Fig. 6B, top panel). 1355 and 1356 anti-peptide antibodies raised against Shank1 are relatively Shank1-specific. 3856 anti-tisation protein antibody and Sh2-15 monoclonal antibody recognize all three Shanks, and 3858 anti-fusion protein antibody recognizes Shank1 and Shank2 but not Shank3. Based on

Ins 2b that contains an alternative translational start codon did not change significantly during development of both cortex and cerebellum (Fig. 4A). At splice site 3, the expression of Ins 3 (VSPWKKKI, Fig. 3B) significantly decreased (p < 0.005, analysis of variance) during development of cortex but not cerebellum (Fig. 4, A and B).

The RT-PCR analysis of Shank1 alternative splicing during postnatal development of cortex and cerebellum. The RT-PCR was performed as explained in Fig. 4. Ins 2 & 3 indicates two closely localized insertions separated by two amino acid residues (Fig. 3B). Ins 4b was found in a human clone (EST191111). To prove the Ins 4b exists in human brain, PCR (P) was performed on human brain Marathon-ready cDNA (CLONTECH).

Fig. 4. RT-PCR analysis of Shank1 alternative splicing during postnatal development of cortex and cerebellum. A, left panels show a schematic of the RT-PCR and the positions of oligonucleotide primers. A given set of primers was used to amplify the relevant RT-PCR products by using total RNAs from the cortex or cerebellum at postnatal day 1 (P1), day 14 (P14), and 6-week-old adult (Ad). Positive (+) (lane 1) and negative (−) (lane 8) control RT-PCRs were performed using the relevant plasmid DNA or water instead of total RNA, respectively. RT-PCR products containing an insert are indicated by arrows, and RT-PCR products without an insert are indicated by arrowheads. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control to show equal amounts of total RNA used for the RT-PCRs. ORF, open reading frame. B, the ratio of the band signal with and without an insert was calculated. The relative strength of the band signals was measured by using the Image-Pro Plus software (Media Cybernetics). Mean ratio and S.D. were obtained from three independent RT-PCRs.

was not performed because only one cDNA clone (h10) for Shank3 was available.

Fig. 5. RT-PCR analysis of Shank2 alternative splicing during postnatal development of cortex and cerebellum. The RT-PCR analysis of Shank2 alternative splicing during postnatal development of cortex and cerebellum (Fig. 4A). At splice site 3, the expression of Ins 3 (VSPWKKKI, Fig. 3B) significantly decreased (p < 0.005, analysis of variance) during development of cortex but not cerebellum (Fig. 4, A and B). At splice site 4, the expression of Ins 4 (containing an in frame stop codon that deletes the C-terminal SAM domain) showed a downward trend that did not reach statistical significance (Fig. 4, A and B).
could be due to the fact that these two Shank1-specific antibodies were raised against two different regions of Shank1 (Fig. 6A).

3856, 3858, and Sh2-15 antibodies (which cross-react with other Shank genes (Fig. 6B)), recognize three additional bands (210, 170, and 160 kDa; Fig. 6C, circles) that are not detected by Shank1-specific antibodies (Fig. 6C, lanes 5–10). Because all three (3856, 3858, and Sh2-15) antibodies recognize all of the additional bands, these bands are likely to represent authentic Shank polypeptides. Regarding the identity of these additional bands, they may represent Shank2 or Shank3 that is not recognized by Shank1-specific antibodies. Alternatively, they could be the Shank1 proteins lacking the binding sites for Shank1-specific (1355 and 1356) antibodies. In fact, both 1355 and 1356 antibodies were raised against the region that are upstream of the splice site 2 of Shank1, making them unable to recognize Shank1 splice variants missing the N-terminal ankyrin repeats and the SH3 domain (Fig. 3A). The patterns recognized by Shank antibodies appear to be specific because they are eliminated by preincubating the antibodies with cognate antigens (Fig. 6C, lanes 11–20). In addition, the presence of multiple Shank bands does not appear to be due to the degradation of membrane proteins because immunoblot analysis of the same samples with PSD-95 antibody does not show any sign of significant degradation (Fig. 7B and C, bottom panels). The band indicated by asterisk (Fig. 6C, lanes 7 and 8) is recognized only by 3858 antibody and may represent a non-specific cross-reacting protein.

To understand the molecular nature of the brain Shank bands, we made Shank1 expression constructs containing the following four splice variants: Shank1a, full-length Shank1; Shank1b, Shank1 lacking the C-terminal SAM domain; Shank1c, Shank1 lacking the N-terminal ankyrin repeats + the SH3 domain; and Shank1d, Shank1 lacking the N-terminal ankyrin repeats + the SH3 domain and the C-terminal SAM domain (Fig. 6D). When run along with the brain Shank proteins, COS Shank proteins migrated to positions ranging in size from 250–180 kDa, which is roughly comparable to the size of brain Shank bands (Fig. 6D). These results indicate that alternative splicing in Shank may be a mechanism to generate diverse Shank proteins in brain.

Spatiotemporal Regulation of Expression and the PSD Enrichment of Shank Proteins—To see whether Shank proteins are differentially expressed in different regions of rat brain, we performed immunoblot analysis using crude synaptosomal membrane proteins from cortex, hippocampus, cerebellum and the rest of the adult rat brain (Fig. 7A). Shank1-specific 1355 antibody did not detect any major differential expression between different regions of rat brain (Fig. 7A, upper panel, lanes 1–4). Similar results were obtained by 1356 antibody (data not shown). On the other hand, Sh2-15 antibody, which recognizes Shank1, Shank2, and Shank3, revealed a differential expression. In particular, the 210-kDa band was seen in all lanes except for cerebellum (Fig. 7A, bottom panel, lanes 1–4).

To see whether there was differential expression of Shank proteins during development, we tried immunoblotting on crude synaptosomal proteins of rat cortex and cerebellum at different developmental stages, postnatal days 1–28 and adult (Fig. 7B). Expression of Shank proteins increased from low levels at birth to high levels at 3–4 weeks, before dropping slightly in adulthood, similar to the profile of PSD-95 protein expression. 1355 could not detect any major differential expression of Shank1 proteins in cortex and cerebellum (Fig. 7B, middle panel). Interestingly, however, Sh2-15 antibody revealed a sudden significant decrease of the expression of two bands in cerebellum. In particular, the 210 and 160 kDa bands...
Regional and developmental expression and PSD enrichment of Shank proteins. 

A, differential expression of Shank polypeptides in different regions of rat brain. Crude synaptosomal proteins from different regions of the rat brain were probed with Shank1-specific peptide antibody (1355) and monoclonal antibody that recognizes Shank1, Shank2, and Shank3. Shank1 antibody reveals the lack of the 210 kDa band in cerebellum (lane 3, bottom panel). He, hippocampus; R, the rest of brain except for cortex, hippocampus, and cerebellum. B, differential expression of Shank polypeptides during development. Crude synaptosomal proteins of the cortex and cerebellum during postnatal development were probed with 1355 and Shank2-15 antibodies. Shank2-15 antibody reveals a significant decrease of the 210 and 160 kDa bands at P21 in cerebellum (middle panel). The same membrane was reprobed with anti-PSD-95 antibodies for comparison (bottom panels). C, enrichment of Shank polypeptides in PSD fractions. Multiple Shank bands are highly enriched in PSD fractions extracted with Triton X-100 once (PSD I) or twice (PSD II) or with Triton X-100 and Sarkosyl (PSD III). The same membrane was reprobed with PSD-95 for comparison (bottom panel). Whole br., whole brain; crude syn., crude synaptosome.

are seen up to P14, but not at P21 and after (Fig. 7B, middle panel, compare lanes 10 and 11). This suggests that these two bands may play an important role in early stages (P1–P14) of rat cerebellar development. Shank2-15 antibody could not detect any differential expression of Shank proteins in cortex (Fig. 7B, middle panel, lanes 1–6). For comparison, the membranes were reprobed with PSD-95 antibody.

To see whether different Shank proteins are differentially associated with the PSD, we performed immunoblot analysis on PSD fractions prepared by extracting the synaptosomal fraction with the detergents of increasing stringency (Fig. 7C). We could not, however, detect any prominent differential enrichment of Shank proteins by both 1355 and Shank2-15 antibodies.

Immunolocalization of Shank1 Proteins at Excitatory Synaptic Sites—Among the two antibodies (1356 and 3856) that work best for immunohistochemistry, the 1356 antibody was chosen because it was specific for Shank1 by immunoblotting. To test whether 1356 is also Shank1-specific in immunohistochemistry, we performed immunostaining on COS cells transfected with HA-tagged Shank1, Shank2, and Shank3. Results show that 1356 specifically recognizes Shank1, but not Shank2 or Shank3 (Fig. 8, A–C). Shank1 antibody stained the dendritic fields of hippocampus (Fig. 8D) and the molecular layer of cerebellum (Fig. 8E) by DAB staining. Preincubation of 1356 with peptide antigen eliminates the staining in brain (cerebellum, shown in Fig. 8F).

To further characterize immunolocalization of Shank1 proteins at subcellular level, we performed immunofluorescence staining on rat brain sections. 1356 antibody was used as the primary antibody (Fig. 9A), the CA1 region of hippocampus (Fig. 9B), and the molecular layer of cerebellum (Fig. 9C). The specific punctate staining is eliminated by preincubation of the antibody with peptide antigen (Fig. 9D, an example from cerebellum). The punctate staining could be obtained when brain sections were treated with limited proteolysis (Fig. 9, A–D and F–H), but not after permeabilization by Triton X-100 (Fig. 9E, cerebellum). This suggests that Shank1 proteins are localized in relatively inaccessible sites for antibodies, such as the PSD (32). By double immunofluorescence staining, the punctate staining of Shank1 colocalizes with PSD-95 (Fig. 9F, cortex), but not with glutamic acid decarboxylase (GAD)(Fig. 9G, cerebellum), suggesting that Shank1 proteins are mainly localized at excitatory synaptic sites. It is noteworthy that double immunofluorescence staining of Shank1 and PSD-95 revealed some punctate stainings in which only one immunoreactivity (Shank1 or PSD-95 alone) is visible (Fig. 9F, arrowheads).

Immunolocalization of Shank1 Proteins in Developing Brain—To test immunolocalization of Shank1 proteins during development, we performed immunofluorescence staining on rat brain sections at different developmental stages, postnatal days 1, 4, 7, 14, and 21 and adult. Punctate staining of Shank1...
Fig. 9. Immunolocalization of Shank1 by confocal fluorescence microscopy in adult and developing brain. Brain sections were stained singly or doubly with the 1356 Shank1-specific antibody and other antibodies (indicated), followed by Cy3- or fluorescein isothiocyanate-conjugated secondary antibodies. Punctate Shank staining was seen in the neuropil of cortex (A), in the CA1 region of hippocampus (B), and in the molecular layer of cerebellum (C). The punctate staining was eliminated by preincubation with the peptide antigen (D) (the example shown here is from cerebellum). Limited proteolysis was used to reveal Shank staining (A–C). Permeabilization with Triton X-100 alone failed to give any Shank staining at synaptic sites (E), as shown by double immunofluorescence for Shank1 (E1) and synaptophysin (E2) in cerebellum. Double label immunofluorescence for Shank1 + PSD-95 in P21 cortex (F1 and F2) or Shank1 + GAD (glutamic acid decarboxylase) in adult cerebellum (G1 and G2), using Cy3-conjugated anti-rabbit and fluorescein isothiocyanate-conjugated anti-guinea pig (PSD-95) or antirabbit (GAD) secondary antibodies. Examples of the extensive colocalization between Shank1 and PSD-95 are indicated by arrows (F1 and F2, insets), and examples of non-colocalization are indicated by arrowheads (F1 and F2, insets). There was no significant colocalization between Shank1 and GAD signals. In developing brain, Shank and PSD-95 colocalized in puncta in cortex at as early as postnatal day 7 (H1 and H2). Size bar, 30 μm.

was seen in cortex (Fig. 9H1) and in the molecular layer of cerebellum (data not shown) at as early as postnatal day 7. In addition, Shank1 colocalizes with PSD-95 (Fig. 9H2), suggesting that Shank1 proteins are localized at synaptic sites. It should be noted, however, that some PSD-95 immunoreactivity is seen at nonsynaptic sites, especially at early stages of neuronal development (33). We could not, however, use synaptophysin as a synaptic marker because the protease treatment of brain sections eliminated the synaptophysin staining (data not shown).

In younger brains at P1 and P4, we could not observe any punctate staining (data not shown). One reason for this could be the lower expression level of Shank1 proteins at P1 as compared with P7 (Fig. 7B, top panel, lanes 1, 2, 7, and 8). Another possibility is that Shank proteins in very early brains are diffusely localized at nonsynaptic sites and digested by protease treatment. On the other hand, staining of brain sections at P1 and P4 after permeabilization with Triton X-100 did not reveal any significant Shank staining (data not shown).

**DISCUSSION**

The Shank family contains three known members, Shank1, Shank2, and Shank3. Each Shank shows distinct tissue distribution of mRNA. Some Shank proteins are differentially expressed in different regions and at different developmental stages of rat brain. Although Shank1, Shank2, and Shank3 share essentially identical domain structure, the long proline-rich regions (900–1000 residues) of Shanks share relatively low amino acid sequence identity (33–40%) as compared with other recognizable domains, including ankyrin repeats, the SH3 domain, the PDZ domain, and the SAM domain (60–90%). Thus, individual Shanks may possess unique functions in addition to common functions. It should also be noted that mRNAs of Shank2 and Shank3 are expressed in some nonneural tissues, which may explain the nonneuronal expression of known Shank-interacting proteins, including Homer/Ves and cortactin (31, 34). Considering the synaptic localization of Shank1 in brain, Shank2 or Shank3 protein expressed in nonneural tissues might function as a molecular scaffold at cell junctions.

The results of the present study suggest that Shank proteins with different domain compositions can be generated by alternative splicing in both Shank1 and Shank2. In Shank1, the N-terminal Ank + SH3 domains or the C-terminal SAM domain can be truncated. In Shank2, the N-terminal Ank + SH3 domain or the C-terminal proline-rich region + SAM domain can be truncated. What would be the significance of alternative splicing of Shank proteins to generate variants containing different domains? A likelihood is that these domains mediate direct protein-protein interactions, and thus the spectrum of Shank-interacting proteins is regulated. For example, the PDZ domain of Shank interacts with the C-terminal QTRL motif of GKAP (also called SAPAP) (22); the motifs in the proline-rich regions interact with cortactin (31) and Homer (22); the SAM domain may mediate self-association (35). In addition, the ankyrin repeats and the SH3 domain, alone or in combination, are known to be the domains for protein-protein interactions (36–38).

One of the most interesting alternative splicings of Shank is the Ins 4b of Shank2 that contains an alternative in frame stop codon and deletes the proline-rich region + SAM domain (Fig. 3). This alternative splicing has two implications. Firstly, the truncation of the proline-rich region eliminates the binding site for Homer (23), which may result in the loss of a potential link between the NMDA receptor-PSD-95-GKAP complex and the mGluR-Homer complex that bind to the PDZ domain and the proline-rich region, respectively. There have been several reports for functional relationship between the two glutamate receptors (39). If Shank is a molecule that may support these functional links, the Ins 4b of Shank2 could be a candidate for modulation. The second implication of Ins 4b comes from the fact that the proline-rich region of CortBP1, a presumptive splice variant of Shank2, interacts with cortactin, an actin-binding protein (31). In other words, Shank2/CortBP1 can mediate the linkage of NMDA receptor-PSD-95-GKAP complex or mGluR-Homer complex to actin. Thus, the truncation of the proline-rich region by Ins 4b has a potential to break the link between the NMDA receptor or mGluR receptor complex to cytoskeleton.

Another alternative splicing to note is the Ins 4 of Shank1. Ins 4 of Shank1 contains an in-frame stop codon and terminates Shank1 protein translation at the end of the proline-rich region, deleting the C-terminal SAM domain. The SAM domain (65–70 amino acid residues long) is a module for protein-protein interaction (35, 40) found in a variety of proteins including the Eph family of receptor tyrosine kinases (41), serine-threo-
nine kinases (42), and the ETS family of transcription factors (43, 44). One of the known functions of the SAM domain is the formation of a homo- or hetero-oligomer (35). Recent x-ray crystallographic analysis of the SAM domain structure of Eph receptors revealed that the SAM domain has structural features suitable for dimer formation, as well as a bigger oligomer formation (45, 46). Moreover, recent in vitro experiments have shown that the SAM domain of Shank3 can form a homomultimer (22). Thus, the SAM domain of Shank may mediate homo- or heteromultimerization of Shank proteins in vivo in a tail-to-tail fashion, potentially making the Shank-related multipeptide complex bigger. Another known function of the SAM domain is its interaction with other proteins that do not contain the SAM domain including leucocyte common antigen-related (47), low molecular weight protein tyrosine phosphatase (48), and Grb10 (49), suggesting the SAM domain of Shank may also interact with non-SAM proteins. Thus, the deletion of the SAM domain in Shank1 may interrupt with Shank oligomerization, as well as its interaction with as yet unknown non-SAM proteins. The next question is whether the expression of the Ins 4 of Shank1 is regulated. In the present study, we could not find a statistically significant change in the expression of Ins 4 of Shank1 during development, although there was a decreasing trend in both cortex and cerebellum (Fig. 4B).

Among all alternative splicings identified in the present study, only two inserts have been actually shown to be significantly regulated during brain development. They are Ins 2a and Ins 3 of Shank1, which are made of only a few amino acids, 9 and 8 residues, respectively (Fig. 3B). The fact that their expressions are significantly regulated during brain development suggests that they may play an important in the maturation of the PSD during brain development. Possibly, these small inserts might function as binding sites for other proteins. For example, a small insertion of agrin that is only 4 residues long is necessary and sufficient for the agrin-heparin interaction (40–52).

There are multiple lines of evidence that Shank may play an important role as scaffold protein in the formation of the PSD. Shank is relatively a big protein (Shank1a, 2087 residues; Shank3, 1740 residues) that is about 2–3 times bigger than the others. Shank proteins show spatiotemporal differential expression during early postnatal brain development. In addition, some of the Shank proteins show spatiotemporal differential expression during this period. Biochemically, Shank proteins are enriched in the PSD. Immunohistochemically, Shank1 staining is seen at excitatory synaptic sites as early as postnatal day 7.

Beijing University, we observed interesting heterogeneity of punctate staining of Shank1 and PSD-95. In addition to the puncta that are doubly labeled with both Shank1 and PSD-95, there were Shank1-only or PSD-95-only puncta in both adult and developing brains. The PSD-95-only synapses could be the ones in which Shank2 or Shank3 protein, instead of Shank1, is expressed, or they could be the ones in which PSD-95 is not linked to Shank. For instance, PSD-95 may interact with the GRAP containing the C-terminal variant that does not interact with Shank (specifically the Y variant instead of the X variant) (24). The Shank1-only synapses could be the ones in which Shank does not interact with the NMDA receptor-PSD-95-GKAP complex.

One obvious future study is the identification of additional binding partners for Shank that will further elucidates the function of alternative splicing. It will be interesting to see whether differential expression of Shank mRNA or proteins occurs at microscopic level, for instance, between different neurons or between different synapses in a given neuron. It will be also interesting to explore the possibilities that Shank functions as a master scaffold protein to link smaller complexes, including the NMDA receptor-PSD-95-GKAP complex and the mGlur-Homer complex, and that alternative splicing of Shank can modulate this.
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