Characterization of the Shank Family of Synaptic Proteins

MULTIPLE GENES, ALTERNATIVE SPLICING, AND DIFFERENTIAL EXPRESSION IN BRAIN AND DEVELOPMENT*

(Received for publication, June 3, 1999)

Sangmi Lim‡, Scott Naisbitt§, Jiyoung Yoon‡, Jong-Ik Hwang¶, Pann-Ghill Suh†, Morgan Sheng‡,†, and Eunjoon Kim‡,**

From the ‡Department of Pharmacology, Pusan National University, Kumjeong-kw, Pusan 609-735, Korea, the §Howard Hughes Medical Institute and Department of Neurobiology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, and the ¶Department of Life Science, Pohang University of Science and Technology, Pohang 790-784, Korea

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., Valtchanoff, 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 PSD-95/Dlg/ZO-1 domain, a sterile a motif 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. 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 [32P]CTP by using the High Prime RNA 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

1 The abbreviations used are: PSD, postsynaptic density; RT, reverse transcription; PCR, polymerase chain reaction; aa, amino acids; HA, hemagglutinin; EST, expressed sequence tag; Ins, insert; PD2, PSD-95/Dlg/ZO-1; SAM, sterile a motif.
and developing rat brains were isolated by using RNAlater total RNA isolation system (Promega). RT-PCR amplifications 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 readings using 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 XbaI, 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 (22) (GW1, British Biotechnology). GW1 Shank1b construct lacking the C-terminal SAM domain was made by replacing the C-terminal AvrII-Sall (nucleotides 4340–7040) fragment of Shank1a (GenBank accession number AF131951) with the AvrII-Sall fragment (nucleotides 4340–6919) from the Shank1 clone 1-3-11 that contains an insertion (81 base pairs) with an in frame stop codon (Ins 4 in Fig. 3B and Fig. 6D). Thus, GW1 Shank1b (total 1863 amino acid residues long) contains aa 1–1499 of Shank1a plus aa 1–14 of Ins 4 of Shank1 (see Fig. 3B). GW1 Shank1b and Shank1c were made by replacing the N-terminal ankyrin repeat + SH3 domain (aa 1–582) of Shank1a and Shank1b with Ins 2b of Shank1a, which contains an alternative translational start site (see Fig. 3B and Fig. 6D). Specifically, a PCR product containing aa 37–88 (see Fig. 3B) of Ins 2b + aa 583–618 of Shank1a was amplified by PCR from the Shank1 clone r11 (22), digested with HindIII and EcoRI, and subcloned into GW1 digested with HindIII and EcoRI. This construct was again linearized by EcoRI digestion and ligated with the EcoRI fragment of GW1 Shank1a containing aa 619–2087 of Shank1a to make GW1 Shank1c, or ligated with the EcoRI fragment of GW1 Shank1b containing aa 619–1863 of Shank1b to make GW1 Shank1d.

To test the specificity of Shank antibodies under Western and immunohistochemical conditions, the HA-tagged Shank1, Shank2, and Shank3 were transfected in COS cells on six-well plates or coverslips coated with polylsine, respectively. COS cell transfections were performed using LipofectAMINE (DNA:LipofectAMINE ratio of 0.4 μg:1.2 μl) and Opti-MEM (Life Technologies, Inc.). After 48 h of transfection, COS cells on six-well plates were harvested and used for immunoblot analysis. For immunohistochemical analysis, COS cells on coverslips were fixed with 2% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 1 min, and stained with anti-HA (Roche Molecular Biochemicals) and 1356 Shank1 antibodies at 1 μg/ml each for 1 h, followed by incubation with fluorescein isothiocyanate-conjugated antimouse and Cy3-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch) at 1:200 and 1: 1000 for 30 min, respectively. The images of COS cells were captured by using LSM510 confocal laser scanning microscope (Zeiss).

**Antibodies**—To make anti-peptide Shank antibodies, two synthetic peptides containing aa 544–559 (EQGRGQRESKDKARKLF for 1355 antibody) and aa 425–440 (CARPSRSGRHPEDARKQ for 1356 antibody) of Shank1a were synthesized, coupled to keyhole limpet hemocyanin, and immunized to rabbits (CGB, Cyteneis, Ltd., Sweden). Cyteneis, Ltd., provided the antigen in a cocktail with BorHi and CIP, a glutathione S-transferase and thiorexin fusion proteins were purified by 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 used as immunogen. Guinea pig PSD-95 antibody was described previously (7). The monoclonal antibodies specific for PSD-95 (K28/58.8.5) and glutamic acid decarboxylase were purchased from Upstate Biotechnologies, Inc. and Sigma, respectively.

**Immunoblot Analysis**—Crude synaptosomal membrane proteins (P2 fractions) from adult and developing rats were used in immunoblot analysis. P2 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% paraformaldehyde, and brain sections were cut by using vibratome. Brain sections were permeabilized by incubating the sections in TE buffer (10 mm Tris- HCl (pH 6.0) + 1 mm EDTA) containing 0.2% Triton X-100. 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.

**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 Naishbit 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 and the r9 clone are splice variants of the same gene, termed Shank2/CortBP1.
Alternative Splicing and Differential Expression of Shank

Fig. 1. Comparison of the domain structure of Shank family proteins and cortBP1. A, schematic diagram of domain organization of Shank family protein. Full-length Shank1 and Shank3 are aligned with the partial clones of Shank2. r9 is a rat clone pulled out by the yeast two hybrid screening. Est191111 is a human EST clone. The recently published CortBP1 (31) is related to Shank1 and Shank3 and is probably a splice variant of Shank2. M and * indicate the methionine start codon and stop codon, respectively. Alternative amino acid sequences are indicated by dashed boxes. Shank1, Shank3, and Shank2/cortBP1 have essentially identical domain structures in their regions of overlap. Note the presence of presumptive splice variations on both sides of the EST19111 clone and at the N terminus of CortBP1. Ank, ankyrin repeats 1–7; SH3, Src homology domain 3; PDZ, PSD-95/Dlg/ZO-1 domain; Pro, proline-rich region; SAM, sterile a motif. Domains are drawn in scale. GenBank™ accession numbers are AF141903 (r9) and AF141901 (Est191111). B, alignment of the amino acid sequences of the SH3 domain of Shanks and Src. The alignment was made using the Pileup program (GCG software) and the GeneDoc program. The amino acid residues identical in four, three, and two sequences are indicated by black, dark gray, and light gray backgrounds, respectively. C, alignment of the amino acid sequences of the PDZ domain of Shanks and PSD-95 (PDZ2). The amino acid sequences are from rat clones r9, Shank1, and Shank3 (22).

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 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 expressed abundantly in rat 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...
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, Ins 1a panel, lanes 2–7). Similar results were obtained for Ins 1b (Fig. 4A). These results indicate that there is no significant differential expression of 5’ untranslated regions during brain development.

At splice site 2, RT-PCR for Ins 2a (combination of the “a” and “r” primers) generates two bands (135 and 108 base pairs, respectively, Fig. 4A, arrow and arrowhead) that contain or lack Ins 2a (Fig. 3B, SLIDGIDSG sequence). Interestingly, the expression these two bands changed markedly during development of cortex (Fig. 4A, lanes 2–4 of Ins 2a, arrow). In particular, the intensity of the upper band increased, whereas the intensity of the lower band decreased. Thus, the ratio of signal of the upper band over the lower band significantly increased during development of cortex (p < 0.005, analysis of variance) (Fig. 4B). In cerebellum, however, the expression of Ins 2a did not change significantly (Fig. 4, A and B). It should be noted that the calculation of the band ratio in a given lane is meaningful because the same set of primers is used to amplify both bands, with and without an insert. The expression level of the

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.

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 partial cDNAs or untransfected COS cells as negative control. 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 antipeptide antibodies raised against Shank1 are relatively Shank1-specific. 3856 antifusion 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). 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 EST191111 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 partial cDNAs or untransfected COS cells as negative control. 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 antipeptide antibodies raised against Shank1 are relatively Shank1-specific. 3856 antifusion 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
Alternative Splicing and Differential Expression of Shank

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 could be due to the fact that these two Shank1-specific antibodies were raised against two different regions of Shank1 (Fig. 6A).
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. Sh2-15 antibody could not detect any differential expression of Shank proteins in cortex (Fig. 7B, middle panels). For comparison, the membranes were probed 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 Sh2-15 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). In rat brain, 1356 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 reveals punctate staining in the neuropil of cortex (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...
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/Vesl 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 examples, 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 multirrotein 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 other putative scaffold proteins, including PSD-95 (724 residues), GUKAP/SAAP1 (666–992 residues), and GRIP (1112 residues). Shank contains diverse domains for protein interaction, and the domain composition of Shank appears to be regulated by alternative splicing. At the protein level, the expression of diverse Shank proteins increases very rapidly 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.

By immunohistochemistry, 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 Shank3, 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 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-R-Homer complex, and that alternative splicing of Shank can modulate this.

REFERENCES

Downloaded from http://www.jbc.org/ by guest on September 23, 2016
Characterization of the Shank Family of Synaptic Proteins: MULTIPLE GENES, ALTERNATIVE SPLICING, AND DIFFERENTIAL EXPRESSION IN BRAIN AND DEVELOPMENT
Sangmi Lim, Scott Naisbitt, Jiyoung Yoon, Jong-Ik Hwang, Pann-Ghill Suh, Morgan Sheng and Eunjoon Kim

doi: 10.1074/jbc.274.41.29510

Access the most updated version of this article at http://www.jbc.org/content/274/41/29510

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 23 of which can be accessed free at http://www.jbc.org/content/274/41/29510.full.html#ref-list-1