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Direct interaction of post-synaptic density-95/Dlg/ZO-1 domain-containing synaptic molecule Shank3 with GluR1 α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor

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Abstract

A class of scaffolding protein containing the post-synaptic density-95/Dlg/ZO-1 (PDZ) domain is thought to be involved in synaptic trafficking of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors during development. To clarify the molecular mechanism of AMPA receptor trafficking, we performed a yeast two-hybrid screening system using the cytoplasmic tail of the GluR1 subunit of AMPA receptor as a bait and identified a synaptic molecule, Shank3/ProSAP2, as a GluR1 subunit-interacting molecule. Shank3 is a PDZ domain-containing multidomain protein and is predominantly expressed in developing neurons. Using the glutathione S-transferase pull-down assay and immunoprecipitation technique we demonstrated that the GluR1 subunit directly binds to the PDZ domain of Shank3 via its carboxyl terminal PDZ-binding motif. We raised anti-Shank3 antibody to investigate the expression of Shank3 in cortical neurons. The pattern of Shank3 immunoreactivity was strikingly punctate, mainly observed in the spines, and closely matched the pattern of post-synaptic density-95 immunoreactivity, indicating that Shank3 is colocalized with post-synaptic density-95 in the same spines. When Shank3 and the GluR1 subunit were overexpressed in primary cortical neurons, they were also colocalized in the spines. Taken together with the biochemical interaction of Shank3 with the GluR1 subunit, these results suggest that Shank3 is an important molecule that interacts with GluR1 AMPA receptor at synaptic sites of developing neurons.

Keywords: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor, development, GluR1 subunit, post-synaptic density-95/ Dlg/ ZO-1 domain, Shank3, synapse.

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Transmission at excitatory synapses is primarily mediated by glutamate acting on three classes of ligand-gated ion channels, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate and NMDA receptors (Wisden and Seeburg 1993; Hollmann and Heinemann 1994). In addition to their role in synaptic transmission, these glutamate receptors (GluRs) have been thought to play a crucial role in many brain functions, including activity-dependent synaptogenesis during development and synaptic plasticity (McDonald and Johnston 1990; Bliss and Collingridge 1993).

Many excitatory synapses in young developing neurons have been found to express only NMDA receptors, which are continuously blocked by magnesium at resting membrane potentials. As no evoked transmission is observed even when glutamate is present, these synapses are referred to as 'silent synapses'. During later development, AMPA receptors are delivered and clustered on the synaptic membrane in an activity-dependent manner, and the synapses subsequently become functionally active (Durand *et al*. 1996; Wu *et al*. 1996; Pickard *et al*. 2000; Liao *et al*. 2001; Isaac 2003). Thus, the clustering of AMPA receptors on the synaptic membrane is an essential event during synaptogenesis.

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Abbreviations used: aa, amino acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; EGFP, enhanced green fluorescent protein; EGFP-GluR1, enhanced green fluorescent protein-fused GluR1 subunit; GluR, glutamate receptor; GST, glutathione S-transferase; mShank3, mouse Shank3; NR2B, NMDA receptor 2B; PBS, phosphate-buffered saline; PDZ, post-synaptic density-95/Dlg/ZO-1; PFA, paraformaldehyde; PSD-95, post-synaptic density-95; SAP-97, synapse-associated protein 97; SDS, sodium dodecyl sulfate; SH3, Src homology 3.

The AMPA receptors are heteromeric complexes of four homologous subunits, GluR1–GluR4 (Wisden and Seeburg 1993; Hollmann and Heinemann 1994). The expression of GluR1–GluR3 subunits increases during development, whereas the expression of GluR4 subunit is seen in earlier development (Zhu *et al*. 2000). Recent studies have shown that induction of long-term potentiation or activation of Ca^{2+}/calmodulin-dependent protein kinase II promoted delivery of GluR1 subunit-containing AMPA receptors to synapses, and that this effect was diminished by mutating the post-synaptic density-95 (PSD-95)/Dlg/ZO-1 (PDZ) domain binding motif at the C-terminal end of the GluR1 subunit (Shi *et al*. 1999; Hayashi *et al*. 2000). This finding indicates that PDZ domain-containing proteins participate in regulating the synaptic localization of GluR1 subunit-containing AMPA receptors; however, this protein remains to be identified. So far, biochemical analysis has shown that GluR1 subunit binds to synapse-associated protein 97 (SAP-97) via its C-terminal PDZ-binding motif (Leonard *et al*. 1998). In developing neurons, however, expression of SAP-97 is distributed more in the somatic region than in the synapses. Thus, SAP-97 is thought to be associated with intracellular AMPA receptors, including GluR1 subunit, and to be involved in the early secretory pathway of AMPA receptor trafficking (Sans *et al*. 2001).

In this study, we used a yeast two-hybrid screening system to search for the protein that interacts with the PDZ-binding motif of GluR1 AMPA receptor, and we identified Shank3/ProSAP2 from the mouse brain cDNA library. Shank3 is a multidomain protein that contains ankyrin repeats, a Src homology 3 (SH3) domain, a PDZ domain, a long proline-rich region and a sterile alpha motif, and is predominantly expressed in spines during synaptogenesis (Lim *et al*. 1999; Naisbitt *et al*. 1999; Sheng and Kim 2000; Böckers *et al*. 2001, 2002). In the present study we found that Shank3 biochemically interacts with the PDZ-binding motif of GluR1 subunit and that Shank3 is colocalized with GluR1 AMPA receptor in the spines. These findings suggest that Shank3 is a candidate clue molecule for clarifying the molecular mechanism of AMPA receptor trafficking to synapses during synaptogenesis.

Materials and methods

Yeast two-hybrid screening

The cDNA fragment encoding the C-terminal 81-amino-acid segment [amino acids (aa) 809–889] of mouse GluR1 subunit was inserted into pLexA to yield a bait plasmid, pLexA-GluR1/C. The bait plasmid was transformed with yeast strain L40 and a mouse brain cDNA library (Clontech, Palo Alto, CA, USA) was used to screen for proteins that interacted with LexA-GluR1/C fusion proteins. Interactions were detected by induction of reporter genes, *HIS3*, *TRP1*, *LUE2* and *LacZ*, which resulted in cell growth and the formation of blue colonies on histidine-, tryptophan- and leucine-depleted yeast synthetic media containing X-gal (80 μg/mL).

Plasmid construction cDNA fragments encoding the full-length mouse Shank3 (mShank3; aa 1–1730), the SH3 and PDZ domains of mShank3 (mShank3/SH3-PDZ; aa 461–671), the PDZ domain (mShank3/PDZ; aa 552–671), the C-terminal fragment containing the PDZ domain (mShank3/PDZ + C; aa 552–1730) and the C-terminal fragment without the PDZ domain (mShank3/C; aa 670–1730) were constructed by PCR and subcloned into a mammalian expression vector, pCMV-Myc (Clontech), to obtain pCMV-myc-mShank3, pCMV-myc-mShank3/SH3-PDZ, pCMV-myc-mShank3/PDZ, pCMV-myc-mShank3/PDZ + C and pCMV-myc-mShank3/C, respectively. Fusion proteins of the regions of mShank3, GluR1 subunit and mouse NMDA receptor 2B (NR2B) (GluRε2) subunit with glutathione S-transferase (GST) (and myc) were constructed by subcloning PCR-amplified DNA fragments into pGEX-4T-2 (Amersham, Piscataway, NJ, USA) containing a thrombin cleavage site or into pGEX-5X-2 (Amersham) containing a factor Xa cleavage site to obtain: pGEX-4T-2, pGEX-mShank3/SH3-PDZ (aa 461–671), pGEX-mShank3/SH3 (aa 461–551), pGEX-mShank3/PDZ (aa 552–671), pGEX-myc-mShank3/SH3-PDZ (aa 461–671), pGEX-GluR1/C (aa 809–889), pGEX-GluR1/del (deletion of four aa from the C-terminal end) (aa 809–885), pGEX-GluR1/mu (exchange of four aa in the C-terminal end, ATGL to AAGA), pGEX-C-terminal segment of NR2B (aa 1279–1456), pGEX-5X-2 and pGEX-5X-GluR1/C (aa 809–889). All of the constructs were confirmed by DNA sequencing.

Cell culture and DNA transfection

Chinese hamster ovary cells or COS7 cells were grown at 37°C in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (Irvine, Santa Ana, CA, USA). For the transient expression studies, the cells were transfected with expression vector by using Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Embryonic day 15–16 mouse cortical primary neurons were prepared as described previously (Hirasawa *et al*. 2003). Briefly, cerebral cortices were dissected, minced and dissociated with a papain. The dissociated cells were plated onto 0.1% polyethylenimine-coated plates at a density of $1.0–1.5 \times 10^4$ cells/cm^2 for immunocytochemistry and 1.0×10^5 cells/cm^2 for pull-down assays, and maintained in Neurobasal medium (Gibco BRL) containing 2% B-27 supplement (Gibco BRL) and 0.5 mM glutamine at 37°C under a humidified 10% CO_2 atmosphere for the periods indicated. For the transient expression studies, the cells were transfected with expression vector by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

The experimental protocols were approved by The Animal Care and Use Committee of the National Institute of Neuroscience.

Purification of recombinant proteins and pull-down assay

The GST fusion proteins were expressed in *Escherichia coli* BL21 and purified on glutathione sepharose 4B (Amersham) according to the manufacturer's protocol. The pull-down assay was performed essentially as described previously (Tu *et al*. 1999). Briefly, the Chinese hamster ovary cells transfected with myc-tagged protein or mouse cortical primary cells were lysed at 4°C for 1 h with a buffer

composed of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, protease inhibitor cocktail (Roche, Penzberg, Germany) and 0.1% Triton X-100 (for Chinese hamster ovary cells) or 1% Triton X-100 (for primary cultured cells). After removing the insoluble material by centrifugation (15 000 *g* for 20 min at 4°C), the protein concentration in the supernatant was determined with a protein assay kit (Pierce, Rockford, IL, USA). Soluble extracts were incubated for 12 h at 4°C with purified GST fusion protein bound to 20 μL of glutathione sepharose in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and protease inhibitor cocktail. The sepharose suspensions were washed four times with lysis buffer. Bound proteins were eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer, separated by SDS–polyacrylamide gel electrophoresis and analysed by immunoblot.

Immunoprecipitation assay

COS7 cells were transfected with each of the expression vectors. At 2 days after transfection, cells were lysed at 4°C for 1 h with immunoprecipitation (IP) buffer composed of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 10% glycerol, 0.5% Triton X-100 and protease inhibitor cocktail. After removing the insoluble material by centrifugation (15 000 *g* for 20 min at 4°C), the protein concentration in the supernatant was determined with a protein assay kit. Soluble extracts were incubated at 4°C for 2 h with rabbit polyclonal anti-enhanced green fluorescent protein (EGFP) antibody bound to protein A- and G-Sepharose beads (Amersham). The immunoprecipitates were washed three times with IP buffer, eluted by boiling in SDS sample buffer, separated by SDS–polyacrylamide gel electrophoresis and analysed by immunoblot.

Antibody production

Rabbit polyclonal antibody against a GST-fused mShank3 fragment (aa 1016–1357) was produced by Biotest Inc. (Tokyo, Japan).

Preparation of the synaptosomal fraction

The synaptosomal fraction was prepared essentially according to the procedures described previously (Carlin *et al*. 1980). In brief, cortex was dissected from mouse at 2 weeks of age and homogenized in 10 volumes of solution A (0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$ and 0.5 M $CaCl_2$) with a Teflon homogenizer. The homogenate was centrifuged (1400 ***g*** for 10 min at 4°C) and the supernatant was saved. The pellet was suspended in solution A and centrifuged (700 ***g*** for 10 min at 4°C). The supernatants were pooled and then centrifuged (13 800 ***g*** for 10 min at 4°C). The resulting pellet was resuspended in solution B (0.32 M sucrose and 1 mM $NaHCO_3$), layered onto a discontinuous sucrose gradient containing 0.8 M/1.0 M/1.2 M sucrose and centrifuged (82 500 ***g*** for 2 h at 4°C). The fraction at the 1.0 M/1.2 M sucrose interface was isolated as the synaptosomal fraction and the protein concentration was determined with a protein assay kit.

Immunoblot analysis

The proteins were separated by electrophoresis through an SDS polyacrylamide gel (Daiichi Pure Chemicals, Tokyo, Japan) and then electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were blocked by incubation for 1 h at room temperature (15∼25°C) with 5% skim milk (Becton Dickinson, Sparks, MD, USA) in buffer A (10 mM Tris-HCl, pH 7.5, 100 mM NaCl and 0.1% Tween 20) and then incubated for 1 h at room temperature with one of the following primary antibodies in buffer A containing 3% skim milk: rabbit polyclonal anti-mShank3 antibody (0.1 μg/mL), mouse monoclonal anti-myc antibody (1 : 1000; Roche), mouse monoclonal anti-PSD-95 antibody (1 : 200; Affinity Bioreagents Inc., Golden, CO, USA), rabbit polyclonal anti-GluR1 antibody (1 : 200; Chemicon, Temecula, CA, USA), rabbit polyclonal anti-GluR2 antibody (1 : 200; Chemicon) or rabbit polyclonal anti-EGFP antibody (1 : 1000; Molecular Probes, Eugene, OR, USA). After three washes in buffer A, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1 : 1000; Amersham) or horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1 : 1000; Amersham) and washed three times with buffer A. Immunoreactive bands were visualized with a chemiluminescence detection system (ECL; Amersham).

Preparation of membrane fraction and coimmunoprecipitation assay

To prepare the membrane fraction, the cortex was dissected from mouse at 2 weeks of age and homogenized with a Teflon homogenizer in PBS containing 0.25 M sucrose and protease inhibitor cocktail. The homogenate was centrifuged at 2000 ***g*** for 10 min to remove nuclei and debris, at 8000 ***g*** for 30 min to remove mitochondria and then at 100 000 ***g*** for 1 h to obtain the pellet as membrane fraction. The membrane proteins were solubilized at 4°C for 2 h with 2% SDS in PBS containing protease inhibitor cocktail and then diluted with five volumes of 2% Triton-X-100 in PBS as described in the previous study in which the binding of Kir 2.3 and PSD-95 was confirmed (Cohen *et al*. 1996). They were subsequently incubated at 4°C for 2 h with rabbit polyclonal anti-Shank3 antibody bound to protein A- and G-Sepharose beads. The immunoprecipitates were washed three times with IP buffer composed of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 10% glycerol, 0.5% Triton X-100 and protease inhibitor cocktail, eluted by boiling in SDS sample buffer, separated by SDS–polyacrylamide gel electrophoresis and analysed by immunoblot.

Immunocytochemistry

Primary cells were fixed with 2% paraformaldehyde (PFA) for 10 min at room temperature. After three washes in PBS, the cells were permeabilized and blocked with 3% goat serum/0.1% Triton X-100 in PBS for 15 min, and then incubated for 1 h at room temperature with one of the following primary antibodies in PBS containing 3% bovine serum albumin (Sigma, St Louis, MO, USA): rabbit polyclonal anti-mShank3 antibody (1.0 μg/mL), mouse monoclonal anti-PSD-95 antibody (1 : 200), mouse monoclonal anti-myc antibody (1 : 500) or rabbit polyclonal anti-EGFP antibody (1 : 500). After three washes in PBS, the cells were incubated for 1 h at room temperature with Alexa Fluor 488 goat anti-mouse IgG (H + L) (1 : 1000; Molecular Probes), Alexa Fluor 488 goat anti-rabbit IgG (H + L) (1 : 1000; Molecular Probes), Alexa Fluor 594 goat anti-mouse IgG (H + L) (1 : 1000; Molecular Probes) or Alexa Fluor 594 goat anti-rabbit IgG (H + L) (1 : 1000; Molecular Probes) in PBS containing 3% bovine serum albumin. After three washes in PBS, the cells were mounted on a glass slide with PermaFluor (Thermo Shandon, Pittsburgh, PA, USA) containing

10% FluoroGuard (Bio-Rad, Hercules, CA, USA) to inhibit photobleaching and examined with a fluorescence microscope (AX70; Olympus, Tokyo, Japan). For surface staining of EGFP-fused GluR1 subunit (EGFP-GluR1), cells were first incubated for 20 min at room temperature with rabbit polyclonal anti-EGFP antibody (1 : 500) in PBS and, after washing in PBS, they were incubated for 20 min at room temperature with Alexa Fluor 488 goat anti-rabbit IgG (H + L) (1 : 1000) in PBS and fixed with 2% PFA for 10 min. They were then permeabilized and blocked for 15 min with 3% goat serum/0.1% Triton X-100 in PBS for subsequent immunostaining.

In situ hybridization

Three cDNA fragments (620 bp, 650 bp and 1 kb) of the unique region of mShank1, mShank2 and mShank3, respectively, were obtained by PCR and subcloned into mammalian expression vector pCMV-SPORT (Gibco BRL). Sense and antisense digoxigenin-labeled probes were produced with SP6 and T7 polymerase, respectively.

Brain specimens were prepared from mice as follows. Anesthetized mice were transcardially perfused with 4% PFA in PBS. Their brains were removed from the skulls and immersed in 4% PFA at 4°C for 2 days, and then in 30% sucrose at 4°C for 2 days. After embedding in optimal cutting temperature compound (Sakura Finetechnical Co. Ltd, Tokyo, Japan), they were quickly frozen in dry ice. Frozen brain sections (14 μm thick) were cut in the sagittal plane with a cryostat (CM-3000; Leica, Nussloch, Germany) and the sections were mounted on 3-aminopropyltriethoxysilane-coated glass slides (Matsunami, Osaka, Japan) and stored at − 80°C until use.

For hybridization, the sections were treated with 4 μg/mL pepsin/0.2 N HCl for 2 min at 37°C, washed twice in PBS containing 0.1% Tween 20 and post-fixed with 4% PFA for 30 min. After washing in PBS containing 0.1% Tween 20 for 5 min, the sections were treated with 0.2% diethylpyrocarbonate in PBS containing 0.1% Tween 20 and hybridized at 65°C for 16 h with 10 μg/mL digoxigenin-labeled probe in a buffer composed of 50% formamide, 5× saline sodium citrate, 1% SDS, 50 μg/mL heparin and 50 μg/mL yeast RNA. They were then washed for 30 min at 65°C in 5× saline sodium citrate containing 50% formamide and 1% SDS, and then twice for 45 min at 65°C in 2× saline sodium citrate containing 50% formamide. After washing in TBS-T (136 mM NaCl, 2.7 mM KCl, 250 mM Tris-HCl, pH 7.5, 0.1% Tween 20) for 20 min at room temperature, they were blocked with 0.2% blocking reagent (Roche) for 40 min and incubated with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (1 : 2000; Roche) diluted in blocking solution at 4°C for 16 h. They were then washed in TBS-T for 20 min at room temperature and alkaline phosphatase-conjugated antibody was visualized by reaction with 35 μg/mL 4-nitroblue tetrazolium chloride/17.5 μg/mL 5-bromo-4-chloro-3-indolyl-phosphate (Roche) in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20 and 2 mM levamisole (Sigma) at room temperature for 1–2 nights in the dark. The color reaction was stopped with deionized water and the sections were dehydrated in a graded ethanol series and mounted with Entellan (Merck, Darmstadt, Germany).

RT-PCR

Total RNA was extracted from mouse cortex with acid guanidinium thiocyanate-phenol-chloroform (Chomczynski and Sacchi 1987) and RT-PCR was performed by using an Advantage RT for PCR kit (Clontech) according to the manufacturer's protocol. The thermocycle profile for PCR amplification was: 30 s at 95°C, 30 s at 55°C, 1 min at 72°C for 30 cycles (mShank1), 33 cycles (mShank2 and mShank3) or 22 cycles (glyceraldehyde-3-phosphate dehydrogenase used as an internal control). The PCR products were separated on a 3% agarose gel and stained with ethidium bromide. The gel images were fed into an image processor (Archiver Eclipse; Fotodyne, Hartland, WI, USA) and quantitatively analysed with NIH imaging software. The primers for PCR analysis were: for mShank1, forward GGCAGGCGTAGGAAGCTCTA and reverse CTCATCCATGTCTGGGTG; for mShank2, forward TATGATGAGCGTCCCCGGCGG and reverse ATCATCAGGGTCTAGATT; for mShank3, forward GGCCCGAAGCGGAAACTTT and reverse ACCATCCTCCTCGGGTTT; and for glyceraldehyde-3-phosphate dehydrogenase, forward GTCATCATCTCCGCCCCTTCTGC and reverse GATGCCTGCTTCACCACCTTCTTG.

Results

Screening for proteins that interact with GluR1 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor

We used a yeast two-hybrid method to identify proteins that interact with GluR1 subunit of AMPA receptor. Using the C-terminal 81-amino-acid segment of GluR1 subunit as yeast two-hybrid bait, we identified several clones from among approximately 1×10^7 mouse brain clones. Sequencing analysis revealed that one of them, clone 321, encoded the C-terminal 1305-amino-acid segment of Shank3, which includes the SH3 and PDZ domains (Fig. 1a). We then cloned full-length Shank3 cDNA from mouse brain by RT-PCR. The deduced mShank3 protein showed 98% sequence identity with rat Shank3.

Shank3 is a multidomain protein that contains ankyrin repeats, an SH3 domain, a PDZ domain, a long proline-rich region and a sterile alpha motif (Fig. 1a), and it scaffolds various proteins, including guanylate kinase-associated protein (GKAP) and Homer, in excitatory post-synapses (Naisbitt *et al*. 1999; Sheng and Kim 2000; Böckers *et al*. 2001). We initially focused on the SH3 and PDZ domains of mShank3, as they are important for interaction with proteins. We investigated the biochemical interaction between mShank3 and GluR1 subunit by GST pull-down assay using the GST-fused SH3 and PDZ domains of mShank3 (GST-mShank3/SH3-PDZ) and extracts from mouse cortical primary cells. GST-mShank3/SH3-PDZ clearly pulled down GluR1 subunit, indicating that mShank3 binds to GluR1 subunit through the SH3-PDZ domains (Fig. 1b). In addition, we examined the other synaptic molecules, GluR2 subunit of AMPA receptor and PSD-95. As shown in Fig. 1(b), GST-mShank3/SH3-PDZ pulled down PSD-95 but not GluR2 subunit.

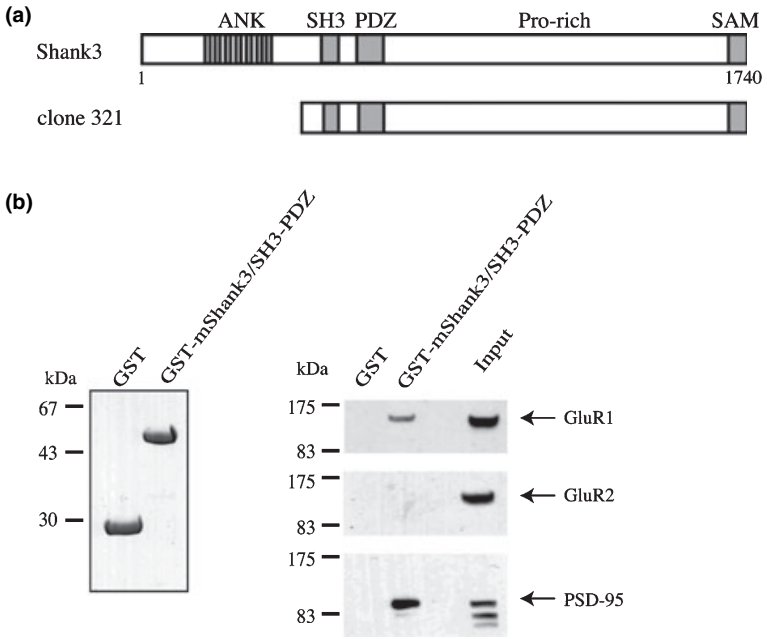

Fig. 1 Schematic structure of Shank3 and the biochemical interaction of mouse Shank3 (mShank3) with GluR1 subunit. (a) The schematic structure of Shank3 and clone 321. ANK, Ankyrin repeats 1–7; SH3, Src homology 3 domain; PDZ, post-synaptic density-95/Dlg/ZO-1 domain; SAM, sterile alpha motif. (b) Pull-down assay. The left panel shows the purified glutathione S-transferase (GST) and GST-fused mShank3/SH3-PDZ separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the right panel shows the immunoblots. GST alone or GST-fused mShank3/SH3-PDZ bound to glutathione sepharose beads was incubated with 1 mg of extract from mouse cortical primary cells cultured for 14 days. After washing, the proteins on the beads were eluted with SDS–PAGE sample buffer and immunoblotted with anti-GluR1 antibody, anti-GluR2 antibody or anti-post-synaptic density-95 (PSD-95) antibody. The input lane was loaded with 25 μg of the primary cell extract. Molecular weight standards are shown on the left.

Post-synaptic density-95/Dlg/ZO-1 domain of mouse Shank3 directly binds to the C-terminal segment of GluR1 subunit

To identify the interactive domain of mShank3, we constructed GST-mShank3/SH3, GST-mShank3/PDZ and GST-mShank3/SH3-PDZ (Fig. 2a), and performed a GST pull-down assay using cell lysates of Chinese hamster ovary cells expressing the myc-tagged C-terminal 81-amino-acid segment of GluR1 (myc-GluR1/C). As shown by SDS–polyacrylamide gel electrophoresis (Fig. 2a), the concentrations of each of the purified GST fusion proteins were almost the same. GST-mShank3/SH3-PDZ and GST-mShank3/PDZ clearly pulled down myc-GluR1/C, whereas no binding of GST-mShank3/SH3 to myc-GluR1/C was detected (Fig. 2a). These results suggest that GluR1 interacts with mShank3 via the PDZ domain.

We next investigated whether the interaction between mShank3/SH3-PDZ and GluR1/C was direct. The GST-fused myc-tagged SH3-PDZ domain of mShank3 (GST-myc-mShank3/SH3-PDZ) was expressed in *E. coli* BL21. After purification with glutathione sepharose, thrombin was reacted at 37°C for 1 h with the GST-myc-mShank3/SH3-PDZ bound to glutathione sepharose beads in PBS and purified myc-tagged mShank3/SH3-PDZ was obtained. GST-GluR1/C was produced using pGEX-5X-2 vector to protect against thrombin digestion and pull-down assays were performed. As shown in Fig. 2(b), purified myc-mShank3/SH3-PDZ bound to GST-GluR1/C, indicating that mShank3/SH3-PDZ binds directly to GluR1/C.

GluR1 subunit binds to the post-synaptic density-95/Dlg/ZO-1 domain of mouse Shank3 through its C-terminal post-synaptic density-95/Dlg/ZO-1 binding motif

As the C-terminal sequence of GluR1 subunit, -ATGL, is a typical PDZ-binding motif, -X-T/S-X-V/L/I (X represents any aa) (Songyang *et al*. 1997), we investigated whether the interaction between the PDZ domain of mShank3 and GluR1 subunit is mediated by the PDZ-binding motif of GluR1 subunit. To precisely identify the C-terminal residues involved in the binding, we prepared mutated GluR1/C carrying AAGA residues instead of ATGL and deleted GluR1/C lacking ATGL residues at its C-terminal end (Fig. 3a), and binding of these GluR1/C mutants to myc-mShank3/SH3-PDZ was almost abolished (Fig. 3b). The NR2B subunit, which binds to the PDZ domain of PSD-95, also has a PDZ-binding motif at its C-terminal end, -ESDV; however, the C-terminal segment of NR2B did not bind to myc-mShank3/SH3-PDZ (Fig. 3b). These findings demonstrate that the final four aa of GluR1 subunit are important for specific binding to the PDZ domain of mShank3.

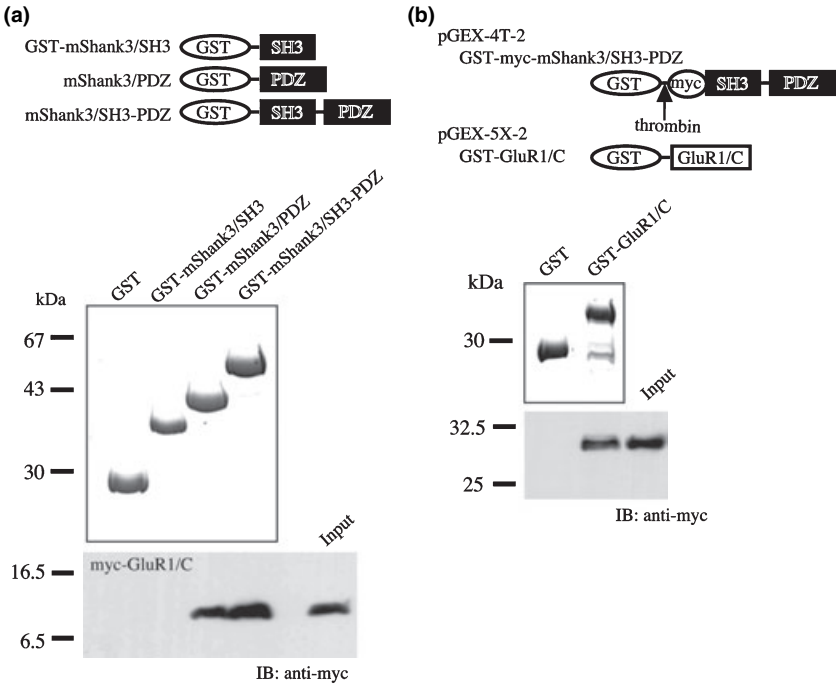

Fig. 2 Direct interaction of mouse Shank3 (mShank3) with GluR1 subunit. (a) Pull-down assay. The schematic structure of glutathione S-transferase (GST)-fused mShank3 mutants is shown at the top. The upper panel shows the purified GST and GST-fused mShank3 proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the lower panels show the immunoblot. GST alone or each of the GST-fusion proteins bound to glutathione sepharose beads was incubated with 400 μg of extract from Chinese hamster ovary (CHO) cells transfected with myc-tagged GluR1/C. After washing, the proteins on the beads were eluted with SDS–PAGE sample buffer and immunoblotted with anti-myc antibody. The input lane was loaded with 30 μg of the CHO cells extract. (b) Pull-down assay. The schematic structure of GST-fused mShank3/SH3-PDZ and GluR1/C is shown at the top. The upper panel shows the purified GST and GST-fused GluR1/C proteins separated by SDS–PAGE and the lower panels show the immunoblots. GST alone or GST-fused GluR1/C bound to glutathione sepharose beads was incubated with purified myc-Shank3/SH3-PDZ. After washing, the proteins on the beads were eluted with SDS–PAGE sample buffer and immunoblotted with anti-myc antibody. The input lane was loaded with 5% of the purified myc-Shank/SH3-PDZ used for the pull-down assay. Molecular weight standards are shown on the left. PDZ, post-synaptic density-95/Dlg/ZO-1; SH3, Src homology 3; IB, immunoblot.

We then investigated the interaction between mShank3 and full-length GluR1 subunit by immunoprecipitation experiments. We constructed myc-tagged mShank3 (myc-mShank3) and the four deletion mutants as shown in Fig. 4(a), and expressed them together with EGFP-GluR1 in COS7 cells. Anti-EGFP antibody coimmunoprecipitated myc-mShank3, myc-mShank3/PDZ + C, myc-mShank3/SH3-PDZ and myc-mShank3/PDZ from cell lysates of COS7 cells expressing EGFP-GluR1 and myc-mShank3 or deletion mutants but not myc-mShank3/C (Fig. 4b). No signals were observed with immunoprecipitates obtained with normal IgG. These results indicate that the PDZ domain of mShank3 is indispensable to the interaction with GluR1 subunit.

Mouse Shank3 is expressed in the spine of the cortical neurons and interacts with GluR1 α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor

To study native mShank3 protein, rabbit polyclonal antibodies were raised against the GST-fused 342-aa segment of mShank3 (aa 1016–1357). To confirm the specificity of the anti-mShank3 antibody, we transiently transfected COS cells with myc-mShank3 to allow the use of anti-myc antibody as a positive control. As shown in Fig. 5(a), the immunoreactive band for anti-mShank3 antibody (240 kDa) was identical to that for anti-myc antibody and no bands were detected in the COS7 cells transfected with myc vector, indicating that the anti-mShank3 antibody specifically detected mShank3 protein. mShank3-immunoreactive bands were observed in mouse cortical lysates and disappeared upon coincubation with antigen GST-fused mShank3 segment but not with GST alone (data not shown). We also confirmed the presence of mShank3 in the synaptosomal fraction as well as GluR1 subunit and PSD-95 (Fig. 5b).

To verify the interaction between mShank3 and GluR1 AMPA receptor *in vivo*, we performed a coimmunoprecipitation assay with membrane fraction prepared from mouse cortex using anti-Shank3 antibody (Fig. 5c). Shank3 antibody efficiently immunoprecipitated endogenous mShank3

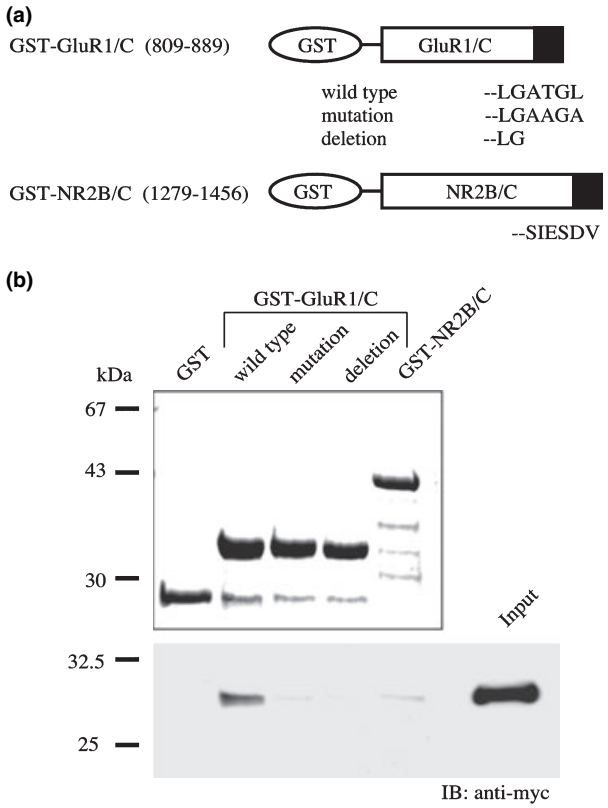

Fig. 3 Role of the post-synaptic density-95/Dlg/ZO-1 (PDZ) binding motif of GluR1 in the interaction with mouse Shank3 (mShank3)/SH3-PDZ. (a) The schematic description of glutathione S-transferase (GST)-fused GluR1/C and C-terminal segment of NMDA receptor 2B (NR2B/C) proteins. (b) Pull-down assay. The upper panel shows the purified GST and GST-fused proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the lower panel shows the immunoblots. GST alone or each of the GST-fusion proteins bound to glutathione sepharose beads was incubated with 200 μg of extract from Chinese hamster ovary (CHO) cells transfected with myc-tagged mShank3/SH3-PDZ. After washing, the proteins on the beads were eluted with SDS–PAGE sample buffer and immunoblotted with anti-myc antibody. The input lane was loaded with 10 μg of the CHO cells extract. Molecular weight standards are shown on the left. SH3, Src homology 3; IB, immunoblot.

protein and coprecipitated GluR1 subunit, while normal IgG immunoprecipitated neither mShank3 nor GluR1 subunit. This finding suggests that mShank3 interacts with GluR1 AMPA receptor in mouse cortex.

We next used this antibody to examine cortical primary neurons for expression of mShank3 and the pattern of mShank3 immunoreactivity was found to be strikingly punctate. To clarify the distribution of mShank3, EGFP was transfected into neurons and its signal was superimposed on the mShank3 staining. As shown in Fig. 5(d), judging from their morphology mShank3 was mainly expressed in the spines. Moreover, the punctate signals of mShank3 closely coincided with those of PSD-95 (Fig. 5e), indicating that mShank3 was colocalized with PSD-95 in the spines.

Mouse Shank3 interacts with membrane-surface GluR1 subunit in the spines

As the GST pull-down and immunoprecipitation experiments demonstrated the biochemical interaction of mShank3 with GluR1 subunit, we investigated the interaction of these proteins in neurons. We constructed a plasmid, EGFP-GluR1, in which EGFP had been fused with the extracellular region of GluR1, and transfected it into cortical neurons cultured for 12 days. When we examined them 2 days later, the EGFP fluorescence perfectly matched the GluR1 staining (data not shown). Under permeant conditions, in which proteins located on both the intracellular and membrane surface are detected, immunostaining with anti-EGFP antibody revealed expression of EGFP-GluR1 in the cell body, neuritis and spines (Fig. 5f, i). By contrast, the EGFP-GluR1 staining was punctate when the anti-EGFP antibody was applied to a living culture under non-permeant conditions to detect EGFP-GluR1 delivered onto the membrane surface (Fig. 5f, ii), indicating that the membrane-surface EGFP-GluR1 forms clusters. Subsequent staining of the same neurons for PSD-95 under permeant conditions showed that the punctate EGFP-GluR1 staining coincided with the PSD-95 staining (Fig. 5f, iii), implying that the membrane-surface EGFP-GluR1 was distributed in the spines. This finding is consistent with previous reports that almost all membrane-surface GluR1 clusters are synaptic (Shi *et al*. 1999). At 2 days after cotransfection with myc-mShank3 and EGFP-GluR1, EGFP-GluR1 staining was performed with anti-EGFP antibody under non-permeant conditions and followed by myc-mShank3 staining with anti-myc antibody under permeant conditions. Many punctate myc-mShank3 staining signals were colocalized with those of EGFP-GluR1 staining (EGFP-GluR1/myc-mShank3 colocalization rate $80.0 \pm 3.8\%$; $n = 5$) (Fig. 5g). At 1 week after cotransfection, the mShank3 staining was as strikingly punctate as native mShank3 staining and merged well with the EGFP-GluR1 staining in the spines (Fig. 5h). These results suggest that mShank3 interacts with membrane-surface GluR1 subunit.

All members of the mouse Shank family are expressed in the cortex and may interact with GluR1 subunit

Previous studies have shown that all three members of the Shank family are expressed in rat cortex. We investigated the expression and distribution of Shank mRNAs in the mouse cortex by *in situ* hybridization during post-natal development on post-natal days 1 and 15 and at 8 weeks of age. Antisense cRNA probes transcribed from each mShank gene specifically recognized particular mShank transcripts whereas sense cRNA probes did not (data not shown). As shown in Fig. 6(a), the hybridization signals for mShank1 and mShank2 mRNAs were detected at all post-natal stages

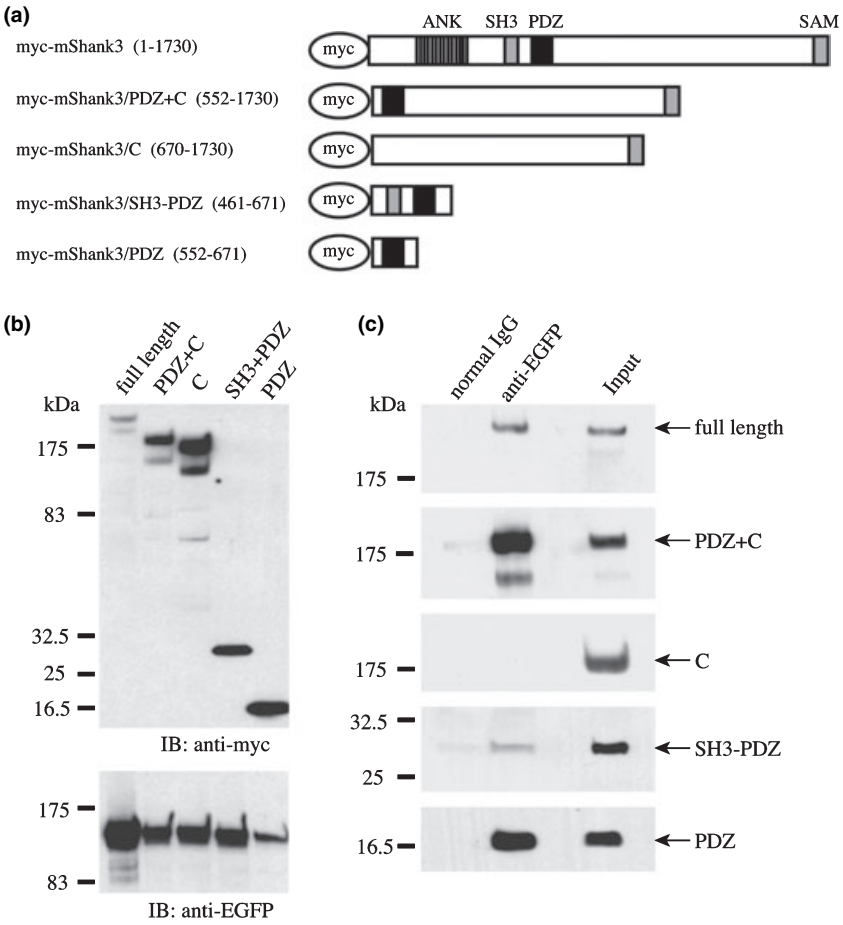

Fig. 4 GluR1 binds to the post-synaptic density-95/Dlg/ZO-1 (PDZ) domain of mouse Shank3 (mShank3). (a) Schematic structure of myc-tagged mShank3 mutants. (b) Immunoblot analysis. COS7 cells were transfected with each of the myc-tagged mShank3 mutants together with enhanced green fluorescent protein-fused GluR1 subunit (EGFP-GluR1). Lysates of transfected COS7 cells (5 μg of proteins) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with anti-myc antibody (upper panel) and with anti-enhanced green fluorescent protein (EGFP) antibody (lower panel). (c) Immunoprecipitation assay. Normal rabbit IgG or anti-EGFP antibody bound to Protein A- and G-Sepharose beads was incubated with 300 μg of extract from COS7 cells cotransfected with EGFP-GluR1 and myc-tagged mShank3 mutant. After washing, the proteins on the beads were eluted with SDS–PAGE sample buffer and immunoblotted with anti-myc antibody. Molecular weight standards are shown on the left. ANK, Ankyrin repeats 1–7; SH3, Src homology 3 domain; SAM, sterile alpha motif; IB, immunoblot.

measured; however, expression of mShank3 mRNA was clearly seen at post-natal days 1 and 15 and the signals at 8 weeks of age were very faint. These results were confirmed by RT-PCT (Fig. 6b) and immunoblot analysis (Fig. 6c). On the other hand, the distribution of the mRNAs of all members of the Shank family in the cortex was not significantly different. Thus, it appears that mShank1, mShank2 and mShank3 are expressed in the same neurons in cortex. Finally, we investigated whether mShank1 and mShank2 interact with GluR1 subunit, because the SH3-PDZ domain is well conserved among all members of the Shank family. As shown in Fig. 6(d), mShank1/SH3-PDZ and mShank2/SH3-PDZ also bound GluR1/C, the same as mShank3/SH3-PDZ.

Discussion

In this study we used a yeast two-hybrid screening system and identified a synaptic molecule that interacts with GluR1 subunit of AMPA receptors. The molecule, named Shank3/ProSAP2, is a multidomain protein localized in PSDs which links cell-surface receptors, including various types of GluRs, to the actin-based cytoskeleton (Lim *et al*. 1999; Naisbitt *et al*. 1999; Sheng and Kim 2000; Böckers *et al*. 2001, 2002). The most striking finding in our study was that GluR1 subunit is capable of directly binding to the PDZ domain of the Shank3 via its C-terminal PDZ-binding motif (four aa sequence, -ATGL), while GluR2 subunit indirectly binds to the SH3 domain of the Shank protein mediated by glutamate receptor-interacting protein (GRIP) (Sheng and Kim 2000). The PDZ domain is a stretch of 80–100 aa residues which plays an important role in protein–protein interaction (Songyang *et al*. 1997). Thus far it has been reported that synaptic proteins containing a type I PDZ-binding motif (the C-terminal four-amino-acid sequence -X-T/S-X-V/L/I, where X represents any aa), such as GKAP/SAP90/PSD-95-associated protein (SAPAP) (-QTRL), the somatostatin receptor 2 (-QTSI) and the calcium-independent α-latrotoxin receptor (-VTSL), bind to the PDZ domain of Shank (Böckers *et al*. 2002) and thus, the PDZ domain of Shank is thought to be a type I PDZ. As expected, no binding of the GluR2 subunit of AMPA receptors was observed in our study, as GluR2 subunit possesses a type II, not type I, PDZ-binding motif. Interestingly, however, little or no binding of the NR2B subunit was observed, even though NR2B subunit has a type I PDZ-binding motif

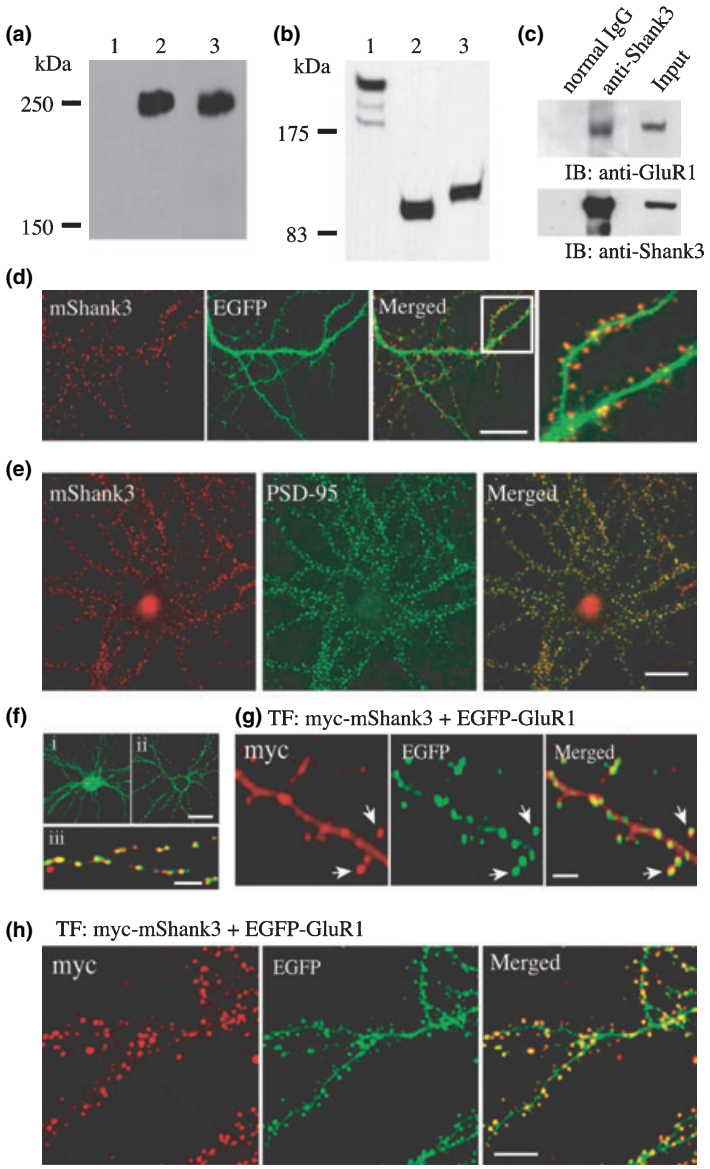

Fig. 5 Expression and distribution of mouse Shank3 (mShank3) in cortical neurons. (a) Immunoblot analysis. Lysates of COS7 cells (5 μg of proteins) transfected with myc (lane 1) or myc-mShank3 (lanes 2 and 3) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with anti-mShank3 antibody (lanes 1 and 2) and with anti-myc antibody (lane 3). (b) Immunoblot analysis. Synaptosomal proteins (30 μg) were separated by SDS–PAGE and immunoblotted with anti-mShank3 antibody (lane 1), anti-post-synaptic density-95 (PSD-95) antibody (lane 2) or anti-GluR1 antibody (lane 3). Molecular weight standards are shown on the left. (c) Immunoprecipitation assay. Normal rabbit IgG or anti-Shank3 antibody bound to Protein A- and G-Sepharose beads was incubated with 1 mg of membrane fraction from mouse cortex. After washing, the proteins on the beads were eluted with SDS–PAGE sample buffer and immunoblotted with anti-GluR1 antibody. The input lane was loaded with 50 μg of membrane fraction. (d) Cortical neurons cultured for 12 days were transfected with enhanced green fluorescent protein (EGFP). At 2 days later, the neurons were fixed and stained with anti-mShank3 antibody. An enlargement of the square field indicated is shown in the right panel. (e) Cortical neurons were cultured for 18 days and double-stained with anti-mShank3 antibody (red) and anti-PSD-95 antibody (green). (f) Cortical neurons cultured for 12 days were transfected with EGFP-fused GluR1 subunit (EGFP-GluR1). After 2 days, the neurons were stained with anti-EGFP antibody under permeant (i) or non-permeant (ii) conditions. The immunoreactive signals for anti-EGFP antibody (green) under non-permeant conditions were matched with those for anti-PSD-95 antibody (red) (iii). (g) Cortical neurons cultured for 12 days were transfected with myc-mShank3 and EGFP-GluR1. At 2 days later, the neurons were stained with anti-EGFP antibody (green) under non-permeant conditions and with anti-myc antibody (red) after fixation. The arrows point to examples of overlapping signals. (h) Cortical neurons cultured for 12 days were transfected with myc-mShank3 and EGFP-GluR1. At 7 days later, the neurons were stained with anti-EGFP antibody (green) and anti-myc antibody (red). Scale bars: 30 μm (d) and 15 μm (e, panels i and ii in f), 10 μm (h) and 5 μm (panel iii in f and g). IB, immunoblot; TF, transfection.

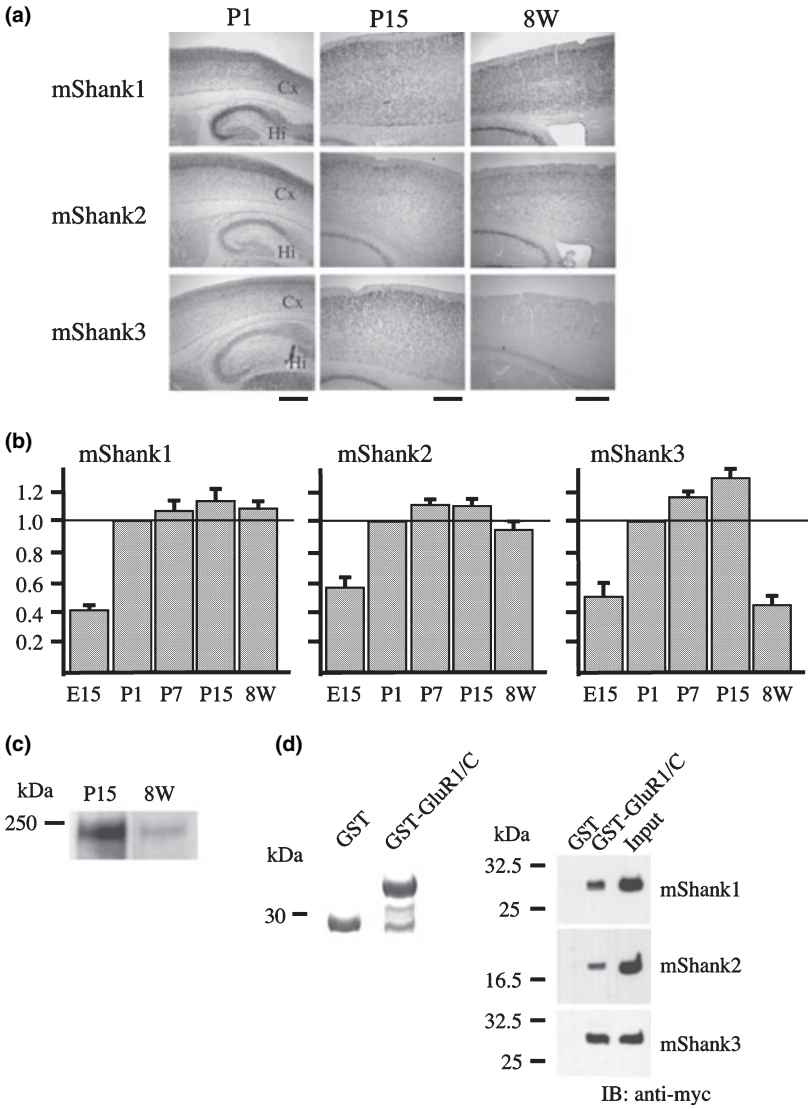

Fig. 6 Expression of mouse Shank (mShank) mRNAs in developing mouse cortex and interaction of mShank proteins with GluR1 subunit. (a) *In situ* hybridization analysis. Distribution of mShank mRNAs in the post-natal mouse cortex. Cx, cortex; Hi, hippocampus. Scale bar, 500 μm. (b) RT-PCR analysis. Amounts of total RNA used for the PCR were normalized to glyceraldehyde-3-phosphate dehydrogenase. The relative strength of the band signals was measured with NIH Imaging software and the ratio was calculated by dividing the value at each stage by the value at post-natal day (P)1. (c) Immunoblot analysis. Lysates of cortex (30 μg) prepared from P15 or 8-week-old mice were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with anti-mShank3 antibody. (d) Pull-down assay. The left panel shows the purified glutathione S-transferase (GST) and GST-fused GluR1/C separated by SDS–PAGE and the right panels show the immunoblots. GST alone or GST-fused GluR1/C bound to glutathione sepharose beads was incubated with 300 μg of extract from Chinese hamster ovary (CHO) cells transfected with myc-tagged mShank/SH3-PDZ. After washing, the proteins on the beads were eluted with SDS–PAGE sample buffer and immunoblotted with anti-myc antibody. The input lane was loaded with 10 μg of the CHO cell extract. Molecular weight standards are shown on the left. PDZ, post-synaptic density-95/Dlg/ZO-1; SH3, Src homology 3 domain; 8W, 8 weeks of age; IB, immunoblot.

(-ESDV) by which NR2B subunit binds to the type I PDZ domain of PSD-95. The above findings suggest that the binding partners of Shank via the PDZ domain are highly selective molecules. A recent analysis of the crystal structure of the PDZ domain of the Shank–peptide ligand (EAQTRL) complex has revealed that the carboxylate binding loop formed by the second β-strand within the PDZ domain is critical for binding to the peptide ligand (Im *et al*. 2003). The aa sequence of the second β-strand is identical in all three members of the Shank family. The recent structural studies support our result showing that GluR1/C not only bound to Shank3 but also to Shank1 and Shank2.

The biochemical interaction of mShank3 with GluR1 subunit was complemented by coimmunoprecipitation assay with membrane fraction prepared from mouse cortex using anti-Shank3 antibody and by transfection experiments showing colocalization of Shank3 with GluR1 subunit in the spines of mouse cultured cortical neurons. When myc-mShank3 was expressed with EGFP-GluR1 in maturing cortical neurons, many myc-mShank3 clusters colocalized with cell-surface EGFP-GluR1. Shi *et al*. (1999) reported that cell-surface recombinant EGFP-GluR1, the same construct as our plasmid, expressed in hippocampal neurons displayed a punctate distribution that colocalized with both surface labeling of endogenous GluR2 subunit and with a pre-synaptic marker, synapsin. Therefore, our results suggest that myc-mShank3 colocalizes with functional AMPA receptors containing GluR1 subunit at synaptic sites.

Recent cumulative evidence has shown that the clustering of AMPA receptors at the post-synaptic membrane is critical for synaptic maturation and plasticity. As trafficking of GluR1 subunit to synapses was diminished by mutating the PDZ interacting region of GluR1 subunit, the trafficking may be mediated by a family of PDZ-domain-containing proteins (Hayashi *et al*. 2000; Shi *et al*. 2001). However, the binding partner of GluR1 subunit remains to be determined. One candidate is SAP-97, a type I PDZ protein homologous to PSD-95, and the biochemical interaction of SAP-97 with GluR1 subunit has actually been demonstrated (Leonard *et al*. 1998). During development, however, expression of SAP-97 is distributed more in the somatic region than in the dendrites, although it is found along dendrites, presumably at synaptic sites, in mature neurons (Valtschanoff *et al*. 2000). Thus, SAP-97 is thought to be associated with intracellular AMPA receptors, including GluR1 subunit, and to be involved in the early secretory pathway, endoplasmic reticulum/cis-Golgi pathway, of AMPA receptors trafficking during development (Sans *et al*. 2001). Shank3, on the other hand, is expressed in post-synaptic sites where the functional AMPA receptors are localized. Roussignol *et al*. (2005) recently showed that transfection of Shank3 into cerebellar granule cells increased the AMPA component of mEPSCs. Thus, Shank3 is an intriguing molecule that interacts with AMPA receptors and regulates their function at post-synaptic sites. The mechanism of activity-depending AMPA receptor trafficking to synapses involving Shank3 needs to be more thoroughly elucidated.

In situ hybridization and RT-PCR analyses have shown that Shank3 mRNA expression in mouse cortex increases after birth and gradually decreases during later development, consistent with previous studies on rat brain (Böckers *et al*. 2001, 2004). Thus, Shank3 may play an important role in neural functions, such as synaptogenesis and synapse maturation, at an early stage of post-natal development rather in adulthood. However, Shank1 and Shank2 mRNAs are also expressed in cortical neurons after birth and their expression levels are sustained into adulthood. Furthermore, the expression patterns of all three Shank mRNAs in the cortex do not significantly differ. The members of the Shank family share essentially the same domain structures, such as the SH3 and PDZ domains, and lower homologous regions, such as the proline-rich region. Thus, it has been thought that the individual members of the Shank family may have unique functions in addition to their common function as scaffold proteins but little is known about their other functions. Further study, especially in Shank knock-out and transgenic mouse, should provide useful insights into the functions of the individual members of the Shank family in regard to synaptogenesis, synapse maturation and subsequent formation of neural networks. Interestingly, recent genetic analyses of the 22q13.3 deletion syndrome, which is characterized by global developmental delay, absent or severely delayed speech and hypotonia, have suggested that the haplo-insufficiency for Shank3 is probably the cause of the pathological state as the Shank3 gene is located on chromosome 22q13.3 (Bonaglia *et al*. 2001; Wilson *et al*. 2003). Thus, clinical studies may lead to elucidation of the particular function of Shank3 and clarification of the specific function of Shank3 may lead to novel strategies for the treatment of this syndrome.

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