Sema4C, a Transmembrane Semaphorin, Interacts with a Post-synaptic Density Protein, PSD-95*

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Semaphorins are known to act as chemorepulsive molecules that guide axons during neural development. Sema4C, a group 4 semaphorin, is a transmembrane semaphorin of unknown function. The cytoplasmic domain of Sema4C contains a proline-rich region that may interact with some signaling proteins. In this study, we demonstrate that Sema4C is enriched in the adult mouse brain and associated with PSD-95 isoforms containing PDZ (PSD-95/DLG/ZO-1) domains, such as PSD-95/SAP90, PSD-93/chapsin110, and SAP97/DLG-1, which are concentrated in the post-synaptic density of the brain. In the neocortex, S4C is enriched in the synaptic vesicle fraction and Triton X-100 insoluble post-synaptic density fraction. Immunostaining for Sema4C overlaps that for PSD-95 in superficial layers I-IV of the neocortex. In neocortical culture, S4C is colocalized with PSD-95 in neurons, with a dot-like pattern along the neurites. Sema4C thus may function in the cortical neurons as a bi-directional transmembrane ligand through interacting with PSD-95.

Semaphorins are a large family of structurally distinct, secreted and transmembrane glycoproteins characterized by the presence of a conserved sema domain of about 500 amino acids (1, 2). Several secreted semaphorins (group 3 semaphorins in vertebrates), such as Sema3A, E, and F (3) induce the collapse of some populations of growth cones in cultured neurons and thus can function as inhibitory and repulsive cues in axonal guidance (1, 4–8). Evidence implicating secreted semaphorins in chemorepulsion came initially from the finding that chick Sema3A can cause the collapse of the chick dorsal root ganglion growth cone (4). Later studies have shown that Sema3A also acts as a chemorepellent for a variety of sensory and motor axons in mammals (5, 8). On the other hand, there is only limited evidence concerning the roles of transmembrane semaphorins in the nervous system. Of group 4 semaphorins, Sema4C (S4C, previously called M-SemaF) is especially expressed in neuronal tissues of embryos, suggesting some function of S4C in the neural tissues, such as directing axon pathfinding, axonal target selection, or synapse formation, although no evidence for such functions has been provided so far (9). Another group 4 semaphorin, Sema4D (previously called M-SemaG or hCD100; Refs. 10 and 11), regulates B lymphocyte proliferation and differentiation, and associates with a serine kinase through its cytoplasmic domain (11). Sema6B forms a complex with c-Src through a proline-rich intracellular domain (12). The cytoplasmic domain of S4C contains a proline-rich region that may interact with Src homology 3 (SH3) domains as well as cytoskeletal proteins, suggesting that the cytoplasmic domain of S4C may interact with some signaling-related proteins. To explore possible functions and the intracellular signaling of S4C, we investigated in which tissues and at which developmental stages S4C is expressed and then attempted to screen proteins that interact with S4C intracellularly. Based on yeast two-hybrid screening, we identified several PSD-95 isoforms as proteins interacting with S4C.

Recently, a number of proteins have been shown to contain PDZ domains, which were originally recognized as repeats of about 90 amino acids in PSD-95/SAP90 and named after three proteins containing such repeats, PSD-95, SAP90, and SAP97. PDZ domains have been shown to mediate the concentration of neurotransmitter receptors and ion channels at the synapses of neurons, as well as the asymmetric distribution of receptors in epithelial cells or their localization at tight junctions (19–21). Moreover, some of these PDZ proteins also serve as scaffolds for the assembly of multiprotein signaling complexes at the membrane (13, 22–25). They mediate protein-protein interactions, generally recognizing short C-terminal motifs, originally described as the (T/S)XV motif (24). Interestingly, one such consensus PDZ binding motif, SSV, is also seen in the C terminus of S4C (9). In the present study we investigated whether PSD-95 interacts with S4C in the central nervous system.

**EXPERIMENTAL PROCEDURES**

Antisera—Antibodies to S4C were raised in rabbits against the product of pGEX5X1 S4Ccd, which is the C-terminal cytoplasmic domain of S4C. Mouse monoclonal antibodies to PSD-95 were purchased from Upstate Biotechnology (Lake Placid, NY) and Transduction Laboratories (Lexington, KY). Mouse monoclonal antibody to HA was obtained from Roche Molecular Biochemicals (Tokyo) and mouse monoclonal antibody to Myc (9E10) was purchased from Transduction Laboratories (Lexington, KY). Mouse monoclonal antibody to HA was obtained from Roche Molecular Biochemicals (Tokyo) and mouse monoclonal antibody to Myc (9E10) was purchased from American Type Culture Collection. Alkaline phosphatase-conjugated goat anti-rabbit IgG

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1 The abbreviations used are: SH, Src homology; PDZ, phosphorylated-sulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; GST, glutathione S-transferase; PSD, post-synaptic density; NMDA, N-methyl-D-aspartate; MAGUK, membrane-associated guanylate kinase.
Spleen; lane 4 tissues of mouse. The homogenates (20 μg of protein/lane) of various tissues were subjected to SDS-PAGE followed by immunoblot analysis using anti-S4C antibodies (1:2,000). Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, muscle; lane 7, kidney; lane 8, placenta. B, immunoblot analysis of S4C in the brain during development. 20-μg protein aliquots of brain homogenates from mice at embryonic day 14 (E14), postnatal days 1 (P1), 7 (P7), and 14 (P14), and 8 weeks (Ad) were analyzed by immunoblotting with anti-S4C antibodies. Arrows in A and B indicate S4C protein of about 100 kDa.

**Immunoblot Analysis**—Embryos and postnatal and adult ddY mice were purchased from SLC (Hamamatsu, Japan). Tissues were homogenized with 20 mM Hepes/NaOH, pH 7.4, 1 mM EDTA, 100 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The protein concentration was measured by the method of Coomassie protein assay reagent (Pierce). Twenty μg of each protein homogenate was fractionated by SDS-PAGE followed by immunoblotting with the indicated antibodies. Primary antisera to S4C, PSD-95, HA, and Myc were used at a 1:2,000 dilution. Immunoreactive proteins were visualized using ECL-Star detection reagent (Amersham Pharmacia Biotech) using the corresponding alkaline phosphatase-conjugated anti-mouse IgG (Santa Cruz) or alkaline phosphatase-conjugated rabbit anti-mouse IgG (New England Bio Labs, Beverly, MA) was used as the second antibody for immunoblot detection.

**Yeast Two-hybrid Screening**—The predicted cytoplasmic domain of the C-terminal amino acids of S4C (S4Ccd) (residues 663–834) was used to screen a rat brain cDNA library using the yeast two-hybrid system (CLONTECH). The yeast two-hybrid libraries in pVP16 were constructed from adult rat brain cDNA (26) were screened using anti-S4C antibodies (1:2000).

**Constructions of Expression Vectors**—Prokaryote and eukaryote expression vectors were constructed in pGEX5X1 (Amersham Pharmacia Biotech), pGBT9 (CLONTECH), pVP16, pCMV, pCMV Myc, and pEF HA (Invitrogen) using standard molecular biology methods (27). The expression vectors were constructed in pGEX5X1 (Amersham Pharmacia Biotech), pGBT9 (CLONTECH), pVP16, pCMV, pCMV Myc, and pEF HA (Invitrogen) using standard molecular biology methods (27).

**In Vitro Binding**—Glutathione S-transferase (GST), GST-fused wild-type S4Ccd and GST-fused C-terminus domain of GST-S4C cd proteins were expressed in Escherichia coli and purified as previously described (26). COS cells were transfected by the DEAE-dextran method with pCMV Myc S4Ccd (PSD-95-1), and various mutant plasmids of S4Ccd inserted into pCMV or pCMV Myc (PSD-95-2, -3, -4, and -5) were prepared using polymerase chain reaction fragments which were inserted into the EcoRI/SalI sites of respective vectors. The prey vectors of pVP16 rat-PSD95-8 (PDZ1+PDZ2+PDZ3), pVP16 SAP102 (PDZ1+PDZ2+PDZ3), pVP16 rat-PSD95 (PDZ1+PDZ2+PDZ3), pVP16 rat-PSD95 (PDZ1+PDZ2+PDZ3), pVP16 mouse-ZO-1 (PDZ1+PDZ2+PDZ3), and pVP16 Drosophila DLG-A (PDZ1+PDZ2+PDZ3) (28) were used for the β-galactosidase assays of yeast transformations with the bait vector pGBTN9-S4Ccd.

**Subcellular Fractions**—Synaptosomal membrane and PSD (PSD1 and 2) fractions were prepared from the cerebral cortex of adult mice as previously described (26, 30) with minor modifications. Briefly, the cerebral cortices from eight adult mice were homogenized in 36 ml of homogenization buffer (4 mM Hepes, pH 7.4, 1 mM EDTA, 0.32 M sucrose, and 2 mM PMSF) and centrifuged at 1,000 g for 15 min. Five hundred μl of the supernatant (original) of GST, GST-S4Ccd, and GST-S4C cd proteins were fixed on 20 μl of glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). After the beads were washed with the extract buffer (flow through), proteins on the beads were subjected to the immunoblot analysis.

**Filter Assay**—The PDZ domains of each MAGUK member were cotransformed with the bait vector pGBTN9-S4Ccd.

**PSD-95 Interacts with Intracellular Domain of Sema4C**

**Fig. 1. Tissue distribution and developmentally regulated expression of S4C protein.** A, immunoblot analysis of S4C in various tissues of mouse. The homogenates (20 μg of protein/lane) of various tissues were subjected to SDS-PAGE followed by immunoblot analysis using anti-S4C antibodies (1:2,000). Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, muscle; lane 7, kidney; lane 8, placenta. B, immunoblot analysis of S4C in the brain during development. 20-μg protein aliquots of brain homogenates from mice at embryonic day 14 (E14), postnatal days 1 (P1), 7 (P7), and 14 (P14), and 8 weeks (Ad) were analyzed by immunoblotting with anti-S4C antibodies. Arrows in A and B indicate S4C protein of about 100 kDa.

**Fig. 2. Yeast cells were cotransformed with a bait vector pGBTN9 S4Ccd and a prey vector pVP16 containing the three PDZ domains of PSD-95/SAP90, SAP102, PSD-93/chapsin-110, ZO-1, or DLG-A. Cotransformants were selected on synthetic agar plates lacking leucine, tryptophan, and histidine. The interactions between S4Ccd and the PDZ domains of each MAGUK member were observed by β-galactosidase activity using colony lift β-galactosidase filter assay according to the manufacturer’s instructions.**

**Notes:**

- The tissue distribution and developmental regulation of S4C protein were studied by immunoblot analysis.
- Yeast two-hybrid screening was used to identify interacting partners for S4C.
- The interactions were confirmed using β-galactosidase assays.
- The PDZ domains of MAGUK proteins were used as prey vectors.
- The interactions were observed using colony lift β-galactosidase filter assay.

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**References:**

- Crouch, D. L., and D. E. Keating. 1996. J. Biol. Chem. 271:4797-4803.
- Kato, M., T. Ohno, and Y. Ohno. 2000. J. Biol. Chem. 275:14,110-14,116.
- Takahashi, M., T. Nishizawa, T. Endo, and M. Furuya. 2001. J. Biol. Chem. 276:26,091-26,096.
supernatant (S) and the PSD pellet (PSD2). The S3 fraction was centriﬁaged at 165,000 \( \times \) g for 2 h, and the resulting pellet (synaptic vesicle fraction) contained synaptic vesicle proteins. 10 \( \mu \)g of protein from each fraction was separated by SDS-PAGE, and immunoblot analysis was performed with the indicated antibodies.

**Fig. 3.** In vitro analysis showing the interaction of PSD-95 with the C terminus of S4C. Three amino acids of the C terminus of S4C, SSV, were substituted by three alanines (S4C*cd). The extracts from COS cells transfectected with pCMV Myc PSD-95 were incubated with GST and GST fusion proteins immobilized on glutathione-Sepharose 4B beads. The original cell extracts (OR), flow through fraction (FT), and beads pellet (PT) were subjected to SDS-PAGE followed by immunoblot analysis with anti-Myc antibody.

**Fig. 4.** In vitro analysis showing the interaction of PDZ domains of PSD-95 with S4C. A, schematic representation of pCMV constructs of wild type (1) and various deletion mutants of PSD-95 (2-5). B, immunoblotting (IB) with anti-PSD-95 or anti-Myc antibodies. Wild type (1) and various mutants of PSD-95 (2-5) were expressed in cells and present in the respective cell lysates (arrows). Many bands of smaller in size than the bands indicated by arrows appears to be degraded PSD-95 proteins. C, interaction with the S4Ccd immobilized on glutathione beads. GST-fused S4Ccd incubated with MycPSD95–1 (lane 1), PSD95–2 (lane 2), PSD95–3 (lane 3), PSD95–4 (lane 4), or MycPSD95–5 (lane 5), was immunoblotted with anti-PSD-95 (lanes 1-4) or anti-Myc antibodies (lane 5). Wild type (lane 1) and deletion mutants of PSD95–2, 3, 4 (lanes 2–4) bound to S4Ccd (indicated by arrows), but mutant PSD-95–5 lacking PDZ1 and PDZ2 (indicated by an arrow) did not (lane 5). Degraded proteins bound to S4C are also observed in lanes 1–4.

Coimmunoprecipitation—HEK 293 cells were cotransfected with pCMV Myc PSD-95-1 and pEF HA S4C or pEF HA S4C* by the calcium phosphate method as previously described (29). The supernatant from the cell extracts prepared by lysis with 20 mM Hepes/NaOH, pH 8.0, 150 mM NaCl, and 1% (w/v) Nonidet P-40, and 1 mM EDTA was diluted with two volumes of 20 mM Hepes/NaOH, pH 8.0, containing 150 mM NaCl and 1 mM EDTA and immunoprecipitated with anti-Myc or anti-HA antibody. The immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting with either anti-S4C or anti-PSD-95 antibody.

The synaptosome fraction prepared from the cerebral cortices of
eight animals was homogenized in 16 ml of 20 mM Hepes/NaOH, pH 7.4, containing 6 mM urea, 1% Triton X-100, and 1 mM PMSF and centrifuged at 100,000 g for 30 min. The supernatant was dialyzed against 3 liters of 20 mM Hepes/NaOH, pH 7.4 and 100 mM NaCl with one exchange of dialysis buffer and centrifuged at 100,000 × g for 30 min to remove the insoluble proteins. 4-ml aliquots of the supernatant (input) were incubated with the monoclonal anti-PSD95 antibody (Upstate Biotechnology) or the normal mouse serum for 3 h, and immune complexes were collected on 20 μl of protein A-Sepharose CL4B. After washing with 20 mM Hepes/NaOH, pH 7.4, 33 mM NaCl and 0.33% Triton X-100, the protein A beads were subjected to immunoblot analysis with anti-PSD-95 antibody (Transduction Laboratories) or the antibodies to S4C.

Immunohistochemistry—HEK 293 cells transfected with pCMV Myc PSRD-95 and pEF HA S4C were stained with the indirect immunofluorescence method. Cells grown on poly-L-lysine-coated coverslips were reacted with monoclonal antibody to Myc and polyclonal antibodies to S4C, nd then incubated with Cy2- or Cy3-labeled second antibodies to rabbit or mouse IgGs (Amersham Pharmacia Biotech). Cortical neurons cultured on the poly-L-lysine-coated coverslips (31) were also stained with the indirect immunofluorescence method using a laser scanning confocal system (Radiance 2000, Bio-Rad). Immunohistochemistry was performed on adult mouse brain sections by the ABC method as previously described (32). Cryostat sections prepared from 4% paraformaldehyde-fixed animals were reacted with the antibodies to S4C either with or without antigen or with the antibody to PSD-95 and then incubated with biotinylated second antibodies to rabbit or mouse IgGs (Amersham Pharmacia Biotech). After reaction with avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame CA), immunoreactivity was visualized with diaminobenzidine (Dotite, Japan).

RESULTS

Tissue and Developmental Stage-specific Expression of S4C Protein—As semaphorins have primarily been found to act as axon guidance molecules during development of the nervous system, it is important to clarify in which tissues and at which developmental stages S4C is expressed. We analyzed the protein expression in homogenates from brain by immunoblot analysis using the protein A-Sepharose generated to the S4Ccd (Fig. 1). Next we analyzed the protein expression in brain homogenates during at various developmental stages (Fig. 1B). S4C was apparent in the brain on embryonic day 14 (E14); thereafter, the protein level markedly increased by birth, and high levels of S4C were detected at birth and during the postnatal developmental stage at postnatal days 7 and 14. The expression was still very strong in the adult brain, suggesting that the S4C functions in the adult brain as well as in the developing brain.

S4Ccd Binds to PDZ-containing Protein, PSRD-95 Family Molecules—In an effort to study the functional mechanism of S4C, we searched for proteins that can bind to S4Ccd. We used the entire cytoplasmic domain of mouse S4C inserted into the GAL4 DNA-binding domain in pGBT9 vector (CLONTECH) as bait to screen an adult rat brain cDNA library in a yeast two-hybrid assay (26). We obtained 37 positive clones from 3 × 10⁶ colonies. Eleven clones were false positives that encoded transcription factors. Sequence analysis revealed nine independent clones among 19 overlapping positive clones that represented three PSD-95 isoforms. Of these nine independent clones, four clones represented PSRD-95/chapsin-110 (GenBank accession numbers; RNU49049, Ref. 33; RNU53368), three clones did PSRD-95/SAP90 (GenBank accession numbers; M96853, Ref. 15; X66474, Ref. 17), and two clones represented SAP97/DLG-1 (HSU13897, Ref. 34; RNU14950, Ref. 35). Each open reading frame was in frame with the GAL4 activation domain. These clones representing PSRD-95 isoforms necessarily contain the complete or partial sequence (longer than two-thirds) of the predicted PDZ1 domain and the complete sequence of PDZ2 but do not necessarily contain the following middle and C-terminal domains, PDZ3, SH3, and GK domains.

To test whether S4Ccd interacts with various MAGUK (PDZ containing membrane-associated guanylate kinase) members including ZO-1, a tight junction protein, and DLG-A, a Drosophila septate junction protein, as well as PSD-95 isoforms, the interactions were monitored as β-galactosidase activity by using the yeast two-hybrid systems. Yeast cells were cotrans-
formed with a bait vector pGBl9 S4Ccd and one of following prey vectors containing a MAGUK member such as pVP16 PSD-95/SAP90, SAP102, PSD-95/chapsin-110, ZO-1, and DLG-A. S4Ccd interact with the N terminus of PSD-95/SAP90, SAP102, and PSD-95/chapsin-110, which contain the three PDZ domains but lack the C-terminal SH3 or GK domains (Fig. 2). In contrast, we observed no interaction of S4Ccd proteins with the PDZ domains of a tight junction protein, ZO-1, or a Drosophila septate junction protein, DLG-A. Thus, there appears to be a specific interaction of the cytoplasmic domain of S4C with the PDZ domains of PSD-95 isoforms of synaptic proteins, such as PSD-95/SAP90, PSD-93/chapsin-110, and SAP102.

\[\text{In Vitro Binding Assay Showing the Interaction between the Cytoplasmic Tail of S4C and the PDZ Domains of PSD-95—Because PSD-95 is the best characterized member of the above identified PSD-95 isoforms, PSD-95 was used for the following studies. The interactions observed above were next explored via “pull-down” assays in which GST fusion proteins bearing wild type (GST-S4Ccd) or C-terminal mutated S4Ccd (GST-S4C*cd) were used to pull down the full-length PSD-95 proteins and various truncated forms of PSD-95 expressed in mammalian cells. Cell extracts from COS cells transfected with pCMV Myc-PSD-95 were incubated with immobilized GST-S4Ccd or GST-S4C*cd proteins. Immunoblot analysis showed no interaction of PSD-95 with GST but showed a strong interaction of PSD-95 with GST-S4Ccd (Fig. 3). Substitution of three amino acid residues with three alanines at the C terminus of S4Ccd (GST-S4C*cd) abolished the interaction with PSD-95. These in vitro binding experiments suggested that the cytoplasmic tail of S4C interacts with PSD-95. Next, we investigated which region of PSD-95 interacts with S4C. Cell extracts from COS cells transiently expressing wild type (PSD95–1) and mutated PSD-95 (PSD95–2) were incubated with immobilized GST-S4Ccd proteins (Fig. 4C). Wild type PSD-95 and three truncated forms, PSD-95-2 (PDZ1 + PDZ2), PSD-95-3 (PDZ1 + PDZ2 + PDZ3), and PSD-95-4 (PDZ1 + PDZ2 + SH3 + GK) bound to GST-S4Ccd, whereas removal of PDZ1 and PDZ2 from PSD-95 (PSD-95-5) almost completely abolished the association with GST-S4Ccd (Fig. 4C). These in vitro binding experiments suggest that the PDZ1 and PDZ2 domains of PSD-95 interact with the C terminus of S4C.]

\[\text{Interaction of PSD-95 and S4C in Mammalian Cells—To confirm the interaction of PSD-95 and full-length S4C in a cellular environment, HEK293 cells were cotransfected with pCMV Myc-PSD-95 and pEF HA S4C wild type or pEF HA S4C*mutant. The cell lysate was immunoprecipitated with anti-HA or anti-Myc antibodies on protein A beads and analyzed by immunoblotting with anti-S4C antibody (Fig. 5, upper right panel) or with anti-PSD-95 antibody (Fig. 5, lower right panel). Anti-Myc antibody precipitated the Myc-PSD-95 protein from transfected cells, and Myc-PSD-95 protein coimmunoprecipitated with HA-S4C protein and vice versa. HA-S4C protein precipitated as a complex containing Myc-PSD-95 protein, whereas HA-S4C* mutant protein failed to precipitate Myc-PSD-95 protein.}

\[\text{Subcellular Distribution—PSD-95 is a synapse-associated protein, enriched in PSD fractions (15) (Fig. 6). If S4C interacts with PSD-95 in the brain, S4C should be enriched in PSD fractions. We explored whether S4C is concentrated in the synaptosome and PSD fractions by immunoblotting analysis using antiserum to S4C (Fig. 6). S4C was enriched in the crude synaptosomal pellet (P2) fraction as compared with the homogenate or cytosolic synaptosomal (S2) fraction. The subfractionation of P2 crude synaptosomal pellet into the synaptic vesicle fraction, the lysis crude synaptosomal membrane (P3) fraction, the synaptosomal membrane fraction, and Triton X-100 insoluble PSD1and 2 fractions showed that S4C was most enriched in the synaptic vesicle and PSD fractions (PSD1 and PSD2), where PSD-95 was also most enriched (Fig. 6). These results suggest that S4C is associated with both pre- and post-synaptic components. S4C enriched in the PSD fraction most likely associates with PSD-95.}

\[\text{Interaction of S4C and PSD-95 in the Brain—To examine whether endogenous S4C interacts with endogenous PSD-95, brain cytosol was immunoprecipitated with an antiserum to PSD-95 (Upstate Biotechnology) and probed with antibodies to S4C by immunoblot analysis. Because PSD-95 and S4C proteins are concentrated in the Triton X-100-insoluble pellet (PSD1 and PSD2) of the synaptosome, urea-containing buffer was used to extract these proteins from the crude synaptosomal pellet fraction (P2), and the extract was immunoprecipitated with normal mouse serum or antibody to PSD-95. The immunoprecipitates were analyzed by immunoblotting with antibody to PSD-95 (Transduction Laboratories) to check the specificity of this antibody (Fig. 7A, left panel) and with antibody to S4C (Fig. 7A, right panel). PSD-95 protein coprecipitated with S4C in the P2 fraction from the cerebral cortex, suggesting the interaction of PSD-95 and S4C in the central nervous system. Immunohistochemical experiments showed that there is partial overlap of S4C and PSD-95 in the superficial layers of the cerebral cortex of the adult mouse (Fig. 7B). S4C was localized to the superficial layers of layers I–IV, whereas PSD-95 was extensively distributed throughout the cortical layers. In the parietal cortex, S4C was detected in the barrel fields, which receive projections especially from cutaneous mechanoreceptors of the mystacial vibrissae (Fig. 7B). This pattern was}
similar to that of PSD-95. This is the first report showing immunohistochemical localization for S4C so far. In primary cultures of neocortical neurons, immunostaining for S4C was found in the neurites of cortical neurons with a dot-like pattern and was colocalized with PSD-95 (Fig. 7C). These results suggest that S4C enriched in the PSD is related to the modulation of neocortical neurons that receive thalamocortical sensory projections.

DISCUSSION

The present study shows that S4C is enriched in the adult brain as well as postnatal developing brain, whereas its level is very low in other tissues, such as heart, lung, liver, kidney, and spleen. Brain-specific expression of S4C protein is almost parallel with that of S4C mRNA (36). Developmentally, expression of S4C mRNA (36) slightly precedes that of the protein. S4C mRNA and protein can be detected at embryonic stage, and they are abundant in postnatal and adult brains. S4C is a component of the PSD and interacts with PSD-95, which is a scaffold protein, that interacts with excitatory neurotransmitter receptors, ion channels, synaptic adhesion, and signaling proteins and that is important molecule coupling the NMDA receptor to signaling pathways that control bi-directional synaptic plasticity and learning (14, 18, 19, 22, 23, 26, 33). Importantly, we showed that S4C is colocalized with PSD-95 in cerebral cortical neurons.

**The PDZ Domains at the N-terminal of PSD-95 Are Responsible for the Interaction with the Cytoplasmic Tail of S4C**—In vitro affinity binding experiments revealed the interaction of PSD-95 with the cytoplasmic domain of S4C expressed in mammalian cells. In vitro binding experiments using mutated PSD-95 and S4C showed that three amino acids of the C terminus of S4C are necessary for binding to the first and second PDZ domains of PSD-95. PSD-95 and its isoforms including PSD-93/Chapsyn-110 and SAP97/DLG-1 are prominent brain-
specific proteins that are enriched in the PSD fraction, and one of the components of a dense thickening of postsynaptic submembranous cytoskeleton observed in electron microscopy. These proteins have three N-terminal PDZ domains, a SH3 domain, and a guanylate kinase domain in their C termini. PSD-95 isoforms interact with NMDA receptors and Shaker-type potassium channels through the first and second PDZ domains, and brain nitric-oxide synthase through the second PDZ domain to induce the clustering of these molecules at the PSD (18, 19, 33–35, 37). Through the third PDZ domain, PSD-95 also interacts with various signaling and adhesion molecules, such as a synaptic adhesion molecule, neurelin (28), a microtubule-associated molecule, CRIP (38), and signaling molecules such as SynGAP (39), a Ras-GTPase activating protein at excitatory synapses that stimulates the GTPase activity of Ras and thus may negatively regulate Ras activity, Citron (40), a target for the activated form of the small GTP-binding protein Rho, and Fyn (41), a member of the Src-family protein-tyrosine kinases implicated in learning and memory that involves NMDA receptor function. A number of PSD-95-binding proteins, including S4C, may be related to each other in their signaling because they are in close proximity to each other because of interacting with PSD-95, although there have been no studies showing direct interactions among PSD-95-binding proteins.

PSD-95 and S4C May Be Involved in Bi-directional Synaptic Plasticity and Learning—In mutant mice lacking PSD-95, the frequency function of NMDA-dependent long term potentiation and long term depression is shifted to produce strikingly enhanced long term potentiation at different frequencies of synaptic stimulation (42). This frequency shift is accompanied by severely impaired spatial learning. However, synaptic NMDA receptor currents, subunit expression, localization, and synaptic morphology are all unaffected in the mutant mice. PSD-95 thus appears to be important in coupling the NMDA receptor to signaling pathways that control bi-directional synaptic plasticity and learning. Several synaptic adhesion molecules such as neurelin and cadherins are thought to be involved in such synaptogenesis and synaptic plasticity (43, 44). Neurelin is a family of synaptic cell adhesion molecules that interact with β-neurexins and form homophilic intercellular junctions (45). Neurexins are primarily known as receptors for α-latrotoxin, which binds to presynaptic nerve terminals and triggers massive neurotransmitter release. Among the neurexins, β-neurexin, an alternatively spliced form, functions as a receptor for neurelin and most likely is involved in bi-directional signaling at excitatory synapses. The cytoplasmic domain of neurelin binds to the third PDZ domain of PSD-95, whereas S4C, NMDA receptors, and Shaker-type potassium channels interact with the first and second PDZ domains. S4C may regulate NMDA signaling by competing with NMDA receptors or potassium channels for binding the first and second PDZ domains of PSD-95.

Interaction of S4C with Other Proteins—A recent study (36) showed the interaction of S4C with GIPC (a protein interacting with the RGS protein GaIP), which has a central PDZ domain and a C-terminal acyl-carrier protein domain. The central PDZ domain of GIPC interacts with GaIP, a GTPase-activating protein for Gα subunits localized on clathrin-coated vesicles. The PDZ domain of GIPC interacts with the C terminus of S4C and neuropilin 1, which is a receptor for secreted group 3 semaphorin, as well as the C terminus of GaIP (36, 46, 47). Because GIPC expression is very low in the brain, GIPC interacts with S4C and neuropilin 1 in the brain but not with GaIP. GIPC mRNA and protein are widely expressed in various tissues such as the pancreas, skeletal muscle, kidney, placenta, lung, and liver, as well as the brain (46, 47). In contrast, S4C expression is predominantly localized in brain tissues, suggesting that S4C most likely functions mainly in brain tissues. GIPC may compete with PSD-95 for binding S4C when GIPC is present at the PSD. However, Wang et al. (36) showed that GIPC is localized in the cell soma of neocortical neurons with large punctal features but not in neurites, which is similar to the findings in transfected HeLa cells (46). Therefore, S4C most likely interacts with PSD-95 at the PSD of the excitatory synapses in the central nervous system, whereas S4C interacts with GIPC mainly in the cell soma of neurons. These results suggest that S4C plays distinct roles depending on its subcellular localization in neurons.

Possible Receptor for S4C—A recent study showed that Sema4D binds to mammalian cells transfected with plexin B1 (48), suggesting that plexin is a candidate for a counterpart receptor for S4C. Plexins (48–51) encode large transmembrane proteins whose cysteine-rich extracellular domains share regions of homology with the scatter factor receptors encoded by the Met gene family (Met-related sequences). The extracellular domain also contains sema domains of about ~500 amino acids. Semaphorins contain cysteine-rich Met-related sequences and sema domains. It has been suggested that these domains were derived from a common evolutionary ancestor with homophilic binding properties (52). The cytoplasmic domain of plexins (~600 amino acids) is highly conserved but shares no homologous regions with the Met tyrosine kinase domain nor with any other known protein. S4C-plexin signaling may be involved in bi-directional NMDA or neurelin-neurexin intercellular signaling, and this can be tested by examining whether S4C is linked to the NMDA receptor or neurelin at synapses through PSD-95.

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REFERENCES
1. Tessier-Lavigne, M., and Goodman, C. S. (1996) Science 274, 1123–1133
2. Kolodkin, A. L., Matthes, D. J., and Goodman, C. S. (1998) Cell 75, 1389–1399
3. Semaphorin Nomenclature Committee (1999) Cell 97, 551–552
4. Luo, Y., Raible, D., and Raper, J. A. (1998) Cell 75, 217–227
5. Adams, R. H., Lohrum, M., Klostermann, A., Betz, H., and Puschel, A. W. (1997) EMBO J. 16, 6077–6086
6. Miyaizaki, N., Furuyama, T., Sakai, T., Fujikura, S., Mori, T., Ohoka, Y., and Nishimoto, I. (1999) J. Neurosci. 19, 243–249
7. Inagaki, S., Furuyama, T., Amasaki, M., Sugimoto, H., Sakai, T., Takeda, N., Kudo, T., and Inagaki, S. (1999) Neurosci. Res. 33, 269–274
8. Kobayashi, H., Koppel, A. M., Luo, Y., and Raper, J. A. (1997) J. Neurosci. 17, 8319–8324
9. Inagaki, S., Furuyama, T., and Iwashita, Y. (1995) FEBS Lett. 370, 269–272
10. Furuyama, T., Inagaki, S., Kasuga, A., Noda, S., Saitoh, S., Ogata, M., Iwashahi, Y., Miyazaki, N., Hamaoka, T., and Tohyama, Y. (1996) J. Biol. Chem. 271, 33376–33381
11. Elbahri, A., Lang, V., Herold, C., Freeman, G. A., Bensusen, A., Bounoua, L., and Bismuth, G. (1997) J. Biol. Chem. 272, 23515–23520
12. Eckhardt, F., Behar, O., Calautti, E., Yonezawa, K., Nishimoto, I., and Fishman, M. C. (1997) Mol. Cell. Neurosci. 9, 409–419
13. Pawson, T., and Scott, J. D. (1997) Science 278, 2075–2080
14. Ponting, C. P., Phillips, C., Davies, K. R., and Blake, D. J. (1997) BioEssays 19, 469–479
15. Cho, K. O., Hunt, C. A., and Kennedy, M. B. (1995) Neuron 9, 929–942
16. Woods, D. F., Bryant, P. J., and Bennett, J. D. (1995) J. Neurochem. 66, 215–223
17. Kistner, U., Wenzel, B. M., Veh, R. W., Cases-Langhoff, C., Garner, A. M., Appeltauer, U., Voss, B., Gundelfinger, E. D., and Garner, C. C. (1993) J. Biol. Chem. 268, 4583–4588
18. Kornau, H. C., Seehagur, P. H., and Kennedy, M. B. (1997) Curr. Opin. Neurobiol. 7, 368–373
19. Sheng, M. (1996) Neuron 17, 575–578
20. Kim, S. K. (1997) Curr. Opin. Cell Biol. 9, 835–839
21. Breit, D. S. (1998) Cell 94, 691–694
22. Ranganathan, R., and Ross, E. M. (1997) Curr. Biol. 7, R770–R773
23. Tsuonda, S., Sierra-Ruiz, J., Sun, Y., Bedner, R., Suruki, E., Becker, A., Sodich, M., and Zuker, C. S. (1997) Nature 388, 243–249
24. Songyang, Z., Fanning, A. S., Fu, C. X., Xu, J., Marfatia, S. M., Chishti, A. H., Crompton, A., Chan, A. C., Anderson, J. M., and Cantley, L. C. (1997) Science 273, 75–77
25. Fanning, A. S., and Anderson, J. M. (1999) Curr. Opin. Cell Biol. 11, 432–439
26. Hata, Y., Butz, S., and Sudhof, T. C. (1996) J. Neurosci. 16, 2488–2494
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A
PSD-95 Interacts with Intracellular Domain of Sema4C

19, 96–108

41. Tsuchiya, T., Umemori, H., Akiyama, T., Nakamichi, S., and Yamamoto, T. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 435–440

42. Migaud, M., Charlesworth, P., Dempster, M., Webster, L. C., Watabe, A. M., Makinson, M., He, Y., Ramsay, M. F., Morris, R. G., Morrison, J. H., O’Dell, T. J., and Grant, S. G. (1998) Nature 396, 433–439

43. Scheiffele, P., Fan, J., Chou, J., Petter, R., and Serafini, T. (2000) Cell 101, 657–669

44. Yagi, T., and Takeichi, M. (2000) Genes Dev. 14, 1169–1180

45. Ichtchenko, K., Hata, Y., Nguyen, T., Ulrich, B., Missler, M., Moomaw, C., and Sudhof, T. C. (1995) Cell 81, 435–443

46. De Vries, L., LouX, Zhao, G., Zheng, B., and Farquhar, M. G. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 12340–12345

47. Cai, H., and Reed, R. R. (1999) J. Neurosci. 19, 6519–6527

48. Tamanogone, L., Artigiani, S., Chen, H., He, S., Ming, G., Song, H., Chedotal, A., Winberg, M. L., Goodman, C. S., Poo, M. M., Tessier-Lavigne, M., and Comoglio, P. M. (1999) Cell 99, 71–80

49. Kameyama, T., Murakami, Y., Sato, F., Kawakami, A., Takagi, S., Hirata, T., and Fujisawa, H. (1996) Biochem. Biophys. Res. Commun. 226, 366–372

50. Maestrini, E., Tamagnone, L., Longati, P., Cremona, O., Gulisano, M., Bione, S., Tamanini, F., Neel, B. G., Tonio, D., and Comoglio, P. M. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 674–678

51. Comeau, M. R., Johnson, R., Dallase, R. F., Petersen, M., Gearing, P., VandenBos, T., Park, L., Fossett, T., Buller, R. M., and Cohen, J. I., (1998) Immunity 8, 473–482

52. Winberg, M. L., Noordermeer, J. N., Tamagnone, L., Comoglio, P. M., Spriggs, M. K., Tessier-Lavigne, M., and Goodman, C. S. (1998) Cell 95, 903–916