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Associates with a Neurite-Outgrowth-Related Protein, SFAP75

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Semaphorin 4C (S4C, previously called M-SemaF) was recently identified as a brain rich transmembrane member of semaphorin family of the vertebrate. In the cytoplasmic domain of S4C there is a proline-rich region suggesting that the cytoplasmic domain may play an important role in Sema4C function. In this study, we have identified the cytoplasmic domain (cd) of M-SemaF(S4C)-associating protein with a Mr of 75 kDa, named SFAP75, from mouse brain. SFAP75 turned out to be the same as the recently reported neurite-outgrowth-related protein named Norbin. Deletion mutants analyses of S4C and SFAP75 revealed that the membrane-proximal region of S4Ccd binds to the intermediate region of SFAP75. Western blot and immunohistochemical analyses with anti-Sema4C and anti-SFAP75 antibodies indicated that S4C and SFAP75 were specially enriched in the brain with a similar distribution pattern to each other. These results suggest that S4C interacts with SFAP75 and plays a role in neural function in brain.

Key Words: **semaphorin; Semaphorin 4C; SFAP75; Norbin; synapse; LTP; juxtamembrane.**

Significant progress has been made recently in understanding the mechanisms of neural network formation (1, 2). Semaphorins are a large family of structurally distinct secreted, glycosylphosphatidylinositol (GPI) anchor and transmembrane glycoproteins characterized by the presence of a conserved sema domain of about 500 amino acids (3, 4). Evidence implicating secreted semaphorins in chemorepulsion came from the finding that Sema3A, Sema3C, and Sema3E act as a chemorepellent to a variety of sensory and motor axons in the chick and mammalians (5–9). On the other hand, there is only limited evidence concerning the roles of transmembrane semaphorins in the nervous system. We have identified Sema4C (S4C, previously called M-SemaF) and Sema4D (S4D, previously called M-SemaG) from mouse brain, which is predicted to encode a transmembrane semaphorin (10, 11). S4C is specifically expressed in both embryonic and adult neuronal tissue, suggesting that S4C functions such as directing axon pathfinding, synapse formation or synapse maintenance. The cytoplasmic domain (cd) of S4C contains a proline-rich region which may interact with Src homology 3 (SH3) domains as well as cytoskeletal proteins (12), suggesting that the cytoplasmic domain of S4C may interact with some signaling related proteins. One way to clarify the mode of action of S4C is to isolate S4Ccd–associating proteins. In the present study, we have isolated here the cytoplasmic domain of M-SemaF(S4C)-associating protein with an Mr of 75 kDa from mouse brain using an affinity column with glutathione S-transferase (GST)-S4Ccd, and named SFAP75. SFAP75 turned out to be the same as a Norbin, which has been identified as neurite-outgrowth-related protein (13, 14). Thus, it is possible that S4C plays a new role in neuronal function with SFAP75 in the central nervous system (CNS).

MATERIALS AND METHODS

Construction. The cDNA of mouse SFAP75 was obtained by PCR using mouse brain cDNA as template. The primers, 5′-CGAATTCATGTCGTGTTGTGACCTG-3′ (sense) and 5′-CGAATTCCTGGTGAATCTGGCC-3′ (antisense) were designed on the basis of rat norbin cDNA (13). Various GST-fused proteins of mouse S4C (GeneBank Accession No. S79463) and SFAP75 were expressed in *Escherichia coli* and purified using standard molecular biology meth-

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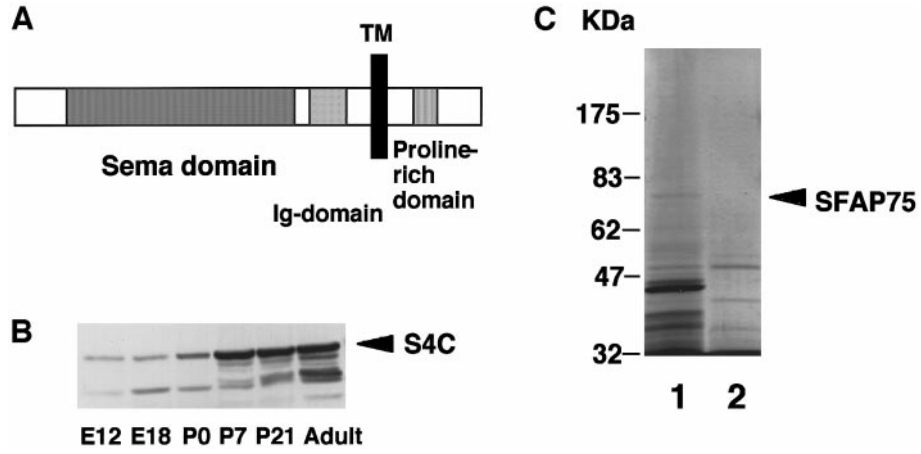

FIG. 1. Identification of S4C cytoplasmic domain-associating protein, SFAP75. (A) Diagrammatic representation of S4C. S4C consists of a sema domain, an immunoglobulin-like domain, a transmembrane domain, and an intracellular domain containing proline-rich region. (B) Expression of S4C in various developmental stages in mouse brain. Each homogenate of developing brain (16 μg of protein each) was prepared from mouse indicated ages and subjected to SDS–PAGE (10.5% polyacrylamide gel) followed by Western blotting with anti-S4C antibody. (C) Affinity purification of SFAP75 by using GST-S4C cytoplasmic domain from mouse brain extracts. Mouse brain extracts were loaded onto a glutathione-Sepharose 4B column containing either GST-S4Ccd (lane 1) or GST (lane 2). The bound proteins were eluted by the addition of SDS–PAGE sample buffer. Aliquots from the elute were subjected to SDS–PAGE (10.5% polyacrylamide gel) and visualized by CBB staining. Arrowhead, SFAP75.

ods (15). pEF-HA-S4C, pCMV-Myc-SFAP75 and its mutants were constructed to express in COS cells or HEK293 cells using standard molecular biology methods (15).

Antiserum. Rabbit polyclonal anti-Norbin/SFAP75 antibody and anti-S4C antibody were raised against GST-SFAP75 (amino acid 1–360) and GST-S4Ccd (amino acid 687–834), respectively. The specificity of the antibodies was confirmed by Western Blotting of the pCMV-Myc-SFAP75 or pEF-HA-S4C-transfected COS cells. Mouse monoclonal antibody to HA was obtained from Boehringer Mannheim (Tokyo) and mouse monoclonal antibody to Myc (9E10) was purchased from American Type Culture Collection. For Western blot analysis, primary antisera to S4C, SFAP75, HA, and Myc was used at a 1:2000 dilution. Immunoreactive proteins were visualized using CDP-Star detection reagent (Amersham Pharmacia Biotech) using the alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Santa Cruz) or AP-conjugated rabbit anti-mouse IgG (New England Bio Labs, Beverly, MA), which were used as second antibodies for immunoblot detection at a 1: 2000 dilution.

Purification of norbin/SFAP75. Twenty mouse brains were homogenized in ice-cold Buffer A (20 mM Tris/HCl at pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM PMSF, and 1%(w/v) Triton X-100). The homogenate was centrifuged at 100,000*g* for 1 h. The supernatant was incubated with a 100 μl of glutathione-Sepharose beads bound with the GST-S4Ccd at 4°C for 1 h and the beads were washed extensively with buffer A and eluted with SDS–PAGE sample buffer. The eluted sample was subjected to SDS–PAGE (10% polyacrylamide gel). The protein band with an Mr of 75 kDa was extracted from the gel and the partial amino acid sequences were determined as previously described (16).

In vitro binding. COS cells were transfected by DEAE-dextran method with pCMV-Myc-SFAP75 and various mutant plasmids of SFAP75 (17). After 48 h, the cells were lysed in 0.5 ml of buffer A, and centrifuged at 100,000*g* for 30 min. The supernatant (original, OR) was incubated with various GST-fused proteins fixed on 20 μl of glutathione-Sepharose 4B beads (Amersham Parmacia Biotech). After the beads were washed with the buffer A (flow through, FT), proteins on the beads were subjected to the immunoblot analysis.

Immunoprecipitation. pCMV-Myc-SFAP75 was cotransfected with pEF-HA-S4C into HEK293 cells by calcium phosphate method (18). After 48 h culture, the cells were lysed with buffer A and centrifugated as described above. The supernatant was incubated with anti-Myc or anti-HA antibodies fixed on protein A-Sepharose beads. After the beads were washed with the extract buffer, proteins on beads were detected with the immunoblottings by the indicated antibodies. Mouse neuroblastoma, Neuro2a cells stably expressing HA-S4C were lysed with buffer A and centrifugated. The supernatant was incubated with anti-HA antibody fixed on protein A-Sepharose beads. After the beads were washed with the extract buffer, proteins on beads were detected with the immunoblottings by the antibodies to S4C or SFAP75.

Immunocytochemistry. Immunohistochemistry was performed on adult mouse brain sections by ABC method as previously described (19). Cryostat sections prepared from 4% paraformaldehyde fixed animals were reacted with the antibodies to S4C, or the antibody to Norbin/SFAP75, and then incubated with biotinylated-second antibodies to rabbit IgGs (Amersham Pharmacia Biotech). After reacted with avidin-biotin-horseradish peroxidase complex (Vecstatin ABC kit, Vector Laboratories, Burlingame CA), immunoreactivity was visualized with diaminobenzidine (Dotite, Japan).

Other procedures. Protein concentration was determined with bovine serum albumin as a reference protein (18). SDS–PAGE and Western blotting were carried out as described previously (20).

RESULTS

Isolation of S4C-Cytoplasmic Domain Binding Protein

S4C is transmembrane semaphorin and the cytoplasmic domain (cd) contains a proline-rich region which may interact with SH3 domains as well as cytoskeletal proteins (Fig. 1A). The expression of SFAP75 is relatively low in early developmental stage and then steadily increased as synaptogenesis occurred in the developing brain suggesting that S4C may play an important role in the central nervous system (Fig. 1B).

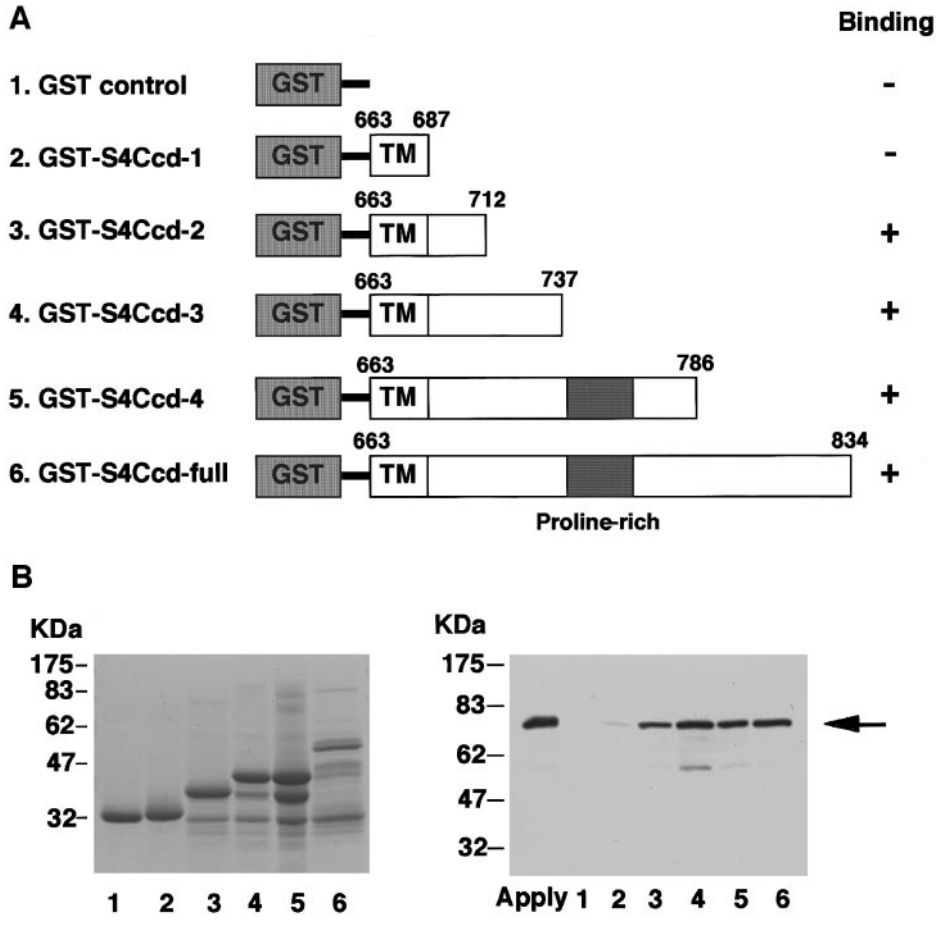

FIG. 2. Binding region of S4C cytoplasmic domain to SFAP75. (A) Schematic representation of deletion mutants of each GST-S4C cytoplasmic domain. GST-S4C cytoplasmic domain binding activity of each mutant is shown at the right. (B) The cell extracts from COS cells transfected with pCMV-Myc-SFAP75 were incubated with glutathione-Sepharose beads containing a series of deletion mutants of GST-S4C cd (left panel). The beads were washed extensively and subjected to SDS–PAGE (10.5% polyacrylamide gel) followed by Western blotting with anti-Myc antibody (right panel).

In an effort to study the functional mechanism of S4C, we searched for proteins that would bind to S4Ccd in mature brain. To detect molecules associating with S4Ccd, the mouse adult brain extracts was loaded onto GST-S4Ccd affinity columns. The proteins bound to the affinity columns were eluted by SDS–PAGE sample buffer. Proteins with a molecular mass of about 75 kDa, named SFAP75, was detected in the elute from the GST-S4Ccd affinity column but not from those containing only GST (Fig. 1C). To identify the S4Ccd associating protein, SFAP75 was subjected to amino acid sequence. Three peptide sequences derived from SFAP75 were determined. There were: (i) TRRRIFPAVG; (ii) LSSWQRNPAL; and (iii) EDAPSLLCK. All three peptide sequences were almost identical to the deduced amino acid sequence of rat Norbin, which is neurite-outgrowth-related protein induced by treatment of tetraethylammonium in rat hippocampal slice culture (13). SFAP75 hardly bound to the cytoplasmic domain of another transmembrane semaphorin such as S4D (previous called as M-SemaG) (data not shown).

Binding Region of S4Ccd and SFAP75

To determine the binding region of the cytoplasmic domain of S4C to SFAP75, we constructed a series of the S4Ccd deletion mutants and examined their binding to SFAP75 (Fig. 2A). The cell extracts of pCMV-Myc-SFAP75 transfected COS cells were incubated with glutathione-Sepharose beads containing a series of deletion mutants of GST-S4Ccd. SFAP75 bound to the cytoplasmic region of S4C deletion mutants 3, 4, 5, and 6 but not 1 and 2, indicating that SFAP75-binding region is located in the proximal region of the transmembrane domain but not proline-rich region in the S4Ccd (Fig. 2B).

Next, to determine the binding region of SFAP75 to the S4Ccd, we constructed a series of SFAP75 deletion mutants and examined their binding to the S4Ccd (Fig.

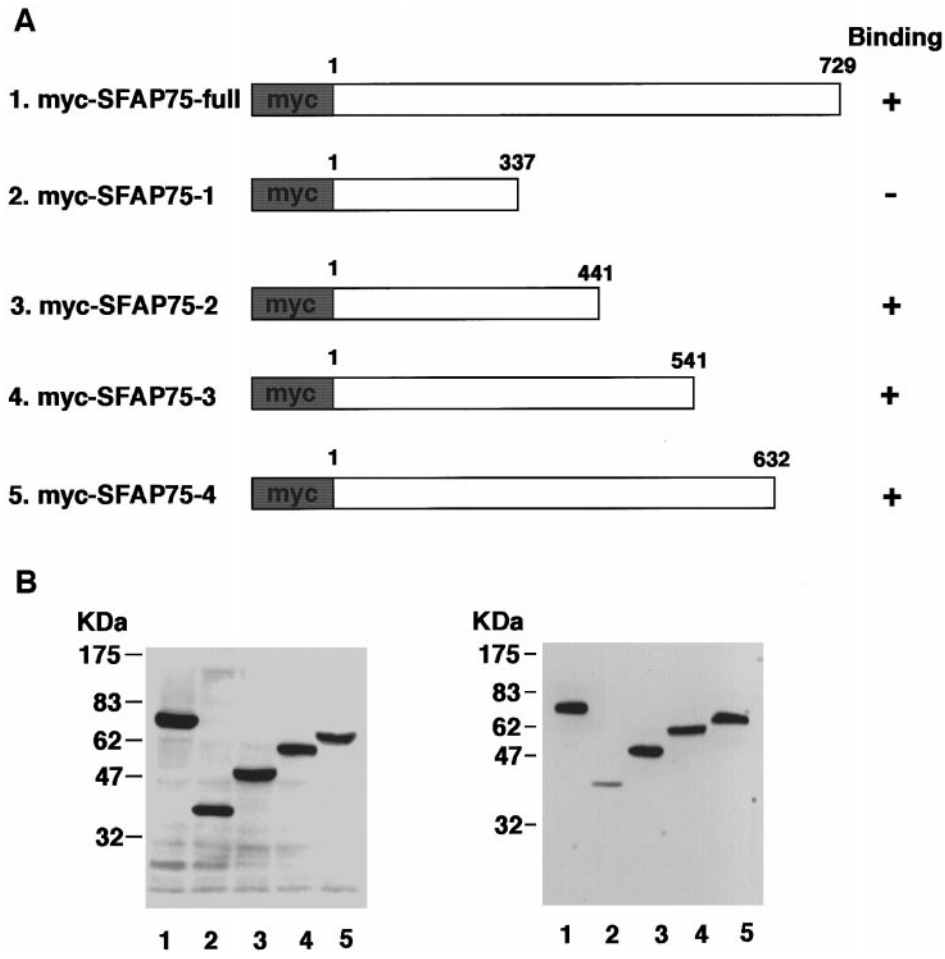

FIG. 3. Binding region of SFAP75 to S4C cytoplasmic domain. (A) Schematic representation of deletion mutants of SFAP75. SFAP75 binding activity of each mutant is shown at the right. (B) The cell extracts from COS cells transfected with pCMV-Myc-a series of deletion mutants of SFAP75 were incubated with glutathione-Sepharose beads containing GST-S4Ccd (left panel). The beads were washed extensively and subjected to SDS–PAGE (10.5% polyacrylamide gel) followed by Western blotting with anti-Myc antibody (right panel).

3A). The cell extracts from COS cells expressing Myc-tagged-SFAP75 and its deletion were incubated with glutathione-Sepharose beads containing GST-S4Ccd. The S4Ccd bound to SFAP75 deletion mutants 3, 4, and 5 but not 2, indicating that the S4Ccd binding region is located in the intermediate region of SFAP75 (Fig. 3B).

Immunoprecipitation of S4C with SFAP75

To confirm the interaction of S4C and Norbin/SFAP75 in a cellular environment, HEK293 cells were cotransfected with pEF-HA-S4C and pCMV-Myc-SFAP75. The cell lysate were immunoprecipitated with anti-Myc or anti-HA antibodies on protein A beads and analyzed by immunoblotting with anti-S4C antibody or anti-SFAP75 antibody (Fig. 4A). The anti-Myc antibody immunoprecipitated not only SFAP75 but also S4C, whereas the normal mouse serum did not. The same results were obtained using anti-HA antibody (data not shown). Since the antibodies to S4C and SFAP75 are not suited for immunoprecipitation, Neuro2a cells expressing HA-tagged S4C were used for immunoprecipitation (Fig. 4B). Endogenous SFAP75 in Neuro2a cells was co-immunoprecipitated with S4C by anti-HA antibody but not the normal mouse serum. These results suggest that SFAP75 is a physiological partner of S4C in neuronal cells.

Overlap Distribution of S4C and SFAP75 in the Brain

We studied the expression and distribution of SFAP75. The developmental expression of SFAP75 in brain is examined (Fig. 5A). SFAP75 was enriched in the adult brain rather than immature brain with a similar developmental pattern to S4C (Fig. 1B). Immunohistochemical experiments with antibodies to S4C and SFAP75 showed their overlapping staining in the cerebral cortex of the adult mouse (Fig. 5B). Immuno-

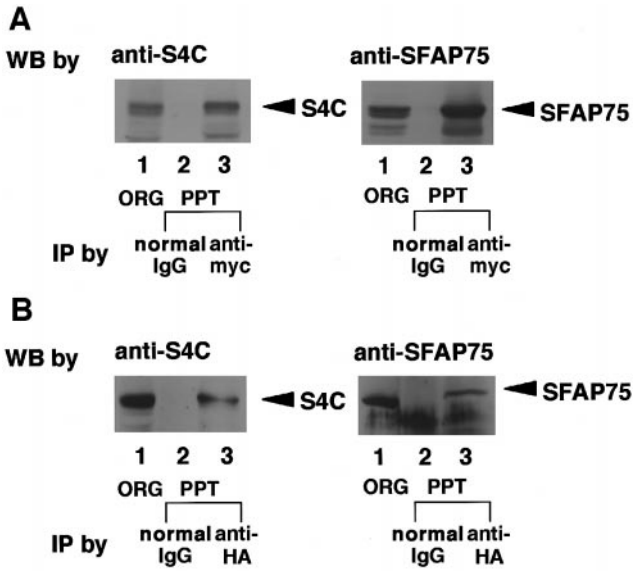

FIG. 4. Coimmunoprecipitation of S4C with SFAP75. (A) The cell extracts of both pEF-HA-S4C and pCMV-Myc-SFAP75 transfected COS cells were incubated with anti-Myc antibody or normal mouse IgG. Protein A-Sepharose beads associated immunocomplexes were subjected to SDS–PAGE (10.5% polyacrylamide gel) followed by Western blotting with anti-S4C (left panel) or SFAP75 (right panel) antibodies. (B) The cell extracts from Neuro2a cells expressing HA-S4C were incubated with anti-HA antibody or normal mouse IgG. Protein A-Sepharose beads associated immunocomplexes were subjected to SDS–PAGE (10.5% polyacrylamide gel) followed by Western blotting with anti-S4C (left panel) or SFAP75 (right panel) antibodies.

staining for S4C and SFAP75 are seen in the surface layer I-IV of the neocortex and S4C and SFAP75 are enriched in the barrels of layer IV, which receive projections from cutaneous mechanoreceptors of especially mystacial vibrissae. These results suggest that S4C and SFAP75 are related to the modulation of neocortical neurons which receive the thalamocortical sensory projections.

DISCUSSION

In this study, we have purified M-SemaF(S4C) cyoplasmic domain-associating protein with an Mr of 75 kDa, SFAP75, from a mouse brain extracts. SFAP75 specifically bind to S4C in cellular environment and immunostaining of SFAP75 overlapped with S4C in the cerebral cortex. The expression of both S4C and SFAP75 increased as synaptogenesis occurred in the developing brain suggesting that S4C may play an important role in the central nervous system. We have identified SFAP75 as Norbin, which is a brain specific protein induced by treatment of tetraethylammonium (TEA) in rat hippocampal slice culture (13, 14). The treatment of rat hippocampal slices with TEA is considered to induce LTP-like synaptic enhancement (21). It has been reported that LTP has become the best studied model for the synaptic plasticity in the mammalian brain (22–24). Although little is known about the mechanisms of induction of the synaptic plasticity, it is stipulated that new synaptic formation might be required in the time course of synaptic plasticity (25). Therefore, neurite-outgrowth-related proteins supposed to be involved in those processes. SFAP75 may play a role in the neurite outgrowth because the overexpression of norbin induced neurite-extension in a neuron-derived cell line, Neuro2a cells (13). Recently, we have demonstrated that S4C is enriched in the crude synaptosome and the C-terminus of S4C associated with PDZ domains of PSD95 family, such as PSD95/SAP90, PSD93/chapsin110, and SAP97/Dlg-A (Inagaki, S., and Ohoka, Y., manuscript in preparation). PSD-95 family sustains the interneuronal signal transmission and the subsequent signal cascade at synaptic junctions by clustering the receptors and signaling associating molecules (26–30). These observations suggest that SFAP75 may be involved in synaptic plasticity with S4C.

A deletion mutant analysis of S4Ccd and SFAP75 revealed that the proline-rich domain of S4Ccd is not responsible for binding to SFAP75. SFAP75 bind to the membrane-proximal region of S4C cytoplasmic domain, in other word, juxtamembrane region. The juxtamembrane region of adhesion molecules such as cadherin family seems to contribute to adhesive function and neurite outgrowth (31–36). A peptide mimicking amino acid sequence in the juxtamembrane region of

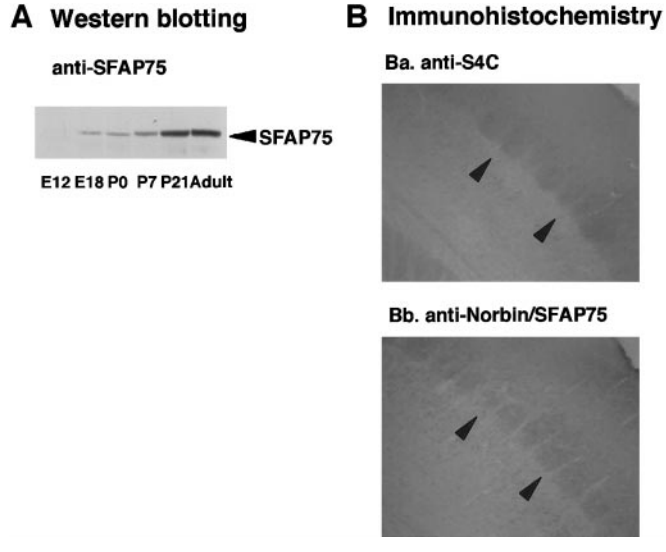

FIG. 5. Developmental expression and immunohistochemical colocalization of S4C and SFAP75. (A) Expression of SFAP75 in various developmental stages in mouse brain. Each homogenate of developing brain (16 μg of protein each) was prepared from mouse indicated ages and subjected to SDS–PAGE (10.5% polyacrylamide gel) followed by Western blotting with SFAP75 antibody. (B) Frontal sections of adult mouse cerebral cortex were stained with anti-S4C or anti-SFAP75 antibodies. Arrowhead, the barrels of layer IV.

N-cadherin inhibits cadherin-mediated cell adhesion and neurite outgrowth (33). In the case of E-cadherin, juxtamembrane region of E-cadherin binding protein, $p120^{ctn}$, modulate E-cadherin-mediated cell adhesion (34–36). Since S4C may be related to cell–cell interaction with its specific ligand, it is likely that SFAP75 bind to juxtamembrane region of S4C and modulates S4C-mediated cell–cell interaction at the synaptic junction. Currently not much is known as to how ligand-induced stimulation of S4C regulates the signal transduction via SFAP75 pathways. Further analysis of interactions between S4C and Norbin are necessary for our understanding of the physiological function of S4C and SFAP75 in central nervous system.

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