Three Isoforms of Synaptic Scaffolding Molecule and Their Characterization

MULTORIZATION BETWEEN THE ISOFORMS AND THEIR INTERACTION WITH N-METHYL-D-ASPARTATE RECEPTORS AND SAP90/PSD-95-ASSOCIATED PROTEIN*

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The synaptic scaffolding molecule (S-SCAM) has been identified as a protein interacting with SAP90/PSD-95-associated protein (SAPAP) (also called guanylate kinase-associated protein/DLG-associated protein). S-SCAM has six PDZ domains, which have numbered six PDZ domains, PDZ-0 to -5, two WW, and one guanylate kinase (GK) domains and interacts with N-methyl-D-aspartate (NMDA) receptor via PDZ-5 and SAPAP via the GK domain. We have identified here shorter isoforms of S-SCAM that start at the 164th or 224th methionine, and we renamed the original one, S-SCAMα, the middle one, S-SCAMβ, and the shortest one, S-SCAMγ. S-SCAMβ and γ have five PDZ (PDZ-1 to -5), two WW, and one GK domains. S-SCAMα interacted with S-SCAMβ and γ through the region containing PDZ-4 and -5. The region containing both of PDZ-4 and -5 is sufficient for the clustering of NMDA receptors and forms a dimer in gel filtration, suggesting that S-SCAM forms multimers via the interaction between the C-terminal PDZ domains and assemblies NMDA receptors into clusters. S-SCAMβ and γ also interacted with SAPAP, suggesting that the N-terminal region of the GK domain is not necessary for the interaction. Finally, we have identified the interaction of the PDZ domains of S-SCAM with the GK domain of PSD-95/SAP90. S-SCAM, PSD-95/SAP90, and SAPAP are colocalized at least in some part in brain. Therefore, S-SCAM, PSD-95/SAP90, and SAPAP may form a complex in vivo.

Neurotransmitter receptors need to be assembled at postsynaptic membrane for efficient neurotransmission. Glutamate is the most representative excitatory neurotransmitter in mammals, and recent studies have revealed that PDZ domain-containing proteins are involved in the assembly of glutamate receptors (reviewed in Refs. 1–3). The PDZ domain is a protein-interacting module that has initially been recognized as a repeat of about 80 amino acids in PSD-95/SAP90, Drosophila Dlg-A, and ZO-1. The PDZ domain is composed of two α helices and six β sheets, and the groove between the second β sheet and the second α helix interacts with the C termini of various proteins (4, 5). PSD-95/SAP90 has three PDZ domains, one SH3, and one guanylate kinase (GK) domains (6, 7). PSD-95/SAP90 binds the C termini of N-methyl-D-aspartate (NMDA) receptor subunits via the first and second PDZ domains and PSD-95/SAP90/PSD-95-associated protein (SAPAP) (also called guanylate kinase-associated protein/DLG-associated protein) via the GK domains (8–11). PSD-95/SAP90 has two cysteine residues at the N terminus and forms a multimer via the disulfide linkage (12). NMDA receptors are conceivably assembled through the multimerization of PSD-95/SAP90. PSD-95/SAP90 also binds kainate receptor subunits (13). The PDZ domain-containing proteins interacting with α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor include glutamate receptor-interacting protein (GRIP), AMPA receptor-binding protein (ABP), and protein interacting with protein kinase C (14–16). GRIP and ABP are composed of seven and six PDZ domains, respectively, and form a heteromultimer via PDZ domains. We have initially identified synaptic scaffolding molecule (S-SCAM) as a protein interacting with SAPAP (17). S-SCAM has six PDZ, two WW, and one GK domain, and we have numbered six PDZ domains, PDZ-0 to -5. S-SCAM interacts with NMDA receptors via the C-terminal PDZ-5 domain and induces the clustering of NMDA receptors when coexpressed in transfected cells. In our previous study, the antibody against S-SCAM recognized multiple signals with various sizes in the synaptic plasma membrane (SPM) fraction, suggesting that S-SCAM has isoforms (17). We have searched for the isoforms and obtained two isoforms, S-SCAMβ and γ. In this paper, we have characterized these isoforms to show that these short isoforms of S-SCAM induce the clustering of NMDA receptors and interact with SAPAP, and revealed the interaction among the isoforms of S-SCAM via PDZ domains. We have also indicated that S-SCAM interacts with PSD-95/SAP90.

MATERIALS AND METHODS

Construction of Expression Vectors—Various expression vectors were constructed by conventional molecular biology techniques and PCR method using pCMV Myc, pCleeo Myc, and pGex4T-1 (Amersham Pharmacia Biotech). pCMV NMDAR1, pCMV NMDAR2A, pCMV SAPAP1, pCleeo Myc S-SCAM-1, -2, -3, and -4, pGex4T-1 S-SCAM-15, and -16 were described previously (10, 17, 18). pCleeo Myc PSD-95–4, -8, -12, and -16 were described previously (10, 17, 18). pCleeo Myc PSD-95–4, -8, -12, and -16 were described previously (10, 17, 18). pCleeo Myc PSD-95–4, -8, -12, and -16 were described previously (10, 17, 18).

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1 The abbreviations used are: GK, guanylate kinase; NMDA, N-methyl-D-aspartate; SAPAP, SAP90/PSD-95-associated protein; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; GRIP, glutamate receptor-interacting protein; ABP, AMPA receptor-binding protein; S-SCAM, synaptic scaffolding molecule; SPM, synaptic plasma membrane; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; and PBS, phosphate-buffered saline.
and pGex4T-1 PSD-95–24 contained the amino acid residues 1–495, 534–724, and 562–613 of PSD-95/SAP90, respectively. The following constructs contain the following amino acids of S-SCAM: pClneo Myc S-SCAM-6, 586–992; pClneo Myc S-SCAM-10, 906–1261; pClneo Myc S-SCAM-12, 1124–1261; and pClneo Myc S-SCAM-17, 906–1221. The eukaryotic expression constructs of S-SCAM are summarized in Fig. 1A, where the positions of the proteins with molecular masses of 180, 155, and 140 kDa, respectively. Arrowheads 1–3 indicate the positions of the proteins with molecular masses of 180, 155, and 140 kDa, respectively.

B, schematic description of the isoforms and various eukaryotic expression constructs of S-SCAM. The numbers on the left and right ends of each model indicate the numbers of the first and last amino acids of each isoform or construct.

Antibodies—The rabbit polyclonal anti-S-SCAM antibody against the WW domain was described previously (17). A rabbit polyclonal antibody against PDZ-0 was raised using glutathione S-transferase (GST)-S-SCAM-22 as an antigen. For simplicity, in this paper we describe the former and latter antibodies as the anti-WW and the anti-PDZ-0 antibodies, respectively. A rabbit polyclonal anti-PDZ-0 antibody was obtained from Upstate Biotechnology, Inc. Rhodamine-conjugated and fluorescein isothiocyanate-conjugated second antibodies for dual labeling were purchased from Chemicon.

Preparation of COS Cell Extracts—COS cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum under 10% CO2 at 37 °C and transfected with various Myc-tagged constructs using the DEAE-dextran method (10). COS cells of two 10-cm dishes were homogenized in 0.5 ml of 50 mM Hepes/NaOH, pH 7.4, containing 100 mM NaCl and 1% (w/v) Triton X-100 and centrifuged at 100,000 × g for 20 min. The supernatant was diluted with 2 volumes of 50 mM Hepes/NaOH, pH 7.4, and used as COS cell extracts.

Subcellular Fractionation of CHO Cells—CHO cells were cultured in DMEM with 10% fetal bovine serum and 40 μg/ml proline under 10% CO2 at 37 °C and transfected with various constructs using TransFast Transfection Reagent (Promega). The subcellular fractionation of CHO cells was performed as described (19). Briefly, cells of one 10-cm dish were collected after 48 h culture and homogenized by sonication in 0.3 ml of 20 mM Hepes/NaOH, pH 7.4. 0.1 ml of the homogenate was kept for analysis, and the remaining samples were centrifuged at 100,000 × g for 30 min to separate the supernatant and the pellet. The pellet was resuspended in 0.2 ml of 20 mM Hepes/NaOH, pH 7.4, containing 1% (w/v) Triton X-100, and 0.05 ml was kept as the first pellet. The remaining sample was centrifuged at 16,000 × g for 10 min to separate the supernatant and the pellet. The pellet was resuspended in 0.15 ml of 20 mM Hepes/NaOH, pH 7.4, containing 1% (w/v) Triton X-100.

Coimmunoprecipitation—Each 0.5-ml aliquot of the extracts of COS cells transfected with pClneo Myc S-SCAM6 and various Myc-tagged constructs of S-SCAM were incubated with 2.5 μl of anti-Myc ascites fixed on 10 μl of protein G-Sepharose Fast Flow beads. After the beads were washed three times with 50 ml Hepes/NaOH, pH 7.4, containing 33 mM NaCl and 0.33% (w/v) Triton X-100, the proteins on the beads were detected by the immunoblotting using the anti-Myc or the anti-WW antibody. Immunoprecipitation from rat crude synaptosomes was performed (20). Briefly, the urea/detergent extracts of rat crude synaptosomes were prepared using 20 mM Hepes/NaOH, pH 8.0, containing 6 mM urea, 100 mM NaCl, and 1% (w/v) Triton X-100 and centrifuged at 100,000 × g for 30 min. The supernatant was dialyzed against 5 liters of 20 mM Hepes/NaOH, pH 8.0, containing 100 mM NaCl, with one exchange, and centrifuged at 100,000 × g for 30 min to remove the precipitate. The aliquots of the extracts were incubated with various
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RESULTS

The anti-WW antibody recognized signals with molecular masses of 180 and 140 kDa and a very faint signal with a molecular mass of 155 kDa in the SPM fraction (Fig. 1A, lane 8). The calculated molecular weight of S-SCAM is 141,068, but the product of the eukaryotic expression construct containing full-length S-SCAM migrated in SDS-PAGE at the same position as the largest signal detected in the SPM fraction (Fig. 1A, lane 2). Therefore, we discussed in the previous report of S-SCAM (17) that the smaller proteins might be degradation products or isoforms. Among 74 clones from the original screen, we detected two independent clones, p3205–4 and -32, which started at the 164th methionine of S-SCAM preceded by the stopping codon and terminated at the same site as the original S-SCAM (Fig. 1B). We named the original S-SCAM, S-SCAMα, and the shorter isoform, S-SCAMβ. We also found two independent clones, p3205–25 and -27, starting at the methionine at the 224th methionine preceded by the stopping codon, although these clones did not contain the termination codon. We named this shortest isoform, S-SCAMγ (Fig. 1B).

antibodies fixed on 10 μl of protein G-Sepharose Fast Flow beads. After the beads were washed three times with 20 mM Hepes/NaOH, pH 8.0, containing 33 mM NaCl and 0.33% (w/v) Triton X-100, the proteins on the beads were incubated with either the anti-Myc or the anti-WW antibody, a, the immunoblot with the anti-Myc antibody; b, the immunoblot with the anti-WW antibody; lanes 1, 3, 5, 7, and 9, the original extract; lanes 2, 4, 6, 8, and 10, the precipitate; lanes 1 and 2, the mock; lanes 3 and 4, pCIneo Myc S-SCAM-2; lanes 5 and 6, pCIneo Myc S-SCAM-3; lanes 7 and 8, pCIneo Myc S-SCAM-4; and lanes 9 and 10, pCIneo Myc S-SCAM-10. Closed arrowhead in Ba indicates the heavy chain of IgG. The products of pCIneo Myc S-SCAM-2 and -10 were partially overlapped by the heavy chain of IgG.

FIG. 4. Interaction between the isoforms of S-SCAM. A, coimmunoprecipitation of S-SCAMα with S-SCAMβ and γ from rat crude synaptosomes. S-SCAMα was immunoprecipitated with the anti-PDZ-0 antibody, and the immunoprecipitate was immunoblotted with the anti-WW antibody. Arrowheads 1–3 indicate the positions of the proteins with corresponding to S-SCAMα, β, and γ, respectively. Lane 1, the original extract; lane 2, the precipitate with the preimmune serum; and lane 3, the precipitate with the anti-PDZ-0 antibody. B, coimmunoprecipitation of S-SCAMβ with various Myc-tagged constructs of S-SCAMα. The extracts of COS cells transfected with pCIneo S-SCAMβ and various pCIneo Myc constructs of S-SCAMα were incubated with the anti-Myc antibody fixed on the glutathione-Sepharose 4B beads, and the proteins on the beads were immunoblotted with either the anti-Myc or the anti-WW antibody. α, the immunoblot with the anti-Myc antibody; b, the immunoblot with the anti-WW antibody; lanes 1, 3, 5, 7, and 9, the original extract; lanes 2, 4, 6, 8, and 10, the precipitate; lanes 1 and 2, the mock; lanes 3 and 4, pCIneo Myc S-SCAM-2; lanes 5 and 6, pCIneo Myc S-SCAM-3; lanes 7 and 8, pCIneo Myc S-SCAM-4; and lanes 9 and 10, pCIneo Myc S-SCAM-10. Closed arrowhead in Ba indicates the heavy chain of IgG. The products of pCIneo Myc S-SCAM-2 and -10 were partially overlapped by the heavy chain of IgG.

In Vitro Binding Experiment Using GST Fusion Proteins and COS Cell Extracts—Each 0.5-ml aliquot of the extracts of COS cells transfected with various pCIneo Myc constructs of S-SCAM were incubated with either GST or GST-PDZ(4 + 5) of S-SCAM which are fixed on glutathione beads, and the proteins on the beads were detected with the anti-Myc antibody. Lanes 1, 4, 7, 10, and 13, the original extract; lanes 2, 5, 8, 11, and 14, the precipitate with GST; lanes 3, 6, 9, 12, and 15, the precipitate with GST-PDZ(4 + 5); lanes 1–3, pCIneo Myc S-SCAM-1; lanes 4–6, pCIneo Myc S-SCAM-2; lanes 7–9, pCIneo Myc S-SCAM-3; lanes 10–12, pCIneo Myc S-SCAM-4; and lanes 13–15, pCIneo Myc S-SCAM-10. B, interaction between PDZ(4 + 5) and PDZ-5. The extracts of COS cells transfected with pCIneo Myc S-SCAM-10 were incubated with GST, GST-PDZ-4, -PDZ-5, or -PDZ(4 + 5), which are fixed on glutathione beads, and the proteins on the beads were detected with the anti-Myc antibody. Lane 1, the original extract; lane 2, the precipitate with GST; lane 3, the precipitate with GST-PDZ-4; lane 4, the precipitate with GST-PDZ-5; and lane 5, the precipitate with GST-PDZ(4 + 5). thrombin in 500 μl of 20 mM Tris/HCl, pH 7.5, and 1 mM CaCl₂ to remove GST. 500 pmol of the cleaved product was charged to a Sephadex 200 PC 3.2/30 column (0.32 × 30 cm) pre-equilibrated with 20 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol. Elution was performed with the same buffer for the equilibration at a flow rate of 50 ml/min, and 48 fractions were collected.

Immunofluorescence Microscopy—The immunofluorescence microscopy of adult rat neural tissue was done as described (21). Briefly, rats were perfused with 4% paraformaldehyde in PBS and postfixed with 4% paraformaldehyde in PBS. Their tissues were sectioned in a cryostat, mounted on glass slides, and then air-dried. The samples were incubated for 12 h with either the rabbit anti-WW, the anti-SAPAP, or the mouse anti-PSD-95 antibody, followed by incubation for 12 h with the Texas Red-conjugated anti-rabbit or anti-mouse antibody.
The anti-WW antibody recognized proteins with molecular masses of 155 and 140 kDa in COS cells transfected with pClneo S-SCAMβ and γ, respectively, in support that S-SCAMβ and γ actually encode proteins in vivo (Fig. 1A, lanes 3 and 4). COS cells transfected with pClneo S-SCAMβ also expressed a protein with a molecular mass of 140 kDa (Fig. 1A, lane 3), suggesting the 224th methionine functions as an initiation site in S-SCAMβ, too. The anti-PDZ-0 antibody recognized the protein with a molecular mass of 180 kDa but not the proteins with molecular masses of 155 and 140 kDa (Fig. 1A, lane 6). Therefore, the proteins with molecular masses of 155 and 140 kDa in the SPM fraction lack PDZ-0 and may be S-SCAMβ and γ, respectively.

Because S-SCAMβ and γ started in the middle of the GK domain, we tested whether S-SCAMβ and γ interacted with SAPAP. SAPAP is resistant to the extraction with Triton X-100 from either the SPM fraction or CHO cells. S-SCAMα was Triton X-100-soluble in CHO cells, but became partially Triton X-100-insoluble, when coexpressed with SAPAP1 (Fig. 2A). Similarly, S-SCAMβ was Triton X-100-soluble and became partially Triton X-100-insoluble in the presence of SAPAP1 in CHO cells (Fig. 2B). The similar result was obtained for S-SCAMγ (data not shown). To confirm further the interaction of S-SCAMβ and γ with SAPAP1, we performed the overlay assay using [35S]methionine-labeled S-SCAMβ and γ. S-SCAMα, -β, and -γ bound to SAPAP1 expressed in COS cells, whereas the probe prepared from pClneo Myc S-SCAM-4 did not (Fig. 3). These findings suggest that the N-terminal portion of the GK domain, which S-SCAMβ and -γ did not contain, was not necessary for the interaction with SAPAP1.

PSD-95/SAP90 and PSD93/chapsyn-110 are reported to form a heteromultimer via the disulfide linkage (12). GRIP and ABP also form a heteromultimer (15). We tested whether the isoforms of S-SCAM formed multimers. The anti-PDZ-0 antibody recognized only S-SCAMα, but the immunoprecipitate with the anti-PDZ-0 antibody contained not only S-SCAMα but also S-SCAMβ and γ in vivo (Fig. 4A), in support that S-SCAMα interacted with S-SCAMβ and γ in vivo. Next, to determine the region involved in the interaction, COS cells were transfected with pClneo S-SCAMβ and various Myc-tagged constructs of S-SCAMα. We immunoprecipitated each Myc-tagged product with the anti-Myc antibody, and we checked whether S-SCAMβ was communoprecipitated. S-SCAMβ bound the product of pClneo Myc S-SCAM-4 or -10 but not the product of pClneo Myc S-SCAM-2 or -3 (Fig. 4B), suggesting that S-SCAMβ interacts with the region containing PDZ-(4 + 5) of S-SCAMα. The GST fusion protein containing PDZ-(4 + 5) of S-SCAMα also interacted with the product of pClneo Myc S-SCAM-1, -4, or -10 (Fig. 5A). Therefore, S-SCAM is likely to form multimers via the self-association between PDZ-(4 + 5). We next tested which of PDZ-4 or -5 was involved in this interaction. PDZ-5 interacted with the product of pClneo Myc S-SCAM-10 containing PDZ-(4 + 5), whereas PDZ-4 did not (Fig. 5B). However, the interaction of GST-PDZ-5 with the product of pClneo Myc S-SCAM-12 containing only PDZ-5 was not detected under the same conditions (data not shown).

To estimate the stoichiometry of the self-association between PDZ-(4 + 5) of S-SCAM, GST-PDZ-(4 + 5) was treated with thrombin to remove the GST tag and then subjected to gel filtration. The calculated molecular weight of PDZ-(4 + 5) is approximately 33,000. In gel filtration, PDZ-(4 + 5) appeared in fraction 30, which corresponded to 78 kDa, and estimated to be a dimer (Fig. 6). Another peak was detected in fraction 38, corresponding to 17 kDa. This peak contained a degradation products of the GST domain, which S-SCAMβ and γ did not contain, was not necessary for the interaction with SAPAP1.

**Fig. 6.** Gel filtration analysis of PDZ-(4 + 5) of S-SCAM. GST-PDZ-(4 + 5) or -PDZ-(3 + 4) of S-SCAM was cleaved with thrombin and subjected to gel filtration analysis. The absorbance at 280 nm is indicated. A, PDZ-(4 + 5); B, PDZ-(3 + 4). The arrowheads indicate the positions of the standard markers as follows: a, γ-globulin (158 kDa); b, chicken ovalbumin (44 kDa); c, myoglobin (17 kDa); and d, vitamin B₁₂ (0.14 kDa).

**Fig. 7.** Clustering of NMDA receptors in COS cells cotransfected with various pClneo Myc constructs of S-SCAM. COS cells were transfected with pCMV NMDAR1 and pCMV NMDAR2A with various pClneo Myc constructs of S-SCAM and double-stained with the anti-NMDAR2A and the anti-Myc antibodies. A, pCMV NMDAR1 and pCMV NMDAR2A alone; B, with pClneo Myc S-SCAMβ; C, with pClneo Myc S-SCAM-10; D, with pClneo Myc S-SCAM-12; and E, with pClneo Myc S-SCAM-8. Upper panel, the staining with the anti-NMDAR2A antibody; and lower panel, the staining with the anti-Myc antibody.
either GST, GST-PDZ-(4
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and the PDZ-(4
5) of S-SCAM, or GST-GK of PSD-95/SAP90, and the proteins on the beads were detected with the anti-Myc antibody. Lane 1, the original extract; lane 2, GST; lane 3, GST-PDZ-(4 + 5) of S-SCAM; and lane 4, GST-GK of PSD-95/SAP90. B, the interaction between the GK domain of PSD-95/SAP90 and the PDZ-(4 + 5) of S-SCAM. The extracts of COS cells transfected with pcDNA Myc PSD-95–8 containing the GK domain were incubated with either GST, GST-PDZ-(4 + 5) of S-SCAM, or GST-GK of PSD-95/SAP90, and the proteins on the beads were detected with the anti-Myc antibody. Lane 1, the original extract; lane 2, the precipitate with GST; lane 3, the precipitate with GST-PDZ-(4 + 5) of S-SCAM; and lane 4, the precipitate with GST-GK of PSD-95/SAP90. C, coimmunoprecipitation of S-SCAM and PSD-95/SAP90. The urea/detergent extracts of rat crude synaptosomes were incubated with the preimmune serum or the rabbit polyclonal anti-PSD-95 antibody, and the immunoprecipitate was immunoblotted with the anti-WW antibody. Lane 1, the original extract; lane 2, the precipitate with the preimmune serum; and lane 3, the precipitate with the anti-PSD-95 antibody.

FIG. 8. Interaction between S-SCAM and PSD-95/SAP90. A, the interaction between the PDZ and GK domains of PSD-95/SAP90. The extracts of COS cells transfected with pcDNA Myc PSD-95–4 containing the PDZ domains were incubated with either GST, GST-PDZ-(4 + 5) of S-SCAM, or GST-GK of PSD-95/SAP90, and the proteins on the beads were detected with the anti-Myc antibody. Lane 1, the original extract; lane 2, GST; lane 3, GST-PDZ-(4 + 5) of S-SCAM; and lane 4, GST-GK of PSD-95/SAP90. B, the interaction between the GK domain of PSD-95/SAP90 and the PDZ-(4 + 5) of S-SCAM. The extracts of COS cells transfected with pcDNA Myc PSD-95–8 containing the GK domain were incubated with either GST, GST-PDZ-(4 + 5) of S-SCAM, or GST-GK of PSD-95/SAP90, and the proteins on the beads were detected with the anti-Myc antibody. Lane 1, the original extract; lane 2, the precipitate with GST; lane 3, the precipitate with GST-PDZ-(4 + 5) of S-SCAM; and lane 4, the precipitate with GST-GK of PSD-95/SAP90. C, coimmunoprecipitation of S-SCAM and PSD-95/SAP90. The urea/detergent extracts of rat crude synaptosomes were incubated with the preimmune serum or the rabbit polyclonal anti-PSD-95 antibody, and the immunoprecipitate was immunoblotted with the anti-WW antibody. Lane 1, the original extract; lane 2, the precipitate with the preimmune serum; and lane 3, the precipitate with the anti-PSD-95 antibody.

FIG. 9. Localizations of S-SCAM, PSD-95/SAP90, and SAPAP in rat retina and cerebellum. Rat retina or cerebellum was immunostained with either the anti-WW, the rabbit polyclonal anti-PSD-95, or the anti-SAPAP antibody and visualized with Texas Red-conjugated second antibody. Left panel, rat retina; and right panel, rat cerebellum. A, S-SCAM; B, PSD-95/SAP90; and C, SAPAP. Bar indicates 10 μm.

product. PDZ-(3 + 4), whose molecular weight was 26,000 appeared in fraction 35, which corresponded to 30 kDa.

We tested whether S-SCAMβ and -γ had the ability to form clusters of NMDA receptors. S-SCAMβ and -γ induced the clustering of NMDA receptors, when coexpressed in COS cells (Fig. 7B and data not shown). We tested which region of S-SCAM was required for the clustering of NMDA receptors. NMDA receptors formed clusters in COS cells, when cotransfected with pcDNA Myc S-SCAM-4 or -10 (Fig. 7C and data not shown) but not with either pcDNA Myc S-SCAM-2, -3, or -12 (Fig. 7D and data not shown). The product of pcDNA Myc S-SCAM-8 lacking PDZ-5 did not induce the clustering of NMDA (Fig. 7E). These findings suggest that the region containing PDZ-(4 + 5) is necessary and sufficient for the clustering of NMDA receptors. The presence of SAPAP1 did not show any effect on the S-SCAM-dependent clustering of NMDA receptors (data not shown).

A recent study has revealed that the region containing three PDZ domains of PSD-95/SAP90 intramolecularly interacts with the GK domain (22). We first confirmed this interaction using the product of pGex4T-1 PSD-95–24 containing 52 amino acids from the GK domain (Fig. 8A). PDZ-(4 + 5) of S-SCAM did not bind the product of pcDNA Myc PSD-95–2 containing the GK domain of S-SCAM (data not shown). Unexpectedly, PDZ-(4 + 5) of S-SCAM interacted with the GK domain of PSD-95/SAP90 (Fig. 8B). To detect the interaction of S-SCAM with PSD-95/SAP90 in vivo, we performed the immunoprecipitation of PSD-95/SAP90. S-SCAM were coimmunoprecipitated with PSD-95/SAP90 from rat brain (Fig. 8C).

In the previous studies, we showed the colocalization of S-SCAM, PSD-95/SAP90, and SAPAP in primary cultured rat hippocampal neurones (10, 17), and here we examined whether S-SCAM, PSD-95/SAP90, and SAPAP were colocalized in vivo. In rat retina, these proteins were localized at inner and outer plexiform layers. In cerebellum, although the localizations of these proteins were not identical, all of S-SCAM, PSD-95/SAP90, and SAPAP were localized at the glomerulus in the granular layer (Fig. 9). Therefore, these three proteins were colocalized at least in some part.

DISCUSSION

In this paper, we have identified smaller isoforms of S-SCAM, S-SCAMβ, and -γ. S-SCAMβ and -γ start at the 164th and 224th methionines, respectively. We previously reported that the region of amino acids 138–185 was the domain involved in the interaction with SAPAP (17). Although S-SCAMβ starts in the middle of this region and S-SCAMγ starts after this region, both of them interact with SAPAP1. The recent version of the simple modular architecture research tool predicts that the region of amino acids 107–291 of S-SCAM is the GK domain (23). Among them, the N-terminal 48 amino acids are rather conserved between the GK domains of PSD-95/SAP90.

Three Isoforms of S-SCAM

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Fig. 10. Alignment of the putative GK domains of PSD-95/SAP90 and S-SCAM.

The conserved amino acids are boxed. The amino acids, which S-SCAMβ and γ contain, are indicated as a gray bar and a black bar, respectively. Open and closed triangles indicate the initiation methionines of S-SCAMβ and γ, respectively. The amino acids encoded by pGex4T-1 PSD-95–24 are shaded. The numbers on the left and right ends indicate the numbers of the amino acid residues of PSD-95/SAP90 and S-SCAMα.

SAP90 and S-SCAM, whereas the middle and C-terminal regions are diverged (Fig. 10), but the interaction of S-SCAMγ with SAPAP suggests that the SAPAP-interacting region of S-SCAM is mapped to the C-terminal region of the GK domain.

The GK domain of PSD-95/SAP90 is reported to interact with the PDZ domains intramolecularly (22). We prepared the GST fusion protein containing only 52 amino acids from the GK domain of PSD-95/SAP90 (Fig. 10, shaded), and we confirmed that this short GK domain still bound the PDZ domains. Interestingly, the putative SAPAP-interacting region of S-SCAM does not contain the amino acids corresponding to these amino acids (Fig. 10, underlined). Although the SAPAP-interacting region of PSD-95/SAP90 needs to be determined, the GK domain of PSD-95/SAP90 may include distinct PDZ-interacting and SAPAP-interacting subdomains.

The physiological significance of the presence of the isoforms of S-SCAM is currently unknown. An unidentified ligand for PDZ-0 could bind only to S-SCAMα. Thus, when S-SCAMα is dominant, such a ligand is assembled with components interacting with other PDZ domains, and when the expression of S-SCAMβ or γ is enhanced, such a ligand is switched off from other components. Such a mechanism might be involved in synaptic plasticity.

The consensus motif for the interaction with PDZ domain was originally known to be the C-terminal sequence Ser/Thr-X-Val (where X is any amino acid). Later, numerous exceptions for the binding motif have been reported (23, 24), and the interactions between PDZ domains have been recognized (15, 25). The PDZ domain of neuronal nitric-oxide synthase interacts with the PDZ domains of PSD-95/SAP90 and syntrophin (25). Recently, the crystal structures of the PDZ domain of neuronal nitric-oxide synthase in complex with that of syntrophin have been studied (26). The β finger that extends beyond the PDZ domain of neuronal nitric-oxide synthase docks into the carboxyl groove of the PDZ domain of syntrophin. In this case, the interaction between the PDZ domains of neuronal nitric-oxide synthase and syntrophin conceivably competes with the binding of the C-terminal peptide to the PDZ domain of syntrophin. GRIP and ABP form a heteromultimer via the PDZ domains (15). It has not been concluded whether the interaction between GRIP and ABP simultaneously occurs with the binding of AMPA receptors to GRIP and ABP. PSD-95/SAP90 has two cysteine residues at the N terminus and forms a tetramer via the disulfide linkage (12). We have found that PDZ-(4 + 5) of S-SCAM also forms a dimer via the PDZ domains. NMDA receptors form clusters when coexpressed with PDZ-(4 + 5) of S-SCAM but not when coexpressed with PDZ-5, suggesting that both of PDZ-4 and -5 are necessary in the interaction or that the insert between PDZ-4 and PDZ-5 is involved in the interaction. S-SCAM also uses PDZ-5 to bind NMDA receptors. We have not yet determined whether a PDZ-(4 + 5) that binds NMDA receptors can still interact with the other PDZ-(4 + 5).

The dimerization of S-SCAM may not be sufficient to induce the clustering of NMDA receptors, because no more than two molecules of NMDA receptors are assembled. In this study, we have detected that PDZ-(4 + 5) of S-SCAM binds the GK domain of PSD-95/SAP90 in vitro and that S-SCAM interacts with PSD-95/SAP90 in vivo. The interaction between S-SCAM and PSD-95/SAP90 may be involved in the clustering of NMDA receptors as well as that between the isoforms of S-SCAM.

S-SCAM, PSD-95/SAP90, and SAPAP. S-SCAM forms dimers and also interacts with the GK domain of PSD-95/SAP90 via its PDZ-(4 + 5). The GK domain of PSD-95/SAP90 interacts with the PDZ domains of PSD-95/SAP90. The GK domains of S-SCAM and PSD-95/SAP90 bind SAPAP. The PDZ domains of S-SCAM and PSD-95/SAP90 interact with NMDA receptors. For simplicity, all interactions are depicted in this model, but it should be noted that it is not clear whether these interactions take place simultaneously.
some part in brain, and these proteins are coimmunoprecipitated from rat brain, suggesting that these proteins interact in vivo. We summarize the interactions proposed in this study (Fig. 11). Although it remains to be clarified whether these interactions take place simultaneously, these interactions may be important to sustain the architecture of the PSD.

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