A Novel Multiple PDZ Domain-containing Molecule Interacting with N-Methyl-D-aspartate Receptors and Neuronal Cell Adhesion Proteins

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At synaptic junctions, pre- and postsynaptic membranes are connected by cell adhesion and have distinct structures for specialized functions. The presynaptic membranes have a machinery for fast neurotransmitter release, and the postsynaptic membranes have clusters of neurotransmitter receptors. The molecular mechanism of the assembly of synaptic junctions is not yet clear. Pioneering studies identified postsynaptic density (PSD)-95/SAP90 as a prototypic synaptic scaffolding protein to maintain the structure of synaptic junctions. PSD-95/SAP90 belongs to a family of membrane-associ- ated guanylate kinases and binds N-methyl-D-aspartate receptors, potassium channels, and neuroligins through the PDZ domains and GKAP/SAPAP/DAP through the guanylate kinase (GK) domain. We performed here a yeast two-hybrid screening for SAPAP-interacting molecules and identified a novel protein that has an inverse structure of membrane-associated guanylate kinases with an NH2-terminal GK-like domain followed by two WW and five PDZ domains. It binds SAPAP through the GK-like domain and NMDA receptors and neuroligins through the PDZ domains. We named this protein S-SCAM (synaptic scaffolding molecule) because S-SCAM may assemble receptors and cell adhesion proteins at synaptic junctions.

Effective neurotransmission requires the precise localization of the receptors for neurotransmitters opposite to the presynaptic active zone. The postsynaptic membrane has a dense thickening of submembranous cytoskeleton, called postsynaptic density (PSD)3 (for review, see Refs. 1 and 2). PSDs play essential roles in the localization of the receptors. Recent studies have identified several molecules that may function in the accumulation and clustering of various receptors at PSDs. PSD-95/SAP90 and its isoforms interact with NMDA receptors and Shaker type potassium channels (3–10), whereas glutamate receptor-interacting protein and Homer interact with α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid and metabotropic glutamate receptors, respectively (11, 12). The common feature for these molecules is that they have a modular PDZ domain for protein-protein interactions (for review, see Refs. 13–16).

PDZ domains were originally recognized as the repeats of about 90 amino acids in PSD-95/SAP90 and named after three proteins containing such repeats, PSD-95/SAP90, Drosophila discs-large tumor suppressor protein (Dlg-A), and a tight junction protein, ZO-1 (3, 4, 17–19). The PDZ domains bind to COOH-terminal sequences of various proteins. The first and second PDZ domains of PSD-95/SAP90 interact with NMDA receptors and Shaker type potassium channels (5, 6). PSD-95/SAP90 is multimerized through the NH2-terminal disulfide linkage and clusters these receptors and channels (20). The third PDZ domain of PSD-95/SAP90 binds to the cytoplasmic domain of neuronal cell adhesion molecules, called neuroligins (21).

PSD-95/SAP90 has one SH3 domain and one guanylate kinase (GK) domain in addition to the PDZ domains. The SH3 domain was defined as a signaling module in Src tyrosine kinase and was later identified in many other proteins (for review, see Ref. 22). The function of the SH3 domain of PSD-95/SAP90 is unknown. The GK domain is homologous to yeast guanylate kinase and is present in various membrane-associated proteins. These proteins are thought to maintain specialized submembranous structures and are named membrane-associated guanylate kinases (for review, see Refs. 23 and 24).

Molecules interacting with the GK domain of PSD-95/SAP90 have been identified by three groups and named GKAP/SAPAP/DAP (25–28). In HEK293 cells, SAPAP is tightly associated with the membrane and recruits PSD-95/SAP90 to the membrane (26). If this observation reflects the event that precedes the formation of PSDs in vivo, SAPAP may be a core molecule of PSDs. The identification of other molecules interacting with SAPAP will yield clues to the roles of SAPAP in the formation of PSDs. We have searched for such molecules and have identified a novel protein that we named S-SCAM (synaptic scaffolding molecule).

Experimental Procedures

DNA Constructs—Various constructs were prepared using pBTM116, pVP16-3, pCMV5, pGex5X-3 (Amersham Pharmacia Biotech), pGexKG, and pClneo Myc. pClneo Myc was constructed by ligating the oligonucleotides etacagcc acctgctcgagg/aattcctcgagcaggtcctcctcgctgataagcttctgctccatgttggggggt into NheI/EcoRI sites of pClneo (Promega). pBTM116 SAPAP1–2 contains residues 397–568 of SAPAP1. The following constructs of S-SCAM were prepared:
P-SCAM, synaptic scaffolding molecule; DRPLA, dentatorubral pallidoluysian atrophy; GST, glutathione S-transferase; SPM fraction, synaptosomal membrane fraction.
RESULTS

Identification of S-SCAM—We screened a rat brain cDNA library by the yeast two-hybrid method using the residues 397–568 of SAPAP1 as a bait. This region contains the potential PSD-95/SAP90-interacting domain and a proline-rich region (Fig. 1) (26). Nine positive clones were obtained. Among them, two clones were PSD-95/SAP90, and one clone was PSD93/chapsyn (7, 32). The remaining six clones had the same novel sequence containing a GK-like domain and a WW domain. The full-length sequence of S-SCAM obtained from a rat brain cDNA library predicted synthesis of a novel protein composed of 1,277 amino acids (Fig. 2). This protein had a GK-like domain at the NH$_2$ terminus followed by two WW and five PDZ domains (Fig. 2). The GK-like domain of S-SCAM is composed of about 50 amino acids and has only a small part of the consensus amino acids of the GK domain. The first WW domain of S-SCAM is the most similar to those of YAP and Nedd-4 (33, 34). The second WW domain is incomplete and lacks the second tryptophan. The PDZ domains are distributed almost evenly from the middle to the COOH-terminal region of S-SCAM. Two sets of amino acid repeats are inserted between the fourth and fifth PDZ domains. One set is composed of four copies of a seven-amino-acid sequence, SPXQAQQX (underlined in Fig. 2). The second set is composed of eight copies of an eight-amino-acid sequence, RQPPXXDY (double-underlined in Fig. 2). The significance of these repeats is unknown. S-SCAM showed high homology to the product of K01A.6 clone of Caenorhabditis elegans, which may be a homolog of S-SCAM. During this study, MAGI-1 with a structure similar to that of S-SCAM was reported (35). MAGI-1 was identified as a K-RasB-interacting protein, and three splice variants were reported. Among them, MAGI-1b is the most similar to S-SCAM and has 48% homology to S-SCAM at the amino acid level. MAGI-1 has a polyglutamine domain containing seven glutamines between the second WW domain and the first PDZ domain, whereas S-SCAM does not. WWP3, a recently identified human WW domain-containing gene (36), also has a sequence highly homologous to that of S-SCAM. The partial sequence of WWP3 was first reported with the accession number U96115, and later the longer sequence was reported with the accession number U80754. U80754 was identified as a gene encoding a protein with WW domains that interacted with atrophin-1 (37, 38). Atrophin-1 is the product of the gene responsible for dentatorubral pallidoluysian atrophy (DRPLA) and has a polyglutamine repeat. The sequence of U80754 also has a polyglutamine domain, and more than 80% of the amino acids are the same as those of MAGI-1, although the polyglutamine domain of WWP3 is larger than that of MAGI-1. Therefore, WWP3 is considered to be a human MAGI-1, and S-SCAM is an isoform of WWP3/MAGI-1.

Tissue Distribution of S-SCAM—Northern blot analysis showed prominent 7.5- and 6.8-kilobase messages only in brain (Fig. 3A). Proteins with molecular masses of 180, 155, and 140 kDa were detected only in brain in Western blots using polyclonal antibodies against the WW domain or NH$_2$-terminal region of S-SCAM (Fig. 3B and data not shown). Three proteins of 180, 155, and 140 kDa were also detected in the extracts of COS cells transfected with pCMV S-SCAM (Fig. 3B), suggesting that the proteins with smaller sizes may be the proteolytic degradation products of S-SCAM or smaller transscripts using other methionines as initiation codons.

Interaction of S-SCAM with SAPAP—To obtain independent evidence for the interaction between SAPAP and S-SCAM, we overlaid blots of extracts of COS cells expressing various SA-
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PAP isoforms with [35S]methionine-labeled S-SCAM. S-SCAM interacted with all of the SAPAP isoforms but not with a control protein, neurabin-l (39) (Fig. 4A). To determine the interacting domain of S-SCAM with SAPAP, probes encoding various regions of S-SCAM were prepared and used in the overlay assay with SAPAP1 (Fig. 4B). Proteins containing the full-length or NH2-terminal region of S-SCAM (pClneo Myc S-SCAM and -2) interacted with SAPAP1, whereas proteins containing the middle or COOH-terminal region of S-SCAM (pClneo Myc S-SCAM-3 and -4) did not (Fig. 4C). Therefore, the NH2-terminal region containing the GK-like domain is required for the interaction with SAPAP. Because PSD-95/SAP90 interacts with SAPAP through the GK domain, S-SCAM and PSD-95/SAP90 may bind to the same region of SAPAP. To test this, SAPAP1 was overlaid with [35S]methionine-labeled S-SCAM in the presence of GST-PSD-95-2 containing the PDZ domains or GST-PSD-95-4 containing the GK domain. GST-PSD-95-4 inhibited the interaction, whereas GST-PSD-95-2 did not (Fig. 4D). Thus, S-SCAM and PSD-95/SAP90 compete for the same binding site on SAPAP, and the GK-like domain of S-SCAM may interact with SAPAP like the GK domain of PSD-95/SAP90.

Interactions of S-SCAM with NMDA Receptors and Neuroligins in Yeast Two-hybrid Method—The PDZ domains of S-SCAM showed the highest homology to those of PSD-95/SAP90 and its isoforms among the PDZ domains of various proteins. Because the PDZ domains of PSD-95/SAP90 interact with NMDA receptors, Shaker type potassium channels, and neuroligins (5, 6, 21), the PDZ domains of S-SCAM may also interact with these molecules. Actually, two prey clones containing amino acid residues 962–1237 and 1038–1237 of NMDAR2C were obtained in yeast two-hybrid screenings with a bait containing the PDZ domains of S-SCAM. As a next step, we tested the interactions of S-SCAM with NMDA receptors and other molecules using the yeast two-hybrid method. The β-galactosidase assays were performed using the COOH termini of NMDAR2A, -2C, neuroligin 1, and Shaker type Kv1.4 potassium channel as baits with various PDZ domains of S-SCAM as preys. The first PDZ domain interacted with neuroligin 1, whereas the fifth PDZ domain interacted with NMDA receptors and weakly with Shaker type Kv1.4 potassium channel (Table I). Therefore, S-SCAM interacts with NMDA receptors and neuroligin 1 via the distinct PDZ domains.

Binding of the PDZ Domains of S-SCAM to the COOH Termini of NMDAR2A and Neurulin 1—To obtain insight into their relative affinities, we studied the interactions of S-SCAM with NMDA receptors and neuroligins in a yeast two-hybrid method. The PDZ domains of S-SCAM showed the highest homology to those of PSD-95/SAP90 and its isoforms among the PDZ domains of various proteins. Because the PDZ domains of PSD-95/SAP90 interact with NMDA receptors, Shaker type potassium channels, and neuroligins (5, 6, 21), the PDZ domains of S-SCAM may also interact with these molecules. Actually, two prey clones containing amino acid residues 962–1237 and 1038–1237 of NMDAR2C were obtained in yeast two-hybrid screenings with a bait containing the PDZ domains of S-SCAM.
with the COOH termini of NMDAR2A and neuroligin 1 by the BIAcore biosensor technology. Synthetic peptides containing the COOH-terminal 15 amino acids of NMDAR2A or neuroligin 1 were fixed on a sensor chip. GST constructs containing various PDZ domains of S-SCAM were superfused on the chip. The first PDZ domain of S-SCAM interacted with the COOH terminus of neuroligin 1 (KD = 5.2 ± 0.5 × 10⁻⁷ M), and the fifth PDZ domain of S-SCAM interacted with the COOH terminus of NMDAR2A (KD = 5.7 ± 0.3 × 10⁻⁷ M). Other PDZ domains interacted with neither NMDAR2A nor neuroligin 1 in this assay (data not shown). The affinities thus determined were similar to those we measured previously for the PDZ domains of PSD-95/SAP90 with the COOH termini of NMDAR2A and neuroligin 1 (21).

Colocalization of S-SCAM with NMDAR2A and Neuroligin 1 on the Plasma Membranes in HEK293 Cells—To test the interactions of S-SCAM with NMDAR2A and neuroligin 1 in intact cells, we transfected HEK293 cells with various combinations of pCMV Myc S-SCAM, pCMV NMDAR2A, and pCMV neuroligin 1. S-SCAM was distributed in the cytosol, when expressed alone (Fig. 5A). NMDAR2A and neuroligin 1 were localized on the membrane, although some signals were also detected in the cytosol (Fig. 5A, B, C, and D).

**TABLE I**

Interactions of the PDZ domains of S-SCAM with NMDA receptors, neuroligin 1, and Shaker type potassium channel in the yeast two-hybrid method

| Bait vector (in pBTM116) | Prey vector (in pVP16–3) | PDZ1 | PDZ2 | PDZ3 | PDZ4 | PDZ5 |
|--------------------------|--------------------------|------|------|------|------|------|
| NR2A                     | ND                       | ND   | ND   | ND   | ND   | 800 ± 20 |
| NR2C                     | ND                       | ND   | ND   | ND   | ND   | 640 ± 30 |
| NL1–1                    | 250 ± 35                 | ND   | ND   | ND   | ND   | ND |
| Shaker                   | ND                       | ND   | ND   | ND   | ND   | 25 ± 1 |

Data list β-galactosidase activities of yeast strain harboring the respective bait and prey plasmids. pVP16–3 S-SCAM PDZ1, PDZ2, PDZ3, PDZ4, and PDZ5 encode each PDZ domain of S-SCAM. pBTM116 NR2A, NR2C, NL1–1, and Shaker encode the COOH termini of NMDAR2A, neuroligin 1, and rabbit Kv1.4 potassium channel. Data shown are nmoles of substrate hydrolyzed/min/mg of protein ± S.D. after background subtraction. ND, no detectable activity.
cytosol (Fig. 5B and data not shown). When S-SCAM was coexpressed with NMDAR2A, S-SCAM was colocalized with NMDAR2A on the membrane (Fig. 5C). When S-SCAM was coexpressed with neurogin 1, S-SCAM was also colocalized with neurogin 1 on the membrane (data not shown). These results suggest that S-SCAM is recruited to the plasma membrane by the interaction with NMDA receptors and neurogin 1 in vivo.

Coimmunoprecipitation of S-SCAM and NMDA Receptors—To determine whether S-SCAM interacts with NMDA receptors in the native environment in neurons, we performed the immunoprecipitation of S-SCAM from the detergent extract of the crude rat brain synaptosomes. Because the monoclonal antibody against NMDAR2A was not available, we used the monoclonal antibody against NMDAR1 and checked whether NMDA receptors were coimmunoprecipitated. The anti-S-SCAM antibody coimmunoprecipitated NMDAR1, whereas the preimmune serum did not (Fig. 6). This result indicates that S-SCAM interacts with NMDA receptors in vivo.

Distribution of S-SCAM in Neurons—To examine the distribution of S-SCAM in neurons, we double-stained primary cultured rat hippocampal neurons on day 14 in the combination of anti-S-SCAM antibody with either anti-NMDAR1 or anti-PSD-95/90 antibody. The staining pattern of S-SCAM was similar to that of PSD-95/90, which was detected in the cell body as well as at the dendrites (Fig. 7A). S-SCAM showed distributions similar to those of NMDAR1 at dendrites (Fig. 7B). Because SAPAP was distributed in a manner similar to that of PSD-95/90 (Fig. 7C), S-SCAM was considered to be distributed in a manner similar to SAPAP.

We also determined the distribution of S-SCAM in the subcellular fractions of rat brain by the immunoblotting with the anti-S-SCAM antibody. The signals detected with the antibody were distributed in both the cytosolic synaptosomal (S2) and crude synaptosomal pellet (P2) fractions (Fig. 8). In the subfractionations of synaptosomes, the signals were the most remarkable in the synaptosomal membrane fraction (SPM). The signals were also detected in the PSD fraction, although S-SCAM was not as enriched as SAPAP (Fig. 8).

**DISCUSSION**

Synaptic junctions are asymmetric interneuronal junctions that transmit signals from a presynaptic neuron to a postsynaptic neuron. The pre- and postsynaptic membranes contain characteristic submembranous structures, active zones, and PSDs. It is thought that the adhesion between the pre- and postsynaptic membranes and the assembly of the components of active zones and PSDs on each membrane lead the formation of synaptic junctions. However, the molecular mechanisms of this process are unclear. Recent studies have revealed that PSD-95/90 and its isoforms interact with receptors and channels through the PDZ domains and assemble them at synaptic junctions (3–6). PSD-95/90 also interacts through
the GK domain with proteins, which are named GKAP/SAPAP/DAP (25–27). Although the functions of GKAP/SAPAP/DAP are not yet clear, they are tightly associated with PSDs and recruit PSD-95/SAP90 to the membranes in transfected cells. Therefore, GKAP/SAPAP/DAP may be a core protein of PSDs, and various components of PSDs may be assembled around GKAP/SAPAP/DAP. In this study, we have obtained a novel protein interacting with SAPAP and named it S-SCAM. S-SCAM is similar to the recently reported protein, WWP3/MAGI-1. In contrast to WWP3/MAGI-1, S-SCAM is expressed only in brain and considered to be a neuronal isoform of WWP3/MAGI-1.

S-SCAM is distributed both in the cytosol and membrane fractions in rat brain. In the further subfractionation, S-SCAM is recovered in the PSD fraction. Even after Triton X-100 extraction, a significant amount of S-SCAM is insoluble and detected in the PSD fraction. The amount of S-SCAM recovered in the PSD fraction is less than that in the SPM fraction, suggesting that S-SCAM is not as tightly attached to PSDs as SAPAP is. Consistently, in rat hippocampal neurons, S-SCAM is also detected in the cell body. The distribution of S-SCAM is reminiscent of that of PSD-95/SAP90. PSD-95/SAP90 is localized in the cytosol and membrane fractions in rat brain and detected in the cell body as well as at the synaptic junctions in rat hippocampal neurons. The reason why some S-SCAM and PSD-95/SAP90 are soluble and others are insoluble is currently unclear. S-SCAM and PSD-95/SAP90 may be peripheral components of PSDs and recruited from the cytosol to PSDs.

The domain structure of S-SCAM at the same time is similar to that of PSD-95/SAP90 and differs from it. Both proteins contain GK-like or GK domains, but these are localized at different ends of the proteins. In both proteins, these domains bind SAPAP. Both S-SCAM and PSD-95/SAP90 have protein modules specialized for binding proline-rich sequences, but in S-SCAM this module is a WW domain and in PSD-95/SAP90 an SH3 domain. The WW domain is reported to bind to a peptide containing PPPXY (40). SAPAP does not have this motif, and the ligand for the WW domain of S-SCAM remains to be identified. Atrophin-1, the product of DRPLA gene, was reported to interact with the WW domains of WWP3/MAGI-1 (37, 38). Because the WW domains are highly conserved between S-SCAM and WWP3/MAGI-1, atrophin-1 is likely to bind to S-SCAM. Another similarity between S-SCAM and PSD-95/SAP90 is that both of the proteins have PDZ domains that bind to NMDA receptors and neuroligins. However, S-SCAM has five PDZ domains, whereas PSD-95/SAP90 has three. The first PDZ domain of S-SCAM interacts with the COOH termini of neuroligins, and the fifth PDZ domain binds to the COOH termini of NMDA receptors. These findings suggest that each PDZ domain has a distinct partner and that the second, third, and fourth PDZ domains may interact with other proteins besides NMDA receptors and neuroligins. Therefore, S-SCAM may function as a novel scaffolding molecule to assemble various components at synaptic junctions and may cooperate with PSD-95/SAP90 to maintain the structure of synaptic junctions.

Several neurodegenerative diseases, including spinobulbar muscular atrophy, Huntington disease, spinocerebellar ataxia, Machado-Joseph disease, and DRPLA, arise from expansion of a CAG repeat-encoding glutamine (41–44). Atrophin-1 with a polyglutamine repeat composed of 14 glutamines was identified to be the gene responsible for DRPLA and reported to interact with the WW domains of WWP3/MAGI-1 (37, 38). Interestingly, WWP3/MAGI-1 also has a polyglutamine repeat, and the expansion mutation in WWP3/MAGI-1 might cause the phenotype similar to DRPLA. As discussed above, S-SCAM is likely to interact with atrophin-1 via the WW domains and may be involved in the pathogenesis of DRPLA.

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