SAPAPs

A FAMILY OF PSD-95/SAP90-ASSOCIATED PROTEINS LOCALIZED AT POSTSYNAPTIC DENSITY*

(Received for publication, November 27, 1996, and in revised form, January 23, 1997)

Masakazu Takeuchi‡, Yutaka Hata‡, Kazuyo Hirao‡, Atsushi Toyoda‡, Mina Irie‡, and Yoshimi Takai‡§§

From ‡Takai Biotimer Project, ERATO, Japan Science and Technology Corporation, c/o JCR Pharmaceuticals Co., Ltd., 2-2-10 Murotani, Nishi-ku, Kobe 651-22 and the §Department of Molecular Biology and Biochemistry, Osaka University Medical School, Suita 565, Japan

PSD-95/SAP90 is a member of membrane-associated guanylate kinases localized at postsynaptic density (PSD) in neuronal cells. Membrane-associated guanylate kinases are a family of signaling molecules expressed at various submembrane domains which have the PDZ (DHR) domains, the SH3 domain, and the guanylate kinase domain. PSD-95/SAP90 interacts with N-methyl-D-aspartate receptors 2A/B, Shaker-type potassium channels, and brain nitric oxide synthase through the PDZ (DHR) domains and clusters these molecules at synaptic junctions. However, neither the function of the SH3 domain or the guanylate kinase domain of PSD-95/SAP90, nor the protein interacting with these domains has been identified. We have isolated here a novel protein family consisting of at least four members which specifically interact with PSD-95/SAP90 and its related proteins through the guanylate kinase domain, and named these proteins SAPAPs (SAP90/PSD-95-Associated Proteins). SAPAPs are specifically expressed in neuronal cells and enriched in the PSD fraction. SAPAPs induce the enrichment of PSD-95/SAP90 to the plasma membrane in transfected cells. Thus, SAPAPs may have a potential activity to maintain the structure of PSD by concentrating its components to the membrane area.

The PSD is a dense thickening of postsynaptic submembranous cytoskeleton observed in electron microscopy. Since PSD has a characteristic structure and is contiguous to the presynaptic active zone, where neurotransmitter release occurs, PSD is proposed to have several functions, such as the stabilization of synaptic junctions, the concentration and regulation of neurotransmitter receptors, and the induction of the transcriptions in response to the synaptic transmission (reviewed in Ref. 1). Many studies have revealed several components of PSD, including fodrin (2), tubulin (3), actin (4), calmodulin (5), Ca\(^{2+}\)/calmodulin-dependent protein kinase II (6), and PSD-95/SAP90 (7–9). However, there still remain many components to be identified and the mechanism how the individual components are assembled to form PSD is unknown.

PSD-95/SAP90, one of the components of PSD, has a characteristic molecular structure composed of three PDZ (DHR) domains, one SH3 domain, and one guanylate kinase domain. Three isoforms, SAP97, PSD-93/chapsyn, and SAP102, have been reported and all isoforms keep the same molecular structure (10–14). Recently, many studies have revealed the function of the PDZ (DHR) domains. The PDZ (DHR) domain is responsible for protein-protein interactions and identified in various proteins (reviewed in Ref. 15). PSD-95/SAP90 and its isoforms interact with NMDA receptors, Shaker-type potassium channels, and brain nitric oxide synthase through the PDZ (DHR) domains to induce the clustering of these molecules at PSD (12–14, 16–20). Thus, PSD-95/SAP90 is important for the concentration of receptors and channels at PSD.

Another line of evidence for the importance of PSD-95/SAP90 at synaptic junctions comes from the recent findings about MAGUKs (reviewed in Ref. 21). MAGUKs are a family of proteins expressed at various submembrane domains. They include Drosophila discs-large tumor suppressor gene (dlg-A) (22), nematode lin-2 (23), palmitoylated erythrocyte membrane protein (p55) (24), ZO-1 (25, 26), ZO-2 (27), CASK (28), PSD-95/SAP90 (7, 8), and the isoforms of PSD-95/SAP90 (10–14). All the members of MAGUKs keep the same molecular structures as that of PSD-95/SAP90. They have the PDZ (DHR), the SH3, and the guanylate kinase domains. Based on the characteristic molecular structure, MAGUKs are considered to play important roles in maintaining the structures of submembrane domains and to be involved in signaling at these domains. Indeed, genetic evidence indicates that MAGUKs are essential for maintenance of the structures of cell junctions. The product of dlg-A is expressed at separate junctions and neuromuscular junctions, and the mutations of this gene lead to neoplastic overgrowth of imaginal discs and morphological changes of synaptic bouton structures (22, 29). Lin-2 expressed at Pn.p cells in nematode is involved in the localization of let-23, a receptor for lin-3, and essential for vulval induction (23). In the context of the general concept about MAGUKs, PSD-95/SAP90 is assumed to play pivotal roles in signaling at synaptic junctions, not only in the clustering of receptors, which may be mediated by domains other than the PDZ (DHR) domains.

In contrast to the accumulation of information about the PDZ (DHR) domains, our knowledge about the SH3 domain and the guanylate kinase domain of PSD-95/SAP90 is limited. The SH3 domain is also responsible for protein-protein interactions and
identified in a wide variety of proteins. However, the molecules interacting with the SH3 domain of PSD-95/SAP90 have not been identified. The guanylate kinase domain, is similar to an enzyme guanylate kinase, has the binding activity for GMP and GDP, but does not show kinase activity (30). The mutations in this domain of dlg-A cause the abnormalities of imaginal discs (22). Thus, this domain should also have some essential role.

To clarify the function of PSD-95/SAP90 and to identify novel components of PSD, we have tried to identify by use of the yeast two-hybrid method the molecules which interact with the region of PSD-95/SAP90 containing the SH3 domain and the guanylate kinase domain, and are localized at PSD. We have obtained novel proteins which are homologous to each other. We named them SAPAPs (SAP90/PSD-95-Associated Proteins). SAPAPs function to induce the translocation of PSD-95/SAP90 from the cytosol to the plasma membrane and may be a new family of proteins involved in signaling at PSD.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening and β-Galactosidase Assays—The bait vector, pBTM116, and the prey vector, pJL115, encoding amino acid residues 430–724 of PSD-95/SAP90 into EcoRI/Sall sites of pBTM116 (31). The yeast two-hybrid library constructed from adult rat brain cDNA was screened using pBTM116 PSD95-3 as a bait, as described previously (28). β-Galactosidase assays were performed as described previously (28).

cDNA Cloning—To obtain full-length clones, rat brain cDNA libraries in λ ZAP II (Stratagene) were screened using the inserts from pPrey1201, pPrey1305, pPrey1310, and pPrey1377 as described previously (28). For pPrey1201, 1 × 10^6 clones were screened and 25 positive clones were obtained. For pPrey1305, 2 × 10^6 clones were screened and 10 positive clones were obtained. For pPrey1310, 1 × 10^6 clones were screened and 60 positive clones were obtained. For pPrey1377, 1 × 10^6 clones were screened and 25 positive clones were obtained. Two at least overlapping clones for each SAPAP were analyzed. DNA sequencing was performed by the dideoxy nucleotide termination method using an ABI373 DNA sequencer.

Constructions of Expression Vectors—Prokaryote and eukaryote expression vectors, bait constructs, and prey constructs were constructed in pGexKg (32), pMalC2 (New England Biolabs), pCMV5 (a gift of Dr. Stanley Hollenberg) as described previously (34). Various GST expression vectors, bait constructs, and prey constructs were constructed using pGex-2t and pMalC2 vectors according to the manufacturer’s protocol. Various antibodies were raised against the GST fusion proteins of SAPAP1 (anti-SAPAP1), -2 (anti-SAPAP2), -3 (anti-SAPAP3), and -4 (anti-SAPAP4) using rabbits. The antigens used for each antibody constructed the following amino acid residues: anti-SAPAP1, 589–689 of SAPAP1; anti-SAPAP2, 569–676 of SAPAP2; anti-SAPAP3, 560–654 of SAPAP3; and anti-SAPAP4, 592–688 of SAPAP4. The amino acid residues used for the antibodies were specific for each SAPAP and each antibody was confirmed to be specific for each SAPAP. The antibody against GST-PSD95/SAP90 was raised against the GST fusion protein containing the full-length of PSD-95/SAP90 using rabbits (anti-PSD-95) or mice (monoclonal anti-PSD-95). The antibody against NMDA receptors 2A/B and various second antibodies were purchased from Chemicon. The monoclonal antibody against the Myc tag, QE10, was obtained from American Type Culture Collection.

Hippocampal Cell Culture and Immunocytochemistry—Embryos were obtained from Wistar rats on the gestation day 20. Hippocampal cells were isolated, dissociated, plated on poly-l-lysine-coated glass coverslips (Matsunami), and cultured in minimal essential medium with 10% horse serum. After 4 days, the medium was replaced with minimal essential medium supplemented with N2 supplement (36), 1 mg/ml ovalbumin, 1 mg/ml pyruvate, and 5 mg/ml cytosine arabinoside. Immunocytochemistry of hippocampal cells and 293 cells was performed using a confocal imaging system (Bio-Rad MRC1024).

Subcellular Fractionation of 293 Cells—293 cells transfected with various pCMV constructs were sonicated in lysis buffer (20 mM Hepes/NaOH pH 7.4, 10 μM (4-aminophenyl)-methanesulfonyl fluoride, 10 mM leupeptin, and 5 mg/ml aprotinin) using 300 μl/10-cm dish. The lysates were centrifuged at 100,000 × g for 30 min at 4 °C. 1 ml of the supernatant was incubated with 30 pmol of various GST fusion proteins immobilized on the glutathione-Sepharose 4B beads for 2 h at 4 °C. The beads were collected by centrifugation, washed with lysis buffer four times, and analyzed through SDS-PAGE and immunoblotting with the monoclonal antibody against the Myc tag.

RESULTS

Isolation of SAPAPs Through the Yeast Two-hybrid Screening—By use of the yeast two-hybrid method, we first searched for the proteins which interacted with the region of PSD-95/SAP90 containing both the SH3 domain and the guanylate kinase domain. 1.4 × 10^6 yeast transformants with a rat brain cDNA library were screened. Nine positive clones were obtained and six clones of them were independent. Four clones

In Vitro Binding Experiment Using the GST Fusion Proteins and the Extracts of COS Cells—COS cells transfected with pCMV Myc SAPAP1 were sonicated in lysis buffer (20 mM Hepes/NaOH, pH 8.0, 150 mM NaCl, 1 mM EDTA, 25 mM N-ethyl-p-glucoside, 1 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, and 5 mg/liter aprotinin) using 500 μl of each 10-cm dish. The lysates were centrifuged at 100,000 × g for 30 min at 4 °C. 1 ml of the supernatant was incubated with 30 pmol of various GST fusion proteins immobilized on the glutathione-Sepharose 4B beads for 2 h at 4 °C. The beads were collected by centrifugation, washed with lysis buffer four times, and analyzed through SDS-PAGE and immunoblotting with the monoclonal antibody against the Myc tag.

Surface Plasmon Resonance Measurements—The interactions of PSD-95/SAP90 and SAPAPs were evaluated by use of Biacore biosensor technology (Pharmacia Biosensor) based on the basic principles and detection methods as described previously (35). The antibodies against GST were covalently fixed on a CM5 research grade sensor chip by the amine coupling kit (Pharmacia) and then the Escherichia coli lysates containing approximately 500 pmol of GST-1201-3, GST-1305-2, and GST-1305-3 contained amino acid residues 315–569 of SAPAP1. GST-1305-2 contained amino acid residues 480–577 of SAPAP2. The chip was equilibrated with phosphate-buffered saline and various doses of various purified MBPs in 200 μl of phosphate-buffered saline were injected across the immobilized surfaces at a flow rate of 20 μl/min. The binding activities (OB) were assured as the baseline value determined 10 s prior to the sample injection and the measurements taken at the indicated time points. All experiments were performed at 25 °C. Data were analyzed using BIAevaluation program 2.1 (Pharmacia).

Antibodies—Polyclonal antibodies were raised against the GST fusion proteins of SAPAP1 (anti-SAPAP1), -2 (anti-SAPAP2), -3 (anti-SAPAP3), and -4 (anti-SAPAP4) using rabbits. The antigens used for each antibody contained the following amino acid residues: anti-SAPAP1, 589–689 of SAPAP1; anti-SAPAP2, 569–676 of SAPAP2; anti-SAPAP3, 560–654 of SAPAP3; and anti-SAPAP4, 592–688 of SAPAP4. The amino acid residues used for the antibodies were specific for each SAPAP and each antibody was confirmed to be specific for each SAPAP. The antibody against PSD-95/SAP90 was raised against the GST fusion protein containing the full-length of PSD-95/SAP90 using rabbits (anti-PSD-95) or mice (monoclonal anti-PSD-95). The antibody against NMDA receptors 2A/B and various second antibodies were purchased from Chemicon. The monoclonal antibody against the Myc tag, QE10, was obtained from American Type Culture Collection.
(pPrey 1201, pPrey1305, pPrey1310, and pPrey1377) contained similar but not the same sequences, which showed no homology to known proteins. The remaining two clones (pPrey1294 and pPrey1314) did not show any homology to known proteins. We focused on pPrey1201, pPrey1305, pPrey1310, and pPrey1377, since the preliminary Northern blot analysis using the inserts from these prey clones showed strong signals only in brain and named the corresponding proteins SAPAP1 to -4.

Structures of SAPAPs—To determine the whole structures of SAPAPs, rat brain cDNA libraries were screened to obtain overlapping cDNA clones for each SAPAP using the insert from the corresponding prey clone as each probe. The sequence contexts of the putative initiator methionines of SAPAP1, -3, and -4 agreed well with the conserved sequence for initiator codons and were preceded by in-frame stop codons (39). The clone of SAPAP2 had another methionine at 237 bases upstream to the putative initiator codon. We identified the putative initiator methionine in the cDNA of SAPAP2 by comparison with those of other SAPAPs. The coding regions of SAPAPs showed 41–52% homology at the amino acid level, while 5' and 3' non-

![Amino acid sequences of SAPAP1s (A), alignment of SAPAPs.](http://www.jbc.org/)

---

**FIG. 1. Amino acid sequences of SAPAPs.**

**A**, alignment of SAPAPs. Sequences are shown in single-letter amino acid code and numbered on the right. Residues conserved among all SAPAPs are shown on a black background. Residues conserved among three SAPAPs are shown on a gray background. The potential interacting domain with PSD-95/SAP90 is underlined. **B**, SAPAPs' sequence homologies. The numbers represent the percentages of the amino acid homologies between the indicated SAPAPs.
Yeast clones cotransfected with the prey pVP16 and the bait pBTM116 vectors in the indicated combinations were selected on the selection plates and grown in selection medium. pBTM116 PSD-95–3, pBTM116 SAP97–5, pBTM116 PSRP1–3, pBTM116 dlg-1, pBTM116 p55–1, pBTM116 CASK-11, and pBTM116 ZO-1–1 contained both the SH3 domain and the guanylate kinase domain of various MAGUKs. pBTM116 PSD-95–9, pBTM116 SAP97–4, pBTM116 PSRP1–2, pBTM116 dlg-2, and pBTM116 CASK-10 contained the guanylate kinase domain of various MAGUKs. pBTM116 PSD-95–11 contained the SH3 domain of PSD-95/SAP90. pVP16 SAPAP1–12 contained the full length of SAPAP1. The β-galactosidase activity (unit/mg protein n = 3 ± S.D.) in the yeast lysates was measured in triplicate. Data represent the mean values with ± S.D.

| Control (pVP16) | SAPAP1 (pPrey1201) | SAPAP2 (pPrey1305) | SAPAP3 (pPrey1310) | SAPAP4 (pPrey1377) | Full-length of SAPAP1 (pVP16 SAPAP1–12) |
|----------------|--------------------|--------------------|--------------------|--------------------|----------------------------------------|
| Control constructs containing the SH3 and guanylate kinase domains | | | | | |
| PSD-95/SAP90 (pBTM116 PSD-95–3) | ND | (5.9 ± 0.1) × 10^2 | (1.7 ± 0.1) × 10^2 | (2.4 ± 0.1) × 10^2 | (1.5 ± 0.2) × 10^2 | (6.9 ± 0.4) × 10^2 |
| SAP97 (pBTM116 SAP97–5) | ND | (3.7 ± 0.4) × 10^2 | (1.4 ± 0.1) × 10^2 | (4.3 ± 0.1) × 10^2 | (2.2 ± 0.1) × 10^2 | NT^6 |
| PSRP1 (pPrey102) (pBTM116 PSRP1–3) | (2.1 ± 0.3) × 10^2 | (3.4 ± 0.1) × 10^2 | (3.3 ± 0.2) × 10^2 | (7.5 ± 0.3) × 10^2 | (2.1 ± 0.3) × 10^2 | NT |
| dlg-A (pBTM116 dlg-1) | ND | (4.3 ± 0.6) × 10^2 | (1.2 ± 0.1) × 10^2 | (5.1 ± 0.1) × 10^2 | (3.1 ± 0.1) × 10^2 | NT |
| p55 (pBTM116 p55–1) | ND | ND | ND | ND | ND | NT |
| CASK (pBTM116 CASK-11) | ND | ND | ND | ND | ND | NT |
| ZO-1 (pBTM116 ZO-1–1) | ND | ND | ND | ND | ND | NT |

| Constructs containing the guanylate kinase domain | | | | | |
| PSD-95/SAP90 (pBTM116 PSD-95–9) | ND | (4.9 ± 0.1) × 10^2 | (1.2 ± 0.1) × 10^3 | (3.4 ± 0.3) × 10^2 | (3.3 ± 0.2) × 10^3 | (3.2 ± 0.4) × 10^10 |
| SAP97 (pBTM116 SAP97–4) | ND | (5.8 ± 0.3) × 10^2 | (8.9 ± 0.8) × 10^2 | (4.4 ± 0.1) × 10^3 | (4.1 ± 0.2) × 10^2 | NT |
| PSRP1 (pPrey102) (pBTM116 PSRP1–2) | (7.7 ± 0.7) × 10^2 | (1.3 ± 0.1) × 10^3 | (4.0 ± 0.1) × 10^2 | (3.8 ± 0.4) × 10^3 | NT |
| dlg-A (pBTM116 dlg-2) | ND | (5.2 ± 0.1) × 10^2 | (1.6 ± 0.1) × 10^3 | (3.8 ± 0.1) × 10^2 | (3.3 ± 0.1) × 10^2 | NT |
| CASK (pBTM116 CASK-10) | ND | ND | ND | ND | ND | NT |

A construct containing the SH3 domain
| PSD-95/SAP90 (pBTM116 PSD-95–11) | ND | ND | ND | ND | ND | NT |

^a ND, not detectable.
^b NT, not tested.

coding regions did not show significant homology (Fig. 1, A and B). SAPAP1 to -4 showed calculated Mr values of 110,172, 110,134, 106,123, and 108,678, and consisted of 992, 980, 977, and 992 amino acids, respectively. The amino acid sequences of SAPAP1 to -4 predicted hydrophilic proteins without a transmembrane region or a signal sequence. All SAPAPs had proline-rich domains in the middle region and the C-terminal region. Data bank searches revealed no significant homology to known proteins. pPrey1201, pPrey1305, pPrey1310, and pPrey1377 contained amino acid residues 315–556 of SAPAP1, 480–577 of SAPAP2, 463–605 of SAPAP3, and 370–617 of SAPAP4, respectively. Based on the homology conserved among the peptides from these four pPrey clones, the domain of SAPAPs essential for the interaction with the region of PSD-95/SAP90 used as the bait can be tentatively concluded to be located in the region of about 80 amino acid residues in the middle portion (Fig. 1A).

To confirm whether the clones of SAPAPs were full-length clones, we constructed eukaryotic expression vectors using these clones and checked the expressions of SAPAPs in COS cells. The polyclonal antibodies were raised against SAPAP1, -2, -3, and -4 using peptides specific for each SAPAP. The antibodies against SAPAP1, -2, and -4 recognized the proteins with Mr of about 140,000 and the antibody against SAPAP3 recognized the protein with a Mr of about 120,000 in COS cells transfected with each pCMV construct (data not shown). The sizes of the proteins detected in the transfected COS cells were different from the calculated sizes based on the sequences. However, these antibodies also recognized the proteins with Mr of 140,000 or 120,000 in brain homogenates (data not shown). Therefore, clones of SAPAP1 to -4 are concluded to be the full-length clones of SAPAPs, and SAPAPs may show abnormal mobilities on SDS-PAGE due to post-translational modifications.

**Interaction of SAPAPs with Other MAGUKs**—We examined by the yeast two-hybrid method whether SAPAPs interact with MAGUKs other than PSD-95/SAP90. Various bait constructs which contained the SH3 domain and the guanylate kinase domain of various MAGUKs were prepared and tested for interactions with original prey clones (Table I). All prey clones of SAPAPs (pPrey1201, pPrey1305, pPrey1310, and pPrey1377) interacted with bait constructs containing both the SH3 domain and the guanylate kinase domain of not only PSD-95/ SAP90, but also SAP97, PSD-95/SAP90-related protein 1 (PSRP1), and dlg-A. The clones did not interact with the bait.
GST fusion protein of PSD-95/SAP90. The extracts of COS cells containing full-length SAPAP1 in contrast to the bait construct containing only the guanylate kinase domains of PSD-95/SAP90, respectively (data not shown). In Western blot analysis using the coding region of SAPAP2 showed a 8.0-kb major hybridizing mRNA and three weak smaller signals, only including heart, spleen, lung, liver, skeletal muscle, and kidney, because of differential polyadenylation. In rat testis, mRNA showed 5.0-kb and 4.2-kb minor hybridizing mRNAs only in brain, possibly containing only the guanylate kinase domains of PSD-95/SAP90, SAPAP97, PSRP1, and dlg-A, form a closely related family different from p55, CASK, and ZO-1. All SAPAPs interacted specifically with members of this closely related family. Region of PSD-95/SAP90 Interacting with SAPAPs—To determine which region of PSD-95/SAP90 is necessary for interaction with SAPAPs, we tested the interactions of the original prey clones and bait constructs containing various regions of PSD-95/SAP90 and its related proteins. The prey clones of SAPAPs interacted with the bait constructs containing only the guanylate kinase domains of PSD-95/SAP90, SAPAP97, PSRP1, and dlg-A, but not with the bait constructs containing the SH3 domain of PSD-95/SAP90 (Table I). Therefore, the guanylate kinase domains of PSD-95/SAP90 and its related proteins are sufficient for interaction with SAPAPs. We also tested the interactions between the bait construct containing the SH3 domain or the guanylate kinase domain of PSD-95/SAP90 and the prey construct containing full-length SAPAP1. The bait construct containing the SH3 domain did not interact even with the prey construct containing full-length SAPAP1 in contrast to the bait construct containing the guanylate kinase domain (Table I). This result suggests that the SH3 domain of PSD-95/SAP90 is not involved in the interaction with SAPAP1.

In Vitro Interaction of SAPAPs with PSD-95/SAP90—To examine the interaction of PSD-95/SAP90 and SAPAPs in vitro, the extracts of COS cells expressing SAPAP1 with the Myc tag at the N terminus were incubated with various GST fusion proteins fixed on glutathione-Sepharose 4B beads. The bound proteins were analyzed by SDS-PAGE followed by immunoblotting with the antibody against the Myc tag. SAPAP1 was bound to GST-PSD-95-1 containing full-length PSD-95/SAP90 and GST-PSD-95-4 containing both the SH3 domain and the guanylate kinase domain (Fig. 2). SAPAP1 was not bound to either GST or GST-PSD-95-2 containing three PDZ (DHR) domains (Fig. 2).

To confirm whether SAPAPs directly interact with PSD-95/SAP90, Biacore biosensor technology was used. GST fusion proteins containing the potential PSD-95/SAP90 interacting region of SAPAPs or GST itself were fixed on the Biacore sensor chip through the antibody against GST. MBP fusion protein containing the guanylate kinase domain of PSD-95/SAP90 was flowed on the chip and the affinities for the GST fusion constructs were measured (Fig. 3). The $K_d$ value of PSD-95/SAP90 for the GST fusion protein of SAPAP1 was calculated to be $2.1 \times 10^{-7}$ M from the association ($k_a = 2.7 \times 10^3$ M$^{-1}$ s$^{-1}$) and dissociation ($k_d = 5.6 \times 10^{-4}$ s$^{-1}$) constants. The $K_d$ value of PSD-95/SAP90 for the GST fusion protein of SAPAP2 was calculated to be $2.3 \times 10^{-7}$ M from the association ($k_a = 2.6 \times 10^3$ M$^{-1}$ s$^{-1}$) and dissociation ($k_d = 5.9 \times 10^{-4}$ s$^{-1}$) constants. The MBP fusion protein of SNAP-25A did not interact with SAPAPs (data not shown). Neither the MBP fusion protein containing three PDZ (DHR) domains nor the MBP fusion protein containing the SH3 domain interacted with SAPAPs (data not shown).

Specific Expression of SAPAPs in Brain—Northern blot analysis using the coding region of SAPAP1 showed 6.4-kb major and 5.2-kb minor hybridizing mRNAs in rat brain, possibly because of differential polyadenylation. In rat testis, mRNA with a smaller size was weakly observed. The significance of this weak signal was unknown. Other rat tissues tested, including heart, spleen, lung, liver, skeletal muscle, and kidney, did not show any detectable signal (Fig. 4A). Northern blot analysis using the coding region of SAPAP2 showed a 8.0-kb major hybridizing mRNA and three weak smaller signals, only in brain (Fig. 4B). Northern blot analyses of SAPAP3 and -4 showed 5.0-kb and 4.2-kb hybridizing mRNAs only in brain, respectively (data not shown). In Western blot analysis using the polyclonal antibody against SAPAP1, the signal was detected only in rat brain (Fig. 4C). Western blot analyses using antibodies against SAPAP2, -3, and -4 showed similar results (data not shown).

Specific Expression of SAPAPs in Neurons—To determine whether SAPAPs are neuronal proteins, we stained these proteins in primary cultured hippocampal neurons from rat embryo using antibodies. The antibody against SAPAP1 stained in the dendrites, the cell bodies, and the nuclei of hippocampal neurons (Fig. 5A). When the antibody was preincubated with an excess amount of GST fusion protein of SAPAP1, the signals detected in the dendrites and cell bodies disappeared, while the signals in the nuclei remained (Fig. 5B). Therefore, signals detected in dendrites and cell bodies were considered to be
specific. This staining pattern was similar to that of PSD-95/SAP90 (data not shown). The glia cells were not stained with the antibody against SAPAP1 (data not shown). Essentially the same results were obtained using the antibodies against SAPAP2, -3, and -4 (data not shown).

Enrichment of SAPAPs in PSD—Western blot analysis of the subcellular fractions of rat brain indicated that proteins detected with antibody against SAPAP1 were enriched in the synaptosome fraction (P2). The subfractionations of synaptosomes into the synaptic vesicle fraction (S3), the lysed synaptosomal membrane fraction (P3), and the synaptosomal membrane fraction (SPM) showed that SAPAP1 was enriched in SPM, in which PSD-95/SAP90 and NMDA receptors 2A/B were also enriched. PSD was prepared by Triton X-100 extraction of SPM. SAPAP1 was further enriched in PSD (Fig. 6). Western blot analyses using antibodies against SAPAP2, -3, and -4 showed essentially the same results (data not shown).

Translocation of PSD-95/SAP90 from the Cytosol to the Plasma Membrane Depending on the Presence of SAPAPs—In the last set of experiments, we expressed SAPAPs and PSD-95/SAP90 in various combinations in 293 cells to examine whether SAPAPs interacted with PSD-95/SAP90 in intact cells. When PSD-95/SAP90 was expressed in 293 cells, PSD-95/SAP90 was located in the cytosol (Fig. 7A). On the other hand, SAPAP1 was located at the plasma membrane in 293 cells (Fig. 7B). Coexpression of PSD-95/SAP90 with SAPAP1 caused the translocation of PSD-95/SAP90 from the cytosol to the plasma membrane where SAPAP1 was located (Fig. 7C). However, when the pCMV construct encoding only three PDZ (DHR) domains of PSD-95/SAP90 (pCMV PSD-95-2) was used, the distribution of the product of this construct was not affected by SAPAP1 (Fig. 7D). Other SAPAPs showed essentially the same effect on the translocation of PSD-95/SAP90 (data not shown).

The translocation of PSD-95/SAP90 depending on the presence of SAPAPs was also verified through subfractionations of transfected 293 cells as described above. SAPAP1 was recovered in the membrane fraction of the transfected 293 cells, both when it was expressed alone and when it was coexpressed with PSD-95/SAP90 (Fig. 8A). PSD-95/SAP90 was mainly recovered in the cytosol fraction in the transfected 293 cells, when it was expressed alone, but was recovered in the membrane fraction, when it was coexpressed with SAPAP1 (Fig. 8B). The protein containing only the PDZ (DHR) domains of PSD-95/SAP90 was recovered in the cytosol fraction, even when coexpressed with SAPAP1 (Fig. 8C). Similar results were obtained using other SAPAPs than SAPAP1 (data not shown).
DISCUSSION

In this article, we have reported four novel proteins which interact with the guanylate kinase domain of PSD-95/SAP90. We have named these proteins SAPAPs. We have raised polyclonal antibodies against each SAPAP and confirmed the expressions of SAPAPs in COS cells. The Mr of SAPAPs detected in COS cells transfected with pCMV constructs containing the coding regions of the clones of SAPAPs are the same as those in brain. Therefore, these clones are considered to be full-length clones.

We have concluded that SAPAPs actually interact with PSD-95/SAP90 based on the following lines of evidence: 1) the recombinant constructs of SAPAPs interact with PSD-95/SAP90 in vitro with the Kd values of about 2 × 10^-7 m, these values are in a reasonable range; 2) SAPAPs are expressed in neurons and highly enriched in the PSD fraction in which PSD-95/SAP90 are enriched; 3) SAPAPs also show interactions with PSD-95/SAP90 in 293 cells and induce the translocation of PSD-95/SAP90 from the cytosol to the plasma membrane; and 4) in the preliminary yeast two-hybrid screenings for SAPAPs-associated proteins, PSD-95/SAP90, SAP97, and PSD-93/chapsyn are obtained reproducibly.2

SAPAPs are about 50% homologous to each other at the amino acid level. The 200 amino acids in the C-terminal region are especially well conserved, although the conserved regions are also observed in the N-terminal region and in the middle region. SAPAPs show no homology to known proteins and the sequences do not suggest the functions of SAPAPs. Since the original prey clones obtained from the yeast two-hybrid screening contain various partial sequences of SAPAPs, we can tentatively map the PSD-95/SAP90-interacting region of SAPAPs. This region is located in the middle region and encompasses about 80 amino acid residues, which do not contain any known motif. The potentially interesting domains are the proline-rich domains conserved in the middle (PPPI/VPP) and C-terminal regions (PPPV/DP and PPI/VIP). The proline-rich domains are generally considered to interact with the SH3 domain. Although the proline-rich domains in the middle regions of SAPAP1, -2, and -4 have the consensus motif of the sequences of the proteins which interact with the SH3 domain of Grb2, the surrounding amino acid contexts of other proline-rich domains do not completely fit to the criteria of the proline-rich domains reported to interact with the SH3 domain (40, 41). It is at present unknown whether these domains function for the interaction with other molecules.

The attempt to identify the SAPAP-interacting region of PSD-95/SAP90 has first been performed using the yeast two-hybrid method. The original prey clones of SAPAPs interact with the bait construct containing the guanylate kinase domain of PSD-95/SAP90 alone, and not the bait construct containing the SH3 domain. Thus, the guanylate kinase domain is sufficient for the interaction. The bait construct containing the SH3 domain alone does not interact with the prey clone containing the full-length of SAPAP1, either. The expression of this bait construct is confirmed, since PSD-95/SAP90 itself is obtained from the yeast two-hybrid screening using this bait construct.2

2 M. Irie, unpublished observation.

---

**FIG. 6. Enrichment of SAPAP1 in the PSD fraction.** Equal aliquots of each subcellular fraction (7 µg of total protein) of rat brain were immunoblotted using the antibody against SAPAP1, PSD-95/SAP90, or NMDA receptors 2A/B. P1, the nuclear pellet fraction; P2, the crude synaptosomal pellet fraction; P3, the lysed synaptosomal membrane fraction; SPM, the synaptosomal membrane fraction; S1, the crude synaptosomal fraction; S2, the cytosolic synaptosomal fraction; and S3, the crude synaptic vesicle fraction. SPM were extracted with 0.5% (w/v) Triton X-100, twice (One Triton and Two Triton). Numbers on the left indicate the positions of Mr markers (in kDa).

**FIG. 7. Interaction of SAPAP1 with PSD-95/SAP90 in intact 293 cells.** 293 cells were transfected with various combinations of pCMV constructs of SAPAP1 and PSD-95/SAP90, fixed, stained with the monoclonal antibody against PSD-95/SAP90 (anti-PSD-95) and the polyclonal antibody against SAPAP1 (anti-SAPAP1), and visualized with rhodamine-conjugated anti-mouse IgG antibody and Cy5-conjugated anti-rabbit IgG antibody. A, transfected with pCMV construct containing the full-length PSD-95/SAP90 (pCMV PSD-95-1); B, transfected with pCMV construct containing full-length SAPAP1 (pCMV SAPAP1); C, transfected with pCMV PSD-95-1 and pCMV SAPAP1; D, transfected with pCMV construct containing the PDZ (DHR) domains of PSD-95/SAP90 (pCMV PSD-95-2) and pCMV SAPAP1.
between neurons, which are not supplied in the primary cultures of hippocampal cells.

Although the functions of SAPAPs are unknown, they may function as anchoring molecules to facilitate the interactions of PSD-95/SAP90 and the molecules located at the plasma membrane, since SAPAPs induce the enrichment of PSD-95/SAP90 at the plasma membrane in 293 cells. However, preliminary experiments suggest that SAPAPs have no effect on the clustering of NMDA receptor 2A by PSD-95/SAP90, although further studies are necessary. As discussed above, not all the proline-rich domains of SAPAPs have the consensus motif for the interacting domains with the SH3 domains. However, there is a possibility that some of the proline-rich domains of SAPAPs may interact with unidentified molecules and SAPAPs may work as an adaptor between PSD-95/SAP90 and these molecules.

Acknowledgments—We thank Dr. D. W. Russell (University of Texas Southwestern Medical Center at Dallas) for pCMV vector, Dr. S. Nakaniishi (Kyoto University, Kyoto, Japan) for cDNA of NMDA receptor 2A, Dr. S. Tsukita (Kyoto University, Kyoto, Japan) for cDNA of mGluR1, Dr. S. Hellenberg (University of Washington, Seattle, WA) for the materials of the yeast two-hybrid method, and Dr. H. Nakaniishi (Takai Biotimer Project, ERATO) for pCMV Myc vector. Some constructs used in this study were produced by Y. H. in the laboratory of Dr. T. C. Sudhof (University of Texas Southwestern Medical Center at Dallas), who generously permitted use of these constructs.

Note added in Proof—After the submission of this work, two groups have independently identified the molecules interacting with the guanylate kinase domain of PSD-95/SAP90 (Kim, E., Naishitt, S., Hausch, Y-P., Rao, A., Rothschild, A., Craig, A. M., and Sheng, M. (1997) J. Cell Biol. 136, 669–678; Sato, K., Yanai, H., Senda, T., Kohu, K., Nakamura, T., Okumura, N., Matsumine, A., Kobayashi, S., Toyoshima, K., and Akiyama, T. personal communication). GKAP, the protein reported by Kim et al. is an alternative splicing isofrom of SAPAP1. DAP1a and DAP2 identified by Sato, K. et al. are the same as SAPAP1 and SAPAP2, respectively. DAP1b is the same as GRAP.

REFERENCES

1. Kennedy, M. B. (1993) Curr. Opin. Neurobiol. 3, 732–737
2. Carlin, R. K., Bartelt, D. C., and Siekevitz, P. (1983) J. Cell Biol. 96, 443–448
3. Walters, B. B., and Matus, A. I. (1975) Nature 257, 496–498
4. Kelly, P. T., and Cotman, C. W. (1978) J. Cell Biol. 79, 173–183
5. Carlin, R. K., Grab, D. J., and Siekevitz, P. (1981) J. Cell Biol. 89, 449–455
6. Kennedy, M. B., Bennett, M. K., and Brodsky, F. M. (1988) Proc. Natl. Acad. U. S. A. 85, 7357–7361
7. Cho, K., Hunt, C. A., and Kennedy, M. B. (1992) Neuron 9, 929–942
8. Kistner, U., Wenzel, B. M., Veh, R. W., Cases-Langhoff, C., Garner, A. M., Appelauer, U., Voss, B., Gundelfinger, E. D., and Garner, C. C. (1993) J. Biol. Chem. 268, 4580–4583
9. Hunt, C. A., Schenker, L. J., and Kennedy, M. B. (1996) J. Neurosci. 16, 1380–1388
10. Lee, R. A., Marfatia, S. M., Branton, D., and Husain-Chishti, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9818–9822
11. Muller, B. M., Kistner, U., Veh, R. W., Cases-Langhoff, C., Becker, B., Gundelfinger, E. D., and Garner, C. C. (1995) J. Neurosci. 15, 2354–2366
12. Brennan, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Wu, Z., Huang, F., Xia, A., Peters, M. P., Frohner, S. C., and Brodsky, F. M. (1996) Cell 84, 757–767
13. Kim, E., Cho, K-O., Rothschild, A., and Sheng, M. (1996) J. Neurosci. 16, 1025–1033
14. Muller, B. M., Kistner, U., Kindler, S., Chung, W. J., Kuhlendahl, S., Fenster, F., Lau, L-F., Veh, R. W., Haganir, B. L., Gundelfinger, E. D., and Garner, C. C. (1997) J. Neurosci. 17, 255–263
15. Ponting, C. P., and Phillips, C. (1995) Trends Biochem. Sci. 20, 102–103
16. Kornau, H., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1996) Science 270, 1737–1740
17. Kim, E., Niethammer, M., Rothschild, A., Jan, Y. N., and Sheng, M. (1995) Nature 375, 85–88
18. Niethammer, M., Kin, E., and Sheng, M. (1996) J. Neurosci. 16, 2157–2163
19. Doyle, D. A., Lee, A., Lewis, J., Kim, E., Sheng, M., and MacKinnon, R. (1996) Cell 85, 1067–1076
20. Cabral, J. H. M., Petrusa, C., Sutcliffe, M. J., Rasa, S., Byron, O., Poy, F., Marfatia, S. M., Chisti, A. H., and Liddington, R. C. (1996) Nature 382, 649–652
21. Anderson, J. M. (1996) Curr. Biol. 6, 382–384
22. Woods, D. F., and Bryant, P. J. (1991) Cell 66, 451–464
23. Hoskins, R., Hajnal, A. F., Harp, S. A., and Kim, S. K. (1996) Development 123, 97–111
24. Ruff, P., Speicher, D. W., and Husain-Chishti, A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6595–6599
25. Itoh, M., Nagafuchi, S., Yonemura, R., Kitani-Yasuda, T., Tsukita, S., and Tsukita, S. (1993) J. Cell Biol. 121, 491–502

FIG. 8. Subcellular fractionations of 293 cells expressing PSD-95/SAP90 and/or SAPAP1. 293 cells transfected with various pCMV constructs described in the legend to Fig. 7 were sonicated in lysis buffer. The lysates were centrifuged and comparable amounts of the cytosol and membrane fractions were analyzed through SDS-PAGE and immunoblotting with either anti-PSD-95 antibody or anti-SAPAP1 antibody. Numbers on the left indicate the positions of Mr (in kDa). A, cytosol and membrane fractions of 293 cells transfected with pCMV SAPAP1 alone (lanes 1 and 2) or with pCMV SAPAP1 and pCMV PSD-95-1 (lanes 3 and 4) were immunoblotted with anti-SAPAP1 antibody; B, cytosol and membrane fractions of 293 cells transfected with pCMV PSD-95-1 alone (lanes 1 and 2) or with pCMV SAPAP1 and pCMV PSD-95-1 (lanes 3 and 4) were immunoblotted with anti-PSD-95; C, cytosol and membrane fractions of 293 cells transfected with pCMV PSD-95-2 alone (lanes 1 and 2) or with pCMV SAPAP1 and pCMV PSD-95-2 (lanes 3 and 4) were immunoblotted with anti-PSD-95 antibody.
26. Willott, E., Balda, M. S., Fanning, A. S., Jameson, B., Itallie, C. V., and Anderson, J. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7834–7838
27. Jesaitis, L. A., and Goodenough, D. A. (1994) J. Cell Biol. 124, 949–961
28. Hata, Y., Butz, S., and Sudhof, T. C. (1996) J. Neurosci. 16, 2488–2494
29. Lahey, T., Gorczyca, M., Jia, X-X., and Budnik, V. (1994) Neuron 13, 823–835
30. Kistner, U., Garner, C. C., and Linial, M. (1995) FEBS Lett. 359, 159–163
31. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1995) Cell 74, 205–214
32. Guan, K. L., and Dixon, J. E. (1991) Anal. Biochem. 192, 262–267
33. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
34. Hata, Y., and Sudhof, T. C. (1995) J. Biol. Chem. 270, 13022–13028
35. Joehnsson, U., Faengerstam, L., Ross, H., Roennberg, J., Sjoelander, S., Sternberg, E., Stahlberg, R., Urbaniczky, C., Oestlin, H., and Malmqvist, M. (1991) BioTechniques 11, 520–527
36. Bottenstein, J. E., and Sato, G. H. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 514–517
37. Jones, D. J., and Matus, A. I. (1974) Biochim. Biophys. Acta 356, 276–287
38. Brose, N., Hofmann, K., Hata, Y., and Sudhof, T. C. (1996) J. Biol. Chem. 270, 25273–25280
39. Kozak, M. (1987) Nucleic Acids Res. 15, 8125–8148
40. Cohen, G. B., Ren, R., and Baltimore, D. (1995) Cell 80, 237–248
41. Sparks, A. B., Rider, J. E., Hoffman, N. G., Fowlkes, D. M., Quilliam, L. A., and Kay, B. K. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1540–1544
SAPAPs: A FAMILY OF PSD-95/SAP90-ASSOCIATED PROTEINS LOCALIZED AT POSTSYNAPTIC DENSITY
Masakazu Takeuchi, Yutaka Hata, Kazuyo Hirao, Atsushi Toyoda, Mina Irie and Yoshimi Takai

J. Biol. Chem. 1997, 272:11943-11951.
doi: 10.1074/jbc.272.18.11943

Access the most updated version of this article at http://www.jbc.org/content/272/18/11943

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 40 references, 19 of which can be accessed free at http://www.jbc.org/content/272/18/11943.full.html#ref-list-1