ProSAP-interacting Protein 1 (ProSAPiP1), a Novel Protein of the Postsynaptic Density That Links the Spine-associated Rap-Gap (SPAR) to the Scaffolding Protein ProSAP2/Shank3*

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ProSAPs/Shanks are a family of proteins that have a major scaffolding function for components of the postsynaptic density (PSD) of excitatory brain synapses. Members of the family harbor a variety of domains for protein-protein interactions, one of which is a unique PDZ domain that differs significantly from those of other proteins. We have identified a novel binding partner for this PDZ domain, termed ProSAPiP1, that is highly enriched in the PSD and shares significant sequence homology with the PSD protein PSD-Zip70. Both molecules code for a Fez1 domain that can be found in a total of four related proteins. ProSAPiP1 is widely expressed in rat brain and co-localizes with ProSAP2/Shank3 in excitatory spines and synapses. ProSAP2/Shank3 co-immunoprecipitates with ProSAPiP1 but not with PSD-Zip70. Both proteins, however, bind and recruit SPAR to synapses with a central coiled-coil region that harbors a leucine zipper motif. This region is also responsible for homo- and heteromultimerization of ProSAPiP1 and PSD-Zip70. Thus, ProSAPiP1 and PSD-Zip70 are founders of a novel family of scaffolding proteins, the “Fezzins,” which adds further complexity to the organization of the PSD protein network.

Spiny excitatory synapses are characterized by a complex network of protein-protein interactions mediated mainly by scaffolding proteins that serve three major functions as follows: (i) the organization of glutamate receptors, (ii) the clustering of synaptic adhesion molecules, and (iii) the coupling of synaptic membrane proteins to intracellular signaling cascades (1, 2). In recent years the structure of the complex network that builds up the postsynaptic density (PSD) 3 has been revealed in more detail (3, 4), and although our understanding is still far from being complete, the picture emerges that a family of proteins termed ProSAP/Shank (5–8), also termed CortBPI (9) or Synamon (10), play a pivotal role as master scaffolding proteins for the structural organization of the PSD (11, 12). ProSAPs/Shanks harbor several protein-protein interaction domains, including ankyrin repeats, an Src homology 3 domain, a PDZ domain, a sterile α-motif domain, and a proline-rich region between the latter two domains. A variety of protein-protein interactions have been ascribed to these domains supporting the view that their multidomain structure underlies their supposed function as master scaffold molecules of the PSD. Many binding partners for ProSAP/Shank identified to date bind to the PDZ domain. These include GKAP/ SAPAPs (6, 7, 10, 13), the calcium-independent calcium latrotoxin receptor (14, 15), the somatostatin receptor subtype 2 (16), and the L-type calcium channels (17, 18). Given that the PDZ domain has a crucial role in synapse assembly and synaptic signaling (19, 20), one can assume that regulated binding of interaction partners to this domain will bring about the flexibility necessary for alterations in the assembly of the PSD in response to extracellular synaptic activation by growth factors, cell adhesion molecules, or the neurotransmitter glutamate. In light of the well documented effects of ProSAPs/Shanks on synapto- and spine morphogenesis (21, 22), the knowledge of alternative binding partners for their protein interaction domains is a prerequisite for a deeper understanding of these fundamental processes. To gain further information about the function of the ProSAP/Shank protein interaction domains, we performed a yeast two-hybrid screen using bait constructs from different regions of the protein (6, 14, 23), including the PDZ domain. In this report we characterize a new protein, termed ProSAPiP1 (ProSAP-interacting Protein 1), that interacts with the PDZ domain of ProSAP2/Shank3. ProSAPiP1 is a synaptic protein that shares significant sequence homology with another recently described PSD protein, termed PSD-Zip70 (24, 25), and the proteins LAPSER1 (26) and N4BP3 (27). Moreover, these proteins are founding a family of altogether four proteins harboring a Fez1 domain with previously unknown function (F37/Eosophageal cancer-related gene coding leucine zipper motif 1 (28)).

We report that the C terminus of PSD-Zip70 does not bind to the same PDZ domain but, like ProSAPiP1, binds the recently identified RapGAP SPAR with a central coiled-coil domain that harbors a leucine zipper motif. Moreover, ProSAPiP1 and PSD-Zip70 can form homo- and heteromultimers. We therefore propose that both proteins constitute a novel family of scaffolding molecules, the “Fezzins.”

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening and Cloning of a Full-length Rat ProSAPiP1 cDNA—Yeast two-hybrid screening was performed according to published methods (23, 29). As bait, the cDNAs coding for the PDZ

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* The abbreviations used are: PSD, postsynaptic density; PBS, phosphate-buffered saline; GFP, green fluorescent protein; aa, amino acid; GST, glutathione S-transferase; PVD, polyvinylidene difluoride; ddH2O, doubly distilled water; DAB, diaminobenzidine hydrochloride; dH2O, distilled water.

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domain of ProSAP2/Shank3 (aa 670–760), full-length ProSAPiP1 (aa 1–704), or the coiled-coil region of ProSAPiP1 (aa 286–475) were fused to the GAL4 DNA binding domain of vector pAS2-1 (Clontech). A rat brain cDNA library cloned into the pACT vector (GAL4 activation domain; Clontech) was screened. By screening with the ProSAP2/Shank3 PDZ domain, three partial cDNA clones for ProSAPiP1 were obtained and sequenced. A full-length cDNA was cloned by several rounds of screening of λ ZAPII (Stratagene, La Jolla, CA) rat hippocampal and total brain cDNA libraries with the 32P-labeled ProSAPiP1 cDNA fragments. Full-length GFP expression constructs were amplified by PCR, cloned into the plasmid pEGFP-C2 (Clontech), and confirmed by DNA sequencing. Similarly, mutant constructs were generated by PCR-based mutagenesis and confirmed by DNA sequencing to determine the ProSAP2/Shank3-binding motif of ProSAPiP1 in a yeast two-hybrid assay approach. Subsequently, yeast two-hybrid screening and assay with the ProSAPiP1 full-length and ProSAPiP1 coiled-coil bait was done as described. Full-length SPAR cDNA was obtained after screening λ ZAPIII (Stratagene) rat hippocampal and total brain cDNA libraries with the partial cDNAs for SPAR (prey).

Antibodies—A partial cDNA of ProSAPiP1 (encoding aa 332–704) was subcloned in the bacterial expression vector pGEX-4T (Amersham Biosciences). A 68.5-kDa glutathione S-transferase (GST)-ProSAPiP1 fusion protein was expressed in Escherichia coli BL 21 and purified on glutathione-Sepharose 4B as recommended by the manufacturer (Amerham Biosciences). The fusion protein was used to generate ProSAPiP1 antibodies in rabbits (Eurogentec, Southampton, UK). The PSD-Zip70 and ProSAP2/Shank3 antibodies have been described previously (32). Transcripts encoding ProSAPiP1 were detected with antisense oligonucleotide purchased from MWG Biotech (Ebersberg, Germany) directed against the 5′ and 3′ ends of the mRNA: 5′-GGC CAG GTG TGC AGG GCC CAT ATG GCT TCT CGA AAT C-3′ (bp 188–148), 5′-GTC TCC AGC TTC GCC ATG ATT AAG CCA GAA GGG CAC TTT C-3′ (bp 274–235), and 5′-CCA GTC CGC AGA CGT CCG GCC TCC AGC TGG TGG-3′ (bp 2179–2144). For Northern blot analysis, a rat multiple tissue Northern blot (Clontech)-hybridized with a C-terminal ProSAPiP1 cDNA probe (bp 1157–end) according to the manufacturer’s protocol (Clontech).

Western Blot Analysis, Isolation of Subcellular Protein Fractions, Overlay and Multimerization Assays, GST Pulldown Assay, and Immunoprecipitation of Brain Lysates—Subcellular fractionation studies were performed as described previously (31). In brief, tissue from adult rats was homogenized in homogenization buffer (320 mM sucrose, 5 mM HEPES, pH 7.4) containing protease inhibitor mixture (Roche Applied Science); cell debris and nuclei were removed by 1000 g centrifugation. The supernatant was spun for 20 min by 12,000 g, resulting in supernatant S2 and pellet P2 (crude membrane fraction). The S2 was centrifuged at 100,000 g for 1 h, and the resulting supernatant was taken as the cytoplasmic fraction (S100). The P2 pellet was further fractionated by centrifugation in a sucrose step gradient (0.85, 1.0, and 1.2 M). For isolation of synaptic junctional proteins (PSD fraction), the synaptosomal fraction of the first gradient was diluted with 5 volumes of 1 mM Tris, pH 8.1. The suspension was kept on ice for 30 min and was centrifuged for 30 min at 33,000 g. The pellet P3 was resuspended in 5 mM Tris, pH 8.1 (1.5 ml/1 g wet tissue), and once again fractionated by centrifugation in a sucrose gradient. The 1.0/1.2 M interface (synaptic junctions) was suspended in 320 mM sucrose, 0.5% Triton X-100, 5 mM Tris, pH 8.1 (120 ml/10 g wet tissue), kept on ice for 15 min, and centrifuged for 30 min at 33,000 g. This PSD fraction was resuspended in 5 mM Tris, pH 8.1, 320 mM sucrose, and 0.5% Triton X-100. The solution was kept on ice for 15 min and centrifuged at 33,000 g. The pellet was resuspended in an equal volume of water.

To study the expression of ProSAPiP1 during postnatal development, P2 fractions from brains of 1-, 4-, 7-, 10-, 14-, 21-, 30-, and 90-day-old rats were resuspended in 500 μl of Laemml buffer. Proteins (20 μg/lane) were separated by SDS-PAGE on 12% gels under fully reducing conditions and transferred on PVDF membranes. For immunodetection, Western blots were incubated with the polyclonal ProSAPiP1 antisemur (dilution 1:2000). Immunoreactivity was visualized using the SuperSignal detection system (Pierce).

For the GST pulldown assay, the last 371 aa of ProSAPiP1 and the PDZ domain of ProSAP2 were cloned into the pGEX4-T2 vector. The GST fusion proteins were coupled to glutathione S-Sepharose (Amerham Biosciences) for 1 h. The crude membrane fraction of rat brain was extracted for 1 h with RIPA at 4°C. After centrifugation (12,000 × g, 20 min, 4°C), the supernatant was incubated with Sepharose contain-
ing the fusion proteins or GST alone overnight at 4 °C. The precipitates were washed twice with RIPA and once with 20 mM Tris, pH 8.0, and analyzed with antibodies against ProSAPiP1 and ProSAP2/Shank3.

For the overlay assay, a full-length GFP-SPAR construct was transfected in COS-7 cells. Cells were harvested, separated on an SDS-polyacrylamide gel, and blotted on a PVDF membrane. The membrane was
**FIGURE 2.** Analysis of ProSAPiP1 expression and ProSAP2-ProSAPiP1 interaction. 

**A,** Northern blot analysis reveals an expression of ProSAPiP1 mRNA at about 4 kb in brain (Br) and kidney (Ki). A very weak signal might also be seen in testis (Te). In all other tissues investigated (muscle (Mu), liver (Li), lung (Lu), and spleen (Sp)), no ProSAPiP1 mRNA expression can be detected. 

**B,** the polyclonal antibody against ProSAPiP1 recognizes a GFP-ProSAPiP1 fusion protein in transfected COS-7 cells at a size of about 105 kDa. In rat brain homogenate the antiserum detects a single band at about 75 kDa.

**C and D,** the Western blot experiments show that ProSAPiP1 is a brain-specific protein because it can only be detected in rat brain and not in kidney or testis. Compared with thalamus (Th), hypothalamus (Ht), and midbrain (MB), the protein expression is higher in cortex (Co), cerebellum (Ce), and in the hippocampus (CA1/CA3/dentate gyrus, DG).

**E,** ProSAPiP1 immunoreactivity appears late during neuronal development. The expression of ProSAPiP1 increases...
incubated overnight at 4°C with a crude membrane fraction of rat brain extracted with RIPA buffer. Membranes were intensely washed, and either GFP or ProSAPiP1 antibodies were used for immunodetection of the antigen. Multimerization assay was performed essentially as described by Tadokoro et al. (32). Recombinant GST-ProSAPiP1 proteins were diluted in 20 mM Tris/HCl, pH 7.5, 50 mM NaCl (0.2 mg/ml) and treated with or without 0.05% glutaraldehyde at 30°C for 30 min, separated by SDS-PAGE (14%), and stained with Coomassie Brilliant Blue R.

For immunoprecipitation the following primary antibodies were used: ProSAPiP1, ProSAP2/Shank3, SPAR, and nonimmune rabbit and nonimmune guinea pig IgG. 50 μl of a 1:1 slurry of protein-A-agarose (Roche Applied Science) was washed three times with PBS and then preincubated with the antibodies for 1 h at 4°C. The crude membrane fraction (P2) of an adult rat brain was solubilized with RIPA for 1 h at 4°C. After centrifugation (20 min, 12,000 × g, 4°C) the supernatant was added to the agarose and incubated overnight at 4°C. The mixture was washed twice with RIPA and once with 20 mM Tris, pH 8.0. Immunoprecipitated proteins were separated by SDS-PAGE and blotted onto PVDF membrane. Immunoreactivity was visualized by using ProSAP1/Shank2, ProSAP2/Shank3, ProSAPiP1, Spar, and horseradish peroxidase-conjugated secondary antibodies and the SuperSignal detection system (Pierce).

Neuronal Cell Culture and COS-7 Cell Transfections—To test the interaction between ProSAPiP1 and ProSAP2/Shank3 in COS-7 cells, ProSAPiP1 was cloned into the red fluorescent protein expression vector (pDsRed2; Clontech) using PCR strategies. Afterward, this expression vector was co-transfected with a GFP-ProSAP2 construct (pEGFP-ProSAP2(333–704)), Cells were grown on glass coverslips, transfected by using Polyfect (Qiagen, Hilden, Germany), and fixed with 4% paraformaldehyde 24 h after transfection.

Cell cultures of hippocampal primary neurons and transfections were performed as described previously (33). Hippocampal neurons were fixed at −20°C for 5 min in methanol. Cells were washed three times with PBS and incubated with 2% bovine serum albumin, 10% horse serum, 5% sucrose, and 0.3% Triton X-100 in PBS to block nonspecific binding and subsequently with primary antibodies for 1–3 h at room temperature with the following dilutions: ProSAPiP1 antiserum at 1:600; ProSAP2/Shank3 antisera at 1:500; SPAR antisera 1:1000; and SAP90/PDS95 antisera at 1:500. Anti-rabbit, anti-guinea pig, and anti-mouse IgGs coupled to Cy2 or Cy3 (DAKO, Glostrup, Denmark) were used to detect the primary antibodies.

RESULTS

Identification of ProSAPiP1 as a PDZ Domain-interacting Protein for ProSAP2/Shank3—By utilizing the yeast two-hybrid system, we screened 1.6 × 10^9 clones of a rat brain cDNA library with the PDZ domain of ProSAP2/Shank 3 as bait. A total of three clones were obtained that encoded partially or full-length a novel protein of 704 aa (EMBL/GenBank™ accession number AJ278801, human homologue KIAA0552; EMBL/GenBank™ accession number NM_014731) termed ProSAPiP1 for ProSAP-interacting Protein 1 (Fig. 1, A and B). ProSAPiP1 is characterized by a coiled-coil domain, a leucine zipper, a C-terminal Fez1 domain (28), and a C-terminal PDZ domain-binding motif —STEI. ProSAPiP1 is localized on rat chromosome 3q36 and encoded by five exons (Fig. 1C). A data base search revealed that ProSAPiP1 is closely related to other Fez1 proteins. We could identify a Fezzin family with a total of four members. The highest sequence identity of ProSAPiP1 is found with the PSD protein PSD-Zip70 (24) (Fig. 1, D and E) and with a putative tumor suppressor molecule named LAPSER1 (26). The sequence homology encompassed the entire open reading frame, including all three putative protein-protein interaction domains, i.e. a coiled-coil domain (aa 348–462), the Fez1 domain, and a PDZ domain-binding motif at the C terminus (either —STEI or —ATEI; Fig. 1, F) with the exception of an N-myristoylation motif that is lacking in ProSAPiP1. Interestingly, the highest degree of similarity for both proteins is found in the coiled-coil and Fez1 domain, raising the possibility that both proteins might bind common binding partners via this region. The N4BP3 (Nedd4-binding protein 3) is a more distinctly and slightly smaller member of this novel Fez1 domain protein family (27).

ProSAPiP1 Is a Brain-Specific Protein Highly Enriched in the PSD—A 4-kb band could be detected on a multiple tissue Northern blot in kidney and brain (Fig. 2A). However, Western blot analysis with a polyclonal antibody directed specifically against ProSAPiP1 as shown, i.e. by GFP-ProSAPiP1 transfection experiments (Fig. 2B), identified the protein-like PSD-Zip70 (24) exclusively in brain but not in other tissues (Fig. 2C). The expression level of the protein increases significantly between day 8 and day 16 in brain (Fig. 2E), and the highest levels of the protein were found in cerebral cortex, the cerebellum, and in the hippocampus (Fig. 2D). In a next set of experiments, we investigated whether ProSAPiP1 is indeed a postsynaptic brain protein that can interact in vivo with ProSAP2/Shank3 in the PSD. Extraction experiments had already shown that ProSAPiP1 is tightly connected to the cytoskeleton and Triton X-100-insoluble (data not shown). Subcellular fractionation aimed to purify protein components of the PSD showed a clear enrichment of the relative amount of ProSAPiP1 immunoreactivity in the PSD fraction as compared with other fractions (Fig. 2F). The increasing amounts of protein during brain development is reflected by a relative increase of protein levels in highly purified PSD fractions following the time course of synapse formation and maturation (Fig. 2G).

C-terminal Amino Acids of ProSAPiP1 Are Crucial for the Interaction with the PDZ Domain of ProSAP2/Shank3—To prove that the C terminus of ProSAPiP1 is necessary for binding to the PDZ domain of ProSAP2/Shank3, we employed a deletion construct lacking the last three amino acids. As expected, this construct showed no binding to the PDZ domain of ProSAP2/Shank3 in yeast (Fig. 2F). Next we determined whether the C terminus of ProSAPiP1 binds in a canonical manner to the PDZ domain of ProSAP2/Shank3 by generating a variety of mutant ProSAPiP1 constructs with point mutations in the last four amino acids, and we tested their binding affinity in a semi-quantitative yeast two-hybrid assay. Inspection of the C terminus of ProSAPiP1 suggests that it significantly between postnatal days 8 and 16. F and G, ProSAPiP1 immunoreactivity is enriched in the insoluble protein fraction (P2). Further subcellular fractionation reveals a tight association with the synaptic cytoskeleton (PSD). S2, soluble protein fraction; P2, membrane fraction; My, myelin fraction; LM, light membrane fraction; Syn, synaptosomal fraction. The increase of ProSAPiP1 labeling during postnatal development (see also D) is in line with the increased immunoreactivity in PSD preparations from cortex and hippocampus of brains from different developmental stages. Higher levels appear first during synaptogenesis (day 16) supporting its pivotal association with synaptic structures. d, days; w, weeks; m, month after birth. H, semi-quantitative analysis of the binding affinity to the PDZ domain of ProSAP2 using constructs with point mutations at different positions. + + + + + + corresponds to dark blue colonies; + + + + + , blue colonies; + + + light blue colonies; +/+, white colonies. Note that substitution of the serine at position 3 to an alanine, which renders the class 1 PDZ-binding motif of ProSAPiP1 identical to those of PSD-Zip70 and LAPSER1, still leads to very weak binding to the PDZ domain of ProSAP2. I GST pulldown and co-immunoprecipitation of ProSAPiP1 and ProSAP2 from rat brain lysates. GST-Septalosome beads were charged with GST-ProSAPiP1 (aa 333–704) or GST alone and incubated with rat brain lysate. Precipitates were immunoblotted against ProSAP2/Shank3. Rat brain extracts were immunoprecipitated with ProSAPiP1, ProSAP2, or control (IgG), and immunoprecipitates were blotted for ProSAP2 and ProSAPiP1. J co-clustering of ProSAPiP1 and ProSAP2 in PSD. COS-7 cells were transfected with DsRed-ProSAPiP1 and GFP-ProSAP2 PDZ domain. GFP-ProSAP2 PDZ domain is evenly distributed in the cytoplasm when transfected alone (ST, single transfection), but it is recruited into ProSAPiP1-positive clusters when co-transfected with DsRed-ProSAPiP1. Co-transfection of GFP-ProSAP2 PDZ with a DsRed-PDZ-Zip70 full-length cDNA construct did show any co-clustering.

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should bind to class 1 PDZ domains with the consensus sequence X(T/S)Y(V/L) (34). Accordingly and as reported previously (7), mutations at positions 0 and −2 led to a drastically reduced binding or no binding in the YTH assay. Substitution of the acidic glutamate residue by a lysine has no negative effect on binding suggesting that acidic residues at this position are not crucial for binding and can be substituted with highly basic residues even with large side chains. This will substantially enlarge the number of potential binding partners for the PDZ domain of Pro-SAPs/Shanks. Notably, the substitution of the serine residue at position −3 to a neutral alanine did not completely abolish binding in the assay.
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This substitution is particularly interesting because it renders the PDZ domain interaction sequence identical to the C terminus of PSD-Zip70, which is a close homologue of ProSAPiP1. Next we examined whether the interaction of ProSAP2/Shank3 occurs in vivo. Immunoprecipitation with an antibody directed against ProSAPiP1 led to the precipitation of ProSAP2/Shank3 immunoreactivity from rat brain homogenate and vice versa (Fig. 2I). Immunoprecipitation of N-terminal Myc-tagged PSD-Zip70 protein transfected into COS-7 cells, however, resulted in no co-immunoprecipitation of ProSAP2/Shank3 and vice versa (data not shown). Moreover, pulldown of ProSAP2/Shank3 with a GST-tagged ProSAPiP1 fusion protein from Triton X-100-extracted rat brain homogenates confirmed the results of the co-immunoprecipitation experiments (Fig. 2I). Further evidence for an interaction between ProSAPiP1 and ProSAP2/Shank3 results from their co-localization after transfection of COS-7 cells. ProSAPiP1 and PSD-Zip70 are found in clusters possibly due to their homodimerization after single transfection. ProSAP2/Shank3 PDZ domain, which is diffusely distributed in COS-7 cells after a single transfection, accumulates at these clusters only in ProSAPiP1 but not in PSD-Zip70 double transfected cells (Fig. 2I).

ProSAPiP1 Is Widely Expressed in Rat Brain and Is Found at Spines and PSDs of Excitatory Synapses—In situ hybridization experiments to rat brain sections at different developmental stages showed that transcript levels steadily increase during postnatal development and reach highest levels in the mature brain (Fig. 3, A and B). The transcript distribution is clearly different from that reported for PSD-Zip70 (24). In contrast to PSD-Zip70, ProSAPiP1 seems to be more abundant in the caudate putamen and especially in cerebellum and hippocampus (Fig. 3, A–C) where PSD-Zip70 is not present or is hardly detectable. The mRNA of both proteins is highly abundant in the cortex (Fig. 2B) (24).

Accordingly, ProSAPiP1 protein levels were found to be highest in cerebral cortex, caudate putamen, cerebellum, and hippocampus (Fig. 3, B and C) and substantially increased from the early postnatal brain to adulthood as assessed by immunoblotting (see Fig. 2E) and immunohistochemical staining. Finally, the transcript distribution of ProSAPiP1 also resembled the distribution pattern of immunoreactivity found after immunostainings of whole rat brain sagittal sections utilizing a rabbit polyclonal antiserum (Fig. 3C). Staining results showed that the proteins could not be detected in cell cytoplasm but showed a punctate staining pattern in the neuropil. Furthermore, the enrichment of ProSAPiP1 immunoreactivity in PSD fractions was supported by the localization of ProSAPiP1 immunoreactivity at the PSD of excitatory synapses employing immunoelectron microscopy of adult rat brain sections (Fig. 3D). ProSAPiP1 and ProSAP2/Shank3 are found in the same cellular and subcellular structures of the brain, thereby providing supporting evidence that the interaction can happen in vivo.

Additional evidence for the relatively late expression and synaptic localization of ProSAPiP1 at synapses (Fig. 2G) was gained from studies in hippocampal primary cultures. An analysis of the expression of ProSAPiP1 in hippocampal neurons revealed that the molecule is localized in dendrites and spines after 2–3 weeks of preparation (Fig. 4A). In older cultures, however, the molecule becomes localized in the tips of spiny protrusions. At that stage counterstaining with ProSAP2/Shank3 antibodies revealed a perfect match of both proteins at postsynaptic densities (Fig. 5A). In older neurons more clusters can be observed that are immunopositive for ProSAP2/Shank3 and SAP90/PSD95 (Fig. 5B).
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ProSAPiP1 Forms Homo- and Heteromultimers—The clustering of ProSAPiP1 and PSD-Zip70 after single transfection in COS-7 cells suggests that both proteins might aggregate through the formation of oligomers. Interestingly, double transfection of COS-7 cells with GFP and DsRed ProSAPiP1 cDNAs in COS-7 cells shows a clear co-localization of the two fusion proteins in clusters (Fig. 6A). The generation of homo-oligomers could also be proven by immunoprecipitation of Myc- and GFP-tagged ProSAPiP1 transfected into COS-7 cells. After precipitation with the anti-Myc antibody, GFP-labeled ProSAPiP1 can be detected. Precipitation with control IgG does not result in a GFP-positive band. C, a multimerization assay with different ProSAPiP1 GST fusion proteins. One fusion protein codes for the coiled-coil domain/leucine zipper (aa 348–462); the C-terminal GST fusion protein codes for the CC and Fez1 domain (aa 332–704). After treatment of different ProSAPiP1 GST fusion proteins with glutaraldehyde (+), several distinctive bands can be identified. Besides the monomers (*), bands at a size for dimers (**), trimers (***) and multimers (****) are to be detected. This is not seen for the GST protein alone (left lanes).

FIGURE 6. Homomeric and heteromeric interaction of ProSAPiP1 and PSD-Zip70. A, co-transfection of GFP- and DsRed-labeled ProSAPiP1 in COS-7 cells shows a perfect co-localization of these constructs in clusters. These results are verified by immunoprecipitation using a GFP- and a Myc-labeled ProSAPiP1 construct (B). The input of proteins is shown in the left lanes. After precipitation of protein with the anti-Myc antibody, GFP-labeled ProSAPiP1 can be detected. Precipitation with control IgG does not result in a GFP-positive band. C, a multimerization assay with different ProSAPiP1 GST fusion proteins. One fusion protein codes for the coiled-coil domain/leucine zipper (aa 348–462); the C-terminal GST fusion protein codes for the CC and Fez1 domain (aa 332–704). After treatment of different ProSAPiP1 GST fusion proteins with glutaraldehyde (+), several distinctive bands can be identified. Besides the monomers (*), bands at a size for dimers (**), trimers (***) and multimers (****) are to be detected. This is not seen for the GST protein alone (left lanes). D, co-transfection of GFP-ProSAPiP1 with myc-PSD-Zip70 shows that both proteins co-localize in identical cluster in COS-7 cells.

ProSAPiP1 and PSD-Zip70 bind to SPAR with their central coiled-coil domain. Because the coiled-coil region seems to be a potential common protein-protein interaction domain in ProSAPiP1 and PSD-Zip70, we sought to identify binding partners for this region utilizing a yeast two-hybrid screen with the full-length and coiled-coil region of ProSAPiP1 as bait. In this screen we identified the RapGAP SPAR (35) as a possible interaction partner (Fig. 7A). We obtained two independent SPAR clones as prey from our ProSAPiP1 screen. The binding region in SPAR was subsequently shown to be largely identical to those responsible in SPAR for binding to the GK domain of SAP90/PSD95 (35) and encompassed amino acid residues 1498–1820. Interestingly, the C-terminus of SPAR also includes a coiled-coil domain with a leucine zipper motif. To substantiate this initial finding, we performed blot-overlay assays and pulldown experiments after transfection of COS-7 cells with GFP-tagged full-length SPAR. Moreover, we performed co-immuno-
FIGURE 7. ProSAPiP1 interacts with SPAR. A, by employing a yeast two-hybrid screen with full-length ProSAPiP1 bait (bait I), we could isolate two preys (P1, aa 1297–1820; P2, aa 1498–1820) coding for the neuronal protein SPAR. Sequence analysis revealed that the SPAR C terminus is organized quite similar to ProSAPiP1. It codes for a coiled-coil domain with a leucine zipper. By using the ProSAPiP1 coiled-coil domain as bait (bait II) in a yeast two-hybrid assay with both preys, we could confirm that this ProSAPiP1 region is sufficient for interaction. Both baits display a strong (+++) interaction with the SPAR preys P1 and P2. B, pulldown experiments show that GST-ProSAPiP1 is able to bind GFP-SPAR expressed in COS-7 cells. Vice versa, overlay experiments with a GFP-SPAR fusion protein blotted on a membrane and incubated with rat brain homogenate show that ProSAPiP1 can be detected at the size of the GFP-SPAR fusion protein. GFP alone is not able to bind ProSAPiP1. C, to test in vivo interaction rat brain extracts were either immunoprecipitated with ProSAPiP1, SPAR, or control (IgG). When SPAR was precipitated a ProSAPiP1-specific double band could be detected (as in PSDs), and ProSAPiP1 precipitation resulted in the specific labeling of the SPAR protein. D, co-transfection of COS-7 cells withDsRed-ProSAPiP1 or DsRed-PSD-Zip70 and GFP-SPAR shows that SPAR is recruited into the ProSAPiP1- or PSDZip70-positive clusters. Transfection of GFP-SPAR alone (ST, single transfection) shows the association of this fusion protein with the actin cytoskeleton as published (35).
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precipitation of the proteins from rat brain homogenate. In all these assays we could show that ProSAPiP1 interacts with SPAR in a specific manner (Fig. 7, B and C). ProSAPiP1 is able to recruit SPAR into synaptic clusters. First we co-transfected DsRed-ProSAPiP1 and GFP-SPAR into COS-7 cells. GFP-SPAR that is associated with the actin cytoskeleton after a single transfection (35) was then recruited into large ProSAPiP1-positive clusters. PSD-Zip70, which shares the highest sequence homology in this coiled-coil and Fez1 domain with ProSAPiP1 and also SAPiP1-positive clusters. PSD-Zip70, which shares the highest sequence interacts with SPAR (25), was also able to recruit SPAR into a cluster in this COS-7 cell assay (Fig. 7D). Finally, we used hippocampal cultures to test this functional role of ProSAPiP1 in neurons. Transfection of GFP-SPAR resulted in the localization of this fusion protein in dendrites and spines. These spines were also positive for endogenous ProSAP2 and ProSAPiP1 protein. GFP-ProSAPiP1 clusters in hippocampal neurons were also heavily stained for endogenous SPAR recruited to these clusters, and the co-transfection of both fusion proteins resulted in ProSAPiP1/SPAR-positive clusters at synaptic sites. In contrast to other tese, and the co-transfection of both myristoylated and nonmyristoylated iso-

most N-terminal AUG in the open reading frame. It needs to be clarified lmost in the future whether both myristoylated and nonmyristoylated iso-

forms of the proteins are synthesized using alternative in-frame start codons. Western blot analysis of rat brain revealed at least in the subcellular fractionation experiments two distinct bands for ProSAPiP1 and PSD-Zip70 (24) with a difference in molecular mass of about 2–3 kDa that might be explained by the usage of different start codons.

Interestingly, the class I PDZ-binding motif of ProSAPiP1 and PSD-Zip70 seems to have a clear specificity for PDZ domain-containing proteins of the PSD. Thus, although both proteins bind SPAR with their central coiled-coil domain, only ProSAPiP1 but not PSD-Zip70, or only with a much weaker affinity, binds to ProSAP2/Shank3. It might well be the case that PSD-Zip70 and possibly LAPSER1, which shares an identi-
tical C terminus, will bind to another PDZ domain and therefore could render the clustered protein complex even more divergent. In turn, this raises the intriguing possibility that both proteins will anchor and recruit SPAR to different PDZ domain-containing molecules of the PSD.

Noteworthy in this regard, PSD-Zip70 has been localized to lipid rafts (24). In neurons this cell compartment is not well characterized, although it was proposed that some PSD proteins, depending upon their myristoylation and acetylation, are localized to this structure (37, 38) and that endocytosis and recycling of synaptic proteins are associated with this membrane microdomain (38, 39). The most accepted view of lipid rafts function is a role in membrane trafficking and concomitant pre-assembly of associated signal transduction and cytoskeletal connections (40). Therefore, it has been speculated that trafficking from lipid rafts to the synapse could be a mechanism to provide pre-assembled macromolecular protein complexes, and the ProSAPiP1/PSD-Zip70 family of proteins could be part of such a pre-assembly process.

Another possibly important difference between both proteins is found in their expression pattern in brain. Although significant overlap exists in the cerebral cortex and in parts of the hippocampus, ProSAPiP1 seems to be more abundant especially in the dentate gyrus and in the caudate putamen and is prominently present in the cerebellum where no transcripts could be found for PSD-Zip70 (24). From this observation it can be concluded that not all excitatory synapses of the brain will contain both proteins.

Strong evidence for a scaffolding function of these proteins at excitatory synapses arises from the presence of a large coiled-coil domain. Such domains are known to provide an interface for protein-protein interactions (41), and we could show that ProSAPiP1 and PSD-Zip70 bind SPAR via this domain (see also below). In addition, it has been shown that they quite frequently serve the multimerization of protein complexes (41). Moreover, ProSAPiP1 and PSD-Zip70 contain a leucine zipper that is known to be involved in protein-protein dimerization via the leucines, which provide a hydrophobic environment for the inter-

action (42). Accordingly, we could show that ProSAPiP1 and PSD-Zip70 are capable of forming homomers and heteromers after hetero-

dogous expression in COS-7 cells. Moreover, we could demonstrate that the ProSAPiP1 C terminus is able to self-oligomerize in a manner similar to PSD-Zip45/Homer 1C (32). This aspect is especially intriguing with regard to proteins of the ProSAP/Shank family, which have been shown to possess the capacity to dimerize and/or multimerize via their C-terminal sterile α-motif domain (7, 13). Recently it could be shown how these huge multimerization complexes are organized on a molecu-

lar level (43). The ProSAP/Shank sterile α-motif domains have the capacity to form large sheets of helical fibers that are arranged in a well ordered array within the PSD. Interestingly, this sheet formation is zinc-dependent, and ProSAP/Shank-interacting proteins can either be positioned toward the subsynaptic membrane or toward the spine neck (43).

Thus, ProSAPiP1 provides another means for cross-linking SPAR to
the ProSAP/Shank platform at excitatory synapses. Our experiments clearly demonstrate that ProSAPiP1 and SPAR interact in vivo and that ProSAPiP1 effectively recruits SPAR to synaptic contacts when overexpressed in hippocampal neurons. At the moment one can only speculate about how postsynaptic zinc concentrations can alter the following: (i) ProSAP/Shank sheet formation, (ii) ProSAPiP1 interaction, and (iii) subsequent recruitment of SPAR to dynamic spines and PSDs. Here one has to refer to the proposed signaling function of SPAR in dendritic spine synapses. SPAR was identified as a RapGAP that signals back via small GTPases to the actin cytoskeleton of the synapse and thereby enlarges dendritic spines by its binding to the guanylate kinase domain of the MAGUK family of proteins (35). In a recent paper, Maruoka et al. (25) report that overexpression or knockdown of PSD-Zip70 interferes with the localization of SPAR to spine synapses and their maturation.

FIGURE 8. ProSAPiP1 recruits SPAR into synaptic clusters in hippocampal cultures. The transfection of GFP-SPAR into hippocampal neurons resulted in the localization of the green fusion protein in neuronal dendrites and spines as indicated by the labeling of postsynaptic densities via ProSAP2 antibodies (red). The GFP-SPAR protein co-localized also perfectly with the wild type ProSAPiP1 proteins as revealed by antibody staining of transfected hippocampal neurons. Transfection of GFP-ProSAPiP1 into neurons resulted in ProSAPiP1 clusters that were strongly positive for endogenous SPAR protein (arrows). Double transfection of hippocampal neurons with GFP-ProSAPiP1 and DsRed-SPAR showed the co-localization of both fusion proteins at synaptic sites. Please note that under these conditions the SPAR fusion protein is solely localized at spines and PSDs (no dendritic staining). Moreover, spines appear large and of mostly round shape (arrows).
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Thus, the SPAR interaction seems to have significant consequences for spine morphology. Because binding to SPAR seems to be a common denominator of putative scaffolding activities of ProSAPIp1 and PSD-Zip70, we therefore propose that the specific binding of both molecules with reference to their PDZ class 1 binding partner could have also functional consequences for the three-dimensional organization of the PSD.

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