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ProSAPiP2, a novel postsynaptic density protein that interacts with ProSAP2/Shank3

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ABSTRACT

The postsynaptic density (PSD) is a highly specialized structure that is located juxtaposed to the presynaptic active zone of excitatory synapses. It is composed of a variety of proteins that include receptors, signaling molecules, cytoskeletal components and scaffolding proteins. ProSAP/Shank proteins are large multidomain proteins that facilitate multiple functions within the PSD. They build large scaffolds that are the structural basis for the direct and/or indirect connection between receptor proteins and the actin based cytoskeleton. Here, we characterize a novel interaction partner of ProSAP2/Shank3, named ProSAP interacting protein 2 (ProSAPiP2) that does not show any close homology to other known proteins. It binds to the PDZ domain of ProSAP2/Shank3 and is highly expressed in the neuronal system. ProSAPiP2 is located in dendrites and spines, is enriched in the PSD and interacts with actin. Therefore ProSAPiP2 could be involved in the linkage between molecules of the PSD and the cytoskeleton.

Introduction

Development, dynamics and maintenance of neuronal networks are mediated by a close and specific interaction between molecules of the outer membrane, i.e. receptors, cell adhesion molecules and ion channels with the highly dynamic actin cytoskeleton [1–3]. The excitatory synapse is a specialized neuronal cell contact that is characterized by the cytomatrix active zone (CAZ) on the presynaptic [4] and an electron dense matrix, called the postsynaptic density (PSD) [5,6], on the postsynaptic side. The morphology of PSDs, spines and synapses can be quickly altered depending on synaptic activity [7,8]. These plastic changes are regulated by several families of PSD proteins including signaling molecules and small GTPases that control the dynamics of the actin cytoskeleton [9–12]. A key molecule is the multidomain scaffolding protein postsynaptic density-95 (PSD-95) that attaches, i.e. transmembranous linkers like neuroligins and/or receptor molecules to the postsynaptic site [5]. In deeper layers of the PSD master scaffolding proteins of the ProSAP/Shank family are mediators between the submembranous layer of synaptic proteins and the actin cytoskeleton, playing a pivotal role in development, maintenance and remodeling of the synaptic compartment [13,14]. Several PDZ domain binding partners of ProSAP/Shank have already been identified including GKAP/SAPAPs, ProSAPiP1 and the calcium-independent latrotoxin receptor [5].

In light of the well documented effects of ProSAP/Shank on synapto- and spine morphogenesis [15,16], 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 (Y2H) screen using the PDZ domain of ProSAP2/Shank3 as a bait. In this report we characterize a new interacting protein at PSDs, termed ProSAPiP2 that is expressed in different brain areas and in neural stem cells. Additionally, ProSAPiP2 interacts with actin, suggesting a role as a linker molecule. In a screening approach the human homologue of ProSAPiP2 has been found to be an interaction partner of TBK1 (Tank binding kinase) in the signaling pathway of NF-κB [17,18], a signaling cascade that is known to be important for neuronal differentiation and synaptic plasticity [19].

Material and methods

Yeast two-hybrid screening and cloning of a full-length rat ProSAPiP2 cDNA. A yeast two-hybrid screen was performed using the Y187 und AH109 yeast strains harboring the reporter genes HIS3

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and ß-galactosidase (ß-gal) under the control of upstream GAL1 activating sequence. As a bait a proline-rich region behind the PDZ domain of ProSAP2/Shank3 (aa 747–804) was fused in frame to the GAL4 DNA binding domain in vector pAS2-1 (Clontech, San Diego, CA) and transformed to screen a rat brain cDNA library cloned into the pACT2 vector (Clontech, San Diego, CA). Moreover, a shortened construct of ProSAPiP2 (aa 281–604) was cloned into the multiple cloning site of pGBKT7 and transformed to screen against a human fetal brain cDNA library cloned into the pACT2 vector (Clontech, San Diego, CA). The two-hybrid screens were carried out according to the manufacturer's protocol. A total of 1.6×10^6–2×10^6 cotransformants were screened, yeast colonies that grew in medium lacking histidine were picked up, and their ß-galactosidase activity was assayed by X-gal Filter-Lift Assays.

Full-length GFP expression constructs of the candidate genes were amplified by PCR, cloned into the plasmid pEGFP-C1 (Clontech) as well as the pMyc CMV and confirmed by DNA sequencing. In the case of ProSAPiP2 as bait 5 putative interaction partners were identified (see Fig. 4).

Antibodies. A partial cDNA of ProSAPiP2 (encoding aa 246–613) was subcloned in the bacterial expression vector pGEX-4T (Amersham Biosciences). A 64-kDa glutathione *S*-transferase (GST)–ProSAPiP2 fusion protein was expressed in *Escherichia coli* BL21 and purified on glutathione–Sepharose 4B as recommended by the manufacturer (Amersham Biosciences). The fusion protein was used to generate ProSAPiP2 antibodies in rabbits (Eurogentec, Southampton, UK). ProSAP2/Shank3 antibodies have been described previously [20].

Culture of primary hippocampal neurons, cell lines and mesencephalic NSCs. All animal experiments were performed in compliance with the guidelines for the welfare of experimental animals issued by the Federal Government of Germany, the National Institutes of Health and the Max Planck Society. Cell culture of rat hippocampal primary neurons (embryonic day 18; E18) was performed as described previously [20]. Hippocampal neurons were seeded on poly-L-lysine (0.1 mg/ml; Sigma–Aldrich, Steinheim, Germany) coated coverslips at a density of 2×10^4 cells/well. Cells were grown in Neurobasal medium, complemented with B27 supplement, 0.5 mM L-glutamine, and 100 U/ml penicillin/streptomycin (all Invitrogen, Karlsruhe, Germany) and maintained at 37 °C in 5% CO_2.

Mesencephalic neural stem cells from rat embryonic brain were prepared as previously described [21]. Tissue was harvested from embryonic day 14.5 rat embryos. After 4–7 days sphere formation was observed. The cells were allowed to adhere to the surface for 4 h before analyzing (24 h including transfection time). COS-7 cells were maintained and transfected as described before [22].

In situ hybridization. In situ hybridization was performed as described previously [23]. Transcripts encoding ProSAPiP2 were detected with an S^{35} labeled cDNA antisense oligonucleotide (AGCTGCGCCAGGGGCTGCTGGGCCCGCAGTGG GCGG; bp 823–787) purchased from MWG-Biotech (Ebersberg, Germany) directed against the 3′ end of the mRNA.

Northern blot analysis. A rat multiple tissue Northern blot (Clontech) was hybridized with a C-terminal ProSAPiP2 cDNA probe (bp 1157-end) according to the manufacturer's protocol (Clontech).

Western blotting. Whole-cell protein extracts were denatured after sonication at 75 °C for 2 min. The proteins were separated and electro-blotted onto nitrocellulose membranes. Specific primary antibodies were: rabbit anti-ProSAPiP2 diluted 1:400. The enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia, Freiburg, Germany) was used for immune detection. To obtain PSD fraction from rat brain a subcellular fractionation procedure was performed as described previously [23].

Immunocytochemical detection. For immunofluorescence detection, cultured cells were fixed with 4% paraformaldehyde (PFA)/

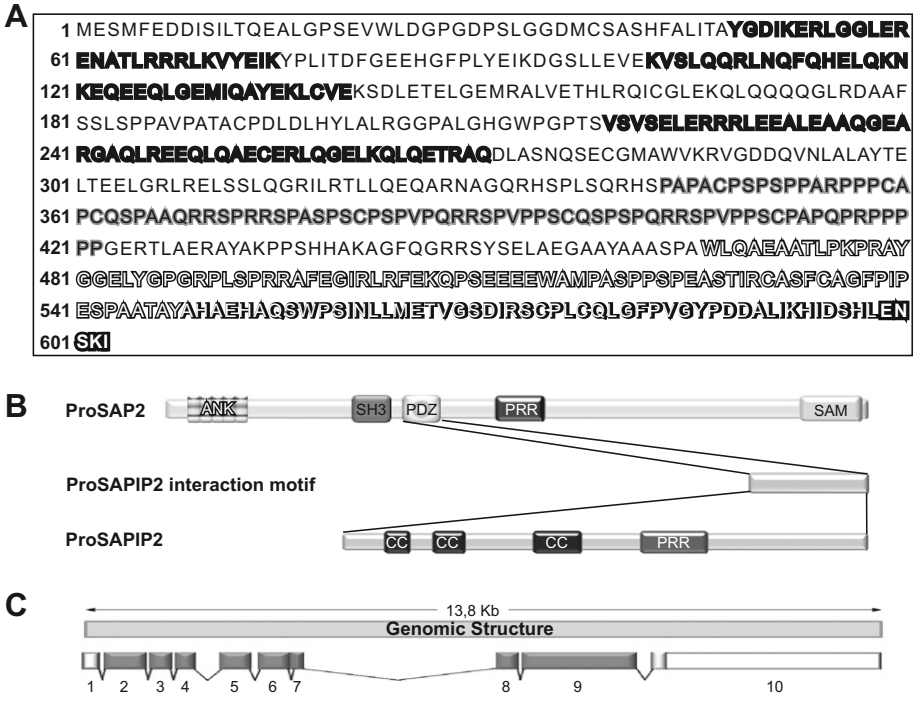

Fig. 1. Structure of the ProSAPiP2 protein. (A) Amino acid sequence of ProSAPiP2 from rat. ProSAPiP2 is a 613 aa long protein and characterized by three central coiled-coil domains (CC, bold letters) and a long proline-rich region (PRR, grey letters). The shortest interacting prey clone from the yeast two-hybrid screen with the ProSAP2/Shank3 PDZ domain is indicated by open letters (aa 468–613) and the putative C-terminal PDZ domain binding motif "ENSKI" is shown (black box). (B) ProSAP2/Shank3 consists of several domains including an ankyrin-repeat region (ANK), an SH3 (Src-homology) domain, a PDZ domain, a proline-rich region (PRR) and a SAM (sterile alpha motif) domain. (C) Chromosomal organization of ProSAPiP2 on rat chromosome 10 (10q31). A 13,798 bp long chromosomal stretch is coding for 10 ProSAPiP2 exons. The start codon is localized on exon 2, the stop is found in exon 9.