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Proline-Rich Synapse-Associated Proteins ProSAP1 and ProSAP2 Interact with Synaptic Proteins of the SAPAP/GKAP Family

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We have recently isolated a novel proline-rich synapse-associated protein-1 (ProSAP1) that is highly enriched in postsynaptic density (PSD). A closely related multidomain protein, ProSAP2, shares a highly conserved PDZ (PSD-95/discs-large/ZO-1) domain (80% identity), a ppI domain that mediates the interaction with cortactin, and a C-terminal SAM (sterile alpha-motif) domain. In addition, ProSAP2 codes for five ankyrin repeats and a SH3 (Src homology 3) domain. Transcripts for both proteins are coexpressed in many regions of rat brain, but show a distinct expression pattern in the cerebellum. Using the PDZ domains of ProSAP1 and 2 as bait in the yeast two-hybrid system, we isolated several clones of the SAPAP/GKAP (SAP90/PSD-95-associated protein/guanylate kinase-associated protein) family. The association of the proteins was verified by coimmunoprecipitation and cotransfection in HEK cells. Therefore, proteins of the ProSAP family represent a novel link between SAP90/PSD-95 bound membrane receptors and the cytoskeleton at glutamatergic synapses of the central nervous system.

Anchoring and clustering of membrane bound receptors and adhesion molecules at specific sites of the cell surface generally is mediated by proteins that are localized underneath the membrane and connected to the cytoskeleton. The postsynaptic density (PSD), a highly specialized sub-membranous network of proteins at synapses of the central nervous system (CNS), includes a variety of adapter proteins that are involved in localizing receptors, adhesion molecules and molecules of the intracellular signaling cascade within the synapse (1, 2). In addition they mediate interaction with the actin-based cytoskeleton that plays an important role in the organization of PSDs (3).

In recent years major efforts have been undertaken to identify the protein components of the PSD (e.g., 4, 5). Identified PSD proteins include the membrane-associated guanylate kinases (MAGuKs) SAP90/PSD-95, chapsyn110/PSD93 and SAP102. This family of proteins moved into the focus of interest because they are involved in recruiting NMDA-type glutamate receptors, potassium channels, the cell adhesion molecule neuroligin and multiple components of the subsynaptic signaling apparatus to the PSD (for review see 6, 7). Members of another family of proteins, named SAPAPs, GKAPs or DAPs, bind to the C-terminal guanylate kinase domain of MAGuKs and are thought to play an important role in recruiting and clustering of MAGuK-based signaling complexes (8–11).

We have recently shown, that ProSAP1, a novel PSD component, is identical with the cortacin-binding protein cortBP1 (12). ProSAP1/CortBP1 harbors a proline-rich ppI motif which interacts with the SH3 domain of the actin filament-associated protein cortactin (13). Here we report that ProSAP1, as well as the related protein ProSAP2, can interact with SAPAP family members and thus may represent the molecular interface between synaptic membrane proteins and the cytoskeleton. While this study was in progress it has been shown by Naisbitt *et al.* (14) that the PDZ-domain of members of the same protein family also interact with the C-terminus of GKAP.

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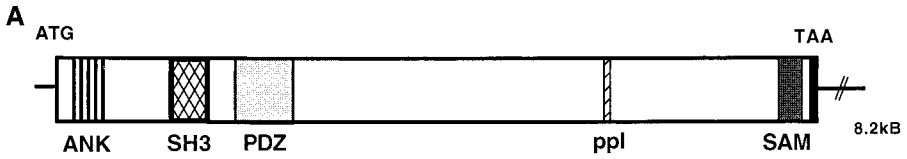

FIG. 1. Structure of ProSAP2 and amino acid sequence alignment of members of the ProSAP family (A) Physical map of the rat ProSAP2 cDNA. The protein coding region is boxed. ProSAP2 is characterized by 5 N-terminal ankyrin repeats (ANK), an SH3, a PDZ and a SAM domain. A proline-rich ppI domain identical with ProSAP1 and thus potentially interacting with the actin binding protein cortactin is also indicated. (B) Alignment of the amino acid sequences as deduced from the cDNA of the rat ProSAP family: ProSAP1 (EMBL/GenBank Acc. No. AJ131899), ProSAP2 (EMBL/GenBank Acc. No. AJ133120) and synamon (EMBL/GenBank Acc. No. AF102855). Identical amino acids are boxed (identity between: ProSAP1/ProSAP2 32%; ProSAP1/Synamon 35%; ProSAP2/Synamon 47%). ANK repeats (- - -), SH3 domain (= = =), PDZ domain (+ + +), ppI motif (* * *) and SAM domain (: : :) are indicated above the aligned sequences.

MATERIALS AND METHODS

Cloning of rat ProSAP2. We used a ProSAP1 ^{32}P labeled partial cDNA coding for the PDZ domain (ProSAP1, bp 647–929, Acc No. AJ 133899) to screen λZAPII rat hippocampal and total brain cDNA libraries (Stratagene, La Jolla, CA) by low stringency hybridization. The sequence information of several independent ProSAP2 clones was used to obtain the full length ProSAP2 cDNA sequence employing a PCR based method for the fast screening of cDNA libraries (15).

In situ hybridization. For *in situ* hybridization rat brains were frozen on dry ice in isopentan at −40°C. The brains were cut with a cryostat in horizontal sections (18 μm), mounted on Superfrost Plus slides (Menzel, Braunschweig, Germany) and stored at −70°C until used. ProSAP mRNAs were detected with cDNA antisense oligonucleotides purchased from MWG-Biotech (Ebersberg, Germany): ProSAP1 (Acc. No. AJ131899), 5′-TTC-TTA-CTG-TCT-GTA-GAG-TTG-GCT-GGT-TGG-CTG-GAG-TTC-3′ (bp 3155–3113); ProSAP2 (Acc. No. AJ 133120), 5′-GTG-GCA-GGT-TCA-CAG-CGA-ATA-CCA-GCT-CTG-GCT-CCT-3′ (bp 4096–bp 4060) and 5′-TCA-GGA-CTG-TGC-ACG-GGT-GTG-GGG-GAC-CGG-GAA-3′ (bp 3644–bp 3612). Both ProSAP2 probes yielded identical hybridization patterns. Hybridization was performed as previously described (12).

Yeast two hybrid screen. The yeast two hybrid screen was performed using the Y190 yeast strain harboring the reporter genes HIS3 and β-galactosidase (β-gal) under the control of upstream GAL1 activating sequence. As a bait the PDZ-domain of ProSAP1 (aa 42–131) and ProSAP2 (aa 670–760) was fused the GAL4 DNA binding domain in vector pAS2-1 Vektor (Clontech, San Diego, CA). A rat brain cDNA library cloned into in the pACT Vektor (GAL4 activation domain, Clontech, San Diego, CA) was screened. Putative protein-protein interactions in yeast were tested by the ability to activate both HIS3 and lacZ gene transcription. To eliminate false positives putative interaction partners the library plasmids were cotransformed with various bait constructs and afterwards candidates were sequenced.

Cell culture experiments and immunoprecipitation. To test the interaction between SAPAPs/GKAPs and ProSAPs in HEK cells, subregions of ProSAP2 cDNA were cloned into the GFP-expression Vektor (pEGFP, Clontech, San Diego, CA) using PCR strategies. GFP-ProSAP2^{PDZ} included aa 638 to 746 and GFP-PDZ$^{C\text{-term.}}$ aa 1022 to 1806 of ProSAP2. Afterwards these expression vectors were cotransfected with expression vectors for GKAP, SAP90 and the Kv1.4 potassium channel (8, 16) into HEK cells using the Lipofectamine method (DAK30, Eurogentec, Belgium). Cells were stained with an anti Kv1.4 antibody (upstate biotechnology, Lake Placid, NY) with Cy3 coupled secondary antibodies. Images were taken using a Leitz DMR XE fluorescence microscope equipped with Leica filter sets N2.1 and L4.

For immunoprecipitation transfected HEK cells were harvested, washed twice with PBS, solubilized in Tris-buffered saline (TBS)/1% deoxycholate/0.1% Triton X-100 (1 h at 4°C) and then centrifuged for 1 h at 40,000*g*. Rat brain membranes (P2) were prepared as described (17) and solubilized using the method of Xia *et al.* (18).

The following antibodies were used in immunoprecipitation and immunodetection experiments: rabbit anti-ProSAP1 (12), monoclonal anti-GKAP (produced by the UAB hybridoma facilities against recombinant GKAP), anti-SAP90/PSD-95 (Transduction Laboratories, UK), and anti-GFP (Clontech). Control rabbit IgG fraction was obtained from Sigma, Munich, Germany). About 20 μl primary antibody was preincubated with 50 μl of a 1:1 slurry of GammaBind-Sepharose (Pharmacia) and the GammaBind-antibody complex was collected as described (18). The supernatant of solubilized P2 fraction (500 μg) or 600 μl of clarified cell lysate were added and incubated overnight at 4°C. The mixture was then washed once with TBS/1% Triton X-100, twice with TBS/1% Triton X-100 plus 350 mM NaCl and finally three times with TBS. Immunoprecipitated proteins were separated by SDS–PAGE and blotted onto nitrocellulose membranes. Immunoreactivity was visualized by HRP-conjugated secondary antibodies and ECL chemiluminescence (Amersham Buchler, Braunschweig, Germany).

RESULTS AND DISCUSSION

ProSAP2 is a member of the ProSAP family of PDZ domain proteins. Several independent ProSAP2 cDNA clones were isolated by low stringency hybridization with a cDNA fragment encoding the PDZ domain of the recently identified PSD protein ProSAP1 (12). The sequence information of these clones was exploited to isolate another 12 independent clones using a PCR-based cloning method. From these clones full-length cDNA could be assembled and complete nucleotide sequence was obtained. The ProSAP2 cDNA harbors an open reading frame for a 1806-aa proline-rich protein (12% prolines). Several structural domains can be predicted (Fig. 1) which are conserved between ProSAP1, ProSAP2 and synamon, the known members of the ProSAP family. The sequence of the latter protein is included in public databases (Acc. No. AF102855). The PDZ domains of these proteins share 80% sequence identity with each other (Fig. 1B), but only moderate similarity with other characterized PDZ domains (12). A C-terminal SAM domain (19) is nearly identical within the protein family. The ppI domain (PPVPPKP) that directly interacts with the cortactin SH3 domain (13) is only conserved between ProSAP1

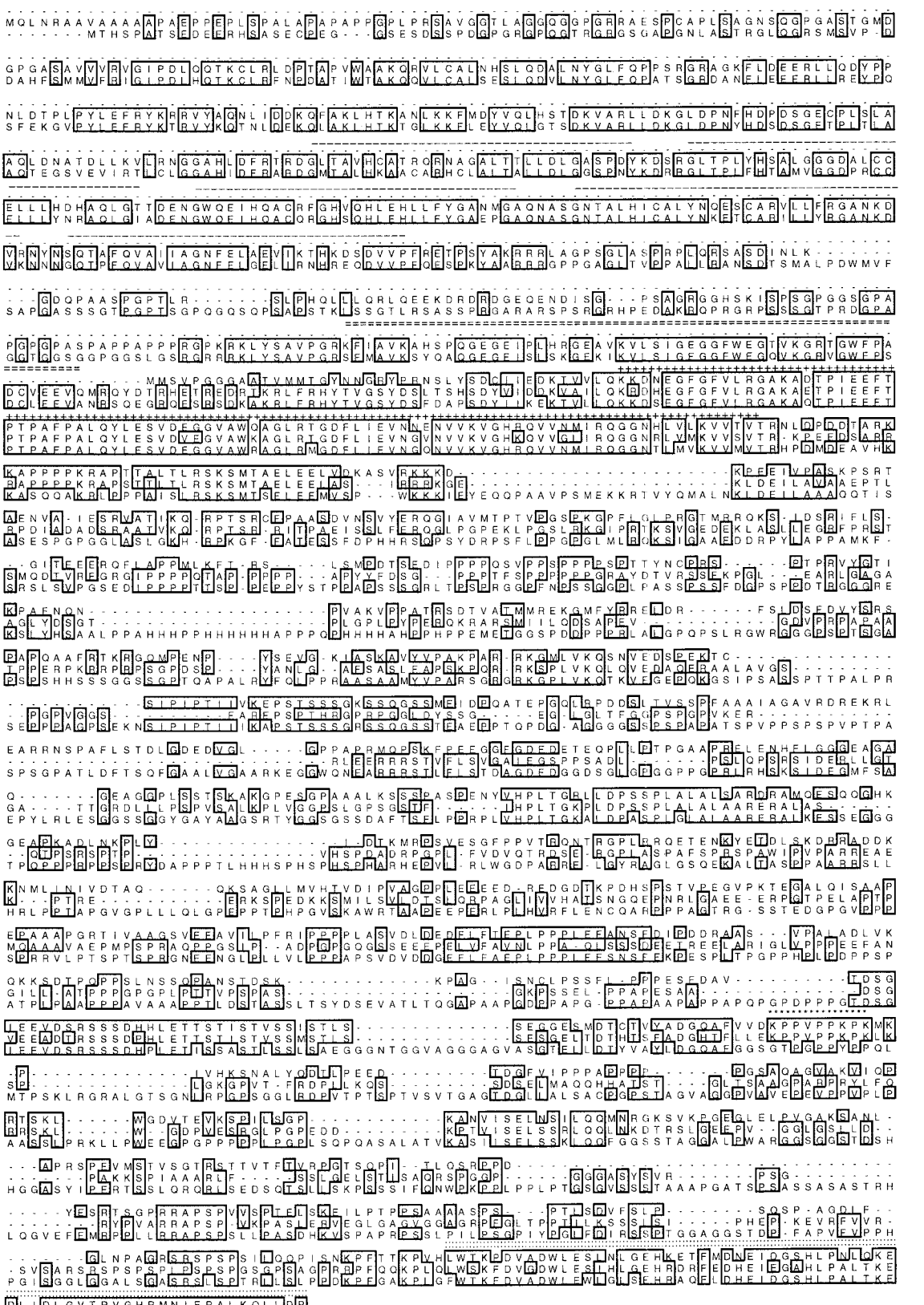

FIG. 1—*Continued*

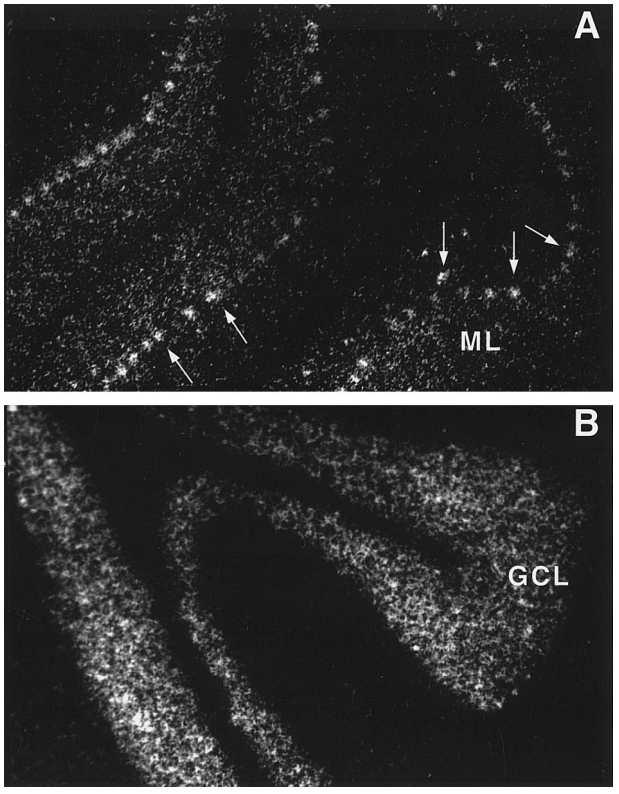

FIG. 2. Distribution of ProSAP1 (A) and ProSAP2 (B) in rat cerebellum *in situ* hybridization to rat brain sections with ^{35}S-labeled ProSAP1 and ProSAP2 antisense oligonucleotides reveals that ProSAP1 is strongly expressed in Purkinje cells (arrows) and shows a moderate labeling in the molecular layer (ML), whereas ProSAP2 is highly expressed in the granular cell layer (GCL). Magnification: 250-fold.

and ProSAP2. At the N-terminus ProSAP2 and synamon contain five N-terminal ankyrin repeats followed by a SH3 domain. This region is missing in ProSAP1 (Fig. 1B). Homology searches revealed that the recently identified proteins of the Shank family of proteins (14) belong to the same family or represent splice variants of the different ProSAP proteins.

Northern analysis suggests that both ProSAP1 and 2 are expressed specifically in the brain (not shown). To examine the spatial distribution of the transcripts we performed *in situ* hybridization studies to horizontal brain sections with antisense oligonucleotides directed against the C-terminal parts of the ProSAP1 and ProSAP2 mRNAs. ProSAP1 transcripts are highly expressed and widely distributed in neurons of the rat brain (12). In most brain regions, including cerebral cortex and hippocampus ProSAP1 and ProSAP2 appear codistributed (data not shown), whereas in the cerebellum a complementary distribution is observed (Fig. 2). ProSAP1 is primarily expressed in Purkinje cells, whereas ProSAP2 transcripts are only found in the granular cell layer of the cerebellum.

Identification of ProSAP interacting proteins. A hallmark of the ProSAP family of multidomain proteins is a new type of PDZ domain that is highly conserved within this family as well as between species (12). Therefore we performed a yeast two-hybrid screen using the ProSAP2 PDZ domain as a bait. Several of the isolated cDNAs encoded SAPAP family members (i.e., 1× SAPAP1/GKAP, 1× SAPAP2, 4× SAPAP3, 1× SAPAP4). In yeast these clones also interacted with baits containing the PDZ domain of ProSAP1. ProSAP1 originally has been isolated as a protein contained in synaptic junctional protein preparations from rat brain (4, 20). Biochemical analysis and ultra-structural localization studies revealed that ProSAP1 is indeed a component of the PSD of excitatory brain (12). Therefore we were sought to analyze in more detail the interaction between ProSAPs and SAPAPs/GKAPs, which are known to be PSD constituents (9). To this end, coexpression studies of different regions of ProSAP2 tagged with GFP with SAPAP1/GKAP, SAP90/PSD-95 and Kv1.4 potassium channels were performed in HEK cells. The latter 3 proteins have been shown to form clusters when coexpressed in COS cells (8). If the PDZ2 domain of ProSAP2 is present, the GFP fluorescence colocalizes with SAPAP1-SAP90-Kv1.4 clusters as detected with anti-Kv1.4 antibodies (Fig. 3A). In contrast, if the C-terminal region of ProSAP2 without the PDZ domain is cotransfected, no colocalization of GFP with SAPAP1/GKAP-SAP90/PSD-95-Kv1.4 clusters is observed. Also GFP-ProSAP2 (PDZ domain) does not cocluster if cotransfected with SAP90/PSD95 + Kv1.4 potassium channel alone (data not shown). Coimmunoprecipitation experiments further support the view that ProSAP2 is recruited to the clusters via its PDZ domain (Fig. 3B). From HEK cells cotransfected with GFP-ProSAP2$^{C\text{-term.}}$, SAPAP1/GKAP, SAP90/PSD-95, Kv1.4, neither GKAP nor SAP90/PSD-95 can be immunoprecipitated with anti-GFP antibodies, whereas both GKAP and SAP90/PSD-95 are coimmunoprecipitated with anti-GKAP and anti-SAP90 antibodies. In the presence of GFP-ProSAP2^{PDZ}, however, anti-GFP antibodies coimmunoprecipitate GKAP and SAP90/PSD-95. To assess whether ProSAPs, SAPAPs and MAGuKs may also interact *in vivo,* we performed immunoprecipitation experiments with anti-ProSAP1 and anti-GKAP antibodies from brain detergent extracts (Fig. 3C). Both antibodies coimmunoprecipitate ProSAP1, SAPAP1/GKAP and SAP90/PSD-95, suggesting that the three proteins are present in naturally occurring protein complexes.

Our experiments indicate that ProSAPs interact with proteins of the SAPAP/GKAP family via their PDZ domain. The yeast two hybrid screen gives also some clue to the region of SAPAPs that mediates this interaction. All 7 SAPAP cDNAs isolated in the screen included the C-terminus. The four SAPAPs share a

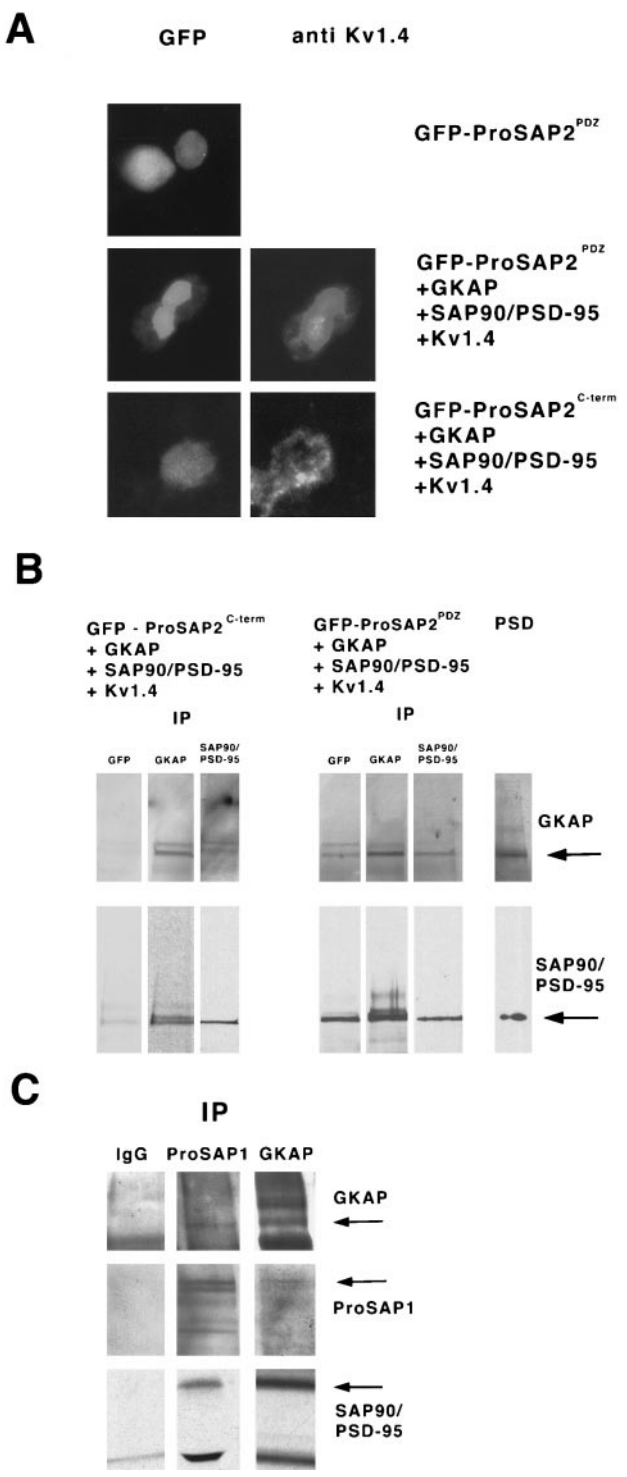

FIG. 3. Coclustering and Coimmunprecipitation of ProSAPs, GKAP, SAP90/PSD-95 and Kv1.4 in transfected HEK cells and in rat brain. (A) HEK cells were transfected with GFP-ProSAP2 (PDZ domain) alone, GFP-ProSAP2 (PDZ-domain) + GKAP + SAP90/PSD95 + Kv.1.4 potassium channel or with GFP-ProSAP2 (C-terminus without PDZ domain) + SAP90/PSD95 + Kv1.4 potassium channel. Cells were stained using a polyclonal antibody generated against Kv1.4 and a CY3 labeled secondary antibody (right panel). GFP-ProSAP2 (PDZ domain) is evenly distributed in the cytoplasm when transfected alone but is recruited into clusters when cotransfected with K.v.1.4 + SAP90/PSD-95 and GKAP. GFP-ProSAP2 (C-terminus without PDZ domain) does not cocluster if cotransfected. GFP-ProSAP2 (PDZ domain). (B) Extracts from transfected HEK cells (ProSAP2/C-term + GKAP + SAP90/PSD-95 + Kv1.4 and ProSAP2/PDZ domain + GKAP + SAP90/PSD-95 + Kv1.4) were immunoprecipitated with anti-GFP, anti-SAP90/PSD-95 and anti-GKAP antibodies as indicated. The immunoprecipitates as well as a rat brain PSD protein fraction were immunoblotted for GKAP and SAP90/PSD-95. Substantial amounts of GKAP and SAP90/PSD-95 can only be detected in GFP-precipitates if the GFP-ProSAP2/PDZ domain construct is present. (C) Rat brain extracts were immunoprecipitated with ProSAP1, GKAP or control (IgG) and immunoprecipitates were immunoblotted for GKAP, SAP90/PSD-95 and ProSAP1. After immunoprecipitation with rabbit anti-ProSAP1 antibodies, GKAP and SAP90/PSD-95 can be detected. Immunoprecipitates with GKAP antibodies contain GKAP, SAP90/PSD95 and ProSAP1 immunoreactivity.

completely conserved C-terminal sequence (-AQTRL) (8, 11), and one of the clones (SAPAP3/26) coded for only the last 40 C-terminal amino acids. Therefore we assume that the interaction motif for the ProSAP PDZ domain is the C-terminus of the SAPAPs. This is consistent with the major mode of target interaction described for other PDZ domains, which has been first described for the interaction of NMDA receptor and potassium channels subunits with the PDZ domains of MAGuKs (21, 22).

SAPAPs/GKAPs are supposed to be essential for the recruitment of MAGuKs, like SAP90/PSD-95, into the synaptic membrane (8, 9, 11) and both proteins may be essential for the synaptic localization of NMDA receptors (23). During synaptogenesis ProSAP1 appears very early at the developing PSD (12). ProSAP1 can be detected in PSD preparations about a week earlier than SAP90/PSD-95 and the NR1 subunit of the NMDA receptor. Here we have shown that the PDZ domain of the ProSAPs interacts directly with the SAPAP/GKAP family of synaptic proteins. This interaction may be an early step in the assembly of the postsynaptic apparatus.

The findings of this paper shed some light on the complexity of the network of proteins linking the synaptic membrane proteins to the cytoskeleton. MAGuKs serve as primary sockets to plug in neurotransmitter receptors, ion channels and synaptic cell adhesion molecules (6, 24–26). Via SAPAPs/GKAPs these complexes are linked to members of the ProSAP protein family. On the other hand ProSAPs interact with the actin cytoskeleton via cortactin (13). Thus these multi-domain proteins constitute a novel family of PSD proteins that are supposed to be placed in the center of the synaptic protein ensemble and may play an important role in the organization of the PSD during synaptogenesis and its reorganization during synaptic plasticity.

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