SYNAPTIC SCAFFOLDING PROTEINS IN RAT BRAIN

ANKYRIN REPEATS OF THE MULTIDOMAIN Shank PROTEIN FAMILY INTERACT WITH THE CYTOSKELETAL PROTEIN α-FODRIN

Received for publication, March 19, 2001, and in revised form, August 15, 2001 Published, JBC Papers in Press, August 16, 2001, DOI 10.1074/jbc.M102454200

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The postsynaptic density is the ultrastructural entity containing the neurotransmitter receptor apparatus of excitatory synapses in the brain. A recently identified family of multidomain proteins termed Src homology 3 domain and ankyrin repeat-containing (Shank), also known as proline-rich synapse-associated protein/somatostatin receptor-interacting protein, plays a central role in organizing the subsynaptic scaffold by interacting with several synaptic proteins including the glutamate receptors. We used the N-terminal ankyrin repeats of Shank1 and -3 to search for interacting proteins by yeast two-hybrid screening and by affinity chromatography. By cDNA sequencing of hippocampal cultures. Our data indicate that the Shank1 and -3 family members provide multiple independent connections between synaptic glutamate receptor complexes and the cytoskeleton.

The clustering of neurotransmitter receptors and cell adhesion molecules at specific sites of the cell membrane (i.e. the postsynaptic membrane) is brought about by a dense network of submembranous proteins that becomes visible as an electron-dense thickening at the postsynaptic membrane (postsynaptic density (PSD)). PSD proteins are able to anchor and cluster membrane-spanning receptors, cell adhesion molecules, or both via protein-protein interaction domains (1, 2).

The search for proteins that are localized at the PSD revealed a family of multidomain proteins termed proline-rich synapse-associated proteins 1 and 2 or somatostatin receptor-interacting protein. For convenience and in accord with the most frequently used nomenclature, we will refer to these proteins as Src homology 3 domain and ankyrin repeat-containing (Shank) proteins (see Scheme 1). These proteins may act as a molecular interface between neurotransmitter receptors and cell adhesion molecules and the actin-based cytoskeleton (5, 7, 9). To date three members of this family, Shank1–3, have been identified (4–11), containing multiple protein-protein interaction domains including ankyrin repeats, an Src homology 3 domain (SH3) domain, a PSD-95/discs large/ZO-1 (PDZ) domain, several proline-rich regions, and a C-terminal sterile α-motif domain (Scheme 1).

The highly conserved PDZ domain has been shown to interact with the C terminus of several different proteins such as the postsynaptic density protein synapse-associated protein-associated protein/guanylate kinase-associated protein (4, 6, 9) and several G-protein-coupled receptors, including the somatostatin receptor subtype 2 (5, 10) and the calcium-independent receptor for α-latrotoxin (12, 13). Via synapse-associated protein-associated protein/guanylate kinase-associated protein, the PDZ domain of the Shank proteins is linked to the N-methyl-D-aspartate receptor-PDZ-95 complex, whereas one of their proline-rich regions serves as a docking site for the metabotropic glutamate receptor-binding protein Homer (11). In addition, the SH3 domain has been proposed to interact indirectly with the α-amino-3-hydroxy-5-methylisoxazole-4-propionate-type glutamate receptor-interacting protein (14). Taken together, these data suggest that the Shank proteins act as a master scaffold for glutamate receptor complexes in postsynaptic specialization (14). Firm contact of this scaffold with the cytoskeleton is then established via the interaction of another proline-rich region of Shanks and the SH3 domain of the F-actin-binding protein cortactin (8).

To date, little is known of potential protein candidates interacting with the N-terminally located ankyrin repeats of Shank proteins.

The abbreviations used are: PSD, postsynaptic density; BSA, bovine serum albumin; EGFP, enhanced green fluorescent protein; HEK, human embryonic kidney; PBS, phosphate-buffered saline; PDZ, PSD-95/discs large/ZO-1; SH3, Src homology 3; Shank, Src homology 3 domain and ankyrin repeat-containing; PAGE, polyacrylamide gel electrophoresis; RIPA, radioimmunoprecipitation assay; Fod, α-fodrin construct.

* This work was supported in part by Deutsche Forschungsgemeinschaft Grants SFB545/B7 (to H.-J. K. and D. R.), SFB426/A1 (to E. D. G.), and KR1879/2–1 (to M. R. K.), European Commission Grant QLG3-CT-1999-00908 (to D. R.), Fonds der Chemischen Industrie (to E. D. G.), and KR1879/2–1 (to M. R. K.), European Commission Grant QLG3-CT-1999-00908 (to D. R.), Fonds der Chemischen Industrie (to E. D. G.).

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Shank1 and -3. First described by Breeden und Nasmyth (15) in two yeast cell cycle regulator proteins (Swi6p and CDC10p), ankyrin repeats were named after repetitive units present in the ubiquitous adapter protein ankyrin (16) and are known for their interaction with many diverse proteins (17). Here we report on a novel interaction of the ankyrin repeats of Shank1 and -3 with a component of the membrane-associated cytoskeleton α-fodrin (also known as brain α-spectrin), another multidomain protein that contains 22 spectrin repeats, one SH3 domain, and two EF-hand motifs proximal to the C terminus of the protein (18–20). Our data indicate that the complexes consisting of α-fodrin and either Shank1 or Shank3 may represent a dynamic substructure of the synapse responsible for alterations of the functional architecture that occur during the organization and reorganization of spines and synapses in the central nervous system (21, 22).

MATERIALS AND METHODS

Expression of Fusion Proteins—a cDNA fragment encompassing the ankyrin repeat region (six repeats) of human Shank1 was cloned into the bacterial expression vector pQE-30 (Qiagen, Hilden, Germany), which allows the expression of His6-tagged fusion proteins. His6-Shank1189–359 was expressed in Escherichia coli strain TopF10 and purified on Ni-chelating Sepharose (Qiagen) following the manufacturer’s instructions. cDNA fragments coding for the C-terminal two spectrin repeats and the EF-hands of α-fodrin as well as the EF-hands alone were cloned into the pGEX-4T-2 glutathione S-transferase fusion protein expression vector (AP Biotech, Freiburg, Germany); proteins were expressed in TopF10 cells and purified on glutathione-Sepharose (AP Biotech).

Purification of Proteins Binding to Shank1—Affinity purification of His6-Shank1189–359-binding proteins followed a protocol essentially as described by Firestein and Bredt (23). After dialysis into 0.1 M NaHCO3 and 0.5 M NaCl, 10 mg of purified protein were coupled to 5 ml of N-hydroxy-succinimidyl-Sepharose (Amersham Pharmacia Biotech). Bovine serum albumin (BSA) was also coupled to N-hydroxy-succinimidyld-Sepharm (5 mg BSA/ml of Sepharose). Brains of 25 adult rats were homogenized in 200 ml of buffer A (20 mM HEPES, pH 7.4, 125 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, and 0.5 M NaCl, 10 mg of purified protein were coupled to 5 ml of N-hydroxy-succinimidyl-Sepharose (Qiagen) following the manufacturer’s instructions. cDNA fragments coding for the C-terminal two spectrin repeats and the EF-hands of α-fodrin as well as the EF-hands alone were cloned into the pGEX-4T-2 glutathione S-transferase fusion protein expression vector (AP Biotech, Freiburg, Germany); proteins were expressed in TopF10 cells and purified on glutathione-Sepharose (AP Biotech).

Diagram 1. SSTRIP, somatostatin receptor-interacting protein; ProSAP, proline-rich synapse-associated protein; CortBP1, cortactin-binding protein 1; Ank, ankyrin repeat; SAM, sterile α-motif.

After staining with Coomassie Brilliant Blue, bands of interest were excised, cut into small pieces, and rinsed exhaustively in water until the pH of the supernatant was neutral. The gel pieces were lyophilized and subsequently rehydrated with 50 ml Tris-HCl, pH 8.5, containing trypsin (10% of the estimated protein content of the band). After digestion overnight at 37 °C, peptides were extracted with 10% trifluoroacetic acid, purified on Sep-Pak C-18 cartridges (Waters, Eschborn, Germany), and lyophilized.

Mass Spectrometry—Samples were dissolved in water; 1 μl was applied on a fast evaporation nitrocellulose/α-cyano-4-hydroxycinnamic acid layer (24) and analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (Bruker Reflex mass spectrometer, reflector mode, pulsed-ion extraction). For protein identification, a data base search using the recorded peptide masses was performed with the program ProFound version 4.10.3 (www.protometrics.com/). The following search parameters were used: data base NCBI Nbr (2000/07/15), entries only from rat (Rattus norvegicus), full mass and pI range, maximum one missed cleavage, protease trypsin, monoisotopic masses M + H, and mass tolerance ±0.3 Da (external calibration only).

Yeast Two-hybrid Screening—A yeast-two-hybrid screen was performed using the Y190 yeast strain harboring the reporter genes HIS3 and β-galactosidase under the control of upstream GAL1-activating sequence. As a bait, the cDNA coding for the six ankyrin repeats of Shank3240–442 were fused to the GAL4 DNA binding domain in vector pAS2-1 (CLONTECH). A brain cDNA library cloned into the pAct vector (GAL4 activation domain; CLONTECH) was screened. Putative protein–protein interactions in yeast were tested by the ability to activate both HIS3 and β-galactosidase gene transcription. To eliminate false-positive putative interaction partners, the library plasmids were cotransformed with various bait constructs, and afterward candidates were sequenced.

To further determine the interaction domain of the initial α-fodrin prey clone, several partial cDNAs coding for one or a combination of different C-terminal α-fodrin domains were amplified by polymerase chain reaction and cloned into the pACT2 vector. Protein–protein interactions in yeast were subsequently tested by the ability to activate both HIS3 and β-galactosidase gene transcription after retransformation in yeast expressing the Shank3240–442 construct as a bait.

Pull-down Assays—Glutathione S-transferase fusion proteins of α-fodrin fragments were expressed and purified; proteins were not eluted but left on the glutathione-Sepharose. 35 μg of His6-Shank1189–359 were added in 50 μl Tris-HCl, pH 7.4, and 0.1% Triton X-100 and incubated at 4 °C for 2 h. After extensive washing in the same buffer, bound fusion proteins were eluted by boiling in SDS sample buffer, separated on 10% SDS-PAGE, and analyzed by Western blotting using an antiserum directed against the ankyrin repeat region of Shank1.

Cell Culture Experiments—COS cells were transfected with a Shank3-enhanced green fluorescent protein (EGFP)-SH3 domain (EGFP-Shank3326–651), an EGFP-ankyrin repeat construct (EGFP-Shank3240–442; pEGFP-vector; CLONTECH), C-terminal α-fodrin cDNA fragments carrying an N-terminal Flag epitope tag (pCMV2-vector; Stratagene, La Jolla, CA), and full-length α-fodrin carrying a C-terminal Flag epitope tag (kindly provided by Drs. J. Morrow and M. Stankewich, Yale University). Cells were grown on glass coverslips treated with poly-D-lysine, fixed with 4% paraformaldehyde, and processed for immunofluorescence detection. Human embryonic kidney (HEK) 293 cells were cotransfected with expression vectors containing a Shank1 fragment coding for the N-terminal Shank11–1288 (5) and the full-length α-fodrin vector. Cells were either processed for immunofluorescence analysis (using a rabbit anti-PDZ domain antibody for...
Shank1 and a mouse monoclonal anti-Flag antibody for the detection of α-fodrin, followed by Cy2-labeled anti-rabbit and Cy3-labeled antimouse secondary antibodies or for immunoprecipitation.

Coimmunoprecipitation Experiments—Membrane fractions from brain were solubilized in radioimmunoprecipitation assay (RIPA) buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl) at 37 °C for 30 min, followed by incubation on ice for 30 min (12). Similarly, transfected HEK cells were incubated with the anti-α-fodrin antibody (5 μg) for 1 h at 4 °C. Immune complexes were collected by incubation (overnight at 4 °C) with Protein A/G-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA). For precipitation with a Shank3 antibody (directed against residues 980–1786 of rat Shank3), solubilization was performed with 1% deoxycholate, 50 mM Tris-HCl, pH 9.0, and 50 mM EDTA. Extracts were incubated with 10 μl of antisera overnight, followed by protein A/G-Sepharose treatment for 2 h. After washing the Sepharose beads extensively in lysis buffer, the immune complexes were eluted from the beads with Laemmli sample buffer, separated on 7% polyacrylamide gels, and electroblotted onto nitrocellulose. The filters were probed with antibodies directed against the Shank1 PDZ domain (5, 10), the C-terminal 800 amino acids of Shank3 or an interacting partner (Orig-Prey); mapping of the interaction site by co-transformation of different prey constructs with the Shank3 bait revealed that spectrin repeat 21 (Fod1) is sufficient to induce bait-prey interaction. Interestingly, spectrin repeats 21 and 22 (Fod2) show only weak interaction that is probably due to misfolding of the prey protein. Constructs that do not contain spectrin repeat 21 (Fod4–Fod9) were negative in the YTH assay. Spec, spectrin repeat (gray oval); EF, common motif of a superfamily of calcium-binding proteins (dark rectangles).

**Fig. 1. Yeast two-hybrid assay with Shank3**. A yeast twohybrid (YTH) assay with the ankyrin repeat region of Shank3 (Shank3399–442) as a bait yielded a partial cDNA clone of α-fodrin as an interacting partner (Orig-Prey); mapping of the interaction site by co-transformation of different prey constructs with the Shank3 bait revealed that spectrin repeat 21 (Fod1) is sufficient to induce bait-prey interaction. Interestingly, spectrin repeats 21 and 22 (Fod2) show only weak interaction that is probably due to misfolding of the prey protein.

**Materials and Methods.**

**Immunohistochemistry of Hippocampal Neurons and Rat Brain Sections—Hippocampal neuronal cultures from 18-day-old embryonic rats were prepared and grown on coverslips as described by Goslin and Banker (25), washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde at room temperature for 15 min, and blocked with 10% horse serum in PBS (0.01% Triton X-100) for 30 min. For double immunofluorescence, the primary cultures were incubated overnight at 4 °C with rabbit anti-Shank3 (1:400) and mouse anti-α-fodrin antibodies (Chemicon International Inc., Temecula, CA; 1:50) in 10% horse serum in PBS (0.91% Triton X-100). After three washes in PBS, coverslips were incubated for 3 h with goat anti-mouse Cy2 and goat anti-rabbit Cy3 (Jackson ImmunoResearch, West Grove, PA). Secondary
antibodies were diluted 1:150 in PBS. Photographs were taken using an Aristoplan photomicroscope (Leitz, Wetzlar, Germany) or a confocal laser scanning microscope (TCS 4D; Leica, Bensheim, Germany).

For immunohistochemistry (26), rat brains were perfusion-fixed with Bouin’s fluid and embedded in Paraplast (Sigma). Subsequently, 15-mum frontal and sagittal brain section were deparaffinized in xylene, rehydrated through a graded ethanol series, and equilibrated in 0.1M Tris-HCl buffer, pH 7.6, for 10 min. After preincubation with 5% normal swine serum in 0.1M Tris-HCl, pH 7.6, and 0.2% Triton X-100 for 30 min, the primary antibodies (Shank3, 1:1000; and -fodrin, 1:100) were applied in preincubation buffer for 22 h at room temperature. Subsequently, sections were incubated with swine anti-rabbit (mouse) IgG (Dako, Hamburg, Germany) diluted 1:50 for 30 min and then with rabbit (mouse) peroxidase-antiperoxidase complex (Dako) diluted 1:100 for 30 min. Antibody binding was visualized by the application of 3,3-dianinobenzidine (0.05%) and H2O2 (0.001%; Sigma) for 6 min. After completion of the staining procedure, sections were dehydrated and mounted in DePeX (Serva, Heidelberg, Germany).

**RESULTS**

**Identification of -fodrin as Interacting Partner of the Ankyrin Repeats of Shank3 and Shank1**—In a yeast-two-hy-
brid experiment, the ankyrin repeats of Shank3, 240–442 were used as bait to screen for potentially interacting proteins in a rat brain cDNA library. Of 1/100,306 clones, four positive candidates were obtained: three clones coded for rat amyloid precursor-like protein 1 and one clone for the C-terminal part of -fodrin (amino acids 2019–2472; Fig. 1). The sequence starts in the middle of the 20th spectrin repeat, followed by spectrin repeats 21 and 22 and two EF-hand regions known to be common motifs of a superfamily of calcium-binding proteins; thus the clone lacks most of the spectrin repeats as well as the SH3 domain (20).

To further map the region of -fodrin interacting with the ankyrin repeats of Shank3, several -fodrin prey constructs were designed, coding for one or a combination of the above-mentioned domains (named Fod1–Fod9; see Fig. 1). The yeast-two-hybrid assay revealed that spectrin repeat 21 alone (Fod1) displayed a strong interaction with the ankyrin repeats of Shank3, whereas the construct containing spectrin repeats 21 and 22 (Fod2) showed only a weak interaction, suggesting that the latter construct may not fold properly in the yeast. Alternatively, intramolecular interactions between different repeats may prevent accessibility of spectrin repeat 21 to the ankyrin repeats of Shank3. A strong interaction was also observed with Fod3 coding for spectrin 21 and 22 plus the first EF-hand motif (Fig. 1), whereas all other prey constructs lacked any interaction with the ankyrin repeats.

In a parallel affinity chromatography experiment, a His6-tagged recombinant Shank1 protein containing the six N-terminally located ankyrin repeats (His6-Shank1189–399) was used as an affinity matrix to search for binding partners of the ankyrin domains of Shank1 in the rat brain (Fig. 2A, lane 1). After eluting the bound proteins from the His6-Shank1189–399-Sepharose beads, three major bands were visible on SDS-PAGE (Fig. 2A, lane 3). Two bands at 45 and 260 kDa were clearly enriched when compared with the supernatant fraction after preadsorption with BSA-Sepharose (lane 2); a third band at 50–55 kDa was also very abundant in the affinity-purified preparation but had about the same intensity as in the initial supernatant fraction (lane 2). These bands were excised from the gel, digested with trypsin, and identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry using the mass fingerprinting technique as -actin (45-kDa band), tubulin (50–55-kDa band), and -fodrin (260-kDa band). A Western blot of the affinity-purified eluate incubated with an antibody confirmed the presence of -fodrin at 260 kDa and some of its major proteolytic breakdown products at 120 and 150 kDa (lane 5).

To determine whether the binding between Shank1 and -fo-
Interaction of Shank1 and -3 with α-Fodrin

α-fodrin in a mammalian system, COS7 cells were transfected either alone or in combination with cDNAs encoding the Flag-tagged α-fodrin<sub>2011-2472</sub> and EGFP-tagged Shank3. Two Shank3 constructs were analyzed, one carrying the ankyrin repeats (Shank3<sub>240-442</sub>) and the other carrying the SH3 domain (Shank3<sub>326-610</sub>). As shown in Fig. 3, in cells transfected only with Shank3<sub>240-442</sub> carrying the ankyrin repeats, the tagged protein was specifically recruited to distinct structures within the cell nuclei (Fig. 3A). This is presumably due to a cryptic nuclear localization signal in the construct (Lys-Arg-Arg-Arg, amino acids 429–432), because full-length Shank3 was not observed to appear in nuclei (data not shown). In contrast, when cells were transfected only with the Shank3<sub>326-610</sub> construct that encodes the SH3 domain (Fig. 3B) or with α-fodrin<sub>2011-2472</sub>, the tagged proteins were diffusely distributed in the cell cytoplasm (Fig. 3C). Although it is unclear at present whether nuclear localization of the Shank3-ankyrin construct is of physiological relevance, it provided us with a unique opportunity to assay for strong protein interactions by looking for changes in the cellular localization of interacting proteins. Thus, when cotransfected in COS7 cells with the Shank3 construct carrying the ankyrin repeats, α-fodrin<sub>2011-2472</sub> undergoes a dramatic redistribution into the same small puncte of the cell nuclei as seen for the tagged Shank3<sub>240-442</sub> alone (Fig. 3, D–F). Similarly, a construct containing only spectrin repeat 21 (α-fodrin<sub>2011-2186</sub>), targeted to the nucleus by interaction with the Shank3 ankyrin repeats (Fig. 3, G–I) underscoring the strong interaction of Shank3 and α-fodrin in mammalian cells. When full-length α-fodrin was coexpressed with the Shank3 ankyrin construct, nuclear redistribution was also observed, but only in ~60% of analyzed cells; an example is shown in Fig. 3, J–L. The lower efficiency of this redistribution with the full-length construct may be due to the numerous other interactions of the α-fodrin molecule, which may require localization in the cytoplasm rather than in the nucleus. This is in agreement with colocalization experiments of full-length α-fodrin and human Shank1 proteins on transfection in HEK cells; here both proteins were mainly colocalized in the cytoplasm (Fig. 4A). The interaction between both proteins in these cells was verified by coprecipitating both proteins using an antibody against α-fodrin (Fig. 4B). Taken together with the mapping analysis performed in the yeast two-hybrid system and the protein purification experiments by the affinity matrix, the in vivo colocalization data demonstrate that the ankyrin repeats of the Shank protein family interact specifically and strongly with spectrin repeat 21 of α-fodrin.

In Vivo Coimmunoprecipitation of α-Fodrin with Shank1 or Shank3—To determine whether the interaction between the ankyrin repeats of Shank1 and α-fodrin can be observed in vivo, coimmunoprecipitation experiments were performed from a brain membrane fraction (Fig. 5). In these experiments we used the anti-Shank1 PDZ domain antibody that recognizes both the 260-kDa (containing the ankyrin repeats; lanes 1–4, asterisks) and the 180-kDa (lacking the ankyrin repeats; lanes 1 and 3, arrowheads) forms of Shank1. The assignment that the 180-kDa protein lacks the ankyrin region was supported by a parallel experiment using an antiserum directed against the ankyrin repeats (Fig. 5, lane 2, and Ref. 5). Both forms, the 260- and 180-kDa proteins, could be partially solubilized in RIPA buffer as shown in Fig. 5, lane 3; precipitation from this lysate using an anti-α-fodrin antibody revealed an immunoprecipitate that when analyzed by Western blotting with the PDZ domain-specific antibody depicted only the larger 260-kDa form of Shank1 (lane 4). The 180-kDa protein was missing in the precipitate, consistent with the notion that α-fodrin specifically interacts with the ankyrin repeats of Shank1 (or Shank3).
α-Fodrin itself could be abundantly detected in the lysate as well as in the precipitate (lanes 8 and 9). When precipitating Shank3 with an antiserum directed against the C-terminal half of the molecule, three bands specific for Shank3 could be detected in the lysate (lane 5) and strongly enriched in the precipitate (lane 7). α-Fodrin was detected in the lysate (lane 10) as well as in the Shank3 precipitate (lane 12), whereas neither Shank3 nor α-fodrin was precipitated with a control antiserum (lanes 6 and 11). Thus our data indicate that ankyrin-containing forms of Shank1 and Shank3 are associated with α-fodrin in the rodent brain in vivo. It should be noted that with both the anti-α-fodrin and the anti-Shank3 antibody, it was not possible to quantitatively precipitate α-fodrin, simply because this cytoskeletal protein is very abundant in brain and is present not only in postsynaptic areas but also in axons, dendrites, and cell bodies (see below). Shank3 and Shank1 proteins instead appear to be largely confined to the postsynaptic regions of glutamatergic synapses, and only there may they be complexed with α-fodrin molecules, explaining the weak bands in our Western blot analysis.

Localization of α-Fodrin and Shank3 by in Situ Hybridization and Immunohistochemistry—To compare the expression pattern of Shank3 and α-fodrin, we performed in situ hybridization and immunohistochemical experiments of cultured neurons and of rat brain sections. The double immunostaining of 3-week-old hippocampal culture neurons with Shank3 and α-fodrin antibodies shows the colocalization of both proteins at a subcellular level (Fig. 6). Although Shank3 is found solely in the postsynaptic densities of these neuronal cells, α-fodrin is more widely distributed in dendrites and PSDs but is clearly enriched toward the postsynapstic. At a higher magnification (insets), the arrows point to overlapping signals at PSDs after staining with Shank3 and α-fodrin antibodies.

To investigate the expression pattern of Shank3 and α-fodrin in rat brain, we used in situ hybridization (Fig. 7A) and immunohistochemistry (Fig. 7B). Shank3 mRNA as well as α-fodrin mRNA are widely expressed throughout the rat brain from day 1 postnatally onward. The expression patterns of both transcripts match at all stages of development. However, although α-fodrin is steadily expressed at a high level during postnatal development, Shank3 expression slightly increases toward days 8–16 and is significantly reduced at adult stages. In contrast to α-fodrin transcripts, which are almost evenly distributed throughout the brain, Shank3 mRNA shows higher expression levels in the outer cortical layers (layers 1 and 2) and the cerebellum compared with other brain regions (Fig. 7A). A similar widespread transcript distribution in rat brain has been observed previously using probes specific for Shank1 (5).

α-Fodrin and Shank3 proteins display a virtually identical distribution pattern throughout the rat brain. Although the cytoplasm of most neurons is devoid of staining, antibodies against both proteins detect their antigen in neurites and as small punctae in the neuropil. α-Fodrin as well as Shank3 is most intensively expressed in the hippocampal formation as well as in the cerebellum (Fig. 7B). In the hippocampal cornu ammonis 1 region, cell bodies in the stratum pyramidale are not stained, but an identical pattern of immunoreactivity is found along the neurites in the stratum radiatum and in the stratum oriens. In the cerebellum, the cytoplasm of the Purkinje cells is nearly devoid of staining, whereas in the molecular layer, dense labeling of the neuropil is observed. In the granular cell layer, the glomeruli, i.e. large synaptic structures connecting mossy fibers from deep cerebellar nuclei with the cerebellar granule cells, are intensely stained (arrows). The cytoplasm of the granule cells is not labeled (Fig. 7B). In conclusion, the widespread colocalization of Shank3 and α-fodrin supports a role for the interaction of these two proteins in vivo.

**DISCUSSION**

In this study we have shown that fodrin interacts with two members of the Shank protein family connecting these scaffold proteins to the brain spectrin family of cytoskeletal proteins. Fodrin consists of α- and β-subunits, which form antiparallel dimers. Only α-fodrin has been found to interact with the N-terminal ankyrin repeats of Shank1 and -3, as has been documented by various means, including affinity chromatography, yeast two-hybrid screening, colocalization analysis, and communoprecipitation experiments from transfected cells and brain extracts. The initial yeast two-hybrid screen indicated that within α-fodrin the interaction domain is located near the C terminus and includes spectrin repeats 21 and 22 and the two Ca²⁺ binding EF-hand motifs. Further mapping experiments revealed that spectrin repeat 21 alone represents the minimal domain structure essential for the protein-protein interaction with the ankyrin repeats of Shank1 and -3. This is particularly remarkable because the amino acid sequence of the spectrin repeat 21 region is not only highly conserved within the α-fodrin molecule itself but also among species (100% identity to human, mouse, and chicken, 81% identity to Drosophila melanogaster, and 79% identity to Caenorhabditis elegans); in contrast, the sequence of the remaining spectrin repeats within the α-fodrin molecule are rather diverse (20–25% identity). On the other hand, ankyrin repeats exhibit
secondary structures in the form of pairs of antiparallel α-helices that are connected by a series of β-hairpin motifs; yet they do not prefer particular motifs, nor do they recognize consensus sequences of the target molecules (17). Thus the finding that the domain structure of ankyrin repeats of Shank1 and -3 recognizes only, preferentially and specifically, spectrin repeat
21 may underline the functional importance of the conserved sequence motif of the cytoskeletal protein α-fodrin.

The structural interaction of Shank3 with α-fodrin is complemented by in situ hybridization and immunocytochemistry experiments demonstrating the coexpression and colocalization of Shank3 with α-fodrin in rat brain. Shank1 displays a similar widespread distribution (3, 5). In situ hybridization at different developmental stages documents that the mRNAs share overlapping expression patterns in all brain areas at all time points investigated. At the protein level, the virtually identical spatial expression of Shank3 and α-fodrin is especially obvious in the hippocampus and cerebellum. Colocalization of both proteins at density. The finding that Shank3 with experiments demonstrating the coexpression and colocalization in situ complemented by Shank3-ankyrin binding) part of widespread distribution (3, 5).

In fact, fodrin has been shown to be a major constituent and -3. In fact, fodrin has been shown to be a major constituent of the cytoskeletal protein aspartate receptor-metabotropic glutamate receptor complex degrades N-methyl-D-aspartate, metabotrobic, and possibly also the fodrin-containing part of the cytoskeleton, accompanied by rapid remodeling of synapses after stimulation.

Acknowledgments—We thank Hans-Hinrich Honck, Gisela Gaede, and Annemie Ahle for excellent technical assistance, Drs. J. Morrow and M. Stankevich (Yale University) for providing an α-fodrin cDNA construct, and Dr. Peter Franke, (Abeitsgruppe Neurochemie, Prof. F. Huch, Institut für Chemie-Biochemie, Freie Universität Berlin) for help with mass spectrometric analysis.

REFERENCES

1. Ziff, E. B. (1997) Neuron 19, 1163–1174
2. Garner, C. C., Nash, J., and Huganir, R. L. (2000) Trends Cell Biol. 10, 274–280
3. Lim, S., Naishiti, S., Yoon, J., Hwang, J. I., Suh, P. G., Sheng, M., and Kim, E. (1999) J. Biol. Chem. 274, 29010–29018
4. Naishiti, S., Kim, E., Tu, J. C., Xiao, B., Sala, C., Valtschanoff, J., Weinberg, J., Worley, P. F., and Sheng, M. (1999) Neuro 23, 589–592
5. Zitter, H., Honck, H. H., Bächner, D., Richter, D., and Kreienkamp, H. J. (1999) J. Biol. Chem. 274, 32997–33001
6. Yarali, H., Hira, K., Deguchi, M., Die, N., Takeuchi, M., and Takay, Y. (1999) J. Biol. Chem. 274, 27463–27466
7. Boeckers, T. M., Kreutz, M. R., Winter, C., Zuschratte, W., Smalla, K. H., Sanmarti-Vila, L., Wex, H., Langenkae, K., Bockmann, J., Garner, C. C., and Gundelfinger, E. D. (1999) J. Neurosci. 19, 6506–6518
8. Du, Y., Weed, S. A., Wen-Chung, X., Marshall, T. D., and Parsons, T. J. (1998) Mol. Cell. Biol. 18, 5858–5861
9. Boeckers, T. M., Winter, C., Smalla, K. H., Kreutz, M. R., Bockmann, J., Seidenbecher, C., Garner, C. C., and Gundelfinger, E. D. (1999) Biochem. Biophys. Res. Commun. 264, 247–252
10. Zitter, H., Richter, D., and Kreienkamp, H. J. (1999) J. Biol. Chem. 274, 18153–18156
11. Tu, J. C., Xiao, B., Naishiti, S., Yuan, J. P., Petralia, R. S., Brekman, P., Deane, A., Askalu, V. K., Lanahan, A. A., Sheng, M., and Worley, P. F. (1999) Nature 401, 230–2309
12. Kreienkamp, H. J., Zitter, H., Gundelfinger, E. D., Richter, D., and Boeckers, T. M. (2000) J. Biol. Chem. 275, 32387–32390
13. Tobaben, S., Sudhol, T. C., and Stahl, B. (2000) J. Biol. Chem. 275, 36204–36210
14. Sheng, M., and Kim, E. (2000) J. Cell Sci. 113, 1851–1856
15. Birken, D., and Nasmyth, K. (1987) Nature 329, 651–654
16. Lux, S. E., John, K. M., and Bennett, V. (1990) Nature 344, 36–42
17. Sedgwick, S. G., and Smorder, S. J. (1999) Trends Biochem. Sci. 24, 311–316
18. Levine, J., and Willard, M. (1981) J. Cell Biol. 90, 631–642
19. Carlin, R. K., Bartelt, D. C., and Verhe, P. (1983) J. Cell Biol. 96, 443–448
20. McMahon, A. P., Giebelhaus, D. H., Champion, J. E., Bailes, J. A., Lacey, S., Carritt, B., Henchman, S. K., and Moon, R. T. (1987) Differentiation 34, 68–78
21. Engert, F., and Benhoeffner, T. (1999) Nature 399, 66–70
22. Lüscher, C., Nicoll, R. A., Malenka, R. C., and Muller, D. (2000) Nat. Neurosci. 3, 545–550
23. Firestein, B. L., and Breit, D. S. (1999) J. Biol. Chem. 274, 10545–10550
24. Vorm, O., Roestoff, P., and Mann, M. (1994) Anal. Chem. 66, 3281–3287
25. Goslin, K., and Banker, G. (1991) in Culturing Nerve Cells (Banker, G., and Goslin, K., eds), pp. 253–281, MIT Press, Cambridge, MA
26. Sternberger, L. A., Hardy, P. H., Cuculis, J. J., and Meyer, H. G. (1970) J. Histochem. Cytochem. 18, 315–333
27. Kennedy, S. P., Warren, S. L., Forget, B. G., and Morrow, J. S. (1991) J. Biol. Chem. 266, 267–277
28. Davis, L. H., and Bennett, V. (1990) J. Cell Biol. 105, 10598–10596
29. Glennon, J. R., Jr., Glennon, P., and Weber, K. (1983) J. Mol. Biol. 167, 257–279
30. Harris, A. S., and Morrow, J. S. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2309–2313
31. Vanderklish, P. W., Kruhsel, L. A., Holst, B. H., Gly, J. A., Crossin, K. L., and Edelman, G. M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2253–2258
32. Siman, R., Baudry, M., and Lynch, G. (1985) Nature 313, 225–228