Synaptic Targeting of PSD-Zip45 (Homer 1c) and Its Involvement in the Synaptic Accumulation of F-actin*

Received for publication, October 22, 2002, and in revised form, December 30, 2002
Published, JBC Papers in Press, January 10, 2003, DOI 10.1074/jbc.M210802200

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PSD-Zip45/Homer 1c, which contains an enabled/VASP homology 1 (EVH1) domain and leucine zipper motifs, is a postsynaptic density (PSD) scaffold protein that interacts with metabotropic glutamate receptors and the Shank family. We studied the molecular mechanism underlying the synaptic targeting of PSD-Zip45 in cultured hippocampal neurons. The EVH1 domain and the extreme C-terminal leucine zipper motif were molecular determinants for its synaptic targeting. The overexpression of the mutant of the EVH1 domain or deletion of the extreme C-terminal leucine zipper motif markedly suppressed the synaptic localization of endogenous Shank but not PSD-95 or GKAP. In contrast, an overexpressed GKAP mutant lacking Shank binding activity had no effect on the synaptic localization of Shank. Actin depolymerization by latrunculin A reduced the synaptic localization of PSD-Zip45, Shank, and F-actin but not of PSD-95 or GKAP. Overexpression of PSD-Zip45 enhanced the accumulation of synaptic F-actin. Additionally, overexpression of PSD-Zip45 and an isoform of Shank induced synaptic enlargement in association with the further accumulation of synaptic F-actin. The EVH1 domain and the extreme C-terminal leucine zipper motif of PSD-Zip45 were also critical for these events. Thus, these data suggest that the PSD-Zip45/Sank and PSD-95/GKAP complexes form different synaptic compartments, and PSD-Zip45 alone or PSD-Zip45-Sank is involved in the synaptic accumulation of F-actin.

As revealed by electron microscopy, the postsynaptic density (PSD) is a dense structure of the excitatory synapses in the central nervous system (1, 2). Recently, numerous studies (3–5) have reported on the identification of PSD scaffold proteins and their involvement in synaptic function. We isolated PSD-Zip45, a novel member of the Homer family, using a PSD-specific monoclonal antibody. This protein contains an enabled/VASP homology 1 (EVH1) domain at its N- and C-terminal leucine zipper motifs (6, 7). The same protein was independently reported as Homer 1c (8) and vesl-1L (9). The EVH1 domain interacts with the PPXXF motif found in group 1 metabotropic glutamate receptors (mGluRs) (7, 8, 10), Shank family members (11, 12), inositol triphosphate receptors, and ryanodine receptors (13). The extreme C-terminal leucine zipper motif is involved in self-multimerization (7). We recently analyzed the dynamic behavior of PSD-Zip45 within dendritic spines using time-lapse confocal microscopy, and we demonstrated its high steady-state turnover rate and assembly/disassembly dynamics, which depend on Ca2+ signals from different sources (14).

The first-discovered PSD scaffold protein, PSD-95 (SAP90), interacts with the NR2 subunits of the NMDA receptor (15–17) and other PSD scaffold proteins such as GKAP (18, 19), signaling molecules, and cell adhesion molecules (4, 20, 21). The Shank family of proteins, which are composed of multiple protein–protein interaction domains, were isolated as GKAP-binding proteins (11) and have also been called ProSAP (22) and synamon (23). One of the Shank family proteins was originally reported as cortactin-binding protein 1, CortBP1 (24). The PDZ and the SH3 domain of Shank family members interact with the extreme C terminus of GKAP (11) and the α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) receptor-interacting protein GRIP (25), respectively. The proline-rich region of Shank includes binding sites for the Homer proteins (26) and the actin-binding protein cortactin (11). Tu et al. (26) demonstrated the macromolecular coupling of the mGluR-Homer and NMDA receptor-PSD-95-GKAP complexes by Shank family members in vitro. Taking these observations together, Xiao et al. (27) proposed that Shank-dependent protein–protein interactions in the postsynaptic protein lattice link together different classes of glutamate receptors and further couple them to the actin cytoskeleton and the Ca2+ sequestering machinery. However, these protein–protein interactions were identified in a piecemeal fashion in vitro. The molecular organization and dynamic interactions of a postsynaptic protein lattice in vivo therefore remain largely unknown. Here we focused on the molecular mechanism underlying the synaptic targeting of PSD-Zip45 and its effect on other PSD scaffold proteins. We demonstrated that both the EVH1 domain and C-terminal leucine zipper motif of PSD-Zip45 are critical for its synaptic targeting and that PSD-Zip45/Sank and PSD-95/GKAP form different compartments in the synapse. In addition, the synaptic localization of Shank is PSD-Zip45-dependent, and their interaction is involved in the enhanced accumulation of synaptic F-actin, and overexpressing these molecules induces synaptic enlargement in association with the further accumulation of synaptic F-actin.
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**EXPERIMENTAL PROCEDURES**

**Antibodies**—Polyclonal antibodies against GKAP and Shank were produced as follows: the cDNAs encoding amino acid residues 287–570 (Shank (19)) and 826–1208 of CortBPI (22), an isoform of the shank family proteins, were amplified by PCR and subcloned into the GST fusion vector pGEX6P1 (Amersham Biosciences). GST-fused GKAP and CortBPI fragments expressed in *Escherichia coli* BL21 were isolated using glutathione-conjugated Sepharose 4B (Amersham Biosciences) and used to immunize New Zealand White rabbits. The antisera against the GST-fused GCB1 fragment were exhaustively preabsorbed with GST protein, followed by purification using a GST-GKAP fragment-coupled Sepharose 4B gel matrix. The antibody against GST-fused CortB1 fragment was purified by the same procedure. The antibody against CortB1 fragment (Fig. 2B) thus obtained specifically cross-reacted with all the isoforms of the Shank family and was used as an anti-shank antibody. Rabbit polyclonal and mouse monoclonal antibodies against GFP (Molecular Probes, Eugene, OR) and other antibodies (anti-PSD-Zip45, anti-PSD-95, anti-synaptophysin, anti-FLAG, and anti-GST antibodies) are described elsewhere (7). These antibodies, a rabbit polyclonal antibody against inositol trisphosphate receptors (Oncogene Research Products, Boston, MA) and mouse monoclonal antibodies against synaptophysin (Sigma) and GABAA receptor (Upstate Biotechnology, Inc., Lake Placid, NY), were used for immunocytochemistry and/or immunoblotting.

**Plasmid Construction**—All constructions were amplified by PCR and subcloned into the GST fusion vector pGEX-6P1 (Amersham Biosciences) or the mammalian expression vector pEGFP (Clontech, Palo Alto, CA), pcDNA3.1 (+) or pcDNA3.1 (−) (Invitrogen, Carlsbad, CA), or pcDNA3.1 (−) modified from pCAGGS (28). Each construct was tagged with GST or FLAG at the N terminus, as indicated. A series of PSD-Zip45 constructs (PSD-Zip45WT, PSD-Zip45ΔCZipA, and PSD-Zip45ΔZipB) was described previously (7), and other PSD-Zip45 variants (PSD-Zip45G98A, PSD-Zip45ΔNterminus, PSD-Zip45Cterminus, PSD-Zip45NterminusG98A) were newly designed. PSD-Zip45N-terminus (residues 1–175) and PSD-Zip45C-terminus (residues 176–366) were amplified by PCR. A glycine-to-alanine point mutation was introduced into PSD-Zip45WT and PSD-Zip45N-terminus at residue 98, yielding the mutants PSD-Zip45G98A and PSD-Zip45NterminusG98A. Each member in a series of PSD-Zip45 variants was subcloned into the pEGFP vector to yield the following constructs: GFP-Zip45WT, GFP-Zip45ΔG98A, GFP-Zip45ΔNterminus, GFP-Zip45ΔCterminus, GFP-Zip45ΔΔZipA, and GFP-Zip45ΔZipB. GFP-Zip45WT, GFP-Zip45G98A, and PSD-Zip45ΔZipB were also subcloned into the pcDNA3.1 (+) or FLAG vectors to yield FLAG-Zip45WT, FLAG-Zip45G98A, and FLAG-Zip45ΔZipB. PSD-Zip45N-terminus and PSD-Zip45NterminusG98A were subcloned into the pGEX-6P1 vector to yield GST-Zip45N-terminus and GST-Zip45NterminusG98A and were used for ligand overlay assays (Fig. 2B). The following GKAP and CortB1 constructs were transfected into cultured hippocampal neurons and used for immunoelectron microscopy. For analysis of the fluorescent localization intensities of the indicated areas in GFP-labeled synapses to those in the adjacent dendrites. For analysis of the fluorescent localization intensities of the indicated areas in GFP-labeled synapses to those in the adjacent dendrites.

**Cell cultures**—Hippocampal neurons were prepared from embryonic rat brains at 18 days of gestation (E18). The hippocampi were dispersed in balanced salt solution, and the cell suspension was plated at a density of 10,000–15,000 cells/cm² onto cover glasses (Matsunami, Osaka, Japan) coated with 1 mg/ml poly-l-lysine. Neurons were cultured in neurobasal medium (Invitrogen) containing 2% B27 supplement (Invitrogen) and 0.5 mm L-glutamine. One-half of the medium was changed once a week. Cultured neurons were used for immunocytochemistry and/or immunoblotting.

Microinjection of plasmid DNA—By using a micromanipulator (Narishige, Tokyo, Japan), plasmid DNAs (low expression, 10 ng/µl; overexpression, 100 ng/µl) were microinjected through glass capillaries into the nuclei of rat hippocampal neurons. After 8–24 h, the neurons were fixed and permeabilized in methanol for 15 min at −20 °C and then treated with blocking solution (10% normal goat serum, 0.2% BSA, and 0.1% Triton X-100 in PBS) for 1 h at 37 °C. In the following experiments, all procedures were carried out at 37 °C. The cultured neurons were then incubated with primary antibodies in the same blocking solution for 2 or 3 h. After incubation, the epitopes in the neurons were incubated with Alexa Fluor™ 488- and/or 546-conjugated secondary antibodies (2 µg/ml, Molecular Probes) in the blocking buffer for 40 min. For triple labeling, the neurons were fixed with 4% paraformaldehyde and 4% sucrose in PBS for 20 min, followed by permeabilization with 0.25% Triton X-100 in PBS for 5 min, treatment with the blocking solution (10% BSA) for 30 min, and incubation with primary antibodies in 3% BSA for 3 h. After labeling with the primary antibodies, the neurons were further incubated with Alexa Fluor™ 350- and 488-conjugated secondary antibodies (2 µg/ml, Molecular Probes) and Alexa Fluor™ 568 phallidin (2 µg/ml, Molecular Probes) in 3% BSA for 40 min. Primary antibodies used for immunocytochemical studies were as follows: anti-PSD-Zip45 (1:150), -PSD-95 (1:400), -GST (1:1,000), -shank (1:2,000), -synaptophysin (1:400), -GABA_R1 (1:200), -GFP (1:1,000), and -FLAG (1:10,000) antibodies. After being washed with PBS, the cover glasses were mounted for observation. Fluorescence images were acquired using a cooled CCD camera (Roper Scientific, Tucson, AZ) mounted on an Olympus IX-70 microscope.

**Quantification**—Quantitative measurements were performed blind. Morphometric studies were performed using Metamorph imaging software. The synapse/dendrite fluorescence intensities (Fig. 2D and Fig. 4B, c) were analyzed as the ratios of the average fluorescence intensities of the indicated areas in GFP-labeled synapses to those in the adjacent dendrites. For analysis of the fluorescent localization intensities of the indicated areas in GFP-labeled synapses to those in the adjacent dendrites.

**RESULTS**

**Molecular Determinants of PSD-Zip45 for Synaptic Targeting**—Previous immunocytochemical and immunoelectron microscopic studies demonstrated the postsynaptic localization of NMDA receptors and PSD scaffold proteins such as PSD-Zip45, Shank, PSD-95, and GKAP in excitatory synapses (7, 8, 11, 26, 30). To reveal the molecular mechanism underlying the synaptic targeting of PSD-Zip45, a small amount (10 ng/µl) of expression plasmids carrying GFP- or FLAG-tagged wild-type PSD-Zip45 (GFP-Zip45WT or FLAG-Zip45WT) was microinjected into the nuclei of cultured hippocampal neurons, and their expression was localized by GFP or FLAG staining (Fig. 1, b and c). GFP-labeled clusters along the dendrites were detectable within 18 h after microinjection. Double labeling for GFP-Zip45WT and endogenous PSD scaffold proteins showed that the GFP immunoclusters along the dendrites overlapped greatly with the PSD-95, GKAP, and Shank clusters (Fig. 1, d–f) but not with clusters of an inhibitory postsynaptic molecule, GABAA receptor (Fig. 1, g). The GFP immunoclusters partially overlapped with clusters of synaptophysin, a presynaptic molecular marker (Fig. 1, h). The same results were obtained using FLAG-Zip45WT (Fig. 1c and data not shown).
Thus, these results indicate that exogenous PSD-Zip45WT proteins are precisely targeted to postsynaptic sites of excitatory synapses in a manner that is indistinguishable from the endogenous protein.

To analyze the molecular determinants of PSD-Zip45 that are required for synaptic targeting, we generated a series of expression plasmids bearing GFP-tagged PSD-Zip45 variants (Fig. 2A) and microinjected small amounts (10 ng/µl) of them into hippocampal neurons. After they were fixed, the neurons were double-labeled with anti-GFP and synaptophysin antibodies (Fig. 2C, a1–a3). In the following experiments, synaptophysin was used to determine the orientation of the synapse. PSD-Zip45 is composed of an N-terminal EVH1 domain and a C-terminal domain containing a coiled-coil structure and two leucine zipper motifs (ZipA and ZipB in Fig. 2A and in Refs. 6 and 7). We prepared four deletion constructs, lacking the EVH1 domain, the coiled-coil structure, ZipA and/or ZipB, and a mutant of the EVH1 domain (Fig. 2A). An overlay binding assay showed that a short form of PSD-Zip45WT, GST-Zip45N-terminus, bound to all the isoforms of the shank family in the PSD fraction, whereas a point mutation of the GST-Zip45N-terminus, GST-Zip45NterminusG89A, lost the shank binding activity, indicating that the EVH1 domain of PSD-Zip45 is the binding site for the shank family members (Fig. 2B).

GFP alone was distributed diffusely through the dendrites to their spines (Fig. 2C, b). Among the six constructs examined, GFP-Zip45WT and GFP-Zip45ΔCCΔZipA formed very bright clusters along the dendrites (Fig. 2C, a and f), and almost all of these clusters partially overlapped with the synaptophysin-positive clusters (Fig. 2C, a1–a3), suggesting a postsynaptic localization of GFP-Zip45WT and GFP-Zip45ΔCCΔZipA. In contrast, the GFP-Zip45Nterminus, -Cterminus, and Δ ZipB derivatives were distributed diffusely (Fig. 2C, c–e and g). To quantify the extent of synaptic targeting (synaptic targeting intensity), we measured the immunofluorescence intensities of the indicated areas that were positive for the GFP-labeled PSD-Zip45 variants in the synapses and of the dendrites immediately adjacent to the synapses (31) as summarized in Fig. 2D. These results indicate that the EVH1 domain and the extreme C-terminal leucine zipper motif (ZipB) of PSD-Zip45 are critical determinants for the synaptic targeting.
Effect of Overexpressed PSD-Zip45 Variants on the Synaptic Localization of Other PSD Scaffold Proteins—To test whether PSD-Zip45 may play a role in the synaptic localization of other PSD scaffold proteins, we microinjected excess amounts (100 ng/μl) of expression plasmids carrying GFP-Zip45WT, GFP-Zip45G89A, and GFP-Zip45ΔZipB into hippocampal neurons. After they were fixed, the neurons were double-labeled with anti-GFP and anti-PSD-95, GKP, or shank antibodies (Fig. 3A). The synaptic localization of exogenously expressed proteins was determined by the orientation of the labeled presynaptic molecule, synaptophysin, in advance (data not shown). When GFP-Zip45WT was overexpressed, endogenous shank,
FIG. 3. Overexpressed GFP-Zip45G89A and GFP-Zip45ΔZipB suppressed the synaptic localization of shank, but not of PSD-95 or GKAP, in cultured hippocampal neurons. Effects of overexpressed PSD-Zip45 variants on the synaptic localization of shank, PSD-95, and GKAP (A). Excess amounts of plasmids (100 ng/pl) carrying GFP-Zip45WT, GFP-Zip45G89A, or GFP-Zip45ΔZipB were microinjected into hippocampal neurons. Each neuron was double-labeled for GFP (a1–i1) and shank (a2–c2), PSD-95 (d2–f2), or GKAP (g2–i2). GFP-labeled images are as follows: GFP-Zip45WT (a1, d1, and g1), GFP-Zip45G89A (b1, e1, and h1), and GFP-Zip45ΔZipB (c1, f1, and i1). The right panels show merged images from the left and middle panels (a3–i3). Enlargements of the boxed regions are shown in the respective lower panels (a1’–i1’, a2’–c2’, and a3’–i3’). Arrows indicate the synapses in transfected neurons, and arrowheads show the synapses in untransfected neurons. Scale bars, 10 μm.

Changes in the synaptic localization of the PSD scaffold proteins induced by the overexpression of PSD-Zip45 variants (B). Six to ten independent experiments were performed for each construct, and 800 synapses from 10 to 20 neurons were randomly selected. The histogram shows the mean fluorescence intensity normalized to the fluorescence intensities of clusters in untransfected neurons stained with the antibodies against the indicated proteins.
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Fig. 4. **Synaptic targeting of GKAP is independent of shank.** A, immunolocalization of GKAP variants and shank. Endogenous GKAP was immunolabeled with an anti-GKAP antibody (a). A small amount (10 ng/μl) of expression plasmids carrying GFP-tagged wild-type GKAP (GFP-GKAP-WT) or its mutant (GFP-GKAP-L694A) was microinjected into cultured hippocampal neurons. The expressed GFP-GKAP-WT (b) and GFP-GKAP-L694A (c) were double-labeled with anti-GFP and shank antibodies (d2 and e2). The right panels (d3 and e3) show merged images from the left (d1 and e1) and middle (d2 and e2) panels. Enlargements of the boxed regions are shown in the respective lower panels (a′–c′). Arrows indicate the synapses in transfected neurons, and arrowheads show the synapses in untransfected neurons. Scale bars, 10 μm. B, summary of the synaptic targeting of GKAP variants and changes in the synaptic localization of shank induced by the overexpression of GKAP variants. The synaptic targeting intensities of the GKAP variants are expressed as the synaptic/dendrite fluorescence intensities as described in Fig. 2D. Four independent experiments were performed for each construct, and 400 synapses from 10 neurons were randomly selected (B, a). Changes in the synaptic localization of shank induced by the overexpression of PSD-95, and GKAP clusters in the synapses were exactly co-localized with expressed GFP-Zip45WT clusters (Fig. 3A, a3, d3, and g3). Notably, when GFP-Zip45G89A or GFP-Zip45 ZipB was overexpressed, the immunofluorescence intensity, but not the number or size, of shank clusters markedly decreased (Fig. 3A, b2 and c2). In turn, overexpressed GFP-Zip45WT, GFP-Zip45G89A, and GFP-Zip45 ZipB had no discernible effects on the intensity, number, and size of endogenous PSD-95 and GKAP clusters (Fig. 3A, d2–e2). The effects of overexpressed PSD-Zip45 variants on the synaptic localization of endogenous PSD scaffold proteins were also quantified by the ratios of the immunofluorescence intensities of labeled synaptic areas in the transfected neurons to those in untransfected neurons (Fig. 3B). Thus, overexpressed GFP-Zip45G89A and GFP-Zip45 ZipB markedly and specifically suppressed the synaptic localization of shank in a dominant-negative fashion.

**GKAP and the Shank Family Are Independently Localized to Synaptic Sites**—The extreme C-terminal sequence (-QTA) of GKAP has been reported to interact with the PDZ domain of Shank in vitro, and their interaction is believed to be involved in the synaptic localization of Shank (11). However, the overexpression analysis shown in Fig. 3 suggests that the synaptic localization of PSD-Zip45 and Shank may not be directly linked to that of GKAP and PSD-95. To test whether GKAP affects the synaptic localization of Shank, we analyzed the synaptic targeting of GFP-tagged wild-type GKAP (GFP-GKAP-WT) and its C-terminal mutant (GFP-GKAP-L694A) that lacked the Shank binding activity (11). A small amount (10 ng/μl) of expression plasmids carrying GFP-GKAP-WT or GFP-GKAP-L694A was microinjected into cultured hippocampal neurons. The orientation of the expressed proteins was also determined by the presynaptic localization of synaptophysin, as demonstrated in Fig. 2. Like endogenous GKAP, GFP-GKAP-WT and GFP-GKAP-L694A were specifically targeted to the synapses (Fig. 4A, a–c). The synaptic targeting intensities of the GFP-GKAP variants were quantified by the same method as described in Fig. 2D (Fig. 4B, a). The synaptic localization of the endogenous Shank was unaffected by overexpressed GFP-GKAP-WT or GFP-GKAP-L694A (Fig. 4A, d2 and e2, and B, b). These results suggest that GKAP and Shank are independently localized to the synaptic sites.

**Involvement of the Actin Cytoskeleton in the Synaptic Localization of PSD-Zip45 and Shank**—As described in the Introduction, some PSD scaffold proteins and F-actin co-localize within the dendritic spines (32). In addition, the proline-rich region of CortBP1 and other Shank family members directly interacts with an actin-binding protein, cortactin (11, 24). The actin cytoskeleton is also involved in the dynamic properties of dendritic spines (33, 34) and the functional regulation of glutamate receptors in postsynaptic sites (35). To test the linkage between the actin cytoskeleton and four PSD scaffold proteins in postsynaptic sites, we compared their synaptic localization in neurons with or without the actin-depolymerizing reagent latrunculin A. This drug stoichiometrically binds to the actin monomer, resulting in the sequestration of F-actin by the depolymerization of actin filaments. Triple staining of F-actin, PSD-Zip45, and Shank in untreated neurons demonstrated the co-localization of these proteins within the synapse (data not shown). Allison et al. (32) reported that treatment with 5 μM

GFP-GKAP-WT or GFP-GKAP-L694A are expressed by the ratios of the fluorescence intensities of shank clusters in transfected neurons to the fluorescence intensities in untransfected neurons. The histogram shows the mean fluorescence intensity normalized to the intensities of the Shank clusters in untransfected neurons. Four independent experiments were performed for each construct, and 400 synapses from 10 neurons were randomly selected (B, b).
Latrunculin A for 24 h depolymerizes most of the F-actin in hippocampal neurons. Consistent with our previous report (14), the same treatment markedly reduced, but did not completely eliminate, the number of F-actin clusters along the dendrites. This may be because of the limitation of this treatment or different latrunculin A resistance of actin filaments with or without actin-binding proteins. In association with this reduction, the numbers of PSD-Zip45- and shank-positive clusters decreased to \(-40\%\) (Fig. 5A). In both cases, the latrunculin A-resistant F-actin clusters were co-localized with the PSD-Zip45- and Shank-positive clusters (Fig. 5A, b1–b3). On the other hand, the synaptic localization of the PSD-95- and GKAP-positive clusters was not affected by the latrunculin A treatment, except for a slight reduction in cluster size (in untreated neurons, PSD-95 clusters (0.93 \pm 0.37 \mu m^2) and GKAP clusters (0.84 \pm 0.39 \mu m^2); in latrunculin A-treated neurons, PSD-95

**Fig. 5.** Changes in the synaptic localization of PSD scaffold proteins induced by depolymerization of F-actin in cultured hippocampal neurons. A, immunolocalization of four PSD scaffold proteins with or without latrunculin A treatment. Neurons treated with 5 \mu M latrunculin A for 24 h (b1–b3) or untreated (a1–a3) were triple-labeled for F-actin (a1 and b1), PSD-Zip45 (a2 and b2), and Shank (a3 and b3). After treatment with latrunculin A (f1, f2, g1, g2, h1, and h2) or without treatment (c1, c2, d1, d2, e1, and e2), the neurons were double-labeled for PSD-95 (c1–h1) and PSD-Zip45 (c2 and f2), Shank (d2 and g2), or GKAP (e2 and h2). Arrows indicate PSD-95-positive and PSD-Zip45- or Shank-positive clusters, and arrowheads show the PSD-95-positive and PSD-Zip45- or Shank-negative clusters, respectively. Each channel is shown in gray scale. Scale bars, 5 \mu m.

The ratios of the numbers of PSD-Zip45-, Shank-, or GKAP-positive clusters to the numbers of PSD-95-positive clusters with or without latrunculin A treatment are summarized (B). Data are derived from the analysis of 600 PSD-95-positive clusters from 20 neurons (*, p > 0.05; **, p < 0.001).
clusters (0.59 ± 0.11 μm²) and GKAP clusters (0.57 ± 0.09 μm²), respectively (Fig. 5A, h1 and h2). These results were obtained by double-labeling with anti-PSD-95 and anti-PSD-Zip45, shank, or GKAP antibodies (Fig. 5A) and then quantifying the labeled clusters based on the number of PSD-95 clusters (Fig. 5B). Thus, our data suggest that the organization of the actin cytoskeleton is closely linked to the synaptic localization of PSD-Zip45 and/or the shank family but not of PSD-95 or GKAP.

To analyze further the relationship between PSD-Zip45 and/or shank and the actin cytoskeleton, we compared the effects of PSD-Zip45 variants with or without overexpressed shank on the synaptic F-actin (Fig. 6A). In our preliminary experiments, we also confirmed that almost all of actin clusters along the dendrites were localized in the synapses as defined by the synaptic cytoskeleton labeling (data not shown). Overexpressed GFP-Zip45 variants, endogenous shank, and F-actin were triple-labeled with anti-GFP and shank antibodies and phalloidin, respectively. Overexpressed GFP-Zip45WT enhanced the synaptic localization of F-actin, whereas overexpressed GFP-Zip45G89A and GFP-Zip45 ΔZipB did not affect the synaptic F-actin (Fig. 6A, a3–c3). Consistent with the results of Fig. 3, GFP-Zip45G89A and GFP-Zip45 ΔZipB, but not GFP-Zip45WT, markedly suppressed the synaptic localization of shank (Fig. 6A, a2–c2). Taken together, these results indicate that an increased PSD-Zip45 level, in the presence of shank, enhances the accumulation of synaptic F-actin, an activity in which the EvH1 domain and the extreme C-terminal leucine zipper motif of PSD-Zip45 are critically important.

Sala et al. (36) recently observed that overexpression of both shank 1B and Homer 1b induces synaptic enlargement in association with an increase in the synaptic F-actin. To determine the domain of PSD-Zip45 that was critical for the synaptic enlargement and the increased localization of synaptic F-actin, we co-transfected PSD-Zip45 variants (FLAG-Zip45WT, FLAG-Zip45G89A, or FLAG-Zip45 ΔZipB) and wild-type CortB1 (GFP-CortB1; shank family) in hippocampal neurons. FLAG-Zip45WT and GFP-CortB1, but not FLAG-Zip45G89A or FLAG-Zip45 ΔZipB, were precisely targeted to the synapses (Fig. 1c and data not shown). Both overexpressed FLAG-Zip45 and GFP-CortB1 were highly concentrated in the enlarged synapses and co-localized with F-actin (Fig. 6A, d1–d3). On the other hand, in neurons that were co-transfected with FLAG-Zip45G89A or FLAG-Zip45ΔZipB and GFP-CortB1, there were less significant changes in the synaptic size and fluorescence intensity of F-actin (Fig. 6A, e3 and f3). The F-actin labeling in the synapse was quantified as demonstrated in Fig. 4C. Thus, these results indicate that both the EvH1 domain and the extreme C-terminal leucine zipper motif of PSD-Zip45 are critical for the PSD-Zip45/shank-dependent synaptic enlargement in association with the enhanced accumulation of synaptic F-actin, and that the synaptic targeting of PSD-Zip45 is prerequisite for these events.

DISCUSSION

Recent studies (31, 37) have demonstrated the synaptic targeting of some PSD scaffold proteins. The first example was the synaptic targeting of PSD-95, in which the N-terminal palmitoylation, the first two PDZ domains, and/or a C-terminal targeting motif are critical. Naisbitt et al. (11) reported that the overexpression of GKAP variant, which lacks the PDZ-binding sequence, caused a marked reduction in the levels of the endogenous shank family members in the synapse. Sala et al. (36) also identified the molecular determinants of the shank family required for their synaptic targeting as their PDZ domain and the flanking sequences and concluded that an interaction between the PDZ domain of shank 1B and the C terminus of GKAP was important for the synaptic localization of shank 1B. Ango et al. (38) analyzed the distribution of exogenous type 5 mGluR (mGluR5) and Homer proteins using cerebellar granule cells in which mGluR5 and Homer 1 proteins were absent, and their findings suggested that the synaptic targeting of mGluR5 is Homer protein-dependent. However, they did not address the molecular determinants of the Homer proteins. We recently reported (14) that PSD-Zip45 that is exogenously expressed in hippocampal neurons is targeted to the synapses and that its targeting is dynamically regulated by Ca²⁺ influxes from different sources. Here, we studied the molecular determinants required for the synaptic targeting of PSD-Zip45 and the effects of PSD-Zip45 variants on the synaptic localization of other PSD scaffold proteins and F-actin.

Synaptic Targeting of PSD-Zip45 and Compartmentalization of Major PSD Scaffold Proteins—Exogenous PSD-Zip45 showed a synaptic localization with other major PSD scaffold proteins, including PSD-95, GKAP, and shank (Fig. 1). We identified both the EvH1 domain and the extreme C-terminal leucine zipper motif of PSD-Zip45 as the molecular determinants for its synaptic targeting (Fig. 2). Furthermore, overexpression of PSD-Zip45G89A or PSD-Zip45 ΔZipB markedly suppressed the synaptic localization of endogenous shank, but not of PSD-95 or GKAP, in a dominant-negative fashion (Fig. 3). These results suggest that the synaptic localization of PSD-Zip45 is a prerequisite for that of shank, and both of PSD-Zip45G89A and PSD-Zip45 ΔZipB cannot recruit shank to the synapses because neither of them is targeted to the synapses. It has been reported that GKAP interacts with the shank family via its PDZ domain and that overexpressed GKAP-L694A, which does not bind shank, suppresses the synaptic localization of shank (11). In contrast, we demonstrated here that whereas GKAP-WT and GKAP-L694A were targeted precisely to the synapses, overexpressed GKAP-WT and GKAP-L694A did not affect the synaptic localization of endogenous shank (Fig. 4). Thus, there is a discrepant result regarding the interaction between GKAP and shank within the synapses. The molecular length of GKAP, clone2–2A, used by us is longer than that by Naisibitt et al. (11). We also constructed GKAP-L694A, a mutant of clone2–2A, that lacks the shank binding activity. Naisibitt et al. (11) used a C-terminal deleted variant of GKAP lacking the shank binding activity. Furthermore, we introduced the plasmids into cultured neurons by the microinjection method, whereas Naisibitt et al. (11) used the transfection method with calcium phosphate. However, it remains unknown whether these different constructs and expression methods might produce the above discrepant result. Our present results indicate that the synaptic localization of shank depends on that of PSD-Zip45 but not of GKAP. It might be inferred from these data that PSD-Zip45-shank and PSD-95-GKAP interactions form different compartments in the synapses. The currently accepted model for a postsynaptic protein lattice is that the shank family members provide molecular bridges between NMDA receptor-PSD-95-GKAP, mGluR-PSD-Zip45, and α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) receptor-GRIP complexes, the actin cytoskeleton, and the Ca²⁺-sequestering machinery (25, 27, 39). Almost all of these interactions, however, have been demonstrated by in vitro assays. We reported previously (14) that although PSD-95 clusters in the synapses of living hippocampal neurons are less dynamic, PSD-Zip45 shows a highly dynamic and rapid redistribution that is regulated by Ca²⁺ influxes from different Ca²⁺ sources. Combining our results, we suggest that PSD-95-GKAP and PSD-Zip45-shank complexes form different compartments in the synapse with respect to their interactions and dynamics.
**Fig. 6.** Enhancement of the synaptic accumulation of F-actin by overexpression of PSD-Zip45 variants with or without the co-expression of shank (GFP-CortBP1) in cultured hippocampal neurons. A, the synaptic localization of shank and F-actin induced by the overexpression of PSD-Zip45 variants with or without shank. Neurons overexpressing GFP-Zip45WT (a1), GFP-Zip45G89A (b1), or GFP-Zip45ΔZipB (c1) alone were triple-labeled for GFP (a1–c1), shank (a2–c2), and F-actin (a3–c3) in cultured hippocampal neurons. Neurons overexpressing FLAG-Zip45WT, FLAG-Zip45G89A, or FLAG-Zip45ΔZipB and GFP-CortBP1 were triple-labeled for FLAG (d1–f1), GFP (d2–f2), and F-actin (d3–f3). Enlargements of the boxed regions are shown in the respective lower panels (a1–f1). Each channel is shown in gray scale. Scale bars, 10 μm. B, summary of changes in the ratio of the fluorescence intensities of synaptic F-actin in transfected neurons to the fluorescence intensities in untransfected neurons as shown in A. Four to six independent experiments were performed for each construct, and 400 synapses from 8 to 10 neurons were randomly selected. The histogram shows the mean fluorescence intensities normalized to the fluorescence intensities of F-actin-positive clusters in untransfected neurons (*, p < 0.001).

**Linkages between PSD-Zip45, Shank, and the Actin Cytoskeleton in the Synapse**—Because the shank family interacts in vitro with cortactin through a cortactin-binding motif in the proline-rich region of the shank proteins (11, 24), it has been postulated that the actin cytoskeleton is involved in the synaptic localization of PSD-Zip45 and shank. In support of this
idea, our recent studies (14) suggest that the synaptic localization of PSD-Zip45 is linked to the polymerization/depolymerization dynamics of the actin cytoskeleton. In this paper, we further compared the linkages between four PSD scaffold proteins and the actin cytoskeleton. Treating hippocampal neurons with the actin-depolymerizing reagent latrunculin A caused a decrease in the synaptic localization of F-actin, PSD-Zip45, and Shank but not of PSD-95 or GKAP (Fig. 5). These results suggest that the synaptic organization of the actin cytoskeleton is closely associated with PSD-Zip45 and Shank.

The PSD-Zip45 Shank complex may link to the actin cytoskeleton through the cortactin Shank interaction or another actin-binding protein that interacts with the PSD-Zip45 Shank complex. The former possibility, however, seems to be unlikely because the synaptic localization of Shank was quite different from that of cortactin (data not shown). Alternatively, there are several pieces of evidence that support the latter possibility.

The N terminus of Cupidin/Homer2a has been reported to interact with F-actin in vitro (40). We did not, however, detect a direct interaction between PSD-Zip45 and F-actin (data not shown). In contrast, we demonstrated that overexpression of PSD-Zip45WT enhances the synaptic accumulation of F-actin without enhancing that of Shank (Fig. 6A, a1–a3). Sala et al. (36) reported that overexpression of both Shank 1B and Homer 1b induces synaptic enlargement in association with an increase in synaptic F-actin. Consistent with this, we found that both of overexpressed PSD-Zip45 and CortBP1 were highly concentrated in the enlarged synapses with F-actin (Fig. 6A, d1–d3). This enhanced accumulation of synaptic F-actin was also observed in the normal-sized synapses in neurons overexpressing PSD-Zip45WT alone (Fig. 6A, a1 and a3). In contrast, overexpressed PSD-Zip45G89A and PSD-Zip45ΔZipB did not affect the synaptic localization of F-actin whether or not CortBP1 was also overexpressed (Fig. 6). These results indicate that the synaptic localization of PSD-Zip45 is a prerequisite for that of Shank, and the complex formation between PSD-Zip45 and Shank is critically linked to the enhanced accumulation of synaptic F-actin. Why did overexpressed PSD-Zip45 alone enhance synaptic accumulation of F-actin without the accumulation of Shank? This may be due to the difference of endogenous Shank and actin pools, in which free Shank is more limited than F-actin. Actually, both of the overexpressed PSD-Zip45 and CortBP1 were highly concentrated in the synapses with the enhanced accumulation of F-actin. Our present results further indicate that increasing the levels of synaptically targeted PSD-Zip45 and Shank through overexpression induces synaptic enlargement in association with the enhanced accumulation of synaptic F-actin. Future study will be required to elucidate the involvement of the PSD-Zip45 Shank and actin interaction in the postsynaptic structure and function.
Synaptic Targeting of PSD-Zip45 (Homer 1c) and Its Involvement in the Synaptic Accumulation of F-actin
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J. Biol. Chem. 2003, 278:10619-10628.
doi: 10.1074/jbc.M210802200 originally published online January 10, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M210802200

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