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Isolation of PSD-Zip45, a novel Homer/vesl family protein containing leucine zipper motifs, from rat brain

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Abstract Using monoclonal antibody against the 45 kDa postsynaptic density protein, we isolated a novel isoform of Homer/vesl. The NH_2-terminal region containing a PDZ domain of this protein is identical to that of Homer/vesl, and the COOH-terminal region containing unique leucine zippers shows self-multimerization. We named this protein PSD-Zip45. In addition to specific binding of PSD-Zip45 mediated by a PDZ domain to the metabotropic glutamate receptors 1α or 5, the distribution of PSD-Zip45 transcripts is highly consistent with that of metabotropic glutamate receptor transcripts. The PSD-Zip45 is, therefore, the first candidate as receptor anchoring proteins containing leucine zipper motifs in the central nervous system.

Key words: Metabotropic glutamate receptor; Receptor clustering; Postsynaptic density; Leucine zipper; PDZ domain

1. Introduction

The postsynaptic density (PSD) is defined by an electron dense structure of postsynaptic sites as revealed by electron microscopy. Numerous proteins have been identified as components of PSD [1,2]. Among them, cytoskeletal proteins are abundant, including actin, brain spectrin (calspectin or fodrin), and α-actinin 2. Deep-etching freeze fracture studies tentatively identified 4 nm filaments as F-actin, which are intermeshed with 8–9 nm filaments below the PSD. These findings suggest that the microfilament lattice may be involved in the mobility and function of neurotransmitter receptors.

Recent studies have revealed a number of neurotransmitter receptor anchoring proteins containing PDZ domains such as PSD-95 for *N*-methyl-D-aspartate (NMDA) receptors or Shaker-type potassium channel [3,4], GRIP for the α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA) receptor [5], and Homer for metabotropic glutamate receptors 1α and 5 (mGluR1α and 5) [6]. PSD-95 and chapsyn-110 have been demonstrated to cluster NMDA receptors and Shaker-type potassium channel in non-neuronal cells [7]. A pair of cysteine residues located near the NH_2-terminus of PSD-95 is suggested to be the disulfide bonding site for tetramerization [8] or the palmitoylation site for membrane sorting [9]. However, the mechanisms responsible for clustering these receptors to postsynaptic specializations remain unclear. The same situation is also found in the mGluR binding protein, Homer. Here, we isolated the first candidate for mGluR anchoring PSD protein which contains unique leucine zipper motifs, and named this protein PSD-Zip45.

2. Materials and methods

2.1. Materials

Polyclonal antibodies against the 2A and 2B subunits of the NMDA receptor (NR2A/B) were purchased from Chemicon (USA).

2.2. Subcellular fractionation and immunoblots

Subcellular fractions were prepared from cerebra of 7-week-old Sprague-Dawley rats by the method of Wu et al. [10]. Each fraction was separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes, incubated with the first antibodies, and then detected by the ECL Western blotting detection kit (Amersham, UK).

2.3. Production of monoclonal antibodies against the PSD proteins and synaptophysin

The PSD proteins were fragmented by cyanogen bromide (CNBr-PSD proteins), emulsified with complete Freund's adjuvant, and then injected intraperitoneally into BALB/c mice. Mice were boosted 2 weeks later, and again after 2 more weeks with CNBr-PSD proteins in saline. Fusion of spleen cells was performed with P3U1 myeloma cells. Hybridoma supernatants were initially screened by Western blots of PSD proteins separated by SDS-PAGE, and supernatants from positive wells were further screened by the same procedure. The antibody (Mab 126H) was purified by protein A affinity chromatography.

Monoclonal antibody against synaptophysin was produced by the same procedures as described above using the synaptosomal fraction as antigens. The obtained antibody (SV96) was characterized by immunoprecipitation, immunoblotting, and immunohistochemistry, and was identified as a synaptophysin-specific antibody.

2.4. cDNA cloning and sequence analysis

A cDNA library was constructed from cerebra of 6-week-old Sprague-Dawley rats using the ZAP Express cDNA synthesis and cloning kit (Stratagene, USA). A total of 3×10^5 clones were screened with Mab 126H according to the manufacturer's instructions. We obtained two clones and rescued plasmids containing insert by the in vivo excision method. Sequencing was performed by SQ5500 DNA Sequencer (Hitachi, Japan).

2.5. Northern blotting

Total RNA was extracted from cerebra of 12-week-old Sprague-Dawley rats by the AGPC method [11]. 10 μg of total RNA was electrophoresed using a 1% formaldehyde-agarose gel and transferred to a nylon membrane. cDNA fragments of PSD-Zip45 (827–2030 nt) and Homer/Vesl (1198–2318 nt) were used as their specific probes, respectively, and were labeled with [α-^{32}P]dCTP by the random priming method. Hybridization was performed overnight at 50°C in a solution containing 50% formamide, 5×SSC, 5×Denhardt's solution, 0.5% SDS, and 100 μg/ml heat-denatured herring sperm DNA. Blots were washed with 0.2×SSC, 0.1% SDS at 48°C, and were subjected to autoradiography.

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2.6. In situ hybridization

In situ hybridization was performed as described previously [12]. Briefly, fresh frozen sections of whole rat brain (7 weeks old) were prepared, fixed in 4% paraformaldehyde/PBS, permeabilized with 5 μg/ml proteinase K, acetylated, and then dehydrated. Specific antisense riboprobes for PSD-Zip45 (840–1405 nt) and Homer/vesl (1198–1789 nt) were transcribed in the presence of [α-^{35}S]UTP (NEN, UK) using the Riboprobe in vitro Translation System (Promega, USA). Hybridization was performed overnight at 55°C, and a high stringency wash followed by RNase A treatment was conducted at 65°C. Data were analyzed by a BAS-5000 phosphor imager (Fujifilm, Japan).

2.7. Multimerization assay

[^{35}S]Methionine labeled PSD-Zip45 and Homer/vesl proteins were prepared using TNT Quick Coupled Transcription/Translation System (Promega, USA). The reaction was conducted for 2 h at 30°C, and was terminated by addition of RNase A. The products were kept overnight, followed by incubation at 30°C for 30 min with or without 0.05% glutaraldehyde. Final products were separated by 14% SDS-PAGE and subjected to autoradiography.

3. Results

Immunoblotting using Mab 126H revealed that the 45 kDa protein that crossreacted with the antibody was specifically concentrated in the PSD fraction (PSDI and II) (Fig. 1). It has been well documented that the 2A and 2B subunits of the NMDA receptor (NR2A/B) [13] and PSD-95 [3] are the PSD protein. In our subcellular fractions, NR2A/B and PSD-95, but not synaptophysin, were densely detected in the PSD fraction. Immunohistochemical analysis also showed the localization of the 45 kDa protein at synaptic buttons in the hippocampus and the cerebellum (in preparation). These results indicate that the 45 kDa protein recognized by Mab 126H is a component of the PSD.

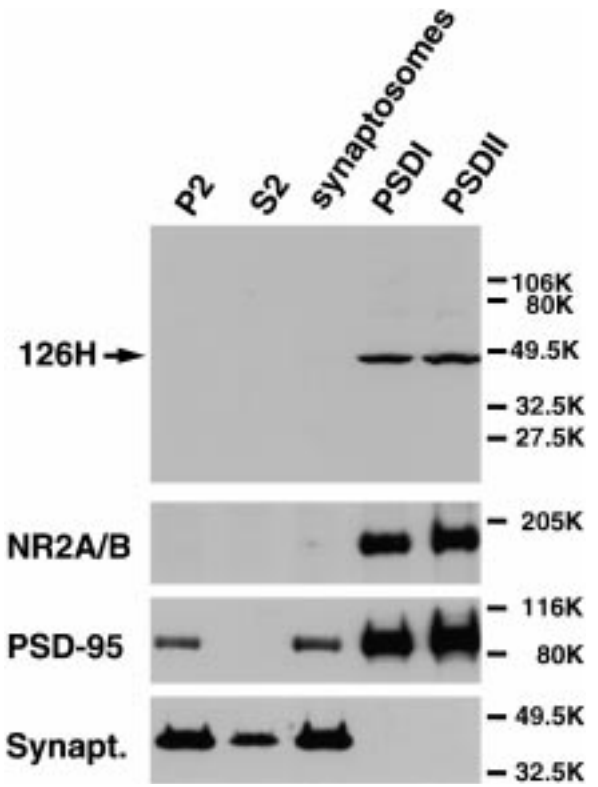

Fig. 1. Subcellular distribution of PSD-Zip45 in the rat cerebrum. 10 μg of crude membrane fraction (P2), soluble protein fraction (S2), synaptosomal fraction, and purified PSD fractions after extraction with Triton X-100 once (PSDI) or twice (PSDII) prepared from 7-week-old rat cerebrum were separated by SDS-PAGE. The transferred membranes were probed with anti-PSD-Zip45 monoclonal antibody (126H), anti-NR2A/B polyclonal antibody, anti-PSD95 polyclonal antibody, and anti-synaptophysin (synapt.) monoclonal antibody, respectively.

A rat cerebrum cDNA library was screened with Mab 126H, and two positive clones were isolated from 3×10^5 clones. These clones showed an identical sequence (GenBank accession number AB017140). Fig. 2a shows the deduced amino acid sequence of positive clones. An open reading frame encodes sequence of 366 amino acids with the calculated M_r of 41 303 (Fig. 2a). Residues 1–175 containing a PDZ domain are completely identical to those for Homer/vesl, which has been recently isolated as an immediately responsible protein against neuronal stimulation [6,14]. This PDZ domain is reported to bind to the COOH-termini of mGluR1a and 5. We also confirmed a specific interaction between the isolated protein and mGluR1α or 5 mediated through a PDZ domain (data not shown). However, the COOH-terminal regions are distinctively different. The Homer/vesl possesses the short COOH-terminus composed of 11 amino residues, while characteristic motifs lie on two leucine zippers in the COOH-terminus of the protein. Five heptad repeats are found in leucine zipper A in which leucine (or valine) residues occur at position *d*, and charged residues at positions *b, c, e, f*, and *g* serve the interface constraint and stabilization (Fig. 2b). Leucine zipper B is composed of four heptad repeats $(abcdefg)_4$, in which leucine (or isoleucine) residues uniquely occur at positions *a, d*, and *g*. Charged residues (glutamic acid, aspartic acid, and lysine) are positioned to make interhelical ion pairs. In spite of these coiled-coil structures, these two zipper motifs are not totally hydrophobic, suggesting no involvement in membrane spanning. Using the same strategy of immunoscreening with the PSD-specific monoclonal antibodies, we also isolated the other PSD proteins containing leucine zipper motifs (in preparation). The leucine zipper motif is, therefore, considered to be one of the common domains in the PSD proteins. We referred to a novel isoform of Homer/vesl as PSD-Zip45.

Northern blotting revealed that PSD-Zip45 mRNA with 4.5 kb and Homer/vesl mRNA with 6.2 kb were hybridized with their respective specific probes (Fig. 3). The PSD-Zip45 mRNA in the cerebrum was more abundant than that in the cerebellum (lanes 1 and 2). Further, expression of PSD-Zip45 mRNA was not affected by tetrodotoxin treatment or tetanic stimuli as revealed by Northern blotting and in situ hybridization, indicating constitutive expression (data not shown). On the other hand, Homer/vesl mRNA in the cerebrum was low and that in the cerebellum was less significant (lanes 3 and 4).

Using these specific probes, the distribution of their transcripts in the whole brain was compared. Intense labeling with the PSD-Zip45 specific probe was found in the cortex, hippocampus, striatum, and olfactory bulb. Moderate and weak labelings were observed in the cerebellum and the brain stem, respectively (Fig. 4a,d). The distribution of PSD-Zip45 mRNA overlapped extensively with that of mGluR1α plus mGluR5 mRNAs as reported in previous studies [15,16]. Moderate labeling of Homer/vesl was detected in the cortex, hippocampus, and olfactory bulb, and a faint signal was found in the striatum (Fig. 4e,h). In the hippocampus, PSD-Zip45 transcripts were detected in the CA1–3 regions and the

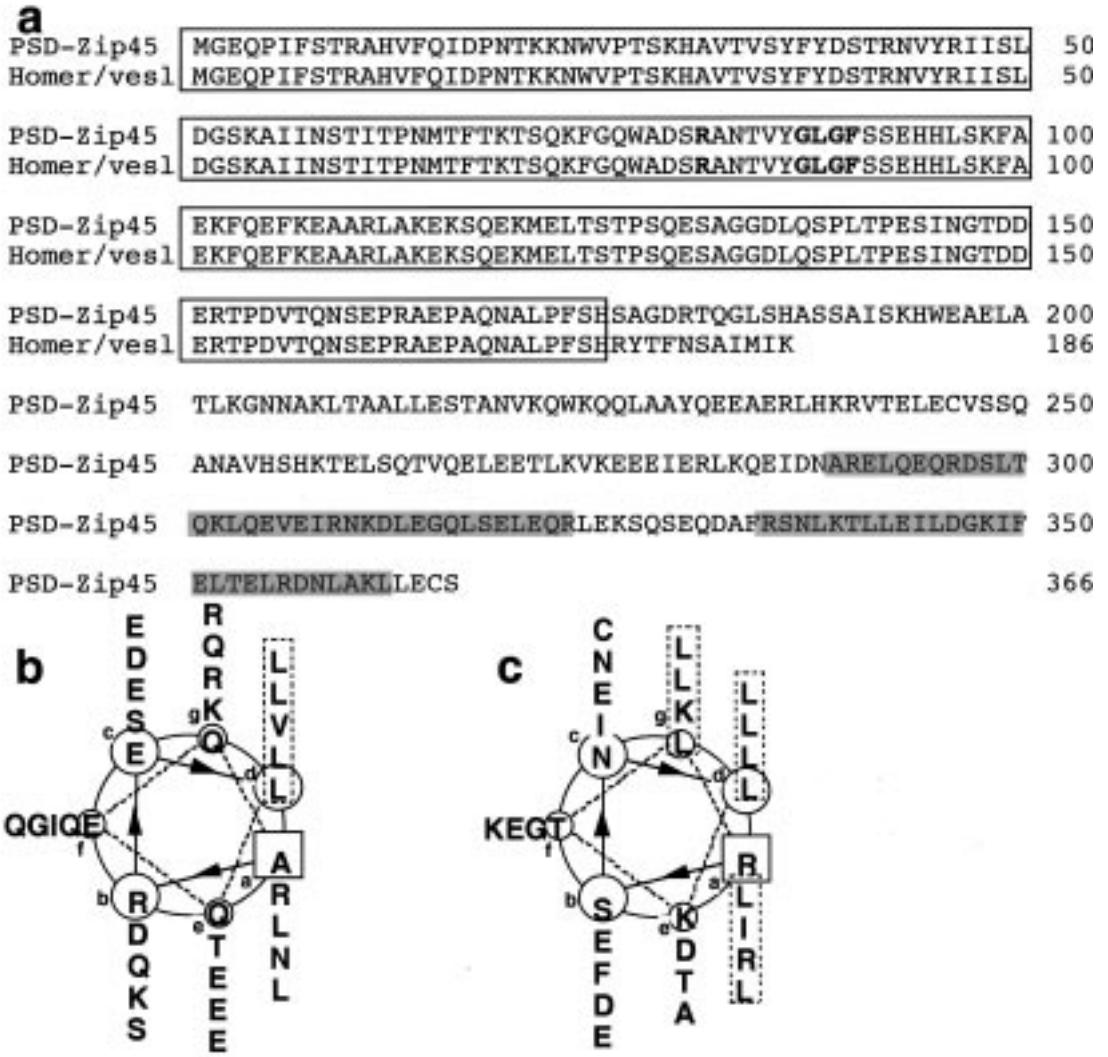

Fig. 2. Primary structure of PSD-Zip45. a: Amino acid sequence alignment of PSD-Zip45 and Homer/vesl. Identical amino acids (1–175 aa) are boxed. Two leucine zipper motifs (289–323, 335–362 aa) are indicated by gray boxes. The GLGF motifs and preceding arginine are represented in bold. b and c: Helical wheel analysis of leucine zippers A and B, respectively. Each strip of leucines (isoleucine or valine) is boxed with a broken line.

dentate gyrus, whereas Homer/vesl transcripts were distributed in the CA1–3 regions, but not the dentate gyrus (Fig. 4b,f).

We examined multimerization of PSD-Zip45 mediated by zipper motifs (Fig. 5). In vitro translated PSD-Zip45 without glutaraldehyde fixation resulted in a comparable banding pattern with the estimated M_r of 45 kDa on 14% SDS-PAGE, indicating SDS-denatured monomer protein. When fixed, more than 70% of PSD-Zip45 translates were retained on the gel top. Even with 5% SDS-PAGE, crosslinked PSD-Zip45 translates were not able to enter the gel, indicating multimerization. The Homer/vesl, which deleted the COOH-terminal domain of PSD-Zip45, did not show such multimerization with or without fixation. These results imply the importance of the COOH-terminal domain containing leucine zippers for multimerization.

4. Discussion

Using the PSD-specific monoclonal antibody, we have isolated a novel isoform of Homer/vesl, which is expressed in response to neuronal stimulation [6,14]. Residues 1–175 containing a PDZ domain of PSD-Zip45 and Homer/vesl are completely identical, whereas the remaining COOH-terminal regions are distinctively different. Characteristic leucine zippers lie in the COOH-terminus of PSD-Zip45. By contrast, Homer/vesl possesses the short COOH-terminus composed of 11 amino acid residues (Fig. 1a). It has been reported that a PDZ domain of Homer/vesl interacts with mGluR1α or 5, but not NMDA receptors [6]. We confirmed the specific binding of mGluR1α or 5 to a PDZ domain of PSD-Zip45 (data not shown). In agreement with these findings, the distribution of PSD-Zip45 transcripts is highly consistent with that of mGluR1α and 5 (Fig. 4a,d). Compared with the immediate expression of Homer/vesl in response to neuronal stimulation [6,14], PSD-Zip45 is constitutively expressed at mRNA and protein levels (Figs. 1 and 4). Based on the primary structures, expression of PSD-Zip45 or Homer/vesl may be explained by alternative splicing of the same mRNA precursor. However, expression patterns of PSD-Zip45 and Homer/vesl are different; the constitutive expression of PSD-Zip45 compares with the neuronal stimulation-induced

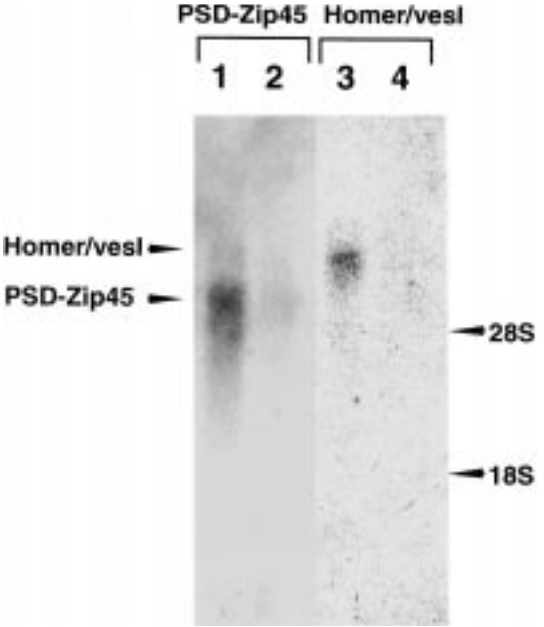

Fig. 3. Northern blot analyses of PSD-Zip45 and Homer/vesl in the rat brain. 10 μg of total RNA from cerebra (lanes 1 and 3) and cerebella (lanes 2 and 4) of 12-week-old rats were hybridized with PSD-Zip45 or Homer/vesl specific radiolabeled probes, respectively. ←

expression of Homer/vesl. Two different genes or two different promoters of one single gene might be necessary to explain these observations. Further study will be required to elucidate these unusual expression patterns.

As shown in Fig. 5, the COOH-terminal region containing leucine zippers of PSD-Zip45 is critical for self-multimerization. Leucine zipper motifs are generally involved in dimer formation by the transcription factor proteins Fos and Jun [17]. Heptad repeats are also present in a variety of cytoplasmic and transmembrane proteins including several cytoskeletal proteins and voltage-gated ion channel [18]. In the neuromuscular PSD, rapsyn (43 kDa protein) containing a leucine zipper motif is involved in acetylcholine receptor (AChR) clustering. Deletion mutants that lack this motif still interact with the membrane and form clusters of rapsyn itself, but are unable to involve in the AChR clustering [19]. However, a

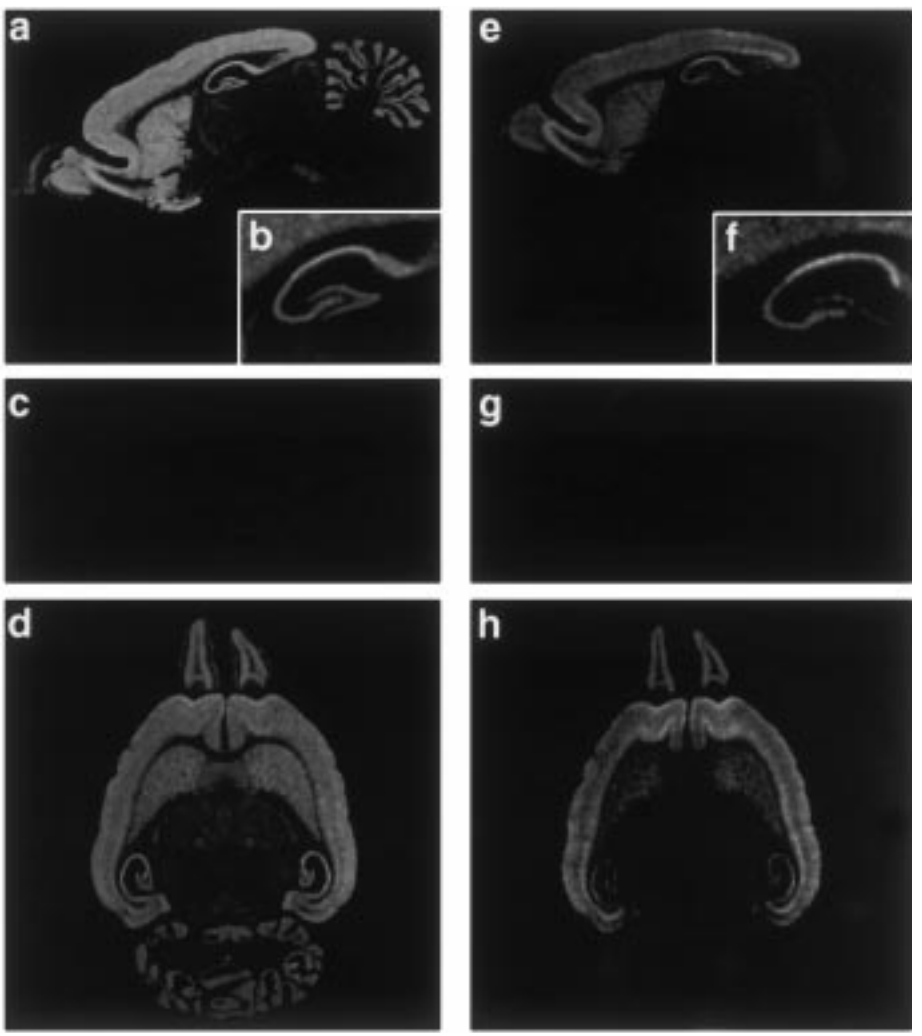

Fig. 4. Distribution of PSD-Zip45 and Homer/vesl mRNAs in rat brain. In situ hybridization of PSD-Zip45 (a–d) and Homer/vesl (e–h) in sagittal (a–c, e–g) or horizontal (d, h) sections of 7-week-old rat brain. The sections were hybridized with either antisense (a, b, d, e, f, and h) or sense riboprobes (c and g). The hippocampus is shown in a higher magnification in b and f.

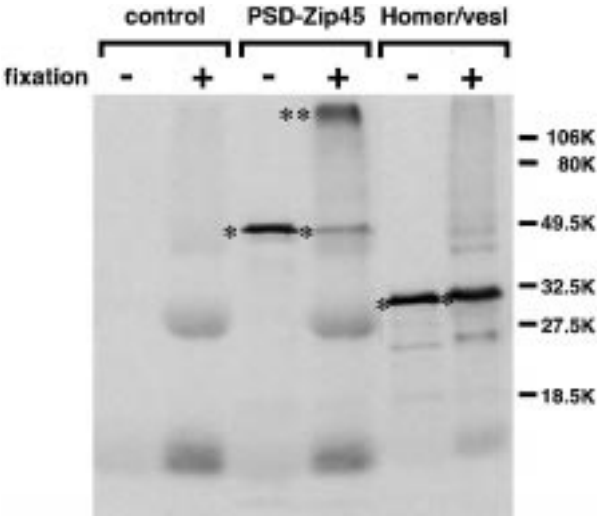

Fig. 5. Multimerization of PSD-Zip45. In vitro translates of PSD-Zip45 and Homer/vesl in the presence of [^{35}S]methionine were incubated with or without 0.05% glutaraldehyde for fixation, separated by 14% SDS-PAGE, and subjected to autoradiography. Control reaction was performed without template DNA. Asterisks indicate monomers of PSD-Zip45 or Homer/vesl translates, respectively. Double asterisks indicate multimers.

new insight has recently been demonstrated into domain mapping of rapsyn that is composed of eight tetratricopeptide repeats (TPRs) forming amphipathic α-helices. Among eight TPRs, TPR1 and 2 are sufficient to mediate rapsyn self-clustering, and TPR8 and extended coiled-coil motif are involved in AChR clustering [20]. In the central nervous PSD, there has been no report regarding proteins containing a leucine zipper motif. The PSD-Zip45 is, therefore, the first structural protein containing leucine zipper motifs.

Electron microscopic analyses demonstrated that metabotropic and ionotropic glutamate receptors are segregated [21–23]. Immunogold labelings of mGluR1α and mGluR5 are localized at the postsynaptic specializations of type I synaptic junctions, and are particularly concentrated at the edge, but not within the main body of anatomically defined synapses [21,22]. By contrast, the AMPA receptors occupy the membrane opposite the active zone in the main body of the same synapses [20]. In 'perforated' synapses, the density of both mGluR immunolabelings is found always in a perisynaptic annulus around the invaginations [23]. These findings suggest that the spatial segregation of metabotropic and ionotropic glutamate receptors might be regulated by their corresponding anchoring proteins. The Homer/vesl is an mGluR binding protein [6]. However, there is no information regarding binding domains except for a single PDZ domain. In addition to colocalization of PSD-Zip45 and mGluR1α plus 5 transcripts and self-multimerization of PSD-Zip45, we have obtained evidence that PSD-Zip45, but not Homer/vesl, can induce clustering of mGluR1α in non-neuronal cells (in preparation). The PSD-Zip45 is, therefore, a potent candidate as a mGluR anchoring protein.

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