Ligand Binding of the Second PDZ Domain Regulates Clustering of PSD-95 with the Kv1.4 Potassium Channel*

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The molecular mechanisms underlying the protein assembly at synaptic junctions are thought to be important for neural functions. PSD-95, one of the major postsynaptic density proteins, is composed of three PDZ domains (PDZ1, PDZ2, and PDZ3), an SH3 domain, and a GK (guanylate kinase) domain. It binds to the N-methyl-D-aspartate glutamate receptor NR2 subunit or to the Shaker-type K⁺ channel, Kv1.4, via the PDZ1 or PDZ2 domain, whereas PDZ3 binds to distinct partners. The intramolecular interaction of these multiple domains has been implicated in efficient protein clustering. We introduced missense and deletion mutations into PDZ1 (PDZ1mΔ) and/or PDZ2 (PDZ2mΔ) of the full-length PSD-95 to disrupt the association of each domain with the target proteins, while preserving the overall structure. The ion channel clustering activities of the PSD-95 mutants were analyzed in COS-1 cells coexpressing each mutant and Kv1.4. The mutant bearing the dysfunctional PDZ2 (PSD-95:2-1mΔ) showed significantly reduced clustering efficiency, whereas the mutant with the dysfunctional PDZ1 (PSD-95:1mΔ-2) exhibited activity comparable with the wild-type activity. Furthermore, we also examined the requirements for the position of PDZ2 in full-length PSD-95 by constructing a series of PDZ1-PDZ2 inversion mutants. Surprisingly, the clustering activity of PSD-95:2-1mΔ was severely defective. Taken together, these findings show that PDZ2, which is endowed with the highest affinity for Kv1.4, is required for efficient ligand binding. In addition, the ligand binding at the position of the second PDZ domain in full-length PSD-95 is prerequisite for efficient and typical cluster formation. This study suggests that the correct placement of the multiple domains in the full-length PSD-95 protein is necessary for the optimal protein activity.

As viewed by an electron microscope, dense thickenings are visible beneath the excitatory postsynaptic membranes in the central nervous system (1). More than 30 proteins have been identified that associate with these specialized submembraneous structures, referred to as postsynaptic density (PSD)‡ (2).

Many of these proteins in the PSD fraction are insoluble in mild detergents such as Triton X-100 (3). Therefore, the PSD is considered to be a tight aggregation of associated proteins that regulate synaptic transmission (4–6).

PSD-95/SAP90, a main component of the PSD fraction (3, 7), is a member of the membrane-associated guanylate kinase (MAGUK) superfamily, which has multiple protein-protein interaction domains. MAGUK proteins, localized mainly beneath the membrane at cell-cell interaction sites, have been implicated as central scaffolds for the protein assembly of specialized membrane domains such as PSDs in neurons, tight junctions in epithelia, and neuromuscular junctions (8–11). Strong support for the role of PSD-95 as a central organizer is found in its ion channel clustering activity. When PSD-95 is expressed heterologously together with the Shaker-type K⁺ channel (Kv1.4) or NMDA glutamate receptors, the expressed proteins become colocalized in plaque-like clusters (12, 13), whereas when the Kv1.4 channel, the NMDA receptor, or PSD-95 is expressed individually, the proteins are localized diffusely throughout the cellular membranes or the cytosol.

PSD-95 contains three N-terminal PDZ domains, a central Src homology-3 (SH3) domain, and a C-terminal guanylate kinase (GK)-like domain (Fig. 1A) (3, 14). SAP97/hdlg (15, 16), chapsyn-110/PSD-93 (13, 17), and SAP102 (18) also share this domain organization. Among the three PDZ domains of PSD-95, PDZ1 and PDZ2 have about 52% homology to each other and interact with the NR2 subunits of the NMDA receptor and the Kv1.4, as well as others, via the -S/TXV sequence motif located at the extreme C terminus (12, 19–21). Additionally, PDZ1 interacts with the kainate-binding glutamate receptor, GluR6, via its C-terminal sequence, ETMA (22). On the other hand, the third PDZ domain (PDZ3) has about 42 and 35% sequence homology to PDZ1 and PDZ2, respectively, and is clearly distinguished from the other two domains by its different sets of binding partners, such as neurogin, CRIP3 (23, 24), and the β1-adrenergic receptor (25). Structure studies of PDZ2 and PDZ3 revealed that both domains consist of a similar six-stranded, anti-parallel β-barrel flanked by two α-helices, forming a ligand binding pocket between βB and αB, with the exception that PDZ1 and PDZ2 have longer variable loops connecting βB and βC than that of PDZ3 (Fig. 1B) (26, 27). Although no binding partner for the SH3 domain has been identified except for a kainate receptor subunit in vitro (22), the GK-like domain binds to the GK domain-associated proteins (GKAP/SAPAP/DAP) (28–31), BEGAIN (32), MAP1A (33), etc., which interact with other postsynaptic proteins.

Extensive studies have revealed that a palmitoylated pair of cysteines in the N-terminal region and PDZ1 or PDZ2 of

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‡ The abbreviations used are: PSD, postsynaptic density; SAP, synapse-associated protein; MAGUK, membrane-associated guanylate kinase; NMDA, N-methyl-D-aspartate; PDZ, PSD-95/discs large/ZO-1; GK, guanylate kinase; GST, glutathione S-transferase; PBS, phosphate-buffered saline; SH3, Src homology-3.
PDZ-95 are essential for membrane targeting (34) and for clustering of Kv1.4 and NR2B in COS cells (35, 36), as well as for synaptic targeting of PSD-95 in hippocampal cultures (37) as shown by deletion analyses of PSD-95. Furthermore, previously reported differences in the biochemical properties between PDZ1 and PDZ2 include the following: (i) PSD-95 PDZ2 exhibits a higher affinity for the target peptides corresponding to the C-terminal sequences of Kv1.4 and NR2B than PDZ1 (24); (ii) a single PDZ protein, neuronal nitric oxide synthase, interacts only with PDZ2 via the PDZ-PDZ interaction (38, 39); and (iii) only PDZ1 binds to the kinase-type GluR6 (22). However, the intramolecular interactions between the SH3 and GK-like domains in the full-length PSD-95 are also required for the clustering of Kv1.4 (40, 41). In addition, the binding of the GK-like domain to MAP1A is stimulated by peptides corresponding to the C-terminal sequences of Kv1.4, NR2B, and CRIP7 (33). Therefore, to understand the molecular mechanisms of PSD-95 and ion channel clustering, the roles of each domain should also be assessed using full-length PSD-95 molecules. Because PDZ1 and PDZ2 in PSD-95 are fundamental to the clustering of Kv1.4 or NR2B, we analyzed the functional differences between PDZ1 and PDZ2 in the channel clustering.

Here, we introduced mutations and deletions into PDZ1 and/or PDZ2 of the full-length PSD-95 based on the reported structure and also constructed a series of PDZ1-PDZ2 inversion mutants, so that the mutated domains were impaired in channel binding but the overall structures of each PDZ domain and the full-length PSD-95 remained more or less intact. These mutants were further evaluated for their Kv1.4 clustering activity in COS-1 cells. The results show that PDZ2 is a key domain for Kv1.4 clustering and that the ligand binding function of PDZ, within the second domain, is essential for developing plaque-like clusters. In addition, the introduced mutations and loop deletions increased the sensitivity to trypsin, suggesting the compact molecular packing of PSD-95. These results imply the importance of the intramolecular interactions of PDZ-95 for ion channel clustering.

**EXPERIMENTAL PROCEDURES**

**DNA Constructions**—The PSD-95, NR2B, and Kv1.4 cDNAs were acquired from mouse whole brain by reverse transcriptase-mediated PCR using appropriate oligonucleotides as primers. The isolated PSD-95 and NR2B cDNAs, and also Kv1.4 cDNA, were subcloned into the XhoI-NotI and XhoI-EcoRI sites of the mammalian expression vector pcDNA3.1 (Invitrogen), respectively. The obtained cDNAs contained several mutations resulting in amino acid substitutions L75F, V496M, L80I, and T1043A in NR2B and E126G, T395P, L465P, and D636E in Kv1.4, compared with the published cDNAs (42, 43). The c-Myc tag (EQKLISEEDL) was inserted between amino acid residues 27 and 28 of the PDZ2 region using oligonucleotide-directed mutagenesis. FLAG-tagged PSD-95 was constructed by inverse PCR using primers encoding the FLAG epitope (DYKDDDDK) and the stop codon. The cDNA was introduced into the NheI and EcoRI sites of pcDNA3.1. The KpnI, SacII, and AflIII sites were introduced before the PDZ1 gene, between the PDZ1 and PDZ2 genes, and after the PDZ2 gene, respectively, to construct a series of PDZ1-PDZ2 inverted PSD-95 mutants. Mutant PDZ domains PDZ1ΔI, PDZ2ΔI, PDZ1Δm, and PDZ2Δm (Fig. 1B) were constructed by a general mutagenesis method using PCR.

To construct the GST-fused PDZ domains, PDZ1, PDZ2, PDZ1Δ, PDZ2Δ, PDZ1Δm, and PDZ2Δm were PCR-amplified and subcloned between the XhoI and EcoRI sites of pGEX-4T3 (Amersham Biosciences, Inc.). GST-fused PDZ domains were expressed in *Escherichia coli* BL21 strains. Bacteria were harvested and lysed in PBS containing 1% Triton X-100, 1 mM EDTA, 10 mM MgCl2, 5 mM dithiothreitol, 10% glycerol, 100 μg/ml lysozyme, 100 μg/ml DNase I, and protease inhibitors. Lysates were incubated at 4°C for 1 h and pelleted at 15,000 × g for 10 min. The bacterial supernatants were incubated with a 200-μl bed volume of glutathione-Sepharose beads (Amersham Biosciences, Inc.) at 4°C for 1 h and washed three times with PBS containing 1% Triton X-100 and 100 mM NaCl, 1 mM MgCl2, 10% glycerol, and 1% Triton X-100 at 4°C.

**Cell Culture and Transfection**— COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and penicillin/streptomycin. Before transfection, COS-1 cells were seeded on serum-coated cover glasses for clustering assays or in 100-mm plates for GST pull-down and immunoprecipitation assays and were cultured overnight.

For clustering assays, cells were transfected by the calcium phosphate precipitation method. The calcium phosphate-DNA mixtures were added within 24 h of seeding and were left for 18–24 h. The cells were shocked with 10% dimethyl sulfoxide (Me2SO) and were incubated at 37°C for additional 24 h.

For the GST pull-down and immunoprecipitation assays, COS-1 cells were transfected with LipofectAMINE reagent (Invitrogen) according to the manufacturer’s procedures.

**GST Pull-down and Immunoprecipitation Assays**—For the GST pull-down assay, NR2B- or Kv1.4-expressing COS cells were lysed in binding buffer 2 days after transfection. The cell lysates were mixed with beads previously bound with either the GST or GST-fused PDZ domain (~30 μg of PDZ1 or 4 μg of PDZ2) and were washed four times with PBS containing 1% Triton X-100 and 10% glycerol. The bound proteins were eluted with SDS sample buffer and analyzed by immunoblotting or silver staining.

For the immunoprecipitation assay, cells expressing Kv1.4 or each PSD-95 protein were solubilized separately in radioimmunoprecipitation assay buffer (200 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 10 μg/ml lysozyme, 100 μg/ml bovine serum albumin, and 0.5% deoxycholate, and 0.1% SDS) containing protease inhibitors. The solubilized supernatants of Kv1.4 and each PSD-95 mutant were mixed and incubated at 4°C for 2 h. Then, monoclonal anti-Kv1.4 antibodies (Upstate Biotechnology) or polyclonal anti-FLAG epitope antibodies (Zymed Laboratories Inc.) (~2 μg) were added to the mixtures, which were further incubated overnight at 4°C. Protein G-Sepharose resin (Amersham Biosciences, Inc.) was added, and the mixtures were incubated for 1 h. The immunoprecipitates were washed, eluted with SDS sample buffer, and analyzed by immunoblotting.

**Limited Digestion of PSD-95 by Trypsin—** Transfected COS-cells expressing each PSD-95 protein were homogenized, and the postnuclear supernatants were further centrifuged at 100,000 × *g* for 30 min. The obtained membrane suspensions (~1.7 mg/ml protein) were treated with trypsin (~25 μg/ml) at 37°C for 5, 60, and 120 min. Twenty μl of each reaction were mixed with SDS sample buffer, fractionated by SDS-PAGE, and analyzed by immunoblotting with a monoclonal anti-PSD-95 antibody (M16) (Transduction Laboratories) with an epitope in the region of 353–504. An anti-FLAG antibody and a monoclonal anti-PSD-95 antibody (7E3-1B8) (Affinity Bioreagents, Inc.) were also used, although the 7E3 antibody did not recognize PDZ2 mutant because of the epitope deletion. Both antibodies gave similar patterns in which the bands larger than ~60 kDa were more sensitive to trypsin than those of the wild-type. For the limited digestion of PSD-95, Pronase, papain, and V8 were also examined. Because of the many digestion sites, we could not detect useful limited fragments under the conditions employed.

**Indirect Immunofluorescence Staining of Transfected COS-1 Cells—** Two days after transfection or on the day after the cells were seeded on growing on chemically defined medium containing with ice-chilled PBS containing 1 mM MgCl2 and 1 mM CaCl2, and were fixed with the same buffer containing 4% paraformaldehyde and 0.1% Triton X-100 for 30 min at 4°C. The cells were then washed with PBS containing 0.1% Triton X-100 and were incubated with the same buffer containing 50 mM glycine to quench the reactions. Nonspecific protein binding was blocked by an incubation with blocking buffer (PBS containing 3% bovine serum albumin and 0.1% Triton X-100) for 1 h at room temperature. Primary antibodies were then added to the blocking buffers, and the mixture was incubated for 3 h at room temperature or overnight at 4°C. The samples were washed with blocking buffer, labeled with fluorescein isothiocyanate-conjugated goat anti-rabbit and Texas Red-conjugated goat anti-mouse antibodies diluted in blocking buffer for 1 h at room temperature, and washed again three times with PBS containing 0.1% Triton X-100. Finally, the coverslips were mounted on slides, and images were obtained with a Zeiss LSM Pascal laser scanning confocal microscope system. The cells shown in Fig. 5, c and f, were observed with an Olympus BX50 microscope.

Clustering assays were repeated 4–5 times. For each assay, the number of cells that formed plaque-like clusters was counted among 50–100 cells coexpressing PDZ-95 and Kv1.4. The clusters in COS cells were judged referring to the published patterns (12, 28, 35, 36, 41) using the following criteria: (i) PSD-95 and Kv1.4 were colocalized; (ii) the localization patterns between the individually expressed PSD-95 or Kv1.4 and the coexpressed PSD-95 and Kv1.4 were different; (iii) the clustered area of PSD-95 and Kv1.4 was larger (~1 μm) than that of just a colocalized dot; (iv) perinuclear signals were not included;
Distinct Roles of PDZ1 and PDZ2 of PSD-95 in Clustering

**RESULTS**

Construction and Characterization of PDZ Domain Mutants—The PDZ1, PDZ2, and PDZ3 domains exhibit distinct ligand specificities, and yet they form similar structures. As compared with PDZ3, PDZ1 and PDZ2 have an additional six-residue insertion in a variable loop connecting βB and βC (Fig. 1B). To disrupt the interactions of PDZ1 and PDZ2 with the Shaker-type K+ channel, Kv1.4, without affecting the overall structure, we first deleted these six residues in the βB/βC loop of PDZ1 (PDZ1Δ) and PDZ2 (PDZ2Δ) to make them resemble PDZ3. Although the interactions of the PDZ domains and NR2B were significantly impaired by the deletions (Fig. 1C), these constructs still exhibited weak but detectable levels of interactions in vitro (Fig. 2B). Therefore, Ser-78 and Ala-80 in the βB of PDZ1, and Ser-173 and Ala-175 in the βB of PDZ2 were further mutated to asparagine and valine, the corresponding residues of PDZ3, respectively (Fig. 1B), because βB is part of a ligand binding pocket and the corresponding N326 in PDZ3 is important for its specific binding to CRIP1 (24).

Each wild-type or mutant PDZ domain, fused to glutathione S-transferase (GST), was examined for an interaction with Kv1.4 in vitro (Fig. 1D). Neither PDZ1Δm nor PDZ2Δm showed detectable binding activity to Kv1.4. Thus, the βB/βC loops and the mutated residues play important roles in the specific binding of PDZ1 and PDZ2 to Kv1.4.

A Single PDZ Domain in PSD-95 Is Sufficient for Binding with Kv1.4 in Vitro—The PDZ1Δ, PDZ2Δ, PDZ1Δm, and/or PDZ2Δm domains were then replaced with the wild-type domains of full-length PSD-95. Fig. 2A shows the mutants constructed in this study. PSD-95:1Δ-2 was constructed by replacing PDZ1 of PSD-95 with PDZ1Δ, whereas PDZ2 was replaced with PDZ2Δm in PSD-95:1-2Δm, and both PDZ domains were replaced in PSD-95:1mΔ-2Δm (Fig. 2A, middle). In addition, a FLAG epitope tag was attached to the C termini of the mutated PSD-95 proteins for their detection. The expression level of the FLAG-tagged PSD-95 was comparable with that of the untagged PSD-95. Hereafter, FLAG-tagged PSD-95 was used as the wild type. The expression levels of these mutants were also comparable with that of the wild-type PSD-95, except for that of PSD-95:1mΔ-2Δm, which was lower.

The ability of these mutant PSD-95 proteins to interact with Kv1.4 in vitro were examined by coimmunoprecipitation assays. The mixture of Kv1.4 and the wild-type or mutant PSD-95 was immunoprecipitated with an anti-Kv1.4 antibody, and the coimmunoprecipitated PSD-95 was analyzed. In addition, the wild-type and mutant PSD-95 proteins were immunoprecipitated with an anti-FLAG epitope antibodies, and the coimmunoprecipitated Kv1.4 was analyzed by immunoblotting. The interactions of PSD-95:1Δ-2, 1-2Δ, 1mΔ-2, and 1mΔ-2Δ mutants with Kv1.4 were similar to those of the wild-type (Fig. 2, B and C), and PSD-95:1Δ-2Δ interacted weakly. However, no Kv1.4 signal was detected in the PSD-95:1mΔ-2Δm immunoprecipitate (Fig. 2C). Thus, PSD-95 was able to associate with Kv1.4 as long as either the PDZ1 or PDZ2 domain remained intact.

Limited Trypsin Digestions of PSD-95 and Mutants—The entire domain assemblies of the PSD-95 mutants were compared with that of the wild-type by limited trypsin digestion.
Distinct Roles of PDZ1 and PDZ2 of PSD-95 in Clustering

Fig. 2. The ability of the wild-type and mutant PSD-95 proteins to interact with Kv1.4 in vitro. A, schematic diagram of the mutant PSD-95 proteins constructed in this study. The representation of each PDZ domain (a white box for PDZ1, a box with oblique lines for PDZ2, and a gray box for PDZ3) is the same as that in Fig. 1A. The mutated domains shown in Fig. 1B are replaced with the wild-type domains in the full-length PSD-95. B and C, communoprecipitation of Kv1.4 with the wild-type and mutant PSD-95 proteins. Kv1.4, the wild-type, and the mutant forms of PSD-95 were expressed individually in COS cells. The extracts from wild-type or mutant PSD-95-expressing cells or from non-transfected cells (the right lane in C) were mixed with the extract from Kv1.4-expressing cells. The mixtures were immunoprecipitated with an anti-Kv1.4 antibody (B) or with an anti-FLAG antibody (C). The Kv1.4 and PSD-95 proteins in the precipitates were detected with anti-Kv1.4 and anti-FLAG antibodies, respectively.

Fig. 3 shows the digestion patterns of the wild-type and mutant proteins in the membrane fractions prepared from COS-1 cells expressing each protein, as analyzed by immunoblotting. The full-length and ~60 kDa bands of the mutants were much more sensitive to trypsin than those of the wild-type, indicating that the βββC loop deletion and/or the mutations in β loosened the molecular packing of PSD-95. It is interesting that the only six-residue deletion in the variable loop and/or the two point mutations of single PDZ domain affect the entire molecular packing of full-length PSD-95. Although the details of entire domain assemblies of the PSD-95 mutants were not clear from this experiment, the patterns of the limited fragments are similar, suggesting that the trypsin-sensitive sites of mutants similar to those of the wild-type were exposed. In addition, because PSD-95:1m-2, which showed wild-type clustering activity, and the other mutants exhibited similar trypsin sensitivity to one another, the deficient clustering activities are not due to the trypsin-sensitive structures. The clustering activities are described later in detail.

PDZ2 Is an Essential Domain for Channel Clustering Activity—The wild-type and mutant PSD-95 proteins were coexpressed with Kv1.4 in COS cells, and their cluster-forming activities were examined by indirect immunofluorescence double labeling of transfected cells. In Fig. 4, a and b, individually expressed PSD-95 and Kv1.4 show diffuse and reticular distributions in the transfected cells, respectively. Fig. 4, c1/c2, shows the cell cotransfected with the wild-type PSD-95 and Kv1.4, in which plaque-like protein clusters are notable, as described previously (12, 28, 35, 36, 41). Although the clustering efficiency of wild-type PSD-95 and Kv1.4 was ~30% of the cotransfected cells in our system, which was considerably lower than the previously reported value (~40–70%) by Kim et al. (13), we tried to count only convincing plaques as clusters using the six criteria described under “Experimental Procedures.” The FLAG tag attached to the C terminus might affect the clustering efficiency. The observed plaque-like protein clusters themselves were remarkable and were easily recognized. The fractions of cells displaying plaque-like protein patch clusters out of cells coexpressing PSD-95 and Kv1.4 were calculated based on the fluorescence microscopy observations and are displayed in Fig. 6 as the clustering efficiencies.

Fig. 4, d and g, shows diffuse localization of PSD-95:1mΔ-2 and PSD-95:1-2mΔ when expressed individually. While coexpression of PSD-95:1mΔ-2 with Kv1.4 resulted in the formation of plaque-like clusters, but with slightly decreased efficiency (~20%) as compared with the wild-type (~30%) (Fig. 4, e1/e2 and f1/f2 and Fig. 6), PSD95:1-2mΔ coexpressed with Kv1.4 was diffusely distributed and showed severely defective clustering efficiency (~1%) (Fig. 4, h1/h2 and i1/i2 and Fig. 6). Thus PDZ2, but not PDZ1, is an essential domain for PSD-95 to form plaque-like protein clusters with Kv1.4 in COS cells.

The Position of PDZ2 Is Important for an Efficient Clustering—To form protein clusters, the N-terminal palmitylated cysteines and an appropriate PDZ domain are indispensable. We considered the importance of the structural arrangement of the PDZ domains in the PSD-95 molecule because the ligand binding of PSD-95 leads to the clustering of Kv1.4. To examine whether the position of PDZ2 in the molecule is important for clustering, a PDZ1-PDZ2 inversion mutant (PSD-95:2-1) was constructed, and its PDZ domains were replaced with PDZ1mΔ and/or PDZ2mΔ as shown in the lower part of Fig. 2A. These mutants were expressed as well as the wild type (data not shown). In addition, their Kv1.4 interacting activities in vitro remained intact except for PSD-95:2mΔ-1mΔ (Fig. 2C). In terms of their clustering activities, PSD-95:2-1 could form typical clusters with an efficiency (~27%) comparable with the wild-type (Fig. 5, b1/b2 and Fig. 6), whereas the clustering activities of PSD-95:2mΔ-1 (~4%) and PSD-95:2-1mΔ (~7%) were significantly reduced (Fig. 5, d1/d2, e1/e2, g1/g2, and h1/h2 and Fig. 6). In addition to diffuse distributions (Fig. 5, d1 and g1), both PSD95:2mΔ-1 and 2-1mΔ also showed prominent localization in perinuclear clusters with Kv1.4 (Fig. 5, e1/e2 and h1/h2) as compared with the other mutants. This observation could be indicative of endoplasmic reticulum localization. These localization patterns of both PSD-95:2mΔ-1 and 2-1mΔ when expressed with Kv1.4 (Fig. 5, e and h) were similar to those of PSD-95 mutants defective in clustering and intramolecular SH3-GK interaction (41). In the case of the clusters of GluR6 and PSD-95 via the PDZ1 domain, dot-like clusters that looked different from plaque-like Kv1.4 clusters were observed (22). There might be differences in the clusters depending upon the composed proteins, their interactions, etc.

The wild-type clustering activity of PSD-95:2-1 suggests that the inversion itself of the PDZ1 and PDZ2 domains does not cause deficiency in the clustering. The significantly reduced activities of PSD-95:2mΔ-1 and PSD-95:1-2mΔ suggest that the PDZ1 alone in the PSD-95 could not confer the efficient clustering activity, whereas the PDZ2 could. In addition, the co-
FIG. 3. Limited trypsin digestion of the wild-type and mutant PSD-95 proteins. The membrane fractions containing the expressed PSD-95 proteins were partially digested. Aliquots were withdrawn at the indicated times and analyzed by immunoblotting with the M16 antibody. The full-length and ~60-kDa bands of the mutant PSD-95 were much more sensitive to trypsin than those of the wild-type. The addition of the ligand peptide (the C-terminal 10 residues of Kv1.4) did not cause any differences in the digestion pattern and the trypsin sensitivity. The bands of the full-length PSD-95 proteins and of the major limited fragments are indicated by arrowheads.

FIG. 4. Kv1.4 clustering by the wild-type and mutant (1mΔ2, 1-2mΔ) PSD-95 proteins. COS cells expressing the wild-type or mutant forms of PSD-95 (a, d, and g), Kv1.4 alone (h), or both (c, e, f, h, and i) were fixed 48 h after transfection. The distributions of PSD-95 and Kv1.4 were examined by immunofluorescence with double labeling with anti-FLAG (green) and anti-Kv1.4 antibodies (red). Transfected cDNAs are indicated at the bottom of each panel, with the colors corresponding to the observed fluorescence. When expressed individually, the wild-type and mutant (1mΔ2, 1-2mΔ) PSD-95 proteins were distributed diffusely throughout the cell (a, d, and g), whereas Kv1.4 was distributed in a perinuclear and a cytoplasmic reticular pattern (b). However, typical plaque-like clusters were formed between Kv1.4 and wild-type PSD-95 (c) or PSD-95:1mΔ2 (e and f) but were not observed with PSD-95:1-2mΔ (h and i). Scale bars, 10 μm.

FIG. 5. Kv1.4 clustering activity of the domain-inverted mutant of PSD-95. COS cells expressing mutant forms of PSD-95 alone (a, c, and f) or with Kv1.4 (b, d, e, g, and h) were fixed 48 h after transfection and labeled fluorescently as described in the legend for Fig. 4. All mutant (2-1, 2mΔ1, and 2-1mΔ) PSD-95 proteins were distributed diffusely throughout the cell when expressed separately (a, c, and f). Kv1.4 could form typical plaque-like clusters with PSD-95:2-1 (b) but not with PSD-95:2mΔ1 (d and e) or 2-1mΔ (g and h). Instead, the localization of Kv1.4 and both PSD-95 mutants was mainly diffuse (d and g). Colocalization of these proteins occurred only in fine scattered puncta or in large perinuclear clusters (e and h). Scale bars, 10 μm.

Comparison of PSD-95:2-1mΔ and PSD-95:1mΔ-2 (Fig. 4, e and f) and Fig. 5, g and h) suggests that the location of PDZ2 in an appropriate position in the multidomain protein, PSD-95, is also required for the efficient clustering activity.

DISCUSSION

A Six-residue Insert in the βB/βC Loop Is Involved in the Ligand Binding of PDZ1 and PDZ2—In this study, we introduced mutations into PDZ1 and/or PDZ2 of PSD-95 to impair the association of each domain with a target protein, while retaining the overall multidomain structure of PSD-95. We also compared the roles of PDZ1 and PDZ2 in the protein clustering of Kv1.4 and PSD-95.

Recently the solution structure of PSD-95 PDZ2 was determined, and the overall structure was quite similar to those of PSD-95 PDZ3 and other PDZ domains (26, 27, 44–47). A sequence alignment revealed that PDZ1 and PDZ2 of PSD-95 have six additional residues in their βB/βC loops, unlike most other PDZ domains. The involvement of this loop in the target peptide binding was suggested by comparing the ligand-free and ligand-bound structures of PDZ2 (26), and we first verified it by biochemical experiments. In our study, the deletion of these six residues from PSD-95 PDZ1 and PDZ2 decreased the Kv1.4 and NR2B binding activities significantly, and additional serine mutations (S78N, S173N) in the βB-sheet further decreased Kv1.4 binding to an undetectable level by immuno-
Precipitation. These residues could form hydrogen bonds with the carboxyl group of Asp/Glu at the −1 position of the target peptide and may be involved in the target selectivity (26, 27). In addition, the N326S mutation of PDZ3 switches its binding specificity from the CRIPT C terminus, QTSV, to the Kv1.4 C terminus sequence, ETDV (24). These results suggest that binding to the target peptide by PDZ domains occurs in a cooperative manner, using the residues in ββ, αα, the variable loop, etc., as shown by structure studies (26, 27), and that the variable ββ/αα loop plays important roles in binding to Kv1.4.

SAP102, one of the MAGUKs, has the same domain architecture as PSD-95. PDZ2 showed the highest affinity for the NR2B C-terminal peptide, as judged by the enzyme-linked immunosorbent assay, whereas PDZ1 showed about one-fifth the affinity, and PDZ3 showed about two orders of magnitude lower affinity than PDZ2 (18). The distinct binding affinities of the three PDZ domains may be determined by binding pockets composed of cooperative, multi-binding sites.

PDZ2, Not PDZ1, of PSD-95 Is a Key Domain for Kv1.4 Clustering—In the neuron, PSD-95 could play a role in accumulating signaling molecules at PSD and thereby regulate synaptic transmission. For the scaffolding function, multivalent protein interacting domains may be essential. However, the functional differences between PDZ1 and PDZ2 of PSD-95 in the clustering of Kv1.4 and NR2B and in synaptic targeting have not been addressed thus far. Deletion of either PDZ1 or PDZ2 or deletions of two PDZ domains such as PDZ1 and PDZ2, PDZ1 and PDZ3, or PDZ2 and PDZ3 from the full-length PSD-95 did not cause any significant defects in its synaptic targeting (48), whereas when a single PDZ domain was retained similarly by deleting the other two domains, the mutants exhibited semiclustered synaptic targeting (37). These results, however, do not prove that PDZ1 and PDZ2 play similar roles in synaptic targeting and in PSD construction. We studied the roles of PDZ1 and PDZ2 in the full-length PSD-95 by mutating the mutual domain. As several lines of previous evidence have indicated (12, 19, 20), our in vitro communoprecipitation assay showed that Kv1.4 and NR2B associated with both PDZ1 and PDZ2 of PSD-95. However, the protein clusters of Kv1.4 could be formed by the mutant PSD-95 carrying intact PDZ2 (1mΔ-2) but not by the PDZ2-mutated PSD-95 (1-2mA) in COS cells. Thus, as far as Kv1.4- and NR2B-type clustering in COS cells is concerned, PDZ1 in the full-length PSD-95 is dispensable, but PDZ2 is not. In contrast, only PDZ1 of PSD-95 binds to GluR6, a subunit of the kainate receptor, via its C-terminal amino acid sequence, ETMA, which leads to the clustering of the kainate receptor and confers the modified channel activity exhibiting incomplete desensitization (22). PDZ2 binds to different sets of partners, such as neurexin (23), CRIPT (24), etc. Thus, an individual PDZ of multivalent domains may be in charge of binding to different types of proteins and may play distinct roles in the PSD, thereby enabling the clustered complex to modulate synaptic transmission. Alternatively, PSD-95, in which both PDZ1 and PDZ2 bind to Kv1.4 or NR2, might exhibit different properties in synaptic transmission from PSD-95 in which PDZ1 and PDZ2 bind to different partners, respectively. This variability and the differential qualities of PSD might be important for synapse stability and plasticity, which were suggested as roles for PSD-95 (49).

Examinations of the synaptic targeting abilities of our mutants and the properties of the PSD fractions are required for a better understanding of the molecular mechanisms of protein clustering by PSD-95 in neurons and its significance in synaptic transmission. In particular, the utilization of PSD-95:1 mΔ-2 and PSD-95:1-2mA would allow the dissection of the PSD functions activated by the NMDA receptor and the kainate receptor.

The Location of PDZ2 in Full-length PSD-95 Is Important for Efficient Clustering—The N-terminal region and the PDZ1 and PDZ2 domains of PSD-95 were sufficient for ion channel clustering as shown by truncation experiments (36). However, the requirements for cluster formation of full-length PSD-95 appear to differ from those of the truncated PSD-95 molecules. Our analysis of the PDZ1-PDZ2 inversion mutants indicates that PDZ2 must be located at its original position in the whole molecule for efficient clustering, based on a comparison of the clustering activities of PSD-95:1mΔ-2 and PSD-95:2-1mA (Figs. 4–6). Ligand binding at the position of the second PDZ domain may be important for PSD-95 to cluster ion channels efficiently. Moreover, the mutated residues and/or the deleted loop may play critical roles in the clustering. PSD-95:2mΔ-1 might be unable to interact sufficiently with Kv1.4 in vitro because of the low affinity of PDZ1 for the target peptide, even though its interaction with Kv1.4 was detected by an in vitro communoprecipitation assay. In contrast, PSD-95:2-1 retained the wild-type clustering activity. This may be because of the first high affinity interaction of PDZ2 with Kv1.4; namely, the first subunit of Kv1.4 interacts with PDZ2. This interaction would allow another subunit to associate more easily with PDZ1 because of the homotetrameric assembly of Shaker K+ channel subunits, like other K+ channels (50), or because of conformational changes that may occur in PSD-95 by binding a ligand to the first PDZ domain. Then, the ligand binding of PDZ2 placed at the second domain in the full-length PSD-95 would lead to the efficient clustering.

Indeed, the reasons why PDZ2 should be located at the second position in the molecule are still unclear. Nevertheless, as shown for the molecular switch mechanism of the c-Src family of tyrosine kinases (51, 52), PDZ2 might interact with other domains such as the SH3 or GK-like domains in the molecule in the ligand-free state, either intramolecularly or intermolecularly, to regulate complex formation, given that PSD-95 alone does not form protein clusters. Then, the ligand

**Fig. 6. Clustering efficiency of mutant PSD-95.** The clustering efficiency levels are expressed as percentages of the cluster-formed cells/cells coexpressing Kv1.4 and the wild-type or mutant PSD-95 proteins. At least three independent dishes were cotransfected, and ~100 cotransfected cells/each transfection were examined for the cluster formation.
binding of PDZ2 may induce a molecular switch to cause allosteric changes in the full-length PSD-95, which lead to cluster formation. The limited digestions of PSD-95 by trypsin showed that the introduced mutations and deletions loosened the entire molecular packing of PSD-95, suggesting the involvement of mutated and/or deleted residues in the intramolecular interaction. The ligand-bound structure of the second PDZ domain might play a fundamental role in inducing a molecular switch for clustering. On the other hand, the structural changes induced by ligand binding could be quite subtle, because the addition of a ligand peptide did not affect the trypsin digestion patterns (data not shown). In the case of the clustering of the kinase receptor by the interaction with PDZ1 of PSD-95, the binding of another subunit, KA2, in the receptor to the SH3 and GK domains may help to induce allosteric changes in the full-length PSD-95 (22).

Additionally, a series of intramolecular interactions among the individual domains have been pointed out in the MAGUK family (33, 40, 41, 53, 54). The intramolecular interaction between the PDZ2/3 domains and the GK-like domain of PSD-95 has been suggested by showing that ligand binding of PDZ2 or PDZ3 enhances the binding activity of the GK-like domain to MAP1A (33). The intramolecular SH3-GK-like domain interaction of PSD-95 was also shown to be responsible for the formation of typical plaque-like coclusters with Kv1.4 (40, 41). The formation of the N-terminal sequences with SH3 in SAP97 has been shown to interfere with the interaction between the SH3 and GK-like domains and to facilitate the binding of the GK-like domain to guanylate kinase-associated protein (53, 54).

Thus, it appears that correct intramolecular interactions in the ligand-free state of PSD-95 and, furthermore, the ligand binding of the second PDZ domain are required to develop efficient clustering of Kv1.4. The combination of three PDZ domains with different properties may allow the MAGUKs to modulate synaptic transmission and neuronal plasticity. Structural studies of the entire PSD-95 protein should help our understanding of the significance of the conserved domain organization of the MAGUKs in synaptic clustering.

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