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Postsynaptic Density-93 Interacts with the δ2 Glutamate Receptor Subunit at Parallel Fiber Synapses

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The glutamate receptor subunit δ2 has a unique distribution at the parallel fiber–Purkinje cell synapse of the cerebellum, which is developmentally regulated such that δ2 occurs at both parallel fiber synapses and climbing fiber synapses early in development but is restricted to parallel fiber synapses in adult animals. To identify proteins that might be involved in the trafficking or docking of δ2 receptors, we screened a yeast two-hybrid library with the cytosolic C terminus of δ2 and isolated a member of the postsynaptic density (PSD)-95 family of proteins, which are known to interact with the extreme C termini of NMDA receptors. We find that δ2 binds specifically to PSD-93, which is enriched in Purkinje cells. In addition, PSD-93 clusters δ2 when they are coexpressed in heterologous cells, and clustering is disrupted by point mutations of δ2 that disrupt the δ2–PSD-93 interaction. Ultrastructural localization of PSD-93 and δ2 shows they are colocalized at parallel fiber synapses; however, PSD-93 also is present at climbing fiber synapses of the adult rat, where δ2 is not found, indicating that the presence of PSD-93 alone is not sufficient for determining the synaptic expression of δ2.

Key words: glutamate receptor; receptor targeting; yeast two-hybrid; synaptic anchor; cerebellum; synaptic receptor regulation

Ionotropic glutamate receptors comprise three subtypes, and each contains multiple subunits that assemble to form functional receptor complexes: NMDA (NR1, NR2A-D), AMPA (GluR1–4), and kainate (GluR5–7; KA1–KA2) (Hollmann and Heinemann, 1994). A fourth subtype, δ, has two known subunits (δ1 and δ2) (Yamazaki et al., 1992; Lomeli et al., 1993). Although both δ1 and δ2 resemble ionotropic glutamate receptor subunits based on sequence similarity, neither forms functional channels when expressed in *Xenopus* oocytes or mammalian cells. For this reason, far less is known about the functional role of δ glutamate receptors than the more extensively characterized NMDA, AMPA, and kainate receptors. Despite their limited functional characterization, genetic studies have identified a critical role for δ2 in cerebellar function. δ2 has a unique distribution, being highly expressed in cerebellar Purkinje cells (Araki et al., 1993), where it is specifically found postsynaptic to parallel fiber synapses (Landsend et al., 1997; Zhao et al., 1997). Because the locus of cerebellar long-term depression (LTD) is the parallel fiber–Purkinje cell synapse, there has been substantial speculation that δ2 might be the glutamate receptor involved in that paradigm of synaptic plasticity. Indeed, mice lacking δ2 display impaired LTD (Kashiwabuchi et al., 1995), and LTD in cultured Purkinje cells requires the δ2 subunit (Hirano et al., 1995; Jeromin et al., 1996). Interest in δ2 has increased recently because of the exciting discovery that the phenotype of the Lurcher mouse, which is characterized by degeneration of cerebellar Purkinje cells, is caused by a point mutation in the third transmembrane domain of δ2 (Zuo et al., 1997). This gain-of-function mutation results in a constitutively open δ2 channel, allowing a large inward current that leads to cell death.

For glutamate receptors to function normally, they must be appropriately targeted to, and retained at, specific postsynaptic sites. Synaptic enrichment of receptors is a complex process requiring many proteins to regulate trafficking of the receptors to dendritic spines and docking at the postsynaptic density (PSD). This complexity is well illustrated by the differential targeting of the δ2 receptor subunit in Purkinje cells. Early in development, δ2 is found at both parallel fiber and climbing fiber synapses but, in the adult, δ2 is restricted to the parallel fiber synapse (Landsend et al., 1997; Zhao et al., 1997). Other ionotropic and metabotropic glutamate receptors are seen at both synaptic populations and do not change with development (Zhao et al., 1998). Very little is known about the mechanisms underlying the differential targeting of receptors; however, recent studies have identified a critical role for the cytoskeleton in the docking and clustering of receptors at postsynaptic sites. The best characterized postsynaptic cytoskeletal anchors are the PSD-95 family of proteins, which are members of the larger family of membrane-associated guanylate kinases (MAGUKs). PSD-95/synapse-associated protein (SAP)-90, PSD-93/chapsyn-110, SAP-97/human discs large, and SAP-102 all contain three conserved PSD-95/discs large/zona occludens-1 (PDZ) domains, an SH3 domain, and a guanylate kinase domain, and all bind to Shaker K^+ channels and NMDA receptors (Sheng and Kim, 1996; Ziff, 1997; Craven and Bredt,

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1998). The binding of PSD-95 and related proteins to NMDA receptors and Shaker K^+ channels requires the last six amino acids of the channels. This region is characterized by a T/SxV binding motif (see Fig. 1) in which the terminal amino acid (0 position) and the residue at the −2 position are critical for binding.

In the present study, we used the δ2 C terminus to screen a rat brain cDNA library using the yeast two-hybrid system to identify proteins that may be involved in the trafficking and/or synaptic localization of δ2. We isolated a member of the PSD-95 family of proteins and, after subsequent characterization, demonstrated that δ2 can bind to PSD-93, PSD-95, and SAP-97. PSD-93 binds to δ2 in several independent assays, induces clustering of δ2 when the two proteins are coexpressed in heterologous cells, and colocalizes with δ2 at the ultrastructural level in parallel fibers of Purkinje cells. Unlike δ2, PSD-93 is expressed at climbing fiber synapses, as well as parallel fiber synapses, suggesting that the PSD-95 family of proteins is not responsible for the initial targeting of receptors to specific synapses but more likely anchors or docks receptors targeted by other mechanisms.

MATERIALS AND METHODS

cDNA constructs. δ2 and δ1 cDNAs in mammalian expression vectors were generously provided by P. Seeburg (Max Planck Institute, Heidelberg, Germany). The PSD-93 cDNA was isolated as described previously (Brenman et al., 1996b). The δ2 wild-type (WT) C terminus (amino acids 852–1008), δ1 WT C terminus (amino acids 852–1009), and δ2 truncated C terminus (amino acids 852–1004) were amplified using PCR and subcloned into the GAL 4 DNA binding domain vector pGBT9 using *Eco*RI and *Bam*HI sites. Point mutations of δ2 were produced using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA), amplified by PCR, and then inserted into pGBT9. The various PDZ constructs of PSD-95 were amplified using PCR and subcloned into the GAL 4 activation domain vector pGAD 424 using *Eco*RI and *Bam*HI sites. The sequences of all PCR products were confirmed by automated sequence analysis.

Screening of the yeast two-hybrid library. The δ2 WT C terminus (amino acids 852–1008) was used to screen a rat brain cDNA library in the activation domain vector pGAD 10 (Clontech, Palo Alto, CA). Approximately 1.94 million clones were screened, yielding five positives. The positive interactors were verified by restreaking and assaying for growth on histidine-deficient plates and β-galactosidase activity.

Yeast two-hybrid assays. Constructs in GAL 4 DNA binding domain vectors and constructs in GAL 4 activation domain vectors were cotransformed into HF7c yeast cells according to the manufacturer's protocol (Clontech). The yeast cells were plated onto synthetic dextrose plates lacking tryptophan and leucine and allowed to grow for ~3 d. Colonies were then resuspended in 10 mM tris and 1 mM EDTA, pH 8.0, and restreaked on dextrose plates lacking tryptophan and leucine and also onto plates lacking tryptophan, leucine, and histidine (His-deficient plates). Growth on His-deficient plates was scored on a − to +++ scale, comparing cotransformations that yielded equivalent growth on plates lacking tryptophan and leucine but containing histidine. Colony lifts were performed on His-deficient plates, and β-galactosidase activity was assayed using X-gal. Color intensity was scored from − to +++.

Cell culture and transfections. Human embryonic kidney (HEK)-293 cells were grown on 10 cm dishes for biochemical analyses. HEK-293 cells were transfected with PSD-93 (5 μg) and δ2 WT or mutant cDNAs (5 μg) using the calcium phosphate coprecipitation method (Blackstone et al., 1992). Transfected cells were analyzed 36 hr after transfection.

HeLa cells were grown on glass coverslips in six-well tissue culture dishes for immunofluorescence microscopy. HeLa cells were transfected with PSD-93 cDNA, along with δ2 WT or mutant cDNAs (4 μg total per well of six-well dish) using the calcium phosphate coprecipitation method (Blackstone et al., 1992). Transfected cells were analyzed 24 hr after transfection.

Antibodies. Antibodies against δ 1/2 (Mayat et al., 1995), GluR2/3 (Wenthold et al., 1992), and PSD-93 (Brenman et al., 1996b) have been characterized previously. Monoclonal antibodies raised against PSD-95 (Kornau et al., 1995) were generously provided by M. Kennedy (California Institute of Technology, Pasadena, CA). We refer to these antibodies as PSD-93/95 because they recognize PSD-93 (see Fig. 5), in addition to PSD-95.

Immunocytochemistry. Transfected HeLa cells grown on coverslips were washed in PBS, fixed in 4% paraformaldehyde in PBS for 20 min, washed in PBS, and permeabilized in 0.25% Triton X-100 in PBS for 5 min. The coverslips were washed in PBS and incubated with primary antibodies in PBS containing 3% normal goat serum (NGS) (PSD-93/95 monoclonal antibodies, 1:1000; δ 1/2 affinity purified antibodies, 1 μg/ml) for 1–2 hr at room temperature, washed in PBS, and incubated with FITC anti-mouse and rhodamine anti-rabbit secondary antibodies in PBS containing 3% NGS (1:500; Jackson ImmunoResearch, West Grove, PA) for 30 min at room temperature, washed three times in PBS, and mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA).

Immunoprecipitations and immunoblot analysis. Transfected HEK-293 cells were collected in PBS and pelleted by centrifugation. The pellets were resuspended in lysis buffer without detergent [50 mM Tris-HCl, pH 7.5, containing protease inhibitors (PMSF, 0.5 mM; leupeptin, 1 μg/ml; pepstatin, 1 μg/ml; and EDTA, 2.5 mM)] and sonicated, and Triton X-100 was added to a final concentration of 1%. The particulate fraction was removed by centrifugation, and the supernatant was incubated with 10 μg of affinity purified δ 1/2 antibodies or 3 μl of PSD-93 guinea pig (GP) antisera bound to protein A Sepharose for 1.5 hr or longer. The beads were washed three times with lysis buffer containing 0.1% Triton X-100, and proteins were eluted by boiling in SDS-PAGE sample buffer.

Frozen rat cerebella were homogenized in lysis buffer without detergent containing protease inhibitors (see above) in a Polytron, and either Triton X-100 or deoxycholate (DOC) was added to a final concentration of 1%. Triton X-100-solubilized tissue was incubated for 30 min at 4°C, and DOC-solubilized tissue was incubated for 30 min at 37°C. The insoluble fraction in each preparation was removed by centrifuging at 100,000 × *g* for 30 min. The DOC samples were then dialyzed overnight against 50 mM Tris-HCl, pH 7.5, containing 0.1% Triton X-100 and centrifuged again to remove insoluble material. Detergent-soluble supernatants were incubated with 10 μg of affinity purified δ 1/2 antibodies or 3 μl of PSD-93 GP antisera bound to protein A Sepharose or to protein A Sepharose alone overnight. The beads were washed three times with lysis buffer containing 0.1% Triton X-100, and proteins were eluted by boiling in SDS-PAGE sample buffer for 3–5 min.

SDS-PAGE was performed using 4–20% gradient gels. Proteins were resolved and transferred onto polyvinylidene difluoride (Immobilon; Millipore, Bedford, MA) membranes and subjected to immunoblot analysis using the antibodies indicated in the figure legends and the appropriate secondary antibodies. Results were visualized using enhanced chemiluminescence (Pierce, Rockford, IL).

Chemical cross-linking. Cerebellar membranes were cross-linked with

δ1	SHG**T**S**I**
δ2	DRG**T**S**I**
NR2A	SIE**S**D**V**
NR2B	SIE**S**D**V**
NR2C	SLE**S**E**V**
NR2D	SLE**S**E**V**
GluR1	LGA**T**G**L**
ShA	SVE**T**D**V**
ShB	SVE**T**D**V**
Kv1.4	AVE**T**D**V**
Kir2.3	RRE**S**A**I**

Figure 1. Alignment of the C termini of glutamate receptors and K^+ channels known to interact with the PSD-95 family of proteins. The terminal six amino acids of each protein are listed with the amino acids in the 0 and −2 positions indicated in *bold type*.

A

Gal 4 DNA binding hybrid	Gal 4 activation hybrid	His 3 activation	β Gal activation
δ2 WT C-terminus (amino acids 852-1008)	PSD-93 (83-421)	+++	+++
δ1 WT C-terminus (851-1009)	PSD-93 (83-421)	+++	+++
δ2 truncated C-terminus (852-1002)	PSD-93 (83-421)	-	-
δ2 C-terminus (852-1008) T 1006 S	PSD-93 (83-421)	-	-
δ2 C-terminus (852-1008) T 1006 P	PSD-93 (83-421)	-	-
δ2 C-terminus (852-1008) I 1008 V	PSD-93 (83-421)	+++	+++
δ2 C-terminus (852-1008) I 1008 A	PSD-93 (83-421)	-	-

B

Figure 2. The C-terminal TxI motif from δ2 or δ1 binds to PSD-93. *A*, Yeast HF7c cells were cotransformed with expression vectors encoding various GAL 4 DNA binding domain fusion proteins and the PSD-93-GAL 4 activation domain fusion protein. The PSD-93 construct includes PDZ domains I and II. Each transformation mixture was plated on synthetic dextrose plates lacking tryptophan and leucine. Interaction was measured by the filter assay (Clontech) as described previously (Fields and Song, 1989) and by growth on histidine-deficient media. Data are representative of experiments repeated two times with similar results. *B*, A schematic of δ2 that indicates the last six amino acids containing the conserved TxI motif and the critical amino acids in the 0 and −2 positions.

A

Gal 4 DNA binding hybrid	Gal 4 activation hybrid	His 3 activation	β Gal activation
δ2 C-terminus (amino acids 852-1008)	PSD-95 PDZ1 (40-124)	-	-
δ2 C-terminus (852-1008)	PSD-95 PDZ2 (158-314)	+	+
δ2 C-terminus (852-1008)	PSD-95 PDZ3 (311-384)	-	-
δ2 C-terminus (852-1008)	PSD-95 PDZ1-2 (40-314)	+++	+++
δ2 C-terminus (852-1008)	PSD-95 PDZ2-3 (158-384)	++	++
δ2 C-terminus (852-1008)	PSD-95 PDZ1-3 (40-384)	+++	+++

B

Figure 3. Interaction of the δ2 C terminus with the PDZ domains of PSD-95. *A*, Yeast HF7c cells were cotransformed with expression vectors encoding the δ2 WT C terminus-GAL 4 DNA binding domain fusion protein and various GAL 4 activation domain fusion proteins. Each transformation mixture was plated on synthetic dextrose plates lacking tryptophan and leucine. Interaction was measured by the filter assay (Clontech) as described previouly (Fields and Song, 1989) and by growth on histidine-deficient media. Data are representative of experiments repeated three times with similar results. *B*, A schematic illustrating the conserved domains of the PSD-95 family of proteins.

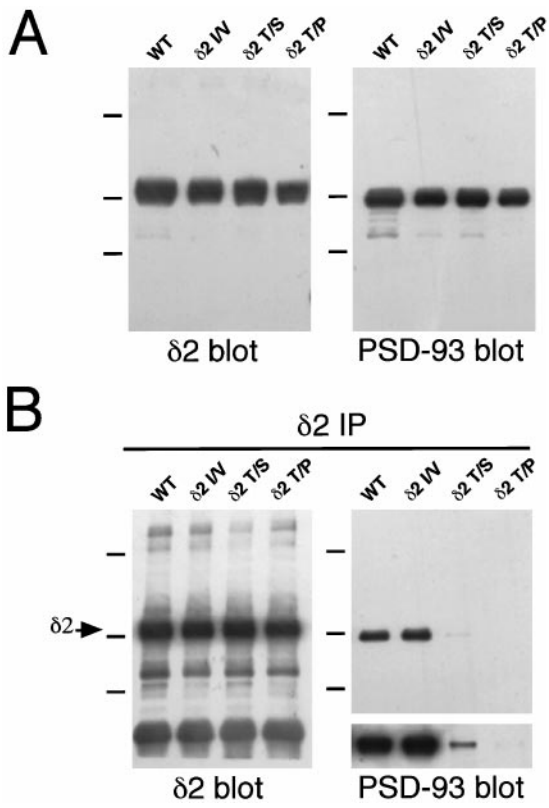

Figure 4. PSD-93 specifically associates with δ2 in heterologous cells. HEK-293 cells were transiently transfected with PSD-93 and δ2 WT, δ2 I 1008 V, δ2 T 1006 S, or δ2 T 1006 P. Cells were solubilized in 1% Triton X-100, and δ2 was immunoprecipitated with δ2 polyclonal antibodies. *A*, Total protein homogenate was resolved by SDS-PAGE and then immunoblotted with δ2 (*left*) or PSD-93 (*right*) antibodies. *B*, δ2 immunoprecipitated samples were resolved by SDS-PAGE and immunoblotted with either δ2 (*left*) or PSD-93 (*right*) antibodies. Overexposure of the PSD-93 blot is included as an *inset* of the *right panel* to illustrate the small amount of PSD-93 that coimmunoprecipitates with the δ2 T 1006 S point mutant. Prestained molecular weight markers are indicated with *bars* corresponding to myosin, phosphorylase B, and bovine serum albumin (M_r of 210, 103, and 71 kDa, respectively). In *B*, the band corresponding to δ2 is indicated with an *arrow*. The additional background bands result from identical antibodies being used for both the immunoprecipitation and immunoblot.

dithiobis(succinimidylpropionate) (DSP) (Pierce) following a modification of a previously described method (Wenthold et al., 1992). DSP, which has a spacer arm length of 12 Å, cross-links primary amines and contains a disulfide bond that can be cleaved by reducing agents. Frozen cerebella were homogenized with a Polytron in 50 mM HEPES, pH 7.5, and centrifuged at 100,000 × *g* for 30 min. The membrane fraction was resuspended in 50 mM HEPES, pH 7.5, and centrifuged again. The pellet was resuspended by sonication in 50 mM HEPES, pH 7.5, at a protein concentration estimated at 6 mg/ml. DSP was prepared in DMSO at 20 mg/ml. It was added to the cerebellar membranes to final concentrations of 0, 200, and 2000 μM, and the samples were incubated with mixing for 30 min at 4°C. Glycine was then added to a final concentration of 150 mM, and the membranes were diluted with 50 mM Tris-HCl, pH 7.5, and centrifuged at 100,000 × *g* for 20 min. The pellet was resuspended in 50 mM Tris HCl, pH 7.5, SDS was added to a final concentration of 1% w/v, and the samples were incubated at 37°C for 30 min. Triton X-100 was added to a final concentration of 2% w/v, and the samples were centrifuged at 100,000 × *g* for 20 min. The supernatants were used for immunoprecipitation. Antibodies to δ 1/2 (10 μg) or PSD-93/95 (2.5 μl) were attached to protein A agarose (25 μl of packed resin), and the cross-linked supernatants were incubated for 2 hr at 4°C. After washing the resin with 50 mM Tris-HCl containing 0.1% Triton X-100, the bound protein was extracted from the resin by boiling in sample buffer containing 5% β-mercaptoethanol for 3 min.

EM analysis. The postembedding immunogold method has been described previously (Wang et al., 1998) and is modified from the method of Matsubara et al. (1996). Briefly, a male Sprague Dawley rat was perfused with 4% paraformaldehyde plus 0.5% glutaraldehyde in 0.1 M phosphate buffer. Two hundred micrometer parasagittal sections of the rostral cerebellum (folia III–V) were cryoprotected in 30% glycerol and frozen in liquid propane in a Leica (Vienna, Austria) EM CPC. Frozen sections were immersed in 1.5% uranyl acetate in methanol at −90°C in a Leica AFS freeze-substitution instrument, infiltrated with Lowicryl HM 20 resin at −45°C, and polymerized with UV light. Thin sections were incubated in 0.1% sodium borohydride plus 50 mM glycine in Tris-buffered saline–0.1% Triton X-100 (TBST), followed by 10% NGS in TBST, primary antibody in 1% NGS–TBST, 10 nm immunogold (Goldmark Biologicals, Phillipsburg, NJ) in 1% NGS–TBST plus 0.5% polyethylene glycol, and finally staining in uranyl acetate and lead citrate. For double labeling, two primary antibodies were combined and two immunogolds were combined (10 nm goat anti-guinea pig and 30 nm goat anti-rabbit; Goldmark Biologicals). Primary antibodies were used at dilutions of 1:100 for PSD-93 and 1:50 for δ 1/2 (rabbit polyclonal).

RESULTS

To identify proteins that interact with δ2 receptors, we screened a yeast two-hybrid rat brain cDNA library (Clontech) with the C-terminal cytosolic domain of δ2 (amino acids 852–1008). We screened ~1.94 million clones and identified five clones that activated transcription of the HIS 3 and β-galactosidase reporter genes. Of these five positives, one was SAP-97 (amino acids 130–662) (Müller et al., 1995), and four encoded a novel protein. The last six amino acids of δ2 are DRGTSI (single letter amino acid code), a sequence that is similar to the well characterized T/SxV motif contained in NMDA receptor subunits and Shaker K^+ channels (Fig. 1) known to interact with the PSD-95 family of proteins. This suggested that δ2 might also interact with members of the PSD-95 family of proteins. Using the yeast two-hybrid system, we found that truncation of the terminal six amino acids of δ2 disrupts the interaction of δ2 and SAP-97 (data not shown). We also determined that δ2 interacts with PSD-93 and PSD-95 (Figs. 2*A*, 3*A*), in addition to SAP-97. δ2 is specifically localized to cerebellar Purkinje cells (Araki et al., 1993; Mayat et al., 1995; Zhao et al., 1997), which express little or no SAP-97 or PSD-95 (Kim et al., 1996). In contrast, PSD-93 is expressed in Purkinje cells (Brenman et al., 1996b; Kim et al., 1996), making it an excellent candidate to interact with δ2 *in vivo*. Therefore, we used PSD-93 in most of the experiments to characterize the interaction with δ2.

Using the yeast two-hybrid system, we analyzed the sequence determinants of δ2 necessary for interaction with PSD-93 and determined whether δ1 interacts with PSD-93 as well. The C termini of both δ1 and δ2 interact with PSD-93, and truncation of the last six amino acids of δ2 disrupts the interaction (Fig. 2*A*). The final amino acid and the amino acid at the −2 position have been shown to be important for binding to MAGUKs (Sheng and Kim, 1996; Ziff, 1997; Craven and Bredt, 1998). Figure 2*B* is a schematic of δ2, indicating the last six amino acids containing the conserved motif and the critical amino acids in the 0 and −2 positions. Mutation of threonine (T) 1006 to proline (P) or mutation of isoleucine (I) 1008 to alanine (A) disrupts the interaction with PSD-93 (Fig. 2*A*). In contrast, changing the terminal isoleucine (1008) to valine (V) has no effect on binding. Interestingly, the mutation of threonine 1006 to serine (S), a conservative substitution, completely disrupts the interaction with PSD-93. Thus, the sequence determinants for the δ2 interaction with PSD-93 differ from those of Shaker K^+ channel interactions with PSD-95 and PSD-93 (Kim et al., 1995; Kim and Sheng, 1996).

We also characterized the specificity of the δ2 interaction with

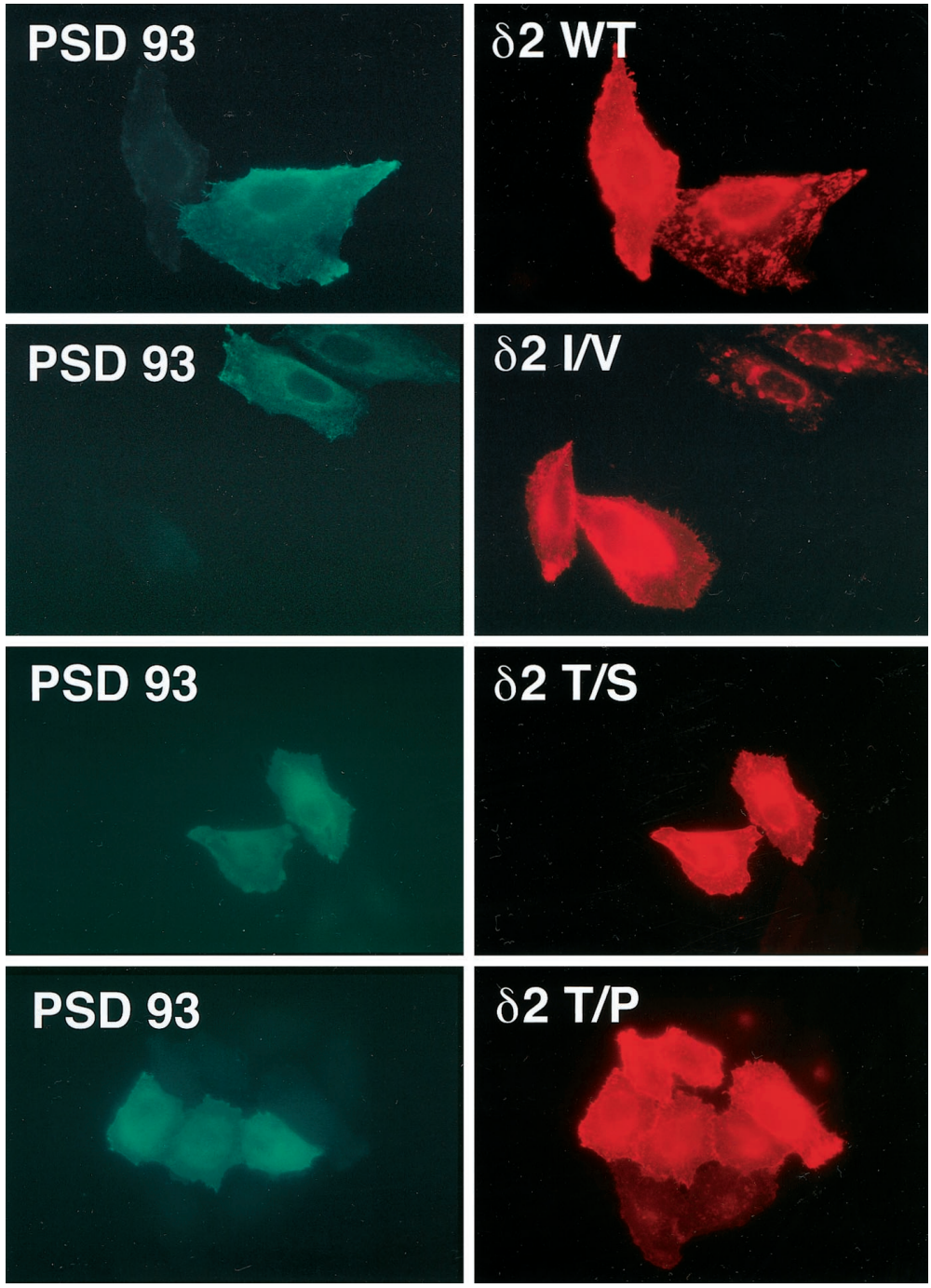

Figure 5. Clustering of δ2 after coexpression with PSD-93. HeLa cells were transiently transfected with PSD-93 and δ2 WT, δ2 I 1008 V, δ2 T 1006 S, or δ2 T 1006 P. Cells were fixed, permeabilized, and incubated with PSD-93/95 monoclonal antibodies and δ2 polyclonal antibodies. Immunoreactivity was visualized using a combination of FITC-conjugated anti-mouse secondary antibodies and rhodamine-conjugated anti-rabbit secondary antibodies. All micrographs were taken using a 63× objective.

various combinations of the three PDZ domains of PSD-95 (Fig. 3*A*), which are highly conserved between members of the PSD-95 family of proteins (Fig. 3*B*). δ2 displayed a robust interaction with PDZ 1–3 or PDZ 1–2, whereas it interacted to a lesser extent with PDZ 2–3, and the binding was diminished further when coexpressed with PDZ 2 alone. δ2 did not interact with PDZ 1 or PDZ 3 alone. Thus, δ2 interacted with PDZ 2 and not PDZ 1 or PDZ 3, yet strongly preferred PDZ 1 and 2 together. This is similar to the binding preferences described for the interaction of certain K^+ channels with PSD-95 (Kim et al., 1995).

We next asked whether full-length δ2 interacts with PSD-93 when the two proteins are coexpressed in heterologous cells. Using a coimmunoprecipitation assay, we found that the two proteins bound robustly when coexpressed in HEK-293 cells (Fig. 4). As detected by yeast two-hybrid, we found that δ2 containing a conservative I 1008 V mutation retained binding to PSD-93, whereas mutating the T 1006 P disrupted binding to PSD-93. The conservative T 1006 S mutation almost completely disrupted the binding of δ2 and PSD-93, as was the case using the yeast two-hybrid assay, although there was some residual binding above background (Fig. 4, *inset*).

The PSD-95 family of proteins has been reported to mediate clustering of receptors at synapses, as well as in heterologous cells (Kim et al., 1995, 1996; Kim and Sheng, 1996; Tejedor et al., 1997). To determine whether PSD-93 clusters δ2, we coexpressed PSD-93 and δ2 in HeLa cells and analyzed the distribution of the

proteins using immunofluorescence. Coexpression of PSD-93 with δ2 resulted in a patchy redistribution of δ2 in HeLa cells, which contrasts dramatically with the diffuse homogenous distribution of δ2 seen in cells expressing δ2 alone (Fig. 5). To confirm the specificity of the clustering, we also cotransfected PSD-93 with several δ2 mutants. We found that δ2 I 1008 V also clustered when coexpressed with PSD-93. In contrast, when either δ2 T 1006 S or δ2 T 1006 P were coexpressed with PSD-93, they maintained the diffuse distribution observed when δ2 was expressed alone. We could not analyze the clustering of the δ2 I 1008 A mutant because this mutation eliminated recognition of the protein by the δ1/2 antibodies, which were raised against the extreme C terminus. Thus, the clustering of δ2 in heterologous cells is dependent on a direct interaction of δ2 with PSD-93. Accordingly, δ2 mutations that disrupt the clustering activity of PSD-93 are mutations that disrupt the direct interaction of δ2 with PSD-93 in the yeast two-hybrid assay (Fig. 2*A*) and in the coimmunoprecipitation assay (Fig. 4).

We performed coimmunoprecipitation experiments from adult rat cerebellum to determine whether endogenous δ2 and PSD-93 interact in brain. We found that PSD-93 does coimmunoprecipitate with δ2 from cerebellum (Fig. 6) and vice versa (data not shown). Interestingly, robust binding of the two proteins is dependent on the detergent, which is used to solubilize the membranes. Although Triton X-100 solubilizes a large amount of PSD-93 and δ2 from cerebellar extracts and it has been shown previously that δ2 can be immunoprecipitated after Triton X-100 solubilization (Mayat et al., 1995), the two do not coimmunoprecipitate, whereas δ2 and PSD-93 coimmunoprecipitate well using DOC-solubilized tissue. This is in sharp contrast to the efficient δ2 and PSD-93 coimmunoprecipitation using Triton X-100 when the two proteins are coexpressed in heterologous cells (Fig. 4). Failure of δ2 and PSD-93 to coimmunoprecipitate from Triton X-100-solubilized cerebellar extracts may indicate that these proteins only interact at postsynaptic densities in brain, which are not soluble in Triton X-100 (Cho et al., 1992).

To address the possibility that the coimmunoprecipitation of δ2 and PSD-93 is caused by an interaction between the two proteins that forms after detergent solubilization rather than indicating an interaction that is present under normal conditions, we cross-linked cerebellar membranes before detergent solubilization using the covalent cross-linker DSP. After cross-linking, the membranes were solubilized with SDS, neutralized with Triton X-100, and immunoprecipitated with antibodies to δ2 and PSD-93. As shown in Figure 7, δ2 coimmunoprecipitates with PSD-93 after cross-linking with 200 μM DSP. Coimmunoprecipitation is not seen in the uncross-linked sample or when cross-linking is done with 2000 μM DSP. At the higher concentration of cross-linker, essentially all of the PSD-93/95 is insoluble, as determined by analyzing the SDS-soluble fraction after cross-linking. A significant amount of the δ2 remains soluble, and this may reflect the cytoplasmic pool of receptor, which is commonly observed for glutamate receptors. GluR2/3 is not cross-linked to PSD-93/95 under these conditions. δ2 was less effective in coimmunoprecipitating PSD-93, although a small amount of coimmunoprecipitation was seen after cross-linking with 200 μM DSP (data not shown). We attribute this to the fact that the δ2 antibody is made to the C terminus, the same region involved in the interaction with PSD-93, and the epitope may not be readily available after cross-linking. However, a significant amount of δ2 was immunoprecipitated, reflecting an uncross-linked pool, indicating that the cross-linking did not affect the C terminus of δ2 in a nonspecific way.

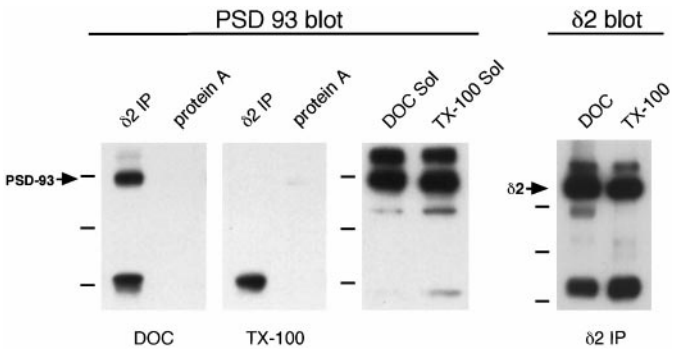

Figure 6. PSD-93 and δ2 interact in rat brain cerebellum. Frozen rat cerebella were solubilized in 1% DOC or 1% Triton X-100, and δ2 was immunoprecipitated using δ2 antibodies. Total soluble protein and immunoprecipitates were resolved by SDS-PAGE and probed with either PSD-93 or δ2 antibodies. Prestained molecular weight markers are indicated with *bars* corresponding to phosphorylase B, bovine serum albumin, and ovalbumin (M_r of 103, 71, and 46 kDa, respectively).

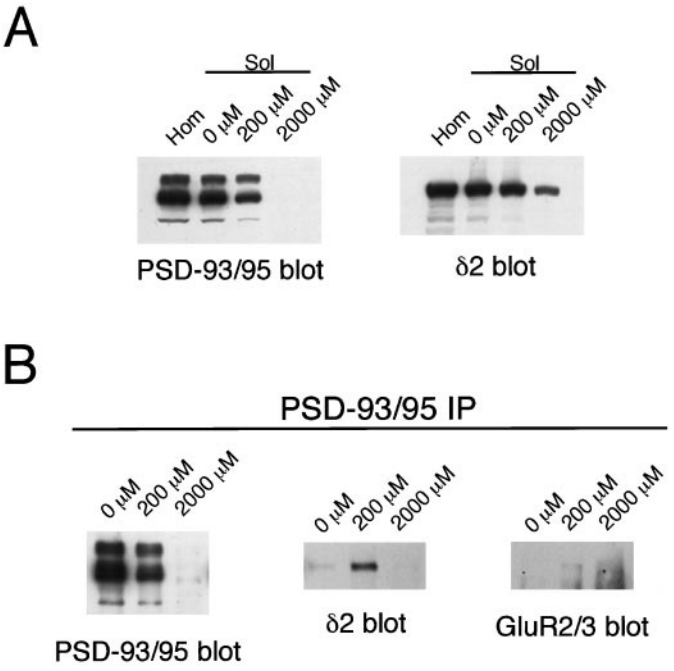

Figure 7. Chemical cross-linking of PSD-93 and δ2 in rat brain cerebellum. Cerebellar membranes were cross-linked with DSP following a modification of a previously described method (Wenthold et al., 1992; see Materials and Methods). DSP (0, 200, or 2000 μM) was used as indicated. After cross-linking, the membranes were solubilized in SDS, followed by Triton X-100, and in total homogenates or soluble fractions (*A*), resolved by SDS-PAGE, and immunoblotted with the antibodies indicated. *B*, PSD 93/95 was immunoprecipitated from the soluble fraction, resolved by SDS-PAGE, and immunoblotted with the antibodies indicated. The GluR2/3 immunoblot was overexposed compared with the PSD-93/95 and δ2 blot to reveal even low levels of cross-linking.

Finally, we characterized the subcellular localization of PSD-93 and δ2 using immunoelectron microscopy. In sections of the molecular layer of the cerebellum, immunogold labeling with a PSD-93 antibody was associated most commonly with the postsynaptic membrane in Purkinje cell parallel and climbing

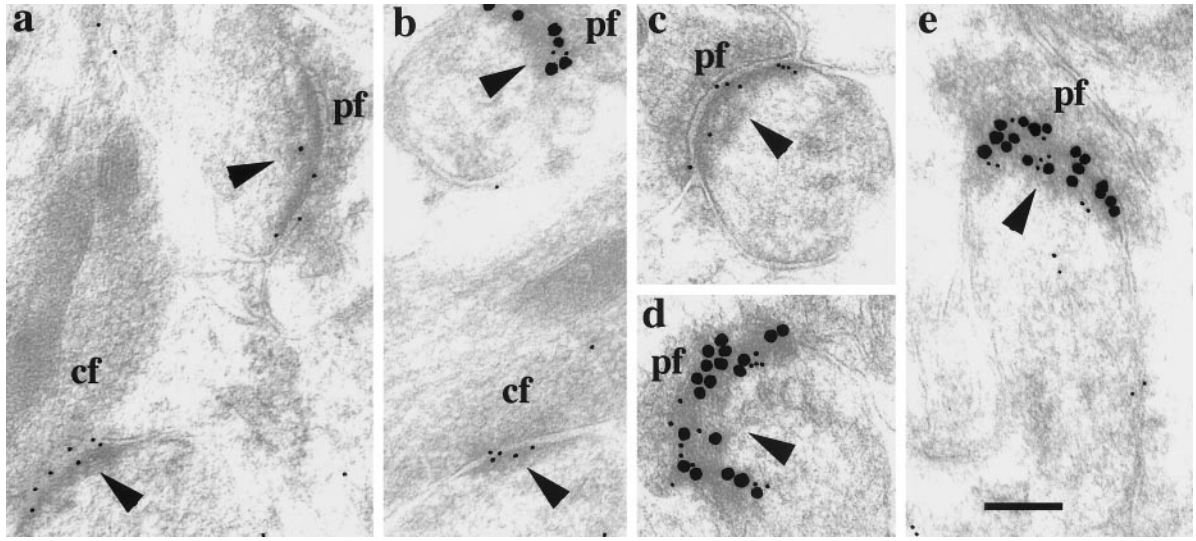

Figure 8. Colocalization of PSD-93 and δ2 at parallel fiber synapses. Sections were single-labeled with PSD-93 antibody (from guinea pig; 10 nm gold) in *a* and *c* and were double-labeled with PSD-93 antibody (10 nm gold) and δ2 1/2 antibody (30 nm gold) in *b*, *d*, and *e*. PSD-93 antibody labels both parallel (*pf*) and climbing (*cf*) fiber synapses (*arrowheads*), whereas δ antibody labels only parallel fiber synapses. Scale bar, 0.2 μm.

fiber synapses (Fig. 8). In sections labeled with both PSD-93 and δ 1/2 antibodies, parallel fiber synapses showed labeling for both antibodies interspersed along the postsynaptic membrane. In contrast, climbing fiber synapses usually showed labeling only for PSD-93. Labeling for PSD-93 in climbing fiber and parallel fiber synapses was confirmed using preembedding immunoperoxidase with the same antibody and using post-embedding immunogold with a second PSD-93 antibody, which was made to a different region of the protein (data not shown).

DISCUSSION

Recent work has demonstrated that several PDZ domain-containing proteins interact with the C termini of glutamate receptors. In addition to the PSD-95 family of proteins that bind to NMDA receptors, AMPA receptors have been shown to interact with glutamate receptor-interacting protein (GRIP) (Dong et al., 1997) and AMPA-binding protein (ABP) (Srivastava et al., 1998), which have multiple PDZ domains. Thus, it has been proposed that different subclasses of glutamate receptors interact specifically with distinct PDZ domain-containing proteins. So far, the only exception to this specificity is a recent report in which the AMPA receptor subunit GluR1 was shown to interact with SAP-97 (Leonard et al., 1998). This finding is unique in that GluR1 specifically interacted with SAP-97 and not the other members of the PSD-95 family.

In the present study, we have demonstrated that the PSD-95 family of proteins interacts with δ receptors, in addition to NMDA receptors. We have specifically shown that δ2 interacts with PSD-93 using the yeast two-hybrid system and that the two proteins coimmunoprecipitate when coexpressed in heterologous cells. We confirmed that this interaction also occurs *in vivo* by coimmunoprecipitating PSD-93 and δ2 from adult rat cerebella and by cross-linking analysis. The interaction of these two proteins depends on the final six amino acids of δ2 (DRGTSI; single letter amino acid code), which generally conform to the T/SxV motif found at the C termini of both Shaker K^+ channels and NMDAR2 subunits. The δ receptors have a terminal isoleucine instead of the terminal valine described for Shaker K^+ channels and NMDA receptors. Thus, they share the T/SxI motif with the inwardly rectifying K^+ channel Kir 2.3, which also binds to the PSD-95 family of proteins (Cohen et al., 1996) (Fig. 1). The δ2 interaction with PSD-93 is strongly dependent on T at the −2 position, which differs from NMDA Shaker K^+ channels which prefer either T or S in this position. This suggests that the surrounding amino acids play a greater role in the interaction than previously recognized. Recently, a similar observation was made by Niethammer et al. (1998).

We also demonstrated that PSD-93 induces the clustering of δ2 in heterologous cells. Thus, PSD-93 can redistribute surface δ2 receptors, just as other members of the PSD-95 family of proteins can cluster NMDA receptors and Shaker K^+ channels. Because we also demonstrated that PSD-93 binds to δ2 in the cerebellum and that these two proteins colocalize at parallel fiber synapses on Purkinje cells, it is likely that PSD-93 plays a role in localization of δ2 at synapses. In addition to the proposed role in the docking of δ2 receptors, it is also possible that PSD-93 links δ2 to multiprotein complexes involved in intracellular signaling via interactions with proteins such as neuronal nitric oxide synthase (Brenman et al., 1996a) and synGAP (Chen et al., 1998; Kim et al., 1998).

Although the expression of δ2 is highest in Purkinje neurons, δ1 and/or δ2 are expressed elsewhere in the brain (Lomeli et al., 1993; Mayat et al., 1995; Petralia et al., 1996). Because both δ and NMDA receptors bind to the PSD-95 family of proteins, it is possible that the two receptors compete for the same synaptic anchors. Interestingly, the developmental profile of functional NMDA receptors in Purkinje cells would support this idea. Functional NMDA receptors are observed only on young Purkinje cells (Crepel and Krupa, 1990; Rosenmund et al., 1992), although expression of both NR1 and NR2 subunits is seen in adult and developing animals (Akazawa et al., 1994). The dramatic increase in δ2 expression beginning at postnatal day 10 approximately coincides with the decrease in functional NMDA receptors. If this is the case, then animals lacking δ2 should express functional NMDA receptors as adults. A competition for synaptic anchors may provide yet another mechanism for regulating the expression of functional receptors.

Although there is growing evidence that the PSD-95 family of proteins is involved in anchoring receptors at synapses, there is little insight into whether or not the presence of the anchor alone is sufficient to determine the expression of the receptor. It has been shown that synaptic clusters of NMDA receptors on cultured hippocampal neurons are always associated with, and preceeded by, clusters of PSD-95 (Rao et al., 1998). If PSD-95 expression mediates clustering of receptors at specific synapses, then all synapses that contain the appropriate anchor would also contain the receptor. In the Purkinje cell, we find that both climbing fiber and parallel fiber synapses express PSD-93, but only parallel fiber synapses express δ2 receptors. This suggests that additional factors play a role in determining the synapse-specific expression of glutamate receptors. One possibility is that receptors are selectively targeted to a synapse and the anchoring proteins dock the receptors in a nonselective manner. In this case, a separate mechanism is required for selectively guiding intracellular organelles with certain cargo to appropriate synapses. A second possibility is that the receptor–anchor interaction is actively regulated. If this is true, then receptors may be targeted to multiple synapses in a single neuron but only selectively retained at the proper sites. In this case, a number of other proteins may be involved in determining both the number of anchors occupied and the nature of the receptors that occupy them. Either scenario is consistent with the fact that PSD-93 and other family members bind to proteins as divergent as NMDA, δ, and AMPA receptors, as well as K^+ channels, which are often expressed in the same neurons.

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