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PSD-93 MEDIATES TYROSINE-PHOSPHORYLATION OF THE *N*-METHYL-D-ASPARTATE RECEPTORS

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Abstract

Src family protein kinases (SFKs)-mediated tyrosine-phosphorylation regulates *N*-methyl-D-aspartate (NMDA) receptor synaptic function. Some members of the membrane-associated guanylate kinase (MAGUK) family of proteins bind to both SFKs and NMDA receptors, but it is unclear whether the MAGUK family of proteins is required for SFKs-mediated tyrosine-phosphorylation of the NMDA receptors. Here, we showed by co-immunoprecipitation that PSD-93, a member of the MAGUK family of proteins, interacts with the NMDA receptor subunits NR2A and NR2B as well as with Fyn, a member of the SFKs, in mouse cerebral cortex. Using a biochemical fractionation approach to isolate subcellular compartments revealed that the expression of Fyn, but not of other members of the SFKs (Lyn, Src, and Yes), was significantly decreased in synaptosomal membrane fractions derived from the cerebral cortex of PSD-93 knockout mice. Interestingly, we found that PSD-93 disruption causes reduction of tyrosine-phosphorylated NR2A and NR2B in the same fraction. Moreover, PSD-93 deletion markedly blocked the SFKs-mediated increase in tyrosine-phosphorylated NR2A and NR2B through the protein kinase C pathway after induction with 4β-PMA in cultured cortical neurons. Our findings indicate that PSD-93 appears to mediate tyrosine-phosphorylation of the NMDA receptors and synaptic localization of Fyn.

Keywords

Src family tyrosine kinases; Fyn; MAGUK; PSD-93; NR2A; NR2B

The *N*-methyl-D-aspartate (NMDA) receptor, a subtype of ionotropic glutamate receptors, contributes to many physiologic and pathologic processes in the central nervous system, including development, neuroplasticity, excitotoxicity, and chronic pain (Choi, 1995; Collingridge and Bliss, 1995; Dougherty and Willis, 1991; Hewitt, 2000; McDonald and Johnston, 1990). Functional NMDA receptors are heteromultimers mainly consisting of NR1 and NR2 (NR2A–NR2D) subunits (Wenthold et al., 2003). The NR2 subunits have long cytoplasmic carboxy-terminal domains that contain sites for phosphorylation and interaction with cytoplasmic proteins. These C-terminal tails of NR2 are essential for maintaining synaptic NMDA receptor functions, as the deletion or site-directed mutagenesis of these residues

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reduces surface expression of NR2 and impairs NMDA receptor-mediated synaptic activity (Lavezzari et al., 2003; Mori et al., 1998; Roche et al., 2001; Sprengel et al., 1998).

PSD-93 is one of a growing superfamily of PDZ (PSD-95, DLG, ZO-1)-domain-containing proteins recently shown to physically link proteins together into macromolecular structures. PSD-93 has structural similarity with three other PDZ-domain-containing proteins, PSD-95, SAP102, and SAP97 (Brenman et al., 1996). These proteins are generically referred to as membrane-associated guanylate kinases (MAGUKs) and contain three tandem PDZ domains (PDZ1–3) at the N-terminal side, a Src homology region 3 (SH3) domain in the middle, and a guanylate kinase-like (GK) domain at the C-terminal end. Via these multiple domains, PSD-95 and PSD-93 bind to a number of membrane and cytosolic proteins. Previous studies have revealed that the first two PDZ domains of PSD-93, PSD-95, and SAP102 specifically bind to the C-termini of the NR2A or NR2B subunits of the NMDA receptors (Brenman et al., 1996; Kim et al., 1996; Kornau et al., 1995; Muller et al., 1996). This PDZ domain-mediated interaction might be critical for NMDA receptor synaptic function, as PSD-93 deletion leads to reduced NMDA receptor-mediated excitatory postsynaptic potentials or currents in central neurons (Tao et al., 2003). However, the underlying mechanisms by which the MAGUK family of proteins regulate synaptic NMDA receptor function are still unclear.

It is known that NMDA receptor function is upregulated by tyrosine phosphorylation (Lu et al., 1999; Salter and Kalia, 2004; Wang and Salter, 1994). The C-terminal tails of NR2A and NR2B subunits also can be tyrosine phosphorylated by members of the Src family of protein tyrosine kinases (SFKs) (Ali and Salter, 2001; Lau and Huganir, 1995; Takasu et al., 2002). Five members of the SFKs, including Src, Fyn, Yes, Lck, and Lyn, are expressed in the mammalian central nervous system, and Src, Fyn, Yes and Lyn are included in the NMDA receptor complex (Kalia and Salter, 2003; Salter, 1998; Yu et al., 1997). Upregulation of the NMDA receptor via phosphorylation by Src has been shown to be required for the induction of NMDA receptor-dependent long-term potentiation (LTP) (Lu et al., 1998). In addition, Fyn deletion blunted NMDA receptor-related LTP in hippocampal CA1 neurons (Grant et al., 1992). These findings suggest that SFK proteins promote post-synaptic NMDA receptor function through tyrosine phosphorylation of NR2A and NR2B.

To elucidate the role of PSD-93 in the regulation of NMDA receptor function, we used the PSD-93 knockout (KO) mouse. We found that PSD-93 binds to both Fyn and NMDA receptor subunits in the cerebral cortex. PSD-93 deletion led to mislocalization of Fyn from the synaptosomal membrane, resulting in the reduction of tyrosine phosphorylation of NR2A and NR2B in the cerebral cortical neurons. PSD-93 might be critical for maintaining the synaptic NMDA receptor complex and be required for tyrosine phosphorylation of NR2A and NR2B.

EXPERIMENTAL PROCEDURES

Animals

Male C57BL/6J wild-type (WT) and PSD-93 KO mice kindly provided by Dr. David S. Bredt (Eli Lilly & Co, Greenfield, IN) weighing 25–30 g were housed up to four per cage on a standard 12-h light/dark cycle, with water and food pellets available *ad libitum*. Animal experiments were carried out with the approval of the Animal Care and Use Committee at Johns Hopkins University and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Cell culture

Cerebral cortices from 12- or 14-day embryonic WT and PSD-93 KO mice were removed and digested for 15 min at 37°C in PBS supplemented with 0.15 unit/mL papain, 2 mM cysteine, 3 mM glucose, and 0.5 mg/mL BSA. After addition of heat-inactivated horse serum, cells were gently dissociated by trituration and centrifuged at 300×g for 3 min. The cells were resuspended in DMEM supplemented with 5% (vol/vol) fetal bovine serum, 5% (vol/vol) horse serum, 1 mM pyruvate, 50 U/mL penicillin, and 25 µg/mL streptomycin, filtered twice through 70-µm and 40-µm filters, and centrifuged 600×g for 10 min. Cells were then resuspended in supplemented DMEM (as described above) and plated at a density of 5×10^5 cells/well in 6-well dishes coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO). Cell cultures were fed every 4 days with Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B-27 and glutamine. Cultures were maintained in a 37°C humidified incubator with a 5% CO₂ atmosphere and used at 10 days in vitro to analyze tyrosine phosphorylation of NR2B.

Cell treatment and protein extraction

Neuronal cultures were washed three times with HEPES controlled salt solution [HCSS: 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 15 mM glucose, and 20 mM HEPES (pH 7.4)]. To examine the effect of the protein kinase C (PKC) signaling pathway, cultured neuronal cells were treated with the PKC activator 4β-phorbol 12-myristate 13-acetate (4β-PMA; ALEXIS Biochemicals Co., San Diego, CA) with the following methods. The cultures were incubated for 20 min at 37°C in 0.1% (vol/vol) DMSO (vehicle) or 0.5 µM SFK inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*]pyrimidine (PP2; Calbiochem, San Diego, CA), and then exposed to 0.1 µM 4β-PMA for 20 min. Treatments were stopped by removing reaction buffer. The cells were washed with HCSS twice and lysed by addition of 50 µL of SDS buffer [1% (wt/vol) SDS, 2 mM sodium orthovanadate, 10 µg/mL leupeptin, 10 µg/mL aprotinin, and 1 mM PMSF]. After the resulting extracts were heated at 90°C for 5 min, 9 vol of dilution buffer [1% (vol/vol) NP40, 1% (wt/vol) CHAPS, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/mL leupeptin, 10 µg/mL aprotinin, and 20 µg/mL pepstatin A] was added, and the samples were centrifuged at 25,000×g for 30 min; supernatants were collected.

Extraction of crude plasma membrane fraction

Mouse cerebral cortex was lysed in homogenizing buffer [10 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 250 mM sucrose, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF, 10 µg/mL leupeptin, 10 µg/mL aprotinin, and 20 µg/mL pepstatin A] with a rotor-stator Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenized solution was centrifuged at 900×g for 15 min. The supernatant was centrifuged again at 37,000×g for 40 min. The precipitate was solubilized with resuspension buffer [500 mM Tris-HCl, 1% (vol/vol) TritonX-100, and 1% (wt/vol) sodium deoxycholate] and centrifuged at 37,000×g for 20 min to precipitate insoluble contents. The supernatant was used as crude plasma membrane fraction.

Co-immunoprecipitation

Rabbit polyclonal PSD-93 antibody (5 µg; Zymed, San Francisco, CA) or rabbit polyclonal Fyn antibody (4 µg; Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with 20 µL of a 1:1 slurry of protein G plus/protein A agarose suspension (Calbiochem) for 2 h. Normal rabbit serum (5 µg; Jackson ImmunoResearch, West Grove, PA) was used as control. Crude plasma membrane fraction (300 µg) extracted from cerebral cortex of WT or PSD-93 KO mice then was added to the antibody-immobilized protein G plus/protein A agarose, and the mixture was incubated for 2–3 hr at 4°C. The agarose beads were washed once with 1% (vol/vol) Triton X-100 in immunoprecipitation buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4

mM KH_2PO_4 , 5 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM NaF, 1 mM PMSF, 10 $\mu\text{g}/\text{mL}$ leupeptin, 10 $\mu\text{g}/\text{mL}$ aprotinin, and 20 $\mu\text{g}/\text{mL}$ pepstatin A), twice with 1% (vol/vol) Triton X-100 in immunoprecipitation buffer plus 300 mM NaCl, and three times with immunoprecipitation buffer. Binding proteins were eluted with SDS-PAGE sample buffer at 95°C. The proteins were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose membrane and detected by rabbit polyclonal anti-PSD-93, mouse monoclonal anti-Fyn (Sigma-Aldrich), rabbit polyclonal anti-NR2A, rabbit polyclonal anti-NR2B, mouse monoclonal anti-PSD-95 (Sigma-Aldrich), mouse monoclonal anti-SAP97 (Stressgen Bioreagents, Victoria, BC, Canada), and mouse monoclonal anti-SAP102 (Calbiochem) antibodies. Thirty micrograms of the cerebral cortex crude plasma membrane fraction were loaded as a positive control (input).

Subcellular fractionation of mouse cerebral cortex

Biochemical fractionation was performed as described previously (Dunah and Standaert, 2001; Lin et al., 1998). Briefly, mouse cerebral cortex homogenate was centrifuged at 1,000×g for 20 min in ice-cold TEVP buffer [10 mM Tris-HCl (pH 7.4), 5 mM NaF, 1 mM sodium orthovanadate, 1 mM EDTA, and 1 mM EGTA] containing 320 mM sucrose to remove cell soma, nuclei, and nuclei-associated membrane (pelleted in P1). The supernatant was collected and centrifuged at 10,000×g for 30 min to produce a pellet (crude synaptosomal fraction). The supernatant was subsequently centrifuged at 160,000×g for 1 h to pellet a light membrane fraction (P3). The crude synaptosomal fraction was lysed hypo-osmotically in water and centrifuged at 25,000×g to pellet a synaptosomal membrane fraction (LP1). The supernatant was centrifuged at 160,000×g for 1 h to pellet a crude synaptic vesicle-enriched fraction (LP2). Twenty micrograms of each fraction were loaded onto 4–20% gradient polyacrylamide gels, separated by SDS-PAGE, and electrophoretically transferred onto nitrocellulose membrane. Immunostaining was carried out with rabbit polyclonal anti-NR2A, rabbit polyclonal anti-NR2B, mouse monoclonal anti-PSD-95, rabbit polyclonal anti-c-Src (Santa Cruz Biotechnology), rabbit polyclonal anti-Fyn (Santa Cruz Biotechnology), rabbit polyclonal anti-Yes (Upstate Biotechnology, Lake Placid, NY), rabbit monoclonal anti-Lyn (Santa Cruz Biotechnology), mouse monoclonal anti-synaptophysin (Sigma-Aldrich), and mouse monoclonal anti- β -actin (Sigma-Aldrich) antibodies.

Detection of phosphotyrosine proteins

For immunoprecipitation, proteins from the LP1 fraction were solubilized under weakly denaturing conditions in resuspension buffer [1% (wt/vol) sodium deoxycholate and 50 mM Tris-HCl (pH 9.0)] at 37°C for 30 min and then diluted with an equal volume of modified RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS, 1 mM sodium orthovanadate, 1 mM PMSF, 10 $\mu\text{g}/\text{mL}$ leupeptin, 10 $\mu\text{g}/\text{mL}$ aprotinin, and 20 $\mu\text{g}/\text{mL}$ pepstatin A]. Protein G plus/protein A agarose-immobilized rabbit polyclonal NR2A antibody (4 μg) or rabbit polyclonal NR2B antibody (3 μg) was incubated with 150 μg of solubilized LP1 fraction or cortical neuronal cell lysate at 4°C for 4 h. Protein G plus/protein A agarose was washed with modified RIPA buffer or dilution buffer three times. Binding proteins were eluted with SDS-PAGE sample buffer at 95°C, loaded onto polyacrylamide gels, and separated by SDS-PAGE. Tyrosine-phosphorylated proteins were detected by phosphotyrosine-specific antibody, clone 4G10 (Upstate Biotechnology).

RESULTS

PSD-93 and Fyn form a complex with NMDA receptors in cerebral cortical neurons

To examine whether PSD-93 interacts with Fyn and NMDA receptors, we used anti-PSD-93 antibody to co-immunoprecipitate proteins from the crude membrane fraction extracted from WT and PSD-93 KO mouse cerebral cortex. As shown in Figure 1A, anti-PSD-93 antibody

immunoprecipitated not only PSD-93 itself but also Fyn, NR2A, and NR2B. As expected, the PSD-93 antibody did not precipitate PSD-93, NR2A, NR2B, or Fyn from the PSD-93 KO mouse extract. To further demonstrate that NR2A/2B, PSD-93, and Fyn form a complex in the cerebral cortex, co-immunoprecipitation was repeated with anti-Fyn antibody. Fyn, PSD-93, NR2A, and NR2B each were pulled down by anti-Fyn antibody in crude membrane extract from WT mice (Fig. 1B), but predictably, the antibody failed to immunoprecipitate PSD-93 from PSD-93 KO cortical extract. NR2A and NR2B were faintly detected in the crude membrane fraction derived from PSD-93 KO mice. These results indicate that Fyn might participate in the NMDA receptor complex through PSD-93, although it might also bind to PSD-95.

SAP97 and SAP102 also might have the potential to associate with Fyn because structural components of PSD-95, PSD-93, SAP97, and SAP102 are almost identical (Hunt et al., 1996; Kim et al., 1996; Muller et al., 1996). Unexpectedly, anti-Fyn antibody did not immunoprecipitate SAP97 or SAP102 in the crude membrane fraction derived from either WT or PSD-93 KO mice (Fig. 2). While the probability that Fyn interacts with SAP97 and SAP102 with lower affinity could not be excluded, Fyn was not shown to interact with SAP97 and SAP102 with the co-immunoprecipitation technique employed.

PSD-93 deletion decreases Fyn in synaptosomal membrane fraction

To examine the effect of PSD-93 deletion on subcellular localization of Fyn in neurons, we extracted four fractions from cerebral cortex of WT and PSD-93 KO mice: cell soma, nuclei and nuclei-associated membrane (P1), light membrane (P3), synaptosomal membrane (LP1) containing pre- and post-synaptic proteins, and a fraction enriched in synaptic vesicles (LP2). PSD-95, NR2A, NR2B, and synaptophysin were detected in each fraction, verifying the specificity of our fractionation procedure, as the subcellular distribution of these proteins has been previously determined (Dunah and Standaert, 2001). Of the SFKs (Fyn, Lck, Lyn, Src, and Yes), all but Lck have been shown to be components of the NMDA receptor complex (Kalia and Salter, 2003). Therefore, we went on to examine the distribution of Fyn, Lyn, Src and Yes. We found that Fyn was highly enriched in the LP1 fraction. Src and Yes were expressed at high levels in the P3, LP1, and LP2 fractions, whereas Lyn was distributed mainly in the P3 fraction (Fig. 3A). These results indicate that Fyn distribution is characteristic in the SFKs. Importantly, Fyn expression in the LP1 fraction from PSD-93 KO mice was lower than that from WT mice. Quantification of the immunoblot density of six-times repeated experiments showed that the amount of Fyn in the LP1 fraction was reduced to $66.9 \pm 4.2\%$ that of WT in the absence of PSD-93. However, no significant change in the amount of Fyn was observed in the total homogenate fraction of the PSD-93 KO mice. The amounts of Lyn, Src, and Yes were not altered by PSD-93 disruption in either the LP1 or total homogenate fraction (Fig. 3A, B). Our results imply that disruption of PSD-93 might alter the subcellular distribution of Fyn, but not other SFK proteins, in the cerebral cortex. It is possible that PSD-93 might be critical for Fyn to anchor at the synapse.

PSD-93 deletion reduces tyrosine-phosphorylation of NR2 subunits

To determine the role of PSD-93 in tyrosine-phosphorylation of NR2 subunits, we compared the amount of tyrosine-phosphorylated NR2A and NR2B in WT and PSD-93 KO mice using an anti-phosphotyrosine antibody. NR2A and NR2B protein were immunoprecipitated from solubilized LP1 fraction of mouse cerebral cortex, and the ratio of tyrosine-phosphorylated NMDA receptor subunit to total NMDA receptor protein was quantified. As shown in Figure 4A, tyrosine-phosphorylated NR2A was significantly reduced by PSD-93 disruption. The ratio of tyrosine-phosphorylated protein in PSD-93 KO mouse to WT was 0.74 ± 0.05 . Tyrosine-phosphorylated NR2B also was significantly decreased in the LP1 fraction extracted from

PSD-93 KO mice (0.51 ± 0.03) compared to that from WT mice. These results indicate that PSD-93 contributes substantially to tyrosine phosphorylation of NR2A and NR2B subunits.

To further test the hypothesis that PSD-93 knockout influences the tyrosine-phosphorylation of NR2B in biological conditions, we stimulated cerebral cortical neuronal cells with the endogenous PKC activator 4 β -PMA. Signal transduction through PKC is the most well-characterized signaling pathway upstream of SFK-mediated NMDA receptor regulation (Lu et al., 1999). Treatment of cerebral cortical neuronal cells with vehicle (DMSO) did not alter the tyrosine-phosphorylation level of NR2B, but 4 β -PMA caused a 2.60 ± 0.56 fold increase in tyrosine-phosphorylated NR2B in the WT neurons (Fig. 5). Pre-incubation with the SFKs inhibitor PP2 completely blocked the increase in tyrosine-phosphorylated NR2B. This result suggests that PKC activation by 4 β -PMA induced the tyrosine-phosphorylation of NR2B through SFKs. Treatment of neurons from PSD-93 KO mice with 4 β -PMA did not increase tyrosine phosphorylation of NR2B as it had in WT neurons (Fig. 5). These results indicate that PSD-93 appears to mediate the tyrosine phosphorylation of NR2B in the signaling pathway that includes PKC and SFKs.

DISCUSSION

In this study, we found that PSD-93 significantly contributes to SFKs-mediated tyrosine phosphorylation of NR2 subunits. We also provided evidence suggesting the possibility that PSD-93 mediates that tyrosine phosphorylation through the formation of a complex with Fyn, NR2A, and NR2B. This finding supports the idea that the PSD-95 family of MAGUK proteins regulates tyrosine phosphorylation of the NMDA receptors (Chen et al., 2003; Hou et al., 2002; Kalia et al., 2006; Tezuka et al., 1999; Yamada et al., 2002).

It has been reported that the MAGUK family of proteins facilitates coupling between NMDA receptors and downstream signaling molecules. For example, the NMDA receptors are coupled to neuronal nitric oxide synthase by PSD-95 or PSD-93. It has been shown that PSD-95 knockdown or disruption of the interaction between NMDA receptors and PSD-95 at cortical synapses blocks Ca²⁺-activated nitric oxide production by NMDA stimulation (Sattler et al., 1999). The present study showed that tyrosine-phosphorylation of NR2A/B was altered by PSD-93 disruption (Fig. 4). The co-immunoprecipitation analysis indicated that PSD-93 interacts with Fyn and NR2A/B (Fig. 1). Additionally, the distribution of Fyn was greatly reduced by PSD-93 KO (Fig. 3). PSD-93 might couple the SFK Fyn to the NMDA receptor complex in cerebral cortex neurons.

Biochemical fractionation indicated that Fyn is uniquely distributed among subcellular fractions and highly expressed in the LP1 fraction (Fig. 3). More importantly, PSD-93 deletion reduced the amount of Fyn, but not the other SFK proteins, in LP1. This phenomenon could be related to its binding with PSD-93 because Src, Lyn, and Yes have been shown not to associate physically with PSD-93 (Kalia and Salter, 2003). Together, these results indicate that PSD-93 is important for regulating, targeting, or localizing Fyn.

Using co-immunoprecipitation with the Fyn antibody, we showed that Fyn interacts with both PSD-95 and PSD-93 (Fig. 2). However, the other SFK proteins interact only with PSD-95 (Kalia and Salter, 2003). This distinction in binding between PSD-95 and PSD-93 might depend on structural differences and post-translational modification. For example, both PSD-93 and PSD-95 are palmitoylated N-terminally, but unlike PSD-95, palmitoylation is not necessary for the PSD-93 postsynaptic targeting (Firestein et al., 2000). Also, PSD-93, but not PSD-95, is tyrosine phosphorylated by Fyn at the insertion sequence between PDZ2 and PDZ3 unique to PSD-93 (Nada et al., 2003). Interestingly, although distribution of PSD-95 was not altered by PSD-93 disruption, PSD-95 was not sufficient to maintain Fyn localization in the

synaptosomal membrane. Despite the structure of PSD-95 being nearly identical to that of PSD-93, PSD-95 did not compensate for the lack of PSD-93.

In this study, we revealed that the presence of PSD-93 regulates the tyrosine phosphorylation of NMDA receptors. The co-immunoprecipitation study (Fig. 1) together with the biochemical fractionation study (Fig. 3) provide strong evidence that Fyn is involved in this PSD-93 mediated tyrosine-phosphorylation pathway. It is widely accepted that PSD-95 plays a role in the tyrosine phosphorylation of NMDA receptors. PSD-95 is reported to interact with SFKs and regulate them diversely. For example, the interaction between the PDZ3 domain of PSD-95 and the SH2 domain of Fyn causes an increase in NR2A tyrosine phosphorylation (Tezuka et al., 1999). Also, the unique sequence at the N-terminus of PSD-95 interacts with the SH2 domain of Src, resulting in the inhibition of Src catalytic activity (Kalia et al., 2006). Whereas PSD-95 is reported to interact with five major SFKs expressed in central nervous system, PSD-93 is able to interact with only Fyn. Although the domain of PSD-93 that interacts with Fyn has not been identified, it is likely that the PDZ3 domain of PSD-93 associates with the SH2 domain of Fyn in a manner similar to that of PSD-95 because the function of Fyn was not prevented by the interaction with PSD-93.

Tyrosine phosphorylation of NMDA receptors has been induced by a variety of triggers, including insulin (Christie et al., 1999), LTP induction (Rostas et al., 1996), peripheral noxious inflammatory and nerve injury insult (Guo et al., 2002; Abe et al., 2005), and ischemia (Besshoh et al., 2005; Cheung et al., 2003; Takagi et al., 1997). However, it remains unclear how the phosphorylation of tyrosine residues in the C-terminal tail of NR2 subunits changes NMDA receptor channel function. One potential role of Fyn-mediated tyrosine phosphorylation of NR2A and NR2B is the trafficking of the receptor to the cell surface. Besshoh *et al.* (Besshoh et al., 2005) recently reported that ischemic challenge promoted an increase in NR2A and NR2B tyrosine phosphorylation, which was positively correlated with an increase in the number of these receptors in the post-synaptic density. In accordance with this finding, Goebel *et al.* (Goebel et al., 2005) established that a tyrosine-phosphatase inhibitor increases NR2A and NR2B tyrosine phosphorylation and membrane surface expression of NMDA receptors in the hippocampus. Moreover, activation of dopamine D₁ receptors enhances phosphorylation of NR2B at Y1472, a Fyn phosphorylation site (Nakazawa et al., 2001), and leads to trafficking of NMDA receptors to the surface membrane in striatal neurons (Dunah et al., 2004; Hallett et al., 2006). Together with our previous data showing that targeted disruption of PSD-93 reduces surface expression of NR2A and NR2B expression (Tao et al., 2003), our current data strongly support these reports that tyrosine phosphorylation is critical for regulation of NR2A and NR2B receptor trafficking.

Several studies have shown that tyrosine phosphorylation is required to recruit downstream signaling proteins. For example, the SH2 domain of signaling proteins binds to a phosphotyrosine-containing sequence (Moran et al., 1990), and tyrosine phosphorylation of NR2A and NR2B was shown to increase the binding affinity for the SH2 domain of phospholipase C- γ (Gurd and Bissoon, 1997). In another example, tyrosine phosphorylation of NR2B by Fyn was found to be necessary for NR2B to interact with the p85 regulatory subunit of phosphatidylinositol 3-kinase (Hisatsune et al., 1999). In addition to this interaction with the SH2 domain, the binding of spectrin and of PSD-95 to NR2A or NR2B was greatly enhanced after these NMDA receptor subunits were first phosphorylated by Fyn or Src (Rong et al., 2001). Tyrosine phosphorylation of NR2A and NR2B expedites the binding of calpaine and promotes the degradation of NMDA receptors by calpaine (Bi et al., 2000; Rong et al., 2001). In support of a role for tyrosine phosphorylation in NMDA receptor trafficking, phosphorylation at the Y1472 site on NR2B prevents the interaction of NR2B with clathrin adaptor protein AP-2 and suppresses the internalization of NMDA receptors (Prybylowski et al., 2005). Alternatively, tyrosine phosphorylation of NMDA receptors might block binding

of other proteins and thereby prevent the assembly of functional signaling proteins such as kinases and phosphatases. Therefore, a full understanding of the role of NMDA receptor tyrosine phosphorylation by Src or Fyn will require further investigation.

Consistent with our previous findings that PSD-93 deletion influences NMDA receptor-mediated postsynaptic response in the spinal cord dorsal horn and forebrain of adult mice (Tao et al., 2003), we showed here that the phosphorylation state of NR2A and NR2B was altered by knockout of PSD-93. Our present study supports the idea that tyrosine-phosphorylation by Fyn is essential for NMDA receptor function and that PSD-93 has the potential to regulate this phosphorylation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

4β-PMA	4β-phorbol 12-myristate 13-acetate
BSA	bovine serum albumin
DMEM	dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
GK	guanylate kinase
HCSS	HEPES controlled salt solution
IP	immunoprecipitation
KO	knockout
LTP	long-term potentiation
MAGUK	membrane-associated guanylate kinase
NMDA	<i>N</i> -methyl-D-aspartate
NP40	nonidet p-40
PDZ	PSD-95/DLG/ZO-1
PKC	protein kinase C
PMSF	

phenylmethylsulfonyl fluoride

PP2

4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*]pyrimidine

PSD

post-synaptic density

SDS

sodium dodecyl sulfate

SDS-PAGE

sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SFKs

Src family protein kinases

SH3

Src homology region 3

SH2

Src homology region 2

WT

wild-type

Fig. 1.

NMDA receptors, PSD-93, and Fyn form a complex in the cerebral cortex. Solubilized crude plasma membrane fraction extracted from wild-type (WT) and PSD-93 knockout (KO) mouse cerebral cortex was immunoprecipitated with anti-PSD-93 antibody (A) or anti-Fyn antibody (B). Immunoprecipitated proteins (300 μ g) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-NR2A, -NR2B, -PSD-93, or -Fyn antibodies. Normal rabbit serum (NRS) was used as a negative control. In the lane marked Input, 30 μ g of protein from the solubilized crude plasma membrane fraction were loaded.

Fig. 2.

The interaction between Fyn and MAGUK family proteins. (A) Wild-type (WT) and PSD-93 knockout (KO) mouse cerebral cortex crude plasma membrane proteins (300 μ g) were immunoprecipitated with anti-Fyn antibody, separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-PSD-95, -SAP97, -SAP102, or -Fyn antibodies. Normal rabbit serum (NRS) was used as a control. In the lane marked Input, 30 μ g of protein from the solubilized crude plasma membrane fraction were loaded.

Fig. 3.

Deletion of PSD-93 decreases Fyn in the synaptosomal membrane fraction. (A) The cerebral cortex from wild-type (WT) and PSD-93 knockout (KO) mice was homogenized immediately after dissection, separated into different biochemical fractions as described in Experimental Procedures, and resolved by SDS-PAGE. H, total homogenate; P1, cell soma, nuclei, and nuclei-associated membrane fraction; P3, light membrane fraction; LP1, synaptosomal membrane fraction; LP2, synaptic vesicle-enriched fraction. (B) Fyn, Lyn, Src, and Yes protein density in the synaptosomal membrane fraction of PSD-93 KO mice was quantified as a percent of WT, which was normalized to 100%. Values represent the mean $\pm$ SEM for Fyn ($66.9 \pm 4.2\%$; $n=6$), Lyn ($100.3 \pm 11.7\%$; $n=6$), Src ($106.2 \pm 6.4\%$; $n=6$), and Yes ($102.2 \pm 7.8\%$; $n=6$); $**P<0.005$ by paired t test.

Fig. 4.

Disruption of PSD-93 reduces tyrosine-phosphorylated NR2B. Protein immunoprecipitated by anti-NR2A antibody (A) and anti-NR2B antibody (B) from synaptosomal membrane fraction (150 μ g) extracted from wild-type and PSD-93 knockout (KO) mice was separated by SDS-PAGE and probed with anti-phosphotyrosine antibody, 4G10 (pTyr). Values were calculated as phosphorylation per protein unit, and the ratio of tyrosine-phosphorylated NR2A or NR2B in PSD-93 KO to those in WT, respectively, is depicted in the histograms. Values represent the mean $\pm$ SEM for NR2A pTyr (0.74 ± 0.05 ; $n=5$), and NR2B pTyr (0.51 ± 0.03 ; $n=5$).

* $P < 0.05$ by paired t test.

Fig. 5.

Lack of PSD-93 affects the phosphorylation state of NR2B in cultured cerebral cortical neurons. To analyze the tyrosine phosphorylation of NR2B, cerebral cortical neurons were pretreated for 20 min in the absence or presence of the Src-family inhibitor PP2 (1 μ M), and then exposed to 0.1 μ M 4 β -PMA for 20 min. After protein extraction in 1% SDS, NR2B was immunoprecipitated with specific antibodies followed by detection with anti-phosphotyrosine antibody, 4G10 (pTyr). The immunoreactive bands were quantified, and values were calculated as phosphorylation per protein unit, and the ratio of tyrosine-phosphorylated NR2B in untreated cells (control) to those in the cells treated with DMSO, 4 β -PMA and PP2/4 β -PMA, respectively, is depicted in the histograms. Values represent the mean $\pm$ SEM for NR2B pTyr in DMSO-treated WT cells (vehicle, 1.13 ± 0.47 ; $n=3$), 4 β -PMA-treated WT cells (2.60 ± 0.56 ; $n=3$), and PP2-pretreated WT cells (0.72 ± 0.13 ; $n=3$), DMSO-treated PSD-93 KO cells (vehicle, 0.78 ± 0.12 ; $n=3$) and 4 β -PMA-treated PSD-93 KO cells (0.66 ± 0.13 ; $n=3$). * $P<0.05$ by paired t test compared with control cells.