Identification of PSD-93 as a Substrate for the Src Family Tyrosine Kinase Fyn*

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In order to study the role of tyrosine kinase signaling in the post-synaptic density (PSD), tyrosine-phosphorylated proteins associated with the PSD-95/NMDA receptor complex were analyzed. The NMDA receptor complex from the mouse brain was successfully solubilized with deoxycholate and immunoprecipitated with anti-PSD-95 or anti-phosphotyrosine antibody. Immunoblot analyses revealed that the predominantly tyrosine-phosphorylated proteins in the PSD were the NR2A/B subunits and a novel 120 kDa protein. Purification and microsequence analysis showed that the 120 kDa protein is mouse PSD-93/Chapsyn-110. Recombinant PSD-93 was phosphorylated by Fyn in vitro, and Tyr-384 was identified as a major phosphorylation site. Tyrosine phosphorylation of PSD-93 was greatly reduced in brain tissue from Fyn-deficient mice compared with wild-type mice. Furthermore, an N-terminal palmitoylation signal of PSD-93 was found to be essential for its anchoring to the membrane, where Fyn is also localized. In COS7 cells, exogenously expressed PSD-93 was phosphorylated, dependent on its membrane localization. In addition, tyrosine-phosphorylated PSD-93 was able to bind to Csk, a negative regulator of Src family kinases, in vitro as well as in a brain lysate. These results suggest that PSD-93 serves as a membrane-anchored substrate of Fyn and plays a role in the regulation of Fyn-mediated modification of NMDA receptor function.

The post-synaptic density (PSD) is an accumulation of opaque material on the cytoplasmic face of the post-synaptic membrane. The density represents the aggregation of neurotransmitter receptors and signaling proteins, which trigger neuronal excitation of the postsynaptic cells. Among the biochemical functions present in the PSD is tyrosine kinase activity, and it is known that neurotransmitter receptors, such as subunits of NMDA receptors, AMPA receptors, and GABA receptors, are phosphorylated on tyrosine. Electrophysiological studies have shown that some tyrosine kinases can modulate the activities of post-synaptic ion channels (1–3), and tyrosine kinase signaling is required for synaptic plasticity (4). Among the NMDA receptors, the NR2A and NR2B subunits have been shown to be tyrosine-phosphorylated. Candidates that may be responsible for this activity include the Src family tyrosine kinases (SFKs), because Src is co-precipitated with the NMDA receptor subunits (3), and the association of the NMDA receptors with PSD-95 could cause promote tyrosine phosphorylation by Fyn (5). Furthermore, Fyn knockout studies have indicated that this kinase is involved in the acquisition of long-term potentiation in the hippocampal CA1 neuron, and in spatial learning (6, 7). Thus, SFKs are believed to play important roles in the regulation of post-synaptic ion channels, although the molecular mechanisms remain unclear.

NMDA receptors are known to form a complex with the PSD protein, PSD-95 and its related proteins. PSD-95 is a member of the membrane-associated guanylate kinase family of proteins (MAGUKs), which have been found to be major structural components of the PSD. Several domains within PSD-95, including three PDZ domains, an SH3 domain, and a guanylate kinase-like domain, have been shown to interact with various membrane proteins and signaling molecules, including the NMDA receptors (8), potassium channels (9), SynGAP (10), DAP/SAPAP/GKAP family (11–13), and SPAR/SPAL (14, 15). In order to identify molecules involved in tyrosine kinase signaling and regulation of NMDA receptors, we here investigated tyrosine-phosphorylated proteins associated with the PSD-95/NMDA receptor complex. The PSD is described as a tightly packed protein complex, and its solubilization under stringent conditions often disrupts protein-protein interactions. However, previous studies showed that solubilization of PSD with deoxycholate did not disrupt the association of PSD-95 with the NMDA receptors (16, 17). Using this procedure we investigated tyrosine-phosphorylated proteins that co-immunoprecipitated with the PSD-95/NMDA receptor complex, and found a novel 120 kDa tyrosine phosphoprotein. The 120 kDa protein was subsequently identified as PSD-93/Chapsyn-110 (18, 19), which is a member of the MAGUK family of proteins. Analysis of Fyn-catalyzed phosphorylation sites revealed that PSD-93 is phosphorylated at a site(s) present in an inserted sequence unique to PSD-93. It was also found that N-terminal cysteine residues of PSD-93 are essential for its membrane localization and phosphorylation. Furthermore, the tyrosine-phosphorylated PSD-93 was found to interact with Csk, a regulator kinase for SFKs, in vitro and in vivo. These findings suggest that PSD-93 serves as a membrane-anchored substrate for Fyn and...
plays a role in the regulation of Fyn-mediated modification of NMDA receptor function.

EXPERIMENTAL PROCEDURES

Materials—The expression vector, pEGFP-N1, pGEX-4T3, and pGEX-2T were obtained from Clontech, Amersham Biosciences, and Agene, respectively. Glutathione-Sepharose and protein G-Sepharose were obtained from Amersham Biosciences. Ni-NTA-agarose was from Qiagen. LipofectAMINE2000 was from Invitrogen. Biotinylated cholera toxin B subunit and Cy3-streptavidine were from Sigma. Recombinant human Fyn tyrosine kinase (amino acids 11–537; M14333) were expressed in Sf9 cells and purified by sequential column chromatography. Fyn antibodies were a gift from B. Novelli (20). The cdna-encoding PSD-93 (U53368) was a gift from Y. Hata.

Antibodies—Rabbit polyclonal antibodies against PSD-95 and PSD-93 were raised against GST fusion proteins containing the PDZ2 domain of PSD-93 to PSD23 regions of PSD-95 (GST-PSD-95-PDZ2123) and PSD-93, respectively. Anti-PSD-93 antisera was passed through a glutathione-Sepharose column to which GST-PSD-95-PDZ2123 was attached to absorb cross-reactive antibodies. Anti-phosphotyrosine antibodies, PY20 and 4G10 were obtained from ICN and Upstate Biotechnology, respectively. Anti-NR2A and anti-NR2B antibodies were from Amersham Biosciences. Anti-phosphotyrosine antibodies, PY20 and 4G10 were used at 1:2,000, or 1:5,000 dilution, respectively. Anti-Fyn antibody was from Upstate Biotechnology. Anti-NR2A and 4G10 were obtained from ICN and Upstate Biotechnology, respectively. Glutathione-Sepharose and protein G-Sepharose were obtained from Amersham Biosciences. Ni-NTA-agarose was from Qiagen. LipofectAMINE2000 was from Invitrogen. Biotinylated cholera toxin B subunit and Cy3-streptavidine were from Sigma. Recombinant human Fyn tyrosine kinase (amino acids 11–537; M14333) were expressed in Sf9 cells and purified by sequential column chromatography. Fyn antibodies were a gift from B. Novelli (20). The cdna-encoding PSD-93 (U53368) was a gift from Y. Hata.

Immunoprecipitation—In Vitro Kinase Assays—Immunoprecipitates of PSD-95 or NR1 were washed once with kinase assay buffer (50 mM PIPES, pH 7.0, 10 mM MgCl2, 0.1 mM Na3VO4) and used as substrates. Recombinant proteins or immunoprecipitates were incubated with 10 ng of purified Fyn in 20 μl of kinase assay buffer with 50 μM cold ATP or 1 μCi (γ32P)ATP for 10 min at 30 °C. Kinase reactions were stopped by adding SDS-sample buffer, and the samples were applied to SDS-PAGE for detection of tyrosine phosphorylation by 4G10 immunoblot or autoradiography. In Fig. 5B, immunoprecipitated Fyn from the brain TNE lysate was used as a kinase source. After the kinase reaction, the reaction mixture was briefly centrifuged and supernatants containing substrate were recovered. Phosphorylated substrates were separated on SDS-PAGE, and immunoblotted with 4G10 to visualize tyrosine phosphorylation in only the substrate proteins.

Treatment of Immunoprecipitates with Endo F or BAP—For Endo (endo glycosidase) F treatment, immunoprecipitates were boiled for 1 min in 20 μl of Endo F digestion buffer (0.25 mM sodium acetate, pH 6.1, 20 mM EDTA, 10 mM 2-mercaptoethanol, 0.1% SDS), and after cooling 1 μl of 20% Nonidet P-40 was added. Digestion was done with 3 units of Endo F (Roche Applied Science) for 2 h at 37 °C. BAP (bacterial alkaline phosphatase) treatment was achieved by incubating immunoprecipitate with 1 unit of BAP (Takara) in 20 μl of BAP buffer (50 mM Tris-HCl, pH 8.0, 1 mM MgCl2) for 2 h at 37 °C.

Preparative Protein Microsequencing—After large scale immunoprecipitation by PY20, precipitated proteins were eluted with SDS buffer. An equal volume of acetone was added to eluate, and proteins were precipitated. The pellet was dissolved in SDS sample buffer and applied to SDS-PAGE, followed by semidry transfer to PVDF membrane. Protein bands were visualized by Ponceau S staining, cut out, and incubated with lysi-endopeptidase at 37 °C overnight. Peptides were recovered from the PVDF membrane and individual peptides were purified by reverse-phase HPLC (C-18). Isolated peptides were further subjected to gas phase sequencing.

Preparation of Recombinant PSD-93—For production of full-length PSD-93 protein in Escherichia coli, the whole open reading frame (ORF) of PSD-93 was PCR-amplified with the primers 5′-ctcctagttgctcatagttgctcagttgctc-3′ (SpfI) and 5′-ccctagttgacttcttttggc-3′ (XhoI), and the amplified fragment was cloned into the SpfI and XhoI sites of pQE30, an N-terminal histidine-tagged expression vector. A full-length PSD-93 expression vector was also constructed from pQE30 in the same way. For fusion proteins with glutathione S-transferase (GST), BamHI and EcoRI-tagged primer sets were designed for the individual regions indicated in Fig. 6C or in Fig. 10, and PCR-amplified fragments were cloned into the corresponding sites of the pGEX-4T3 or pGEX-6P1 vector. These vectors were introduced into the E. coli TG1 strain, and expression of recombinant proteins was induced with isopropyl-β-D-thiogalactopyranoside. His- or GST-tagged proteins were expressed as soluble forms, and were purified with Ni-NTA-agarose or glutathione-Sepharose, respectively, by standard procedures. Mutagenesis was achieved by nested PCR procedure. For example, mutation of Tyr-348 to phenylalanine was accomplished using a mutated Y348F reverse primer, 5′-cagtttattcacagtgctgaaaaca-3′, and a forward primer, 5′-agcctagttgacttcttttggc-3′. PCR products amplified by the SphI and XhoI primers. The resultant PCR product containing the Y348F reverse primer and the XhoI and F348 forward primers were double co-immunoprecipitated from wild-type and PSD-93 mutant mouse forebrains were prepared as described (17) in a buffer containing 1% (w/v) deoxycholate. The first co-immunoprecipitation was done by adding 150 μg of a sheep anti-NR1 antibody to a 25-ml extract, incubation at 4 °C for 4 h, followed by an addition of a 50% slurry of 300 μl of protein G-Sepharose and a further incubation at 4 °C for 8 h. Unbound material was removed by washing the resin in 50 ml of cold extraction buffer, and bound proteins were released from the resin by adding 300 μl of SDS-buffer containing 4% SDS, 50 mM Tris, pH 9.0, and boiling the whole sample for 30 min at 95 °C. The supernatant was then removed and 10-μl aliquots were diluted with 0.7 ml of Nonidet P-40 buffer containing 1% Nonidet P-40, 50 mM Tris, and 0.1 mM NaCl. These samples were then used for a second immunoprecipitation using an antibody against either phosphotyrosine (2 μl) (anti-PY20, from Upstate Biotechnology), anti-PSD-93 (4 μl) (APZ-002), anti-NR2A (1 μl) or anti-NR2B (1 μl). After an incubation for 3 h at 4 °C, 30 μl of a 50% slurry of protein G-Sepharose was added and incubated for a further 8 h at 4 °C. Unbound material was removed, and the resin was washed four times with 0.5 ml cold Nonidet P-40 buffer each, and bound material was released from the resin by boiling it in 30 μl of SDS buffer for 5 min at 95 °C.

In Vitro Kinase Assays—Immunoprecipitates of PSD-95 or NR1 were washed once with kinase assay buffer (50 mM PIPES, pH 7.0, 10 mM MgCl2, 0.1 mM Na3VO4) and used as substrates. Recombinant proteins or immunoprecipitates were incubated with 10 ng of purified Fyn in 20 μl of kinase assay buffer with 50 μM cold ATP or 1 μCi (γ32P)ATP for 10 min at 30 °C. Kinase reactions were stopped by adding SDS-sample buffer, and the samples were applied to SDS-PAGE for detection of tyrosine phosphorylation by 4G10 immunoblot or autoradiography. In Fig. 5B, immunoprecipitated Fyn from the brain TNE lysate was used as a kinase source. After the kinase reaction, the reaction mixture was briefly centrifuged and supernatants containing substrate were recovered. Phosphorylated substrates were separated on SDS-PAGE, and immunoblotted with 4G10 to visualize tyrosine phosphorylation in only the substrate proteins.
lysate was recovered as the supernatant after centrifugation of and the homogenate was diluted into 3.34 ml Triton buffer. The SDS min at 4 proteins were sequentially extracted as follows. A mouse brain was first recovered as the ODG lysate after centrifugation of 20,000 pellet was again lysed in 4 ml of ODG buffer, and the supernatant was applied to SDS-PAGE. 

were washed four times with TNE buffer, solubilized with SDS sample buffer, and applied to SDS-PAGE. Sequential Extraction of Mouse Forebrain Proteins—Mouse forebrain proteins were sequentially extracted as follows. A mouse brain was first lysed in 8 ml of TNE buffer, and the supernatant was recovered as the TNE lysate after centrifugation of 20,000 g for 20 min at 4 °C. The pellet was once again lysed with ODG buffer, and the supernatant was recovered at each step as the TNE lysate and the DOC lysate. The precipitated PSD fraction by the use of deoxycholate as suggested previously (16, 17). Mouse brain was lysed once with TNE buffer containing 1% Nonidet P-40, and the insoluble fraction containing PSD was recovered by centrifugation. The precipitated PSD fraction was then solubilized with DOC buffer containing 1% deoxycholate. By this differential solubilization procedure, we found that a major portion of PSD-95 was solubilized in DOC buffer from adult mouse brain. We then investigated the expression of PSD-95 and tyrosine-phosphorylated proteins in the PSD during brain development (Fig. 1A). In the newborn mouse brain, PSD-95 was detected only in the TNE fraction (Fig. 1A, stage (w) 0). One week after birth, PSD-95 was detected equally in the TNE and DOC fractions. From 2 weeks on, the majority of PSD-95 was detected in the DOC fraction. More dramatic changes were observed for tyrosine-phosphorylated proteins. Following brain development, the total tyrosine-phosphorylated protein content decreased in the TNE fraction. In contrast, total tyrosine-phosphorylated protein content increased in the DOC fraction. These changes took place within 3 weeks after birth. A tyrosine-phosphorylated protein with a mobility of ~180 kDa, already known to contain NMDA receptor subunit NR2A/B, appeared at the 1 week stage in the DOC fraction and then dramatically increased during the next 2 weeks. In the DOC fraction, tyrosine-phosphorylated SFKs including Fyn were detected as strong 60-kDa bands at the newborn stage, and the signals gradually decreased, indicating that SFKs are associated with the PSD. The band corresponding to SFKs could be detected in the TNE fraction only at early stages.

Next we conducted immunoprecipitation assays using anti-PSD-95 antibody to detect tyrosine-phosphorylated proteins associated with the PSD-95/NMDA receptor complex (Fig. 1B). Two major tyrosine-phosphorylated proteins, pp180 and pp120 were detected in the immunoprecipitates. It is well known that one of the major ligands for the PDZ1 and PDZ2 domains of PSD-95 is the 180-kDa NMDA receptor subunits NR2A/B (8) and that these NR2 molecules are tyrosine-phosphorylated.
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Therefore, the identity of pp180 was likely to be NR2. On the other hand, there was no obvious candidate for the tyrosine-phosphorylated 120 kDa protein.

Characterization of pp120—We first tested whether pp120 was NR1, which is a major component of the NMDA receptor complex and has a molecular size of about 120 kDa. Since NR1 is characterized by glycosylation, we initially examined the glycosylation status of pp120. The pp120 was immunoprecipitated with anti-PD-95 or anti-PY antibody, and the immunoprecipitates were treated with Endo F to remove N-glycosylation moieties. Endo F treatment produced a mobility shift of the apparent molecular size of NR2 and pp120, as indicated by arrowheads. E, Fyn phosphorylates the PSD-95/NMDA receptor complex in vitro. The PSD-95/NMDA receptor complex was immunoprecipitated with either anti-NR1 or anti-PD-95 antibody from the adult brain DOC lysates. Immunoprecipitates were incubated with or without Fyn in kinase assay buffer containing [γ-32P]ATP. Phosphorylation of pp180 and pp120 and autophosphorylation of Fyn were detected by autoradiography.

Next we conducted an in vitro kinase assay using pp120 as the substrate. It is known that SFK Fyn is able to phosphorylate NR2 (23). We therefore tested whether Fyn would phosphorylate pp120 or not. The PSD-95/NMDA receptor complex was immunoprecipitated with either anti-NR1 or anti-PD-95 antibody, and the immunoprecipitates were incubated with Fyn and ATP. As shown in Fig. 2E, both NR2 and pp120 were phosphorylated in a Fyn-dependent manner. Like NR2, this result indicates that pp120 could serve as a substrate for Fyn, at least in vitro.

Identification of pp120 as PSD-93—As shown in Fig. 2, we found that pp120 protein could be effectively recovered by immunoprecipitation with anti-PD-95 antibody. We thus performed a large scale immunopurification of pp120 with anti-PY antibody to obtain sufficient protein for microsequencing, as described in the “Experimental Procedures.” We succeeded in obtaining more than 10 pmol of pp120 (Fig. 3A). A broad band of pp120 was transferred onto a PVDF membrane and processed for peptide microsequencing. We first obtained a partial amino acid sequence of a peptide, ’’DGLRQGDRLLMNVN…’’ that is completely identical to that of mouse PSD-93. We next subjected the other nine peptides to five cycles of sequencing reactions, and these sequences were all found to correspond to sequences in PSD-93 (Fig. 3B). Furthermore, we confirmed that pp120 was immunoprecipitated by anti-PD-93 antibody (Figs. 8 and 10). Taken together, these findings show that pp120 is mouse PSD-93.

Tyrosine Phosphorylation of PSD-93 in Vitro—Tyrosine phosphorylation of the PSD-95 family of MAGUKs has not been reported. Thus, we carefully evaluated tyrosine phosphorylation of PSD-93 using recombinant PSD-93 protein. His-tagged PSD-93 and PSD-95 were produced in E. coli, and subjected to in vitro kinase assays with purified Fyn. As shown in Fig. 4A, PSD-93, but not PSD-95, was tyrosine-phosphorylated in a Fyn-dependent manner. We next tried to locate the phosphorylation sites in PSD-93 using GST fusion proteins containing various regions of PSD-93 as substrates. As shown in Fig. 4, B and C, GST fusion proteins containing a region encompassing amino acids 331–417 were tyrosine-phosphorylated, while a GST fusion protein containing PDZ1–3 of PSD-95 was not. Alignment of the amino acid sequences of the PSD-95 family members reveals that there is an insertion unique to PSD-93 at amino acids 343–417 (Fig. 5A), suggesting that this region might contain the phosphorylation site(s). Of five tyrosine residues within this insertion, two residues, Tyr-348 and Tyr-364, are potential phosphorylation sites, because the sequences Tyr-Ser-Thr-Val- and Tyr-Ser-Pro-Val conform to the SH2 binding motif. Mutants in which either Tyr-348 or Tyr-364 was replaced with Phe (Y348F and Y364F) were generated and sub-
jected to in vitro kinase assay with Fyn (Fig. 5B). Mutant Y364F was significantly phosphorylated, whereas Y348F was not, indicating that Fyn specifically phosphorylates Tyr-348 in vitro.

Identification of Tyrosine-phosphorylated pp180 and pp120 Proteins—We next examined the in vivo phosphorylation of NR1, NR2A, NR2B, PSD-95, and PSD-93 in the NMDA receptor complex using a 2-step immunoprecipitation approach (Fig. 6). NMDA receptor complexes were isolated from mouse brain extracts using anti-NR1 antibodies, then denatured by boiling in 4% SDS and individual proteins then isolated in a second immunoprecipitation using specific antibodies. In the second step immunoprecipitation, the proteins were isolated using anti-phosphotyrosine or specific antibodies and this was followed by immunoblotting with the antibodies mentioned. These experiments showed that pp180 comprised both tyrosine-phosphorylated NR2A and NR2B and pp120 comprised PSD-93. Here we found another minor tyrosine-phosphorylated band at around 95 kDa, and it was also revealed a possibility that pp95 compromised PSD-95. Tyrosine phosphorylation of pp95 was a relatively weak signal and PSD-95 mutant mice (24) were used to confirm the identity of this band as extracts from these mutants are devoid of any signal at 95 kDa. However, PSD-95 was not a good substrate of Fyn at least in vitro (Figs. 2E and 4A). It should be required further observation about pp95. Similar experiments using anti-NR1 in the second step failed to reveal any detectable tyrosine phosphorylation (data not shown).

Membrane Localization of PSD-93 Requires Its N-terminal Region—In order to examine the in vivo interaction between PSD-93 and Fyn, the intracellular localization of these proteins were assessed using GFP fusion proteins expressed in COS7 cells. Fyn was found to be enriched in the plasma membrane, particularly in a membrane microdomain referred to as lipid rafts. This localization required N-terminal myristoylation and palmitoylation. In the N-terminal portion of PSD-95 and PSD-93, there are potential palmitoylated cysteine residues that have been suggested to be necessary for membrane localization and clustering (25, 26) (Fig. 7A). GFP fusion proteins containing the N-terminal region of PSD-93 and its mutant carrying Ala-5 and Ala-7 instead of Cys (PSD-93 NCA) were expressed in COS7 cells, and visualized with a confocal laser-scanning microscope. GFP fusion proteins containing the N-terminal region of Fyn or PSD-95 and wild-type GFP were also expressed as controls. To visualize the membrane domain, biotin-labeled cholera toxin B fragment (CTX) was used as a probe for the membrane-specific marker GM1 ganglioside, and detected with Cy3-streptavidine. As shown in Fig. 7B, GFP fusion proteins containing the N terminus of Fyn, PSD-93 and PSD-95 were all observed at the cell-surface membrane and vesicles. In contrast, mutants of PSD-93 and PSD-95 containing a cysteine to alanine change were distributed in the cytosol similar to wild-type GFP. These GFP fusion proteins were repeatedly and uniformly observed in transfected cells. Thus, tyrosine phosphorylation of PSD-93 by Fyn is not inconsistent from their membrane localizations.

Tyrosine Phosphorylation of PSD-93 in COS7 Cells and in Vivo—In order to evaluate the tyrosine phosphorylation of PSD-93 under physiological conditions, PSD-93 and its mu-
tants were expressed in COS7 cells and their tyrosine phosphorylation levels were compared (Fig. 8A). Wild-type PSD-93 was efficiently phosphorylated in COS7 cells. The Y348F mutant was also phosphorylated, although the phosphorylation of Y348F was apparently lower than that of wild-type PSD-93. In contrast, Y364F was phosphorylated to a similar or lower extent than wild-type PSD-93. Phosphorylation of the Y348F/Y364F double mutant was at an almost equivalent level to that of Y348F. Phosphorylation of the PSD-93 NCA mutant lacking palmitoylation sites was barely detected, implicating the im-

**Fig. 4. In vitro tyrosine phosphorylation of PSD-93 by Fyn.** A, recombinant full-length PSD-95 and PSD-93 were produced and used as substrates for Fyn. PSD-95 or PSD-93 was incubated with or without Fyn. After the kinase reaction, samples were subjected to immunoblot analysis with anti-PY (4G10) antibody. In the PSD-95 lanes, a weak nonspecific band was detected. B, the domain arrangement of PSD-93 is shown. GST fusion proteins with partial PSD-93 sequences at their C termini were constructed. Individual regions are indicated as a bar with amino acid residue numbers at both ends. C, in vitro kinase assay with various regions of PSD-93 (shown in B) as substrates. The PDZ1 to PDZ3 region of PSD-95 fused to GST, or the PSD-93 construct was incubated with immunoprecipitated Fyn immobilized to Sepharose-beads. After the kinase reaction, the substrate fraction was recovered in the supernatant and subjected to anti-PY (4G10) immunoblot (upper) or silver staining (lower). Each substrate protein band is indicated with asterisk. Tyrosine phosphorylation was detected in GST fusion constructs containing amino acids 331–417 of PSD-93 (constructs 1, 4, and 5).
portance of membrane localization of PSD-93 for phosphorylation. These results indicate that Tyr-348 is a major phosphorylation site even in COS7 cells. However, it is possible that PSD-93 could be phosphorylated at other sites as well.

To examine whether PSD-93 is phosphorylated by Fyn in vivo, tyrosine phosphorylation of PSD-93 in a Fyn-deficient mouse was analyzed. The TNE and DOC fractions were prepared from Fyn-mutant and wild-type mouse brains. In both the TNE and DOC fractions, total tyrosine phosphorylation levels were reduced in the Fyn mutant (Fig. 8B). In the DOC fraction, it was obvious that tyrosine phosphorylation of both pp120 and pp180 was decreased in the Fyn mutant. Although the amount of immunoprecipitated PSD-93 was not changed, its tyrosine phosphorylation was greatly reduced in the Fyn mutant (Fig. 8C). Tyrosine phosphorylation was not detected in PSD-93 immunoprecipitates from the TNE fraction for either genotype, suggesting that PSD-93 is phosphorylated only when it is associated with PSD or that only the phosphorylated form of PSD-93 can be integrated into PSD.

Association of Csk with the Phosphorylated Form of PSD-93—We found that there is a striking homology between the phosphorylation site of PSD-93 and of the membrane phosphoprotein Cbp: these sites are VY(348)STVNK and MY(317)SSVNK (27), respectively. In the case of Cbp, phosphorylation of Tyr-317 has been shown to be a key event for the binding of Csk, a kinase that is an essential negative regulator of the SFKs (28). Thus, we asked if there is an interaction between PSD-93 and Csk.

First we tested the in vitro binding of Csk with recombinant PSD-93 by a pull-down assay. GST fusion protein containing the PSD-93-specific insertion (amino acid 343–417; Fig. 9A) was phosphorylated by Fyn and incubated with purified Csk...
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In vitro phosphorylation of NR2A, NR2B, and PSD-93. Samples were prepared by double co-immunoprecipitation as described in the text using either forebrain from wild-type mice (psd-95+/+) or PSD-95 mutant mice (psd-95−/−). Initial co-precipitation was done using a sheep anti-NR1 antibody (lanes 3 and 4), followed by a second co-precipitation of the bound material using an antibody against phosphotyrosine (lanes 5 and 6) or using specific antibodies (lanes 7 and 8) against NR2A (panel A, pp180), NR2B (panel B, pp180), PSD-93 (panel C, pp120), and PSD-95 (panel D, pp95). Unbound material after the two co-immunoprecipitations for each specific protein is shown as a control for efficiency of the second co-precipitation (lane 9, ub). Whole extracts are shown for comparison (lanes 1 and 2). The samples were then separated on a 6% SDS-PAGE gel and Western blotting was done using antibodies against phosphotyrosine (PY), NR2A, NR2B, PSD-93, and PSD-95 as marked on the right of the figure. Representative blot is shown, and the results were reproducibly observed.

After incubation, GST fusion proteins were separated, and the precipitates were subjected to immunoblot analysis with anti-GST or anti-Csk antibodies (Fig. 9B). We clearly observed that Csk was co-precipitated with PSD-93 in a phosphorylation-dependent manner.

We next examined the binding of Csk to PSD-93 expressed in COS7 cells. Exogenously expressed PSD-93 or Y348F were immunoprecipitated and their phosphorylation was examined by anti-PY immunoblotting. Co-precipitation of PSD-93 was detected as the same PY band in anti-Csk immunoprecipitates from the cell lysate containing wild-type PSD-93, but not that containing Y348F, as shown in Fig. 9C.

We further tested the interaction between Csk and PSD-95 in mouse brain lysates. The brain tissues were fractionated into TNE, ODG, and SDS fractions by sequential extraction (see “Experimental Procedures”), and the fractions were either subjected directly to immunoblot analysis or used for immunoprecipitation with anti-Csk or anti-PSD-93 antibodies. As shown in Fig. 10, A and B, tyrosine-phosphorylated proteins were found to be concentrated in the SDS fraction. PSD-93 and NR2B were mainly recovered in SDS fractions, although small amounts of PSD-93 were still detectable in the TNE and ODG fractions. We also observed that PSD-93 in the SDS fraction had a slower mobility and a broad band on SDS-PAGE, indicating a possible difference of phosphorylation status of PSD-93 at not only tyrosine but also serine/threonine residues among these fractions. Fyn and Csk were also detected in the SDS fraction, demonstrating their strong binding to the ODG-insoluble protein complexes. In the anti-Csk immunoprecipitates from the SDS fraction, PSD-93 was readily detected by anti-PSD-93 antibody (Fig. 10C). The pp120 and pp180 bands, corresponding to PSD-93 and NR2A/B, respectively, were also detected in the anti-Csk as well as anti-PSD-93 immunoprecipitates. It is noteworthy that there is another tyrosine-phosphorylated protein of around 80 kDa in the anti-Csk immunoprecipitates from the ODG fraction (Figs. 9C and 10C). In previous studies, we have shown that Csk interacts with the phosphoprotein Cbp present in the detergent-insoluble membrane fraction (27). Thus it is very likely that the 80 kDa protein is Cbp localized in the PSD fraction of mouse brain. These results suggest that Csk is able to bind to tyrosine-phosphorylated PSD-93 in vivo.

DISCUSSION

In this study, we identified PSD-93 as a tyrosine-phosphorylated protein that is tightly associated with the NMDA receptor complex. In vitro and in vivo studies showed that PSD-93 is one of the functional membrane targets of Fyn, and that Fyn phosphorylation of PSD-93 promotes its interaction with Csk, a negative regulator of SFKs. In addition, we found that association of PSD-93 with the PSD is coincident with its tyrosine phosphorylation, and that the phosphorylation of PSD-93 is functionally correlated with phosphorylation of the NMDA receptor subunits NR2A/B during brain development. These findings raise the possibility that PSD-93 play a role(s) in the regulation of Fyn-mediated modulation of PSD function.

In non-neural cells, the SFK member Fyn is highly enriched in lipid rafts, a membrane microdomain considered to serve as starting platform for intracellular signaling. Fyn acts as a critical molecular switch involved in the regulation of various cellular functions, including cell adhesion, immune response, differentiation, and proliferation. The characteristics of PSD, such as its insolvibility to non-ionic detergents and the accumulation of many signaling molecules including the SFKs, led us to hypothesize that the PSD in neuronal cells functions as a raft-like platform for SFK signaling. Indeed, it has already been shown that excitatory postsynaptic proteins NR2A/B are phosphorylated by Fyn. In order to elucidate the role(s) of tyrosine phosphorylation events in the regulation of PSD functions, we first analyzed tyrosine-phosphorylated proteins in the PSD-95/NMDA receptor complex.

To study the PSD-95/NMDA receptor complex, we first needed to efficiently solubilize the complex from the tightly packed PSD complex. For the solubilization of lipid rafts, n-octyl-β-D-glucoside (ODG) is widely used as a detergent. However, this agent was ineffective in solubilizing the PSD-95/NMDA receptor complex. Therefore, in the current study, we used 1% deoxycholate, as described in a previous report (16), and succeeded in efficient immunoprecipitation of the receptor complex with either anti-PSD-95 or anti-PY antibody. We found from in vitro kinase assay that this complex contains at least two major Fyn substrates, pp180 and pp120. It was already known that the NMDA receptor subunits NR2A/B are endogenous substrates for Fyn (23) and interact with PSD-95 (8). Indeed, we confirmed by immunoprecipitation assay that...
pp180 is a mixture of NR2A/B (Fig. 6). As regards pp120, we identified it as mouse PSD-93/Chapsyn-110 by microsequencing and immunoprecipitation assays with anti-PSD-93 antibody. It is quite likely that PSD-93 is present in the PSD-95/NMDA receptor complex, because PSD-93 is a member of the PSD-95 family of MAGUKs and known to interact with the NMDA receptors (19). However, tyrosine phosphorylation of the PSD-95 family MAGUKs in vivo had not been observed. Within the PSD-95 family, PSD-93 is quite unique in that it has an inserted sequence between the PDZ2 and PDZ3 domains. In this study we found that a major tyrosine phosphorylation site, Tyr-348, is located within this inserted sequence unique to PSD-93, suggesting that tyrosine phosphorylation is a specific feature of PSD-93.

In this study we showed that the N-terminal region of PSD-93 is essential for its membrane localization. This membrane localization was dependent on the cysteine residues, Cys-5 and Cys-7, which were shown to be sites for palmitoylation (26). Indeed, palmitoylation was found to be essential for tyrosine phosphorylation of PSD-93 in COS7 cells (Fig. 8A). Sequential solubilization revealed a coincidence between detergent insolubility and tyrosine phosphorylation of PSD-93. Although
PSD-93 was present in both Nonidet P-40-soluble and insoluble fractions, the Nonidet P-40-soluble form was found to be non-phosphorylated. Tyrosine phosphorylated PSD-93 in the NMDA receptor complex was insoluble in ODG buffer, while that in COS7 cells was soluble in ODG buffer. This suggests that there may be some specific partners for tyrosine-phosphorylated PSD-93 in the brain that facilitates its association with PSD. It is also interesting that changes in the tyrosine-phosphorylated PSD-93 during brain development were correlated with the maturation of PSD. The expression of PSD-93 was observed at least from an embryonic day 15 (18), while its phosphorylation became detectable from 2 weeks after birth.
Fig. 1. At which time synaptic formations are actively taking place. It was also found that tyrosine phosphorylation of NR2A/B occurred with the same time course as that of PSD-93 during brain development (Fig. 1B), suggesting that these two phosphorylations are regulated by a common mechanism. These observations implicate tyrosine phosphorylation of PSD-93 as well as NR2A/B in the formation or maintenance of the PSD structure. Alternatively, it is possible that these proteins are tyrosine-phosphorylated only after they are integrated into PSD by another mechanism.

Concerning the physiological aspects of the tyrosine phosphorylation of PSD-93, determination of the phosphorylation site allowed the identification of its binding partner. The amino acid sequence around the major phosphorylation site, Tyr-348, is consistent with the Csk binding sequence found in Cbp (27). Subsequently, we confirmed that Csk is able to bind tyrosine-phosphorylated PSD-93 in vitro and in vivo. This suggests that PSD-93 is a membrane-bound Fyn substrate that can recruit Csk to the membrane after being phosphorylated. Thus it is possible that, like Cbp, PSD-93 is involved in a negative feedback mechanism to suppress activated SFKs (29). This result also raises the possibility that the switching on and off of the SFKs occurs in the PSD. Accumulating information shows the importance of actin-cytoskeletal reorganization in the dendritic spines for the formation of the PSD and for synaptic plasticity (30, 31). Various protein complexes, including the PSD-95 family of MAGUKs, are envisioned to play a role in this process (14, 32). In parallel, it has been suggested that SFKs participate in the regulation of the actin cytoskeleton in the PSD via the SFKs-N-WASP pathway (33, 34). Thus, tyrosine phosphorylation...
tion of PSD-93 may serve to terminate these SFK-mediated processes.

In this study, we reported tyrosine phosphorylation of a member of the PSD-95 family of MAGUKs, PSD-93, and its possible involvement in the regulation of SFKs. To elucidate their physiological relevance to the function of the NMDA receptor complex, further studies under physiological conditions are now being undertaken.

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