Increased tyrosine phosphorylation of PSD-95 by Src family kinases after brain ischaemia

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PSD (postsynaptic density)-95, a scaffold protein that tethers NMDA (N-methyl-D-aspartate) receptors to signal molecules, is implicated in pathological events resulting from excitotoxicity. The present study demonstrates that brain ischaemia and reperfusion increase the tyrosine phosphorylation of PSD-95 in the rat hippocampus. PP2, a specific inhibitor of SrcPTKs (Src family protein tyrosine kinases), prevents the ischaemia-induced increases not only in the tyrosine phosphorylation of PSD-95, but also in the interaction between PSD-95 and Src kinases. PSD-95 is phosphorylated either by purified Src/Fyn kinases in vitro or by co-expression of constitutively active Src/Fyn in COS7 cells. The results suggest that SrcPTKs are involved in PSD-95 phosphorylation. The single Tyr523 mutation to phenylalanine (Y523F) reduces the Src/Fyn-mediated phosphorylation of PSD-95 in COS7 cells and in vitro. As shown with a rabbit polyclonal antibody against phospho-PSD-95 (Tyr523), Tyr523 phosphorylation is responsible for the increased tyrosine phosphorylation of PSD-95 induced by ischaemia in the rat hippocampus. In cultured hippocampal neurons, overexpression of PSD-95 Y523F; but not PSD-95 Y533F, abolishes the facilitating effect of PSD-95 on the glutamate- or NMDA-mediated currents, implying that PSD-95 Tyr523 phosphorylation contributes to the post-ischaemic over-activation of NMDA receptors. Thus the present study reveals an additional mechanism for the regulation of PSD-95 by tyrosine phosphorylation. This mechanism may be of pathological significance since it is associated with excitotoxicity in the ischaemic brain.

Key words: brain ischaemia, N-methyl-D-aspartate (NMDA) receptor, postsynaptic density-95 (PSD-95), Src, tyrosine phosphorylation.

INTRODUCTION

PSD (postsynaptic density)-95, also referred to as SAP (synapse-associated protein)-90, belongs to the PSD-95 subfamily of MAGUKs (membrane-associated guanylate kinases) and serves as a scaffold protein in excitatory synapses of the brain [1,2]. All PSD-95 subfamily members, including PSD-95, PSD-93, SAP-97 and SAP-102, share an organized domain structure, consisting of three N-terminal PDZ domains, an SH (Src homology) 3 domain, and a C-terminal GK (guanylate kinase) domain. With these distinct structural domains, PSD-95 subfamily proteins bind to the membrane-associated excitatory glutamate receptors, as well as to various downstream signal molecules, including CaMKII (Ca2+/calmodulin-dependent protein kinase II) [3,4], SrcPTKs (Src family protein tyrosine kinases) [5,6] and Pyk2 (proline-rich tyrosine kinase 2) [7,8].

The NMDA (N-methyl-D-aspartate) subtype of glutamate receptors co-localizes with PSD-95 in the excitatory PSD [9]. Functional NMDA receptor channels are hetero-oligomeric protein complexes composed of necessary NMDA receptor subunit 1, regionally localized NMDA receptor subunit 2 (A–D) and, more rarely, NMDA receptor subunit 3 (A or B) [10,11]. In the hippocampus, NMDA receptor subunits 2A and 2B are the major subtypes of NMDA receptor subunit 2 and determine the functional properties of NMDA receptors [12,13]. PSD-95 associates with the C-terminal of NMDA receptor subunits 2A and 2B by the PDZ1/2 domains [9,14]. As an organizer of the excitatory synapse, PSD-95 is involved in the NMDA-receptor-dependent excitatory synaptic transmission and synaptic plasticity [1,15,16]. At the same time, PSD-95 has been implicated in the pathological events resulting from brain ischaemia and excitotoxicity mediated by over-activation of NMDA receptors [17–19].

SrcPTKs, the largest family of membrane-associated non-receptor tyrosine kinases, interact with the PDZ3 domain of PSD-95 via its SH2 domain [5]. There are five members of SrcPTKs (Src, Fyn, Yes, Lck and Lyn) in the mammalian central nervous system. Both Src and Fyn possess high enzyme activity and are highly expressed in the brain [20]. PSD-95 is a substrate for the intracellular serine/threonine kinases CaMKII [3,4], cyclin-dependent kinases [21], p38/ and ERK (extracellular-signal-regulated kinase) 1/2 [22]. Although PSD-95 binds to SrcPTKs, there is little information related to its tyrosine phosphorylation.

Previous work by our laboratory has indicated that brain ischaemia and reperfusion enhance the interactions between PSD-95 and Src/Fyn kinases [23,24]. In the present study we found that, in the rat hippocampus, PSD-95 is tyrosine-phosphorylated after brain ischaemia, suggesting a pathological role of such phosphorylation. This process is mediated by SrcPTKs in vivo and in vitro. The mutation of Tyr523 in PSD-95 not only decreases Src/Fyn-dependent phosphorylation of PSD-95 in COS7 cells and in vitro, but also eliminates the facilitating effect of PSD-95 on glutamate/NMDA-mediated currents in rat hippocampal neurons, implicating the tyrosine phosphorylation of PSD-95 in the up-regulation of NMDA receptors. These results contribute to the

Abbreviations used: CaMKII, Ca2+/calmodulin-dependent protein kinase II; DMEM, Dulbecco’s modified Eagle’s medium; EGFR, enhanced green fluorescent protein; GK, guanylate kinase; AG, PSD-95 with GK domain deleted; KLH, keyhole-limpet haemocyanin; NMDA, N-methyl-D-aspartate; PSD, postsynaptic density; pY, phosphorysotyrosine; Pyk2, proline-rich tyrosine kinase 2; RT-PCR, reverse transcription-PCR; SAP, synapse-associated protein; SH, Src homology; ASG, PSD-95 with SH3 and GK domains deleted; SrcPTK, Src family protein tyrosine kinase.

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elucidation of the molecular mechanisms underlying ischaemic excitotoxicity.

**EXPERIMENTAL**

**Antibodies**

Mouse monoclonal anti-PSD-95 antibody (clone 7E3-1B8), mouse monoclonal anti-pY (phosphotyrosine) antibody (clone PT-66), rabbit polyclonal anti-c-Myc antibody, alkaline-phosphatase-conjugated secondary antibodies and non-specific mouse IgG and rabbit IgG were from Sigma. Rabbit polyclonal anti-Src antibody, mouse monoclonal anti-Fyn (Fyn15) antibody and rabbit polyclonal anti-actin antibody were from Santa Cruz Biotechnology. Rabbit polyclonal anti-[phospho-PSD-95 (Tyr18)] antibody was raised in New Zealand White rabbits with a KLH (keyhole-limpet haemocyanin)-conjugated pY-containing synthetic peptide (REDSVLSpYETVT) as the immunogen, and the antibody was purified by two-step immunoadfinity chromatography using a column conjugated to the non-phosphopeptide to eliminate antibodies against non-phosphorylated PSD-95, followed by purification with another column conjugated with the immunogen (AbMART).

**Plasmids**

The vector pcDNA3.1 (+) (Invitrogen) was used for the expression of Src, Fyn, PSD-95 and various mutant constructs. SrcY529F- and SrcK297R-pcDNA3.1, encoding constitutively active and inactive forms of Src, were subcloned from SrcY529F- and SrcK297R-pUSEamp (Upstate Biotechnology) respectively. The cDNAs of Fyn Y531F and Fyn K299M, encoding constitutively active and inactive forms of Fyn respectively, were amplified by RT-PCR (reverse transcription-PCR) and overlap extension. The cDNAs of Myc–ΔSG (PSD-95 with SH3 and GK domains deleted) and Myc–ΔG (PSD-95 with GK domain deleted), encoding SH3/GK-domain-deletion mutants of PSD-95, were PCR amplified from Myc–PSD-95-GW1 (a gift from Dr M. Sheng, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA, U.S.A.). The cDNAs of full-length PSD-95 and PSD-95 single tyrosine mutants Y432F, Y439F, Y523F, Y533F, Y573F, Y580F, Y604F, Y609F, Y701F and Y715F were amplified by RT-PCR and overlap extension respectively. All constructs were verified by DNA sequencing.

**Brain ischaemia and drug treatments**

Brain ischaemia was induced by the four-vessel occlusion method as described previously [23]. Briefly, adult male Sprague–Dawley rats (220–270 g) were anaesthetized with chloral hydrate (300 mg/kg, intraperitoneal) and vertebral arteries were electrically coagulated. On the following day, ischaemia was induced for various time periods by the occlusion of both common carotid arteries with aneurysm clips. Animals not meeting the following criteria were excluded: completely flat bitemporal EEG (electroencephalogram), maintenance of dilated pupils or absence of a corneal reflex. Rat rectal temperature was maintained at 37–37.5 °C during ischaemia and 2 h of reperfusion. Sham-operated rats received the same treatments, except for occlusion of the carotid artery. PP2 or PP3 (3 μg/μl in DMSO; Calbiochem) was infused into the rat cerebral ventricle (from the bregma: anteroposterior, −0.8 mm; lateral, 1.5 mm; depth, 3.5 mm) through a stepmotored microsyringe (Stoelting) 30 min before occlusion. All experiments were performed in accordance with the guidelines of the local Animal Care Committee.

**Tissue preparation**

Rats were decapitated at indicated times after ischaemia, and the hippocampi were removed and quickly frozen in liquid nitrogen. Samples were homogenized in ice-cold homogenization buffer [50 mM Mops (pH 7.4), 320 mM sucrose, 100 mM KCl, 0.5 mM MgCl₂, 0.2 mM dithiothreitol and phosphatase and protease inhibitors (20 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 50 mM NaF, 1 mM each of EDTA, EGTA, sodium orthovanadate, p-nitrophenyl phosphate, PMSF and benzamidine, and 5 μg/ml each of aprotinin, leupeptin and pepstatin A)]. The homogenates were centrifuged at 800 g for 10 min at 4 °C, and the supernatants were collected. Protein concentrations were determined by the method of Lowry [24a]. Samples were stored at −80 °C until use.

**Immunoprecipitation**

The protein samples were diluted 4-fold with immunoprecipitation buffer containing 50 mM Hepes (pH 7.1), 10% (v/v) glycerol, 150 mM NaCl, 1 mM ZnCl₂, 1.5 mM MgCl₂, 1% Triton X-100, 0.5% NP-40 (Nonidet P40) and protease and phosphatase inhibitors as described above. The samples were incubated with 1–2 μg of the indicated antibodies for 4 h or overnight at 4 °C. Protein A–Sepharose (25 μl) was added and the incubation was allowed to continue for 2 h at 4 °C. Immunoprecipitates were collected by centrifugation at 10000 g for 2 min at 4 °C, and the bound proteins were eluted from Protein A–Sepharose by boiling for 5 min in Laemmli sample buffer [62.5 mM Tris/HCl (pH 6.8), 2% (w/v) SDS, 1% 2-mercaptoethanol, 10% (v/v) glycerol and 0.003% Bromophenol Blue]. After centrifugation at 10000 g for 1 min, the supernatants were used for immunoblotting or for kinase assays in vitro.

**Immunoblotting**

As described previously [23], the immunoprecipitates or equal amounts of protein samples were separated by SDS/PAGE and then transferred on to nitrocellulose membranes (Amersham Pharmacia). After blocking with 3% (w/v) BSA for 3 h, the membranes were incubated with primary antibodies or alkaline-phosphatase-conjugated secondary antibodies and developed with the colour substrate, BCIP (5-bromo-4-chloroindol-3-yl phosphate) and NBT (Nitro Blue Tetrazolium) (Promega) following the manufacturer’s protocol.

**In vitro kinase assay**

Immunoprecipitates of PSD-95 proteins were incubated with 3 units of purified Src or 5 m-units of Fyn kinase (Upstate Biotechnology) and ATP (100 μM) in kinase reaction buffer [40 mM Hepes (pH 7.4), 25 mM MgCl₂, 5 mM MnCl₂, 0.4 mM EGTA, 0.5 mM sodium orthovanadate and 0.4 mM dithiothreitol] for 30 min at 30 °C. The kinase reaction was initiated by the addition of ATP and was stopped by the addition of Laemmli sample buffer. After boiling for 5 min, the reaction mixtures were centrifuged at 10000 g for 1 min and the supernatants were subjected to immunoblot analysis with an anti-pY antibody to visualize the tyrosine phosphorylation of PSD-95.

**Hippocampal synaptosome preparation**

Rat hippocampal samples were homogenized in ice-cold homogenization buffer (see above) and were centrifuged at 800 g for 15 min at 4 °C. The collected supernatants were centrifuged at 14000 g for 15 min at 4 °C. The pellets were lysed in ice-cold homogenization buffer and then subjected to immunoprecipitation and kinase assays in vitro.
The tyrosine phosphorylation of PSD-95 was detected by immunoprecipitation (IP) followed by immunoblot (IB) analysis. The expression of target proteins was detected by immunoblotting. The results shown are representative of at least three independent experiments. (a) The tyrosine phosphorylation of PSD-95 after 15 min of brain ischaemia. The samples from the sham- or ischaemia-operated rats were immunoprecipitated with anti-PSD-95, anti-pY or non-specific mouse IgG (Mo.IgG), followed by blotting with anti-pY or anti-PSD-95 antibodies respectively. (b1) The tyrosine phosphorylation of PSD-95 after 5, 15 or 30 min of ischaemia (b1), or various times of reperfusion following 15 min of ischaemia (b2). Immunoprecipitation was performed using an anti-pY antibody, and blotting was performed using an anti-PSD-95 antibody (upper panels). Blotting of hippocampal samples using an anti-PSD-95 antibody was also performed (lower panels). (c) The effects of PP2 on PSD-95 tyrosine phosphorylation and on the Src–PSD-95 interaction after brain ischaemia. Rats were dosed with 15 μg of PP2, PP3 or with the same volume of DMSO (5 μl) for 30 min before 15 min of ischaemia. The Src–PSD-95 interaction was detected by co-immunoprecipitation. Immunoprecipitation was performed using an anti-pY antibody or anti-Src antibody, and immunoblotting was performed using an anti-PSD-95 antibody. Immunoblotting of hippocampal samples using an anti-PSD-95 antibody and an anti-Src antibody was also performed.

RESULTS

Tyrosine phosphorylation of PSD-95 after brain ischaemia

Since brain ischaemia promotes the PSD-95–Src/Fyn interactions [23, 24], the tyrosine phosphorylation of PSD-95 was measured after transient (15 min) brain ischaemia in the rat hippocampus, one of the most vulnerable brain regions. As demonstrated by immunoprecipitation with a mouse anti-PSD-95 antibody followed by immunoblotting with a mouse anti-pY antibody, or by immunoprecipitation with an anti-pY antibody and then immunoblotting with an anti-PSD-95 antibody, a 95 kDa protein band was observed, the amount of which was greater in the ischaemia groups when compared with the sham-operated groups (Figure 1a). In contrast, immunoprecipitation with a non-specific mouse IgG resulted in no bands observed at 95 kDa. Immunoblot analysis showed no difference in the expression level of PSD-95 between the ischaemia and sham-operated groups (Figure 1a).

The tyrosine phosphorylation of PSD-95 increased consistently during 5, 15 and 30 min of brain ischaemia (Figure 1b1). There was a decrease after reperfusion following 15 min of ischaemia, then tyrosine phosphorylation of PSD-95 increased again to a maximum at 6 h, and remained evident at 24 h reperfusion (Figure 1b2).

To clarify the role of SrcPTKs in the tyrosine phosphorylation of PSD-95 after brain ischaemia, a selective SrcPTK inhibitor, PP2, was administered to rats 30 min before ischaemia. PP3, an inactive derivative of PP2, was used as a negative control. Pre-treatment with PP2, but not with the same dose of PP3 or with vehicle (DMSO), attenuated the increased tyrosine phosphorylation of PSD-95 in the enhanced interaction between PSD-95 and Src. These results suggest that SrcPTKs are involved in ischaemia-induced tyrosine phosphorylation of PSD-95 (Figure 1c).
Src/Fyn-mediated phosphorylation of PSD-95 in vitro and in COS7 cells

The anti-PSD-95 antibody (clone 7E3-1B8) slightly cross-reacts with PSD-93, a member of the PSD-95 subfamily [26]. PSD-93 has been reported to be a substrate for Fyn kinases [27]. Therefore active Src or Fyn kinases were used in vitro and in COS7 cells to verify the tyrosine phosphorylation of PSD-95. For the in vitro kinase assay, PSD-95 proteins immunoprecipitated from the hippocampal synaptosome fractions or COS7 cells transfected with PSD-95-pcDNA3.1 were used as the substrates. The results showed that PSD-95 proteins from synaptosomes or COS7 cells were phosphorylated after incubation with purified active Src or Fyn kinases for 30 min (Figure 2a). In COS7 cells, the construct for full-length PSD-95 was co-transfected with plasmids encoding constitutively active (Src Y529F/Fyn Y531F) or inactive (Src K297R/Fyn K299M) forms of Src or Fyn. The results showed that PSD-95 was tyrosine-phosphorylated only when it was co-expressed with active Src or Fyn, but not with the inactive kinases (Figure 2b).

Identification of tyrosine phosphorylation sites in PSD-95 by Src/Fyn

To locate the phosphorylation sites in PSD-95 by Src/Fyn, recombinant proteins expressing Myc-tagged full-length PSD-95, ΔSG (amino acids 1–424 of PSD-95) and ΔG (amino acids 1–533 of PSD-95) were generated (Figure 3a). In all constructs, the PDZ3 domain was preserved for binding to Src and Fyn. In COS7 cells, phosphorylation was observed when Myc–PSD-95 and Myc–ΔG were co-expressed with constitutively active Src or Fyn kinases, but Myc–ΔSG was not phosphorylated; and the interactions between Myc–PSD-95, Myc–ΔG or Myc–ΔSG and Src/Fyn were not affected (Figure 3b). In vitro, Myc–ΔG, but not Myc–ΔSG, was phosphorylated in a Src- or Fyn-dependent manner (Figure 3c). These results suggest that the major phosphorylation sites of PSD-95 for Src and Fyn are located in amino acids 425–533. However, amino acids 534–724 of PSD-95 cannot be excluded as a location for tyrosine-phosphorylation sites because of possible multi-site phosphorylation.

There are four tyrosine residues in amino acids 425–533 of PSD-95 (Figure 4a). In COS7 cells, the single tyrosine mutants of full-length PSD-95, including Y432F, Y439F, Y523F or Y533F, were co-expressed with constitutively active Src or Fyn kinases. A reduction in tyrosine phosphorylation was evident with PSD-95 Y523F compared with full-length PSD-95 (Figures 4b1 and 4b2). The single Tyr523 mutation did not alter the interactions between PSD-95 and Src or Fyn kinases (Figure 4b3). Furthermore, PSD-95 Y523F could not be efficiently phosphorylated by Src or Fyn kinases in vitro relative to PSD-95 (Figure 4c). Thus Tyr523 is the major phosphorylation site in PSD-95.

There are six tyrosine residues located in amino acids 534–724 of PSD-95 (Figure 4a). Each of the single PSD-95 tyrosine mutants, Y573F, Y580F, Y604F, Y609F, Y701F and Y715F, displayed a similar phosphorylation level to wild-type full-length PSD-95 when co-expressed with constitutively active Src or Fyn kinases (Figure 4d), suggesting that there are no phosphorylation sites in the C-terminal 200 amino acids of PSD-95.

PSD-95 Tyr523 phosphorylation in COS7 cells and rat hippocampus

To evaluate the phosphorylation of Tyr523 in vivo, a rabbit polyclonal antibody which selectively recognizes Tyr523-phosphorylated PSD-95 was raised using the pY-containing synthetic peptide KLH-CREDSVLSpYETVT as the immunogen (Figure 5a).

Figure 2 Src/Fyn induces tyrosine phosphorylation of PSD-95 in vitro and in COS7 cells

The results shown are representative of at least three independent experiments. (a) Kinase assay of tyrosine phosphorylation of PSD-95 by purified Src (a1) or Fyn (a2) in vitro. The immunoprecipitates (IP) from immunoprecipitation using anti-PSD-95 or non-specific mouse IgG (Mo.IgG) from hippocampal synaptosomes or COS7 cells over-expressing PSD-95 were used as substrates. Immunoblotting (IB) using anti-pY and anti-PSD-95 antibodies was performed. (b) The tyrosine phosphorylation of PSD-95 by co-expressing active Src (b1) or Fyn (b2) in COS7 cells. The expression of target proteins was confirmed by immunoblotting with anti-PSD-95, anti-Src (b1) or anti-Fyn (b2) antibodies as indicated. Actin was detected as an internal control. (−), untransfected COS7 cells. V, transfection of empty vector.
Post-ischaemic tyrosine phosphorylation of PSD-95

Figure 3 Various degrees of tyrosine phosphorylation of the ΔG and ΔSG PSD-95 mutants by Src/Fyn in COS7 cells and in vitro

The results shown are representative of at least three independent experiments. (a) Schematic diagram of Myc-tagged full-length PSD-95, ΔG and ΔSG mutant constructs. a.a, amino acid. (b) Myc-tagged ΔG or PSD-95, but not ΔSG, is phosphorylated by co-expressed constitutively active Src (b1) or Fyn (b2) in COS7 cells. The associations between Myc fusion proteins and Src/Fyn were assessed by co-immunoprecipitation. The tyrosine phosphorylation of target proteins was examined by immunoprecipitation (IP) with an anti-c-Myc, followed by blotting with an anti-pY antibody. The expression of target proteins was confirmed by immunoblotting (IB) using anti-c-Myc, anti-Src and anti-Fyn antibodies as indicated. V, transfection with empty vector. (c) In vitro kinase assay of the phosphorylation of Myc-tagged ΔG and ΔSG by purified Src (c1) or Fyn (c2). The immunoprecipitates (IP) of anti-c-Myc or non-specific rabbit IgG (Ra.IgG) from COS7 cells over-expressing Myc-tagged ΔG or ΔSG were used as substrates. Immunoblotting (IB) was performed using anti-pY and anti-c-Myc antibodies.

Immunoblot analysis with an anti-[phospho-PSD-95 (Tyr523)] antibody showed that Tyr<sup>523</sup> was phosphorylated in COS7 cells co-transfected with PSD-95 and constitutively active Fyn, but not with inactive Fyn kinases; the mutation of Tyr<sup>523</sup> to Phe<sup>523</sup> eliminated the phosphorylation of PSD-95 Tyr<sup>523</sup> (Figure 5b). The results establish the specificity of the anti-[phospho-PSD-95 (Tyr<sup>523</sup>)] antibody. In the rat hippocampus, PSD-95 Tyr<sup>523</sup> was phosphorylated, especially in the ischaemia groups; but there was only a weak signal in the sham-operated groups (Figure 5c). These results indicate that, in the rat hippocampus, the phosphorylation of Tyr<sup>523</sup> is responsible for the increased tyrosine phosphorylation of PSD-95 after brain ischaemia.

Effects of PSD-95 Tyr<sup>523</sup> on glutamate/NMDA-mediated currents

PSD-95 has been implicated in the up-regulation of NMDA-receptor function, but the role of PSD-95 Tyr<sup>523</sup> phosphorylation in the function of NMDA receptors is not clear. Whole-cell patch-clamp experiments were performed to record the glutamate- or NMDA-stimulated whole-cell currents in the cultured primary hippocampal neurons with over-expressed EGFP-tagged PSD-95, PSD-95 Y523F, PSD-95 Y533F or with EGFP alone (Figure 6a). EGFP was used for identifying the candidate cells. The capacitance is directly proportional to the surface membrane area of a cell; the amplitude of glutamate- and NMDA-induced signals is proportional to the number of PSD-95 molecules. The results showed that the over-expression of PSD-95 Tyr<sup>523</sup> increased the glutamate- or NMDA-stimulated currents compared to the control EGFP alone.
Figure 4  PSD-95 Tyr\(^{523}\) is a principal phosphorylation site for Src/Fyn in COS7 cells and in vitro

The tyrosine phosphorylation of target proteins in vivo was examined by immunoprecipitation (IP) followed by immunoblotting (IB) with an anti-pY antibody. The expression of target proteins was confirmed by immunoblotting. The results shown are representative of at least three independent experiments. (a) Schematic diagram of the position of the tyrosine residues in the C-terminal of PSD-95 [amino acids (a.a.) 400–724]. (b) The tyrosine phosphorylation of full-length PSD-95 and its single tyrosine mutants (Y432F, Y439F, Y523F and Y533F) by co-expression with constitutively active Src (b1) or Fyn (b2) in COS7 cells. The associations between PSD-95, PSD-95 Y523F and PSD-95 Y533F with Src/Fyn were assessed by co-immunoprecipitation (b3). Immunoprecipitations were performed using anti-PSD-95 (b1 and b2), anti-Src and anti-Fyn antibodies (b3), and immunoblotting was performed using anti-pY, anti-PSD-95, anti-Src and anti-Fyn antibodies as indicated. Actin was detected as an internal control. (−), untransfected COS7 cells. V, transfection with empty vector. (c) In vitro kinase assay of the phosphorylation of PSD-95 and PSD-95 Y523F by purified Src/Fyn kinases. The immunoprecipitates isolated from COS7 cells over-expressing PSD-95 or PSD-95 Y523F using an anti-PSD-95 antibody were used as substrates. Immunoblotting with anti-pY and anti-PSD-95 antibodies was performed. (d) The tyrosine phosphorylation of PSD-95 and the PSD-95 tyrosine single mutants (Y573F, Y580F, Y604F, Y609F, Y701F and Y715F) co-expressed with active Src (d1) or Fyn (d2) in COS7 cells. Immunoprecipitation was performed using an anti-PSD-95 antibody and immunoblotting with anti-pY, anti-PSD-95, anti-Src, anti-Fyn and anti-actin antibodies was performed as indicated.
The phosphorylation of Tyr<sup>523</sup> is responsible for the increased tyrosine phosphorylation of PSD-95 after brain ischaemia

The results are representative of at least three independent experiments. (a) Amino acid sequence of the KLH-conjugated synthetic peptide used as an immunogen for developing the anti-[phospho-PSD-95 (Tyr<sup>523</sup>)] antibody. Y523, Tyr<sup>523</sup>. (b) Immunoblot (IB) analysis of PSD-95 Tyr<sup>523</sup> phosphorylation in COS7 cells over-expressing PSD-95 or PSD-95 Y523F together with active/inactive Fyn. Immunoblotting was performed using anti-[phospho-PSD-95 (Tyr<sup>523</sup>)] (pY523), anti-PSD-95 and anti-Fyn antibodies. (c) Immunoblot analysis of PSD-95 Tyr<sup>523</sup> phosphorylation in the hippocampus of rats subjected to a sham operation or to 15 min of ischaemia. Immunoblotting was performed using anti-[phospho-PSD-95 (Tyr<sup>523</sup>)] (pY523) and anti-PSD-95 antibodies.

currents, on the other hand, relates to the total number of functional NMDA receptors. Therefore the current density (defined as the current divided by the capacitance) provides an estimate of the activity of functional NMDA receptors in different neurons. The results showed that the over-expression of full-length wild-type PSD-95 led to an increase in current density stimulated by glutamate or NMDA; over-expression of the PSD-95 Y<sub>533</sub>F mutant had an effect similar to that observed for wild-type PSD-95; but PSD-95 Y523F did not affect glutamate- or NMDA-induced currents (Figures 6b and 6c). These results reveal that Tyr<sup>523</sup> is involved in the facilitating effect of PSD-95 on the activity of glutamate receptors, including NMDA receptors.

**DISCUSSION**

Excitotoxicity is implicated in the pathogenesis of neurodegenerative disorders resulting from brain ischaemia. After an ischaemic episode, excessively released glutamate causes the over-activation of glutamate receptors, mainly of the NMDA subtype, and activates intracellular calcium-dependent signal cascades that eventually lead to neuronal cell death, a process known as excitotoxicity [28]. However, the clinical application of NMDA-receptor antagonists is limited by severe side effects. The postsynaptic molecular mechanisms associated with the over-activation of NMDA receptors may provide a potential target for the therapy of ischaemic stroke. PSD-95 is involved in the up-regulation of NMDA receptors, but the regulation of PSD-95 after brain ischaemia remains to be elucidated.

Reversible protein phosphorylation is a ubiquitous mechanism for the regulation of biological function of proteins and the control of signal transduction. Although an interaction between PSD-95–Src/Fyn is established, the regulation of PSD-95 by tyrosine phosphorylation has not been reported previously. The reason may be the result of a relatively weak signal under physiological conditions [27]. In the post-ischaemic brain, PSD-95 is highly tyrosine-phosphorylated by SrcPTKs, suggesting a pathological association of such phosphorylation. PSD-93, another member of the PSD-95 subfamily in the hippocampal postsynapse, is a major tyrosine-phosphorylated protein in the NMDA receptor complex.
of the normal mouse brain [27]. Immunoprecipitation with an anti-p-Y antibody, followed by blotting with a specific antibody against PSD-93 (catalogue number 34-4700; Invitrogen) showed that PSD-93 had a high basal level of tyrosine phosphorylation in the rat hippocampus; and in comparison with sham-operated animals, the tyrosine phosphorylation of PSD-93 was not changed following 15 or 30 min of brain ischaemia (results not shown). Thus the tyrosine phosphorylation of PSD-93 may correlate with its physiological function.

The present study provides evidence that Tyr$^{523}$ is a major phosphorylation site \textit{in vitro} and \textit{in vivo}. Since mutation of Tyr$^{523}$ of PSD-95 did not eliminate Src/Fyn-mediated phosphorylation, screening for other tyrosine phosphorylation sites in PSD-95 was performed. Every tyrosine mutant construct in amino acids 425–724 except for Y523F displayed a tyrosine phosphorylation level similar to that for wild-type PSD-95, suggesting that the additional tyrosine phosphorylation sites exist among amino acids 1–424. However, the ASG mutant, containing amino acids 1–424, could not be efficiently phosphorylated by active Src/Fyn kinases, revealing the possibility that amino acids 425–533 of PSD-95 are required for the phosphorylation of PSD-95 by SrcPTKs.

Pyk2, an upstream activator of SrcPTKs, binds to the SH3 domain of PSD-95, and SrcPTKs bind to the PDZ3 domain of PSD-95. This structural adjoin allows SrcPTKs to be activated by Pyk2 [29]. Tyr$^{523}$ is located between the SH3 and GK domains of PSD-95. There is a direct intramolecular interaction between the SH3 domain and the GK domain in the PSD-95 subfamily [30]. Protein phosphorylation usually results in a conformational change in the modified protein. It is possible that phosphorylation of PSD-95 at Tyr$^{523}$ opens up this stable intra-molecular structure, facilitates the association of Pyk2 with the SH3 domain of PSD-95 and increases the activation of SrcPTKs.

Brain ischaemia induces the increase in the phosphorylation of PSD-95 Tyr$^{523}$. The mutation of Tyr$^{523}$ not only reduces the phosphorylation of PSD-95, but also abolishes the effect of PSD-95 on the up-regulation of NMDA receptor function. The results imply that PSD-95 Tyr$^{523}$ phosphorylation contributes to the excessive openess of NMDA receptors after brain ischaemia, but the mechanism needs to be clarified. The tyrosine phosphorylation of NMDA receptor subunit 2A by SrcPTKs enhances the activity of NMDA receptors [31], and PSD-95 allows Src to potentiate the NMDA receptor subunit 1/NMDA receptor subunit 2A currents [32]. Previous work has shown that the antisense oligonucleotide-mediated down-regulation of PSD-95 expression inhibits NMDA receptor subunit 2A tyrosine phosphorylation and diminishes hippocampal CA1 pyramidal cell death after brain ischaemia [19,33]. The tyrosine phosphorylation of PSD-95 appears to precede that of NMDA receptor subunit 2A after transient brain ischaemia. Thus phosphorylation of PSD-95 Tyr$^{523}$ could promote the tyrosine phosphorylation of NMDA receptor subunit 2A. On the basis of this concept, the rapid phosphorylation of PSD-95 Tyr$^{523}$ induced by brain ischaemia could open up the intramolecular SH3–GK interaction and facilitate the activation of SrcPTKs by Pyk2, which would result in increased tyrosine phosphorylation of NMDA receptor subunit 2A and subsequent over-activation of NMDA receptors. This hypothesis remains to be evaluated.

In conclusion, the present study reveals an additional mechanism for the regulation of PSD-95 involving tyrosine phosphorylation. Such phosphorylation, accomplished by SrcPTKs, occurs after brain ischaemia, indicating that it has a pathological significance. Tyr$^{523}$ in PSD-95 is a main phosphorylation site for Src/Fyn kinases, and it contributes to the up-regulation of NMDA receptors. Elucidation of the molecular mechanisms responsible for the over-activation of NMDA receptors through tyrosine phosphorylation of PSD-95 may provide a novel targets for the therapy of ischaemic stroke.

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