

Striatal Enriched Phosphatase 61 Dephosphorylates Fyn at Phosphotyrosine 420*

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A family of protein tyrosine phosphatases enriched within the central nervous system called striatal enriched phosphatase (STEP) has been implicated in the regulation of the *N*-methyl-D-aspartate receptor. STEP₆₁, a membrane-associated isoform located in the postsynaptic densities (PSDs) of striatal neurons, contains two transmembrane domains, two proline-rich domains, and a kinase-interacting motif. This study demonstrates that STEP₆₁ associates with Fyn, a member of the Src family kinases that is also enriched in PSDs. By using human embryonic kidney 293 cells for co-transfection, we determined that a substrate-trapping variant (STEP₆₁ CS) binds to Fyn but not to other members of the Src family present in PSDs. In a complementary experiment, myc-tagged Fyn immunoprecipitates STEP₆₁ CS. STEP₆₁ binds to Fyn through one of its proline-rich domains and the kinase-interacting motif domain, whereas Fyn binds to STEP₆₁ through its Src homology 2 domain and the unique N-terminal domain. STEP₆₁ CS pulls down Fyn when the Tyr⁴²⁰ site is phosphorylated. *In vitro*, wild-type STEP₆₁ dephosphorylates Fyn at Tyr⁴²⁰ but not at Tyr⁵³¹. These results suggest that STEP regulates the activity of Fyn by specifically dephosphorylating the regulatory Tyr⁴²⁰ and may be one mechanism by which Fyn activity is decreased within PSDs.

Dynamic regulation of protein tyrosine phosphorylation requires a balance between the activity of protein tyrosine kinases and protein tyrosine phosphatases (PTPs).¹ One of the best-characterized families of protein tyrosine kinases is the Src kinase family, which consists of several members including Src, Fyn, Lyn, Lck, and Yes. These proteins are highly conserved in their amino acid sequences and their regulatory domains. Each member also contains a unique domain at the N terminus that distinguishes it from other family members (see review, Ref. 1).

Src and Fyn are present within postsynaptic densities of central nervous system neurons (2, 3). Their presence at the

PSD and their direct association with both receptors and key anchoring proteins such as PSD-95 (4) suggest that they regulate signaling events within these neurons. Indeed, several recent studies show that members of the Src kinase family modulate synaptic transmission by regulating the level of tyrosine phosphorylation of proteins within the PSD including the NR2 subunit of the *N*-methyl-D-aspartate (NMDA) receptor (5, 6).

The Src family kinases are themselves regulated by tyrosine phosphorylation. They contain two conserved tyrosine residues that are phosphorylated (tyrosine 420 and tyrosine 531; numbering according to the amino acid sequence of human Fyn). Phosphorylation of tyrosine 531 of Fyn by C-terminal Src kinase leads to its inactivation (7, 8). Dephosphorylation of this site results in a conformational change and activation of the protein through autophosphorylation at the second site, Tyr⁴²⁰. The dynamic balance in the level of phosphorylation at these two sites contributes to the overall level of Fyn kinase activity. Therefore, the identification of tyrosine phosphatases that lead to the dephosphorylation of one regulatory site or the other would add to the understanding of how these kinases are regulated.

The striatal enriched phosphatase (STEP) family of PTPs is enriched within specific subsets of neurons in the central nervous system (9, 10). STEP₆₁ is one member of this family. It contains two polyproline domains that meet the consensus sequence for motifs involved in protein-protein interactions (11). In addition, STEP₆₁ contains a kinase-interacting motif (KIM) that has been identified as a domain required for the binding of STEP to extracellular signal-regulated kinase (ERK) 1/2 *in vitro* (12). STEP₆₁ is regulated, in part, by dopamine signaling through a D1/cAMP/protein kinase A-mediated phosphorylation of the serine residue within the KIM domain. Phosphorylation at that site leads to a decrease in its enzymatic activity against substrates (13). In contrast, this serine is dephosphorylated after the activation of NMDA receptors, leading to an increase in its phosphatase activity² and inhibition of ERK1/2-mediated signaling pathways within these neurons.

STEP₆₁ is present in the PSDs of neurons that receive both dopaminergic and glutamatergic synaptic input (14–16). STEP₆₁ was recently shown to co-immunoprecipitate as part of the NMDA complex of proteins (17). Application of STEP to the cytoplasmic side of membrane patches from embryonic spinal cord neurons led to a depression in NMDA-mediated activity. In contrast, application of functionally inhibiting STEP antibodies led to an increase in NMDA receptor synaptic currents. Finally, application of STEP postsynaptically prevented the induction of long-term potentiation by tetanic stimulation in the CA1 region of hippocampal neurons (17). Thus, STEP ap-

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We dedicate this work to the memory of Dr. Akira Okamura.

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¹ The abbreviations used are: PTP, protein tyrosine phosphatase; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; HEK, human embryonic kidney; KIM, kinase-interacting motif; NMDA, *N*-methyl-D-aspartate; PSD, postsynaptic density; STEP, striatal enriched phosphatase; SH, Src homology; PBS, phosphate-buffered saline.

² S. Paul, A. C. Nairn, P. Wang, and P. J. Lombroso, submitted for publication.

appears to counter the activity of Src family members known to up-regulate synaptic transmission at excitatory synapses (18). The exact mechanisms by which this occurs remain unclear.

Recent studies have shown that the receptor-like PTP, PTP α , plays a role in modulating the activity of Src and Fyn by dephosphorylating phosphotyrosine 531 both *in vivo* and *in vitro* (19, 20). The identification of additional PTPs that might participate in regulating these kinases was the goal of the present study. Because there are two regulatory tyrosine residues among Src family members, it was hypothesized that a second PTP might independently regulate the phosphorylated tyrosine at amino acid 420.

Here, we demonstrate that STEP₆₁ binds to Fyn both *in vitro* and *in vivo*. The domains in both proteins that govern this interaction were identified. In STEP₆₁, both the KIM domain and the N-terminal proline-rich domain are involved. In Fyn, the SH2 domain and the unique N-terminal domain are responsible for the observed interaction. Finally, tyrosine 420, but not tyrosine 531, is dephosphorylated by STEP₆₁. These results suggest that STEP₆₁ participates in regulating Fyn activity and that this is one mechanism by which STEP regulates signaling events at excitatory synapses.

EXPERIMENTAL PROCEDURES

Differential Centrifugation of Rat Brain—All experimental procedures used in the present study were approved by the Animal Care and Use Committee at Yale University School of Medicine. Adult, female Long Evans rats were purchased from Charles River Laboratories (Wilmington, MA).

Subcellular fractionation was performed with modifications to the methods described in previous publications (11, 15). In brief, rats were euthanized, and the striatum was dissected on ice and homogenized in 10-fold (w/v) cold buffer (320 mM sucrose, 4 mM HEPES, pH 7.4, and complete protease inhibitor mixture tablets (Roche Molecular Biochemicals)). Homogenized tissue was centrifuged at 800 $\times g$ for 10 min to form S1 and P1 fractions. The S1 fraction was further centrifuged at 9,000 $\times g$ for 15 min to form a crude synaptosomal fraction (P2). This fraction was then washed in homogenization buffer and centrifuged at 10,200 $\times g$ for 15 min. The resulting pellet was lysed in a 10 \times volume of cold water and immediately buffered to 1 mM HEPES, pH 7.4. Lysed synaptosomes were centrifuged at 25,000 $\times g$ for 20 min to form LS1 and LP1 fractions. The LP1 fraction was resuspended in 250 mM sucrose and HEPES, pH 7.4, and additional protease inhibitors were added before overlaying the sample on a gradient consisting of 4 ml of 0.8 M sucrose, 1 ml of 1.0 M sucrose, and 4 ml of 1.2 M sucrose. The gradient was centrifuged at 65,000 $\times g$ for 2 h. Samples from the 1.0–1.2 M sucrose interface were collected and washed in a 10 \times volume of cold PBS at 48,000 $\times g$ for 10 min. The resulting PSDs were resuspended in PBS and frozen at -80°C until needed.

Affinity Column Chromatography—pGEX-STEP46 was transformed into BL21 cells and grown at 37 $^\circ\text{C}$ until log phase before induction with 0.1 mM isopropyl- β -D-thiogalactopyranoside. Cells were harvested, resuspended in 0.01 culture volume with PBS, and lysed in B-Per according to manufacturer's instructions (Pierce). Cell lysate was centrifuged, and the resulting supernatant was passed through a glutathione-Sepharose column (Amersham Biosciences). The column was washed extensively with PBS, and recombinant protein was eluted by using 10 mM glutathione in 50 mM Tris-HCl, pH 8.0. STEP-GST recombinant protein was concentrated with a Centrion-30 column (Millipore) before passing through Sephadex G-50 equilibrated with PBS to remove the glutathione peptide. GST-STEP fusion protein was coupled to *N*-hydroxysuccinimide-activated Sepharose-4 Fast Flow (Amersham Biosciences) overnight at 4 $^\circ\text{C}$. The next day, unconjugated protein was removed in cold PBS, and nonreactive groups were blocked with ethanolamine buffer (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3) for 1 h at room temperature. Uncoupled protein was alternatively washed with ethanolamine buffer and acetate buffer (0.1 M acetate, 0.5 M NaCl, pH 4.0) before a final wash with cold PBS. The GST-STEP₄₆-conjugated Sepharose matrix was then used to pack an affinity column.

The rat brain P2 fraction was solubilized in 1% Triton X-100, followed by centrifugation to remove insoluble material. The supernatant was collected and diluted 5-fold with cold PBS before passing through the GST-STEP₄₆ affinity column, which was then extensively washed with PBS-1% Triton X-100 followed by PBS without detergent with

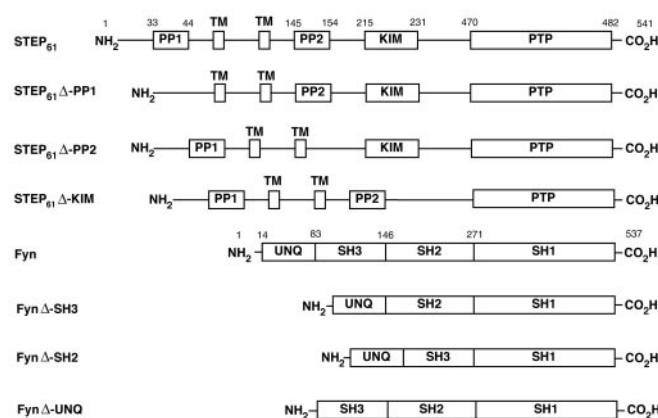


FIG. 1. Schematic drawing of STEP₆₁ and Fyn DNA constructs. Deleted domains are designated by Δ . Drawing is not to scale. *PP1* and *PP2*, proline-rich domains; *TM*, transmembrane domains; *UNQ*, unique domain. Numbering on STEP₆₁ and Fyn full-length constructs represents the amino acid sequence for the STEP₆₁-deleted domains is as follows: Δ -*PP1*, ³³PPPPPPSPSPSEP⁴⁴; Δ -*PP2*, ¹⁴⁵PPEPPALPP¹⁵⁴; and Δ -*KIM*, ²¹⁵LQERRGSNVSLTDMCT²³¹. Amino acid numbering for the Fyn-deleted domains is as follows: Δ -*UNQ*, 14–83; Δ -*SH3*, 82–146; and Δ -*SH2*, 146–271.

normal (150 mM) or high (500 mM) NaCl concentrations. Any bound protein was eluted with low pH buffer (0.2 M glycine, pH 2.5) and quickly neutralized in the presence of 1.0 M Tris, pH 9.0. Eluted material was pooled, concentrated with Centrion-10 (Millipore), and analyzed by using SDS-PAGE and Western blotting.

Plasmid DNA Constructs—STEP cDNA was constructed in pGEX-2T vector as described previously (21). The GST-STEP open reading frame was amplified by using PCR with primers GST-*KpnI* (5'-CACGGGGTACCACCATGGCCCTATACTAGGTTATTGG-3') and STEP *NotI* (5'-ACGATGAAGCGGCCGCTCACTCTGAGGACTGGAGGGAC-3') (where the italic letters denote the restriction sites for *KpnI* and *NotI* and the underlined letters denote the Kozak sequences), in which *KpnI* and *NotI* restriction sites were added, and cloned into pcDNA3 (Invitrogen). In addition, a Kozak sequence was added within the GST-*KpnI* primer for enhanced expression in mammalian cells. The control vector expressing only GST was also made using the same GST-*KpnI* primer and a different GST-*NotI* primer (5'-ACGATGAAGCGGCCGCTCAGATCCACGCGGAACCAG-3').

Human Fyn cDNA (pCMV-hFyn) was a generous gift of M. D. Resh (Memorial Sloan-Kettering Cancer Center). The open reading frame of Fyn was amplified by PCR and subcloned into pCMV-Myc-tagged vector (Stratagene). Site-directed mutagenesis was performed to generate constitutively active or inactive mutant Fyn by using Turbo *Pfu* DNA polymerase (Stratagene). STEP and Fyn deletion constructs were performed according to instructions (Excite; Stratagene) (see Fig. 1 for DNA constructs). PCR products were cleaned with the Qiagen PCR clean kit, phosphorylated with T4 kinase, and ligated by using T4 ligase (New England Biolab).

Cell Culture and Transfection—HEK293 cells were obtained from American Type Culture Collection (CRL-1573) and maintained in minimum Eagle's medium supplemented with 10% heat-inactivated horse serum. Transfections were performed as described previously (15). In brief, a day before transfection, HEK293 cells were seeded at 1×10^6 cells/60-mm dish. The next day, DNA was mixed with FuGENE 6 (1:3), incubated briefly, and applied directly onto cells.

Immunoprecipitation—All steps were done either on ice or at 4 $^\circ\text{C}$, unless otherwise specified. Transfected cells were solubilized in IP buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM Na₃VO₄, 1% Brij-96, 2 mM EDTA, and 10 $\mu\text{g/ml}$ of both leupeptin and aprotinin). Samples were centrifuged at 10,000 $\times g$ for 10 min. The supernatant was precleared with 50 μl of protein G-Sepharose (Amersham Biosciences) for 1 h and then incubated with 5 μg of anti-myc antibody overnight. An aliquot of 50 μl of protein G-Sepharose was added the next day to the sample and incubated for 1 h. The beads were washed four times in IP buffer. Proteins were eluted with 50 μl of SDS-PAGE buffer and analyzed on 8% SDS-PAGE.

For pull-down experiments, HEK293 cells were co-transfected with pcDNA-GST-STEP, pCVM-hFyn, or control vector pCMV. Thirty-six h after transfection, cells were lysed with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Brij-96, 2 mM EDTA, 0.2 mM sodium vanadate (Na₃VO₄),

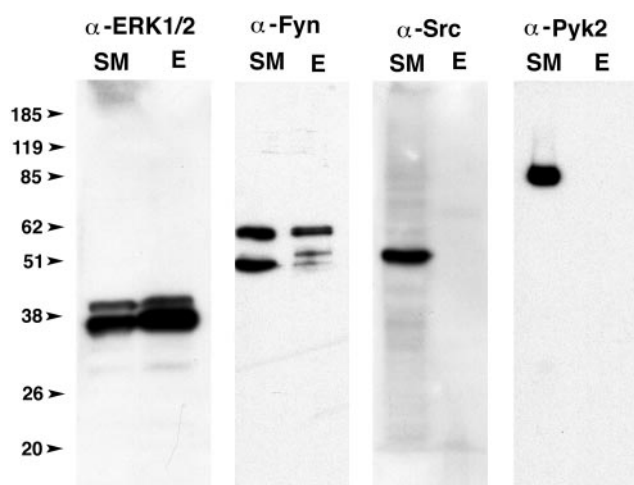


FIG. 2. Fyn binds to STEP₄₆ affinity column chromatography. Rat brain homogenates were passed over and eluted from a STEP₄₆-GST affinity column, and the eluates were processed using SDS-PAGE and Western blot analyses. Antibodies against ERK1/2, Fyn, Src, and Pyk2 were used to compare the amounts of these proteins in both starting material (SM) and eluate (E).

and 10 μ g/ml of both aprotinin and leupeptin. The lysate was vortexed briefly and centrifuged to remove insoluble material. Fifty μ l of glutathione-Sepharose 4 CL beads were added to the supernatants and incubated for 1 h, followed by four washes with lysis buffer. Proteins were eluted from the beads with 50 μ l of SDS sample buffer and boiling.

In Vitro Dephosphorylation—HEK293 cells were transfected with pCMV-myc-Fyn wild-type plasmid DNA. Thirty-six h after transfection, cells were harvested and lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and complete protease inhibitor mixture tablet (Roche Molecular Biochemicals). Immunoprecipitation was performed as described above. After the last wash, antigen-antibody-protein G-Sepharose complex was washed with 50 mM imidazole, pH 7.4, and 5 mM EDTA. Purified STEP₆₁-GST was added to Sepharose beads and incubated at 30 °C for 30 min. Beads were washed once in lysis buffer containing 1 mM Na₃VO₄ and boiled in SDS-PAGE buffer. Samples were analyzed by immunoblotting.

Immunoblotting—SDS-PAGE was performed as described by Laemmli (22). Proteins were blotted onto polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked with 5% nonfat dry milk in PBS-0.05% Tween 20 (PBST) for 30 min and incubated with primary antibody in PBST either overnight at 4 °C or for 1 h at room temperature and then incubated with peroxidase-conjugated secondary antibodies (1:40,000) for 1 h at room temperature. The membrane was reacted with chemiluminescent substrate from Pierce. Anti-Fyn monoclonal antibody (Chemicon) was used at 1:400 with an overnight incubation. Anti-phospho-Src-Y420 antibodies were obtained from Cell Signaling, whereas anti-phospho-Fyn Y531 was obtained from BioSource. The monoclonal antibody against STEP (23E5) was used at 1:1000 in PBST at room temperature as described previously (10, 15). For reprobing, membranes were stripped for 30 min at room temperature with stripping buffer (62.5 mM Tris-HCl, pH 6.8, 100 mM 2-mercaptoethanol, and 2% SDS) and then reacted with chemiluminescent to check for the completeness of stripping.

RESULTS

Fyn Binds to STEP—A substrate trapping strategy was used in an initial attempt to identify physiologically relevant substrates of STEP. Similar strategies have been used to identify proteins that associate with a number of PTPs after critical amino acids are mutated in order to make the PTP enzymatically inactive (23, 24). Rat brain samples were passed over a catalytically inactive STEP₄₆ CS-GST column and washed extensively under moderately stringent conditions (*i.e.* 150 mM NaCl and 1% Triton X-100). Bound proteins were eluted with low pH buffer and analyzed for the presence of STEP-associated proteins. Mitogen-activated protein kinase has previously been reported to associate with STEP and served as a positive control for these experiments (25) (Fig. 2). We next looked for

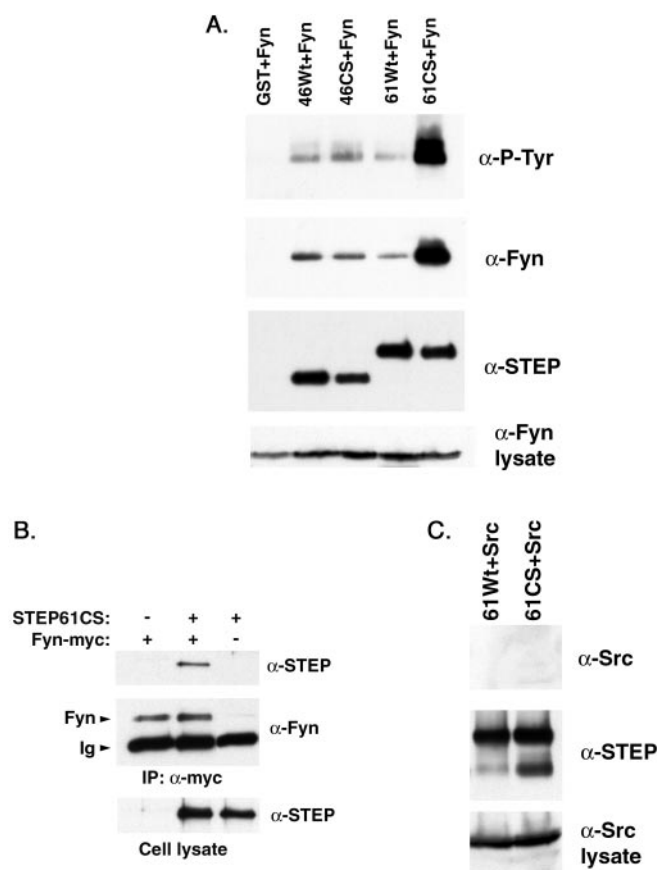


FIG. 3. A, Fyn associates with STEP after co-expression in HEK293 cells. HEK293 cells were co-transfected with Fyn and STEP-GST constructs. Cells were lysed 36 h later, and insoluble material was removed. Glutathione-Sepharose beads were added to the supernatant to pull down STEP-GST fusion proteins. Beads and protein complex were boiled and processed by SDS-PAGE and Western blot analyses. The membrane was initially probed with anti-phosphotyrosine antibody (*top panel*), followed by rabbit anti-Fyn (*second panel*) and a monoclonal antibody against STEPs (*third panel*). The amount of Fyn in the starting material was determined (*bottom panel*). **B, Fyn-myc-pCMV cDNA** was co-transfected with STEP₆₁ CS-pCMV. Fyn-myc was immunoprecipitated with anti-myc monoclonal antibody, and the samples were analyzed for the presence of STEP (*top panel*). Blots were stripped and reprobed for the presence of Fyn (*middle panel*). The amount of STEP in the starting material was determined (*bottom panel*). **C, neuronal Src** was co-transfected with wild-type (61Wt) or inactive (61CS) STEP₆₁-GST. Glutathione-Sepharose beads were used to isolate STEP, and the resultant pellet was analyzed for the presence of Src (*top panel*) and STEP (*middle panel*). The amount of Src in the starting material was determined (*bottom panel*).

the association of STEP with members of the Src kinase family, including Fyn, Src, and Lyn. Western blot analysis determined that, of these proteins, only Fyn was present in the eluate. Pyk2, a calcium-dependent tyrosine kinase present within PSDs, was also analyzed. Parallel analyses indicated that neither Src or Pyk2 bound to the column (Fig. 2). Immunoblotting for Lyn showed results similar to those for Src and Pyk2 (data not shown). These results indicate that under the conditions used in these experiments, Fyn binds to a substrate-trapping construct of STEP.

STEP₆₁ Binds Preferentially to Tyrosine Phosphorylated Fyn—The next experiments determined whether the association of Fyn with STEP could be replicated in cell lines and whether one or another of the various STEP isoforms bound preferentially to Fyn. Various GST-STEP constructs were co-transfected with Fyn into HEK293 cells, and pull-down experiments were performed (Fig. 3A). The results indicate that Fyn was pulled down to some degree by all STEP isoforms (STEP₄₆

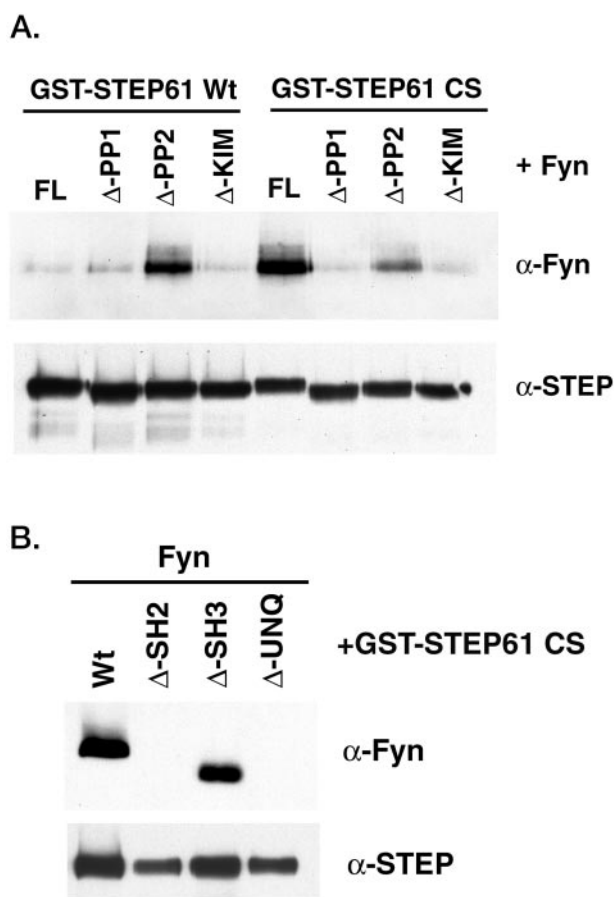


FIG. 4. *A*, the polyproline domain and kinase-interactive motif of STEP are necessary for the interaction of STEP with Fyn. Fyn was co-transfected with different STEP constructs into HEK293 cells. Both wild-type (lanes 1–4) and inactive (lanes 5–8) STEP deletions were used in these experiments as indicated. The deletions were polyproline domain 1 (Δ -PP1), polyproline domain 2 (Δ -PP2), and the kinase-interactive motif (Δ -KIM). Glutathione-Sepharose beads were used to pull down STEP, and the resultant pellets were analyzed for the presence of Fyn. Full-length (FL) STEP₆₁ constructs were used as controls. *B*, the SH2 and the unique domain of Fyn are necessary for the interaction of Fyn with STEP. Full-length Fyn (lane 1) or the deletion mutants Δ -SH2 (lane 2), Δ -SH3 (lane 3), and the unique domain Δ -UNG (lane 4) were co-transfected with STEP₆₁ CS-GST. Glutathione-Sepharose beads were used to pull down STEP, and the resultant pellets were analyzed for the presence of Fyn (top panel) and STEP (bottom panel).

wild-type, the inactive CS mutant, STEP₆₁ wild-type, and its inactive variant). The control GST failed to pull down any Fyn. STEP₄₆ is an alternatively spliced variant within the STEP family of proteins. Both STEP₄₆ and STEP₆₁ isoforms contain the KIM domain. They differ by the presence in STEP₆₁ of a novel 172-amino acid region at the N terminus that contains two transmembrane and two polyproline domains. STEP₄₆ pulling down Fyn in the co-transfected cell lines is consistent with the observation from the STEP₄₆ affinity column chromatography. Moreover, the substrate-trapping, inactive STEP₆₁ variant pulled down more Fyn than its active isoform. In addition, the highest levels of tyrosine-phosphorylated Fyn were associated with the inactive STEP₆₁ CS variant. These results suggest that Fyn associates most strongly with STEP₆₁.

Complementary pull-down experiments were performed. Inactive STEP₆₁ was co-transfected with a myc-tagged Fyn. Anti-myc tag antibody was used to immunoprecipitate myc-Fyn, and the samples were analyzed for the presence of STEP (Fig. 3B). STEP₆₁ CS was detected with the myc tagged-Fyn sample but was not detected in the control lanes. Similar co-transfection of STEP₆₁ and Src was performed. Src was not detected to asso-

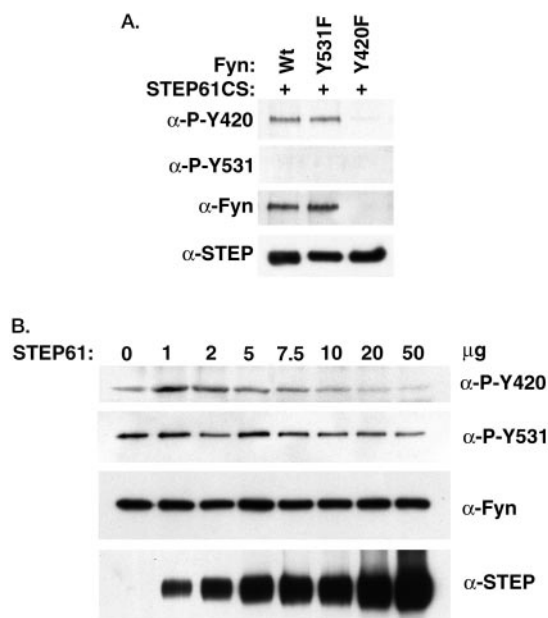


FIG. 5. *A*, STEP₆₁ CS association with Fyn increases when Fyn is phosphorylated at phosphotyrosine 420. STEP₆₁ CS-GST-pcDNA3 was co-transfected with either wild-type Fyn (lane 1), Fyn with a mutation at Y531F (lane 2), or Fyn with a mutation at Y420F (lane 3). Glutathione-Sepharose beads were used to pull down STEP, and the resultant pellets were analyzed for the presence of Fyn. The blot was first incubated with anti-phospho-Tyr⁴²⁰ antibodies (top panel) and then reprobed with anti-phospho-Tyr⁵³¹ antibodies (second panel), followed by anti-Fyn monoclonal antibody (third panel) and, finally, anti-STEP (bottom panel). *B*, STEP₆₁ dephosphorylates Fyn *in vitro* at Tyr⁴²⁰ but not Tyr⁵³¹. HEK293 cells were transfected with myc-tagged Fyn for 36 h. Cells were harvested, lysed, cleared with protein G-Sepharose beads, and immunoprecipitated with anti-myc monoclonal antibody. The resultant pellet was evenly divided into eight samples. Purified active STEP₆₁-His was added to the protein-bead complex for an *in vitro* phosphatase assay. Samples were analyzed on Western blots with anti-phospho-Tyr⁴²⁰ (top panel), anti-phospho-Tyr⁵³¹ (second panel), anti-Fyn (third panel), and anti-STEP (bottom panel).

ciate with either the wild-type or the substrate-trapping mutant (Fig. 3C). Taken together, these results suggest that STEP₆₁ interacts with the tyrosine kinase Fyn.

STEP₆₁ Binds to Fyn through Two Polyproline Domains and the KIM—These experiments were designed to determine the Fyn binding domain in STEP₆₁. cDNA constructs that contained deletions of either of the two polyproline domains (PP1 and PP2) or the KIM domain (see Fig. 1) were co-transfected with Fyn cDNA. The ability of the resulting mutants to associate with Fyn was assessed (Fig. 4A). Full-length inactive STEP₆₁ CS once again associated with Fyn. Deletion of either the PP1 or the KIM domain in the inactive variant decreased the amount of Fyn pulled down. Interestingly, deletion of PP2 in the wild-type variant enhanced the binding of STEP to Fyn. These results suggest that the KIM and polyproline domains are involved in the binding of STEP₆₁ with Fyn.

In a complementary series of experiments, different Fyn domains were deleted and co-transfected with full-length STEP₆₁ CS. As expected, the inactive STEP₆₁ isoform bound with full-length Fyn. It also bound with the Fyn construct that contained an SH3 deletion. The Fyn construct with either an SH2 or the unique domain deletion failed to bind to STEP₆₁ (Fig. 4B). These results suggest that the SH3 domain of Fyn is not critical for the binding to STEP₆₁, whereas the SH2 and the unique domains of Fyn appear to be necessary for the observed interactions with STEP.

STEP₆₁ Dephosphorylates Fyn at Phosphotyrosine 420—Two regulatory tyrosines are phosphorylated in Fyn and regulate

its activity. These two tyrosine residues serve as potential targets for STEP. In an initial series of experiments, we compared the relative association of the substrate-trapping STEP₆₁ CS isoform to wild-type Fyn, compared with its association to the Fyn variants that contain mutations at either tyrosine 420 (Y420F) or tyrosine 531 (Y531F).

After co-transfection, STEP₆₁ fusion protein was pulled down and analyzed for the presence of Fyn (Fig. 5A). For these experiments, antibodies were used that specifically recognize each of the tyrosine-phosphorylated sites. STEP₆₁ CS pulled down wild-type Fyn and the Y531F mutant. The latter construct is not phosphorylated at the 531 site but is constitutively phosphorylated at the Tyr⁴²⁰ residue (Fig. 5A, *top panel*). In contrast, when tyrosine 420 was mutated, STEP₆₁ was no longer able to associate with Fyn (Fig. 5A, *bottom panel*). These results suggest that phosphorylation at tyrosine 420 of Fyn is necessary for the observed association with the substrate-trapping STEP₆₁ CS variant.

We next determined whether Fyn was preferentially dephosphorylated at either of these sites by STEP. These experiments were performed in the presence of EDTA to prevent autophosphorylation of Fyn. Active GST-STEP₆₁ fusion protein was added to the immunoprecipitated wild-type myc-Fyn. The phosphotyrosine level at each site was then detected by using anti-phospho-Tyr⁴²⁰ and anti-phospho-Tyr⁵³¹ antibodies (Fig. 5B). Anti-Fyn was used to demonstrate equal loading in each lane, and anti-STEP was used to show the increasing amounts of STEP₆₁ fusion protein. These results indicate a clear decrease in phospho-Tyr⁴²⁰ levels with increasing amounts of STEP protein. In contrast, there was no difference in phospho-Tyr⁵³¹ levels, suggesting that STEP₆₁ is specifically recognizing and dephosphorylating phospho-Tyr⁴²⁰ *in vitro*.

DISCUSSION

Glutamate is the major excitatory neurotransmitter in the central nervous system (26). The NMDA receptor is one of several glutamate-responsive receptors that have been implicated in key signaling pathways including synaptic plasticity, neuronal development, and excitotoxicity (27–29).

Tyrosine phosphorylation of NMDA receptors is one mechanism by which the activity of these receptors can be regulated. Tyrosine phosphorylation up-regulates NMDA receptors and increases ion flow through these channels. Several members of the Src family of tyrosine kinases have been implicated in these processes, including Src, Fyn, and abl (6, 30–34). These kinases are themselves phosphorylated on two regulatory tyrosine residues that have opposing functions (one inhibits kinase activity, and the other enhances kinase activity). Thus, the identification of tyrosine phosphatases that act specifically on one or the other phosphorylation site would add an additional degree of control of the kinases involved in tyrosine-phosphorylating key regulatory proteins, receptors, and other substrates within the PSD.

The major finding in the present study was that STEP associated with Fyn and dephosphorylated one of its two regulatory tyrosine residues (Tyr⁴²⁰). Several lines of evidence support this model. The initial affinity chromatography experiment using rat brain homogenates as the starting material showed that Fyn bound to a substrate-trapping variant of STEP. A positive control for the experiment was finding that ERK1/2 also bound to the column because these proteins had previously been shown to interact directly with STEP (12, 25). In the present study, the eluate was further analyzed for the specificity of the results by looking for other kinase members also present in PSDs, such as Src, Lyn, or Pyk2; these proteins are not detected.

By using co-transfection experiments, we demonstrated that STEP and Fyn interact in mammalian cells. These experiments show that some STEP isoforms associate more strongly than others. In particular, STEP₆₁ pulled down tyrosine-phosphorylated Fyn more effectively than STEP₄₆. The complementary series of experiments pulled down myc-tagged Fyn and demonstrate a similar interaction with STEP₆₁. The substrate-trapping variant of STEP₆₁ bound to the wild-type and to the Y531F form of Fyn, but not the Y420F isoform, suggesting that under the conditions used in these experiments, there is some degree of specificity in that STEP requires one but not the other tyrosine residue to be present and phosphorylated in order to bind to Fyn.

We also mapped specific binding domains within STEP₆₁ and Fyn. Both the N-terminal proline-rich domain (PP1) and the KIM domain in STEP₆₁ appear to be necessary for binding because constructs with a deletion of either fail to interact with Fyn. The KIM domain had previously been identified as a binding domain in STEP required for association with mitogen-activated protein kinase (12). The present results suggest that this domain may also be necessary for interaction with additional proteins, including the tyrosine kinase Fyn.

The domains in Fyn that are necessary for binding to STEP₆₁ appear to include the SH2 and the unique N-terminal domains. When the unique domain of Fyn is deleted, Fyn fails to associate with STEP₆₁. This observation is in line with our initial experiment indicating that both Src and Lyn with different unique domains fail to associate with STEP isoforms.

These results suggest that Fyn is in an active state or “open” conformation when associated with STEP. In an inactive state or “closed” conformation, the SH2 domain binds to its phosphorylated Tyr⁵³¹ through an intramolecular association. Upon stimulation, the phosphorylated Tyr⁵³¹ is dephosphorylated by a nearby PTP. Fyn autophosphorylates the Tyr⁴²⁰ site and releases the SH2 domain from intramolecular association. Subsequently, active STEP₆₁ dephosphorylates Tyr⁴²⁰. This permits the free SH2 domain to bind to other phospho-tyrosine and possibly to the PTP catalytic domain of STEP (35, 36). The PP1 domain and the KIM motif of STEP₆₁ stabilize the association between STEP and Fyn.

In summary, our results suggest that STEP binds to Fyn and specifically dephosphorylates the regulatory tyrosine at amino acid 420. A recent finding demonstrated that PTP α also binds to Fyn but in this case specifically dephosphorylates Tyr⁵³¹ (20). The binding of PTP α to Fyn is independent of its phosphatase domain and requires an interaction between tyrosine-phosphorylated PTP α and the SH2 domain of Fyn (19, 37). It remains to be determined whether the further regulation of Fyn by STEP described in this study is responsible for the regulation of NMDA receptors at the PSD.

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