Characterization of Fyn-mediated tyrosine phosphorylation sites on GluRε2 (NR2B) subunit of the $N$-methyl-$D$-aspartate receptor

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The abbreviations used are: NMDA, $N$-methyl-$D$-aspartate; GluR, glutamate receptor; NR1, NMDA receptor subunit 1; PTK, protein tyrosine kinase; LTP, long-term potentiation; EPSP, excitatory postsynaptic potential; BAP, bacterial alkaline phosphatase; PY, phosphotyrosine; HA, influenza hemagglutinin; GST, glutathione-S-transferase.
SUMMARY

The N-methyl-d-aspartate (NMDA) receptors play critical roles in synaptic plasticity, neuronal development, and excitotoxicity. Tyrosine phosphorylation of NMDA receptors by Src-family tyrosine kinases such as Fyn is implicated in synaptic plasticity. To precisely address the roles of NMDA receptor tyrosine phosphorylation, we identified Fyn-mediated phosphorylation sites on the GluRε2 (NR2B) subunit of NMDA receptors. Seven out of 25 tyrosine residues in the C-terminal cytoplasmic region of GluRε2 were phosphorylated by Fyn in vitro. Of these seven residues, Tyr1252, Tyr1336, and Tyr1472 in GluRε2 were phosphorylated in human embryonic kidney fibroblasts when co-expressed with active Fyn, and Tyr1472 was the major phosphorylation site in this system. We then generated rabbit polyclonal antibodies specific to Tyr1472-phosphorylated GluRε2, and showed that Tyr1472 of GluRε2 was indeed phosphorylated in murine brain using the antibodies. Importantly, Tyr1472 phosphorylation was greatly reduced in fyn mutant mice. Moreover, Tyr1472 phosphorylation became evident when hippocampal long-term potentiation (LTP) started to be observed and its magnitude became larger in murine brain. Finally, Tyr1472 phosphorylation was significantly enhanced after induction of LTP in the hippocampal CA1 region. These data suggest that Tyr1472 phosphorylation of GluRε2 is important for synaptic plasticity.

INTRODUCTION

The N-methyl-d-aspartate (NMDA) subtype of excitatory glutamate receptors (GluRs)
play central roles in synaptic plasticity (1), neuronal development (2), and excitotoxicity (3). The receptors are formed by assembly of two classes of subunits, a principal subunit GluRζ1 (NR1 in rat) and modulatory subunits GluRε1–GluRε4 (NR2A-NR2D in rat) (4-6). The GluRζ1 subunit is essential for the function of NMDA receptor channels, whereas GluRε1–GluRε4 subunits determine the characteristics of NMDA receptor channels by forming different heteromeric configurations with the GluRζ1 subunit (4-6). The physiological importance of these subunits has been demonstrated by gene targeting. Mice with a null-mutation in GluRζ1 die neonatally (7, 8). Mutant mice in which GluRζ1 is deleted only in hippocampal CA1 pyramidal cells reach adulthood but lack NMDA receptor-mediated postsynaptic currents and long-term potentiation (LTP) in the hippocampal CA1 region (9). In GluRε1 mutant mice, hippocampal LTP is reduced, and spatial learning is impaired (10). Disruption of GluRε2 results in neonatal death and impairment of hippocampal long-term depression (11).

GluRε subunits have unusually long C-terminal tails that are extended into the cytoplasm (4-6). Several PDZ domain-containing proteins, such as PSD95 and chapsyn-110/PSD93, interact with the NMDA receptor through the C-terminal PDZ binding motif (12). Importance of the C-terminal tails of GluRε subunits is also demonstrated by gene targeting. Phenotypic defects in mice expressing C-terminally truncated GluRε1 or GluRε2 are similar to those in mice lacking entire GluRε1 or GluRε2 (13, 14). C-terminal tails of GluRε subunits are likely to participate in their synaptic localization or regulation of NMDA receptor functions. Furthermore, GluRε2 is phosphorylated at its C-terminal tails by Src-family of nonreceptor protein-tyrosine kinases, such as Fyn, cyclic AMP-dependent protein kinase, protein kinase C, and calcium/calmodulin-dependent protein kinase II (CaMKII) (15-19). However, the physiological roles of these phosphorylation events remain unclear.
In the NMDA receptor complex, GluRε1, GluRε2, and GluRε4 are tyrosine-phosphorylated in the brain (20-22), and Fyn significantly contributes to phosphorylation of GluRε1 and GluRε2 (15, 18, 19). Among these subunits, GluRε2 is the major tyrosine-phosphorylated protein in the forebrain synapse (21). Protein tyrosine phosphorylation regulates NMDA channel receptor activity. NMDA receptor-mediated currents are potentiated by Src-family tyrosine kinases and suppressed by tyrosine phosphatases (23, 24). In addition, taste-learning increases tyrosine phosphorylation of GluRε2 in the rat insular cortex and tyrosine phosphorylation of GluRε2 is increased after induction of LTP in the dentate gyrus of anesthetized adult rats (25-27). It is reported that Src and Fyn do not potentiate the current through recombinant GluRζ1–GluRε2 channels in 293 cells (28). The p85 subunit of phosphatidylinositol 3-kinase, phospholipase Cγ, SHP2, and brain spectrin interact with GluRε2 in a tyrosine phosphorylation-dependent manner (15, 29-31). These data suggest that GluRε2 tyrosine phosphorylation may at least in part be involved in intracellular signaling in murine brain.

The physiological importance of Fyn in the nervous system has been suggested by analyses of fyn-mutant mice. These mice show various neural defects including defective LTP, impaired spatial memory, impaired myelination, and altered ethanol sensitivity (32-34). In addition, up-regulation of Src is observed after spatial maze learning, suggesting involvement of other Src-family kinases in synaptic plasticity (35). In this paper, to precisely understand the roles of tyrosine phosphorylation of GluRε2 by the Src-family kinases, we set out experiments in which Fyn-mediated tyrosine phosphorylation sites of GluRε2 were determined. By showing that Tyr1472 of GluRε2 is phosphorylated in murine brain, we propose that Tyr1472 phosphorylation plays important roles for synaptic plasticity.
EXPERIMENTAL PROCEDURES

Antibodies—Rabbit polyclonal antibodies against GluRε2 were raised against a glutathione-S-transferase (GST) fusion protein with mouse GluRε2 (amino acids 1034-1119) and affinity purified. The anti-GluRε2 antibodies did not cross-react with rat
NR2A (data not shown). Rabbit polyclonal antibodies against phospho-Tyr1472 of GluRε2 were raised using a keyhole limpet hemocyanin-conjugated synthetic peptide with the sequence CSNGHV(phosphoY)EKLSSI as immunogen. The antibodies were purified from sera of the immunized rabbits by successive affinity chromatography, using a column of NHS-activated Sepharose 4B resin (Amersham Pharmacia Biotech) conjugated to the GST-C3 protein (to subtract IgGs against non-phosphorylated GluRε2) followed by a column conjugated to the immunogen. The antibodies did not recognize NR2A (GluRε1) expressed in 293T cells together with active Fyn (data not shown). Anti-influenza hemagglutinin (HA) monoclonal antibody (mAb) (12CA5) was purchased from Roche Molecular Biochemicals. Anti-phosphotyrosine mAb (RC20) and anti-NR2B mAb were from Transduction Laboratories. Rabbit anti-Fyn (Fyn3) and rabbit anti-NR2B antibodies were from Santa Cruz Biotechnology and Chemicon, respectively.

Baculoviral expression and purification of GST-Fyn-Sf9 insect cells were maintained in Sf-900 medium (GIBCO) containing 10% fetal bovine serum at 27 °C. Adherent cells were infected with recombinant baculovirus carrying the GST-human Fyn cDNA. 72 h after infection, cells were lysed in TNE buffer [1% (wt/vol) Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 5mM EDTA, 0.2 mM Na3VO4, with aprotinin at 50 units/ml]. GST-Fyn fusion protein was purified on glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the supplier’s instruction.

Purification of GST-C1, C2, and C3 proteins-pGEX-C1, C2, and C3 were described previously (15). Fusion proteins were expressed in Escherichia coli BL21. Purification of GST fusion proteins was described above.

Tryptic peptide mapping analysis-To prepare the in vitro phosphorylated GluRε2 proteins, the GST-GluRε2 fusion proteins (2 µg) were phosphorylated by 1 µg of baculovirally expressed and purified GST-Fyn in 40 µl kinase buffer (20 mM Hepes-NaOH (pH 7.2), 10
mM MgCl₂, 3 mM MnCl₂) in the presence of 100 • M ATP and 5 µCi of [γ⁻³²P]ATP at 30 •C for 30 min. The reactions were terminated by the addition of 20 µl of 3 × Laemmli sample buffer and then resolved by 10% SDS-polyacrylamide gel. ³²P-labeled GST-GluRe2 proteins were excised from the gel and then subjected to peptide mapping analysis according to Boyle et al. (36). Briefly, tryptic peptide samples were electrophoresed for 40 min at 1.0 kV in pH 1.9 buffer using the HTLE7000 apparatus (CBS Scientific); the plates were air dried and then placed in tanks for ascending chromatography using phosphochromatography buffer. After ascending chromatography, the plates were air dried, and then exposed.

Construction of cDNAs-For epitope-tagging of GluRe2, GluRe2 cDNA (4) was inserted with the oligonucleotides encoding an HA-epitope-containing sequence DYPYDVPDYASLV at XmaI site that encoded amino acid residues 66 and 67. The resultant cDNA, HA-GluRe2, was subcloned to pME18S (37). The expression plasmids pME-FynY531F and PSD-95 were described previously (19, 37). Various YF mutants of GluRe2 were generated by oligonucleotide-mediated site-directed mutagenesis (38). Mutations were verified by dideoxynucleotide sequencing.

Cell culture and transient transfection-HEK 293T cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 •C in 5% CO₂. Cells (1.5 × 10⁶) were transfected with combinations of expression plasmids (5 µg each) by the standard calcium phosphate method. The amount of DNA transfected was adjusted in each experiment by using a control expression vector pME18S (37). Two days after transfection, cells were collected for protein extraction.

Preparation of lysates, immunoprecipitation, and immunoblotting-For preparation of lysates of 293T cells, cells were washed with phosphate-buffered saline, and then lysed with 1ml of TNE buffer. Typically, 800 µl of lysates were used for immunoprecipitation.
For preparation of whole-cell lysates of telencephalons, samples were homogenized in 0.2 vol. (ml/g tissue) of RIPA buffer [1% (wt/vol) Nonidet P-40, 1% (wt/vol) sodium deoxycholate, 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 5 mM EDTA, 0.2 mM/Na,VO₄, with aprotinin at 50 units/ml] containing 0.5% SDS. The lysates were boiled for 5 min to dissociate the NMDA receptor complex and diluted with 4 vol. of RIPA buffer. For immunoprecipitation, lysates were cleared by centrifugation with an excess amount of Protein G Sepharose (Amersham Pharmacia Biotech) and then incubated with indicated antibodies on ice for 1 h. Immune complexes were collected on Protein G Sepharose and washed five times with lysis buffer. Immunoprecipitates or lysates were resolved by SDS/7.5% PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). Then the membranes were blocked and probed with antibodies indicated. When necessary, the antibodies were stripped from the membranes by incubation in 62.5 mM Tris (pH 7.4), 2% SDS and 0.7% 2-mercapto-ethanol at 60°C for 40 min, then the membranes were reprobed with antibodies indicated. For quantification, the immunoreacted protein bands were analyzed with NIH image software.

Phosphatase treatment-100 µl of brain lysates from wild-type and fyn-mutant mice, which contained about 150 µg of proteins, were immunoprecipitated with anti-GluRε2 antibodies. Immune complexes were collected on Protein G Sepharose (Amersham Pharmacia Biotech) and washed five times with lysis buffer, followed by washing twice with bacterial alkaline phosphatase (BAP) buffer [Tris-HCl (pH 9.0), 1 mM MgCl₂]. Bound proteins were incubated in 100 µl of the BAP buffer with or without 5 units of BAP (Takara) at 37°C for 6 h. After the BAP reaction, the beads were washed 3 times and then subjected to Western blot analysis.

Electrophysiology-Extracellular field potential recordings were performed essentially as described (39). Hippocampal slices (400 µm thick) were prepared from 6- to 8-week old
mice and placed in an interface-type holding chamber for at least 1 h. A slice was then transferred to the recording chamber and submerged beneath continuously perfusing artificial cerebrospinal fluid (119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO₄, 2.5 mM CaCl₂, 1.0 mM NaH₂PO₄, 26.2 mM NaH₂CO₃, 11 mM glucose) that had been saturated with 95% O₂ and 5% CO₂. All the perfusing solutions contained 100 μM picrotoxin to block GABAₐ receptor-mediated inhibitory synaptic responses. The CA3 region was surgically separated from the CA1 region to prevent invasion of epileptiform activity. A glass recording electrode (containing 3 M NaCl) and a tungsten bipolar stimulating electrode were placed in the stratum radiatum. The test stimulation was applied to Schaffer collateral fibers at 0.1 Hz. The stimulus strength was adjusted to get the initial excitatory postsynaptic potential (EPSP) slope value of 0.10-0.15 mV/ms. To induce LTP, we applied four trains of tetanic stimulation (100 Hz for 1 s) at an interval of 10 s. Axopatch 1D amplifier (Axon Instruments) was used, and the signal was filtered at 1 kHz, digitized at 10 kHz, and stored in an IBM-compatible computer equipped with a TL-1 DMA analog-to-digital board (Axon Instruments). All experiments were done at 25°C. All data are presented as the means ± SEMs. A statistical evaluation was made by use of paired t test.

RESULTS

Phosphopeptide mapping of the C-terminal cytoplasmic region of the GluRε2 subunit in vitro-The GluRε2 subunit contains 25 tyrosine residues in the intracellular C-terminal region. To determine the site(s) of Fyn-mediated GluRε2 phosphorylation, we constructed glutathione-S-transferase (GST) fusion proteins containing truncated segments of the intracellular C-terminal region of the GluRε2 subunit (termed GST-C1, C2, and C3 proteins) (Fig. 1A). GST-C1, C2, and C3 fusion proteins were bacterially expressed, purified, and then phosphorylated in vitro with baculovirally expressed GST-Fyn in the
presence of [γ-32P]ATP. The phosphorylated proteins were subjected to tryptic phosphopeptide mapping. As shown in Fig. 1, highly phosphorylated peptides P1-P4, P5 and P6, and P7-P9 were generated from GST-C1, GST-C2, and GST-C3 fusion proteins, respectively. To identify the tyrosine residues that were phosphorylated in GST-C3 protein, we constructed Y1252F (conversion of Tyr1252 to Phe1252)-, Y1336F-, and Y1472F-GST-C3 proteins by site-directed mutagenesis. Conversion of Tyr1472 to Phe1472 resulted in generation of a tryptic phosphopeptide map that lacked phosphopeptide P9 (Fig. 1C). Similarly, phosphopeptide P7 was absent in the phosphopeptide map of Y1252F-GST-C3 protein, and phosphopeptide P8 was absent in the phosphopeptide map of Y1336F-GST-C3 protein (data not shown). Two-dimensional tryptic phosphopeptide map of GST-C1 fusion protein displayed highly phosphorylated peptides, P1-P4 (Fig. 1D), and map of GST-C2 fusion protein showed highly phosphorylated peptides P5 and P6 (Fig. 1E). Phosphopeptides corresponding to P1-P4 were absent in two-dimensional tryptic phosphopeptide maps of GST-C1 having Y932F, Y1039F, Y1070F, or Y1109F mutations, and phosphopeptides corresponding to P5 and P6 were missing in maps of GST-C2 having Y1109F or Y1252F mutations (data not shown). These results indicate that Tyr932, Tyr1039, Tyr1070, Tyr1109, Tyr1252, Tyr1336, and Tyr1472 are Fyn-mediated phosphorylation sites in GluRε2 in vitro.

**Tyr1472 as the principal Fyn-mediated phosphorylation site in human embryonic kidney (HEK) 293T cells**-In vivo phosphorylation of the seven tyrosine residues was examined using GluRε2 transfected HEK 293T cells. First, 293T cells were transfected with an expression plasmid encoding HA-tagged wild-type GluRε2 alone or together with plasmids encoding Fyn Y531F, which is a constitutively active form of Fyn (37), and PSD-95. PSD-95 promotes Fyn-mediated tyrosine phosphorylation of GluRε2 (data not shown) as well as NR2A (GluRε1) (19). The cells were lysed, and HA-tagged GluRε2 was immunoprecipitated from
cellular lysates and subjected to immunoblotting with an anti-phosphotyrosine (PY) antibody. GluRε2 was prominently tyrosine-phosphorylated only when it was co-expressed with Fyn Y531F (Fig. 2A). Next, 293T cells were transfected with expression plasmids for either HA-tagged wild-type GluRε2 or one of the seven single YF mutants of GluRε2 described above together with Fyn Y531F and PSD-95 expression plasmids. As shown in figure 2B, Y1472F mutation resulted in the significant reduction of the tyrosine-phosphorylation level of GluRε2. There was no reduction in the tyrosine phosphorylation levels of GluRε2 in the other mutants. Moreover, phosphorylation of GluRε2 Y1252F/Y1472F and Y1336F/Y1472F double mutants was less than that of GluRε2 Y1472F mutant (Fig. 2C lanes 2, 3, and 4), and phosphorylation of GluRε2 Y1252F/Y1336F/Y1472F triple mutant was nearly eliminated in 293T cells (Fig. 2C lanes 1 and 5). These results suggest that Tyr1252, Tyr1336, and Tyr1472 of GluRε2 are phosphorylated in 293T cells when active Fyn is co-expressed. Phosphorylation of four other residues, Tyr932, Tyr1039, Tyr1070, and Tyr1109 was under detectable level.

Characterization of antibodies against Tyr1472-phosphorylated GluRε2-Antisera that recognize Tyr1472-phosphorylated GluRε2 were raised by immunizing rabbits with phosphotyrosine-containing synthetic peptides corresponding to the amino acid sequence of GluRε2 surrounding Tyr1472 (Fig. 3A). Antisera were extensively preabsorbed with non-phosphorylated GST-C3 fusion protein that contains Tyr1472 and then affinity-purified. The purified antibodies, termed anti-phospho-Tyr1472 antibodies, showed selective immunoreactivity with GST-C3 protein phosphorylated by Fyn in vitro but not to non-phosphorylated GST-C3 protein and phosphorylated GST-C3 proteins treated with bacterial alkaline phosphatase (BAP) (Fig. 3B). Immunoreactivity was blocked by preincubation of the antibodies with the antigen (data not shown). To examine whether the purified antibodies recognize Tyr1472-phosphorylated GluRε2, 293T cells were transfected with expression plasmids encoding Fyn Y531F, PSD-95, and either HA-tagged wild-type
GluRε2 or GluRε2 Y1472F mutant. Western blots of HA-tagged GluRε2 immunoprecipitates with anti-phospho-Tyr1472 antibodies showed their specific reactivity with tyrosine-phosphorylated wild-type GluRε2 but not with tyrosine-phosphorylated GluRε2 Y1472F mutant or non-phosphorylated GluRε2 (Fig. 3C). The level of tyrosine phosphorylation of GluRε2 Y1472F mutant was lower than that of wild-type GluRε2, but was clearly detectable using anti-PY antibody (Fig. 2C and data not shown). The amount of immunoprecipitated GluRε2 and the expression levels of Fyn and PSD-95 were similar in each experiment (Fig. 3C and data not shown). Thus, the anti-phospho-Tyr1472 antibodies specifically recognized Tyr1472-phosphorylated GluRε2. The antibodies did not recognize tyrosine-phosphorylated NR2A (GluRε1) expressed in 293T cells (data not shown).

*Phosphorylation of Tyr1472 of GluRε2 in murine brain*- To examine whether Tyr1472 of GluRε2 is phosphorylated in murine brain, GluRε2 was immunoprecipitated from telencephalons, which had been boiled to dissociate the NMDA receptor complex, and subjected to immunoblotting with anti-phospho-Tyr1472 antibodies. The antibodies reacted with a protein corresponding to Tyr1472-phosphorylated GluRε2, but this immunoreactivity was completely abolished when immunoprecipitated GluRε2 were extensively dephosphorylated with BAP (Fig. 4A). The amount of GluRε2 in each lane was similar. The results demonstrated that GluRε2 was phosphorylated at Tyr1472 in murine brain. Importantly, the immunoreactivity of anti-phospho-Tyr1472 antibodies for immunoprecipitated GluRε2 from telencephalon of *fyn*-mutant mice was much less than that from wild-type mice (Fig. 4B). Therefore, we concluded that Tyr1472 of GluRε2 was phosphorylated in murine brain and that Fyn contributed significantly to the phosphorylation events.

Because expression of GluRε2 is developmentally regulated (40), the profile of Tyr1472 phosphorylation during postnatal development was examined. GluRε2 immunoprecipitates from lysates of telencephalons of mice at postnatal (P) days 3, 7, 16, 28, and 56 were
subjected to immunoblotting with anti-phospho-Tyr1472 antibodies and anti-phosphotyrosine antibody. The level of Tyr1472 phosphorylation was low at P3 and P7 but gradually increased at P16, P28, and P56 (Fig. 5). Overall tyrosine phosphorylation of GluRε2 during the same developmental period, which was determined by blotting with an anti-PY antibody, was similar to Tyr1472 phosphorylation. Similar amount of immunoprecipitated GluRε2 was loaded in each lane. Results suggest that phosphorylation of Tyr1472 of GluRε2 in murine brain is developmentally regulated.

*Increase in phosphorylation of Tyr1472 of GluRε2 after LTP induction in the hippocampal CA1 region*- LTP in the CA1 region of the hippocampus, which is a cellular model for learning and memory, is induced by activation of postsynaptic NMDA receptors (41, 42). Because tyrosine phosphorylation of postsynaptic NMDA receptors is implicated in the expression of LTP at the Schaffer collateral-commissural-CA1 synapse (43), alteration in the level of Tyr1472 phosphorylation after induction of LTP was examined. Excitatory postsynaptic potentials (EPSPs) were recorded in the hippocampal CA1 region by extracellular field potential recording techniques. Tetanic stimulation of the afferent fibers (100 Hz for 1 s, repeated 4 times at 10-sec intervals) gave rise to LTP of excitatory synaptic transmission in C57BL/6 mice (184.4 ± 8.8% of baseline, n=10) (Fig. 6A). Sixty minutes after tetanic stimulation, the ratio of the Tyr1472 phosphorylation of GluRε2 in the stimulated slices to that in the non-stimulated slices was 1.50 ± 0.13 (p < 0.002) (Fig. 6C and representative data are shown in Fig. 6B). Five minutes after tetanic stimulation, the level of Tyr1472 phosphorylation of GluRε2 in the stimulated slices was almost the same as that in the control slices (data not shown). The data suggest that Tyr1472 phosphorylation of GluRε2 is involved in the expression of LTP.
DISCUSSION

Here we showed that Tyr1472 of GluRε2, the most prominently phosphorylated site in 293T cells, was phosphorylated in murine brain and that Tyr1472 phosphorylation was significantly reduced in fyn-mutant mice. We also showed that the level of Tyr1472 phosphorylation was developmentally regulated and enhanced after induction of LTP. We found that 7 out of 25 tyrosine residues in the intracellular C-terminal region of GluRε2 were significantly phosphorylated by Fyn in vitro. Among these residues, Tyr1252, Tyr1336, and Tyr1472 of GluRε2 were phosphorylated in HEK 293T cells when active Fyn was co-expressed. Tyr1472 was the most prominently phosphorylated site in this system. Moreover, using anti-phospho-Tyr1472 antibodies, we showed that Tyr1472 of GluRε2 was phosphorylated in murine brain. When examined in vitro, the level of Tyr1472 phosphorylation was similar, relative to that of other major Fyn-mediated phosphorylation sites. However in 293T cells,
active Fyn mainly phosphorylated Tyr1472 in comparison to other six tyrosine residues identified in vitro. This difference may be due in part to an excess phosphorylation reaction in vitro or the presence of protein tyrosine phosphatases in 293T cells. To our knowledge, Tyr1472 of GluRe2 is a residue identified firstly as a tyrosine phosphorylation site of NMDA receptors. There are cognate sites to Tyr1252, Tyr1336, and Tyr1472 of GluRe2 in GluRe1 (4-6). To further understand how tyrosine phosphorylation regulates NMDA receptor function, determination of Fyn-mediated phosphorylation sites of GluRe1 is also important.

The best characterized form of LTP occurs in the hippocampal CA1 region. LTP is initiated by transient activation of NMDA receptors and is expressed as a persistent increase in synaptic transmission through α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (41, 42). Induction of LTP produces rapid activation of Src within 1-5 minutes and prior application of Src-specific inhibitors prevents induction of LTP in this region (43). We observed enhancement of Tyr1472 phosphorylation of GluRe2 after 60 minutes of LTP induction. This suggests that Src-family kinases may phosphorylate GluRe2 slowly, similar to the phosphorylation of the GluR1 subunit of AMPA receptors by CaMKII (44). Although CaMKII is activated within 1 minute after induction of LTP (45), the potentiation of AMPA receptor-mediated currents by CaMKII reaches a maximum 15-30 minutes after induction of LTP (44, 46, 47). Similar changes are seen in the overall NR2B (GluRe2) tyrosine phosphorylation, which is enhanced 15 minutes after induction of LTP in the dentate gyrus of anesthetized adult rats (26, 27). Moreover, we observed that the level of Tyr1472 phosphorylation as well as overall tyrosine phosphorylation of GluRe2 was low during embryonic (data not shown) and early developmental stages (P3 and P7). This may be partly due to the low expression of PSD-95, which promotes Fyn-mediated phosphorylation of GluRe2 as well as GluRe1 (NR2A) (19), during the early developmental stages (48). The low level of Tyr1472 phosphorylation in P3 and P7 mice may cause small LTP in the early
developmental stages (49, 50). The level of Tyr1472 phosphorylation was significantly reduced in fyn-mutant mice, which show impaired hippocampal LTP and spatial learning (32), suggesting that reduced Tyr1472 phosphorylation of GluRε2 may partly explain the defects in LTP and spatial learning in fyn-mutant mice. These observations suggest that Tyr1472 phosphorylation may be required for the expression of LTP and, therefore, is important for synaptic plasticity in the hippocampus. Since NMDA receptors are phosphorylated not only by Src-family kinases but also serine/threonine kinases such as PKC, PKA, and CaMKII (15-19), phosphorylation of GluRε2 by various kinases may be involved in multiple forms of synaptic plasticity as in the case of GluR1 in which Ser831 phosphorylation of CaMKII and Ser845 phosphorylation by PKA differentially contribute to hippocampal synaptic plasticity (51).

It is reported that Src-family kinases do not potentiate recombinant NR1-NR2B channels (GluRζ1–GluRε2 channels in mice) expressed in HEK 293 cells (28). However, since NMDA receptor protein complexes from murine brain are composed of a variety of postsynaptic proteins (52), some of which are missing in HEK 293 cells, contribution of Tyr1472 phosphorylation of GluRε2 to the channel activity of NMDA receptor in vivo should be examined. As protein tyrosine phosphorylation regulates protein-protein interactions (53), Tyr1472 phosphorylation by Src-family kinases may induce intracellular signal transduction pathways by recruiting Src homology 2-containing proteins (15). This might contribute to the biochemical changes required for modulation of synaptic transmission and synaptic plasticity. Indeed, many signaling pathways regulated by tyrosine kinases, such as the Ras-mitogen-activated protein kinase pathway, are involved in modulation of synaptic transmission and long-term memory (54). In addition, clustering of receptors at the postsynaptic membrane is crucial for rapid and efficient synaptic signaling (55). Acetylcholine receptor clustering at neuromuscular junctions is regulated by tyrosine phosphorylation of the
receptor (56). Similarly, NMDA receptor clustering at synapses may be regulated by Tyr1472 phosphorylation of GluRε2.

In summary, we report Fyn-mediated phosphorylation sites of GluRε2 showing that Tyr1472 of GluRε2 is a major phosphorylation site. Our data suggest that Tyr1472 phosphorylation may modulate hippocampal synaptic plasticity. In order to further establish the physiological importance of Tyr1472 phosphorylation of GluRε2, it is interesting to investigate electrophysiological and behavioral changes in GluRε2 Y1472F knock-in mice where Y1472F mutant of GluRε2 replaces wild-type GluRε2.

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Legends to figures

FIG. 1. **Identification of tyrosine residues of GluRε2 phosphorylated by Fyn in vitro.**

A, Schematic diagram of GST-fusion proteins containing the intracellular C-terminal region of GluRε2. B, C, D, and E, Two-dimensional tryptic phosphopeptide maps of GST-C3 (B), GST-C3 Y1472F mutant (C), GST-C1 (D), and GST-C2 (E). Purified GST-fusion proteins were phosphorylated by GST-Fyn in vitro. The phosphorylated proteins were separated by SDS-polyacrylamide gel electrophoresis, excised from the gels, and digested with trypsin. The resulting tryptic peptides were separated in the first dimension by electrophoresis and in the second dimension by chromatography, as indicated by the arrows. The dot in each map shows the origin of electrophoresis. The phosphopeptides in the individual maps are indicated by P1-P9.

FIG. 2. **Phosphorylation of Tyr1252, Tyr1336, and Tyr1472 of GluRε2 by active Fyn in HEK 293T cells.**

A, Tyrosine phosphorylation of GluRε2 by active Fyn in 293T cells. B, Identification of Tyr1472 as the major Fyn-mediated phosphorylation site in 293T cells. C, Phosphorylation of Tyr1252, Tyr1336, and Tyr1472 by active Fyn in 293T cells. 293T cells were transfected with combinations of expression plasmids for GluRε2, various GluRε2 YF mutants, PSD-95, and FynY531F. The cells were lysed in TNE buffer. GluRε2 immunoprecipitates (IP) from the lysates were subjected to immunoblotting (Blot) with the anti-PY antibody RC20 (A-a, B-a, and C-a). The filter used in a was reprobed with...
anti-GluR\(\varepsilon\)2 antibodies (A-b, B-b, and C-b). The expression levels of FynY531F and PSD-95 were confirmed by immunoblotting (A-c, B-c, C-c, and data not shown). All experiments were performed more than three times. Positions and sizes (kDa) of standard protein markers are indicated on the left. The positions of GluR\(\varepsilon\)2 (180 kDa), GluR\(\varepsilon\)2 YF mutants (180 kDa), and FynY531F (59 kDa) are indicated by arrowheads.

**FIG. 3.** Characterization of rabbit anti-phospho-Tyr1472 polyclonal antibodies. A, The synthetic peptide containing phospho-Tyr 1472 used for immunogen. B, Detection of the *in vitro* phosphorylated GST-C3 protein with the anti-phospho-Tyr1472 antibodies. GST-C3 fusion protein was phosphorylated by GST-Fyn (lanes 2 and 3) and followed by treatment with bacterial alkaline phosphatase (BAP) (lane 3). The proteins were subjected to immunoblotting (Blot) with anti-phospho-Tyr1472 antibodies (a). The filter used in a was reprobed with anti-GluR\(\varepsilon\)2 monoclonal antibody (mAb) (b). C, Detection of Tyr1472-phosphorylated GluR\(\varepsilon\)2 in 293T cells using the anti-phospho-Tyr1472 antibodies. 293T cells were transfected with combinations of expression plasmids encoding GluR\(\varepsilon\)2, GluR\(\varepsilon\)2Y1472F, PSD-95, and FynY531F. GluR\(\varepsilon\)2 immunoprecipitates (IP) from the lysates were subjected to immunoblotting (Blot) with anti-phospho-Tyr1472 antibodies (a). The filter used in a was reprobed with anti-GluR\(\varepsilon\)2 mAb (b). Expression levels of FynY531F and PSD-95 were confirmed by immunoblotting (c and data not shown). Although the data are not presented, immunoblotting of the filter used in a with anti-PY antibody showed basically the same pattern of immunoreactive signals as shown in Fig. 2Ca with respect to wild-type GluR\(\varepsilon\)2 and GluR\(\varepsilon\)2 Y1472F mutant. All experiments were performed more than three times. Positions and sizes (kDa) of standard protein markers are indicated on the left. The positions of GST-C3 (51 kDa), GluR\(\varepsilon\)2 (180 kDa), GluR\(\varepsilon\)2 Y1472F mutant (180 kDa), and FynY531F (59 kDa) are indicated by arrowheads.
FIG. 4. Fyn-mediated phosphorylation of Tyr1472 of GluRε2 in murine brain. A, Phosphorylation of Tyr1472 of GluRε2 in the murine brain. Telencephalons from wild-type mice were homogenized in RIPA/0.5% SDS buffer, boiled, and then diluted with 4 vol of RIPA buffer. GluRε2 immunoprecipitates (IP) from the lysates were treated with (lane 2) or without bacterial alkaline phosphatase (BAP) (lane 1) and were subjected to immunoblotting (Blot) with anti-phospho-Tyr1472 antibodies (a). The filter used in a was reprobed with anti-GluRε2 mAb (b). B, Reduced level of Tyr1472 phosphorylation of GluRε2 in fyn-mutant mice. Telencephalons from wild-type mice and fyn-mutant mice were homogenized in RIPA/0.5% SDS buffer, boiled, and then diluted with 4 vol of RIPA buffer. GluRε2 immunoprecipitates (IP) from the lysates of wild-type and fyn-mutant (fyn−/−) mice were subjected to immunoblotting (Blot) with anti-phospho-Tyr1472 antibodies (a). The filter used in a was reprobed with anti-GluRε2 mAb (b). All experiments were performed more than three times. Positions and sizes (kDa) of standard protein markers are indicated on the left. The positions of GluRε2 (180 kDa) are indicated by arrowheads.

FIG. 5. Developmental change of Tyr1472 phosphorylation in murine brain. Telencephalons from P3 to P56 wild-type mice were homogenized in RIPA/0.5% SDS buffer, boiled, and then diluted with 4 vol of RIPA buffer. The GluRε2 immunoprecipitates (IP) from the lysates were subjected to immunoblotting (Blot) with anti-phospho-Tyr1472 antibodies (a). The filter used in a was reprobed with the anti-PY antibody (b) and anti-GluRε2 mAb (c). All experiments were performed more than three times. Positions and sizes (kDa) of standard protein markers are indicated on the left. The positions of GluRε2 (180 kDa) are indicated by arrowheads.
FIG. 6. **Increased Tyr1472 phosphorylation of GluR\(\varepsilon\)2 in the hippocampal CA1 region after induction of LTP.** A, Representative recording of LTP. In the inset, sample traces (average of 10 consecutive EPSPs) are shown, which were recorded at the times indicated by the numbers in the figure. A tungsten bipolar stimulating electrode was placed in the stratum radiatum, and Schaffer collateral-commissural fibers were stimulated 4 times at 100 Hz for 1 s at an interval of 10 s to induce LTP. B, Representative blot of the GluR\(\varepsilon\)2 immunoprecipitates from non-stimulated control or stimulated slices with anti-phospho-Tyr1472 antibodies (\(a\)) and then with anti-GluR\(\varepsilon\)2 mAb (\(b\)). Hippocampal CA1 regions from non-stimulated control (Ctrl) or stimulated (LTP) slices were homogenized in RIPA/0.5% SDS buffer, boiled, and then diluted with 4 vol of RIPA buffer. GluR\(\varepsilon\)2 immunoprecipitates (IP) from the lysates were subjected to immunoblotting (Blot) with anti-phospho-Tyr1472 antibodies (\(a\)). The filter used in \(a\) was reprobed with anti-GluR\(\varepsilon\)2 mAb (\(b\)). Positions and sizes (kDa) of standard protein markers are indicated on the left. The positions of GluR\(\varepsilon\)2 (180 kDa) are indicated by arrowheads. C, Quantification of Tyr1472 phosphorylation in non-stimulated control and stimulated slices (\(n=10\) from each of 4 mice). Open bar indicates the level of Tyr1472 phosphorylation in non-stimulated slices (Ctrl). Closed bar indicates the level of Tyr1472 phosphorylation in stimulated slices 60 min after induction of LTP (LTP). An asterisk indicates statistically significant difference from control (\(p < 0.002\)).
Figure 2

A

a

\[ \begin{array}{c}
250 \\
160 \\
\end{array} \]

\[ \rightarrow \]

IP: α-GluRε2

Blot: α-PY

b

\[ \begin{array}{c}
250 \\
160 \\
\end{array} \]

\[ \rightarrow \]

IP: α-GluRε2

Blot: α-GluRε2

c

\[ \begin{array}{c}
50 \\
1 \\
2 \\
3 \\
\end{array} \]

\[ \rightarrow \]

Fyn

B

a

\[ \begin{array}{c}
250 \\
160 \\
\end{array} \]

\[ \rightarrow \]

IP: α-GluRε2

Blot: α-PY

b

\[ \begin{array}{c}
250 \\
160 \\
\end{array} \]

\[ \rightarrow \]

IP: α-GluRε2

Blot: α-GluRε2

c

\[ \begin{array}{c}
50 \\
1 \\
2 \\
3 \\
4 \\
5 \\
6 \\
7 \\
8 \\
\end{array} \]

\[ \rightarrow \]

Fyn

C

a

\[ \begin{array}{c}
250 \\
160 \\
\end{array} \]

\[ \rightarrow \]

IP: α-GluRε2

Blot: α-PY

b

\[ \begin{array}{c}
250 \\
160 \\
\end{array} \]

\[ \rightarrow \]

IP: α-GluRε2

Blot: α-GluRε2

c

\[ \begin{array}{c}
50 \\
1 \\
2 \\
3 \\
4 \\
5 \\
\end{array} \]

\[ \rightarrow \]

Fyn
Figure 3
Figure 4
Figure 6

A

Normalized EPSP slope vs. Time (min)

B

Ctrl LTP

IP: α-GluR2

Blot: α-phospho-Tyr1472

GluR2

(kDa)

250

160

a

2

b

1

Normalized EPSP slope

Normalized EPSP slope vs. Time (min)

C

% phosphorylation

Ctrl LTP

Normalized EPSP slope vs. Time (min)
Characterization of Fyn-mediated tyrosine phosphorylation sites on GluR\(\epsilon\) subunit of the N-methyl-D-aspartate receptor

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