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Tyrosine Phosphorylation of ErbB4 is Enhanced by PSD95 and Repressed by Protein Tyrosine Phosphatase Receptor Type Z

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Protein tyrosine phosphatase receptor type Z (Ptprz/PTPζ/RPTPβ) is a receptor-like protein tyrosine phosphatase (RPTP) preferentially expressed in the brain. ErbB4 is a member of the ErbB-family tyrosine kinases known as a neuregulin (NRG) receptor. Both are known to bind to postsynaptic density-95 (PSD95) on the second and the first/second PDZ (PSD95/Disc large/zona occludens1) domains, respectively, through the PDZ-binding motif of their carboxyl termini. Here we report a functional interaction between Ptprz and ErbB4. An intracellular carboxyl-terminal region of Ptprz pulled-down PSD95 and ErbB4 from an adult rat synaptosomal preparation. ErbB4 and Ptprz showed co-localization in cell bodies and apical dendrites of neurons in the prefrontal cortex. In HEK293T cells, phosphorylation of ErbB4 was raised by co-expression of PSD95, which was repressed by additional expression of Ptprz. *In vitro* experiments using the whole intracellular region (ICR) of ErbB4 also showed that PSD95 stimulates the autophosphorylation of ErbB4, and that the ICR of Ptprz dephosphorylates ErbB4 independent of the presence of PSD95. Taken together with the finding that the tyrosine phosphorylation level of ErbB4 was increased in *Ptprz*-deficient mice, these results suggest that Ptprz has a role in suppressing the autoactivation of ErbB4 by PSD95 at the postsynaptic density in the adult brain.

Key words: ErbB4, PDZ domain, PSD-95/SAP90 family, Ptprz/PTPζ/RPTPβ, tyrosine phosphorylation.

Abbreviations: ICR, intracellular region; LTP, long-term potentiation; MALDI-TOFMS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; NMDA, N-methyl-D-aspartate; PDZ, PSD95/Disc large/zona occludens1; PSD, postsynaptic density; PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; RPTP, Receptor-like PTP; RTK, Receptor PTK.

Receptor-like protein tyrosine phosphatases (RPTPs) are a structurally and functionally diverse family of enzymes comprised of eight subfamilies (*1*, *2*). Most RPTPs contain two tandem PTP domains, with the catalytic activity retained in the first, the membrane-proximal domain (PTP-D1). Little is known about the function of the second, the membrane-distal domain (PTP-D2), although the structural integrity of the D2 domain is important for the activity and stability of the RPTP (*1*, *2*). Protein tyrosine phosphatase receptor type Z (Ptprz, also called PTPζ or RPTPβ) is a RPTP expressed predominantly in the central nervous system (*3*), but also in peripheral tissues including the stomach (*4*). Ptprz has two receptor isoforms, Ptprz-A and Ptprz-B, the latter of which has a deletion in the extracellular region relative to type A (Fig. 1A). Among the 21 RPTPs (*1*, *2*), only the R5 subfamily, Ptprz/PTPζ and Ptprg/PTPγ, have a canonical PDZ-binding motif (-S-L-V) at their carboxyl-termini (*5*). We previously reported that the carboxyl-terminus of Ptprz binds to the second PDZ domain of postsynaptic density-95 (PSD95) (*6*), which is the major synaptic scaffolding protein involved in synaptic plasticity. In adult rat brains, Ptprz and PSD95 are both distributed in the dendrites of pyramidal neurons of the hippocampus and neocortex, and abundant in the PSD fraction (*6*). In addition to the PSD95 family, we subsequently revealed that Ptprz also interacts with other PDZ domain-containing proteins such as membrane associated guanylate kinase, WW and PDZ domain-containing -1/3 (MAGI-1/-3), Veli-3, synaptojanin 2 binding protein (Synj2bp), syntrophin acidic 1 and basic 1 (Snta1 and Sntb1) and multiple PDZ protein 1 (Mupp1) (*7*).

We recently reported a genetic method to screen for PTP substrates which we have named the 'yeast substrate-trapping system (*7*, *8*).' This method is based on the yeast two-hybrid system with two essential modifications: the conditional expression of a protein tyrosine kinase (PTK) to tyrosine-phosphorylate the prey protein, and screening using a substrate-trap PTP mutant (*9*) as bait. Using this method, we identified several substrates for Ptprz, such as G protein-coupled receptor kinase-interactor 1 (Git1), p190 RhoGAP, golgi-associated PDZ and coiled–coil motif containing (GOPC/

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PIST), and MAGI-1 (*7*, *8*). On the other hand, other groups reported that β-catenin (*10*) and sodium channel α subunit (*11*) are also substrates. Of note is that these molecules are not included in the substrate clones in our screening. Interestingly, β-catenin is known to interact with the fifth PDZ domain of MAGI-1 in epithelial cells (*12*), suggesting that Ptprz and β-catenin form a stable enzyme–substrate complex on the MAGI-1 scaffold. Likewise, the interaction between sodium channels and Ptprz is presumably mediated by syntrophin in neuronal cells, because the first pleckstrin homology (PH) domain and the syntrophin-unique (SU) domain of syntrophin interact with sodium channels (*13*), and the PDZ domain of syntrophin with Ptprz (*7*).

These findings suggest that RPTPs have at least two different modes of selective interaction with a substrate. One is based on direct interaction between the PTP-D1 domain and the tyrosine-phosphorylated substrate molecules (*1*), which can be detected with the yeast substrate-trapping system (*7*, *8*). The other mode is indirect, and some molecules could become specific substrates with the help of scaffold proteins like PDZ proteins. There is a possibility that the latter was passed over in our screening. To further identify substrates for Ptprz, we performed pull-down experiments using the carboxyl terminal region of Ptprz from synaptosomal fractions of the adult rat cerebrum. We successfully identified a battery of molecules which may include substrates for Ptprz, together with PSD95. Among them, we focused on ErbB4. ErbB4 is a member of the ErbB receptor family of RTKs, which is activated by neuregulins (NRG) and other growth factors (*14*, *15*). ErbB4 is known to be a receptor tyrosine kinase concentrated at the PSD of excitatory synapses through binding to PSD95 (*16*). In the present study, we found that ErbB4 is a novel substrate for Ptprz.

MATERIALS AND METHODS

Animals—Adult rats (Sprague-Dawley, 3-month-old), and adult wild-type and *Ptprz*-deficient mice (*17*) were used. All animal experiments were performed according to the guidelines for Animal Care with approval by the Committee for Animal Research, National Institutes of Natural Sciences.

GST Pull-Down Experiment—A glutathione *S*-transferase (GST) fusion protein with the D2 domain and the carboxy terminal tail of Ptprz (amino acid residues 2,030–2,316 of rat Ptprz-A; GenBank accession no. U09357; GST-Ptprz-D2; Fig. 1A) was expressed from an expression plasmid, pGEX-Ptrprz-D2, in *Escherichia coli* strain BL21, and purified by glutathione affinity chromatography using a GSTrap column attached to a chromatography apparatus (AKTA prime plus, GE Healthcare). pGEX-Ptprz-D2 was prepared by subcloning the appropriate cDNA from pZeoPTPζ (*8*) into pGEX-6P (GE Healthcare). *GST-Ptprz-D2-SA*, a mutant in which serine 2,314 is substituted with alanine, was expressed from pGEX-Ptprz-D2-SA: pGEX-Ptprz-D2-SA was generated from pGEX-Ptrprz-D2 by using a QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene).

For pull-down experiments, GST fusion proteins (50 μg) were covalently linked to glutathione Sepharose beads (500 μl) by using GST Orientation Kit (Pierce), and then incubated overnight at 4°C with synaptosomal lysate (50 mg protein/ml beads) prepared from the adult rat cerebrum as described (*16*). Once the beads were washed, the bound proteins were eluted with a sample buffer containing 2% SDS in 0.1 M Tris–HCl, pH 6.8 and subjected to acetone precipitation. The proteins were then separated by SDS–PAGE, and stained with Coomassie brilliant blue. The specific bands were excised, subjected to in-gel tryptic digestion and then subjected to matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOFMS) (Reflex III, Bruker Daltonics) The peptide mass fingerprinting was performed by Mascot search (http://www.matrixscience.com/) against the NCBI non-redundant protein database.

Immunohistochemistry—The double staining experiment was performed as described (*6*) with slight modifications. Sections were incubated overnight with a mouse monoclonal antibody against the intracellular region of Ptprz (anti-RPTPβ, BD transduction laboratories) and rabbit polyclonal antibodies against the intracellular region of ErbB4 (sc-283, Santa Cruz Biotechnology). The binding antibodies were visualized with fluorescent Alexa dye-conjugated secondary antibodies (Molecular Probes), and observed by confocal microscopy (LSM510, Carl Zeiss).

Cell Culture and DNA Transfection—Human embryonic kidney 293T (HEK293T) cells (6×10^5) were cultured overnight on a 6 cm dish coated with rat tail collagen in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in a humidified incubator at 37°C in 5% CO_2. The cells were transfected with 5 μg of expression plasmid using calcium phosphate precipitation. Twelve hours after the transfection, the cells were detached with trypsin-EDTA, replated at about 2×10^4 cells/cm^2 on collagen-coated plates and further cultured for 36 h to allow protein expression. Expression constructs for rat Ptprz-B (pZeo-PTPζ) (*8*), a PTP-inactive DA mutant of Ptprz-B (pZeo-PTPζ-D1902A) (*8*) and human ErbB4 (pcDNA3.1/Zeo-ErbB-4: JM-a CYT-1 isoform; kindly provided by Dr S. Yokoyama, RIKEN Genomic Sciences Center) (*18*) have been described previously. The expression plasmid of PSD95 (pcDNA3.1-PSD95) was prepared as follows: The EcoRI–SphI fragment encoding the full-length rat PSD95 was excised from pGAD424-PSD95 (*6*), and inserted into pcDNA3.1 (Invitrogen) in between the EcoRI and EcoRV sites.

Tyrosine Phosphorylation of ErbB4 in HEK293T Cells and Mouse Brain—Transfected cells (replated on 3.5 cm dishes and cultured for 24 h) were starved in serum-free DMEM for 12 h. The cells were lysed with 300 μl of 1% NP-40 in TBS (10 mM Tris–HCl, pH 7.4 and 150 mM NaCl) containing 1 mM DTT, 1 mM vanadate, 10 mM NaF and protease inhibitors (complete EDTA-free, Roche) for 30 min on ice, and the supernatant was collected by centrifugation at 10,000*g* for 15 min. In HEK293T cells, endogenous ErbB2, which can form a heterodimer with ErbB4 (*19*), was strongly detected (data

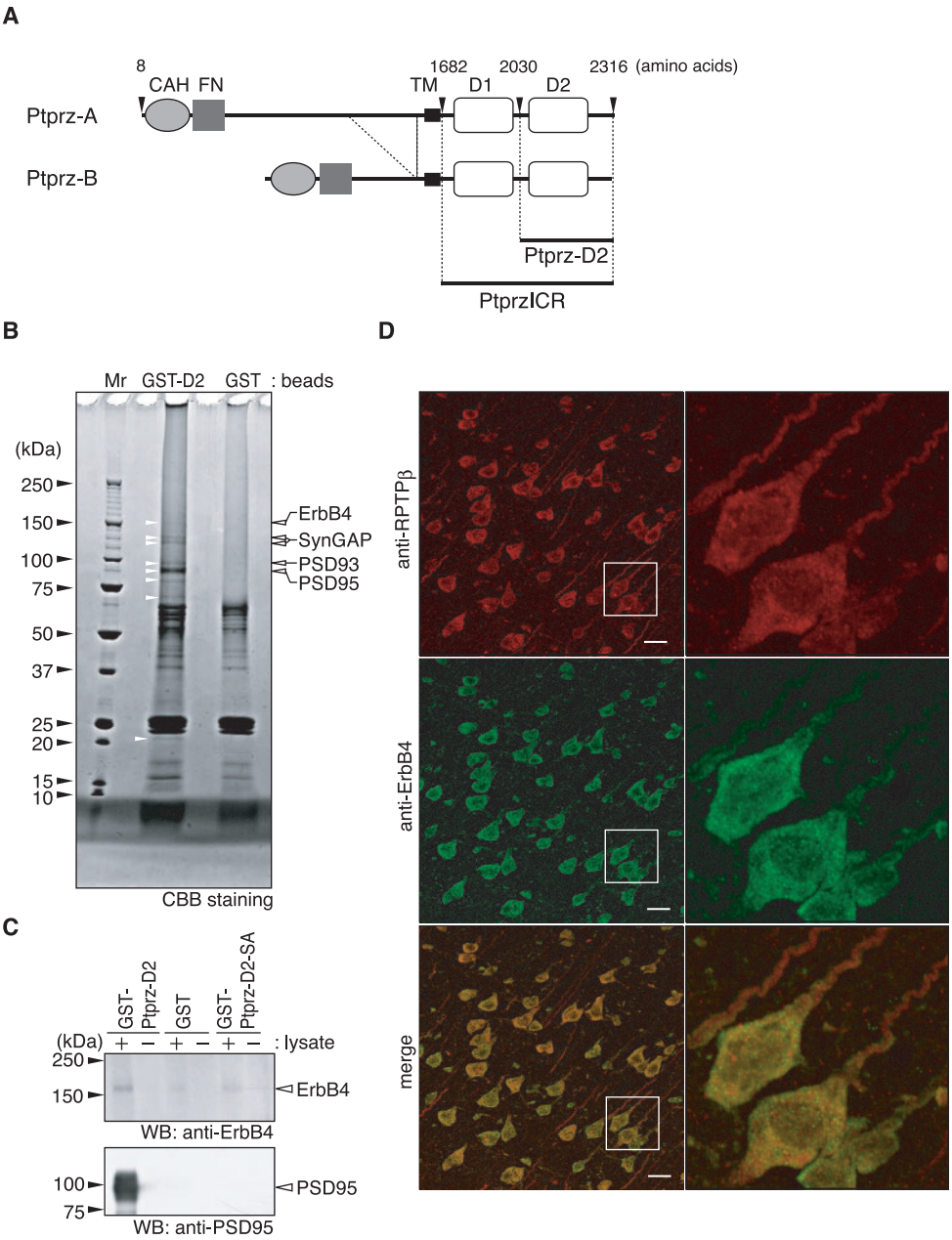

Fig. 1. **Identification of binding molecules for Ptprz and co-localization of Ptprz with ErbB4 in the adult rat brain.** (A) Schematic diagram of rat Ptprz-A and Ptprz-B isoforms. The regions used for the pull-down experiment (GST-Ptprz-D2) and for the *in vitro* dephosphorylation assay (GST-PtprzICR) are indicated by underlines. Ptprz-A and -B are composed of an N-terminal carbonic anhydrase-like (CAH) domain, a fibronectin type III (FN) domain, a serine, glycine-rich domain for chondroitin-sulfate attachment, a transmembrane segment (TM), two PTP domains (D1 and D2) and the PDZ-binding motif at the C-terminus. (B) GST pull-down assays. Arrowheads indicate the specific bands that were detected in the pull-down samples using GST-Ptprz-D2. (C) Western blot analyses of the pull-down samples using different fusion proteins with GST. (D) Co-localization of Ptprz and ErbB4 in the adult rat prefrontal cortex. The sections were immunostained with anti-RPTPβ mouse monoclonal antibody and anti-ErbB4 rabbit polyclonal antibodies. No immunofluorescence was observed without the primary antibodies (data not shown). Right panels are enlargements of the area enclosed by squares in the left panels. Scale bars, 20 μm.

not shown). Because ErbB2 and ErbB4 were difficult to distinguish by western blotting due to their similar molecular sizes, we disrupted the protein complexes in the supernatants by adding SDS to a final concentration of 1% and boiling for 5 min. After dialysis against TBS containing 1% NP-40, 1 mM DTT, 1 mM vanadate and 10 mM NaF, the supernatants (200 μl) were incubated overnight with 4 μl of anti-ErbB4 (sc-283) at 4°C. The immunocomplexes were precipitated using 10 μl of Protein G Sepharose 4FF (GE Healthcare) for 2 h, washed four times with the lysis buffer and subjected to SDS–PAGE followed by western blotting. Antibodies

against phosphotyrosine (PY20, GE Healthcare), the extracellular region of Ptprz (anti-Ptprz-S polyclonal antibodies) (*3*), ErbB4 (sc-283) and PSD95 (7E3-1B8, Calbiochem) were used. The signal intensity of the bands was quantified by densitometry, and statistically analysed by using Stat View software (version 5.0J; SAS Institute, Cary, NC).

For the *in vivo* analysis, mice (>2.5-months-old) were habituated for at least 2 h to a new homecage and then decapitated. The cerebral cortex was rapidly dissected out on ice, weighed and immediately used for the preparation of the crude synaptosomes as described (*16*) with slight modifications. Briefly, the pooled tissues (from two mice per group) were homogenized with 20 volumes of ice-cold 0.32 M sucrose in 4 mM HEPES, pH 7.4, containing 1 mM vanadate and 10 mM NaF. Crude synaptosomes were separated by centrifugation as described (*16*), and then subjected to the immunoprecipitation experiments as above.

In Vitro Phosphorylation Assay—GST-ErbB4ICR (the whole intracellular region of ErbB4) was purchased from Cell Signaling Technology. GST-PSD95 was expressed in *E. coli* from pGEX-PSD95 harbouring the full-length cDNA of rat PSD95 (*6*) in pGEX-6P, and purified by glutathione affinity chromatography (GSTrap FF), anion-exchange chromatography (Hitrap Q FF) and then gel chromatography (Superose 6 10/300 GL). GST-ErbB4ICR (1.25 ng) for the autophosphorylation was pre-incubated with indicated amounts of GST-PSD95 (or control GST) in 10 μl of 60 mM HEPES, 5 mM $MgCl_2$ and 5 mM $MnCl_2$, pH7.2, containing 1.25 mM DTT and 100 μg/ml bovine serum albumin (PTK buffer) for 15 min on ice. The phosphorylation reaction was started by adding 5 μl of ATP solution (30 μCi/ml [γ-^{32}P]ATP and 30 μM non-labelled ATP in the PTK buffer) at 30°C: [γ-^{32}P]ATP (~6,000 Ci/mmol) was from GE Healthcare. The reaction was terminated by adding sample buffer for the SDS–PAGE. The samples were separated by SDS–PAGE, and the signals on the gel were detected with an imaging plate (BAS-MS 2025) using BAS5000 Bio-image analyzer (Fuji Photo Film).

In Vitro Dephosphorylation Assay—GST-PtprzICR (the whole intracellular region of rat Ptprz-A) was prepared as described (*8*). For *in vitro* dephosphorylation, we first prepared the autophosphorylated ErbB4 as the substrate. GST-ErbB4ICR (300 ng) was autophosphorylated with 10 μCi/ml of [γ-^{32}P]ATP and 10 μM of non-labelled ATP in 100 μl of the PTK buffer as above. ATP and cofactors were removed by gel filtration (Sephadex G-25, GE Healthcare) with 10 mM Tris–HCl, pH 7.0, containing 5 mM DTT, 5 mM EDTA and 100 μg/ml bovine serum albumin (PTP buffer), and the phosphorylated preparation was stored at −85°C until used: The rate of recovery of the ^{32}P-labelled GST-ErbB4ICR was 54% based on radioactivity after the gel filtration. Before the dephosphorylation assay, indicated amounts of GST-PtprzICR and GST-PSD95 were pre-incubated in 5 μl of the PTP buffer for 15 min on ice. The reaction was then started by adding 10 μl of ^{32}P-labeled GST-ErbB4ICR (~2 ng) at 30°C. The samples were separated by SDS–PAGE followed by autoradiography as earlier.

RESULTS

Identification of Molecules Binding to Ptprz in the Synaptosomal Fraction of the Adult Rat Brain—We first performed pull-down experiments using a GST fusion protein comprising the second PTP domain and the carboxy terminal tail (GST-Ptprz-D2; Fig. 1A), with deoxycholate extracts of adult rat cerebral synaptosomes. As shown in Fig. 1B, eight specific bands were found in the fraction pulled down with GST-Ptprz-D2 beads, but not in that pulled down with GST beads. Mass spectrometry revealed that the major band at 95 kDa is SAP90A (rat orthologue of PSD95), and that the 180-kDa band is ErbB4, a member of the ErbB receptor tyrosine kinase (RTK) family known as a receptor for NRG: the others were synaptic Ras GTPase activating protein (SynGAP), and PSD93/chapsyn-110 (data not shown). We confirmed the specific interaction between PSD95, ErbB4 and Ptprz-D2 in the pull-down experiment by western blotting (Fig. 1C). Notably, neither ErbB4 nor PSD95 was pulled down using a Ptprz mutated at the PDZ-finding motif (GST-Ptprz-D2-SA) which cannot bind to PSD95 (*6*) (Fig. 1C). This suggests that ErbB4 and Ptprz form a complex through a process mediated by PSD95.

It has been reported that ErbB4 binds to PSD95 through interaction between the carboxyl terminus (-T-V-V) and the first or second PDZ domain (*16*). Both are abundant in the PSD fractions of the adult rat brain. Indeed, double immunostaining indicated the co-localization of Ptprz and ErbB4 at the cellular level in the adult rat brain (Fig. 1D). The immunoreactivity for Ptprz was associated with the dendrites and cell bodies of a population of neurons in the prefrontal cortex as reported previously (*6*); the immunoreactivity for ErbB4 was mainly detected in the cell bodies of the same neurons.

Effects of PSD95 and Ptprz on Phosphorylation of ErbB4 in HEK293T Cells—To determine functional interactions among Ptprz, ErbB4 and PSD95, we transiently transfected human embryonic kidney (HEK) 293T cells with the expression plasmids, and examined the tyrosine phosphorylation of ErbB4 by western blotting following the immunoprecipitation. In this experiment, we used Ptprz-B (Fig. 1A), which is the major receptor isoform expressed in the adult brain (*3*) and abundant in the PSD fraction (*6*). Although the overall tyrosine phosphorylation pattern of the cellular proteins of HEK293T cells did not differ among samples (Fig. 2A), co-expression of Ptprz-B only slightly decreased the tyrosine phosphorylation of ErbB4 as compared with that in cells mock-transfected, whereas co-expression of the PTP-inactive DA mutant form of Ptprz-B significantly enhanced the tyrosine phosphorylation level (Fig. 2B).

The tyrosine phosphorylation of ErbB4 showed a 2-fold increase with the co-expression of PSD95. Under those conditions, interestingly, the basal phosphorylation of ErbB4 raised by PSD95 was significantly decreased by co-expression of Ptprz-B, but not further increased by co-expression of the DA mutant of Ptprz-B (Fig. 2B). These results suggest that PSD95 induces activation of ErbB4 through dimerization, and the trans-autophosphorylated tyrosine residues in ErbB4 were

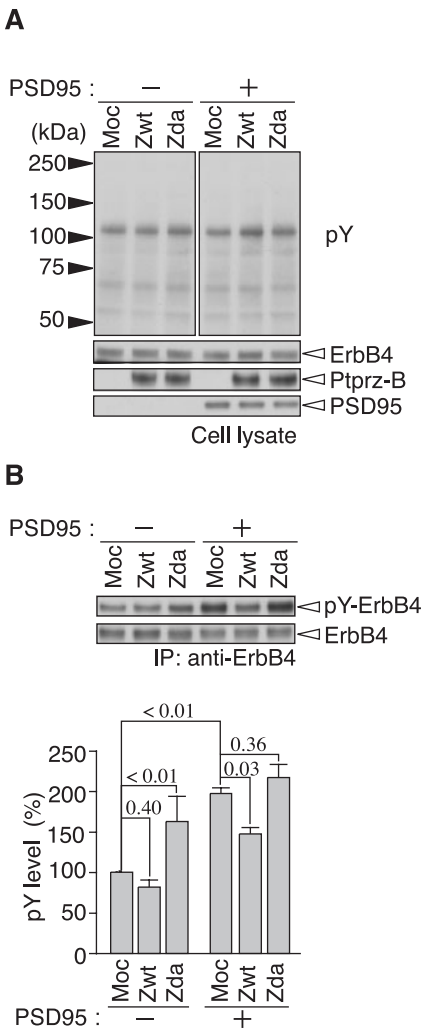

Fig. 2. **Tyrosine phosphorylation of ErbB4 and its dephosphorylation Ptprz in HEK293T cells.** (A) HEK293T cells transfected with indicated expression plasmids were starved in serum-free medium for 12 h, and lysed with 1% NP-40 in TBS. The tyrosine phosphorylation of cellular proteins and protein expression from the plasmids in HEK293T cells were analysed by western blotting. (B) The tyrosine phosphorylation of ErbB4 was analysed by immunoprecipitation from the cell lysates followed by western blotting. Signal intensities were quantified by densitometry and are shown as a percentage of control cells (Mock, lane 1). Data are the means ± SE ($n = 4$). *P* values shown above the bars were calculated with Fisher's PLSD *post hoc* tests.

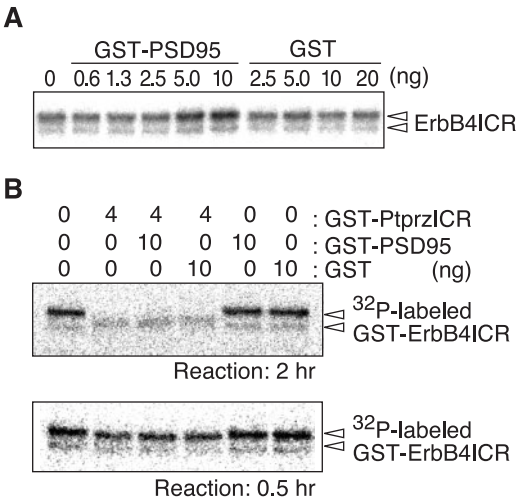

Fig. 3. ***In vitro* assays for phosphorylation of ErbB4 by PSD95 and its dephosphorylation by Ptprz.** (A) *In vitro* phosphorylation assay of ErbB4. GST-ErbB4 (1.25 ng) pre-incubated with indicated amounts of GST-PSD95 or GST was subjected to autophosphorylation with 10 μCi/ml of [γ-^{32}P]ATP and 10 μM of unlabeled ATP in a 15 μl volume for 30 min at 30°C. The reaction was terminated by adding SDS–PAGE sample buffer, and then analysed by SDS–PAGE followed by autoradiography. (B) *In vitro* dephosphorylation assay of ErbB4. To exclude the effect of the kinase activity of ErbB4 itself on this assay, ATP and cofactors (Mg^{2+} and Mn^{2+}) were removed from the ^{32}P-labelled GST-ErbB4ICR sample by gel filtration. After indicated amounts of GST-PtprzICR, GST-PSD95 and GST were mixed and preincubated in a 5 μl volume for 15 min, 10 μl of the ^{32}P-labelled GST-ErbB4ICR (~2 ng) was added and incubated for 2 h (upper) or 0.5 h (lower) at 30°C. The reaction was terminated by adding SDS–PAGE sample buffer, and then analysed as above.

specifically dephosphorylated by Ptprz. It is also conceivable that clustering of Ptprz with ErbB4 on the PSD95 scaffold facilitates the dephosphorylation of ErbB4 by Ptprz because of the steric advantage.

Effects of PSD95 and Ptprz on Phosphorylation of ErbB4 In Vitro—To further gain insight into the regulation of the autophosphorylation of ErbB4 by PSD95 and its dephosphorylation by Ptprz, we performed *in vitro* phosphorylation and dephosphorylation assays using recombinant proteins. Our earlier experiments indicated that the tyrosine phosphorylation of the entire intracellular region (ICR) of ErbB4 reaches a plateau within 5 min and maintains this level with no apparent drop during 60 min of incubation (data not shown). Therefore, we carried out the kinase reaction for 30 min. As shown in Fig. 3A, the tyrosine phosphorylation of ErbB4ICR was dose-dependently increased by GST-PSD95, but not by control GST, consistent with the enhancement of the phosphorylation of ErbB4 by PSD95 in HEK293T cells (Fig. 2B). Here, we verified that ErbB4ICR does not phosphorylate GST or GST-PSD95 (data not shown).

We then examined the dephosphorylation activity of PtprzICR (Fig. 1A) using ^{32}P-labelled ErbB4ICR *in vitro*. To eliminate the kinase activity of ErbB4ICR itself in the dephosphorylation assay, we removed ATP from the substrate preparation by gel filtration (see Materials and Methods section). PtprzICR showed prominent dephosphorylation activity in 2 h against ErbB4ICR *in vitro* (Fig. 3B, upper). In contrast to the autophosphorylation activity of ErbB4ICR, the dephosphorylation activity of PtprzICR against ErbB4ICR was not affected by GST-PSD95 (Fig. 3B, upper). The same result was obtained with a short incubation (0.5 h), and there was no influence upon the dephosphorylation activity of PtprzICR in the presence of PSD95 (Fig. 3B, lower). We also examined PTP activity using *p*-nitrophenyl phosphate (pNPP), a low-molecular-mass compound which

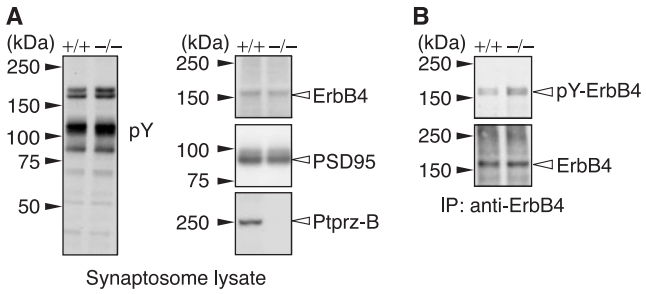

Fig. 4. **Comparison of the tyrosine phosphorylation of ErbB4 between wild-type and *Ptprz*-deficient mice.** (A) Cerebral synaptosomes were prepared from wild-type (+/+) and *Ptprz*-deficient mice (–/–). Using the lysates, the overall tyrosine phosphorylation pattern and protein expression in the synaptosomes were analysed by western blotting. Ptprz-B was detected after the synaptosomal lysates were treated with chondroitinase ABC as described (*3*), because all splicing isoforms of Ptprz are expressed as chondroitin-sulphate proteoglycans (*3*). (B) The tyrosine phosphorylation of ErbB4 was analysed by immunoprecipitation from the synaptosomal lysates followed by western blotting. The tyrosine phosphorylation of ErbB4 was increased in *Ptprz*-deficient mice. This is consistent with the results obtained in the heterologous cells (Fig. 2B).

binds directly to the active site of the PTP domain, but the hydrolysis of pNPP by PtprzICR was not changed by addition of GST-PSD95 (data not shown). These results indicate that the binding of PSD95 does not influence the dephosphorylation activity of Ptprz itself.

Increased Tyrosine Phosphorylation of ErbB4 in the Synaptosomal Fractions of Ptprz-Deficient Mice—Finally, we compared the tyrosine phosphorylation level of ErbB4 in the synaptosome fractions of the neocortex between wild-type and *Ptprz*-deficient mice (*17*). No significant differences in the overall tyrosine phosphorylation pattern (Fig. 4A, left), along with the same expression levels of ErbB4 and PSD95 (Fig. 4A, right, top and middle), were observed. Ptprz-B was specifically detected in the synaptosomes prepared from the wild-type mice as expected (Fig. 4A, right, bottom). The tyrosine phosphorylation of ErbB4 was significantly increased in *Ptprz*-deficient mice compared with wild-type mice (1.3-fold increase, $P = 0.04$, $n = 7$ by unpaired Student's *t*-test, Fig. 4B). This result was expected and consistent with the results obtained by selective expression using the heterologous cells (Fig. 2B). Taken together, these results clearly indicate that Ptprz is involved in the regulation of phosphorylation (activation) of ErbB4 in the brain.

DISCUSSION

In this study, we identified ErbB4 and Ptprz in a protein complex containing PSD95 from adult rat synaptosomes. Here, PSD95 appears to be a scaffold protein for interaction between ErbB4 and Ptprz. In heterologous cells, PSD95 enhanced tyrosine phosphorylation of ErbB4 without ligand stimulation, which was repressed by further co-expression of Ptprz. Ptprz(ICR) dephosphorylated the tyrosine phosphorylated ErbB4(ICR) *in vitro*. Furthermore, the tyrosine phosphorylation of ErbB4 was increased in the cortical synaptosomal fractions in adult *Ptprz*-deficient mice as compared with wild-type mice. All these results indicate that ErbB4 is a novel substrate for Ptprz.

We previously found that the carboxyl-terminus (-S-L-V) of Ptprz associates with the second PDZ domain of PSD95 (*6*), which is the best characterized component at the PSDs of excitatory synapses (*20*). ErbB4 also reportedly binds to the first and second PDZ domain of PSD95 through the carboxyl terminal tail (-T-V-V) of ErbB4 (*16*). The cytoplasmic portion of ErbB4 has a conserved tyrosine kinase domain flanked by regulatory sequences that become tyrosine autophosphorylated at multiple sites by ligand-induced dimerization (*14*). It has been reported that PSD95 forms multimers *via* amino-terminal 'head-to-head' interaction (*21*) and thereby clusters many signalling molecules at the PSD. Such clustering may induce the dimerization and autophosphorylation of ErbB4 without ligand stimulation. The entire ICR of epidermal growth factor receptor (EGFR/ErbB1) is also activated by aggregation induced by a variety of agents (*22*, *23*). Moreover, a very recent study indicated that EGFR kinase can be activated by increasing its local concentration through the formation of an asymmetric dimer (*24*). Here, we found that PSD95 significantly promotes the phosphorylation of ErbB4 in the serum-starved HEK293T cells without ligand stimulation (Fig. 2B). This activation of ErbB4 by PSD95 also occurred *in vitro* (Fig. 3A). Taken together with the previous findings, PSD95 presumably raises the basal level of phosphorylation (activation) of ErbB4, and may further enhance the ErbB4 signalling by NRG (*16*).

In the absence of PSD95, the tyrosine phosphorylation of ErbB4 was not decreased by wild-type Ptprz, although it was enhanced by the substrate-trapping DA-mutant form of Ptprz [Ptprz(DA)]. On the other hand, in the presence of PSD95, wild-type Ptprz did affect the tyrosine phosphorylation, but Ptprz(DA) did not. This could be explained by the presence of another phosphatase for the phosphorylated tyrosine residues on ErbB4 under the conditions without PSD95 in HEK293T cells. The dominant-negative effect of the Ptprz(DA) mutant could be a result of the protection of ErbB4 from this phosphatase by substrate trapping. One of the candidates for the phosphatase is histidine acid phosphatase ACPT which is reportedly expressed in the brain together with ErbB4 (*25*). Relevantly, it is known that co-expression of PSD95 increases the amount of ErbB4 included in a Triton X-100 insoluble fraction in HEK293 cells, probably due to its translocation to lipid rafts, specialized domains of the plasma membrane (*26*). Thus, there is also the possibility that ErbB4 is sequestered from ACPT in lipid rafts.

We recently showed that Ptprz is inactivated through oligomerization in cultured cells using the full-length Ptprz-B (*27*). The dephosphorylation activity of PtprzICR for ^{32}P-labelled ErbB4ICR was not affected by PSD95 *in vitro* (Fig. 3B). This suggests that phosphatase activity of Ptprz is not influenced by the binding to PSD95. Also in the situation *in vivo*, the binding of Ptprz to PSD95 *via* the carboxy terminal tail appears to maintain the dephosphorylation activity of Ptprz for ErbB4 (Fig. 2B). Thus, it is likely that Ptprz functions as a modulator of

ErbB4 signalling through inhibition of its excessive activation by PSD95. This would be an important mechanism to control the ErbB4 pathway *via* stimulation by the ligand (NRGs) at the PSD.

We found that the tyrosine phosphorylation of ErbB4 is consistently increased in the cortical synaptosomal fractions in adult *Ptprz*-deficient mice (Fig. 4B). ErbB4 associates with PSD95 together with NMDA receptors at the PSD of excitatory synapses in the brain (*16*, *28*). Some recent studies indicated that NRG-ErbB4 signalling is functionally important for the maintenance and/or regulation of synaptic plasticity. We demonstrated that *Ptprz*-deficient mice show age-dependent impairments of spatial learning along with enhanced hippocampal long-term potentiation (LTP) in the CA1 region (*29*) and impairments in hippocampus-dependent contextual fear memory (*30*). On note, NRG1 and its receptor ErbB4 have been implicated in the etiology of schizophrenia (*31*, *32*). A recent study suggests that enhanced NRG1-ErbB4 signalling contributes to NMDA hypofunction in this disorder (*32*). This study indicated that a substantial increase in NRG1-induced ErbB4 activation was dependent on the enhancement of ErbB4-PSD95 interaction in the *post-mortem* prefrontal cortex in schizophrenia, although the underlying molecular mechanisms have not been elucidated. The data in the present study suggest that a decrease in the activity, or expression level, of Ptprz contributes to the enhanced ErbB4 signalling in such psychiatric disorders.

In contrast to the RPTP family, in which only two R5 subfamily members (Ptprz and Ptprg) have the canonical PDZ-binding tail, a significant number of RTK family members can bind to the various PDZ proteins *via* their carboxy tails or intracellular regions (*5*). Because the -S-L-V motif at the carboxy terminal of Ptprz can interact with various PDZ proteins (*6*, *7*), Ptprz (and Ptprg) supposedly regulate other RTKs on different PDZ protein scaffolds. In fact, we found that the tyrosine phosphorylation of TrkA, a member of the Trk receptor tyrosine kinase family which binds to the PDZ domain of GIPC (*33*), is efficiently decreased with the co-expression of Ptprz in cultured cells (our unpublished data). Therefore, further systematic study should be conducted to examine such possibilities. In conclusion, our findings suggest that organized clustering of RTKs and RPTPs in the synapse through PDZ proteins is important to prevent the unnecessary activation of RTKs and to maintain (or recover) a state responsive to extracellular ligands.

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