Neuroscience 164 (2009) 1020–1030

FUNCTIONAL DIFFERENCE OF RECEPTOR-TYPE PROTEIN TYROSINE PHOSPHATASE ζ/β ISOFORMS IN NEUROGENESIS OF HIPPOCAMPAL NEURONS

H. ASAI,[a1] S. YOKOYAMA,[a1] S. MORITA,[a] N. MAEDA[b] AND S. MIYATA[a]*

[a]*Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606–8585, Japan*

[b]*Department of Developmental Neuroscience, Tokyo Metropolitan Institute for Neuroscience, Musashidai, Fuchu, Tokyo 183–8526, Japan*

Abstract—Receptor-type protein tyrosine phosphatase ζ/β (RPTPζ) is a transmembrane chondroitin sulfate proteoglycan (CSPG) and has been shown to play crucial roles in controlling axonal growth and neuronal migration. The RPTPζ has two transmembranous isoforms, shorter receptor form of RPTPζ (sRPTPζ) and full-length receptor form of RPTPζ (fRPTPζ), but no studies have been reported about functional difference of these two isoforms. In the present study, therefore, we examined whether or not two RPTPζ isoforms have different role in controlling dendritic morphology and synaptic number in cultured hippocampal neurons using the quantitative morphometrical analysis. Confocal microscopic observation showed that the immunoreactivity of RPTPζ was observed throughout cells such as axons, growth cones, and dendrites at the early stages of neuronal culture, while it was seen predominantly on dendrites at the late stages. Western blotting analysis revealed that fRPTPζ was mainly expressed at the early stages of culture and both RPTPζ isoforms were expressed at late stages of culture. The overexpression of sRPTPζ in hippocampal neurons increased the dendritic arborization without altering the average length of dendritic branches, whereas that of fRPTPζ decreased the dendritic arborization and increased the average length of dendritic branches. The RNA interference of fRPTPζ expression increased the dendritic arborization without altering the average length of dendritic branches. The overexpression of fRPTPζ decreased the density of hippocampal dendritic synapses, but that of sRPTPζ had no effects. Pleiotrophin, a ligand for RPTPζ to interfere the phosphatase activity, increased the density of hippocampal dendritic synapses. Thus, the present study demonstrates that two transmembranous RPTPζ isoforms have different functions for regulating dendritogenesis and synaptogenesis.

Key words: brain, extracellular matrix, glycosaminoglycan, phosphorylation, protein kinase, culture.

Receptor-type protein tyrosine phosphatase ζ/β (RPTPζ) is a chondroitin sulfate proteoglycan (CSPG) containing protein-tyrosine-phosphatase activity (for reviews see, Margolis and Margolis, 1997; Oohira et al., 2000). Molecular cloning studies have demonstrated the existence of two isoforms of transmembrane RPTPζ (Levy et al., 1993; Maurel et al., 1994): full-length receptor form of RPTPζ (fRPTPζ), which consists of the extracellular carbonic anhydrase domain, fibronectin type III domain, attachment region of CS (chondroitin sulfate) glycosaminoglycans, transmembrane segment, and intracellular phosphatase domains; shorter receptor form of RPTPζ (sRPTPζ), in which a greater part of the attachment region of CS glycosaminoglycans is deleted. In addition to transmebranous RPTPζ isoforms, there is a secreted isoform, named as phosphacan, which lacks intracellular phosphatase domains.

It is known that RPTPζ mediates the signal transduction by interacting with the growth factors such as pleiotrophin (PTN) and midkine (Maeda and Noda, 1998; Maeda et al., 1996, 1999). PTN-induced inactivation of the tyrosine phosphatase activity of RPTPζ increases the tyrosine phosphorylation of β-catenin in U373-MG cells (Meng et al., 2000). Membrane-associated guanylate kinase with inverted orientation (Magi) proteins are scaffolding molecule to interact with C-terminal tail of RPTPζ at plasma membrane (Adamsky et al., 2002; Fukada et al., 2005). The yeast two-hybrid system demonstrates the interaction between β-adducin and the intracellular domain of RPTPζ, and the tyrosine phosphorylation of β-adducin is sharply increased in HeLa cells by PTN stimulation (Pariser et al., 2005a,b). The C-terminal sequence of RPTPζ is able to bind to the postsynaptic density protein (PSD)-95 family protein via the second PDZ domain (Kawachi et al., 1999). Fyn, a Src family member tyrosine kinase, interacts with the intracellular domain of RPTPζ and the tyrosine phosphorylation of Fyn is increased in HeLa cells by PTN stimulation (Pariser et al., 2005a). An immunohistochemical study has revealed that RPTPζ and G-protein-coupled receptor kinase-interactor (Git1) are co-

[1] These authors contributed equally to this work.

*Corresponding author. Tel: +81-75-724-7796; fax: +81-75-724-7796.
E-mail address: smiyata@kit.ac.jp (S. Miyata).

Abbreviations: ADBL, average dendritic branch length; CHO, chinese hamster ovary; CS, chondroitin sulfate; CSPGs, chondroitin sulfate proteoglycans; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle's medium; DNGR, Delta/Notch-1 like epidermal growth factor; E18, embryonic day 18; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; fRPTPζ, full-length form of receptor-type protein tyrosine phosphatase ζ; GAP-43, growth-associated protein 43; Git1, G-protein-coupled receptor kinase-interactor; Grlf1, glucocorticoid receptor DNA binding factor 1; Magi, membrane-associated guanylate kinase with inverted orientation; MAP 2, microtubule-associated protein2; PB, phosphate buffer; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.3% Triton X-100; PDN, primary dendritic number; PFA, paraformaldehyde; PSD-95, postsynaptic density protein-95; RPTP, receptor-type protein tyrosine phosphatase ζ; sRPTPζ, shorter receptor form of receptor-type protein tyrosine phosphatase ζ; TBST, 50 mM Tris-buffered saline containing 0.5% Tween-20; TDBTN, total dendritic branch tip number.

0306-4522/09 $ - see front matter © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2009.09.012

localized in hippocampal and neocortical pyramidal dendrites and PTN increases the tyrosine phosphorylation of Git1 in B103 cells (Kawachi et al., 2001). The RPTPζ is shown to regulate the activity of Rho by controlling the phosphorylation state of glucocorticoid receptor DNA binding factor 1 (Grlf1) in neuroblastoma cells (Tamura et al., 2006).

Considering the target molecules for phosphatase of the RPTPζ as mentioned above, it is plausible that RPTPζ controls the neuronal morphology such as axonal elongation, dendritogenesis, and synaptogenesis, since β-catenin (Yu and Malenka, 2003), β-adducin (Rabenstein et al., 2005), Git1 (Zhang et al., 2003), Grlf1 (Sfakianos et al., 2007), and Fyn (Morita et al., 2006) play important roles in actin dynamics of cytoskeletons. Until now, however, only a few studies have been reported whether or not neuronal RPTPζ regulates the dendritic morphogenesis, although the expression of RPTPζ is demonstrated in neurons themselves *in vivo* (Shintani et al., 1998; Kawachi et al., 1999; Hayashi et al., 2005b) and *in vitro* (Maeda et al., 1996; Maeda and Noda, 1998; Hayashi et al., 2005a,b). It is shown that the signaling of RPTPζ regulates the morphogenesis of cerebellar Purkinje dendrites via modulating GLAST activity of adjacent Bergmann glial cells (Tanaka et al., 2003). PTN-RPTPζ signaling controls subcellular localization of Delta/Notch-1like epidermal growth factor (DNGR) and thereby regulates dendritic development of Purkinje neurons (Fukazawa et al., 2008). More importantly, no attempt has been made about functional differences of two transmembranous isoforms, sRPTPζ and fRPTPζ, in such neuronal morphogenesis. Therefore, we designed to investigate the effects of sRPTPζ and fRPTPζ overexpression or RNA interference of fRPTPζ on the dendritic morphology and dendritic synapse number using cultured hippocampal neurons. The present study reveals that two RPTPζ isoforms have different functions in controlling neurogenesis in hippocampal neurons.

EXPERIMENTAL PROCEDURES

Animals

Wistar rats were used in the present experiment. They were housed under standard conditions with a 12:12 h dark–light cycle and were given free access to food and water. All experimental protocols were performed in accordance with the guidelines for animal research of Neuroscience Society of Japan and approved by the committee of Kyoto Institute of Technology.

Cell culture

Neuronal cultures were prepared from the hippocampus of Wistar rats (E18) according to our previous method (Miyata et al., 2005). Dissociated hippocampus cells were plated on polyethyleneimine-coated glass coverslips at a density of 25,000 cells/cm^2 and grown in Neurobasal medium (Invitrogen, Japan, Tokyo, Japan) containing B27 supplement (Invitrogen, Japan). To eliminate glial cells, 2.5 μM Ara-C (Sigma-Aldrich, Japan, Tokyo, Japan) was added to the Neurobasal medium for 12 h on 2 days after plating. In some experiment, hippocampal neurons were cultured in the presence of 100 ng/ml PTN (Sigma-Aldrich, Japan). In some experiment, hippocampal neurons were treated with 0.5% Triton X-100 in 20 mM phosphate buffer (PB; pH 7.4) for 10 min at either 4 or 37 °C to examine the detergent solubility. Chinese hamster ovary (CHO) cells (100,000 cells/well) were grown in Dulbecco's modified Eagle's (DMEM) medium (Nissui, Tokyo, Japan) containing 10% fetal bovine serum and penicillin and streptomycin (Invitrogen, Japan).

Vector constructs and transfection

For overexpression, both sRPTPζ and fRPTPζ cDNAs were subcloned into the mammalian expression vector, pcDNA3.1 (+) (Invitrogen, Japan), in which constitutive expression is directed from the cytomegalovirus promoter (Nishiwaki et al., 1998). The product was digested with HindIII (Takara, Ohtsu, Japan) and NotI (Takara, Japan) for fRPTPζ and with EcoR V (Toyobo, Osaka, Japan) and NotI (Takara, Japan) for sRPTPζ and cloned into pcDNA3.1 vector.

For RNA interference, pRNAT-U6.1/Neo vectors (GenScript, Piscataway, NJ, USA) were digested with *BamH*I and HindIII and a DNA construct (5′-GATCCGTCTCGTATTGGTCTAGCTGAGTGTGCTGTCCAGCTAGACCAATACGAGACTCTTTTTA-3′), which was designed to include sense and antisense strands of rat fRPTPζ (nucleotide sequence 4972–4994), was cloned into the vector. This fRPTPζ siRNA vector is able to express the short hairpin RNA, which is rapidly processed into double strand RNA to suppress the expression of fRPTPζ, in transfected mammalian cells. All plasmid vectors were purified with endotoxin-free column, NucleoBond EF plasmid purification kit (Macherey-Nagel, Düren, Germany).

Hippocampal neurons at 7 days *in vitro* (DIV) and CHO cells were transfected using Lipofectamine 2000 (Invitrogen, Japan). DNA/lipid complexes were formed by the incubation of 2 μl Lipofectamine 2000 with 1 μg pcDNA3.1 vectors containing sRPTPζ or fRPTPζ construct and 1 μg pIRES-hrGFP-1a vectors (Stratagene, CA, USA) in 100 μl Opti-MEM medium (Invitrogen, Japan) without serum and antibiotics, and added to hippocampal neurons. For siRNA expression, 1 μl Lipofectamine 2000 and 1 μg pRNAT-U6.1/Neo vectors for fRPTPζ were used. After 5 h incubation at 37 °C, the medium was replaced with culture medium and antibiotics, and cells were kept for 7 days (hippocampal neurons) before the analysis. CHO cells were screened for 2 weeks using 1 mg/ml geneticin after the transfection.

Immunocytochemistry

Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) in 0.1 M PB (pH 7.5) for 20 min on 1, 3, 7, 14, and 21 DIV. Fixed cells were rinsed with PBS, treated with 25 mM glycine in PBS for 10 min, methanol at −20 °C for 2 min, and 0.3% Triton X-100 in PBS (PBST) for 15 min, and incubated with 5% normal goat serum in PBST for 30 min. To examine the localization of RPTPζ immunoreactivity as neuronal culture development, chondroitinase ABC (0.01 U/ml, Seikagaku Corp, Tokyo, Japan) was added into culture medium 3 h before fixation. The cells were then incubated with a 6B4 rabbit IgG antibody against RPTPζ (dilution 1:500) in PBST containing 5% NGS at 4 °C overnight. The 6B4 proteoglycan were prepared from rat brains by anion exchange chromatography and CsCl density gradient centrifugation, which was identified as RPTPζ by judging from its molecular weight, phosphatase activity, and HNK-1 epitope (Maeda et al., 1994, 1995). The characterization of this antibody was well documented as described previously (Nishiwaki et al., 1998; Maeda et al., 1998). They were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (KPL, Gaithersburg, MD, USA 10 μg/ml) in PBST containing 5% normal goat serum at 37 °C for 1 h and then incubated with one of following antibodies in PBST containing 5% NGS at 37 °C