Involvement of Receptor-like Protein Tyrosine Phosphatase ζ/RPTPβ and Its Ligand Pleiotrophin/Heparin-binding Growth-associated Molecule (HB-GAM) in Neuronal Migration

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Abstract. Pleiotrophin/heparin-binding growth-associated molecule (HB-GAM) is a specific ligand of protein tyrosine phosphatase ζ (PTPζ)/receptor-like protein tyrosine phosphatase β (RPTPβ) expressed in the brain as a chondroitin sulfate proteoglycan. Pleiotrophin and PTPζ isoforms are localized along the radial glial fibers, a scaffold for neuronal migration, suggesting that these molecules are involved in migratory processes of neurons during brain development. In this study, we examined the roles of pleiotrophin-PTPζ interaction in the neuronal migration using cell migration assay systems with glass fibers and Boyden chambers. Pleiotrophin and poly-L-lysine coated on the substratums stimulated cell migration of cortical neurons, while laminin, fibronectin, and tenascin exerted almost no effect. Pleiotrophin-induced and poly-L-lysine–induced neuronal migrations showed significant differences in sensitivity to various molecules and reagents. Polyclonal antibodies against the extracellular domain of PTPζ, PTPζ-S, an extracellular secreted form of PTPζ, and sodium vanadate, a protein tyrosine phosphatase inhibitor, added into the culture medium strongly suppressed specifically the pleiotrophin-induced neuronal migration. Furthermore, chondroitin sulfate C but not chondroitin sulfate A inhibited pleiotrophin-induced neuronal migration, in good accordance with our previous findings that chondroitin sulfate constitutes a part of the pleiotrophin-binding site of PTPζ, and PTPζ-pleiotrophin binding is inhibited by chondroitin sulfate C but not by chondroitin sulfate A. Immunocytochemical analysis indicated that the transmembrane forms of PTPζ are expressed on the migrating neurons especially at the lamellipodia along the leading processes. These results suggest that PTPζ is involved in the neuronal migration as a neuronal receptor of pleiotrophin distributed along radial glial fibers.

Key words: PTPζ • pleiotrophin • neuronal migration • receptor-like protein tyrosine phosphatase • proteoglycan

NEURONAL migration is a prerequisite for the development of the cortical structures in the central nervous system (CNS).[1] Migrations of CNS neurons are guided by a radial glial fiber system, in which postmitotic neurons generated in the proliferative ventricular zone translocate to their final positions along the radial glial fibers (Rakic, 1971). The process of neuronal migration is dynamic and depends on orchestration of multiple molecular events involving cell adhesion molecules, extracellular matrix molecules, ion channels, and cell surface receptors (Rakic et al., 1994). Recently, several molecules have been shown to play roles in the neuronal migration in CNS. β_1 integrin is required for the migratory process of neurons in the chicken optic tectum (Galileo et al., 1992). Astrotactin is a ligand expressed on neurons mediating neuron-glia binding during neuronal migration (Zheng et al., 1996). Reelin is a glycoprotein secreted by Cajal-Retzius cells in the developing cerebral cortex, which is essential for the establishment of the inside-out pattern of neuronal migration (D'Arcangelo et al., 1997). Furthermore, it has been suggested that the interaction between glial erbB receptor and its ligand, neuregulin on cerebellar granule cells is required for the radial glia formation and neuronal migration (Rio et al., 1997). The rate of granule cell migration is considered to be regulated by Ca^{2+} influx through N-type Ca^{2+} channels

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[1] *Abbreviations used in this paper*: anti-NFH, anti-highly phosphorylated neurofilament; CMF-HBSS, Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution; CNS, central nervous system; E, embryonic day; HB-GAM, heparin-binding growth-associated molecule; MAP, microtubule-associated protein; PTP, protein tyrosine phosphatase; RPTP, receptor-like PTP; SA-HRP, streptavidin-conjugated horseradish peroxidase.

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and NMDA receptors (Komuro et al., 1993, 1996). Although these molecules were identified as functionally important, little is known about the signal transduction mechanism of neurons underlying neuronal migration.

Recently, we and others identified a proteoglycan-type protein tyrosine phosphatase, protein tyrosine phosphatase ζ (PTPζ)/receptor-like PTPβ (RPTPβ), specifically expressed in the brain (Krueger and Saito, 1992; Levy et al., 1993; Barnea et al., 1994; Maeda et al., 1994; Maurel et al., 1994). In the early cortical development, PTPζ has been localized along radial glial fibers and on migrating neurons, suggesting that this receptor-type phosphatase is involved in neuronal migration (Canoll et al., 1993; Maeda et al., 1995). PTPζ consists of an NH_2-terminal carbonic anhydrase-like domain, a fibronectin type III domain, a large cysteine-free region, a transmembrane segment, and two tyrosine phosphatase domains (Krueger and Saito, 1992; Levy et al., 1993). There exist three splice variants of this molecule: (*a*) the full-length PTPζ (PTPζ-A), (*b*) the short form of PTPζ, in which most of the cysteine-free region is deleted (PTPζ-B), and (*c*) the secreted form (PTPζ-S), which corresponds to the extracellular region of PTPζ-A and is also known as 6B4 proteoglycan/phosphacan (Maeda et al., 1992, 1994; Maurel et al., 1994). Here, we refer to these three molecules as PTPζ as a whole. All these splice variants are synthesized as chondroitin sulfate proteoglycans in the brain, suggesting that the chondroitin sulfate portion is essential for the receptor function (Maeda et al., 1994; Nishiwaki et al., 1998). PTPζ has been shown to bind various cell adhesion and extracellular matrix molecules such as F3/contactin, N-CAM, L1, TAG1, and tenascin (Grumet et al., 1994; Milev et al., 1994, 1996; Peles et al., 1995). Furthermore, we found that PTPζ binds pleiotrophin/heparin-binding growth-associated molecule (HB-GAM) (Maeda et al., 1996*a*). Chondroitin sulfate and the protein portion of PTPζ together constitute the binding site of pleiotrophin, and various glycosaminoglycans inhibit PTPζ-pleiotrophin binding (Maeda et al., 1996*a*). Pleiotrophin has a family member midkine that shows 50% similarity. These molecules have mitogenic and neurite outgrowth-promoting activities and constitute a new growth factor gene family (Kadomatsu et al. 1988; Li et al., 1990; Merenmies and Rauvala 1990). *N*-syndecan also has been shown to bind pleiotrophin, and PTPζ and *N*-syndecan are considered to be the pleiotrophin receptors responsible for the pleiotrophin-induced neurite outgrowth (Raulo et al., 1994; Kinnunnen et al., 1996; Maeda et al., 1996*a*; Rauvala and Peng, 1997).

In addition to the mitogenic and differentiation promoting activities, pleiotrophin and also midkine are thought to be functional in cell–cell interaction and migration (Kurtz et al., 1995). In the early cerebral cortex, pleiotrophin is synthesized by radial glial cells and is deposited on radial glial fibers during neuronal migration (Matsumoto et al., 1994*a,b*; Rauvala et al., 1994). This raises the possibility that the ligand–receptor mechanism between PTPζ and pleiotrophin plays a role in the neuronal migration. In this study, we addressed this possibility using two cell migration assay systems in vitro: migration assay on glass fibers (Fishman and Hatten, 1993) and Boyden chamber cell migration assay (Kim et al., 1997). In the both assay systems, pleiotrophin potently induced cell migration of cortical neurons. Pleiotrophin-induced neuronal migration was inhibited by glycosaminoglycans, and the effectivity of their inhibition matched well with that on pleiotrophin-PTPζ binding. Furthermore, polyclonal antibodies against the extracellular domain of PTPζ and low concentrations of soluble PTPζ-S added in the culture medium inhibited pleiotrophin-induced neuronal migration. Finally, protein tyrosine phosphatase inhibitor, sodium vanadate, suppressed the pleiotrophin-induced neuronal migration. From these observations, we suggest that PTPζ on migrating neurons acts as a receptor of pleiotrophin on radial glial fibers by transducing its signal to induce neuronal migration.

Materials and Methods

Materials

PTPζ-S (6B4 proteoglycan) was purified as described previously (Maeda et al., 1995). Polyclonal antibodies against purified PTPζ-S (anti-6B4 PG), antiserum 31-5 (anti-31-5), and mAb 6B4 were described previously (Maeda et al., 1992, 1994). Anti-RPTPβ was purchased from Transduction Laboratories (Lexington, KY). Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology Inc. (Lake Placid, NY). A cocktail of monoclonal antibodies to phosphorylated neurofilaments SMI312 was obtained from Sternberger Monoclonals Inc. (Baltimore, MD). Antiserum against microtubule-associated protein 2 (MAP2) was a generous gift from Dr. Niinobe (Osaka University, Japan; Niinobe et al., 1988). Biotinylated anti–mouse Ig, biotinylated anti–rabbit Ig and streptavidin-conjugated alkaline phosphatase were purchased from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). TSA-Indirect kit was obtained from DuPont NEN (Boston, MA). Vectastain ABC kit and Fluorescein Avidin DCS were from Vector Labs, Inc. (Burlingame, CA). PermaFluor was from Immunon (Pittsburgh, PA). Tenascin purified from human glioma cell line u-251MG was from Chemicon International, Inc. (Temecula, CA). Fibronectin was purchased from Nitta Gelatin (Chiba, Japan). Laminin, heparin, poly-L-lysine ($M_r > 300 \times 10^3$), and rabbit IgG were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium, F12 medium, and B-27 supplement were from Gibco BRL (Gaithersburg, MD). Chondroitin sulfate A from whale cartilage, chondroitin sulfate C from shark cartilage, and heparan sulfate from bovine kidney were purchased from Seikagaku, Inc. (Tokyo, Japan). Transwell™ was obtained from Corning Coster Corp. (Cambridge, MA). Micro BCA kit was from Pierce Chemical Co. (Rockford, IL). HiTrap Protein G was from Pharmacia Biotechnology, Inc.

Herbimycin A and erbstatin analogue were obtained from Research Biochemicals International (Natick, MA). Lavendustin A was purchased from Life Technologies, Inc. (Gaithersburg, MD). These tyrosine kinase inhibitors were solubilized in DMSO and stored at −20°C until use.

Sodium orthovanadate was activated by depolymerization. In brief, 10 mM sodium orthovanadate was adjusted to pH 10 using 1 M NaOH. The resulting yellow solution was boiled until it became clear. The solution was readjusted to pH 10 and boiled again. This procedure was repeated until the solution remained clear with a stable pH.

Preparation of Dissociated Neurons

Cerebra were dissected from embryonic day-17 (E17) Sprague-Dawley rats, and the meninges were removed. The tissues were incubated in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (CMF-HBSS) containing 0.1% trypsin for 15 min at 37°C. After three washes with CMF-HBSS, the tissues were triturated with Pasteur pipettes in CMF-HBSS containing 0.025% DNAase I, 0.4 mg/ml soy bean trypsin inhibitor, 3 mg/ml BSA, and 12 mM $MgSO_4$. The cell suspension was centrifuged at 160 *g* for 5 min at 4°C, and the pelleted cells were washed once with CMF-HBSS. The cells were suspended in culture medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and F12 medium containing 2% B-27 supplement (DF/B-27 medium). Cell suspensions were used for cell migration assays as described below.

Cell Migration Assay on Glass Fibers

Cell migration assay on glass fibers was carried out according to the method described by Fishman and Hatten (1993) with slight modifications. Whatman GF/A glass fiber filters were autoclaved and then shattered by vortexing in distilled water. Fibers were pelleted by microcentrifugation and coated first with 7 μg/ml poly-L-lysine for 1 h at room temperature. Fibers were washed three times with distilled water, and then coated with 30 μg/ml laminin or pleiotrophin diluted in 5 mM Tris-HCl, pH 8.0, for 2 h at room temperature. The fibers were washed with DF/B-27 medium and used for migration assay.

Wells of 48-well plates were coated with 20 μg/ml poly-L-lysine, to which glass fibers were added together with 200 μl of DF/B-27 medium. Cortical neurons (50,000 cells in 20 μl of DF/B-27 medium) were added into the each well and cultured for 15–20 h at 37°C under 5% CO_2. Then, cultures were monitored for migration by time-lapse video recording using Zeiss Axiovert 135M microscope equipped with Zeiss ZVS-3C75DE CCD camera (Carl Zeiss, Inc., Thornwood, NY) and Sony LVR-3000AN video disk recorder (Sony Corp., Tokyo, Japan).

Fields containing neurons bound to the glass fibers were randomly selected, and their images were recorded at 5-min intervals for 2 h at low magnification (×20). For each group of differently treated fibers, migration assay experiments were performed at least three times. 5–10 independent fields were analyzed in each experiment, and the migration speeds of all the neurons on glass fibers were measured. The migrations of at least 100 neurons in total were analyzed for each group of the treated fibers.

Boyden Chamber Cell Migration Assay

Boyden chamber cell migration assays were performed using Transwells™ (Corning Costar Corp.) containing polycarbonate membranes (tissue culture treated, 6.5-mm diam, 10-μm thickness, 3-μm pores). The under surface of the membrane was coated with 12 μl of various concentrations of proteins solubilized in 5 mM Tris-HCl, pH 8.0, for 2 h at room temperature. For the routine assay, membranes were coated with 35 μg/ml pleiotrophin or 20 μg/ml poly-L-lysine. After washing three times with PBS, the membranes were placed in the wells of a 24-well plate containing 0.5 ml of DF/B-27 medium. The amounts of pleiotrophin bound to the filters increased linearly from the concentration of 10 to 100 μg/ml (data not shown), and precoating with poly-L-lysine was not necessary. The binding was stable and no decrease in the bound material was observed during overnight incubation in the culture medium.

Cortical neurons (100,000 cells in 0.2 ml DF/B-27 medium) were added to the upper chamber and incubated for 20 h at 37°C under 5% CO_2. Glycosaminoglycans, PTPζ-S, antibodies, and inhibitors were added to both the upper and lower chambers. In the experiments where PTPζ-S was added, pleiotrophin- or poly-L-lysine-coated membranes were additionally blocked with 0.2% BSA/DF medium for 3 h at 37°C to prevent the nonspecific adsorption of PTPζ-S to the membranes. Cells were fixed with 4% paraformaldehyde and 0.1 M sodium phosphate, pH 7.4, and then the upper surface of the membrane was wiped with a cotton-tip applicator to remove nonmigratory cells. The cells migrated were stained for 30 min with 1% crystal violet, 5% ethanol, and 0.1 M borate, pH 9.0. The membrane was mounted between two glass slides with 50% glycerol, and the numbers of nuclei of the migrated cells per a microscopic field (×25) were counted. Each assay was performed in triplicate and the experiments were repeated on at least three separate isolations of cortical neurons.

Neurite Extension Assay

Effects of vanadate and tyrosine kinase inhibitors on neuronal cell differentiation were examined as follows. Glass coverslips (9 mm in diameter) were coated with 5 μg/ml pleiotrophin for 5 h at 4°C, and then washed five times with PBS. Neurons (2×10^4 cells in 20 μl of DF/B-27 medium) were plated on the coverslips. After incubation for 1 h at 37°C under 5% CO_2, 150 μl per well of DF/B-27 medium containing inhibitors was added. Cells were cultured for another 20 h and then fixed and double stained with anti-MAP2 and anti-phosphorylated neurofilament antibodies SMI 312, as described previously (Maeda et al., 1996*b*).

Detection of Tyrosine Phosphorylated Proteins

Suspended cells (3.5×10^6 cells in 1 ml of DF/B-27) were seeded onto poly-L-lysine–coated 35-mm dishes. After incubation for 2 h at 37°C under 5% CO_2, 1 ml of DF/B-27 containing various inhibitors was added to the dishes. Cells were incubated for a further 20 h, then washed two times with 1 ml of cold DF medium, and solubilized by adding 0.5 ml of 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM pepstatin A, 5 mM EDTA, 2 mM sodium vanadate, 10 mM sodium pyrophosphate, 1% NP-40, 0.1% SDS, and 50 mM Tris-HCl, pH 7.5. The solutions were centrifuged at 2,000 *g* for 5 min and the supernatants were applied to 7.5% SDS-PAGE according to the method of Laemmli (1970). After electrophoresis, proteins were transferred to PVDF membranes. The membranes were blocked with 5% nonfat dried milk in PBS, incubated for 30 min with 4G10 anti-phosphotyrosine monoclonal antibody (1/1,000), and washed three times with PBS. The membranes were then incubated for 30 min with biotinylated anti–mouse Ig (1/200), washed three times with PBS, and incubated for 30 min with streptavidin-conjugated alkaline phosphatase (1/1,000). After washing three times with PBS, the membranes were treated with 0.3 mg/ml nitroblue tetrazolium, 0.18 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 0.1 M NaCl, 50 mM $MgCl_2$, 0.1 M Tris-HCl, pH 9.5.

Immunocytochemistry

Cells on Boyden chamber membranes were washed once with PBS and incubated for 20 min in 4% paraformaldehyde, and 0.1 M sodium phosphate, pH 7.4. Fixed cells were washed three times with TBS, incubated in 2.5% H_2O_2/PBS for 60 min and permeabilized with 0.2% Triton X-100/PBS for 30 min. After blocking with 2% BSA/4% goat serum/PBS for 30 min, cells were incubated for 2 h with anti-MAP2 antiserum (1/2,000). After three washes with PBS, cells were incubated for 30 min in biotinylated anti–rabbit Ig solution (1/200), washed three times with PBS, and incubated for 30 min in avidin-biotin-peroxidase complex solution. After three washes with PBS, cells were incubated in 0.1% diaminobenzidine/0.02% H_2O_2/PBS.

Immunocytochemical staining with anti-RPTPβ was performed using TSA Indirect kit. Cells were fixed as above, washed three times with TBS, incubated for 30 min in 0.3% H_2O_2/methanol, washed three times with TBS, and permeabilized with 0.05% Triton X-100/TBS for 10 min. Cells were blocked with 2% BSA, 4% goat serum, and TBS for 30 min, and incubated overnight with anti-RPTPβ (1/100) at 4°C. After three washes with TBS, cells were incubated in biotinylated anti-mouse Ig (1/200) for 30 min, and then processed with TSA Indirect kit according to the supplier's protocol. Finally, cells were incubated with Fluorescein Avidin DCS (1/50) for 30 min. After three washes with TBS, cells were mounted in PermaFluor and observed with a Zeiss fluorescence microscope.

Immunohistochemistry

After ether anesthesia, Sprague-Dawley rats were perfused with PBS and then with a solution containing 4% paraformaldehyde and 0.1 M sodium phosphate buffer, pH 7.4, via the left ventricle. The solution was washed out from the right atrium, and the brains were dissected out and embedded in paraffin after dehydration through a graded alcohol series. Paraffin-embedded samples were cut into sections 6 μm thick, which were then deparaffinized and equilibrated in PBS. The sections were incubated sequentially in the following solutions: (*a*) 2.5% H_2O_2/PBS for 60 min; (*b*) 1% BSA and 4% goat serum for 30 min; (*c*) anti-RPTPβ (1/100) for 24 h at 4°C; (*d*) biotinylated anti–mouse Ig solution for 60 min; (*e*) 0.5% Dupont blocking reagent and TBS for 30 min; (*f*) streptavidin-conjugated horse radish peroxidase (SA-HRP) solution for 30 min; (*g*) biotinyl tyramide solution for 8 min; (*h*) SA-HRP solution for 30 min; (*i*) 0.1% diaminobenzidine/0.02% H_2O_2 and PBS. TSA-Indirect kit was used according to the supplier's protocol.

Immunohistochemical staining with mAb 6B4 was performed as described previously (Maeda et al., 1995).

Other Methods

Protein concentration was determined using a MicroBCA kit using BSA as a standard. IgG fractions from anti-6B4 PG and anti–31-5 antisera were prepared with HiTrap Protein G according to the supplier's protocol. The amounts of proteins attached to the glass fibers or Boyden chamber membranes were measured using ^{125}I-labeled proteins as described previously (Maeda and Noda, 1996*b*).

Results

Pleiotrophin Induces Neuronal Migration on Glass Fibers

Immunohistochemical studies indicated that pleiotrophin is distributed along radial glial fibers, suggesting that this molecule is involved in neuronal migration (Matsumoto et al., 1994). To test this possibility in vitro, cell migration of cortical neurons on glass fibers coated with pleiotrophin was assayed according to the method of Fishman and Hatten (1993; Fig. 1 *A*). Since glass fibers have a similar geometry to radial glial fibers, protein-coated glass fibers are suitable substratum to examine whether a protein induces neuronal migration. On uncoated glass fibers, cortical neurons showed rounded shape, and no neuronal migration was observed (*N* = 100), indicating that only the glial fiber-like geometry is not sufficient to promote migration (data not shown).

Glass fibers were pretreated with poly-L-lysine to promote the binding of proteins. The pretreated glass fibers were then coated with 30 μg/ml pleiotrophin or laminin. Laminin was used as a control protein because it potently promoted migration of cerebellar granule cells on glass fibers (Fishman and Hatten, 1993). The amounts of pleiotrophin and laminin attached to the fibers were 216 ± 63 and 85 ± 33 ng/mg fibers, respectively. For each group of glass fibers coated with poly-L-lysine only (PLL fiber), poly-L-lysine + pleiotrophin (PTN fiber), or poly-L-lysine + laminin (LN fiber), neurons attached to the fibers within several hours, and >85% of the cells displayed spindle shapes on the fibers. The migration consisted of periods of movement interspersed with stationary periods (Fig. 1 *A*), which was similar to the migration of cerebellar granule cells on glass fibers coated with astroglial membranes (Fishman and Hatten. 1993).

On the PLL fibers, 22% of bound cells migrated at the rate of >4 μm/h in an observation period of 2 h and the

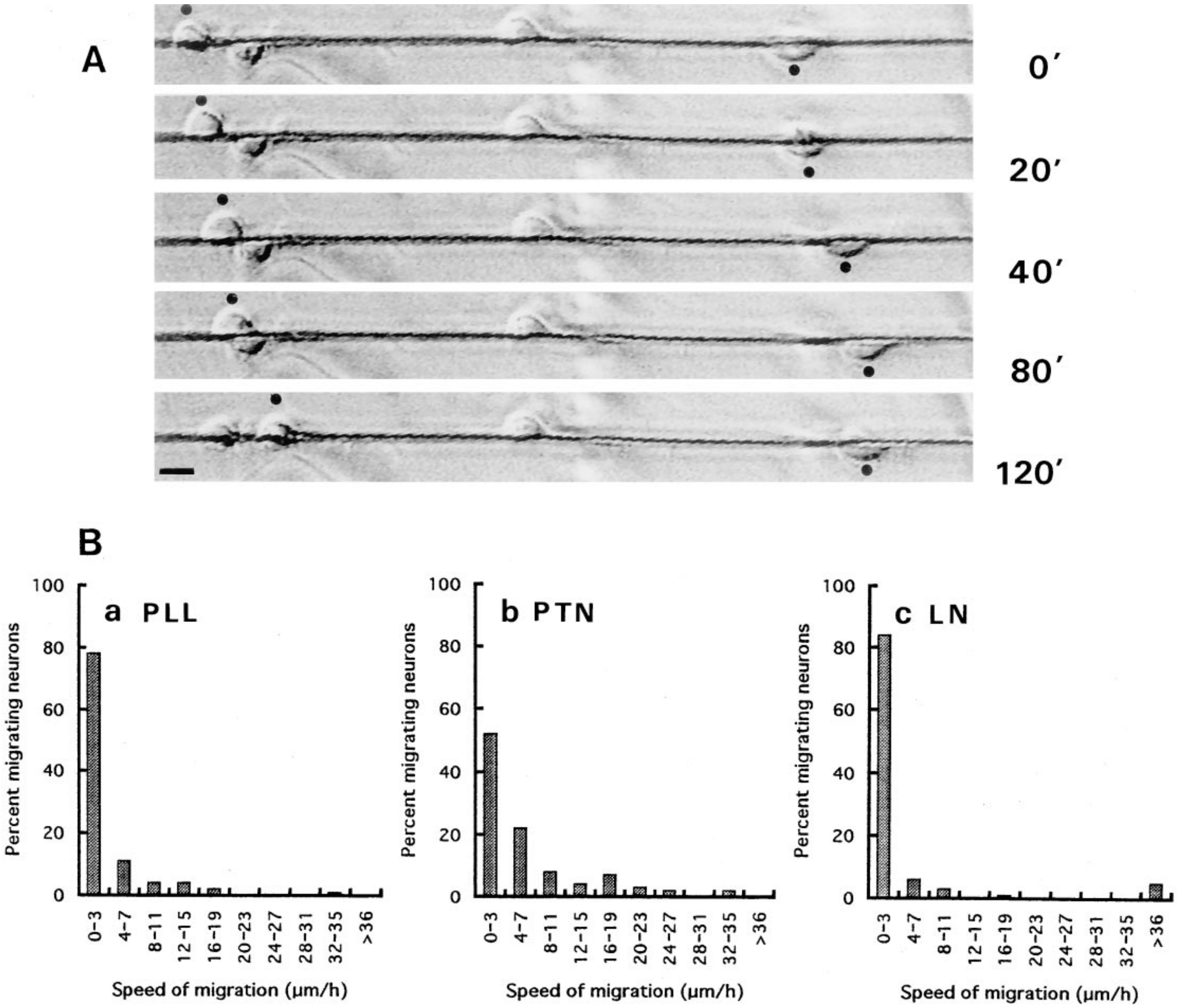

Figure 1. Pleiotrophin induces cell migration of cortical neurons. (*A*) Time-lapse video microscopy revealed several neurons (indicated by filled dots) migrating on a PTN fiber. Both neurons migrated ∼25 μm along the fiber during a 2-h observation. The cell located in the middle appeared to stay in the stationary period. (*B*) The percentage distributions of migration rates of cortical neurons on PLL (*a*), PTN (*b*) and LN (*c*) fibers are shown. On PLL fibers, 22% of the cells migrated at >4 μm/h, and the average speed of migration among them was 9.5 μm/h. On PTN fibers, 48% of the cells migrated at more than 4 μm/h, and their average speed of migration was 11.0 μm/h. On LN fibers, 18% (13%) of the cells migrated at >4 μm/h, and their average speed was 26.3 μm/h (6.8 μm/h); the values in parentheses are those when the rapidly migrating population (>36 μm/h) was not included. Bar, 10 μm.

maximum rate was 33 μm/h (Fig. 1 *B*, *a*, $N = 101$). About 67% of the cells showed movement of <1.5 μm/h, which corresponded to the baseline level of motility of most living cells in culture. On the other hand, 48% of the cells migrated at the rate of >4 μm/h on the PTN fibers, and the maximum rate was 33 μm/h (Fig. 1 *B*, *b*, $N = 107$), indicating that pleiotrophin promoted migration additionally to the activity of poly-L-lysine. Only 21% of the cells stayed stationary with the migration rate of <1.5 μm/h. The migrating neurons had leading processes and showed caudal positioning of nucleus as observed for neurons on radial glial fibers (Rakic, 1971). The shape of the migrating neurons on PTN fibers was indistinguishable from that on PLL fibers.

In contrast to the PTN fibers, the percentage distribution of migration rates of neurons on the LN fibers was similar to that on PLL fibers (Fig. 1 *B*, *c*, $N = 116$; 18% of the cells were at the rate of >4 μm/h). However, it is noteworthy that ~5% of cells displayed very rapid migration on LN fibers (>36 μm/h) with the maximum rate of 75 μm/h. The shape of these cells was more rounded than those of migrating neurons on PLL and PTN fibers and of more slowly migrating neurons on LN fibers, suggesting that there was a small subpopulation of cells that could specifically respond to laminin. These results indicated that pleiotrophin and poly-L-lysine promote the migration of cortical neurons on glass fibers, but the activity of laminin is low except for to a minor population.

Pleiotrophin Induces Neuronal Migration in a Dose-dependent Manner

Cell migration assay on glass fibers is rather time consuming and is not suitable for a series of perturbation experiments. Therefore, we adopted an alternative assay system, Boyden chamber cell migration assay, because essentially similar results were obtained (see below). Membranes were coated with proteins and/or poly-L-lysine as described in Materials and Methods. Our culture was estimated to be 98% pure neurons that expressed PTPζ (Maeda et al., 1996*b*), and it was expected that most of the migrated cells were neurons. This was further confirmed here by the results that the migrated cells were intensely stained with anti-MAP2 (Fig. 2). The neurons migrating the pleiotrophin-coated filters were spindle-shaped with a tapered leading process, and resembled the neurons migrating along radial glial fibers (Fig. 2 *A*). After migration, the neurons resumed a rounded body shape and extended actively long axon-like processes with numerous varicosities (Fig. 2 *B*). Thus, it seems likely that this assay system mimics the processes of migration and subsequent differentiation of neurons occurring in the cerebral cortex during brain development.

Fig. 3 *A* shows that pleiotrophin potently induced the cell migration of cortical neurons in a dose-dependent manner. 520 ± 110 ng/cm^2 of pleiotrophin bound to the filters when coating was carried out at 50 μg/ml. After 20 h of incubation, ~12% of the cells migrated through the filters when underside was coated with >50 μg/ml of pleiotrophin. No further migration was observed during the next 20 h, indicating that the migratory process was completed during 20 h of incubation. Percentage of the migrated cells was relatively low probably because of the developmentally heterogenous nature of neurons at this stage. The neurons that failed to contact with the pores of filters when they were plated, made large cell aggregates and would not migrate anymore. This may be another reason for the low percentage of migrated cells. One cannot directly compare the values of percentage of the migrated cells between Boyden chamber assay and glass fiber assay, because only the cells attached to the fibers were analyzed in the latter assay system. When uncoated Boyden chamber membranes were used, no cell migration occurred, and in each culture condition, no cells dropped through the pores into the lower chamber. When both sides of the membranes were coated with pleiotrophin, <1% of neurons migrated to the undersurface of the filters (data not shown), indicating that pleiotrophin does not activate random migration.

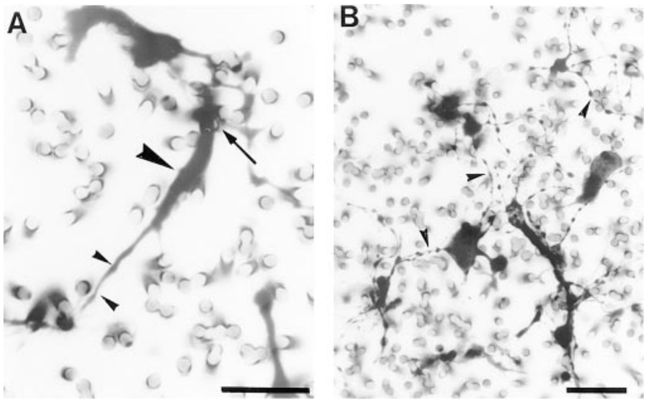

Figure 2. Neurons migrated on the pleiotrophin-coated Boyden chamber membranes. (*A*) A spindle-shaped neuron (*large arrowhead*) passing through a pore (*arrow*) of the filter had a leading process (*small arrowheads*). (*B*) After migration to the lower side, neurons resumed a rounded shape and extended long axon-like processes (*arrowheads*). In these samples, the migrated neurons were immunostained with anti-MAP2, after nonmigratory cells on the upper surface of the filters were removed by wiping with a cotton-tip applicator. Bar, 20 μm.

Poly-L-lysine significantly induced neuronal migration on Boyden chamber membranes, although the effective concentration range was limited (Fig. 3 *A*). On the other hand, laminin exerted little effect on the migration of cortical neurons also in the Boyden chamber cell migration assay (Fig. 3 *A*). 240 ± 81 ng/cm^2 of laminin bound to the filters when coating was carried out at 50 μg/ml, excluding the possibility that laminin did not bind to the membrane effectively. In addition, even when the filters pretreated with poly-L-lysine were used, laminin did not show significant additive effect on neuronal migration (data not shown). These observations suggest that cell migrations on glass fibers and Boyden chamber membranes are based on the common cellular mechanisms. This is also the case for cerebellar neurons. In Boyden chamber assay of cerebellar neurons from P7 rats that were mostly granule cells, >50% of the cells migrated through the filters coated with 30 or 100 μg/ml of laminin. In contrast, <1% of the cells migrated on the filters coated with 10 or 30 μg/ml of poly-L-lysine, and even when coating was done with 100 μg/ml

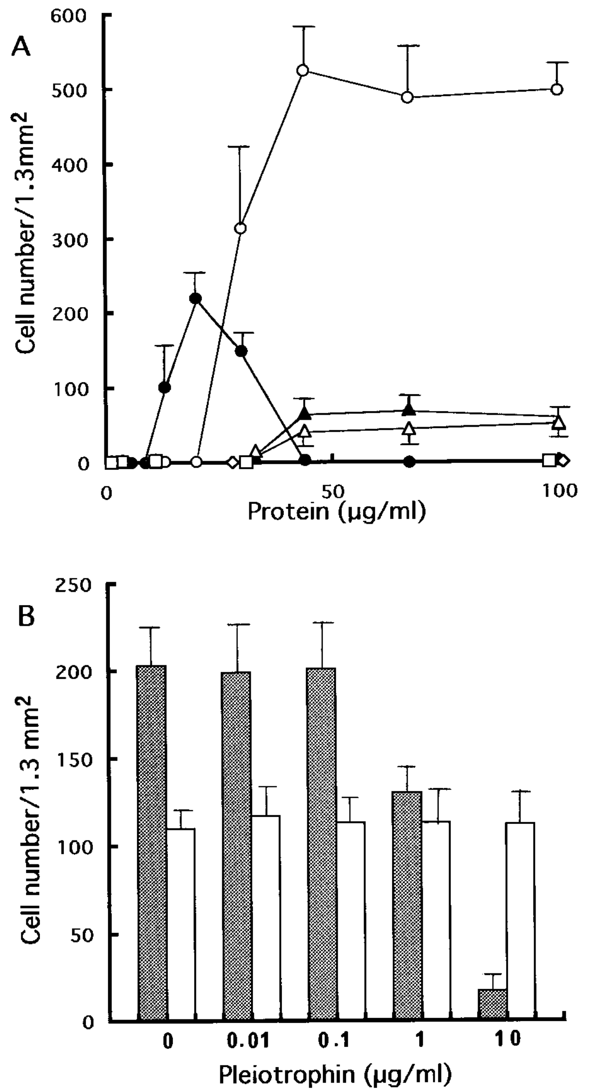

Figure 3. Boyden chamber cell migration of cortical neurons. (*A*) Cortical neurons were analyzed by Boyden chamber cell migration assay using membranes coated with various concentrations of pleiotrophin (○), poly-L-lysine (●), fibronectin (▲), laminin (△), tenascin (□) and PTPζ-S (◇). Pleiotrophin and poly-L-lysine significantly stimulated neuronal migration, whereas the other proteins exhibited low activities. Each point represents the mean ± SD of triplicate values. (*B*) Cortical neurons were subjected to Boyden chamber cell migration assay using membranes coated with 30 μg/ml pleiotrophin (*shaded column*) or 15 μg/ml poly-L-lysine (*open column*) in the presence of various concentrations of soluble pleiotrophin in the lower chamber. Soluble pleiotrophin suppressed the pleiotrophin-induced neuronal migration, but not the poly-L-lysine-induced migration. Each bar represents the mean ± SD of quintuplicate values.

poly-L-lysine, <5% of the cells migrated (data not shown). Consistently, Fishman and Hatten (1993) reported that the LN fibers coated even with 2.5 μg/ml laminin potently promoted migration of granule cells, but PLL fibers were totally inactive. On the other hand, ~8% of the cerebellar neurons migrated through the Boyden chamber membranes coated with 30 μg/ml pleiotrophin or fibronectin in our experiments. This is also consistent with the observations of Fishman and Hatten (1993) that fibronectin moderately induced migration of granule cells on glass fibers. Taken together, these findings suggested that cortical neurons and cerebellar granule cells use different receptor systems for migration.

The extracellular matrix molecules such as fibronectin, tenascin, and PTPζ-S had little or no effect on the migration of cortical neurons (Fig. 3 *A*), although substantial amounts of proteins bound to the filters (data not shown).

To evaluate the chemotaxic activity of pleiotrophin, we examined the migration on pleiotrophin-coated filters in the presence of various concentrations of soluble pleiotrophin in the lower chamber. As shown in Fig. 3 *B*, migration was not augmented, but rather inhibited by the high concentrations of soluble pleiotrophin, suggesting that only substrate-bound pleiotrophin is active ligand for migration. Next we examined the migration on poly-L-lysine–coated filters in the presence of soluble pleiotrophin in the lower chamber. In contrast to the results of pleiotrophin-induced migration (Fig. 3 *B*, *shaded column*), soluble pleiotrophin exerted no effect on the poly-L-lysine-induced neuronal migration (Fig. 3 *B*, *open column*), suggesting that poly-L-lysine–induced migration is based on the receptor system different from that of pleiotrophin-induced migration. This also supports the notion that pleiotrophin has no chemotaxic activity. In the following experiments, we examined the effects of various reagents on the pleiotrophin-induced migration of cortical neurons using Boyden chamber cell migration assay. We used poly-L-lysine–induced neuronal migration as a control of pleiotrophin-induced migration to discriminate between the general effects and specific effects of substances on cell migration.

Effects of Glycosaminoglycans on the Pleiotrophin-induced Neuronal Migration

Previously, we indicated that heparin, heparan sulfate, and chondroitin sulfate C but not chondroitin sulfate A inhibited binding of pleiotrophin to PTPζ-S (Maeda et al., 1996*a*). The effects of various glycosaminoglycans on the neuronal migration was examined by adding glycosaminoglycans into the culture medium (Fig. 4). Heparin and heparan sulfate potently inhibited pleiotrophin-induced neuronal migration (Fig. 4 *A*), however, these glycosaminoglycans also inhibited poly-L-lysine–induced migration (Fig. 4 *B*), suggesting that the inhibitory effects of these substances were at least partly due to the general influences on neurons. In contrast, chondroitin sulfate C specifically inhibited pleiotrophin-induced neuronal migration (Fig. 4, *A* and *B*). Chondroitin sulfate A, on the other hand, exerted no effect on either type of migration. Pleiotrophin-induced neurite extension was also inhibited by chondroitin sulfate C but not by chondroitin sulfate A (data not shown). These observations raised a possibility that pleiotrophin–PTPζ interaction is physiologically involved in the neuronal migration.

Involvement of Neuronal PTPζ in Pleiotrophin-induced Neuronal Migration

When PTPζ on the neuronal cell surface functions as a receptor for pleiotrophin in this cell migration, soluble PTPζ-S added into the culture medium should competitively in-

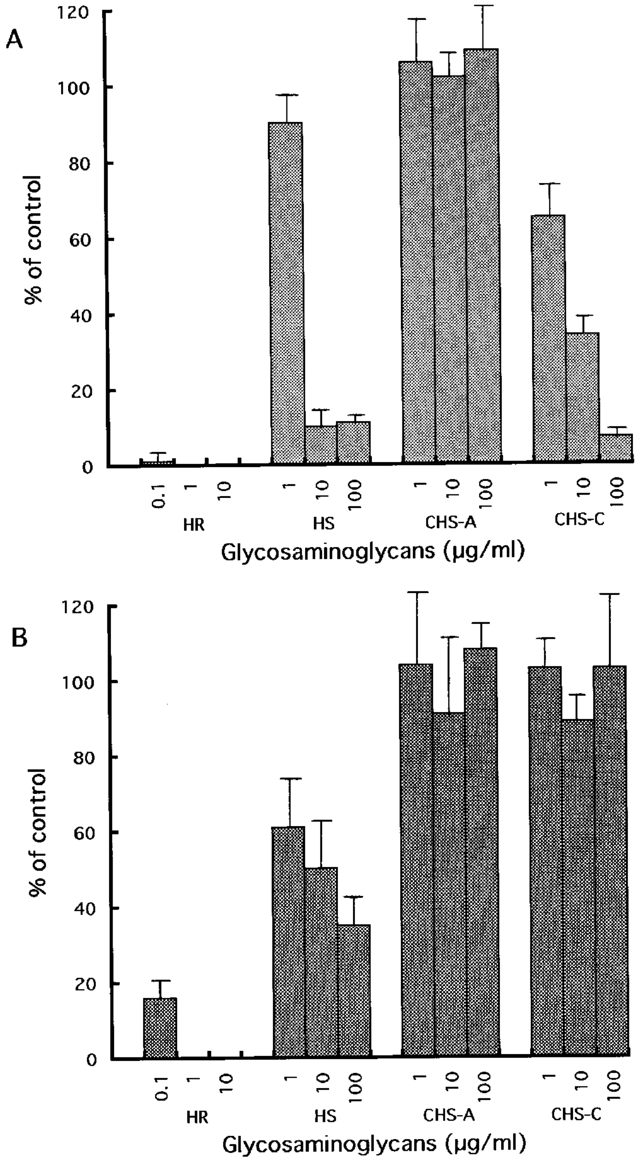

Figure 4. Effects of glycosaminoglycans on pleiotrophin-induced neuronal migration. Cortical neurons were analyzed by Boyden chamber cell migration assay using pleiotrophin-coated (*A*) and poly-L-lysine–coated (*B*) membranes. Neurons were cultured in the presence of heparin (*HR*), heparan sulfate (*HS*), chondroitin sulfate A (*CHS-A*) and chondroitin sulfate C (*CHS-C*). Heparin and heparan sulfate suppressed both pleiotrophin- and poly-L-lysine–induced neuronal migration. In contrast, chondroitin sulfate C inhibited only pleiotrophin-induced neuronal migration. Each bar represents the mean ± SD of triplicate values.

hibit the pleiotrophin-induced neuronal migration. Fig. 5 shows that low concentrations of PTPζ-S (IC_{50} = ~0.5 μg/ml as protein) indeed suppressed the pleiotrophin-induced migration, whereas the poly-L-lysine–induced migration was not affected. Furthermore, polyclonal antibodies against PTPζ-S (anti-6B4 PG), which also recognize the extracellular domains of the two transmembrane forms of PTPζ, inhibited the pleiotrophin-induced neuronal migration (Fig. 6). This effect was observed at concentrations of over 200 μg/ml and 80% inhibition was attained at 300 μg/ml anti-6B4 PG. By contrast, polyclonal antibodies against the COOH-terminal portion of PTPζ-S (anti–31-5), which also react with PTPζ-A, exerted no effect as control rabbit IgG. On the other hand, poly-L-lysine–induced neuronal migration was not influenced by any of these antibodies (Fig. 6). These results suggest that PTPζ on neurons is involved in the pleiotrophin-induced neuronal migration as a pleiotrophin receptor.

Effects of Protein Tyrosine Phosphatase and Kinase Inhibitors on Pleiotrophin-induced Neuronal Migration

Signal transduction of PTPζ has been postulated to be mediated by modification of the tyrosine-phosphorylation levels of intracellular proteins. It has been reported that pleiotrophin stimulated tyrosine phosphorylation of a 200-kD protein in NIH 3T3 and NB41A3 cell lines (Li and Deuel, 1993). Therefore, we examined the effects of tyrosine phosphatase and kinase inhibitors on pleiotrophin-induced neuronal migration. To reduce the possible artifacts of inhibitors, we evaluated first the effects of a range of concentrations of inhibitors on cell viability and differentiation (Fig. 7). Sodium vanadate has been used as an inhibitor of protein tyrosine phosphatases (Gordon, 1991), and herbimycin A inhibits Src family tyrosine kinases (Uehara et al. 1988). Lavendustin A and erbstatin analogue have been reported to inhibit EGF receptor kinase (Onoda et al., 1989; Umezawa et al., 1990).

Cortical neurons from E17 rat embryos were cultured on pleiotrophin-coated coverslips in the presence or absence of inhibitors, and double-stained with anti-MAP2 and anti-highly phosphorylated neurofilament (anti-NFH) after 20 h of culture. MAP2 is a microtubule-associated protein characteristic of dendrites, and NFH is enriched in axons (Lafont et al., 1992; Maeda et al., 1996*b*). Neurites of cultured neurons initially express both MAP2 and NFH, and then differentiate into dendrites and axons expressing either of the two proteins after 3 d in vitro. In the control culture, neurons extended several thick and smooth MAP2-positive neurites, and most of them were also stained with anti-NFH (Fig. 7, *A* and *B*). NFH-positive and MAP2-negative axon-like processes with numerous varicosities were also often observed (Fig. 7 *B*, *arrowheads*). DMSO used as a solvent of kinase inhibitors had no effect on the morphogenesis of neurons. At up to 100 μM for sodium vanadate, 2 μg/ml for herbimycin A, 5 μg/ml for lavendustin A, and 1 μg/ml for erbstatin analogue, no decrease in the cell viability was observed during 20 h of incubation (data not shown). In the presence of 100 μM sodium vanadate, NFH expression in the neurites was significantly suppressed, and almost no axon-like process was found, although anti-MAP2 staining was comparable to that of control (Fig. 7, *C* and *D*). At 33 μM, the effects of sodium vanadate on NFH expression were much less evident. Herbimycin A at 0.2–2 μg/ml suppressed the neurite extension, and the MAP2-positive neurites were thin (Fig. 7 *E*) and only faintly stained with anti-NFH (data not shown). Lavendustin A at 1–5 μg/ml exerted similar effects on the neurite outgrowth (data not shown). Erbstatin analogue at 0.3–1 μg/ml inhibited differentiation of neurons, in which cells showed flattened shape with numerous short filopodial processes (Fig. 7 *F*). When neurons were cultured for 24 h in the absence of

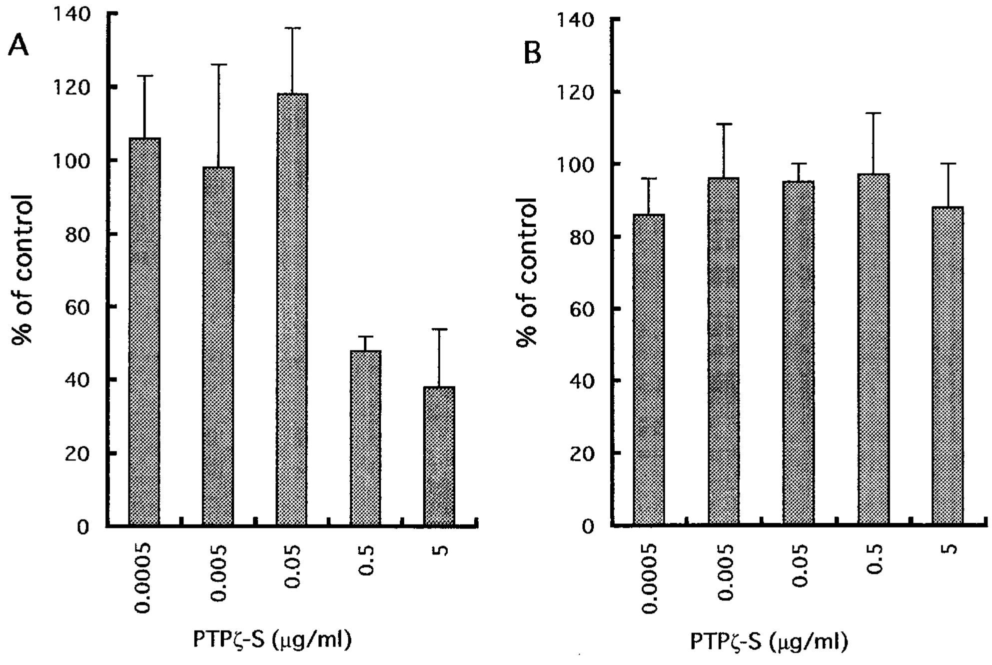

Figure 5. Effects of PTPζ-S on the pleiotrophin-induced neuronal migration. Boyden chamber cell migration assay was performed using (*A*) pleiotrophin- and (*B*) poly-L-lysine–coated membranes in the presence of various concentrations of PTPζ-S added into the culture medium. PTPζ-S significantly inhibited pleiotrophin-induced neuronal migration, whereas poly-L-lysine–induced migration was not affected. Each bar represents the mean ± SD of quintuplicate values.

inhibitors after the 20 h incubation with inhibitors, the neurites reextended with normal morphology, indicating that the effects of inhibitors were reversible in the concentration ranges used (data not shown).

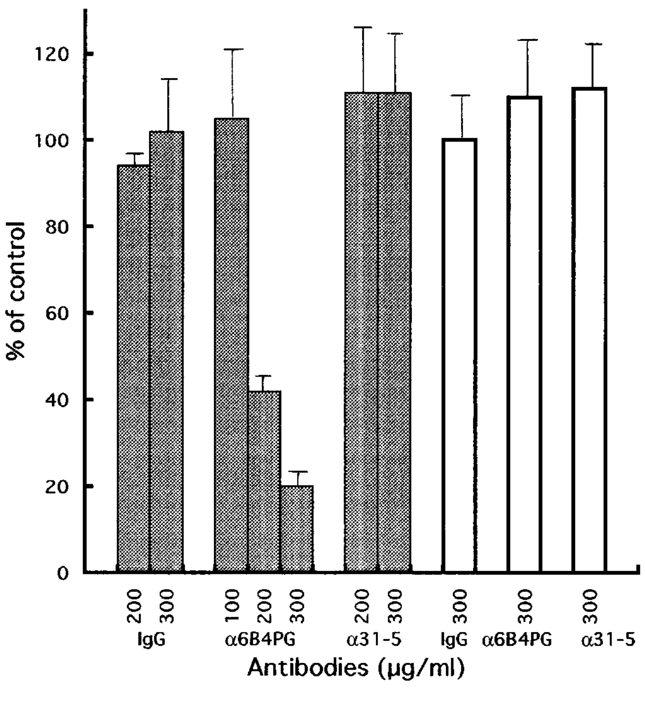

Figure 6. Polyclonal antibodies against PTPζ inhibit pleiotrophin-induced neuronal migration. Boyden chamber cell migration assay was performed using pleiotrophin- (*shaded column*) and poly-L-lysine– (*open column*) coated membranes in the presence of rabbit IgG (IgG), anti-6B4 PG (α6B4PG) and anti-31-5 (α31-5). Anti-6B4 PG inhibited pleiotrophin-induced neuronal migration, whereas poly-L-lysine–induced migration was not affected. Each bar represents the mean ± SD of triplicate values.

Next, we analyzed the tyrosine phosphorylation levels under these culture conditions by immunoblot analysis with anti-phosphotyrosine antibody (Fig. 8). Sodium vanadate strongly stimulated the tyrosine phosphorylation of the intracellular proteins (Fig. 8 *B*). In particular, tyrosine phosphorylation of 230-, 160-, 100-, 80-, and 60-kD proteins was markedly increased. Tyrosine-phosphorylation of major proteins was not influenced by lavendustin A and erbstatin analogue (Fig. 8, *D* and *E*). In contrast, herbimycin A suppressed selectively the tyrosine phosphorylation of 190- and 130-kD proteins (Fig. 8 *C*).

Fig. 9 shows the effects of inhibitors on the neuronal migration. Tyrosine kinase inhibitors exerted essentially the same effects on pleiotrophin- and poly-L-lysine–induced neuronal migrations. Herbimycin A completely inhibited migration, while lavendustin A had no effect up to 5 μg/ml. By contrast, erbstatin analogue potently stimulated migration at 0.3 μg/ml. The stimulatory effect of erbstatin analogue decreased at 1 μg/ml, which might be due to cytotoxic effects of this reagent. The effective concentration ranges of herbimycin A and erbstatin analogue for the migration are comparable to those for the inhibition of mitogenic activities (Uehara et al., 1988; Umezawa et al., 1990). In contrast to these tyrosine kinase inhibitors, sodium vanadate specifically inhibited pleiotrophin-induced neuronal migration. More than 70% inhibition of pleiotrophin-induced migration was observed at 100 μM sodium vanadate, although a normal level of migration was retained on poly-L-lysine even at this concentration. These results suggest that protein tyrosine kinases are involved in assembly of the general machinery for cell locomotion, and tyrosine phosphatase activity is essential for the signal transduction of pleiotrophin.

Immunohistochemical Localization of PTPζ in the Cortical Neurons

In a previous study, we demonstrated that anti-6B4 PG epitopes were present on cortical neurons especially at

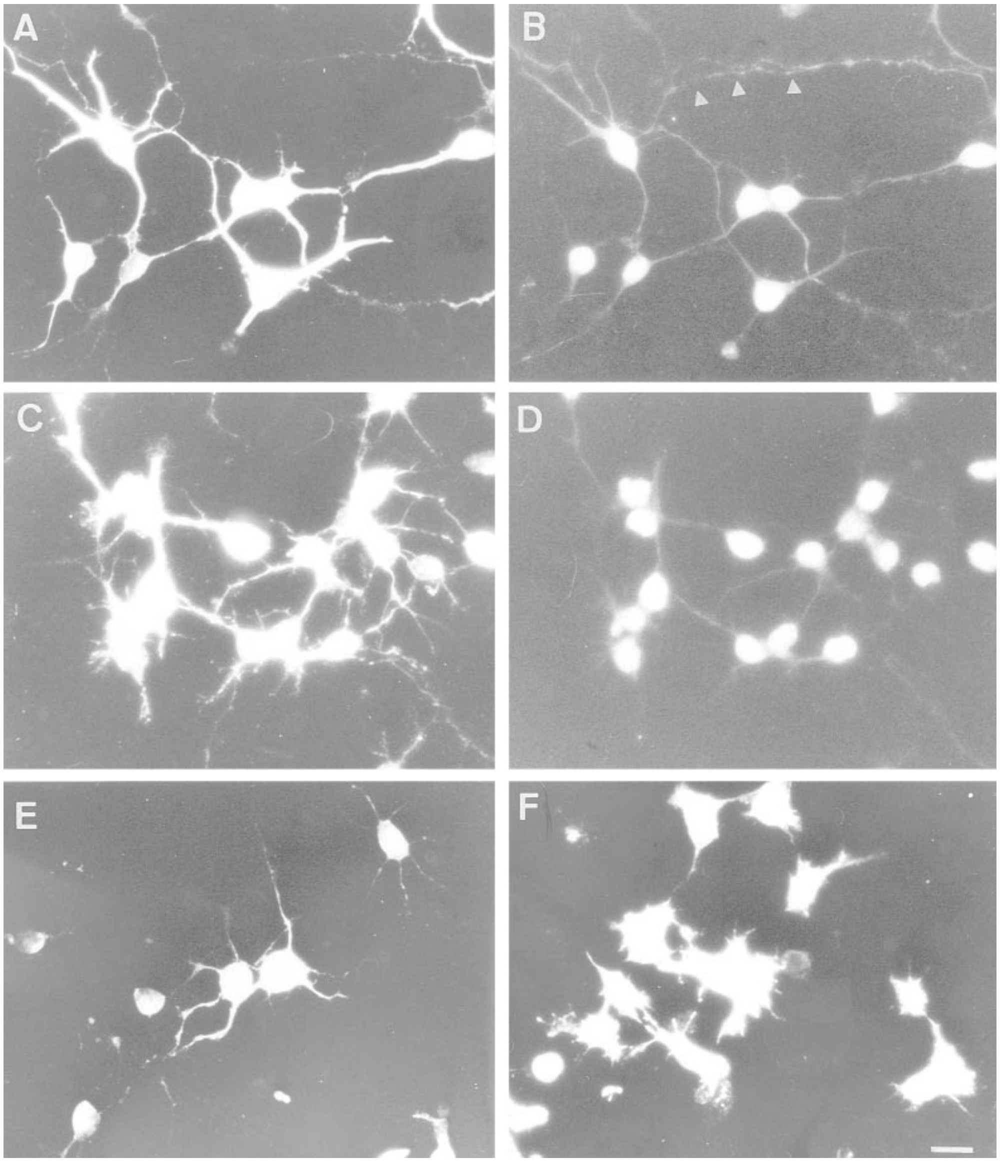

Figure 7. Effects of vanadate and protein tyrosine kinase inhibitors on the morphological differentiation of cortical neurons. Cortical neurons were cultured on pleiotrophin-coated coverslips in the presence of 0.02% DMSO (*A* and *B*), 100 μM sodium vanadate (*C* and *D*), 2 μg/ml herbimycin A (*E*), and 1 μg/ml erbstatin analogue (*F*). After 20 h in vitro, the neurons were double-immunostained with anti-MAP2 (*A, C, E*, and *F*) and anti-NFH (*B* and *D*). Arrowheads in *B* indicate the NFH-positive and MAP2-negative process. Bar, 20 μm.

growth cones (Maeda et al., 1996*a*). Because anti-6B4 PG recognizes all the three isoforms of PTPζ, it was not evident which isoforms are expressed. Therefore, we used a monoclonal antibody against intracellular D2 domain of PTPζ (anti-RPTPβ) to reveal the distribution of receptor forms of PTPζ. Immunocytochemical staining of migrating neurons on pleiotrophin-coated filters revealed that immunoreactivity to anti-RPTPβ was broadly distributed on the leading processes (Fig. 10, *A* and *B*). Immunoreactivity was also broadly detected on the lamellipodia, which occurred along the entire length of the leading processes (Fig. 10 *B*). When neurons cultured on poly-L-lysine–coated coverslips were analyzed, it became evident that at the growth cones of extending neurites, the rims of lamellipodia specifically showed relatively strong anti-RPTPβ-immunoreactivity (Fig. 10, *C* and *D*). A subset of filopodial processes on the growth cones also showed the positive immunostaining (Fig. 10 *C*). These results indicated that cortical neurons expressed transmembrane forms of PTPζ not only in the migrating stage but also in the differentiated stage changing the subcellular localization.

Fig. 11 *A* shows the anti-RPTPβ immunoreactivity in the E18 cortex. The immunoreactivities were observed at all the layers with relatively dense staining in the cortical plate and the superior portion of marginal zone. At higher magnification, most of the neurons in the cortical plate showed staining along cell surface (Fig. 11 *B*, *arrows*), but in addition to the cell surface staining, a subset of neurons displayed intracellular reticular staining (Fig. 11 *B*, *arrowheads*). Although we do not know about the functional significance of these intracellular molecules, they might correspond to the precursor forms stored in the endoplasmic reticulum. Similar intracellular distribution of PTPζ was also observed in the L cell transfectants expressing the full-length form of this molecule (Nishiwaki et al., 1998). In contrast, mAb 6B4 immunostaining, which mainly corresponds to the presence of PTPζ-S (Maeda et al., 1995), distributed along radial glial fibers in the superior part of the cortical plate (Fig. 11 *C*, *arrowheads*) and at the marginal zone. In the inferior part of cortical plate, dense staining was observed also around neurons (Fig. 11 *C*, *arrows*).

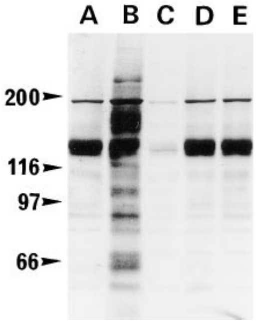

Figure 8. Analysis of protein tyrosine phosphorylation in cortical neurons treated with vanadate and kinase inhibitors. Cortical neurons were cultured for 20 h in the absence (*A*) or presence of 100 μM sodium vanadate (*B*), 1 μg/ml herbimycin A (*C*), 3 μg/ml lavendustin A (*D*), and 0.3 μg /ml erbstatin analogue (*E*). Cells were solubilized, and the proteins were separated by 7.5% SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine monoclonal antibody 4G10. The positions of molecular mass markers (in kD) are shown on the left.

Discussion

In this study, we examined the neuronal migration induced by pleiotrophin using two kinds of cell migration assay systems and obtained experimental results indicating that pleiotrophin-PTPζ interaction is involved in the migration. Cell migration is classified into several types of directed and random movement, i.e. chemotaxis, haptotaxis, and chemokinesis (Kim et al., 1997). Chemotaxis is the directed movement of cells toward a concentration gradient of a soluble attractant. Haptotaxis is a migration of cells on a substrate-bound substance in solid phase. Chemokinesis is random cell motion. The Boyden chamber assay results of our study suggest that pleiotrophin induces neuronal migration by haptotaxis, because immobilized pleiotrophin promotes migration but soluble pleiotrophin rather inhibited the neuronal migration on pleiotrophin-coated filters probably through competitive inhibition of the receptor binding. Haptotaxis requires cell adhesion, but cell adhesion is not sufficient to account for the neuronal migration. In fact, fibronectin is a good substrate for the adhesion of cortical neurons, but it showed very low activity for neuronal migration. In addition, laminin was not a good substrate for migration of cortical neurons in our assay systems.

Neurons migrating along radial glial fibers show characteristic morphology, i.e., bipolar shape, extension of a tapered leading process and caudal positioning of the nucleus (Rakic, 1971; Gregory et al., 1988). In this study, those neurons migrating on the pleiotrophin-coated glass fibers clearly displayed the bipolar shape with a leading process and a caudally positioned nucleus. The neurons migrating on the pleiotrophin-coated Boyden chamber membranes also displayed similar morphology. After migrating across the membrane, cortical neurons recovered their rounded shape and extended multiple neurites. These findings suggest that the physical surface structure of the substrate is important for the determination of the cell morphology. The small pores of a membrane as well as glass fibers might become a mechanical stimulus to neurons, causing the morphological changes and inducing migration upon specific substrate molecules.

Glycosaminoglycans including heparin, heparan sulfate, and chondroitin sulfate strongly inhibit pleiotrophin-PTPζ binding (Maeda et al., 1996*a*). The important feature is that chondroitin sulfate C but not chondroitin sulfate A inhibits the binding (Maeda et al., 1996*a*). Pleiotrophin-

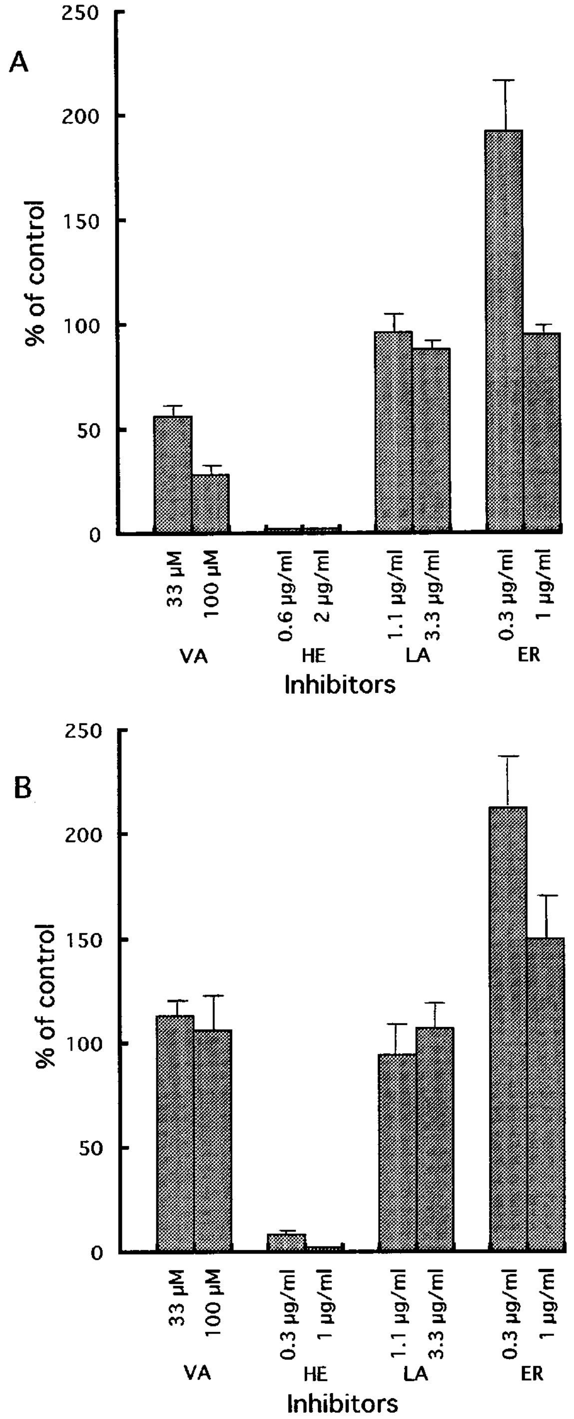

Figure 9. Effects of vanadate and protein tyrosine kinase inhibitors on neuronal migration. Boyden chamber cell migration assay was performed using the membranes coated with pleiotrophin (*A*) or poly-L-lysine (*B*) in the presence of sodium vanadate (*VA*), herbimycin A (*HE*), lavendustin A (*LA*), and erbstatin analogue (*ER*). Sodium vanadate specifically suppressed the pleiotrophin-induced neuronal migration. On the other hand, tyrosine kinase inhibitors exerted essentially the same effects on both pleiotrophin- and poly-L-lysine–induced neuronal migration. Each bar represents the mean ± SD of triplicate values.

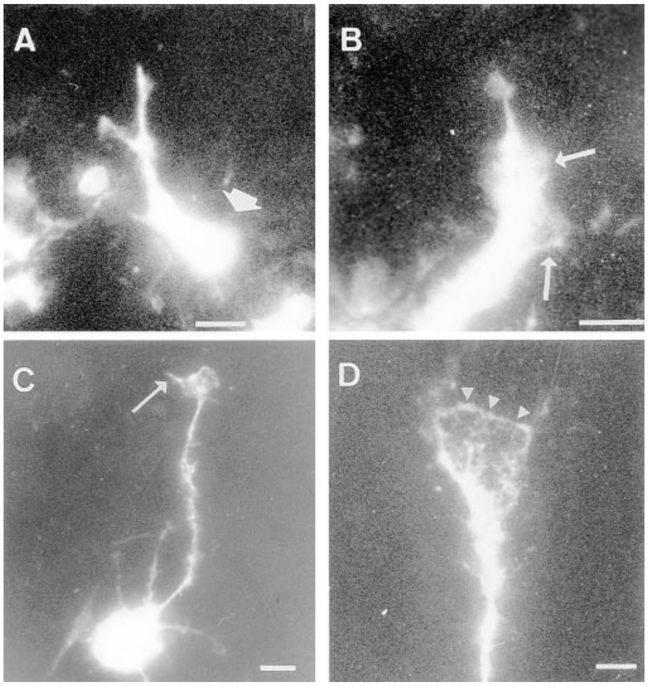

Figure 10. Presence of transmembrane forms of PTPζ in cortical neurons. (*A* and *B*) Cortical neurons migrating on the pleiotrophin-coated filters (the underside) were fixed and immunostained with anti-RPTPβ, which recognizes intracellular D2 domain of PTPζ. An arrow in *A* points the position of a pore on the membrane, through which a neuron was migrating. The immunostaining was distributed broadly on neurons. Lamellipodia extended around the leading process were also stained with anti-RPTPβ (*B*, *arrows*). (*C* and *D*) Cortical neurons were cultured for 20 h on poly-L-lysine-coated coverslips, fixed and immunostained with anti-RPTPβ. Most cortical neurons were stained with anti-RPTPβ, although expression levels were variable. Cell bodies and neurites were intensely stained. At the growth cones, a subset of filopodias (*C*, *arrow*) and the rim of lamellipodia (*D*, *arrowheads*) were stained. When preimmune IgG was used instead of anti-RPTPβ, only weak background staining was observed (data not shown). Bars: (*A, B*, and *D*) 5 μm; (*C*) 10 μm.

induced neuronal migration was again inhibited by heparin, heparan sulfate, and chondroitin sulfate C, but not by chondroitin sulfate A. In contrast, neither type of chondroitin sulfate influenced the poly-L-lysine–induced neuronal migration, although heparin and heparan sulfate moderately inhibited it. It has been reported that heparin and heparan sulfate bind neuronal cell surfaces and are then internalized (Lafont et al., 1992). Thus, it is conceivable that the effects of heparin and heparan sulfate were at least partly due to their direct effects on neurons. Importantly, similar sensitivities to glycosaminoglycans were observed in the effect on neurite outgrowth of cortical neurons. Chondroitin sulfate C but not A suppressed the pleiotrophin-induced neurite outgrowth, while neither affected the poly-L-lysine–induced neurite outgrowth (data not shown). This suggests that pleiotrophin-induced neurite outgrowth and neuronal migration are based on the same receptor system.

PTPζ-S corresponds to the extracellular domain of the full-length receptor form, PTPζ-A (Maeda et al., 1994; Maurel et al., 1994). Biochemical analysis indicated that both molecules are modified in the same fashion with glycosaminoglycans and oligosaccharides (Maeda et al., 1994; Hamanaka et al., 1997). Therefore, PTPζ-S is expected to competitively inhibit the ligand binding and the subsequent signal transduction of transmembrane forms of PTPζ. PTPζ-S added in the culture medium actually suppressed the pleiotrophin-induced neuronal migration, but again poly-L-lysine–induced migration was not influenced. The IC_{50} of the inhibitory effect was about 0.5 μg/ml (2.8 nM), which roughly matched the K_d value of pleiotrophin–PTPζ binding ($K_d = 0.25$ and 3 nM; Maeda et al., 1996*a*). It is notable that the inhibition by PTPζ-S is only partial (Fig. 5). This suggests that alternative pleiotrophin receptors such as *N*-syndecan (Rauvala and Peng, 1997) could also be involved in the pleiotrophin-induced neuronal migration.

Anti-6B4 PG polyclonal antibodies also inhibited pleiotrophin-induced neuronal migration, but poly-L-lysine–induced migration was not affected. Polyclonal antibodies against the COOH-terminal portion of PTPζ-S (anti–31-5) exerted no effect on either type of neuronal migration, suggesting that the NH_2-terminal portion of PTPζ is involved in the pleiotrophin-induced neuronal migration. We previously reported that pleiotrophin-induced neurite extension was also inhibited by anti-6B4 PG but not by anti–31-5 (Maeda et al., 1996*a*). Taken together, these findings suggest that PTPζ acts as a receptor for pleiotrophin in the pleiotrophin-induced neuronal migration as well as in the pleiotrophin-induced neurite extension.

Although PTPζ is involved in the neuronal migration, it is not evident whether protein tyrosine phosphatase activity of PTPζ is essential for the migration to occur. A protein tyrosine phosphatase inhibitor, sodium vanadate inhibited pleiotrophin-induced neuronal migration. This effect is not due to the nonspecific cytotoxicity, because poly-L-lysine–induced neuronal migration was not influenced by sodium vanadate, and the viability of neurons was not affected in the range of concentrations used in the experiments. In addition, sodium vanadate exerted almost no effect on the MAP2-positive neurite formation, although extension of axon-like processes was significantly suppressed (Fig. 7). These are supporting evidence that protein tyrosine phosphatase activity of PTPζ is essential for the pleiotrophin-induced neuronal migration, however, further studies using a dominant negative form of PTPζ are necessary to answer this question.

In contrast to sodium vanadate, protein tyrosine kinase inhibitors exerted similar effects on both pleiotrophin- and poly-L-lysine–induced neuronal migrations. Herbimycin A strongly suppressed the tyrosine phosphorylation in the neurons and pleiotrophin-induced neurite extension, and completely inhibited neuronal migration. On the other hand, lavendustin A exerted no effect on the neuronal migration, although it clearly suppressed the pleiotrophin-induced neurite formation. Interestingly, erbstatin analogue strongly promoted neuronal migration, but suppressed the morphological differentiation of cortical neurons on the pleiotrophin-coated coverslips. The treated cells showed a spread-shape with no neurites even after 20 h of incubation. In the absence of inhibitors, cortical neurons plated on pleiotrophin-coated coverslips firstly show

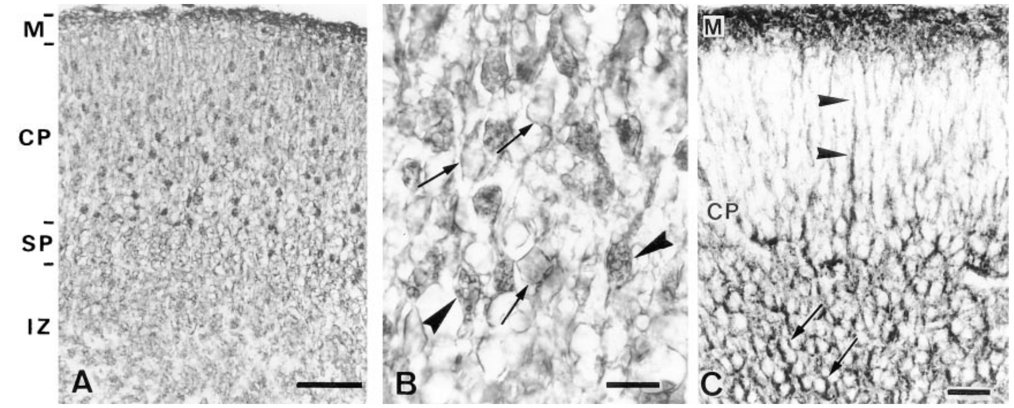

Figure 11. Immunohistochemical localization of PTPζ in the developing cerebral cortex. (*A* and *B*) Frontal sections from E18 rat brains were immunohistochemically stained with anti-RPTPβ which recognizes transmembrane forms of PTPζ. Immunostainings were observed at all the layers including marginal zone (*M*), cortical plate (*CP*), subplate (*SP*), and intermediate zone (*IZ*). Higher magnification figure of cortical plate shows that the stainings were distributed along the cell surface of neurons (*B*, *arrows*) and a subset of neurons also displayed intracellular reticular stainings (*B*, *arrowheads*). (*C*) Strong mAb 6B4 immunostaining, which mainly corresponds to the presence of PTPζ-S, was observed on the marginal zone. In the superior part of cortical plate, the stainings were observed along radial glial fibers (*C*, *arrowheads*), and in the inferior part, surroundings of neurons were also stained (*C*, *arrows*). Bars: (*A*) 50 μm; (*B*) 10 μm; (*C*) 20 μm.

a spread-shape (phase I), and then the cell bodies become rounded and dendrite- and axon-like processes are extended (phase II). It seems that the transition from phase I to phase II is inhibited in the presence of erbstatin analogue. It is also conceivable that phase I is a migratory phase of cortical neurons, and the activity of erbstatin-sensitive protein tyrosine kinase is necessary for the transition from the migratory phase to the differentiation phase. These observations also suggest that pleiotrophin-induced neuronal migration and neurite extension use different signal transduction pathways, even if pleiotrophin receptor is common.

In the early cerebral cortex, pleiotrophin was synthesized by radial glial cells and was deposited on radial glial fibers in the cortical plate especially between the migrating neurons and the radial glial processes (Matsumoto et al., 1994*b*; Rauvala et al., 1994). However, in the intermediate zone pleiotrophin displayed a wider distribution and was present also on developing axons (Matsumoto et al., 1994). On the other hand, the distribution of PTPζ isoforms is controversial. Based on in situ hybridization experiments, Cannol et al. (1993, 1996) reported that RPTPβ/PTPζ was synthesized predominantly by glia, whereas detailed analyses by Snyder et al. (1996) and ourselves (Maeda et al., 1995, 1996*a*) demonstrated that both neurons and glial cells synthesized PTPζ isoforms and their expressions are dynamically regulated during development. By immunohistochemical analysis using a monoclonal antibody to the D2 domain of PTPζ (anti-RPTPβ), we showed that the transmembrane forms of PTPζ were present on migrating neurons in the cortical plate (Fig. 11). Moreover, very recently, we generated mice in which PTPζ gene was replaced by the LacZ gene by gene targeting. Analysis of the LacZ expression in heterozygous PTPζ-targeted mice (PTP$\zeta^{+/-}$) clearly indicated that cortical neurons as well as glial cells expressed PTPζ from the embryonic stage to the adulthood (Shintani et al., 1998). These observations suggest that the ligand–receptor relationship between pleiotrophin on radial glia and PTPζ on neurons plays a role in the neuronal migration at cortial plate.

In contrast to the immunostaining with anti-RPTPβ, mAb 6B4 stained strongly the radial glial fibers and a part of cortical plate neurons. From the content of each PTPζ isoforms, mAb 6B4 immunostainings are mainly considered to correspond to the presence of PTPζ-S (Maeda et al., 1995). PTPζ-S distributed along radial glial fibers and on cortical plate neurons might regulate the strength of the signal transduction of pleiotrophin by competitive inhibition of pleiotrophin binding to the transmembrane forms of PTPζ. In addition to the radial glial fibers, strong immunoreactivity to mAb 6B4 was observed at the marginal zone. When migrating neurons reach the marginal zone, they detach from their glial guide and form adhesive interactions with ambient neurons (Rakic et al., 1994). When neurons encounter the marginal zone, high concentrations of PTPζ-S in this zone might switch off the signal of pleiotrophin and stop the neuronal migration.

It has been considered that cell motility depends on the cytoskeletal organization. Using cerebellar granule neurons, Rakic et al. (1996) observed that the positive ends of microtubules in the leading process face the growing tip, and suggested that the dynamics of polymerization and depolymerization of oriented microtubules create the forces that displace the nucleus and cytoplasm within the leading process. On the other hand, Rivas and Hatten (1995) indicated that disruption of actin filaments with cytochalasin B inhibited the migration of cerebellar granule neurons. They also demonstrated that lamellipodia with a ruffled appearance were common along the leading process, in which actin filaments were concentrated. L cell transfectants expressing transmembrane-forms of PTPζ displayed a specific localization of this receptor phosphatase at the lamellipodia especially at ruffling membranes, where PTPζ was colocalized with actin filaments and an actin-binding protein, cortactin (Nishiwaki et al., 1998), suggesting that PTPζ is involved in the organization of actin filaments. Furthermore, the transmembrane forms of PTPζ were observed on the lamellipodia along leading processes of migrating neurons (Fig. 10). Ligand binding of PTPζ might induce reorganization of actin filaments by dephosphorylating specific substrates, which leads to the neuronal migration. Identification of specific substrates of PTPζ is requisite to reveal the molecular mechanism of pleiotrophin-induced neuronal migration.

Neuronal migration is a complex process regulated by the interplay of multiple signal transduction pathways. This study indicated that PTPζ is a neuronal receptor involved in the pleiotrophin-based neuronal migration. Elucidations of the signal transduction pathway of PTPζ and its cross talk with the other signaling systems including cell adhesion molecules are essential to reveal the molecular mechanism of neuronal migration.

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References

Barnea, G., M. Grumet, P. Milev, O. Silvennoinen, J.B. Levy, J. Sap, and J. Schlessinger. 1994. Receptor tyrosine phosphatase β is expressed in the form of proteoglycan and binds to the extracellular matrix protein tenascin. *J. Biol. Chem.* 269:14349–14352.

Cannol, P.D., G. Barnea, J.B. Levy, J. Sap, M. Ehrlich, O. Silvennoinen, J. Schlessinger, and J.M. Musacchio. 1993. The expression of a novel receptor-type tyrosine phosphatase suggests a role in morphogenesis and plasticity of the nervous system. *Dev. Brain Res.* 75:293–298.

Cannol, P.D., S. Petanceska, J. Schlessinger, and J.M. Musacchio. 1996. Three forms of RPTP-β are differentially expressed during gliogenesis in the developing rat brain and during glial cell differentiation in culture. *J. Neurosci. Res.* 44:199–215.

D'Arcangelo, G., K. Nakajima, T. Miyata, M. Ogawa, K. Mikoshiba, and T. Curran. 1997. Reelin is a secreted glycoprotein recognized by the CR-50 monoclonal antibody. *J. Neurosci.* 17:23–31.

Fishman, R.B., and M.E. Hatten. 1993. Multiple receptor systems promotes CNS neural migration. *J. Neurosci.* 13:3485–3495.

Galileo, D.S., J. Majors, A.F. Horwitz, and J.R. Sanes. 1992. Retrovirally introduced antisense integrin RNA inhibits neuroblast migration in vivo. *Neuron.* 9:1117–1131.

Gordon, J.A. 1991. Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. *Methods Enzymol.* Vol. 201:477–482.

Gregory, W.A., J.C. Edmondson, M.E. Hatten, and C.A. Mason. 1988. Cytology and neuron-glial apposition of migrating cerebellar granule cells in vitro. *J. Neurosci.* 8:1728–1738.

Grumet, M., P. Milev, T. Sakurai, L. Karthikeyan, M. Bourdon, R.K. Margolis, and R.U. Margolis. 1994. Interaction with tenascin and differential effects on cell adhesion of neurocan and phosphacan, two major chondroitin sulfate proteoglycans of nervous tissue. *J. Biol. Chem.* 269:12142–12146.

Hamanaka, H., N. Maeda, and M. Noda. 1997. Spatially and temporally regulated modification of the receptor-like protein tyrosine phosphatase ζ/β isoforms with keratin sulfate in the developing chick brain. *Eur. J. Neurosci.* 9: 2297–2308.

Kadomatsu, K., M. Tomomura, and T. Muramatsu. 1988. cDNA cloning and sequencing of a new gene intensely expressed in early differentiation stages of embryonal carcinoma cells and in mid-gestation period of mouse embryogenesis. *Biochem. Biophys. Res. Commun.* 151:1312–1318.

Kim, H.J., C.A. Henke, S.K. Savik, and D.H. Ingbar. 1997. Integrin mediation of alveolar epithelial cell migration on fibronectin and type I collagen. *Am. J. Physiol.* 273:L134–L141.

Kinnunen, T., E. Raulo, R. Nolo, M. Maccarana, U. Lindahl, and H. Rauvala. 1996. Neurite outgrowth in brain neurons induced by heparin-binding growth-associated molecule (HB-GAM) depends on the specific interaction of HB-GAM with heparan sulfate at the cell surface. *J. Biol. Chem.* 271: 2243–2248.

Komuro, H., and P. Rakic. 1993. Modulation of neuronal migration by NMDA receptors. *Science.* 260:95–97.

Komuro, H., and P. Rakic. 1996. Intracellular Ca^{2+} fluctuations modulate the rate of neuronal migration. *Neuron.* 17:275–285.

Krueger, N.X., and H. Saito. 1992. A human transmembrane protein-tyrosine-phosphatase, PTPζ, is expressed in brain and has an N-terminal receptor domain homologous to carbonic anhydrases. *Proc. Natl. Acad. Sci. USA.* 89: 7417–7421.

Kurtz, A., A.M. Schulte, and A. Wellstein. 1995. Pleiotrophin and midkine in normal development and tumor biology. *Crit. Rev. Oncog.* 6:151–177.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.

Lafont, F., M. Rouget, A. Triller, A. Prochiantz, and A. Rousselet. 1992. In vitro control of neuronal polarity by glycosaminoglycans. *Development.* 114: 17–29.

Lafont, F., A. Prochiantz, C. Valenza. M. Petitou, M. Pascal, M. Rouget and A. Rousselet. 1994. Defined glycosaminoglycan motifs have opposite effects on neuronal polarity in vitro. *Dev. Biol.* 165:453–468.

Levy, J.B., P.D. Canoll, O. Silvennoinen, G. Barnea, B. Morse, A.M. Honegger, J.-T. Huang, L.A. Cannizzaro, S.-H. Park, T. Druck, et al. 1993. The cloning of a receptor-type protein tyrosine phosphatase expressed in the central nervous system. *J. Biol. Chem.* 268:10573–10581.

Li, Y.-S., P.G. Milner, A.K. Chauhan, M.A. Watson, R.M. Hoffman, C.M. Kodner, J. Milbrandt, and T.F. Deuel. 1990. Cloning and expression of a developmentally regulated protein that induces mitogenic and neurite outgrowth activity. *Science.* 250:1690–1694.

Li, Y.-S., and T.F. Deuel. 1993. Pleiotrophin simulates tyrosine phosphorylation in NIH 3T3 and NB41A3 cells. *Biochem. Biophys. Res. Commun.* 195:1089–1095.

Maeda, N., F. Matsui, and A. Oohira. 1992. A chondroitin sulfate proteoglycan that is developmentally regulated in the cerebellar mossy fiber system. *Dev. Biol.* 151:564–574.

Maeda, N., H. Hamanaka, T. Shintani, T. Nishiwaki, and M. Noda. 1994. Multiple receptor-like protein tyrosine phosphatases in the form of chondroitin sulfate proteoglycan. *FEBS Lett.* 354:67–70.

Maeda, N., H. Hamanaka, A. Oohira, and M. Noda. 1995. Purification, characterization and developmental expression of a brain-specific chondroitin sulfate proteoglycan, 6B4 proteoglycan/phosphacan. *Neuroscience.* 67:23–35.

Maeda, N., T. Nishiwaki, T. Shintani, H. Hamanaka, and M. Noda. 1996*a*. 6B4 proteoglycan/phosphacan, an extracellular variant of receptor-like protein-tyrosine phosphatase ζ/RPTPβ, binds pleiotrophin/heparin-binding growth-associated molecule (HB-GAM). *J. Biol. Chem.* 271:21446–21452.

Maeda, N., and M. Noda. 1996*b*. 6B4 proteoglycan/phosphacan is a repulsive substratum but promotes morphological differentiation of cortical neurons. *Development.* 122:647–658.

Matsumoto, K., A. Wanaka, T. Mori, A. Taguchi, N. Ishii, H. Muramatsu, T. Muramatsu, and M. Tohyama. 1994*a*. Localization of pleiotrophin and midkine in the postnatal developing cerebellum. *Neurosci. Lett.* 178:216–220.

Matsumoto, K., A. Wanaka, K.Takatsuji, H. Muramatsu, T. Muramatsu, and M. Tohyama. 1994*b*. A novel family of heparin-binding growth factors, pleiotrophin and midkine, is expressed in the developing rat cerebral cortex. *Dev. Brain Res.* 79:229–241.

Maurel, P., U. Rauch, M. Flad, R.K. Margolis, and R.U. Margolis. 1994. Phosphacan, a chondroitin sulfate proteoglycan of brain that interacts with neurons and neural cell-adhesion molecules, is an extracellular variant of a receptor-type protein tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA.* 91: 2512–2516.

Merenmies, J., and H. Rauvala. 1990. Molecular cloning of the 18-kD growth-associated protein of developing brain. *J. Biol. Chem.* 265:16721–16724.

Milev, P., P. Maurel, M. Häring, R.K. Margolis, and R.U. Margolis. 1996. TAG-1/axonin-1 is a high-affinity ligand of neurocan, phosphacan/protein-tyrosine phosphatase-ζ/β, and N-CAM. *J. Biol. Chem.* 271:15716–15723.

Milev, P., D.R. Friedlander, T. Sakurai, L. Karthikeyan, M. Flad, R.K. Margolis, M. Grumet, and R.U. Margolis. 1994. Interactions of chondroitin sulfate proteoglycan phosphacan, the extracellular domain of a receptor-type protein tyrosine phosphatase, with neurons, glia, and neural cell adhesion molecules. *J. Cell Biol.* 127:1703–1715.

Niinobe, M., N. Maeda, H. Ino, and K. Mikoshiba, 1988. Characterization of microtubule-associated protein 2 from mouse brain and its localization in the cerebellar cortex. *J. Neurochem.* 51:1132–1139.

Nishiwaki, T., N. Maeda, and M. Noda. 1998. Characterization and developmental regulation of proteoglycan-type protein tyrosine phosphatase ζ/RPTPβ isoforms. *J. Biochem.* 123:458–467.

Onoda, T., H. Iinuma, Y. Sasaki, M. Hamada, K. Isshiki, H. Naganawa, T. Takeuchi, K. Tatsuka, and K. Umezawa. 1989. Isolation of a novel tyrosine kinase inhibitor, lavendustin A, from *Streptomyces griseolavendus*. *J. Nat. Prod.* 52:1252–1257.

Peles, E., M. Nativ, P.L. Campbell, T. Sakurai, R. Martinez, S. Lev, D.O. Clary, J. Schilling, G. Barnea, G.D. Plowman, et al. 1995. The carbonic anhydrase domain of receptor tyrosine phosphatase β is a functional ligand for the axonal cell recognition molecule contactin. *Cell.* 82:251–260.

Rakic, P. 1971. Mode of cell migration to the superficial layers of fetal monkey neocortex. *J. Comp. Neurol.* 145:61–84.

Rakic, P., R.S. Cameron, and H. Komuro. 1994. Recognition, adhesion, transmembrane signaling and cell motility in guided neuronal migration. *Curr. Opin. Neurobiol.* 4:63–69.

Rakic, P., E. Knyihar-Csillik, and B. Csillik. 1996. Polarity of microtubule assemblies during neuronal cell migration. *Proc. Natl. Acad. Sci. USA.* 93: 9218–9222.

Raulo, E., M.A. Chernousov, D.J. Carey, R. Nolo, and H. Rauvala. 1994. Isolation of a neuronal cell surface receptor of heparin binding growth-associated molecule (HB-GAM). *J. Biol. Chem.* 269:12999–13004.

Rauvala, H., A. Vanhala, E. Castren, R. Nolo, E. Raulo, J. Merenmies, and P. Panula. 1994. Expression of HB-GAM (heparin-binding growth-associated molecules) in the pathways of developing axonal processes in vivo and neurite outgrowth in vitro induced by HB-GAM. *Dev. Brain Res.* 79:157–176.

Rauvala, H., and H.B. Peng. 1997. HB-GAM (heparin-binding growth-associated molecule) and heparin-type glycans in the development and plasticity of neuron-target contacts. *Prog. Neurobiol.* 52:127–144.

Rio, C., H.I. Rieff, P. Qi, and G. Corfas. 1997. Neuregulin and erbB receptors play a critical role in neuronal migration. *Neuron.* 19:39–50.

Rivas, R., and M. Hatten. 1995. Motility and cytoskeletal organization of migrating cerebellar granule neurons. *J. Neurosci.* 15:981–989.

Shintani, T., E. Watanabe, N. Maeda, and M. Noda. 1998. Neurons as well as astrocytes express proteoglycan-type protein tyrosine phosphatase ζ/RPTPβ: analysis of mice in which the *PTPζ/RPTP*β gene was replaced with the *LacZ* gene. *Neurosci. Lett.* 247:135–138.

Snyder, S.E., J. Li, P.E. Schauwecker, T.H. McNeill, and S.R.J. Salton. 1996. Comparison of RPTPζ/β, phosphacan, and trkB mRNA expression in the developing and adult rat nervous system and induction of RPTPζ/β and phosphacan mRNA following brain injury. *Mol. Brain Res.* 40:79–96.

Uehara, Y., Y. Murakami, S. Mizuno, and S. Kawai. 1988. Inhibition of transforming activity of tyrosine kinase oncogenes by herbimycin A. *Virology.* 164:284–298.

Umezawa, K., T. Hori, H. Tajima, M. Imoto, K. Isshiki, and T. Takeuchi. 1990. Inhibition of epidermal growth factor-induced DNA synthesis by tyrosine kinase inhibitors. *FEBS Lett.* 260:198–200.

Zheng, C., N. Heintz, and M.E. Hatten. 1996. CNS gene encoding astrotactin, which supports neuronal migration along glial fibers. *Science.* 272:417–419.