6B4 Proteoglycan/Phosphacan, an Extracellular Variant of Receptor-like Protein-tyrosine Phosphatase \( \zeta/RPTP\beta \), Binds Pleiotrophin/Heparin-binding Growth-associated Molecule (HB-GAM)*

(Received for publication, March 8, 1996, and in revised form, May 20, 1996)

Nobuaki Maeda, Taeko Nishiwaki, Takafuli Shintani, Hiroki Hamanaka, and Masaharu Noda

From the Division of Molecular Neurobiology, National Institute for Basic Biology, and the Department of Molecular Biomechanics, The Graduate University for Advanced Studies, Okazaki 444, Japan

A major chondroitin sulfate proteoglycan in the brain, 6B4 proteoglycan/phosphacan, corresponds to the extracellular region of a receptor-like protein-tyrosine phosphatase, PTP\( \beta \). Here, we purified and characterized 6B4 proteoglycan-binding proteins from rat brain. From the CHAPS (3-{[3-cholamidopropyl]dimethylammonio}-1-propanesulfonic acid) extract of brain microsomal fractions, 18-, 28-, and 40-kDa proteins were specifically isolated using 6B4 proteoglycan-Sepharose. N-terminal amino acid sequencing identified the 18-kDa protein as pleiotrophin/heparin-binding growth-associated molecule (HB-GAM). Scatchard analysis of 6B4 proteoglycan-pleiotrophin binding revealed low \( K_z \) (3 nm) and high \( K_x \) (0.25 nm) affinity binding sites. Chondroitinase ABC digestion of the proteoglycan decreased the binding affinities to a single value \( K_x = 13 \text{ nm} \) without changing the number of binding sites. This suggested the presence of two subpopulations of the proteoglycan with different chondroitin sulfate structures. Heparin potently inhibited binding of 6B4 proteoglycan to pleiotrophin \( (IC_{50} = 3.5 \text{ ng/mL}) \). Heparan sulfate and chondroitin sulfate C inhibited moderately \( (IC_{50} = 150 \text{ and } 400 \text{ ng/mL}, \text{ respectively}) \), but, in contrast, chondroitin sulfate A and keratan sulfate were poor inhibitors \( (IC_{50} > 100 \text{ ng/mL}) \). Immunofluorescence and immunoblotting analyses indicated that both 6B4 proteoglycan and PTP\( \beta \) are located on cortical neurons. Anti-6B4 proteoglycan antibody added to the culture medium suppressed pleiotrophin-induced neurite outgrowth of cortical neurons. These results suggested that interaction between 6B4 proteoglycan and pleiotrophin is required for the action of pleiotrophin, and chondroitin sulfate chains on 6B4 proteoglycan play regulatory roles in its binding.

The expression of the phosphate-buffered saline (PBS\(^{1}\))-soluble brain-specific chondroitin sulfate proteoglycan, 6B4 proteoglycan/phosphacan, with a 300-kDa core glycoprotein, is dynamically regulated in the developing rat brain \((1, 2)\). Cloning the cDNA for 6B4 proteoglycan revealed that it is an extracellular variant of a receptor-like protein-tyrosine phosphatase, PTP\( \beta \) \((3, 4)\). We then demonstrated that PTP\( \zeta \) is also present in the form of chondroitin sulfate proteoglycan with a 380-kDa core glycoprotein in the rat brain \((3)\). The 6B4 proteoglycan is composed of an N-terminal carboxy anhydrase (CAH)-like domain, a fibronectin type III domain, and a C-terminal serine, glycine-rich domain that is thought to be the chondroitin sulfate attachment region \((3, 4)\). In PTP\( \gamma \), the serine, glycine-rich domain is followed by a membrane-spanning region and two tyrosine phosphatase domains \((5, 6)\). PTP\( \zeta \) and 6B4 proteoglycan are widely distributed in the developing nervous system and may play roles in neuronal cell migration, neurite extension, axonal outgrowth, and development of the cerebellar mossy fiber system \((1, 2, 7-10)\). We demonstrated that 6B4 proteoglycan is a repulsive substrate for cell adhesion but promotes neurite extension and differentiation of cortical neurons \((11)\). These results suggest that 6B4 proteoglycan as well as PTP\( \zeta \) participate in various aspects of brain development by regulating the tyrosine phosphorylation level of intracellular proteins. However, little is known about the signal transduction system coupled extracellularly or intracellularly with these proteoglycan molecules.

Several proteins bind 6B4 proteoglycan/phosphacan and PTP\( \zeta/RPTP\beta \) \((8, 9, 12)\). Screening various extracellular matrix and cell adhesion molecules revealed that N-CAM, Ng-CAM, and tenascin bind to phosphacan \((8)\). Contactin was recently identified as PTP\( \zeta/RPTP\beta \)-binding protein by screening an expression cDNA library using the CAH domain as probe \((12)\). These studies indicated that 6B4 proteoglycan and PTP\( \zeta \) bind various extracellular and cell surface molecules. At present, however, the specific ligands that regulate the phosphatase activity of PTP\( \zeta \) upon their binding are unknown. In this study, we investigate ligands that bind to 6B4 proteoglycan and PTP\( \zeta \) by means of affinity chromatography using a matrix coupled with intact or chondroitinase ABC-digested 6B4 proteoglycan. Several proteins bound to 6B4 proteoglycan-Sepharose dependently or independently of chondroitin sulfate moiety. An 18-kDa protein, which bound to 6B4 proteoglycan-Sepharose independently of chondroitin sulfate, was identified as pleiotrophin.

Pleiotrophin \((13)\), also known as heparin-binding growth-associated molecule (HB-GAM) \((14)\), or heparin-binding neurite-promoting factor, HBGF \((15)\), is a mitogenic and neurite-enhancing factor that modulates the growth and differentiation of neuronal cells. The cellular region of a receptor-like protein-tyrosine phosphatase [PTP(\( \beta \))](8, 9, 12) binds pleiotrophin. At present, however, the specific ligands that regulate the phosphatase activity of PTP(\( \beta \)) upon their binding are unknown. In this study, we investigate ligands that bind to 6B4 proteoglycan and PTP(\( \beta \)) by means of affinity chromatography using a matrix coupled with intact or chondroitinase ABC-digested 6B4 proteoglycan. Several proteins bound to 6B4 proteoglycan-Sepharose dependently or independently of chondroitin sulfate moiety. An 18-kDa protein, which bound to 6B4 proteoglycan-Sepharose independently of chondroitin sulfate, was identified as pleiotrophin.

*This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan and from Mizutani Foundation for Glycoscience. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed: Division of Molecular Neurobiology, National Institute for Basic Biology, and the Department of Molecular Biomechanics, The Graduate University for Advanced Studies, Okazaki 444, Japan. Tel.: 81-564-55-7590; Fax: 81-564-55-7595.

1 The abbreviations used are: PBS, phosphate-buffered saline; PTP, protein-tyrosine phosphatase; CAH, carbonic anhydrase; HB-GAM, heparin-binding growth-associated molecule; BSA, bovine serum albumin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.
promoting factor isolated from the brain. Pleiotrophin shows 50% homology to midkine, and they are considered to constitute a new growth factor gene family (16). Syndecan-3/N-syndecan binds pleiotrophin (17), but the signal-transducing receptor for this factor is unknown. In this study, we examine the function of 6B4 proteoglycan and PTPζ as a possible receptor of pleiotrophin.

**EXPERIMENTAL PROCEDURES**

**Materials—**Human recombinant pleiotrophin was purchased from R&D Systems. Recombinant human αFGF was from Intergen. Tenascin purified from human glioma cell line u-251MG was from Chemicon International Inc. Fibronectin was purchased from Nitta Gelatin. Lamini- nin was obtained from Iwaki. Chondroitin sulfate A from whale cartilage, chondroitin sulfate C from shark cartilage, heparan sulfate from bovine Sprague-Dawley rat whole brains under hypoxic conditions, heparin was obtained from Iwaki. Chondroitin sulfate A from whale cartilage, keratansulfate from bovine cornea, heparitinase I, heparitinase II, and protease-free chondroitinase ABC were purchased from Seikagaku Corp. Heparin, poly-l-lysine, and rabbit IgG were from Sigma. CNBr-activated Sepharose 4B, Protein G Sepha- rose 4FF, and HitTrap Protein G were purchased from Pharmacia Bio- tech Inc. A mixture of monoclonal antibodies to phosphorylated neuro- filament, SMi 312, was obtained from Sternberger Monoclonals Inc. Texas Red Avidin D and Vectastain ABC kit were obtained from Vector Labs. Fluorescein isothiocyanate-conjugated anti-mouse Ig was from J Jackson Immunoresearch. Biotinylated anti-rabbit Ig was from Amer- phar Inc. A mixture of monoclonal antibodies to phosphorylated neurofilament heavy chain, IGF-1, IGF-2, and HiTrap Protein G were purchased from Pharmacia Biotech. Tripolyphosphate, Tris-HCl, pH 7.2, and then added 125I-6B4 proteoglycan (15,000 cpm/5 μl for the usual assay and 2,000–80,000 cpm/35 μl for Scatchard analyses) to the coated wells and incubated the plates for 5 h at room temperature. The wells were washed three times with PBS and then blocked with 1% BSA/PBS for 1 h at room temperature. We diluted 125I-6B4 proteoglycan in 0.5% BSA, 2 mM CaCl2, 2 mM MgCl2, 0.1% CHAPS, 0.15 M NaCl, 10 mM sodium acetate, 10 mM Tris-HCl, pH 7.5, to a final concentration of 0.2 μg/ml. Various concentrations of protease- free chondroitinase ABC and heparitinases I and II were then added to the samples, and the solutions were incubated for 1 h at 30°C for use in binding assays.

**Preparation of Dissociated Cortical Neurons—**Cerebra were dissected from embryonic 16- (E16) or 17-day-old Sprague-Dawley rats, and the tissue was triturated in a culture dish. The tissue was treated with Ca2+ - and Mg2+-free Hanks’ balanced salt solution (CMF-HBSS) containing 0.1% trypsin for 15 min at 37°C. Then, three washes with CMF-HBSS, the cells were triturated with Pasteur pipettes in CMF-HBSS containing 0.025% DNase I, 0.4 mg/ml soybean trypsin inhibitor, 3 mg/ml BSA, and 12 mM MgSO4. The cell suspension was centrifuged at 160 × g for 6 min at 4°C, and the pellets were washed once more with CMF-HBSS. Cells were resuspended in culture medium consisting of a 1:1 mixture of Dulbecco’s modified Eagle’s medium and F12 medium (DF medium) containing 2% B-27 supplement (DF/B-27 medium) and were seeded as described below.

**Cell Culture—**Glass coverslips (13 mm in diameter) were incubated in 0.002% poly-l-lysine washed three times with distilled water, and then air dried. Cells (40 μl of a 1 × 106 cells/ml suspension) were plated on coverslips in 24-well plates. After a 1-h incubation at 37°C under 5% CO2, 0.5 ml per well of DF/B-27 medium was added. The cells were cultured overnight and processed for immunocytochemistry as described below.

**For the neurite extension assay, wells of Nunclon Immuno- plates (Nunc) were coated overnight with 2 μg/ml pleiotrophin or 2 μg/ml poly-l-lysine at 4°C. The wells were washed three times with distilled water and then blocked with 0.25% BSA for 2 h at 37°C. The solutions were removed, and then 40 μl of DF/B-27 medium containing antibodies at various concentrations were added to the wells followed by 1.5 × 104 cortical neurons (10 μl in the medium) per well. Phase-contrast micro- graphs of cultures were taken after a 50-h incubation, and neurite length distri- bution was analyzed as described (11).

**Immunocytochemistry—** Cultures were washed once with PBS and then fixed with 4% paraformaldehyde, 0.1% sodium phosphate buffer, pH 7.5, for 15 min. Fixed cells were rinsed three times with Tris-buffered saline, permeabilized with 0.2% Triton X-100/PBS for 30 min, then blocked with 2% BSA, 4% goat serum in PBS. Cells were then incubated for 2 h with the mixture of anti-phosphorylated neurofilament antibodies, SMi 312 (1/500), and anti-6B4 proteoglycan antisera (1/500). After three washes with PBS, the cells were incubated for 30 min with fluorescein isothiocyanate-conjugated anti-mouse IgG (1/100) and biotinylated anti-rabbit Ig (1/200), followed by three washes with PBS, and then incubated once more for 30 min with Texas Red Avidin D (1/1000). The cells were washed three times with PBS, mounted, and observed using a Zeiss fluorescence microscope. All solutions were di- luted with PBS containing 0.05% BSA. Incubations were at room temperature.

**Immunoprecipitation—** Cortical neurons (105 cells) in DF/B-27 me- dium were plated on 6-cm culture plates coated with poly-l-lysine as described above. After 3 days of culture under 5% CO2, 37°C, the cells were extracted for 2 h at 4°C with 15 ml of 0.2% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, 10 μM pepstatin A, 10 μM leupeptin, 1 mM EDTA, 50 mM Tris-HCl, pH 7.5 (buffer A). After centrifugation at 15,000 × g for 15 min, 500-μl aliquots of the supernatant were adsorbed for 1 h at 4°C with 25 μl of Protein G Sepharose 4F. After a brief
proteins that eluted with EDTA seemed to be aggregated in the presence of divalent cations because similar proteins were also eluted with EDTA from a control Sepharose CL-4B column (data not shown). Three major proteins (18-, 28-, and 40-kDa proteins) were eluted with 1 mM NaCl from the 6B4 proteoglycan-Sepharose column but not from Sepharose CL-4B. Divalent cations (5 mM each) were not essential, but in their absence, these proteins did not bind efficiently to the column.

Next, chondroitinase ABC-digested 6B4 proteoglycan was coupled to a Sepharose gel for affinity chromatography and tested as described above. Under these conditions, 40- and 28-kDa proteins flowed through the column, and only the 18-kDa protein was eluted with 1 mM NaCl, suggesting that the two former proteins are chondroitin sulfate-binding proteins (data not shown). Gas phase amino acid sequencing indicated that the N-terminals of the 28- and 40-kDa proteins were blocked, since the N-terminal amino acid was undetectable. Amino acid sequences obtained from the CNBr-digested samples could not be found in the sequence of proteins that have been reported to date (data not shown). On the other hand, the N-terminal amino acid sequence of the 18-kDa protein obtained was GKKKEPKKKVKKSDGXEXQXXVXPT, which faithfully corresponded to that of pleiotrophin except for the four X positions (13).

Binding of 6B4 Proteoglycan to Pleiotrophin—The results of the affinity chromatography suggested that pleiotrophin specifically binds to 6B4 proteoglycan. We therefore measured the binding of 125I-6B4 proteoglycan using ELISA plates coated with various proteins. Among these, pleiotrophin and tenascin significantly bound to 6B4 proteoglycan, whereas aFGF, fibroblastin, and laminin did not (Fig. 2A). Tenascin reportedly binds phosphacan (8). The binding of 125I-6B4 proteoglycan to pleiotrophin was dependent on the concentration of the proteoglycan, and the nonspecific binding was negligibly low (Fig. 2B). These results indicate that 6B4 proteoglycan bound to pleiotrophin.

Effects of Glycosaminoglycans on the Binding of 6B4 Proteoglycan to Pleiotrophin—Pleiotrophin is a heparin-binding protein that binds to the heparan sulfate chains of syndecan (17, 20). The 6B4 proteoglycan preparation did not contain any detectable amount of heparan sulfate proteoglycan. It was also confirmed that the preparation did not contain N-syndecan by immunoblotting analysis using anti-N-syndecan monoclonal antibody H5 (18) (data not shown). Next, we analyzed the effects of various glycosaminoglycans on 6B4 proteoglycan-pleiotrophin binding. As shown in Fig. 3, heparin potently inhibited the binding of 6B4 proteoglycan to pleiotrophin (IC50 = 3.5 ng/ml), and heparan sulfate moderately suppressed the binding (IC50 = 150 ng/ml). Chondroitin sulfate C also had a moderate effect (IC50 = 400 ng/ml), but in contrast, chondroitin sulfate A scarcely influenced the 6B4 proteoglycan-pleiotrophin binding (IC50 > 100 μg/ml). However, chondroitinase ABC digests of chondroitin sulfate A and C (unsaturated disaccharides) had no effect on the binding.

Characterization of the Binding of 6B4 Proteoglycan to Pleiotrophin—The roles of chondroitin sulfate chains in the 6B4 proteoglycan-pleiotrophin interaction were tested by digesting 125I-6B4 proteoglycan with various concentrations of chondroitinase ABC, and then we assayed the binding (Fig. 4A). Digestion with increasing amounts of enzyme reduced the binding to about 60% of the control. Additional heparitinase digestion did not affect the binding (Fig. 4A, open square), which was consistent with the finding that 6B4 proteoglycan does not contain heparan sulfate (2). Scatchard analysis of the binding of native 6B4 proteoglycan to pleiotrophin indicated that there are low (Kd = 3.0 mM) and high (Kd = 0.25 mM)
affinity binding sites (Fig. 4, B and C). We then performed a binding assay using the chondroitinase ABC-digested 125I-6B4 proteoglycan (Fig. 4B). Scatchard analysis indicated that chondroitinase ABC digestion of the 6B4 proteoglycan reduced the affinity of both sites ($K_d = 13 \text{ nM}$) without changing the number of binding sites (Fig. 4C).

Presence of 6B4 Proteoglycan and PTPζ on Cortical Neurons—Dissociated cortical neurons from E16 rats were doubly stained with anti-6B4 proteoglycan antibody (anti-6B4 PG) and anti-phosphorylated neurofilament monoclonal antibody (anti-P-NF). As shown in Fig. 5, P-NF-positive neurons were stained with anti-6B4 PG. The cell bodies and a portion of neurites showed strong immunoreactivity to anti-6B4 PG. It is notable that the rims of the growth cones and the filopodial processes were 6B4 proteoglycan-positive. A polyclonal antibody against the C-terminal portion of 6B4 proteoglycan (anti-31–5) (3) gave similar results.

Immunoprecipitates with anti-6B4 PG were prepared from extracts of cultured cortical neurons and analyzed by immunoblotting with anti-6B4 PG (Fig. 6). Two core protein bands of chondroitin sulfate proteoglycan (300 and 380 kDa) were detected after chondroitinase ABC digestion. Judging from the molecular weight and enzyme activity, the former was 6B4 proteoglycan and the latter was PTPζ (3). There were two additional minor bands of 230 and 260 kDa, which were not sensitive to chondroitinase ABC digestion. These proteins are probably the precursor form of the proteoglycans in the cells, which are not yet glycosylated with chondroitin sulfate, or dvPTPζ, which is another splicing variant of PTPζ (6). Because this culture was estimated to be 98% pure neurons (11), these results suggest that cortical neurons synthesize both 6B4 proteoglycan and PTPζ.

Effect of Anti-6B4 Proteoglycan Antibody on Pleiotrophin-induced Neurite Outgrowth—Pleiotrophin stimulates neurite outgrowth (13, 14). As shown in Fig. 7, neurons aggregated without neurites on the uncoated ELISA plates after 20 h. By contrast, cortical neurons actively extended long neurites on the pleiotrophin-coated plates (Fig. 7B). To examine whether 6B4 proteoglycan-pleiotrophin interaction is involved in the neurite outgrowth, anti-6B4 PG was added to the culture medium. Anti-6B4 PG significantly suppressed the pleiotrophin-induced neurite outgrowth of cortical neurons at a concentration of over 200 μg/ml, in contrast to the control IgG (Fig. 7, C and D). On the other hand, poly-L-lysine-induced neurite outgrowth was not influenced by anti-6B4 PG, indicating that the effect of the antibody is not a nonspecific cytotoxic activity (Fig. 7D). In fact, the cell viability determined by trypan blue exclusion was not influenced by adding the antibody to the medium (data not shown). However, it was revealed that anti-6B4 proteoglycan (200 μg/ml) did not interfere with 6B4 proteoglycan-pleiotrophin binding by the solid phase binding assay (data not shown). This result suggests that anti-6B4 proteoglycan does not directly mask the pleiotrophin binding site but affects the signal transfer mechanism of PTPζ and 6B4 proteoglycan.

**DISCUSSION**

This study provides evidence that 6B4 proteoglycan binds pleiotrophin. The 6B4 proteoglycan had high and low affinity binding sites for pleiotrophin. Chondroitinase ABC digestion of
Scatchard plot of intact proteoglycan has high (Kd = 0.25 nM) and low (Kd = 3.0 nM) affinity binding sites, but chondroitinase ABC-digested proteoglycan has a single low affinity binding site (Kd = 13 nM).

Our Scatchard analysis indicated that there were two binding sites with different affinities to pleiotrophin. This may be explained by the presence of two populations of 6B4 proteoglycan in the brain bearing chondroitin sulfate chains with different structures. In fact, Rauch et al. (21) have reported that monoclonal antibodies distinguished two subpopulations of phosphacan with different chondroitin sulfate structures. We showed here that chondroitin sulfate C was a potent inhibitor of 6B4 proteoglycan-pleiotrophin binding as well as heparan sulfate. In contrast, chondroitin sulfate A was a poor inhibitor. From this point of view, it is also notable that the structure of chondroitin sulfate chains on 6B4 proteoglycan changes during development of the brain. In the early developmental stages, substantial amounts of chondroitin sulfate C unit are found, but later, the chondroitin sulfate chains of 6B4 proteoglycan are virtually composed of only chondroitin sulfate A units (2, 21). This developmental change in the structure of chondroitin sulfate may change the binding affinity of the proteoglycan to pleiotrophin.

The contribution of chondroitin sulfate to the protein function is similar in thrombomodulin (22). Digesting thrombomodulin with chondroitinase abolished the inhibitory effect of this molecule on thrombin-induced fibrinogen clotting, indicating that chondroitin sulfate constitutes part of the active site.

Raulo et al. (17) purified N-syndecan from the membrane fractions of the rat brain and from the cultured neurons as a proteoglycan that reduced the affinity of the proteoglycan to pleiotrophin without changing the number of binding sites. These results suggested that the core protein of 6B4 proteoglycan together with chondroitin sulfate chains constitute the binding sites for pleiotrophin. Among the various glycosaminoglycans, heparin potently inhibited the binding of 6B4 proteoglycan to pleiotrophin, consistent with the fact that pleiotrophin has high affinity for heparin (13, 15). N-Syndecan/syndecan-3 and syndecan-1 were reported to bind pleiotrophin through heparan sulfate chains (17, 20). Heparitinase digestion of 6B4 proteoglycan did not affect the binding activity to pleiotrophin, showing that heparan sulfate is not involved in the binding. This is consistent with the finding that 6B4 proteoglycan does not contain heparan sulfate (2).

Our Scatchard analysis indicated that there were two binding sites with different affinities to pleiotrophin. This may be explained by the presence of two populations of 6B4 proteoglycan in the brain bearing chondroitin sulfate chains with different structures. In fact, Rauch et al. (21) have reported that monoclonal antibodies distinguished two subpopulations of phosphacan with different chondroitin sulfate structures. We showed here that chondroitin sulfate C was a potent inhibitor of 6B4 proteoglycan-pleiotrophin binding as well as heparan sulfate. In contrast, chondroitin sulfate A was a poor inhibitor. From this point of view, it is also notable that the structure of chondroitin sulfate chains on 6B4 proteoglycan changes during development of the brain. In the early developmental stages, substantial amounts of chondroitin sulfate C unit are found, but later, the chondroitin sulfate chains of 6B4 proteoglycan are virtually composed of only chondroitin sulfate A units (2, 21). This developmental change in the structure of chondroitin sulfate may change the binding affinity of the proteoglycan to pleiotrophin.

The contribution of chondroitin sulfate to the protein function is similar in thrombomodulin (22). Digesting thrombomodulin with chondroitinase abolished the inhibitory effect of this molecule on thrombin-induced fibrinogen clotting, indicating that chondroitin sulfate constitutes part of the active site.
major binding protein for pleiotrophin. The 6B4 proteoglycan is quite soluble, and most of it is recovered in the soluble fraction of the brain extract. Moreover, there is much less PTP than N-syndecan. This may be why Raulo et al. (17) did not detect 6B4 proteoglycan as pleiotrophin-binding protein. They indicated that adding anti-N-syndecan antibody or digesting the neurons with heparitinase suppresses the pleiotrophin-induced neurite outgrowth (17, 23), suggesting that N-syndecan is involved in the signal transduction of pleiotrophin. The K_d value of the binding of N-syndecan to pleiotrophin is 0.6 nM, which is in a similar range to those of 6B4 proteoglycan (0.25 and 3 nM). The content of pleiotrophin in the developing brain is extremely high, being about 10–15 μg/g of wet tissue (24). This is also true for 6B4 proteoglycan or N-syndecan in the brain. About 10–18 μg of 6B4 proteoglycan and 10 μg of N-syndecan can be purified from 1 g of brain (2, 17). Thus, 6B4 proteoglycan and N-syndecan have an apparently equivalent binding capacity to pleiotrophin, and 6B4 proteoglycan and PTPζ may compose another signal transduction pathway for pleiotrophin.

Immunoelectron microscopy indicated that pleiotrophin is located not only on the cell surface but also in the extracellular matrix, suggesting that pleiotrophin binds to some extracellular matrix components (25). Our studies indicated that 6B4 proteoglycan is localized at extracellular matrix as well as cell surface (10). Thus, 6B4 proteoglycan may serve as an extracellular reservoir of pleiotrophin. In addition to the soluble 6B4 proteoglycan, an alternatively spliced variant PTPζ is located on the neuronal cell surface as a receptor-like protein-tyrosine phosphatase. Although it is not confirmed whether this molecule binds pleiotrophin, its carbohydrate modification is quite similar to that of 6B4 proteoglycan, including the chondroitin sulfate (3). Therefore, it is conceivable that PTPζ is a cell surface signal-transducing receptor for pleiotrophin.

The developmental expression of pleiotrophin is dynamically regulated during development of the brain (13, 23, 25–29). In the rat cerebral cortex, the expression of pleiotrophin begins at around E12–E14, peaks at the perinatal period, and persists at a lower concentration into adulthood (23, 25). In the early cerebral cortex, pleiotrophin is located along radial glial fibers, a scaffold for neuronal migration, and in the subplate and the marginal zone (23, 25). Later, in the early postnatal period, pleiotrophin is more widely expressed in the cerebral cortex including the pathways of the developing axon (23, 25). These expression profiles are quite similar to those of 6B4 proteoglycan and PTPζ (2, 7), suggesting that these molecules concertedly function together in the development of the cortex, especially in neuronal migration and axonal outgrowth.

Increasing evidence indicates that pleiotrophin and 6B4 proteoglycan promote neurite extension (2, 11–13, 23, 24). Pleiotrophin attached to the substrate induces neurite outgrowth from cortical neurons in vitro. 6B4 proteoglycan itself does not promote neurite extension but rather exerts repulsive effects on neurons. However, plates coated with 6B4 proteoglycan together with poly-L-lysine, fibronectin, or tenascin promoted neurite extension from cortical neurons (2, 11). The CAH region of PTPζ/PTPβ coated on a substrate promotes neurite extension from tectal neurons (12). Here, we demonstrated that anti-6B4 proteoglycan antibody suppressed the pleiotrophin-induced neurite outgrowth, suggesting that 6B4 proteoglycan and PTPζ are the functional receptors for pleiotrophin.

The mechanism by which 6B4 proteoglycan regulates neurite outgrowth seems to be rather complex. Milev et al. (30) have reported that complex-type N-linked oligosaccharides mediate the binding of phosphacan to Ng-CAM, N-CAM, and tenascin. The CAH region of phosphacan binds contactin (12). Matrix-bound 6B4 proteoglycan/phosphacan may be recognized by neuronal cell surface receptors such as contactin and N-CAM, which trigger the neurite outgrowth. On the other hand, 6B4 proteoglycan and PTPζ on the neuronal cell surface may bind to matrix-bound ligands such as pleiotrophin, which also leads to neurite outgrowth.

An immunohistochemical analysis of the cultured cortical neurons revealed immunoreactivity to anti-6B4 proteoglycan antibody on the rims of the growth cones and the filopodial processes, which are actin-rich structures that play important roles in axonal path finding and neurite outgrowth. Since this antibody recognizes both 6B4 proteoglycan and PTPζ (3), it is not clear which protein is responsible for this immunoreactivity. However, our recent analysis of cDNA transformants, stably expressing in the mouse fibroblast L cells, indicated that PTPζ was localized at cell cortical structures such as membrane ruffles and lamellipodia. The cell cortex is rich in actin filaments and actin-binding proteins and plays important roles in cell locomotion and cell-cell and cell-substrate interaction.

Fig. 7. Antibody against 6B4 proteoglycan suppressed the pleiotrophin-induced neurite outgrowth of cortical neurons. A–C, cortical neurons from E17 rat embryos were cultured on the Nunc Maxisorp plates. Plates used were uncoated (A) or coated with 2 μg/ml pleiotrophin in the absence (B) or presence (C) of 300 μg/ml anti-6B4 proteoglycan antibody. Phase-contrast micrographs were taken after 20 h in vitro. Scale bar, 50 μm. D, cortical neurons from E17 rat embryos were cultured on Nunc Maxisorp plates coated with 2 μg/ml pleiotrophin in the presence of 300 μg/ml of control rabbit IgG (PTN + IgG) or anti-6B4 proteoglycan antibody (PTN + anti6B4). Neurons were also cultured on the plates coated with 2 μg/ml poly-L-lysine in the presence of 300 μg/ml of rabbit IgG (PLL + IgG) or anti-6B4 proteoglycan antibody (PLL + anti6B4). The average length of neurites extending from 100 neurons was measured after 50 h in vitro. Neurite length from neurons cultured on the plates coated (PTN) or uncoated (NONE) with 2 μg/ml pleiotrophin was also measured. This experiment was performed three times, and the results of a typical experiment are shown.

2 H. Hamanaka, N. Maeda, and M. Noda, unpublished observation.

3 T. Nishiwaki, N. Maeda, and M. Noda, unpublished observation.
We therefore postulate that PTPζ functions as a receptor that regulates the organization of actin filaments and that the ligand binding to this receptor leads to the reorganization of cytoskeleton in the growth cones and filopodial processes, resulting in neurite outgrowth and cell locomotion. Further studies are required to elucidate the involvement of pleiotrophin and PTPζ in the regulation of the neuronal cytoskeleton.

Acknowledgments—We thank Sonoko Oosawa (Center for Analytical Instruments, National Institute for Basic Biology) for performing amino acid sequence analysis and Akiko Kodama for secretarial assistance.

REFERENCES

1. Maeda, N., Matsui, F., and Oohira, A. (1992) Dev. Biol. 151, 564–574
2. Maeda, N., Hananaka, H., Oohira, A., and Noda, M. (1995) Neuroscience 67, 23–35
3. Maeda, N., Hananaka, H., Shintani, T., Nishiwaki, T., and Noda, M. (1994) FEBS Lett. 354, 67–70
4. Maurice, P., Rauch, U., Flad, M., Margolis, R. K., and Margolis, R. U. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 2512–2516
5. Krueger, N. X., and Saito, H. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 7417–7421
6. Levy, J. B., Candoll, P. D., Silvennoinen, O., Barnea, G., Morse, B., Honegger, A., Huaq, J.-T., Cannizzaro, L. A., Park, S. H., Druck, T., Hubeck, K., Sap, J., Ehrlich, M., Musachio, J. M., and Schlessinger, J. (1993) J. Biol. Chem. 268, 10573–10581
7. Candoll, P. D., Barnea, G., Levy, J. B., Sap, J., Ehrlich, M., Silvennoinen, O., Schlessinger, J., and Musachio, J. M. (1993) Dev. Brain Res. 75, 235–288
8. Milev, P., Friedlander, D. R., Sakurai, T., Karthikeyan, L., Flad, M., Margolis, R. K., Grumet, M., and Margolis, R. U. (1994) J. Cell Biol. 127, 1703–1715
9. Grumet, M., Milev, P., Sakurai, T., Karthikeyan, L., Bourdon, M., Margolis, R. K., and Margolis, R. U. (1994) J. Biol. Chem. 269, 12142–12146
10. Nishizuka, M., Ikeda, S., Araki, M., Noda, M., and Noda, M. (1996) Neurosci. Res. 24, 345–353
11. Maeda, N., and Noda, M. (1996) Development 122, 647–658
12. Peles, E., Nativ, M., Campbell, P. L., Sakurai, T., Martinez, R., Lev, S., Clay, O. D., Schilling, J., Barnea, G., Plowman, G. D., Grumet, M., and Schlessinger, J. (1995) Cell 82, 251–260
13. Li, Y.-S., Milner, P. G., Chauhan, A. K., Watson, M. A., Hoffman, R. M., Kodner, C. M., Milbrandt, J., and Deuel, T. F. (1990) Science 250, 1680–1684
14. Rauvala, H., and Pihlaskari, R. (1987) J. Biol. Chem. 262, 16625–16635
15. Kretschmer, P. J., Fairhurst, J. L., Decker, M. M., Chan, C. P., Gluzman, Y., Böhm, P., and Kovess, I. (1991) Growth Factors 5, 99–114
16. Kadozuma, K., Tomomura, M., and Muramatsu, T. (1988) Biochem. Biophys. Res. Commun. 151, 1312–1318
17. Raulo, E., Chernousov, M. A., Carey, D. J., Nido, R., and Rauvala, H. (1994) J. Biol. Chem. 269, 12999–13004
18. Watanabe, E., Matsui, F., Keino, H., Ono, K., Kushima, Y., Noda, M., and Oohira, A. (1996) J. Neurosci. Res. 44, 84–96
19. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350–4354
20. Mitsiades, T. A., Salmivirta, M., Muramatsu, T., Muramatsu, H., Rauvala, H., Lehtonen, E., Jalaskan, M., and Theila, J. (1995) Development 121, 37–51
21. Rauch, U., Gao, P., Janetcko, A., Flaccus, A., Hiligtenberg, L., Tekotte, H., Margolis, R. K., and Margolis, R. U. (1991) J. Biol. Chem. 266, 14785–14801
22. Bourin, M.-C., and Lindahl, U. (1990) Biochem. J. 270, 419–425
23. Rauvala, H., Vanhala, A., Castrén, E., Nido, R., Raulo, E., Merenmies, J., and Panula, P. (1994) Dev. Brain Res. 79, 157–176
24. Rauvala, H. (1989) EMBO J. 8, 2933–2941
25. Matsumoto, K., Wania, A., Takatsui, K., Muramatsu, H., Muramatsu, T., and Tohyama, M. (1994) Dev. Brain Res. 79, 229–241
26. Bloch, B., Nomand, E., Kovess, I., and Böhm, P. (1992) Dev. Brain Res. 70, 267–278
27. Wania, A., Carrol, S. L., and Milbrandt, J. (1993) Dev. Brain Res. 72, 133–144
28. Matsumoto, K., Wania, A., Mori, T., Taguchi, A., Ishii, N., Muramatsu, H., Muramatsu, T., and Tohyama, M. (1994) Neurosci. Lett. 175, 216–220
29. Wewetzer, K., Rauvala, H., and Unsicker, K. (1995) Brain Res. 693, 31–38
30. Milev, P., Meyer-Puttlitz, B., Margolis, R. K., and Margolis, R. U. (1995) J. Biol. Chem. 270, 24650–24653
31. Bretscher, A. (1991) Annu. Rev. Cell Biol. 7, 337–374
6B4 Proteoglycan/Phosphacan, an Extracellular Variant of Receptor-like Protein-tyrosine Phosphatase ζ/RPTPβ, Binds Pleiotrophin/Heparin-binding Growth-associated Molecule (HB-GAM)
Nobuaki Maeda, Taeko Nishiwaki, Taka funny Shintani, Hiroki Hamanaka and Masaharu Noda

J. Biol. Chem. 1996, 271:21446-21452.
doi: 10.1074/jbc.271.35.21446

Access the most updated version of this article at http://www.jbc.org/content/271/35/21446

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 31 references, 14 of which can be accessed free at http://www.jbc.org/content/271/35/21446.full.html#ref-list-1