Heterogeneity of the Chondroitin Sulfate Portion of Phosphacan/6B4 Proteoglycan Regulates Its Binding Affinity for Pleiotrophin/Heparin Binding Growth-associated Molecule*

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PTPγ is a receptor-type protein-tyrosine phosphatase that is synthesized as a chondroitin sulfate proteoglycan and uses pleiotrophin as a ligand. The chondroitin sulfate portion of this receptor is essential for high affinity binding to pleiotrophin. Here, we purified phosphacan, which corresponds to the extracellular domain of PTPγ from postnatal day 7 (P7) and P12 rat cerebral cortex (PG-P7 and PG-P12, respectively) and from P20 rat whole brain (PG-P20). The chondroitin sulfate of these preparations displayed immunologically and compositionally different structures. In particular, only PG-P20 reacted with the monoclonal antibody MO-225, which recognizes chondroitin sulfate containing the 1–3GalNAc(6S) disaccharide unit (D unit). Analysis of the chondroitinase digestion products revealed that Glcβ1–3GalNAc(4S) disaccharide unit (A unit) was the major component in these preparations and that PG-P20 contained 1.3% D unit, which was not detected in PG-P7 and PG-P12. Interaction analysis using a surface plasmon resonance biosensor indicated that PG-P20 had ~5-fold stronger affinity for pleiotrophin (dissociation constant (K_D) = 0.14 nM) than PG-P7 and PG-P12, although all these preparations showed similar low affinity binding to pleiotrophin after chondroitinase ABC digestion (K_D = 1.4–1.6 nM). We also found that shark cartilage chondroitin sulfate D containing ~20% D unit bound to pleiotrophin with moderate affinity (K_D = 2.7 nM), whereas whale cartilage chondroitin sulfate A showed no binding to this growth factor. These results suggest that variation of chondroitin sulfate plays important roles in the regulation of signal transduction in the brain.

Recent studies reveal that proteoglycans play pivotal roles in various developmental processes such as cell migration, recognition, and morphogenesis (1–4). Accumulating evidence suggests that glycosaminoglycans with specific sequences selectively bind with a wide range of proteins including growth factors, morphogens, and proteases, regulating their biological activities (1–4). Although this paradigm is principally derived from the findings on heparan sulfate proteoglycans, increasing evidence suggests that chondroitin sulfate proteoglycans also play such roles (3).

PTPγ2 is a receptor-type protein-tyrosine phosphatase abundantly expressed in the brain as a chondroitin sulfate proteoglycan (5, 6). The extracellular domain of this receptor is secreted in the brain as a major soluble chondroitin sulfate proteoglycan called phosphacan/6B4 proteoglycan/DSD-1-PG (6–8). PTPγ binds to pleiotrophin/heparin-binding growth-associated molecule with high affinity (K_D = 0.25–3 nM) (9–11). The binding of pleiotrophin to PTPγ depends on the chondroitin sulfate portion of this receptor, and removal of chondroitin sulfate resulted in a drastic decrease in the binding affinity and signal transduction (9–11). This binding was inhibited strongly by squid cartilage chondroitin sulfate E (CS-E) and shark cartilage CS-D, moderately by shark cartilage CS-C, and very weakly by whale cartilage CS-A (9, 10).

An in vitro cell migration assay indicated that pleiotrophin induces migration of cortical neurons (10). This activity of pleiotrophin was also inhibited by CS-C, -D, and -E but not by CS-A. Furthermore, using an organotypic slice culture system of the rat cerebellum, we revealed that disruption of PTPγ–pleiotrophin signaling leads to the aberrant morphogenesis of Purkinje cells (12). The number of Purkinje cells with abnormal dendrites such as multiple and disoriented dendrites markedly increased when cerebellar slices were treated with polyclonal antibodies against PTPγ, chondroitinase ABC, CS-C, CS-D, or CS-E, whereas CS-A was also ineffective in this system (12). These findings suggested that a specific structural motif containing oversulfated portion in chondroitin sulfate is involved in the high affinity binding between PTPγ and pleiotrophin and their signal transduction.

Recently, Deepa et al. (13) report that squid cartilage CS-E interacted with various heparin binding growth factors. CS-E bound strongly to pleiotrophin, midkine, fibroblast growth factor-16 (FGF-16), FGF-18, and heparin binding epidermal growth factor-like growth factor with comparable affinities to those of heparin (13). Zou et al. (14) also found that chondroitin sulfate artificially enriched with E unit (Glcβ1–3GalNAc(4S,6S)) bound strongly with midkine, which shows a 45% amino acid sequence identity to pleiotrophin, forming a heparin binding growth factor family together with this pro-

* The abbreviations used are: PTP, protein-tyrosine phosphatase; CS, chondroitin sulfate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TBS, Tris-buffered saline; P, postnatal day; HPLC, high performance liquid chromatography; ABC, avidin-biotin-peroxidase complex.

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tein. However, these chondroitin sulfate preparations contained unusually high amounts of oversulfated disaccharide units (more than 60% was E unit), which are only minor components in the brain (15). In fact, Ueoka et al. (15) report that chondroitin sulfate proteoglycan from embryonic day 18 rat brain contained only 1.7% D (Glca2Sβ1→3GalNAcE) and 1.2% E units. Thus, to evaluate the contribution of the chondroitin sulfate structure to the signal transduction of phosphacan/PTPγ, it is necessary to use proteoglycan samples purified from the brain.

It has been suggested that phosphacan is composed of several subpopulations of molecules bearing chondroitin sulfate chains with different structures (16, 17). In this study we found that the immunological and compositional structure of chondroitin sulfate of phosphacan changed dramatically during development of the brain. Using a BIAcore system we observed that the binding affinity of phosphacan for pleiotrophin highly depends on the structure of chondroitin sulfate, especially on the presence of D unit. Our observations suggest that structural variation of chondroitin sulfate plays important roles in the regulation of signal transduction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant pleiotrophin was purchased from R&D systems (Minneapolis, MN). CS-A from whale cartilage (average molecular mass 34 kDa), CS-B from pig skin (average molecular mass 32 kDa), CS-C (average molecular mass 64 kDa) and CS-D (average molecular mass 32 kDa) from shark cartilage, and cartilage (average molecular mass 70 kDa), chondroitinase ABC, chondroitinase AC-II, unaturated chondroitin-sulfate A (2H6), and monoclonal anti-chondroitin sulfate D (MO-225) were purchased from Seikagaku Corp. (Tokyo, Japan). Heparin from porcine intestinal mucosa (average molecular mass 14 kDa) was obtained from Calbiochem. YMC pack PA-03 column was purchased from YMC Co (Kyoto, Japan). Monoclonal anti-chondroitin sulfate (CS-56), QuantiiPro BCA assay kit, and actinase E were purchased from Sigma. Vectastain ABC kit was from Vector Labs (Burlingame, CA). Immobilon membrane and Microcon YM-30 were purchased from Millipore (Bedford, MA). Streptavidin-conjugated alkaline phosphatase and Sepharose CL-4B were purchased from Amersham Biosciences. A research grade sensor chip CM5 and an amine coupling kit containing N-hydroxysuccinimid, N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide hydrochloride, and 1 M ethanolamine hydrochloride were purchased from BiaCore AB. Human recombinant pleiotrophin was purchased from R&D systems. CS-A from whale cartilage (average molecular mass 70 kDa), chondroitinase ABC, chondroitinase AC-II in a total volume of 30 μl of 50 mM sodium acetate buffer, pH 5.0, at 37 °C for 30 min. The reaction was terminated by heating at 100 °C for 1 min. The samples were dried and derivatized with 2-aminoethylmaleimide and the excess reagent was removed by paper chromatography. An aliquot of the 2-amino- benzamide-derivative (1 nmol as GlcUA) was subjected to HPLC on an amine-bound silica PA-03 column (4.6 × 250 mm) using a linear gradient of NaH2PO4 from 16 to 530 mM over 60 min at a flow rate of 1 ml/min. Eluates were monitored by fluorescence intensity with excitation at 380 nm and emission wavelengths of 390 and 420 nm, respectively.

**Immobilization of Pleiotrophin on the Sensor Surface**—Pleiotrophin was immobilized on the surface of a CMS sensor chip by amine coupling, where the primary amino groups on the protein were coupled to the carboxymethylated dextran on a sensor surface. The carboxymethylated dextran surface on the sensor chip was activated by injection of 35 μl of a mixture of N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide hydrochloride/N-hydroxysuccinimide (0.2/0.05 M). Then 10 μl/ml pleiotrophin in 10 mM maleate buffer, pH 6.0, was injected onto the activated sensor surface. The remaining unreacted sites were blocked by injecting 35 μl of 1 mM ethanolamine, pH 8.5. The amounts of pleiotrophin immobilized onto the sensor surface were controlled within the range 1000–4500 resonance units by changing the injection buffer. All steps were carried out in a continuous flow of a solution containing 10 mM HEPES, pH 7.4, 0.15 mM NaCl, 3 mM EDTA, 0.005% Tween 20 (HBS running buffer) at a flow rate of 5 μl/min.

**Results**

**Surface Plasmon Resonance Analysis**—All experiments were carried out at flow rate of 20 μl/min at 25 °C. Buffer exchange of phosphacan solution was performed using Microcon YM-30 by repeated concentration and reconstitution with HBS running buffer. The phosphacan preparations (10 μl of a 200 μg/ml solution) were treated with 25 μl of protease-free chondroitinase ABC for 1 h at 37 °C. Each sample was diluted in HBS running buffer and injected onto the sensor surface. The sensor surfaces were regenerated between each injection by washing with 0.2% H2O2, PBS for 3 min, the solution was washed out with 50 μl of 3 M NaOH at room temperature for 24 h. After neutralization with glacial acetic acid, the sample was digested with 5 μg/ml actinase E at 50 °C for 24 h in the presence of 0.5 μl Tris-HCl, pH 7.8. After heating at 100 °C for 3 min, the solution was applied to a Sepharose CL-4B column (7 mm × 30 cm), and the chondroitin sulfate was eluted with 0.4 mM ammonium acetate. The size of chondroitin sulfate was estimated from the Kav values as described by Wasteson (19). Analysis of the disaccharide composition of chondroitin sulfate was performed by the method of Yoshida et al. (20) after chondroitinase ABC digestion of the phosphacan preparations. The disaccharide composition of the preparations were also confirmed by the method reported by Kinoshita and Sugahara (21). Briefly, phosphacan preparations (30 nmol as hexuronate) were incubated with 10 μl of chondroitinase AC-II in a total volume of 30 μl of 50 mM sodium acetate buffer, pH 6.0, at 37 °C for 30 min. The reaction was terminated by heating at 100 °C for 1 min. The samples were dried and derivatized with 2-aminoethylmaleimide, and the excess reagent was removed by paper chromatography. An aliquot of the 2-amino- benzamide-derivative (1 nmol as GlcUA) was subjected to HPLC on an amine-bound silica PA-03 column (4.6 × 250 mm) using a linear gradient of NaH2PO4 from 16 to 530 mM over 60 min at a flow rate of 1 ml/min. Eluates were monitored by fluorescence intensity with excitation at 380 nm and emission wavelengths of 390 and 420 nm, respectively.

**Characterization of Chondroitin Sulfate**—Purified phosphacan (100 nmol as hexuronate) was treated with 0.2% NaOH at room temperature for 2 h. After neutralization with glacial acetic acid, the sample was digested with 5 μg/ml actinase E at 50 °C for 24 h in the presence of 0.5 μl Tris-HCl, pH 7.8. After heating at 100 °C for 3 min, the solution was applied to a Sepharose CL-4B column (7 mm × 30 cm), and the chondroitin sulfate was eluted with 0.4 mM ammonium acetate. The size of chondroitin sulfate was estimated from the Kav values as described by Wasteson (19). Analysis of the disaccharide composition of chondroitin sulfate was performed by the method of Yoshida et al. (20) after chondroitinase ABC digestion of the phosphacan preparations. The disaccharide composition of the preparations were also confirmed by the method reported by Kinoshita and Sugahara (21). Briefly, phosphacan preparations (30 nmol as hexuronate) were incubated with 10 μl of chondroitinase AC-II in a total volume of 30 μl of 50 mM sodium acetate buffer, pH 6.0, at 37 °C for 30 min. The reaction was terminated by heating at 100 °C for 1 min. The samples were dried and derivatized with 2-aminoethylmaleimide, and the excess reagent was removed by paper chromatography. An aliquot of the 2-amino- benzamide-derivative (1 nmol as GlcUA) was subjected to HPLC on an amine-bound silica PA-03 column (4.6 × 250 mm) using a linear gradient of NaH2PO4 from 16 to 530 mM over 60 min at a flow rate of 1 ml/min. Eluates were monitored by fluorescence intensity with excitation at 380 nm and emission wavelengths of 390 and 420 nm, respectively.

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**RESULTS**

**Immunohistochemical Localization of Chondroitin Sulfate Epitopes in the Developing Mouse Brain**—To analyze systematically the changes in chondroitin sulfate structure during development of the brain, we immunohistochemically stained the mouse brain sections using three kinds of monoclonal an-
tibodies against native chondroitin sulfate: CS-56, 2H6, and MO-225. Among these monoclonal antibodies, MO-225 specifically recognizes tetrasaccharides containing D unit (23). CS-56 strongly reacts with whale cartilage CS-A and shark cartilage CS-C (24), and 2H6 selectively recognizes whale cartilage CS-A (25); however, the structures of their epitopes are unknown.

Fig. 1 shows the immunohistochemically stained sagittal sections from the brains of postnatal day 7 (P7), P12, and P20 mice. 2H6 and CS-56 epitopes were highly expressed in the P7 cerebral cortex, but their expression in this region decreased thereafter (Fig. 1, Cx). On the other hand, the expression of MO-225 epitope was very low in the cerebral cortex during these postnatal ages. In contrast, the cerebellum was strongly stained by MO-225 from P7 to P20 (Fig. 1, Ce). The expression of CS-56 epitopes was also observed in the P7 and P12 cerebellum, but the expression disappeared in the P20 cerebellar cortex. 2H6 epitope was barely detected in the postnatal cerebellar cortex. These results indicated that the chondroitin sulfate structure changes regionally and developmentally in the mouse brain. Similar results were obtained using sections from the rat brain (data not shown), and a detailed description of the immunohistochemical analysis will be reported elsewhere.

Purification of Phosphacan Bearing Chondroitin Sulfate with Different Structures—From the immunohistochemical analysis described above, we anticipated that phosphacan bearing chondroitin sulfate chains with different structures could be purified from the rat brain by selecting appropriate regions and ages, because this molecule is the major chondroitin sulfate proteoglycan in the brain. Our expectations were as follows. 1) Phosphacan samples with low content of D unit would be purified from P7 and P12 cerebral cortex. 2) Phosphacan bearing chondroitin sulfate chains with a substantial amount of D unit would be purified from P20 whole brain. 3) These three phosphacan samples would show different overall chondroitin sulfate structures.

Fig. 2 shows the immunoblot analysis of phosphacan samples from P7 cerebral cortex (P7), P12 cerebral cortex (P12), and P20 whole brain (P20). Analysis of the purified phosphacan with the polyclonal antibodies against core protein (anti-6B4 PG) indicated that the sizes of native proteoglycan and core glycoprotein were indistinguishable among these preparations (Fig. 2A). Reactivities to CS-56 and 2H6 monoclonal antibodies were highly detected in PG-P7, moderately in PG-P12 and barely in PG-P20 (Fig. 2, B and C). On the other hand, the MO-225 epitope was highly expressed in PG-P20 but was not detected in PG-P7 and PG-P12 (Fig. 2D).

Analysis of the disaccharide composition of each preparation indicated that PG-P20 contained 1.3% D unit, which was not detected in PG-P7 and PG-P12 (Table I). The contents of A unit (GlcA1–3GalNAc(4S)) and C unit (GlcA1–3GalNAc(6S)) were variable among these preparations. In particular, the contents of C unit drastically decreased from P7 to P20 (Table I). Despite the large difference in the disaccharide composition, the chain length of chondroitin sulfate and GlcA contents of the each preparation were not significantly different from each other (Table I).

Analysis of Interaction between Phosphacan and Pleiotrophin with the BIAcore System—Real-time analysis of the interaction between phosphacan and pleiotrophin was performed using the BIAcore system. Various concentrations of phosphacan samples were passed over the CM5 sensor chip containing immobilized pleiotrophin, and the change in response was evaluated as a function of time (Fig. 3). The binding of phosphacan to pleiotrophin was monitored by the increase in the response with time up to 12 min, which corresponds to the association phase. This phase was followed by a dissociation phase generated by the stream flowing over the sensor chip containing only running buffer. The affinity of phosphacan for pleiotrophin was quantified by determining the dissociation constant $K_D$ ($K_D = k_d / k_a$), where $k_d$ and $k_a$ represent dissociation and association rate constants calculated from the dissociation and association phases, respectively.
The sensorgrams of the bindings of PG-P7 and PG-P12 to pleiotrophin were very similar to each other (Fig. 3, A and C), and showed $K_D$ values of 0.66 and 0.57 nM, respectively (Table II). PG-P20 exhibited a higher affinity for pleiotrophin ($K_D$ = 0.14 nM) than PG-P7 and PG-P12 (Fig. 3E). The association rate constant for the PG-P20-pleiotrophin interaction ($k_a = 2.0 \times 10^6$ M$^{-1}$ s$^{-1}$) was 3–4-fold faster than those for PG-P7- and PG-P12-pleiotrophin interactions ($k_a = 5.3$ and $6.1 \times 10^5$ M$^{-1}$ s$^{-1}$, respectively) (Table II). On the other hand, their dissociation rate constants were not significantly different from each other ($k_d = 2.7 - 3.5 \times 10^{-4}$ s$^{-1}$).

When the proteoglycan samples were treated with chondroitinase ABC to remove chondroitin sulfate, their affinities for pleiotrophin remarkably decreased (Fig. 3, B, D, and F), and all the preparations showed similar, relatively low affinity binding to pleiotrophin ($K_D = 1.4 - 1.6$ nM) (Table II). At higher concentrations of phosphacan, the binding of intact proteoglycans reached near equilibrium in the association phase, whereas chondroitinase ABC-treated proteoglycans exhibited a nearly linear increase in responses during the association phase even at the concentration of 1.3 $\mu$g/ml (Fig. 3). This was caused mainly by the decrease in the association rate constants of phosphacan-pleiotrophin interaction after chondroitinase ABC digestion (Table II). On the other hand, the values of the dissociation rate constant were barely influenced by the chondroitinase ABC digestion (Table II). All the phosphacan preparations displayed similar values of association and dissociation rate constants after chondroitinase ABC digestion ($k_a = 1.6 - 2.0 \times 10^5$ M$^{-1}$ s$^{-1}$ and $k_d = 2.2 - 3.2 \times 10^{-4}$ s$^{-1}$). These results indicated that the presence of chondroitin sulfate critically influences the association rate, and the value of this rate constant is regulated by the structural variation of chondroitin sulfate chains.

It is noteworthy that the calculated values of $R_{\text{max}}$, which correspond to the maximum responses reached after equilibrium, increased ~2-fold after chondroitinase ABC digestion of the phosphacan preparations. This might be caused by the decrease in steric hindrance after chondroitinase ABC digestion, where intact proteoglycans cannot efficiently use the ligands immobilized in the carboxymethylated dextran matrix because of their large molecular size.

### Table I

| Phosphacan | $\Delta\text{HexA-GalNAc}$ | $\Delta\text{HexA-GalNAc}(4S)$ | $\Delta\text{HexA-GalNAc}(6S)$ | $\Delta\text{HexA/(2S)}-\text{GalNAc}(6S)$ | GlaA content | Average size of CS |
|------------|-----------------|-----------------|-----------------|-----------------|--------------|-----------------|
| PG-P7      | 4.3             | 63.6            | 32.1            | ND              | 1.7          | 17              |
| PG-P12     | 2.7             | 83.2            | 14.1            | ND              | 1.8          | 18              |
| PG-P20     | 6.9             | 85.8            | 6.0             | 1.3             | 1.6          | 20              |

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was observed even at 1.3 nM (Fig. 4C). Among these chondroitin sulfate samples, CS-E showed the strongest affinity for pleiotrophin with a $K_D$ of 0.76 nM (Fig. 4E and Table III). Deepa et al. (13) previously reported that CS-E bound pleiotrophin with a lower affinity ($K_D = 11.4$ nM). The cause of this difference is unknown, but they observed the association between soluble pleiotrophin and immobilized CS-E. This might result in the different affinity of pleiotrophin-CS-E interaction. In this study, we found for the first time that CS-D also had strong affinity for pleiotrophin with a $K_D$ of 2.7 nM (Fig. 4D).

CS-B had ~45-fold less affinity ($K_D = 34$ nM) in comparison with CS-E. However, it should be noted that the sensogram of this type of chondroitin sulfate showed a two-phase reaction. There was a small, rapid rise in the responses (Fig. 4B, arrow) followed by a slow, large increase. Although the former phase was observed even at 1.3 $\mu$g/ml of CS-B, the latter became evident at higher concentrations (Fig. 4B), suggesting that this preparation is composed of a small population of high affinity components and a large population of low affinity components with a $K_D$ of 34 nM, the value of which was calculated by neglecting the first phase of binding. Vacherot et al. (26) also report that CS-B binds with pleiotrophin/heparin affinity regulatory peptide with a $K_D$ of 51 nM, although they did not detect the first phase. CS-C also showed specific binding to pleiotrophin; however, we could not determine the $k_{on}$ and $k_{off}$ values, because the reaction was too slow to make that determination (Fig. 4C). In contrast, CS-A showed no binding even at 50 $\mu$g/ml (Fig. 4A).

The sensogram of the binding of heparin with pleiotrophin was similar to that of CS-E (Fig. 4F), where heparin showed strong affinity for pleiotrophin with a $K_D$ of 1.7 nM (Table III). Previously, we reported that various types of chondroitin sulfate and heparin inhibited the binding of phosphacan to pleiotrophin (9). The IC$_{50}$ values for these inhibitory effects roughly coincided with the $K_D$ values obtained in this study (Table III), supporting the reliability of our BIAcore analysis. These results indicated that the affinity of chondroitin sulfate for pleiotrophin is highly dependent on the structural variation of this type of glycosaminoglycan.

**DISCUSSION**

In this study, we demonstrated dynamic changes in the chondroitin sulfate structure in the developing brain. This regional and developmental change in the chondroitin sulfate structure was reflected in the chondroitin sulfate portion of phosphacan. Real-time analysis of the interaction between pleiotrophin and various phosphacan preparations indicated that the differences in the chondroitin sulfate structure on phosphacan markedly influence their binding affinity for pleiotrophin. This suggests that the ligand binding affinity and the strength of signal transduction of phosphacan/PTP$_{\xi}$ are regulated by the structural heterogeneity of its chondroitin sulfate portion.

The three phosphacan preparations purified in this study showed immunologically and compositionally different chondroitin sulfate structures. PG-P7 strongly reacted with CS-56 and 2H6 but was not recognized by MO-225. PG-P12 showed medium reactivity to CS-56 and 2H6 without reactivity to MO-225. PG-P20 strongly reacted with MO-225 with low reactivities to CS-56 and 2H6. MO-225 recognizes chondroitin sulfate containing D unit (23), which is consistent with our data that D unit was only detected in PG-P20 by an HPLC analysis of the chondroitinase digestion products. On the other hand, it was reported that 2H6 strongly reacted with whale cartilage CS-A (25), and this antibody is sold as anti-chondroitin sulfate A. However, there was no correlation between the contents of A unit in phosphacan preparations and their reactivities to 2H6. In fact, PG-P7 with the lowest content of A unit (64%) showed the strongest reactivity to 2H6, and PG-P20 bearing chondroitin sulfate containing 86% A unit displayed very low reactivity to this antibody, suggesting that the epitope of 2H6 is not a simple stretch of A unit but some complex sequences in chondroitin sulfate. These observations suggest that chondroitin sulfate chains of phosphacan have a differential combination of structural motifs depending on the regions and developmental stages of the brain.

Analysis of the interaction between pleiotrophin and phosphacan preparations with the BIAcore system revealed that differences in the chondroitin sulfate structure on phosphacan lead to a large difference in the binding affinity for pleiotrophin. PG-P20 displayed high affinity binding to pleiotrophin ($K_D = 0.14$ nM), and PG-P7 and PG-P12 showed 4–5-fold lower affinity for pleiotrophin ($K_D = ~0.6$ nM) than PG-P20. The difference in affinity disappeared after chondroitinase ABC digestion, and all the preparations showed $K_D$ values of 1.4 – 1.6 nM. This indicated that chondroitin sulfate chains of PG-P7 and PG-P12 increased the binding affinity of phosphacan ~2.5-fold, whereas those of PG-P20 increased the affinity more than 11-fold.

From experiments using various midkine mutants, we previously suggested that there is a hierarchy with three steps in the binding between phosphacan and midkine: 1) low affinity binding between midkine and core glycoprotein ($K_D = ~8$ nM); 2) medium affinity binding between midkine and phosphacan bearing a general structure of chondroitin sulfate ($K_D = ~3$ nM); and 3) high affinity binding between midkine and phosphacan bearing a specific structural motif of chondroitin sulfate ($K_D = ~0.5$ nM), which involves a specific contribution of Arg$^{26}$ of midkine (27). An *in vitro* cell migration assay indicated that the third high affinity binding mediated by Arg$^{26}$ is necessary for the full signal transduction of PTP$_{\xi}$ (27). It seems highly possible that the binding of pleiotrophin to phosphacan...
follows a similar hierarchy, because the characteristics of the binding between these molecules are very similar to those of phosphacan and midkine (9, 27), which shares the basic structural motif with pleiotrophin (28). According to this scheme, it is likely that the chondroitin sulfate chains of PG-P7 and PG-P12 correspond to the general structure, and those of PG-P20 correspond to the polysaccharide with a specific structural motif. At present, we do not know what components contribute to the low affinity binding of chondroitinase ABC-treated phosphacan to pleiotrophin. Kurosawa et al. (29) indicate that midkine bound specifically to sulfatide with a $K_D$ of 5.1 nM, suggesting that midkine could bind with sulfated monosaccharides. Unsaturated disaccharides of chondroitin sulfate chains of PG-P7 and PG-P12 had an $a$-sulfated glucuronic acid (16, 30, 31) might be involved in the low affinity binding of chondroitin sulfate and are consistent with the view that oversulfated chondroitin sulfate has strong affinity for pleiotrophin.

Recently, Deepa et al. (13) report that squid cartilage CS-E interacted with various heparin binding growth factors including pleiotrophin and midkine. However, squid CS-E contains a substantial amount of unusual 3-O-sulfated glucuronic acid. Accordingly, Zou et al. (14) prepared artificial CS-E without 3-O-sulfation of glucuronic acid using N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase and demonstrated that this preparation also bound strongly with midkine. These findings suggested that oversulfated portions in chondroitin sulfate contribute to the strong binding with heparin binding growth factors. In this study, we revealed that, in addition to CS-E, shark cartilage CS-D strongly bound with pleiotrophin. Shark cartilage CS-C also showed binding to a pleiotrophin-immobilized sensor chip. CS-C contains substantial amounts of D unit (9.6% for CS-C and 21.2% for CS-D) (32), which could contribute to the binding to pleiotrophin. On the other hand, no binding of whale cartilage chondroitin sulfate A to pleiotrophin was observed by our BIAcore analysis. Our results revealed unexpectedly diverse characteristics of each type of chondroitin sulfate and are consistent with the view that oversulfated chondroitin sulfate has strong affinity for pleiotrophin.

Although chondroitin sulfate chains of PG-P7 and PG-P12 displayed different A unit/C unit ratios (2.0 and 5.9, respectively), their affinities for pleiotrophin were not significantly different from each other ($K_D$ = 0.66 and 0.57 nM, respectively). On the other hand, chondroitin sulfate chains of PG-P20 had an A unit/C unit ratio of 14.3 and contained 1.3% D unit. From the
observations that oversulfated chondroitin sulfate had a strong affinity for pleiotrophin and whale cartilage CS-A did not bind to pleiotrophin, we suggest that D unit in the chondroitin sulfate chains of PG-P20 contributes to the high affinity binding of this proteoglycan to pleiotrophin. Clement et al. (17) report that chondroitin sulfate chains of DSD-1-PG contained 5% D unit (17). DSD-1-PG corresponds to the subpopulation of phosphacan changes dynamically during development (4, 12, 17, 35). In this study, we demonstrated that the structure of chondroitin sulfate affects the interaction between phosphacan and pleiotrophin. This suggests that the signal transduction system of the brain concomitant with the changes in the affinity for pleiotrophin is one of the molecular sensors that distinguishes differences in the chondroitin sulfate structure. Further studies are required to characterize the nature of this sensor to elucidate the molecular mechanism involved in neurite extension.

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