The Binding of Chondroitin Sulfate to Pleiotrophin/Heparin-binding Growth-associated Molecule Is Regulated by Chain Length and Oversulfated Structures*  

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Pleiotrophin is an 18-kDa heparin-binding growth factor, which uses chondroitin sulfate (CS) proteoglycan, PTPζ as a receptor. It has been suggested that the D-type structure (GlcA(2S)β1–3GalNAc(6S)) in CS contributes to the high affinity binding between PTPζ and pleiotrophin. Here, we analyzed the interaction of shark cartilage CS-D with pleiotrophin using a surface plasmon resonance biosensor to reveal the importance of D-type structure. CS-D was partially digested with chondroitinase ABC, and fractionated using a Superdex 75pg column. The ≥18-mer CS fractions showed significant binding to pleiotrophin, and the longer fractions had stronger affinity for pleiotrophin than the shorter ones. The ~46-mer CS fraction bound to densely immobilized pleiotrophin with high affinity (KD = ~30 nM), and the binding reactions fitted the bivalent analyte model. However, when the density of the immobilized pleiotrophin was lowered, the strength of affinity remarkably decreased (KD = ~2.5 μM), and the reactions no longer fitted the model and were considered to be monovalent binding. The 20–24-mer fractions showed low affinity binding to densely immobilized pleiotrophin (KD = 3–20 μM), which seemed to be monovalent. When ~22-mer CS oligosaccharides were fractionated by strong anion exchange HPLC, each fraction differed in affinity for pleiotrophin (KD = 0.36 ~ >10 μM), and the affinity correlated with the amount of D- and E-type (GlcAβ1–3GalNAc(4S,6S)) type oversulfated structures. These results suggest that the binding of pleiotrophin to CS is regulated by multivalency with CS ~20 mer as a unit and by the amounts of oversulfated structures.

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2 The abbreviations used are: HB-GAM, heparin-binding growth-associated molecule; HARP, heparin affin regulatory peptide (HARP), is an 18-kDa growth factor that shows 45% amino acid sequence identity with midkine (1, 2); Pleiotrophin and midkine share many biological activities such as the promotion of neurite outgrowth and migration of embryonic neurons and osteoblasts (1, 2). Furthermore, it has been revealed that both use a common signal-transducing receptor, receptor-type protein-tyrosine phosphatase ζ (PTPζ) (3–7). PTPζ is synthesized as a membrane-bound chondroitin sulfate (CS) proteoglycan, and its extracellular variant, which is generated by alternative splicing, is secreted as a major soluble CS proteoglycan in the brain, phosphacan (8, 9).

The binding of phosphacan to pleiotrophin and midkine depends on the CS portion of this proteoglycan, and the removal of CS resulted in a remarkable decrease in the binding affinity (9, 10). It was revealed that pleiotrophin inactivated the tyrosine phosphatase activity of this receptor leading to an increase in the tyrosine phosphorylation levels of specific substrates such as cat-1 and β-catenin (4, 11). Several researchers suggested that pleiotrophin induces the dimerization of PTPζ, which results in the inactivation of its enzymatic activity (4, 11).

On the other hand, midkine and pleiotrophin easily formed noncovalently bound multimers, and it has been suggested that multimers larger than dimers are the active forms (12–15). Furthermore, both growth factors were cross-linked by transglutaminases forming covalently bound multimers (12–15), and this multimerization process was remarkably promoted by heparin and CS (13, 15). These observations raise the possibility that multimers of pleiotrophin and midkine bind with PTPζ through CS inducing the dimerization of this receptor.

Previously, we demonstrated that the structure of CS on phosphacan changed during development of rat brain, and even at the same developmental stage, the CS structure varied depending on the region of the brain (16). An analysis using a surface plasmon resonance biosensor indicated that phosphacan bearing the CS with D unit (GlcAβ1–3GalNAc(6S)) had higher affinity for pleiotrophin than that without this structure (16). The binding of phosphacan with pleiotrophin and midkine was inhibited by various CS preparations (9, 10). The binding was inhibited strongly by squid cartilage CS-E and shark cartilage CS-D, moderately by shark cartilage CS-C and very weakly by whale cartilage CS-A (9, 10). CS-E and CS-D contain large amounts of E-type (GlcA(2S)β1–3GalNAc(6S)) type oversulfated structures, respectively. C unit (GlcAβ1–3GalNAc(6S)) is the major component of CS-C, which additionally contains moderate amounts of D unit. On the other hand, the amounts of the oversulfated structures are very small in CS-A, in which A unit (GlcAβ1–3GalNAc(4S)) is the major component. These findings suggested that D- and E-type oversulfated structures in CS chains play important roles in the determination of the affinity of pleiotrophin and midkine for PTPζ.

However, the contribution of the D unit in CS to the binding with these growth factors is yet to be clearly demonstrated. Some researchers reported that CS-E but not CS-D strongly bound with midkine and suggested that the E-type structure was the critical determinant of the binding affinity for midkine (17–19). Other studies, however, indicated that CS-E strongly bound with pleiotrophin, but CS-D also bound to
this growth factor with high affinity (16, 20, 21). In the present study, we analyzed the interaction of CS-D with pleiotrophin to reveal the structural determinants involved in the pleiotrophin-CS interaction. We suggest that three parameters: chain length, amounts of oversulfated structures, and pleiotrophin multimerization, play important roles in determination of the binding affinity between CS and pleiotrophin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant pleiotrophin and anti-human pleiotrophin antibody were purchased from R&D systems (Minneapolis, MN). CS-D from shark cartilage was provided by Dr. Nobuo Sugii (Seikagaku Corp., Central Research Laboratories, Tokyo, Japan). Chondroitinase ABC, protease-free chondroitinase ABC and an unsaturated chondro-disaccharide kit were purchased from Seikagaku Corp. (Tokyo, Japan). A YMC pack PA-03 column was obtained from YMC Co. (Kyoto, Japan). Bio-Gel P-10 was purchased from Bio-Rad. A Spherosph S5 SAX column was obtained from Waters Corp. (Milford, MA). Horseradish peroxidase-conjugated anti-rabbit IgG, an ECL Plus Western blotting detection system, a HiLoad 16/60 Superdex 75pg column, and a HiTrap desalting column were purchased from Amersham Biosciences. A research grade sensor chip CM5 and an amine coupling kit containing N-hydroxysuccinimide, N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide hydrochloride, and 1 M ethanolamine hydrochloride were obtained from Biacore AB (Uppsala, Sweden). Maxisorp immunoplates were purchased from Nunc (Rockester, NY). The biorecognizing system used for surface plasmon resonance measurements consisted of a BIAcore 1000 and BIAevaluation software 4.1 (Biacore AB). Disuccinimidyl suberate (DSS) was obtained from Pierce. Phosphacan/6B4 proteoglycan was purified from 20 rat whole brain as described previously (22).

**Preparation and Fractionation of CS Oligosaccharides**—First, 120 mg of shark cartilage CS-D (O unit: GlcAβ1–3GalNAc): A unit:C unit:D unit:E unit = 1.5:25:9.5:4:18:8:3.4; average molecular mass ~20 kDa) was dissolved in 1.5 ml of 30 mM sodium acetate, 0.1 M Tris-HCl, pH 7.8. The solution was incubated with 30 milliunits of protease-free chondroitinase ABC at 30 °C until the absorbance at 232 nm of the sample reached 8.5% of the value obtained after complete digestion. The sample was heated at 100 °C for 5 min and then applied to a column of Bio-Gel P-10 (1.6 × 90 cm) equilibrated with 0.4 M ammonium acetate. The fractions corresponding to Kav values of 0.16–0.61 and 0.45–0.77 were pooled. After lyophilization, the samples were applied to a Superdex 75pg column (1.6 × 60 cm) and eluted with 0.2 M NH4HCO3 at a flow rate of 1 ml/min. The fractions were lyophilized and then dissolved in distilled water. The 22-mer fraction was further chromatographed using a Spherosph S5 SAX column (4.6 × 250 mm) with a linear gradient of NaH2PO4 from 0.8 M to 1.35 M at pH 3.5 at a flow rate of 0.5 ml/min. The eluates were monitored by absorbance at 232 nm. Chain lengths of CS (up to 24 mer) were determined using a Voyager DE-STR MALDI-TOF MS spectrometer. The chain lengths of CS larger than 24 mer were estimated from the Kav value of each fraction on the Superdex 75pg column with a standard curve generated using 6–20 mer of CS oligosaccharides.

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**Materials**—Phosphacan/6B4 proteoglycan was immobilized on the surface of a CM5 sensor chip by amine coupling, where the primary amino groups on the protein were coupled to the carboxymethylated dextran on a sensor chip surface. The carboxymethylated dextran on the sensor chip was activated by injection of 35 μl of N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide hydrochloride-N-hydroxysuccinimide (0.2/0.05 M). Then, 20 μg/ml of 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 M ethanolamine, pH 8.5. The amounts of pleiotrophin immobilized on the sensor surface were controlled within the range of 2,900 and 6,300 resonance units (RU) by changing the injection volume. All steps were carried out in a continuous flow of a solution containing 10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% Tween 20 (HBS running buffer) at 5 μl/min.

**Binding of CS Oligosaccharides to Pleiotrophin**—Previously, we demonstrated that shark cartilage CS-D bound to pleiotrophin with high affinity using the BIAcore system (16). Many of the chondroitin sulfate-dependent activities of pleiotrophin such as promotion of neurite exten-
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sion and migration of embryonic neurons are mediated by substrate-bound form of this growth factor (1–3). Thus we immobilized pleiotrophin on the CM5 sensor chips by the amine coupling method because it seems that this closely models the active form of pleiotrophin. However, there was a possibility that this coupling method could destroy lysine residues that are essential for binding with CS. So we firstly immobilized pleiotrophin preincubated with excess amounts of CS oligosaccharides to protect CS binding basic amino acids. However, it became apparent that the sensograms of the binding of various CS preparations to pleiotrophin were essentially the same, whether or not pleiotrophin was preincubated with CS oligosaccharides (data not shown). Thus in the following experiments, pleiotrophin was coupled to the sensor chips without preincubation with CS oligosaccharides.

To characterize the CS structure required for binding to pleiotrophin, we fractionated CS-D partially digested with protease-free chondroitinase ABC by Superdex 75 gel permeation chromatography. Each fraction (10 μg/ml as hexuronate) was applied to a CM5 sensor chip containing immobilized pleiotrophin (Fig. 1). In this assay, a saturated amount of pleiotrophin was immobilized on the sensor chip (5,942 RU) to maximize the sensitivity. As shown in Fig. 1A, CS fractions bound to pleiotrophin, but the binding decreased sharply as the chain length shortened. Although the 18-mer fraction showed slight binding to pleiotrophin, we could not detect any binding for ≤16-mer oligosaccharides in this assay system (Fig. 1B).

In a previous study, we demonstrated that the binding of phosphacan to pleiotrophin was strongly inhibited by CS-D (10). Using a solid phase binding assay, we next examined the inhibitory effects of CS oligosaccharides on the binding of phosphacan to pleiotrophin. The binding was inhibited efficiently by ≥20-mer oligosaccharides, moderately by 16-mer and 18-mer oligosaccharides, and not by 14-mer oligosaccharides (Fig. 2). These findings suggest that 16–18 mer was the basic functional unit required for the binding to pleiotrophin.

Multivalent Binding of CS to Pleiotrophin—The above experiments indicated that longer CS bound more efficiently to pleiotrophin than shorter ones. This suggests that the affinity of CS for pleiotrophin was increased by multivalent binding of long polysaccharides to multiple pleiotrophin immobilized on the sensor chip. To test this possibility, the ~46-mer CS fraction (F-a, Fig. 1A) was applied to sensor chips immobilized with various densities of pleiotrophin. Three sensor chips were prepared, on which low (2,461 RU; low density condition), medium (2,905 RU; medium density condition), and high (4,753 RU; high density condition) amounts of pleiotrophin were immobilized. During the immobilization process, the amounts of immobilized pleiotrophin increased rapidly to ~4,000 RU (~4 ng/mm²). Then, the reaction became very slow with a maximum immobilization of ~6,000 RU (~6 ng/mm²) (data not shown). This suggests that pleiotrophin was immobilized sparsely on the sensor chips under the low and medium density conditions, and the density of immobilized pleiotrophin was semisaturated under the high density condition.

Fig. 3 shows the binding of fraction F-a to the sensor chips immobilized with three different densities of pleiotrophin. Under the high density condition (Fig. 3A), CS associated relatively slowly with pleiotro-
phin, and the dissociation also proceeded gradually. In contrast, under the low density condition (Fig. 3E), fraction F-a associated with pleiotrophin very quickly and also dissociated quickly, showing “box-shaped" sensorgrams. The sensorgrams were intermediate between the two under the medium density condition (Fig. 3C).

We analyzed these sensorgrams using various binding models in BIAevaluation software 4.1. Although the 1:1 (Langmuir) binding model is most commonly used, these sensorgrams did not fit this model well. On the other hand, the sensorgrams shown in A and C but not E fit the bivalent analyte model. The samples were injected at 120 s, and the dissociation phase began at 430 s. The sensorgrams of A, C, and E were analyzed using Scatchard plots in B, D, and F, respectively.

![Figure 3](image.png)

**TABLE 1**

| PTN | Scatchard analysis, $K_D$ | Bivalent analyte model |
|-----|-------------------|----------------------|
|     | $k_{a1}$ | $k_{d1}$ | $K_D1$ | $k_{a2}$ | $k_{d2}$ |
| 4753 RU | $0.062 \pm 0.008$ | $(1.4 \pm 0.6) \times 10^7$ | $0.07 \pm 0.02$ | $0.5 \pm 0.3$ | $(2.1 \pm 0.5) \times 10^{-5}$ | $(10 \pm 1) \times 10^{-4}$ |
| 2905 RU | $0.4 \pm 0.1$ | $(4.1 \pm 2.0) \times 10^7$ | $0.09 \pm 0.02$ | $2.2 \pm 1.5$ | $(4 \pm 3) \times 10^{-5}$ | $(6 \pm 2) \times 10^{-4}$ |
| 2461 RU | $2.5 \pm 0.8$ | $-\cdots$ | $-\cdots$ | $-\cdots$ | $-\cdots$ | $-\cdots$ |

*The values could not be determined because the sensorgrams did not fit the bivalent analyte model.*

The affinity of CS for pleiotrophin depends on the density of pleiotrophin immobilized on the sensor chip. The CM5 sensor chips were immobilized with 4,753 RU (A), 2,905 RU (C), and 2,461 RU (E) of pleiotrophin, and various concentrations of fraction F-a (~46 mer) were applied to the sensor chips. The responses were recorded as a function of time (gray shaded lines). The sensorgrams were fit to the bivalent analyte model, which is depicted as solid lines (A and C). The sensorgrams shown in A and C but not E fit the bivalent analyte model. The samples were injected at 120 s, and the dissociation phase began at 430 s. The sensorgrams of A, C, and E were analyzed using Scatchard plots in B, D, and F, respectively.

Although $K_D$ represents the strength of the interaction between CS and one pleiotrophin unit, we cannot know from this value the overall strength of the binding between these molecules in a bivalent reaction. However, using a steady state analysis (Scatchard plot), we can reveal overall binding strength between CS and the immobilized pleiotrophin ($K_{p2}$ avidity), in which the sum of the binding strength of reaction 1 and reaction 2 is calculated. So, the sensorgrams were then analyzed by plotting steady state binding responses against concentrations of CS (Fig. 3B, D, F). As shown in Table 1, the $K_{p2}$ values for the interaction under the high, medium, and low density conditions were calculated to...
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be 62 nM, 0.4 μM, and 2.5 μM, respectively. The $K_D$ values for the interactions under high and medium density conditions were 6–8-fold smaller than the $K_{D1}$ values, suggesting that bivalent binding of CS to two pleiotrophin units led to the stronger interaction between these molecules. The sensorgrams of the low density condition could not be analyzed with the bivalent model, and only the results of the Scatchard analysis were available (Fig. 3F).

Chain Length-dependent Binding of CS to Pleiotrophin—The CS oligosaccharides of various chain lengths were applied to sensor chips immobilized with 5,942 RU of pleiotrophin. In this experiment, a saturated level of pleiotrophin was immobilized on the sensor chips to improve sensitivity for the binding of short oligosaccharides. The sensorgrams of the long CS (Fig. 4) showed box-shaped sensorgrams of the low density condition could not be analyzed with the bivalent model, suggesting that these short CS oligosaccharides interacted with only one pleiotrophin unit.

Next, we cross-linked pleiotrophin with DSS in the presence of ~34-mer (F-a), ~24-mer (F-g), and 10-mer fractions (Fig. 5). In the presence of cross-linker alone, almost no dimers were observed (Fig. 5g). Also in the presence of the 10-mer fraction, almost no dimers were observed (Fig. 5, e and f). In contrast, the F-g fraction induced dimer formation of pleiotrophin (Fig. 5, c and d), and in the presence of the F-a fraction, trimers and tetramers were additionally observed (Fig. 5, a and b). These results indicated that ~46- and ~24-mer fractions accommodate four and two pleiotrophin molecules, respectively and further suggested that pleiotrophin dimer was the basic functional unit.

Oversulfated Structures in CS Regulate the Affinity for Pleiotrophin—To examine the significance of structural variations of CS in pleiotrophin-CS interaction, we fractionated F-h oligosaccharides (~22 mer) with a Spherisorb SS SAX column (Fig. 6). The separated fractions (F-h-1–11) were applied to a sensor chip immobilized with 6,300 RU of pleiotrophin, and the steady state binding of CS oligosaccharides was measured (Fig. 7). Whereas F-h-1–4 showed very weak binding to pleiotrophin, the binding responses increased from F-h-5 to F-h-11. Because these fractions were all 20–24-mer oligosaccharides (Table 3), it was concluded that the differences in the binding response were caused by the structural variation of CS. F-h-1–6 contained 10–21% D unit with almost no E unit (Table 3). On the other hand, F-h-7–11 contained 1–6% D unit with 22–25% E unit. Several reports suggested that D- and E-type structures are the major determinants of the affinity of CS for pleiotrophin (16–19). So, we plotted amounts of these structures in Fig. 7, revealing that the affinity of CS oligosaccharides for pleiotrophin correlated well with the contents of these oversulfated structures. It should be noted that F-h-10 and F-h-11 showed markedly enhanced binding to pleiotrophin compared with F-h-8 and F-h-9 (Fig. 7). Although these fractions contained comparable amounts of D unit, F-h-10 and F-h-11 contained higher amounts of E unit than F-h-8 and F-h-9. Bao et al. (21) reported that CS oligosaccharides containing E unit showed stronger affinity for pleiotrophin than CS oligosaccharides containing D unit but without E unit. Thus, the differences in the binding efficiency of F-h-8–11 might be explained by the difference in the

![FIGURE 4. The affinity of CS for pleiotrophin depends on the chain length. The CM5 sensor chip was immobilized with 5,942 RU of pleiotrophin, and various concentrations of fractions F-a (A, ~46 mer), F-c (B, ~34 mer), and F-e (C, ~28 mer) were applied to the sensor chip. The samples were injected at 75 s, and the dissociation phase began at 375 s.](image)

**TABLE 2**

| Fraction  | Scatchard analysis, $K_D$ | Bivalent analyte model | $K_{D1}$ | $K_{D2}$ | $K_{D1}$ | $K_{D2}$ | $K_{D1}$ | $K_{D2}$ |
|-----------|---------------------------|------------------------|---------|---------|---------|---------|---------|---------|
|           |                           |                        | $k_{a1}$ | $k_{a2}$ | $k_{a1}$ | $k_{a2}$ | $k_{a1}$ | $k_{a2}$ |
| F-a (46 mer) | 0.030 ± 0.005             | 1/100                 | 0.06 ± 0.02 | 0.4 ± 0.2 | $30 ± 3$ | $10 ± 1$ |
| F-b (36 mer) | 0.10 ± 0.05               | 1/10                  | 0.06 ± 0.02 | 0.9 ± 0.4 | $11 ± 3$ | $6 ± 1$  |
| F-c (34 mer) | 0.17 ± 0.03               | 1/0.1                 | 0.08 ± 0.04 | 1.1 ± 0.6 | $6.9 ± 0.5$ | $5 ± 1$  |
| F-d (32 mer) | 0.35 ± 0.07               |                        |           |         |         |         |         |         |
| F-e (28 mer) | 0.55 ± 0.08               |                        |           |         |         |         |         |         |
| F-f (26 mer) | 2.1 ± 0.1                 |                        |           |         |         |         |         |         |
| F-g (24 mer) | 3.2 ± 0.6                 |                        |           |         |         |         |         |         |
| F-i (20 mer) | 21 ± 8                    |                        |           |         |         |         |         |         |

*a* The values could not be determined because the sensorgrams did not fit the bivalent analyte model.
amounts of E unit. However, there also is a possibility that this was caused by the difference in the CS sequences.

The sensorgrams of these oligosaccharides were all box-shaped, and the \( K_d \) values were calculated using a Scatchard analysis (Fig. 8). F-h-6 with 21% D and no E structures showed a \( K_d \) value of 2.7 \( \mu \)M, and F-h-12 with 25% D and 5% E structures had a \( K_d \) value of 0.36 \( \mu \)M (Table 3). These results suggested that the amounts of D- and E-type structures contribute to the determination of the affinity of CS for pleiotrophin.

**DISCUSSION**

In this study, we demonstrated that the interaction between CS and pleiotrophin is regulated by three parameters: 1) chain length, 2) amount of oversulfated structures of CS, and (3) multivalency of the interaction. This suggests that the structural heterogeneity of CS plays important roles in the signal transduction of pleiotrophin.

**Multivalent Interaction of CS with Pleiotrophin**—Pleiotrophin and midkine are composed of two domains: N- and C-terminal halves (1, 2). The C-terminal half of these growth factors contains two clusters of basic amino acids (Clusters I and II), which have been shown to function as heparin binding sites for midkine (1, 14). The basic amino acid residues in Clusters I and II are conserved or type-conserved in pleiotrophin and midkine except for one arginine residue in Cluster II (14). We observed no difference between the bindings of pleiotrophin and midkine to phosphacan probably because of such common structural features (9, 10).

Based on an NMR analysis of midkine in solution, Iwasaki et al. (14) suggested that midkine forms a head-to-head dimer in the presence of heparin oligosaccharides. In this dimeric structure, two Cluster IIs from each midkine fused to form a heparin binding site at the dimer interface, and the Cluster Is work as separate heparin binding sites on the distal sides of the dimer (Fig. 9B). These heparin binding sites of the midkine dimer were considered to fit the three sulfate group clusters formed in heparin 20 mer (14). Furthermore, Kaneda et al. (23) indicated that heparin 22 mer but not 12 mer inhibited midkine-induced neurite outgrowth. These observations suggest that the midkine dimer is the basic functional unit, which interacts with heparin 20 mer. Previously, we indicated that a mutation at Arg78 in Cluster I of midkine resulted in the loss of CS-dependent high affinity binding to phosphacan, suggesting that heparin binding sites in midkine serve as CS binding sites (10).

On the other hand, the heparin binding site of pleiotrophin was not as clearly identified as that of midkine. Despite the highly conserved structure of pleiotrophin and midkine, it has been reported that both N-terminal and C-terminal halves of HB-GAM (pleiotrophin) strongly bound with heparin (24). Whereas Cluster II of midkine contains 3 basic amino acid residues, that of HB-GAM contains only 2 basic amino acids (14). This structural difference might result in the different heparin binding mechanism between these molecules. Although the heparin binding sites of pleiotrophin and midkine might be somewhat different, the HB-GAM monomer was considered to bind with the heparin decasaccharide like midkine (25, 26). Quite recently, Bao et al. (21) demonstrated that CS octasaccharides containing D unit weakly bound to a pleiotrophin-conjugated affinity column, suggesting that pleiotrophin monomer accommodate CS shorter than 8 mer. Based on these observations, we assume that the pleiotrophin monomer has two heparin binding sites, which also serve as CS binding sites (Fig. 9B).

Fig. 1 indicated that CS oligosaccharides longer than 18 mer bound significantly to pleiotrophin immobilized on the sensorchips. We could not detect the binding of CS oligosaccharides shorter than 16 mer, although CS 8–10 mer could bind to pleiotrophin-conjugated affinity column (21). Because affinity chromatography can separate very weak affinity substances, it seems that the affinities of CS oligosaccharides shorter than 16 mer were too weak to be detected using BIAcore system.
The 46-mer fraction showed high affinity binding to pleiotrophin (30 nM) under the same condition (Table 2), in which a saturated amount inhibited by KD
Interaction between pleiotrophin and CS 22-mer fractions with various contents of D and E units

TABLE 3

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The KD values were calculated from the sensorgrams using six or more concentrations of analyte (CS) in two or three independent experiments.

| Fraction | Composition | Chain length | KD (µM) |
|----------|-------------|--------------|---------|
| F-h-1    | ΔDi-4S 14.5  | 22 mer       | 2.2     |
| F-h-2    | ΔDi-6S 75.8  | 22 mer       | 2.2     |
| F-h-3    | ΔDi-diS 9.7  | 22 mer       | 2.2     |
| F-h-4    | ND          | 20 mer       | 2.2     |
| F-h-5    | 21.6        | 20, 22, 24 mer| 2.2     |
| F-h-6    | 20.5        | 20, 22, 24 mer| 2.2     |
| F-h-7    | 20.5        | 20, 22, 24 mer| 2.2     |
| F-h-8    | 26.4        | 20, 22, 24 mer| 2.2     |
| F-h-9    | 29.2        | 20, 22, 24 mer| 2.2     |
| F-h-10   | 30.6        | 20, 22, 24 mer| 2.2     |
| F-h-11   | 31.0        | 20, 22, 24 mer| 2.2     |
| F-h-12   | 31.7        | 20, 22, 24 mer| 2.2     |

a ND, not detected.

b The values could not be determined because of the low affinity of these CS fractions.

On the other hand, the binding of phosphacan to pleiotrophin was inhibited by ≥16-mer CS oligosaccharides (Fig. 2), suggesting that the CS 16–18 mer was the basic functional unit, which could presumably interact with the pleiotrophin dimer. Because pleiotrophin was immobilized on the sensor chip through a flexible 100-nm carboxymethylated dextran, it would move fairly freely, and the pleiotrophin molecules would be able to associate with each other. Thus, it was expected that CS could interact with pleiotrophin multimers on the sensor surfaces. Although the 18-mer fraction showed significant binding to pleiotrophin, its binding affinity was very low (KD > 20 µM). On the other hand, the 46-mer fraction showed high affinity binding to pleiotrophin (KD = 30 nM) under the same condition (Table 2), in which a saturated amount of pleiotrophin was immobilized on the sensor chips (~6,000 RU). Whereas the sensorgrams of the interaction of pleiotrophin with 34–46-mer CS polysaccharides fitted the bivalent analyte model well, CS fractions shorter than 28 mer did not fit this model (Fig. 4 and Table 2). This suggested that ≥34-mer CS polysaccharides could bind with two pleiotrophin units.

When a semisaturated amount of pleiotrophin was immobilized on the sensor chips (4,753 RU; high density condition), the 46-mer fraction showed high affinity binding to pleiotrophin (KD = 62 nM) (Fig. 3). The affinity of the 46-mer fraction for pleiotrophin remarkably decreased (KD = 2.5 µM), when the density of immobilized pleiotrophin was lowered (2,461 RU; low density condition). When a moderate amount of pleiotrophin was immobilized on the sensor chip (2,905 RU; medium density condition), the affinity was also intermediate between the two conditions (KD = 0.4 µM). Analysis of the sensorgrams shown in Fig. 3A using the bivalent analyte model indicated that KD was 0.5 µM, the value of which was ~8-fold larger than that of KD (Table 1). Based on the observation that CS 46 mer accommodated 4 pleiotrophin molecules (Fig. 5), we suggest that the 46-mer CS fraction bound first to the pleiotrophin dimer with a KD of 0.5 µM, and then to a second pleiotrophin dimer, resulting in a stronger overall binding (KD = 62 nM) (Fig. 9B, a)). Under the medium density condition, the KD and KD values were calculated to be 0.4 and 2.2 µM, respectively (Table 1). This KD value was close to the KD value in the high density condition, suggesting that CS interacted with two pleiotrophin molecules under the medium density condition (Fig. 9B, b)). On the other hand, the sensorgrams under the low density condition did not fit the bivalent analyte model, suggesting that CS bound with one molecule of pleiotrophin. The KD value was calculated to be 2.5 µM, which was close to the KD value under the medium density condition (KD = 2.2 µM). These results suggested that the KD values for the interaction of the 46-mer CS fraction with the monomer, dimer, and tetramer were ~2,500, ~500, and ~60 nM, respectively (Fig. 9B). However, we cannot exclude the possibility that the reaction 1 under the medium density condition (KD = 2.2 µM) and the binding under the low density condition (KD = 2.5 µM) were mediated by pleiotrophin dimers.

Importance of Oversulfated Structures in the Interaction of CS with Pleiotrophin—When the 22-mer CS fraction was subjected to strong anion exchange HPLC, CS oligosaccharides were separated depending on the amounts of D- and E-type structures (Table 3). Fractions F-h-1 and -2, which were estimated to contain only one D unit per chain, showed no binding to pleiotrophin. Fractions F-h-3 and -4, which were considered to be composed of a mixture of CS oligosaccharides containing 1 or 2 D units per chain, slightly bound to pleiotrophin, but the affinity was very low. On the other hand, F-h-6, which was estimated to
of shark cartilage CS-D was characterized by a high frequency of A-D-containing sequences such as A-D-A, A-D-C, and D-A-D-A, and no D-D tetrasaccharide sequence was found (27, 28). These A-D-containing regions seemed to be separated by sequences without a D unit such as C-C, C-A, C-C-C, C-C-A, C-C-C-C, and C-C-C-A (27, 28). Combinations of these sequences would generate CS 22 mer containing 1, 2, or 3 D units. Because of this structural limitation, CS-D would not contain sequences with densely clustered D units. On the other hand, E units could be highly clustered in the CS sequences. Zhou et al. (18) demonstrated that an artificial CS-E structure, of which up to 95% was E units, could be formed in various CS preparations using squid or human N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase. The affinity of these artificial CS-E for midkine correlated with the amounts of E unit, and the CS-E with a dense E unit cluster bound quite strongly with midkine (18). The clustered E units in CS-E might interact with each of Cluster Is and IIs on the midkine dimer, leading to strong interaction. Such different features of D- and E-type structures might play important roles in the determination of the binding affinity of CS for pleiotrophin and midkine, leading to the different functions of D unit- and E unit-rich CS.

Hikino et al. (29) demonstrated that shark cartilage CS-D and squid cartilage CS-E exerted distinct effects on cultured embryonic hippocampal neurons. Whereas CS-D promoted the outgrowth of short dendrite-like neurites, CS-E stimulated the outgrowth of long axon-like processes (29). Using various CS/dermatan sulfate (DS) preparations, they found that D- and/or iD- (IdoA(2→3)GalNAc(6S)) type structures were involved in the promotion of dendrite-like processes. On the other hand, E and/or iE (IdoAα1→3GalNAc(4S,6S))-type structures were suggested to play roles in the promotion of the outgrowth of axon-like neurites (29). Bao et al. (20) also reported that CS/DS hybrid chains purified from embryonic pig brain efficiently bound with pleiotrophin. These CS/DS hybrid chains were separated on a pleiotrophin-conjugated column into unbound, low affinity, and high affinity fractions. The latter two fractions promoted the outgrowth of dendrite- and axon-like neurites by cultured embryonic hippocampal neurons, respectively (20). Pleiotrophin expressed by glial cells mediated the dendrite-promoting activity of the low affinity CS/DS fraction, but this growth factor was not involved in the axon-promoting activity of the high affinity fraction (20). These low and high affinity CS/DS fractions showed high contents of D/iD and E/iE units, but the latter fraction was more heavily sulfated and contained a larger amount of the E-type structure than the former one (20). The low affinity binding of D unit-rich CS with pleiotrophin might play important roles in the formation of dendrites.

**CS and PTPζ Signaling**—Midkine is multimerized by tissue transglutaminase, and this process is highly promoted by glycosaminoglycans such as heparin (13). Pleiotrophin is also easily multimerized by this enzyme under similar conditions. Kojima et al. (13) indicated that multimerization of midkine is required for its enhancing effects on plasminogen activator activity in bovine aortic endothelial cells. These observations suggest that pleiotrophin/midkine multimers, but not monomers, activated the receptors. At present, N-syndecan, anaplastic lymphoma kinase (ALK) and PTPζ have been identified as receptors for pleiotrophin/midkine (3, 4, 9, 30, 31). Among them, the signal transduction mechanism of PTPζ has begun to be elucidated, and it has been proposed that tyrosine phosphatase activity of PTPζ is inactivated by pleiotrophin probably through dimerization of this receptor (4, 11). Recently, we demonstrated that PTPζ-pleiotrophin signaling is involved in the morphogenesis of cerebellar Purkinje cells (32). In the postnatal

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3 N. Maeda, unpublished observation.
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cerebellar cortex, PTPζ pleiotrophin and D unit-rich CS were accumulated in the molecular layer, and it has been considered that these molecules cooperatively played roles in the dendrite formation of Purkinje cells (32, 33). Transglutaminase type 2 was also reported to be expressed in the molecular layer (12), suggesting that these components constitute the extracellular signaling complex involved in the development of Purkinje cell dendrites.

In the postnatally developing rodent cerebellum, phosphacan-bearing CS rich in D unit was abundantly expressed (33). This CS proteoglycan might trap pleiotrophin through D unit-rich CS and stimulate the multimerization of pleiotrophin by transglutaminase. The multimerized pleiotrophin might move to CS of PTPζ multimers were present, pleiotrophin would bind CS molecules cooperatively played roles in the dendrite formation of Purkinje cells, leading to the cross-linking of the receptor molecules. The efficiency of this cross-linking process could be highly dependent on the CS structure of PTPζ and pleiotrophin multimerization. If CS of PTPζ contained a cluster of oversulfated disaccharide units and pleiotrophin multimers were present, pleiotrophin would bind CS chains of two PTPζ and stably cross-link the receptor molecules leading to strong signaling. In this case, a higher pleiotrophin multimer should cause more stable cross-linking of PTPζ. If the level of oversulfated disaccharide units was low and/or only a lower pleiotrophin multimer such as a dimer was present, the dimerization of PTPζ, and the signaling might be transient and weak.

We previously indicated that phosphacan with the CS-containing D unit showed ~5-fold stronger affinity for pleiotrophin ($K_D = 0.14$ nM) than phosphacan without D unit (16). This difference in the affinity appeared after chondroitinase ABC treatment of phosphacan, and both preparations showed a similar affinity cross-linking to pleiotrophin. This is consistent with the present results that the affinity of CS for pleiotrophin depends on the D unit content. However, it should be noted that the core protein portion of phosphacan could also contribute to the binding to pleiotrophin (9, 16). Although we do not know the mechanism of the binding between pleiotrophin and phosphacan core protein, the affinity of phosphacan core protein for pleiotrophin was relatively high ($K_D = 1.5$–$13$ nM) (9, 16). This suggests that there is cooperative interaction among the core protein, CS, and pleiotrophin. In this case, a lower pleiotrophin multimer such as a dimer and CS with few D units might be sufficient to cause stable cross-linking of PTPζ.

In this study, we demonstrated that the binding of pleiotrophin with CS was dependent on the chain length and amount of oversulfated structure in CS and on the pleiotrophin multimerization. At present, it is unknown whether specific CS sequences are required for the binding with pleiotrophin. Future study is required to study this problem.

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