Demonstration of the Pleiotrophin-binding Oligosaccharide Sequences Isolated from Chondroitin Sulfate/Dermatan Sulfate Hybrid Chains of Embryonic Pig Brains*

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Mammalian brains contain significant amounts of chondroitin sulfate (CS), dermatan sulfate (DS), and CS/DS hybrid chains. CS/DS chains isolated from embryonic pig brains (E-CS/DS) promote the outgrowth of neurites in embryonic mouse hippocampal neurons in culture by interacting with pleiotrophin (PTN), a heparin-binding growth factor. Here, we analyzed oligosaccharides isolated from E-CS/DS, which showed that octasaccharides were the minimal size capable of interacting with PTN at a physiological salt concentration. Five and eight sequences were purified from fluorescently labeled PTN-bound and -unbound octasaccharide fractions, respectively, by enzymatic digestion followed by PTN-affinity chromatography. Their sequences were determined by enzymatic digestion in conjunction with high performance liquid chromatography, revealing a critical role for oversulfated D and/or iD disaccharides in the low affinity for PTN, which is required for neuritogenesis. The critical D and iD units are GlcUA(2-6)-GalNAc(4, 6-O-sulfate) and IdoUA(2-O-sulfate)α1-3GalNAc(6-O-sulfate), respectively, where IdoUA represents i-iduronic acid. In contrast, high affinity interactions with PTN required decasaccharides with E units (GlcUAβ1–3GalNAc(4, 6-O-sulfate)), B units (GlcUA(2-O-sulfate)β1–3GalNAc(4-O-sulfate)), and/or their IdoUA-containing counterparts (iE and iB) in addition to D/iD units, although the biological significance of such strong interactions remains to be investigated. Thus, chain size and composition are crucial to the interaction with PTN, and PTN binds to multiple sequences in E-CS/DS chains with distinct affinity. Notably, not only heparan sulfate but also CS/DS hybrid chain structures of mammalian brains contain a high degree of microheterogeneity with a cluster of oversulfated disaccharides and appear to play roles in regulating the functions of PTN.

Chondroitin sulfate (CS)§ and dermatan sulfate (DS), a class of glycosaminoglycan (GAG)-type polysaccharides, occur covalently attached to proteoglycan (PG) core proteins (1). Like heparan sulfate (HS), another class of GAGs, CS/DS chains are abundant at cell surfaces and in the extracellular matrices. They regulate cell division, adhesion, and morphogenesis through direct binding to secreted signaling proteins, thereby modulating their activities, or through interactions with extra-cellular matrix molecules (2–6, for reviews, see a Ref. 7). CS and DS typically have backbones consisting of repeating disaccharide units of -(GlcUA-GalNAc- or -IdoUA-GalNAc-), respectively, where IdoUA represents i-iduronic acid. Notably, hybrid chains, composed of both units in varying proportions, also exist (8). These units are modified during chain elongation by specific sulfotransferases at C-2 of GlcUA/IdoUA and/or C-4 and/or C-6 of GalNAc in various combinations, thereby producing characteristic sulfation patterns and enormous structural diversity. These structural characteristics are strictly regulated, as revealed by the compositional analysis of CS/DS chains from various organs and by immunohistochemical staining using antibodies recognizing different CS/DS epitopes.

In the mammalian brain, CS/DS have been implicated in neural development by regulating neuronal adhesion and migration, neurite formation, and axonal guidance etc. (9, 10). However, the reported functions of brain CS/DS in neuritogenesis are controversial. CS/DS chains are generally considered to play inhibitory roles in neurite extension and axonal growth, a contention supported by studies in vivo showing that removal of CS chains by chondroitinase ABC treatments permits axonal regeneration after nigrostriatal tract axotomy and spinal cord injury (11–13). On the other hand, DSD-1-PG, the mouse homologue of rat phosphacan, which carries a functional domain (the so-called DSD-1 epitope) in the CS/DS side chains, promoted the outgrowth of neurites toward embryonic rat hippocampal neurons in culture (14). Further studies have shown that oversulfated CS and DS chains, and hybrid CS/DS chains from various marine organisms exhibit neuritogenic activity (Refs. 15–19, for a review, see Ref. 7). Importantly, we have recently demonstrated that a unique iD (IdoUA(2-O-sulfate)-GalNAc(6-O-sulfate))-containing epitope is spatiotemporally expressed in particular regions of the developing mouse brain (20) and appears to be involved in the formation of neurites in cultured embryonic mouse
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hippocampal neurons. In that study, we used a monoclonal antibody, 2A12, which was raised against DS from ascidian. Thus, it is reasonable to assume that the inhibitory and promoting activity of CS/DS chains is probably defined by specific carbohydrate sequences with characteristic sulfation patterns in a particular microenvironment.

Structural studies have demonstrated that the disaccharide composition and the GlcUA/IdoUA ratio of the brain CS/DS chains change during development (21, 22), and that certain CS/DS epitopes are found only in specific regions of the mammalian brain (23, 24). These findings suggest that subpopulations of CS/DS chains play distinct roles during development. Most interestingly, CS/DS hybrid chains (E-CS/DS) isolated from embryonic pig brain were found to promote the outgrowth of neurites toward embryonic mouse hippocampal neurons in culture (22). Further investigation (25) has demonstrated that a small proportion of E-CS/DS subpopulations bind a heparin-binding growth factor pleiotrophin (PTN). These chains show neuritogenic activity by interacting with endogenously expressed PTN and presenting it to the neuronal cell surface, thereby facilitating the neuritogenic function of this growth factor. Preliminary results (25), regarding the structure-function relationship, have suggested important roles for oversulfated disaccharides such as the D/iD unit (HexUA(2S)-IdoUA(6S)), the E/iE unit (HexUA-GalNAc(4S,6S)) and IdoUA-containing structures in the interaction of E-CS/DS with PTN, where HexUA, 2S, 4S and 6S represent hexuronic acid, and 2-O-, 4-O-, and 6-O-sulfate, respectively.

In the present study, we isolated and characterized a series of PTN-bound and -unbound oligosaccharides from E-CS/DS with novel structures. The results demonstrated that PTN binds with distinct affinity to multiple sequences in E-CS/DS chains, and that both the size and composition of sugar chains are crucial for the interaction. In addition, we show the first direct evidence that CS/DS chains of the mammalian brain are characterized by a high degree of microheterogeneity and highly sulfated domains containing a cluster of oversulfated disaccharides.

EXPERIMENTAL PROCEDURES

Materials—The E-CS/DS chains were purified from a phosphate-buffered saline homogenate of embryonic pig brains as described previously (22, 25). Hyaluronidase SD from Streptococcus dysgalactiae, chondroitinases ABC (conventional and protease-free preparations), AC-I, AC-II, and B, and heparitinase were purchased from Seikagaku Corp. (Tokyo, Japan). Recombinant human PTN for interaction assays was obtained from RELIA Tech GmbH (Braunschweig, Germany). Hexuronate-2-O-sulfatase (abbreviated as Hexuronate-2-sulfatase) was a gift from K. Yoshida, Seikagaku Corp. A PTN-bound affinity column was prepared by coupling 0.5 mg of recombinant human PTN, a gift from K. Yoshida, Seikagaku Corp. (Tokyo, Japan). Recombinant human PTN for interaction assays was obtained from RELIA Tech GmbH (Braunschweig, Germany). Hexuronate-2-O-sulfatase (abbreviated as Hexuronate-2-sulfatase) was a gift from K. Yoshida, Seikagaku Corp. A PTN-bound affinity column was prepared by coupling 0.5 mg of recombinant human PTN, which was produced in yeast, to a HiTrap column (4.6 × 250 mm) was purchased from Amersham Biosciences. An amine-bound silica PA-03 column (4.6 × 250 mm) was purchased from YMC Co. (Kyoto, Japan).

Other chemicals and reagents were of the highest quality available.

Inhibition Assays Using a BIAcore System—The inhibitory activity of sugar chains against the interaction of PTN with immobilized E-CS/DS chains was examined using a BIAcore system (BIAcore AB, Uppsala, Sweden). Biotinylated E-CS/DS chains were immobilized onto the streptavidin-derivatized surface of a sensor chip as described (22). To investigate the effects of treatments with GAG-degrading enzymes on the inhibitory activity of E-CS/DS, equal amounts of E-CS/DS (1.35 μg) were individually incubated with chondroitinase ABC (5 international milliunits), AC-I (2 international milliunits), or B (2 international milliunits), hyaluronidase SD (2.5 international milliunits), or heparitinase (1 international milliunit), in an appropriate buffer at 37 °C for 1 h, and the resulting digest was then mixed with 200 ng of PTN in a volume of 130 μl and co-injected onto the surface of the sensor chip. The same amount of PTN and the intact E-CS/DS was run as a control. Response curves were recorded, and the maximal response of each reaction was used for calculation. Inhibitory activity was expressed as a percentage relative to the response obtained from an injection of PTN (200 ng) without sugar chains.

In the case where oligosaccharides, generated from E-CS/DS, were used as inhibitors, certain amounts of oligosaccharides (8–80 pmol) were mixed with 50 ng of PTN and co-injected into the BIAcore system. The same amount of PTN only was run as a control. The inhibitory efficiency was calculated as a percentage relative to the response obtained from the control.

Fragmentation of E-CS/DS—An exhaustive digestion with chondroitinase B was used to selectively dissect the E-CS/DS chains. The digestion of E-CS/DS (100 μg) was initiated with 50 international milliunits of chondroitinase B in 100 μl Tris-HCl buffer, pH 8.0 (26), in a total volume of 30 μl at 30 °C and run for 4 h. Thereafter the same amount of the enzyme was added, and the incubation continued for 12 h. After boiling at 100 °C for 1 min to stop the reaction, the digest was lyophilized and derivatized with a fluorophore 2-amino benzamide (2AB), and then the excess 2AB was removed by paper chromatography (27). The 2AB derivatives were subjected to gel filtration on a column (10 × 300 mm) of Superdex Peptide using 0.2 M NH₄HCO₃ as an eluant at a flow rate of 0.3 ml/min (22). Each resolved fraction as indicated in Fig. 2A was collected, rechromatographed under the same conditions, and desalted by repeated lyophilization. Size determination of the compounds in each fraction was achieved by comparison of the elution positions with those of size-defined oligosaccharide standards derived from CS-C or CS-D and by a mass spectrometric (MS) analysis as described below.

Fractionation of E-CS/DS Oligosaccharides on a PTN Affinity Column—The PTN affinity column (1 ml) contained 0.35 mg of the protein. Before sample application, the column was washed with 3 ml of 10 mM Tris-HCl buffer, pH 7.4 (Buffer A), containing 2.0 mM NaCl, and then equilibrated with 5 ml of 0.15 M NaCl-containing Buffer A as above. 2AB-derivatized oligosaccharide fractions were each dissolved in 250 μl of 0.15 M NaCl-containing Buffer A and applied to the PTN column. To maximize the absorbance, loading was repeated six times by recycling each unbound fraction. Oligosaccharides (typically 50 pmol) were subjected to the affinity chromatography, and the column was washed stepwise with 3 ml of Buffer A containing 0.15, 0.2, 0.3, 0.4, 0.5, 0.7, or 2.0 mM NaCl for analytical runs. For preparative purposes, up to 2500 pmol of oligosaccharides were applied to the affinity column, which was then washed with Buffer A containing 0.15 mM NaCl and 0.7 mM NaCl successively. The 0.15 mM NaCl-eluted fraction was again subjected to affinity fractionation under the same conditions as described above. The 0.15 mM NaCl-eluted subfraction obtained from the second fractionation was considered the unbound subfraction, whereas the combined 0.7 mM NaCl-eluted subfraction was considered the bound subfraction of the parent fraction.

For desalting and quantification, subfractions were subjected to gel filtration on a Superdex Peptide column as described above. Calculation of the relative abundance among subfractions of a certain fraction was achieved by comparing the overall fluorescence intensity of the oligosaccharide peak(s) detected in each subfraction. A niton-exchange Chromatography—Separation of the PTN-unbound (400 pmol) and -bound (230 pmol) oligosaccharides in the chondroitin-

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nase B-resistant octasaccharide fraction, and analysis of the enzymatic digests with bacterial chondroitinases were carried out by anion-exchange high performance liquid chromatography (HPLC) with an amine-bound silica PA-03 column. The identification and quantification of the unsaturated di- and tetrasaccharides generated by chondroitinases were achieved by making comparisons with the authentic unsaturated di- and tetrasaccharides from CS and DS chains: \( \Delta \text{HexUA} \alpha 1-3 \text{GalNAc} (\Delta \text{Di-DS or } \Delta \text{O unit}), \Delta \text{HexUA} \alpha 1-3 \text{GalNAc} (6S) (\Delta \text{Di-6S or } \Delta \text{C unit}), \Delta \text{HexUA} \alpha 1-3 \text{GalNAc} (4S) (\Delta \text{Di-4S or } \Delta \text{A unit}), \Delta \text{HexUA} (2S) \alpha 1-3 \text{GalNAc} (6S) (\Delta \text{Di-di} \text{S}_6 \text{or } \Delta \text{D unit}), \Delta \text{HexUA} (2S) \alpha 1-3 \text{GalNAc} (4S) (\Delta \text{Di-di} \text{S}_4 \text{or } \Delta \text{B unit}), \Delta \text{HexUA} \alpha 1-3 \text{GalNAc} (4S,6S) (\Delta \text{Di-di} \text{S}_6 \text{or } \Delta \text{E unit}), \Delta \text{HexUA} (2S) \alpha 1-3 \text{GalNAc} (4S,6S) (\Delta \text{Di-Tr} \text{S} \text{or } \Delta \text{T unit}) \) (28), \( \Delta \text{HexUA} \alpha 1-3 \text{GalNAc} (6S) \beta 1-4 \text{GlcUA} \beta 1-3 \text{GalNAc} (6S) (\Delta \text{C-C}) \) (29), \( \Delta \text{HexUA} \alpha 1-3 \text{GalNAc} (6S) \beta 1-4 \text{GlcUA} \beta 1-3 \text{GalNAc} (4S) (\Delta \text{C-A}) \) (29), \( \Delta \text{HexUA} \alpha 1-3 \text{GalNAc} (4S) \beta 1-4 \text{GlcUA} \beta 1-3 \text{GalNAc} (6S) (\Delta \text{A-C}) \) from a chondroitinase ABC digest of 2AB-labeled \( \Delta \text{HexUA} \alpha 1-3 \text{GalNAc} (6S) \beta 1-4 \text{GlcUA} \beta 1-3 \text{GalNAc} (6S) (\Delta \text{C-C-A-C}) \), \( \Delta \text{HexUA} \alpha 1-3 \text{GalNAc} (4S) \beta 1-4 \text{GlcUA} \beta 1-3 \text{GalNAc} (4S) (\Delta \text{A-A}) \) (29), \( \Delta \text{HexUA} (2S) \alpha 1-3 \text{GalNAc} (6S) \beta 1-4 \text{GlcUA} \beta 1-3 \text{GalNAc} (6S) (\Delta \text{D-C}) \) (29), \( \Delta \text{HexUA} (2S) \alpha 1-3 \text{GalNAc} (6S) \beta 1-4 \text{GlcUA} \beta 1-3 \text{GalNAc} (4S) (\Delta \text{D-A}) \) (29), \( \Delta \text{HexUA} \alpha 1-3 \text{GalNAc} (4S) \beta 1-4 \text{GlcUA} (2S) \beta 1-3 \text{GalNAc} (6S) (\Delta \text{AA-D}) \) (30), and \( \Delta \text{HexUA} \alpha 1-3 \text{GalNAc} (4S,6S) \beta 1-4 \text{GlcUA} \beta 1-3 \text{GalNAc} (4S) (\Delta \text{E-A}) \) (30).

Delayed Extraction Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry—Dried oligosaccharides (2–4 pmol of individual oligosaccharides, and 10–20 pmol of mixed oligosaccharide fractions) were first mixed with 1–2 \( \mu \text{mol of (Arg-Gly)} \) (5–20 pmol) and then 1 \( \mu \text{mol of gentisic acid (1 mg/ml)} \) (31, 32). Each mixture was spotted on a plate for MS analysis using a mixture of (Arg-Gly) (5–20 pmol) and gentisic acid as a control. The analysis was run in a positive mode using the HCD1001 method according to the manufacturer’s instructions. The MS spectra were recorded on a Voyager DE-PRO-Pro (PerSeptive Biosystems, Framingham, MA) using the linear mode.

Enzymatic Treatments—For the analysis of disaccharide composition, oligosaccharides (20–100 pmol as disaccharides) were incubated with 5 international milliunits of chondroitinase ABC for 2 h and then chondroitinase AC-II (1 international milliunit) or ABC (a conventional preparation, 1 international milliunit, respectively). To characterize the disaccharides at the non-reducing ends of the isolated oligosaccharides, aliquots (0.5–2 pmol) of each 2AB-labeled oligosaccharide subfracton were incubated with chondroitinase AC-II (1 international milliunit) or ABC (a conventional preparation, 1 international milliunit, respectively). To characterize the disaccharides at the non-reducing ends of the isolated oligosaccharides, the PTN-unbound and -bound subfractions (1–3 pmol) were subjected to digestions with a protease-free preparation of chondroitinase ABC (1 international milliunit) for 15 min or with chondroitinase AC-II (1 international milliunit) for 60 min, respectively, followed by a derivatization with 2AB. In one case, the disaccharide at the reducing end was examined by incubation of the oligosaccharide subfracton (~2 pmol) with \( \Delta \text{Hexuronate-2-sulfatase} \) (4 \( \mu \text{IU}) \) as described (15, 34). All the enzymatic reactions were carried out at 37 \(^\circ\)C if not specified, and heating at 100 \(^\circ\)C for 1 min was used to terminate the reactions. Each digest was subjected to analyses by anion-exchange HPLC and/or gel filtration as described above.

RESULTS

Chondroitinase B-resistant Structures Contain the Major PTN-binding Epitopes of E-CS/DS—The E-CS/DS chains had a disaccharide composition of \( \Delta \text{O, } \Delta \text{C, } \Delta \text{A, } \Delta \text{D, } \Delta \text{B, and } \Delta \text{E at a molar percentage of 14.4:32.9:60.1:1.7:}:<0.1:0.9, as evaluated by chondroitinase ABC digestion, and contained ~9% IdoUA-containing disaccharides, which were cleavable by chondroitinase B and largely scattered along the chains (22, 25). Here, to characterize the epitopes in E-CS/DS for binding to PTN, we first assayed the effects of various treatments with chondroitinases or hyaluronidase on the inhibitory activity of E-CS/DS against the interaction between PTN and immobilized E-CS/DS in the BIACore system. As shown in Fig. 1, E-CS/DS at a concentration of 10 \( \mu \text{g/ml inhibited 75% of the interaction. However, treatment with chondroitinase ABC or AC-I abolished the inhibitory activity of E-CS/DS almost completely, indicating that both treatments destroyed most of the PTN-binding epitopes in the parent polymer. In contrast, neither hyaluronidase SD nor heparitinase treatment affected the inhibitory activity of E-CS/DS. The results of the hyaluronidase digestion suggested that long and consecutive sequences of non-sulfated disaccharides are not the structural elements in E-CS/DS critical for the binding to PTN. The results of the heparitinase digestion confirmed that the E-CS/DS preparation used in this study was free of contamination by HS. To our surprise, a chondroitinase B digest of E-CS/DS exhibited 65% of the inhibitory activity of the intact E-CS/DS, indicating that the chondroitinase B-resistant structures of E-CS/DS contained the majority of the PTN-binding epitopes.

Gel filtration analysis of the digests of E-CS/DS obtained with chondroitinase B or hyaluronidase SD after labeling with 2AB revealed that no chondroitinase B digestion generated a series of oligosaccharides ranging from di- to >20-mer oligosaccharides (Fig. 2A). In contrast, the hyaluronidase SD digest contained disaccharides and non-separable

\( S. S. \text{ Deepa, S. Fukui, and K. Sugahara, manuscript submitted.} \)
large oligosaccharides (>12-mer) in addition to small amounts of tetra- and hexasaccharides (data not shown).

Isolation of Minimal Oligosaccharides for PTN Binding from a Chondroitinase B Digest of E-CS/DS—The parent E-CS/DS chains were digested using chondroitinase B for the isolation of PTN-binding structures, taking advantage of the specificity of the enzyme, which not only retained the major PTN-binding structures in the digest but also provided structural information on the type of uronic acid (IdoUA) at the cleavage site. E-CS/DS was exhaustively digested with chondroitinase B, derivatized with 2AB, and fractionated by gel filtration. The effluent fractions (F1 to F8) were collected as indicated in Fig. 2A, and the molecular size of the major component in each fraction was determined by MALDI-MS analysis.

To investigate which fraction contained the minimal structures required for the binding to PTN, each fraction was subjected to affinity chromatography on a PTN column, which was eluted with a salt gradient. Oligosaccharides in the effluent of each parent fraction (F3 to F8) were determined based on their fluorescence intensity, which corresponds to their relative molar percentage. As illustrated in Fig. 2B, essentially all the fluorescent materials (2AB-derivatized oligosaccharides) (99.6%) in F4 were detected in the flow-through fraction (F3 also, data not shown), whereas small yet significant proportions (7.0–16.5%) of the fluorescent materials were found in the subfractions (F5 to F7) eluted with higher concentrations of salt (≥0.2 M NaCl) (F8 also, data not shown). The results indicated that an octasaccharide fraction, F5, appeared to contain the minimal structures needed for binding to immobilized PTN at a physiological salt concentration.

The results from the affinity fractionation were verified by inhibition assays. The flow-through subfraction (F5-ub) and bound subfraction (F5-b) of F5, which were eluted with 0.15 and 0.7 M NaCl, respectively, were used as inhibitors against the binding of PTN to immobilized E-CS/DS. A smaller amount (8 pmol) of F5-b inhibited ~53% of the interaction, whereas 80 pmol of F5-ub failed to show significant inhibition (Fig. 2C). These results confirmed that the high (0.7 M) and low (0.15 M) salt-eluted subfractions of F5 from the PTN column enriched the PTN-bound and -unbound oligosaccharides in the parent fraction, respectively. Similar results were also observed for the bound and unbound subfractions of F6 (data not shown). In addition, the good separation of the PTN-bound and -unbound oligosaccharides by affinity fractionation suggested that the 2AB tag at the reducing end of each oligosaccharide had little influence on the interaction with PTN at a physiological salt concentration.

Disaccharide compositions of the chondroitinase B-resistant fractions and their subfractions obtained from the affinity fractionation are summarized in Table One. Fractions F1 to F4, which did not bind to the PTN column, contained non-, 6-O- and/or 4-O-sulfated disaccharides in different proportions, but no detectable oversulfated disaccharides. In contrast, the bound subfractions of both F5 and F6 (F5-b and F6-b) contained considerable amounts of oversulfated disaccharides such as D/iD (26.9–20.4%) and E/iE (6.7–8.6%), whereas no or small amounts (0–2.6%) of such disaccharides were found in the corresponding unbound subfractions. Notably, much higher (2.9- to 4.3-fold) proportions of non-sulfated disaccharides were detected for the unbound subfractions than the bound subfractions of F5, F6, and F7. Apparently, these results revealed a strong correlation between the charge density and disaccharide composition of oligosaccharides and their PTN-binding activity.

To investigate and compare the structures of PTN-unbound and -bound oligosaccharides in detail, both F5-ub and F5-b were separated by anion-exchange HPLC. Eight subfractions (F5-ub-a to -h) were series.
subfractions (F5-b-I to -VI) in addition to seven minor subfractions. Notably, the presence of the small peaks between 35 to 48 min resolved for F5-ub (Fig. 3A), whereas six main well separated bound subfractions (F5-b-1 to -7) were obtained from F5-ub (Fig. 3B).

Structural Characterization of the Isolated PTN-unbound Octasaccharide Subfractions F5-ub-a to F5-ub-h—The oligosaccharides, isolated by anion-exchange HPLC, were characterized by MALDI-TOF-MS analysis and enzymatic digestions in conjunction with HPLC. The MS analysis indicated that three subfractions, F5-ub-a, -b, and -c, contained trisulfated octasaccharides, and that the main components in the other five subfractions, F5-ub-d, -e, -f, -g, and -h, were tetrasulfated octasaccharides (supplementary Table IS). Each subfraction was subjected to digestions with chondroitinase AC-II, chondroitinase ABC (a conventional or protease-free preparation), or a mixture of chondroitinases ABC (unless specified, chondroitinase ABC means a conventional preparation), and AC-II as described under “Experimental Procedures.” All components in these subfractions were digested completely by chondroitinase AC-II, indicating that all the internal uronic acid residues of each component are GlcUA.

The results obtained from the enzymatic characterization of F5-ub-a to -h are summarized in TABLE TWO. Except F5-ub-g, which contained two components in comparable amounts, these subfractions mainly contained a single predominant component. The enzymatic analysis of subfraction F5-ub-h is described below as an example. A sequential digestion with chondroitinases ABC and AC-II gave rise to ΔC and ΔA units in a molar ratio of 1:3 (Fig. 4A), suggesting that the predominant component in F5-ub-h is composed of one C and three A units. It is known that treatment with chondroitinase ABC of an oligosaccharide (≥6-mer) with a 2AB tag at the reducing end results in an unsaturated tetrasaccharide tagged with 2AB in addition to free disaccharide(s), irrespective of the structure of the parent oligosaccharide (27). As shown in Fig. 4B, chondroitinase ABC generated a predominant fluorescent peak corresponding to ΔA-2AB, suggesting that the predominant component in F5-ub-h has an A-A structure at the reducing end. This was verified by the observation that ΔA-2AB was the only fluorescent peak detected in a chondroitinase AC-II digest (Fig. 4C) of this subfraction, suggesting that the reducing end of the predominant oligosaccharide in F5-ub-h is an A unit. Moreover, the disaccharide at the non-reducing end was clarified by a partial digestion of F5-ub-h with

| Oligosaccharide fractions | Content | Disaccharides | Sulfation degree |
|---------------------------|---------|---------------|-----------------|
|                           | %       | ΔO⁺ | ΔC | ΔA | ΔD | ΔB | ΔE | ΔT | mol% |
| F1                        | 6.3     | —   | —    | 100 | —   | —   | —   | —   | 1.00 |
| F2                        | 10.7    | 35.4 | 27.1 | 37.6 | —   | —   | —   | —   | 0.65 |
| F3                        | 13.6    | 12.6 | 61.2 | 26.2 | —   | —   | —   | —   | 0.87 |
| F4                        | 21.8    | 22.2 | 37.1 | 40.7 | —   | —   | —   | —   | 0.78 |
| F5-ub                     | 17.5    | 25.7 | 26.1 | 48.2 | —   | —   | —   | —   | 0.74 |
| F5-b                      | 1.3     | 8.9  | 24.3 | 33.2 | 26.9 | —   | 6.7  | —   | 1.25 |
| F6-ub                     | 10.0    | 20.4 | 33.9 | 43.0 | 1.9  | —   | 0.7  | —   | 0.82 |
| F6-b                      | 2.2     | 4.7  | 20.6 | 39.2 | 20.4 | 1.8  | 8.6  | 4.7  | 1.35 |
| F7-ub                     | 13.9    | 28.4 | 29.0 | 41.5 | 0.7  | —   | 0.4  | —   | 0.73 |
| F7-b                      | 2.2     | 8.2  | 31.5 | 51.0 | 6.0  | —   | 3.3  | —   | 1.01 |
| F8                        | 0.5     | ND   | ND   | ND   | ND   | ND   | ND   | ND   | NC  |

a The names refer to the peaks and fractions designated in Fig. 2 (A and B). “ub” and “b” denote the unbound and bound subfractions, respectively.

b The relative percentage was calculated based on the fluorescence intensity of each fraction shown in Fig. 2 (A and B).

c For abbreviations including ΔA (ΔDi-4S), ΔB (ΔDi-diSB), ΔC (ΔDi-6S), ΔD (ΔDi-diSE), ΔE (ΔDi-diSD), and ΔT (ΔDi-TriS) for unsaturated disaccharide units, see “Experimental Procedures.”

d Sulfation degree was calculated as the average number of sulfate groups/disaccharide unit.

e “—”, not detected.

f ND, not due to the limited amounts obtained.

g NC, not calculated.

FIGURE 3. Separation of F5-ub and F5-b by anion-exchange chromatography. F5-ub (400 pmol) and F5-b (230 pmol) were separated by anion-exchange HPLC on an amine-bound silica column as described in “Experimental Procedures.” Each fraction derived from E-CS/DS was digested with chondroitinase ABC and chondroitinase AC-II, and the products were identified and quantified by MS analysis and enzymatic digestions in conjunction with HPLC. The MS analysis indicated that three subfractions, F5-ub-a, -b, and -c, contained trisulfated octasaccharides, and that the main components in the other five subfractions, F5-ub-d, -e, -f, -g, and -h, were tetrasulfated octasaccharides (supplementary Table IS). Each subfraction was subjected to digestions with chondroitinase AC-II, chondroitinase ABC (a conventional or protease-free preparation), or a mixture of chondroitinases ABC (unless specified, chondroitinase ABC means a conventional preparation), and AC-II as described under “Experimental Procedures.” All components in these subfractions were digested completely by chondroitinase AC-II, indicating that all the internal uronic acid residues of each component are GlcUA.

The results obtained from the enzymatic characterization of F5-ub-a to -h are summarized in TABLE TWO. Except F5-ub-g, which contained two components in comparable amounts, these subfractions mainly contained a single predominant component. The enzymatic analysis of subfraction F5-ub-h is described below as an example. A sequential digestion with chondroitinases ABC and AC-II gave rise to ΔC and ΔA units in a molar ratio of 1:3 (Fig. 4A), suggesting that the predominant component in F5-ub-h is composed of one C and three A units. It is known that treatment with chondroitinase ABC of an oligosaccharide (≥6-mer) with a 2AB tag at the reducing end results in an unsaturated tetrasaccharide tagged with 2AB in addition to free disaccharide(s), irrespective of the structure of the parent oligosaccharide (27). As shown in Fig. 4B, chondroitinase ABC generated a predominant fluorescent peak corresponding to ΔA-2AB, suggesting that the predominant component in F5-ub-h has an A-A structure at the reducing end. This was verified by the observation that ΔA-2AB was the only fluorescent peak detected in a chondroitinase AC-II digest (Fig. 4C) of this subfraction, suggesting that the reducing end of the predominant oligosaccharide in F5-ub-h is an A unit. Moreover, the disaccharide at the non-reducing end was clarified by a partial digestion of F5-ub-h with...
TABLE TWO

Enzymatic analyses of subfractions of the PTN-unbound octasaccharides

| Subfractions | Purity | CSase ABC/AC-II followed by 2AB labeling* | CSase ABC | CSase AC-II | Non-reducing | Sequences end units† | Parent structures† |
|--------------|--------|------------------------------------------|-----------|------------|-------------|-------------------|-----------------|
|               | mol%   | ratio                                   |           |            |             |                   |                  |
| F5-ub-a       | 84.0   | ∆O + ∆C (1:3)                           | ∆C-C     | ∆C        | ∆C          | ∆C-O-C-C−      | iC-O-C-C-iX−    |
| F5-ub-b       | 75.4   | ∆O + ∆C + ∆A (1:2:1)                    | ∆C-C     | ∆A        | ∆A          | ∆A-O-A-C−      | iA-O-A-C-iX−    |
| F5-ub-c       | 94.8   | ∆C + ∆A (1:3)                           | ∆C-A     | ∆A        | ∆A          | ∆A-O-A-A−      | iA-O-A-A-iX−    |
| F5-ub-d       | 86.5   | ∆C (∼95%)                               | ∆C-C     | ND*        | ND*         | ∆C-C-C-C−      | iC-C-C-C-iX−    |
| F5-ub-e       | 85.8   | ∆C + ∆A (3:1)                           | ∆C-C     | ∆C        | ∆C          | ∆C-C-C-C−      | iA-C-C-C-iX−    |
| F5-ub-f       | 93.0   | ∆C + ∆A (3:1)                           | ∆C-C     | ∆C        | ∆C          | ∆C-C-C-C−      | iC-C-C-C-iX−    |
| F5-ub-g       | 60.40  | ∆C + ∆A (1:1)                           | ∆C-C + ∆A (3/2, mol/mol) | ∆C + ∆A (1/1, mol/mol) | ∆A | ∆C-C-C-C− (60%)† | iA-C-C-C-iX−    |
|               | 96.0   | ∆C + ∆A (1:3)                           | ∆A-A     | ∆A        | ∆A          | ∆C-C-A-A−      | iC-A-A-A-iX−    |

* The unsaturated disaccharides were detected in the 2AB-derivatized digest of each subfraction with CSases ABC and AC-II, O, C, and A stand for GlcUA, GalNAc, GlcUA-GalNAc(6S), and GlcUA-GalNAc(4S), respectively, whereas the symbol ∆ denotes the unsaturation of the GlcUA residue at the non-reducing end.  
† The non-reducing end units refer to the major disaccharide generated from each 2AB-tagged octasaccharide subfraction by a controlled treatment with a protease-free preparation of chondroitinase ABC, which exhibits an exolytic action (15, 30), as shown in Fig. 4D.  
‡ ND, not done.  
§ The “i” stands for an i-l-iduronate residue, and iX represents any disaccharides with an IdUA residue including iA, iC, iD, iE, iB, or iT, except for iO (for the abbreviations, see the “Experimental Procedures”).  
δ Novel structures.

FIGURE 4. Chromatograms of the enzymatic characterization of F5-ub-h and F5-b-V.

Oligosaccharide fractions, typically 1–3 pmol, were digested with given enzymes and analyzed by anion-exchange HPLC on a PA-03 column. In some cases, the enzymatic digests were derivatized with a fluorophore 2AB before being applied to HPLC. A and E, F5-ub-h (A) and F5-b-V (E) were sequentially treated with chondroitinase ABC and chondroitinase AC-II, labeled with 2AB, and analyzed by HPLC. B and F, F5-ub-h (B) and F5-b-V (F) were individually treated with chondroitinase ABC and analyzed by HPLC. C and G, F5-ub-h (C) and F5-b-V (G) were individually treated with chondroitinase AC-II, labeled with or without 2AB, and analyzed by HPLC. D, F5-ub-h was partially digested with chondroitinase ABC (a protease-free preparation), labeled with 2AB, and analyzed by HPLC. H, F5-b-V was digested with chondroitinase AC-II and labeled with 2AB, further digested with chondroitinase ABC, and then labeled again with 2AB, and analyzed by HPLC. Peaks eluted before 10 min are attributable to free 2AB and some unknown side reaction products of the 2AB labeling. The peaks marked by asterisks were derived from impurities or the buffers used for the enzymatic treatments. The elution position of authentic 2AB-derivatized unsaturated di- and oligosaccharides are indicated by arrows. 1, ∆Ω-DS (∆O); 2, ∆Ω-5S (∆C); 3, ∆Ω-4S (∆A); 4, ∆Ω-Di-5S; 5, ∆Ω-Di-5S (∆E); 6, ∆Ω-Tris (∆T); 7, ∆A-A; 8, the predominant component of F5-ub-h (∆C-A-A-A, see “Results”); 9, ∆A-D; and 10, the hexasaccharide at the reducing end of the predominant component of F5-b-V (∆A-D-D or ∆D-A-D, see “Results”).

a protease-free preparation of chondroitinase ABC, taking advantage of strong tendency of the protease-free preparation (15, 30) to exert an exolytic effect when acting on oligosaccharides such as hexa- and octasaccharides. Fig. 4D shows that ∆C was the major disaccharide generated by a controlled digestion of F5-ub-h with chondroitinase ABC (a protease-free preparation), suggesting that a ∆C unit is located at the non-reducing end of the predominant oligosaccharide of this subfraction. Based on these results, it was concluded that the subfraction F5-ub-h contained a predominant component with the sequence ∆C-A-A-A.

Structural analysis of all the other PTN-unbound octasaccharides revealed that they are composed of different combinations of O, C, and A but no di- or trisulfated disaccharide, consistent with the results obtained from an analysis of the disaccharide composition of F5-ub (TABLE ONE). None of these octasaccharides bound to the PTN column at a physiological salt concentration, implying important roles for oversulfated (di- or trisulfated) disaccharides in the binding of oligosaccharides to PTN. Notably, except for ∆C-C-C-C (F5-ub-d) and ∆C-C-A-C (F5-ub-f), which have been isolated from shark cartilage CS-C− and CS-D (15) or CS-C−, respectively, all the other sequences were isolated for the first time from natural CS/DS chains. It is also noteworthy that these octasaccharides are derived from the corresponding parent decasaccharide sequences flanked by two IdUA residues in E-CS/DS (TABLE TWO) and that iC units were discovered in some of these sequences. Although sulfotransferase that can produce iC units have been detected in bovine serum (35), iC units were never demonstrated previously in native DS chains.

Structural Characterization of the Isolated PTN-bound Octasaccharide Subfractions F5-b-I to F5-b-VI—A similar strategy was employed to characterize the structures of the main subfractions (F5-b-I to -VI) of F5-b, which were separated as shown in Fig. 3B. They were considered to contain the smallest oligosaccharides, which could interact with PTN. Among these six subfractions, five were determined by MS to share a common octasaccharide core but with different numbers of sulfate groups (supplementary Table IS). The MS analysis of F5-b-VI was not successful probably due to its greater molecular size and higher degree of sulfation as evidenced by the disaccharide composition analysis (TABLE THREE). Hence, an enzymatic analysis was carried out for the other five subfractions (F5-b-I to -V), and the results are summarized in TABLE THREE.
Pleiotrophin-binding Sulfated Oligosaccharides

TABLE THREE

Enzymatic analyses of subfractions of the PTN-bound octasaccharides

| Subfractions | Purity | CSases ABC/AC-II followed by 2AB labeling | CSase ABC | CSase AC-II | CSase AC-II/2AB labeling | Sensitivity to 2-O-sulfatase | Sequences | Parent structures |
|--------------|--------|------------------------------------------|-----------|-------------|-------------------------|-----------------------------|-----------|------------------|
|              |        | mol% | mol ratio |            |            |                         |                           |           |                  |
| F5-b-I       | 81.7   | ΔC + ΔD (2.6:1) | ΔD-C | ΔC | ND | ND | ΔC-C-D-C \* | iC-C-D-C-iX * |
| F5-b-II      | 68.9   | ΔA + ΔC + ΔD (1:1.7:1.1) | ΔD-C | ΔC | ND | ND | ΔC-C-D-C or ΔC-A-D-C | iA-C-D-C-iX * |
| F5-b-III     | 79.1   | ΔC + ΔD (1:1.2) | ΔD-C | ΔC | ND | ND | iC-D-C-iX *  |
| F5-b-IV      | 75.4   | ΔD + ΔE (1.0:1.9) | ΔD-C | ΔC | ND | ND | ΔC-D-C or C-D-iD-C | iC-D-iC-iX * |
| F5-b-V       | 82.7   | ΔD + ΔE + ΔA-D (1.2:1:0.1) | ΔA-D | ΔE | ND | ND | ΔE-D-A-D' or ΔE-D-A-D or iE-D-DA-iX | iE-D-iA-D-iX |
| F5-b-VI      | 82.7   | ΔA + ΔT + ΔTetra (3:0:1:0:1:8) \(b\) | ΔTetra | — | ND | ND | ND | ND |

* The unsaturated di- and tetrasaccharides were detected in the 2AB-derivatized digest of individual subfractions with CSases ABC and AC-II. C, A, D, E, and T stand for the following disaccharide units: C, GlcUA-GalNAc(6S); A, GlcUA-GalNAc(4S); D, GlcUA(2S)-GalNAc(6S); E, GlcUA-GalNAc(4S,6S); T, GlcUA(2S)-GalNAc(4S,6S). The symbol Δ denotes the unsaturation of the GlcUA residue at the non-reducing end.

b For the definitions of "C" and "T", see footnote d to TABLE TWO.

c ND, not determined because of the very limited amounts available.

d Novel sequences.

e Plus (+) indicates sensitivity to treatment with 4Hexuronate-2-O-sulfatase.

f No appreciable amount of unsaturated disaccharide was detected in a CSase AC-II digest of the corresponding subfraction.

g ΔTetra stands for an unidentified tetrasaccharate.

A sequential digestion with chondroitinase ABC and chondroitinase AC-II generated different sets of combinations of various disaccharides containing ΔA, ΔC, ΔD, and ΔE units for each subfraction. The chromatogram for F5-b-V is shown in Fig. 4E. It should be noted that the 2AB-tagged ΔC-D structure is resistant to treatment with chondroitinase AC-II.\(^6\) Similar results were obtained for other 2AB-tagged CS tetrasaccharides with a D unit at the reducing end such as ΔC-D\(^3\) and D unit-enriched CS hexasaccharides such as ΔD-D-C.\(^6\) Analysis of the chondroitinase ABC digest revealed that the predominant components of four subfractions (F5-b-I to -IV) contained a common tetrasaccharide D-C or iD-C at their reducing ends, which resulted in ΔD-C-2AB, whereas that of F5-b-V was found to be A-D or iA-D, which resulted in ΔA-D-2AB (Fig. 4F). F5-b-I, -II, and -III were completely degraded by chondroitinase AC-II, and all produced one predominant peak corresponding to ΔC-2AB. These findings indicate that all the internal uronic acid residues of the predominant components of these three subfractions are GlcUA, and that all these main components have a C unit at their reducing ends. These results are consistent with those obtained from the digestion with chondroitinase ABC.

In contrast, chondroitinase AC-II did not degrade F5-b-IV or -V into di- or tetrasaccharides from the reducing ends (TABLE THREE). However, 2AB derivatization of the chondroitinase AC-II digest of F5-b-IV or F5-b-V showed only one predominant disaccharide ΔC and a hexasaccharide or ΔE and a hexasaccharide (Fig. 4G), respectively. The results suggested that the predominant components of F5-b-IV and -V have ΔC and ΔE at their non-reducing ends, respectively. Moreover, sequential digestion with chondroitinase AC-II and then chondroitinase ABC of these two subfractions followed by labeling with 2AB gave rise to ΔC and ΔD disaccharides for F5-b-IV, whereas the same treatment of F5-b-V yielded ΔD and ΔE (Fig. 4H) in a molar ratio of ~1:1. These results demonstrated that the tetrasaccharides at non-reducing ends of the predominant components in F5-b-IV and -V have ΔC-D and ΔE-D structures, respectively.

In addition, F5-b-III was sensitive to treatment with 4Hexuronate-2-sulfatase (data not shown), which removes a sulfate group only from the C-2 position of a HexUA located at the non-reducing terminus (34). The results suggest that the predominant component of F5-b-III has a ΔD unit at the non-reducing terminus, because this subfraction contained only two kinds of disaccharide units, C and D.

Taking the results obtained from the enzymatic analyses and MS together, it was concluded that the predominant components in F5-b-I to -V have the following sequences: ΔC-C-D-C (F5-b-I), ΔA-C-D-C or ΔC-A-D-C (F5-b-II), ΔD-C-D-C (F5-b-III), ΔC-D-D-C or ΔC-D-iD-C (F5-b-IV), and ΔE-D-A-D or ΔE-D-A-D (F5-b-V) (the "i" in iA and iD stands for IdoUA) (TABLE THREE).

Thus, all five of the oligosaccharides isolated from the PTN-bound subfraction F5-b contained one or more disulfated disaccharides such as D, iD, E, and iE, which is in marked contrast to the oligosaccharides separated from the PTN-unbound (F5-ub) subfractions, where no disulfated disaccharide was detected. These D/iD-enriched oligosaccharides were eluted before 0.3 M NaCl from the PTN column (Fig. 2B), suggesting that these units played an important role in the low yet significant affinity for CS/DS-PTN interaction. All the sequences identified here, except for the ΔC-A-D-C structure (15), are novel. These octasaccharides are derived from the corresponding parent decasaccharide sequences flanked by two IdoUA residues in E-CS/DS (TABLE THREE) as in the case of the PTN-unbound octasaccharides (TABLE TWO). Notably, we have now verified iD units, whose existence in the mouse cerebellum was previously suggested based on the immunostaining with the iD-specific monoclonal antibody, 2A12, raised against ascidian DS (20).

Characterization of the PTN-bound and -Unbound Subfractions of Decasaccharide Fraction F6—A significant proportion (16.5%) of F6 was retained on the PTN column after a wash with the equilibration buffer: more than that of F5 (7%) but comparable to that of F7 (15.1%) (Fig. 2B). More significantly, the amount of materials eluted with high salt (0.4 and 0.5 M NaCl) was significantly larger in F6 (4.1%) than in F5 (less than 0.3%) or F7 (1.6%) (Fig. 2B). These results suggested that F6 contained

K. Kalayamamitra, P. Kongtamelert, and K. Sugahara, manuscript in preparation.
patterns of F6-b and its chondroitinase AC-II digest revealed that the chondroitinase AC-II-resistant structures in F6-b constituted most of the materials originally eluted in the high salt-eluted subfractions (0.4 and 0.5 M NaCl-eluted) of F6-b (Fig. 5B), suggesting that the components, which strongly interacted with PTN, were abundant in the chondroitinase AC-II-resistant structures.

To investigate the structural features of the chondroitinase AC-II-resistant components of F6-b, a chondroitinase AC-II digest of F6-b was separated by gel filtration, and four subfractions (F6-b-I, -II, -III, and -IV) were isolated as indicated in panel a of Fig. 5A. F6-b-I and Fb-b-II contained mainly deca- and hexasaccharides, respectively, as assessed by MALDI-TOF-MS analysis (Fig. 6, A and B), whereas F6-b-III and -IV consisted of trisulfated tetrasaccharides and monosulfated disaccharides, respectively, as determined by anion-exchange HPLC (TABLE FOUR). Subfractions of F6-b-I and -II were completely degraded into di- and tetrasaccharides by treatment with a mixture of chondroitinases ABC and AC-II (panel d of Figs. 5A, 6C, and 6D). Molar ratios of the resultant disaccharides and tetrasaccharides are summarized in TABLE FOUR. Intriguingly, only 40% of F6-b-I was resistant to a mixture of chondroitinases AC-I and AC-II (panel c in Fig. 5A), suggesting that 60% of F6-b-I, which corresponded to 21% of F6-b, was sensitive to chondroitinase AC-I, and thus might contain IdooUA-containing structures (see "Discussion").

Next, the interaction with PTN of these four subfractions resolved by gel filtration was assessed by affinity chromatography using the PTN column. Neither F6-b-II, -III, nor -IV bound to the column, although F6-b-II contained a ΔD-A-D or ΔD-iA-D hexasaccharide as the predominant component (70% in molar percentage) and F6-b-III contained a mixture of ΔA-D and ΔE-A tetrasaccharides (TABLE FOUR), indicating that these hexa- and tetrasaccharides could not bind PTN at a physiological salt concentration. In contrast, F6-b-I was the only subfraction that bound to the PTN column, and therefore the affinity fractionation pattern of F6-b must represent that of F6-b-I (Fig. 5B). Thus, F6-b-I most likely contained the high affinity PTN-binding components of F6-b. Comparison of the disaccharide compositions of F6-b-I (TABLE FOUR) and F6-b (TABLE ONE) revealed that E or iE and B or iB, but not D/iD units, in F6-b were selectively concentrated in F6-b-I, suggesting that these units are involved in the high affinity interaction between E-CS/DS and PTN.

**DISCUSSION**

It has been recently demonstrated that a subpopulation of the E-CS/DS chains promotes the outgrowth of neurites through interaction with PTN and that oversulfated disaccharides, such as DiD and E/iE units, in combination with IdooUA-containing structures are required for the interaction of E-CS/DS with PTN (25). In the present study, the major PTN-binding epitopes in E-CS/DS were identified. Our results support the previous findings and provided further insight into the oligosaccharide-binding specificity of PTN.

Contrary to the treatment with chondroitinase ABC or AC-I, digestion with chondroitinase B reduced the PTN-binding activity of E-CS/DS only to a small extent (Fig. 1), which allowed us to isolate the major PTN-binding epitopes in E-CS/DS from a digest with chondroitinase B. The smallest oligosaccharides in the chondroitinase B digest of E-CS/DS, which bound to PTN at a physiological salt concentration, were octasaccharides, although stronger interactions were observed with decasaccharides or larger oligosaccharides. This size dependence is reminiscent of the interactions of GAG oligosaccharides with other heparin-binding growth factors such as fibroblast growth factor (FGF) 1

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**FIGURE 5. Characterization of the PTN-bound and -unbound subfractions of F6. A, gel filtration chromatograms of the enzymatic digests of PTN-bound (F6-b) and -unbound (F6-ub) subfractions.**  
F6-b was subjected to treatments with chondroitinase AC-II (panel a), chondroitinases AC-II and AC-I (panel c), or a mixture of chondroitinases AC-II and ABC (panel d), respectively, and then analyzed by gel filtration on a column of Superdex Peptide. The gel filtration pattern of the chondroitinase AC-II digest of F5-ub is shown in b for comparison. Sizes of resolved peaks were determined by MALDI-TOF-MS analysis (Fig. 6, A and B), indicating that these hexa- and tetrasaccharides could not bind PTN at a physiological salt concentration. In contrast, F6-b-I was the only subfraction that bound to the PTN column, and therefore the affinity fractionation pattern of F6-b must represent that of F6-b-I (Fig. 5B). Thus, F6-b-I most likely contained the high affinity PTN-binding components of F6-b. Comparison of the disaccharide compositions of F6-b-I (TABLE FOUR) and F6-b (TABLE ONE) revealed that E or iE and B or iB, but not D/iD units, in F6-b were selectively concentrated in F6-b-I, suggesting that these units are involved in the high affinity interaction between E-CS/DS and PTN.
Pleiotrophin-binding Sulfated Oligosaccharides

The PTN-bound decasaccharide fraction isolated from E-CS/DS was digested with chondroitinase AC-II and separated by gel filtration on a Superdex peptide column into F6-b-I to -IV (Fig. 5A). The resolved peaks were subjected to analyses for molecular size, disaccharide composition and activity to bind a PTN-coupled affinity column as described below.

### Table Four

Properties of the chondroitinase AC-II-resistant subfractions of PTN-bound decasaccharides

| Subfractions | Size | CSases ABC/AC-II followed by 2AB labeling | Deduced major component | Binding activity |
|--------------|------|-----------------------------------------|-------------------------|-----------------|
| F6-b-I       | 10-mer 75 and 85S | ΔC + ΔA + ΔD + ΔB + ΔE + ΔA-D + ΔT + ΔE-A (22.8:26:2:14.1:4.6:11.3:6.5:3:4:11.1) | ND | + |
| F6-b-II      | 6-mer 5S    | ΔC + ΔA + ΔD + ΔE + ΔA-D + ΔT + ΔE-A (4.7:9:9:31.4:8:3:27.5:10:6:7) | ΔD-A-D or ΔD-iA-D (~70) | - |
| F6-b-III     | 4-mer 3S    | ΔA-D + ΔE-A (58.2:41.8) | ΔA-D (60) + ΔE-A (40) | - |
| F6-b-IV      | 2-mer 1S    | ΔC + ΔA (46:45:3.6) | ΔC (46) + ΔA (54) | - |

* The sizes of F6-b-I and -II were assessed by MALDI-TOF-MS (see the legend to Fig. 6) and those of F6-b-III and -IV were determined by gel filtration with an amino-bond silica PA-33 column. The elution positions of 2AB-derivatized authentic unsaturated di- and oligosaccharides are indicated by arrows. 1, ΔO, 2, ΔC, 3, ΔA, 4, ΔD, 5, ΔB, 6, ΔE, 7, ΔD-B, 8, ΔT; and 9, ΔE-A (for the abbreviations, see the legend to Fig. 4). The peaks marked by asterisks were derived from the buffers used for the enzymatic treatments, and those marked by # symbols were from the column.

(36), FGF2 (36, 37), FGF4 (38), FGF7 (37), and hepatocyte growth factor (39).

Comparison of the oligosaccharides isolated from the PTN-bound (F5-b) and -unbound (F5-b) octasaccharide subfractions revealed a pivotal role for the oversulfated disaccharides, particularly the D or iD unit, in the binding of E-CS/DS oligosaccharides to PTN with a low yet significant affinity (Fig. 2B and TABLES TWO and THREE). This finding is consistent with our previous results that a low affinity fraction of E-CS/DS, eluted with 0.4 M NaCl from the PTN column, was rich in D and/or iD units among the unbound (0.15 M NaCl-eluted), low affinity (0.4 M NaCl-eluted), and high affinity fractions (1.0 M NaCl-eluted) of E-CS/DS (25). This is also in agreement with a recent report (24) that CS-D from shark cartilage binds PTN, and a small proportion of D (1.3%) in the CS chains of phosphacan is required for effective binding to PTN. Our results demonstrated that certain octasaccharide sequences with at least one D or iD unit, derived from E-CS/DS, are required for the binding to PTN at a physiological salt concentration. The failure to bind PTN of a hexasaccharide fraction (F6-b-II), which contained the ΔD-A-D and/or ΔD-iA-D structure as a major component (70%), suggested that not only a D or iD unit but size is critical for E-CS/DS oligosaccharides to interact with PTN (TABLE FOUR).

Previous findings (24, 25, 40) suggested that E- or iE-containing structures have higher affinity for PTN than D- or iD-containing structures. This study, the chondroitinase AC-II digest of PTN-bound decasaccharides contained high affinity components and was enriched with E and/or iE and B and/or iB units, but not D or iD units, from the PTN-bound decasaccharide fraction (F6-b) (Fig. 5B and TABLES ONE and FOUR). These findings support previous results and suggest the involvement of these particular disulfated disaccharide units in the high affinity interaction between E-CS/DS and PTN.
hybrid chains with this enzyme generates structures with a backbone of \( \Delta \text{HexUA-GalNAc-IdoUA-GalNAc-GlcUA/IdoUA-GalNAc} \), \( n \geq 0 \) (30, 41). In this study, the chondroitinase AC-II-resistant components (F6-b-I) were large (decasaccharides) and had a moderate proportion (18%) of D and/or iD units. The results suggest that these compounds might contain IdoUA-containing disaccharides at the penultimate position from their non-reducing ends, although this enzyme has limited ability to digest small CS oligosaccharides (hexa- or tetrasaccharides) that contain a cluster of D units. \(^7\) This assumption was supported by the observation that a large portion (60%) of this chondroitinase AC-II-resistant PTN high affinity subtraction (F6-b-I) was cleavable by chondroitinase AC-I (Fig. 5A). This observation suggested that the resistance to chondroitinase AC-II was not due to the clustering of D units, because chondroitinase AC-I has no activity to cleave D unit-containing structures (41). Hence, it is reasonable to assume that the chondroitinase AC-I-sensitive compounds of the chondroitinase AC-II-resistant decasaccharides shown in Fig. 5A contained unique IdoUA-containing disaccharides, which were resistant to chondroitinase B as well.

Chondroitinase B is the only enzyme currently available to cleave the galactosaminic linkages bound to IdoUA in DS or CS/DS hybrid chains, although its substrate specificity is not yet fully understood. This enzyme is known to efficiently generate \( \Delta \) units from various DS preparations but does not work on non-sulfated dermatan (41). Although commercial porcine skin CS-B (or DS) results in \( \Delta \text{C, D, and E} \) in a molar ratio of 5.6:86:8.4 upon chondroitinase ABC digestion, sequential digestions with chondroitinases AC-I and AC-II, and then chondroitinase B produced tetrasaccharides (8%) and disaccharides (92%) (data not shown), suggesting that chondroitinase B cleaves most, but not all, the galactosaminic bonds linked to IdoUA in DS or CS/DS hybrid chains. In addition, it has been reported that chondroitinase B generates \( \Delta \text{E} \) unit from Flagfish notochord CS-H, which is rich in E/iE and T/iI units (18, 42), and \( \Delta \text{B} \) unit from DS of bovine aorta (41) and ascidian *Halocynthia roretzi*, which is rich in iB units, \(^7\) respectively. These findings indicate that the galactosaminic bond linked to iE and iB units were sensitive to this enzyme. In contrast, DS from ascidian *Ascidia nigra*, which contains iD units and iC units as a major (\( \sim 80\% \)) and a minor (\( \sim 20\% \)) component, respectively, was totally resistant to chondroitinase B (20). Nevertheless, the sequences obtained in this study clearly indicate that chondroitinase B cleaved E-CS/DS chains at the non-reducing sides of some, if not all, iC and iD units as well as iA and iE units (TABLES TWO and THREE). These results together indicate that chondroitinase B does not cleave all galactosaminic linkages bound to IdoUA in DS or CS/DS hybrid chains, but the size and sulfate pattern of the substrates could be important for the recognition and digestibility by this enzyme. This may explain why the chondroitinase B-resistant oligosaccharides obtained in this study still appeared to contain some IdoUA-containing structures, which were involved in the high affinity interaction between E-CS/DS oligosaccharide and PTN. The sequences of such unique oligosaccharide structures remain to be determined.

Oversulfated disaccharides, such as D/iD, B/iB, and E/iE, have been implicated in the development of the brain (7, 17, 28). However, the analytical discrimination of D, B, and E units from their idD, iB, and iE counterparts in CS/DS chains remains difficult but necessary for a full understanding of the structure-function relationship of the CS/DS chains. Our recent study (20) demonstrated the presence of functional iD-containing domains in the developing mouse brain using a monoclonal antibody, 2A12, against the DS from ascidian. This has been confirmed in this study by the structural determination of F5-b-III, whose predominant component has a \( \Delta \text{D} \) unit at the non-reducing end. The \( \Delta \text{D} \) unit should be derived from an iD-containing structure in the parent E-CS/DS polymers in view of the specificity of chondroitinase B used for fragmentation of E-CS/DS. Similarly, the isolation of the structure \( \Delta \text{E-D-A-D} \) or \( \Delta \text{E-D-A-D} \) (F5-b-V) from E-CS/DS indicated the presence of iE unit in the mammalian brain. Interestingly, among the five identified oligosaccharides isolated from the PTN-bound octasaccharides, three contained two or more D/iD and/or E/iE units. This finding indicates that these rare oversulfated disaccharides (\( \sim 3\% \)) in E-CS/DS are not distributed randomly but tend to form clusters, which appear to be involved in the binding of E-CS/DS to PTN with high affinity and may be involved also in the binding of other growth factors. In addition, the high affinity subtraction (F6-b-I) contained a mixture of at least four decasaccharide sequences with six to nine sulfate groups (TABLE FOUR and Fig. 6A), suggesting that these decasaccharides contained one to four disulfated disaccharides in addition to monosulfated disaccharides.

The results altogether demonstrate that the E-CS/DS chains possess multiple minimal sequences capable of binding PTN, as reported also for the binding of HS chains to FG1, FG2, FG4, FG7, and FG8b (36, 38). The presence of multiple structurally distinct sequences with different affinity for a given growth factor or heparin-binding protein may be a general tendency, which is applicable not only to HS but also to CS/DS chains. The multiple PTN-binding sequences may be relevant in the sliding of PTN along a long CS/DS chain from a lower affinity to a higher affinity binding site as has been hypothesized for the binding of heparin-binding growth factors to multiple binding sequences on an HS chain (43). In addition, jumping of PTN from a lower affinity binding site of a CS/DS or HS chain to a higher affinity binding site of another CS/DS or HS chain may also be possible as has been suggested for HS-binding growth factors and HS chains (43).

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