Neuritogenic Activity of Chondroitin/Dermatan Sulfate Hybrid Chains of Embryonic Pig Brain and Their Mimicry from Shark Liver

INVolvement of the Pleiotrophin and Hepatocyte Growth Factor Signaling Pathways

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Accumulating evidence suggests the involvement of chondroitin sulfate (CS) and dermatan sulfate (DS) hybrid chains in the brain's development and critical roles for oversulfated disaccharides and IdoUA residues in the growth factor-binding and neuritogenic activities of these chains. In the pursuit of sources of CS/DS with unique structures, neuritogenic activity, and therapeutic potential, two novel CS/DS preparations were isolated from shark liver by anion exchange chromatography. The major (80%) low sulfated and minor (20%) highly sulfated fractions had an average molecular mass of 3.8–38.9 and 75.7 kDa, respectively. Digestion with various chondroitinases (CSases) revealed a large panel of disaccharides and IdoUA residues along the polysaccharide chains in both of the fractions. The higher with either GlcUA or IdoUA scattered along the polysaccharide chains in both of the fractions. The higher M₄ fraction, richer in IdoUA(2-O-sulfate)α1-3GalNAc(4-O-sulfate) and GlcUAβ/IdoUAα1-3GalNAc(4,6-O-disulfate) units, exerted greater neurite outgrowth-promoting (NOP) activity and better promoted the binding of various heparin-binding growth factors, including pleiotrophin (PTN), midkine, recombinant human heparin-binding epidermal growth factor-like growth factor, VEGF₁₆₅, fibroblast growth factor-2, fibroblast growth factor-7, and hepatocyte growth factor, revealing the involvement of the HGF and PTN signaling pathways in the activity.

Glycosaminoglycan (GAG) side chains of proteoglycans, namely chondroitin sulfate (CS)/dermatan sulfate (DS) are widely expressed at the cell surface and in extracellular matrices (1, 2). CS and DS chains consist of repeating disaccharide units of -GlcUA-GalNAc- and -IdoUA-GalNAc- with various sulfation patterns, respectively, and often exist as CS/DS co-polymers (3, 4). There is evidence to suggest that CS/DS plays crucial roles in biological events, such as the development of the central nervous system (5–8), wound repair (9, 10), viral attachment (11–13), growth factor signaling (14, 15), morphogenesis (16), and cytokinesis (17–19). Among these events, the development of the central nervous system, involving neuronal adhesion, migration, and neurite formation, has recently attracted attention in terms of the functions of CS/DS (4, 20).

The disaccharide composition of CS/DS chains in the brain shows developmental changes (21–23), suggesting that these chains differing in the ratio of GlcUA and IdoUA and in the sulfation profile may exert distinct functions during the brain's development. The proportion of oversulfated disaccharides and the presence of IdoUA are two crucial factors for the neurite outgrowth-promoting (NOP) activity of CS/DS chains (24–28), and the CS/DS chains expressed at the surface of neuronal cells or immobilized on the matrices exert NOP activity and bind various heparin-binding growth factors in vitro.

Our laboratory has recently shown that endogenous...
pleiotrophin (PTN) (29) or heparin-binding growth-associated molecule (30), which is a neuritogenic growth factor (31), is recruited by the CS/DS chains from pig embryonic brain (E-CS/DS) and mediates the NOP activity of the CS/DS chains (32). Five octasaccharide sequences with at least one GlcUA(2S)β1–3GalNAc(6S) (D) or IdoUA(2S)α1–3GalNAc(6S) (iD) disaccharide, where 2S and 6S stand for 2-O- and 6-O-sulfate, respectively, have been isolated from a subfraction with low affinity for PTN after enzymatic fragmentation of the polysaccharides, followed by affinity chromatography using a PTN-immobilized column (33). To search for sources of CS/DS chains with therapeutic potential, CS/DS hybrid chains were purified from shark liver and found to have a unique structure and strong NOP activity. Further study revealed the molecular mechanism of the NOP activity to involve the signaling pathway of not only PTN but also hepatocyte growth factor (HGF).

**EXPERIMENTAL PROCEDURES**

**Materials**—Livers of Prionace glauca (blue shark) were provided by Maruha Group Inc., Central Research Institute (Tsukuba-City, Japan). Pregnant ddY mice were purchased from SLC Inc. (Shizuoka, Japan). CS-A from whale cartilage; CS-B from porcine skin; CS-C and CS-D from shark cartilage; CS-E from squid cartilage; and standard unsaturated disaccharides, chondroitinases (CSases) ABC (EC 4.2.2.4), AC-I (EC 4.2.2.5), and AC-II (EC 4.2.2.5), and hyaluronidase SH (EC 4.2.2.1) from Streptomyces hyalurolyticus were purchased from Seikagaku Corp. (Tokyo, Japan). CSase B was obtained from IBEX Technologies (Montreal, Canada). Embryonic pig brain-derived CS/DS (E-CS/DS) and its high affinity fraction (E-CS/DS-H) were prepared as described previously (32). Recombinant human (rh) pleiotrophin (PTN) expressed in E. coli and rh-vascular endothelial growth factor-165 (VEGF_{165}) expressed in insect cells were from RELIA Tech GmbH (Braunschweig, Germany). rh-Midkine (MK) expressed in Ershicherichia coli and rh-fibroblast growth factor (FGF)-1 (or acidic FGF) expressed in E. coli were from PeproTech EC Ltd. (London, UK). rh-FGF-2 (or basic FGF) expressed in E. coli was from Genzyme TECHNE (Minneapolis, MN). rh-Heparin-binding epidermal growth factor-like growth factor (HB-EGF) and rh-hepatocyte growth factor/scatter factor (HGF/SF) expressed in Sf21 insect cells, rh-keratinoctye growth factor (KGF/FGF-7) expressed in E. coli, and anti-mouse HGF receptor IgG were obtained from R&D Systems (Minneapolis, MN). 1,9-Dimethylmethylene blue was from Aldrich. Polyclonal goat anti-rh-PTN IgG and polyclonal goat anti-rh-MK IgG were obtained from Genzyme/Techné (Cambridge, MA). Anti-FGF-2/basic FGF, clone bFM-1, was from Upstate Biotechnology, Inc. (New York, NY). Polyclonal goat IgG against mouse anaplastic lymphoma kinase (ALK T-18) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal anti-rat HGF rabbit IgG was provided by Prof. Toshikazu Nakamura (Osaka University, Osaka, Japan). Purified serum IgG from mouse, goat, and rabbit were obtained from Sigma. Actinase E was from Kaken Pharmaceutical Co. (Tokyo, Japan). All other chemicals and reagents were of the highest quality available.

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**Materials**—Livers of blue shark (P. glauca), were dehydrated and delipidated by extraction with acetone, air-dried, and used for extraction of GAGs essentially as described previously (28) with some modifications. Briefly, 65 g of the acetone powder, corresponding to 163 g of the wet tissue, was treated with actinase E, followed by 5% trichloroacetic acid to precipitate residual proteins and peptides and with ether to extract trichloroacetic acid. To extract GAGs exhaustively, the precipitate obtained with trichloroacetic acid was treated with 0.5 M NaOH at 4 °C for 20 h and then neutralized with 1 M acetic acid before being precipitated with trichloroacetic acid, extracted with ether, and combined with the GAG extract obtained by actinase digestion. A crude GAG fraction was recovered from the combined extract by precipitation with 80% ethanol containing 5% sodium acetate at 4 °C overnight. The yield was 9 g, containing 416 mg of GAG based on the carbazole reaction.

**Purification of SL-CS/DS**—The crude GAG fraction (1.5 g) was loaded on a DEAE-Sephadex column (15 × 300 mm) pre-equilibrated with 0.3 M phosphate buffer, pH 6.0, containing 0.2 M NaCl. After the column was washed with the equilibration buffer, GAGs were eluted with the same buffer containing 2.0 M NaCl, dialyzed against water, and concentrated to dryness (the yield was 25 mg). This sample was subjected to a nitrous acid treatment (pH 1.5) to remove heparin/HS as described previously (34), and the resultant HS fragments were removed by gel filtration on a Superdex® 75 column (10 × 300 mm; Amersham Biosciences) eluted with 0.2 M NH₄HCO₃ at a flow rate of 0.4 ml/min. The elution was monitored by absorption at 210 nm. The fraction eluted in the void volume was pooled and freeze-dried repeatedly by reconstituting in water to remove NH₄HCO₃. This CS/DS preparation was fractionated by anion exchange chromatography on an Accell QMA Plus cartridge (Waters, Milford, MA) and eluted stepwise with 0.3 M phosphate buffers (pH 6.0) containing 0.2, 1.0, 1.5, and 2.0 M NaCl. The fractions obtained by elution with 1.0 and 1.5 M NaCl, referred to as SL-CS/DS (1.0 M) and SL-CS/DS (1.5 M), respectively, were desalted using a PD-10 column (Amersham Biosciences), and each fraction was analyzed by the carbazole reaction for the amount of CS/DS (35). Finally, the SL-CS/DS preparations were passed through a Sep-PakC₁₈ cartridge (Waters) to remove peptides.

**Determination of the Disaccharide Composition and Molecular Mass**—An aliquot (1 µg as GAG) of SL-CS/DS (1.0 M) or (1.5 M) was subjected to digestion with CSase ABC, a mixture of CSases AC-I and AC-II, or CSase B. Each digest was labeled with 2-amino-6-benzenamine (2AB) and subjected to anion exchange HPLC on an amine-bound silica PA-03 column (YMC-Pack PA, Kyoto, Japan) as described previously (28). To determine the molecular mass, an aliquot (5 µg as GlcUA) of SL-CS/DS (1.0 and 1.5 M fractions) was chromatographed by gel filtration on a Superdex™ 200 column (10 × 300 mm; Amersham Biosciences), which had been calibrated using a series of size-defined commercial polysaccharides (36). The sample was eluted with 0.2 M ammonium bicarbonate at a flow rate of 0.3 ml/min for 90 min. Fractions were collected at 3-min intervals, freeze-dried, and dissolved in 100 µl of water. An aliquot was utilized for estimating the total amount of GAGs using 1,9-di-
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methyImethylene blue according to the method of Chandrasekhar et al. (37), except that the absorbance was recorded at 525 nm.

**Interaction Analysis**—Inhibition of PTN binding to a PTN high affinity fraction derived from embryonic pig brain (E-CS/DS-H) was examined using a BIACore® system (BIACore AB, Uppsala, Sweden). E-CS/DS-H was immobilized on a sensor chip as previously described (32). PTN (100 ng) was mixed with 0.5 μg of each tested GAG preparation (SL-CS/DS fractions or commercial CS/DS preparations) and incubated for 15 min at room temperature prior to injection onto the surface of an E-CS/DS-H-immobilized sensor chip. Results are expressed as relative percentages of inhibition based on the binding of PTN to E-CS/DS-H in the absence of inhibitor as 100%. To examine the interaction with various growth factors, SL-CS/DS (1.5 m) was immobilized on a sensor chip as reported earlier (38). For kinetic analysis, various concentrations of growth factors were injected onto the surface of this sensor chip in the running buffer (HBS-Ep, pH 7.4, BIACore AB) with a medium flow rate (30 μl/min) as per the manufacturer’s protocol. Each growth factor was allowed to interact with SL-CS/DS (1.5 m) for 2 min each for association and for dissociation, after which the sensor chip was regenerated by injecting 1 μl NaCl for 2 min before each injection. The kinetic parameters were evaluated with BIACalculator® software (BIACore AB) using a 1:1 binding model with mass transfer. To investigate the structural characteristics of the putative functional epitopes of SL-CS/DS (1.5 m) for the binding to various growth factors, an aliquot (10 μg as GAG) was digested with 10 mIU each of CSase A, a mixture of CSases AC-I and AC-II, or CSase B and labeled with 2-AB as described above. Each digest was dissolved in 200 μl of 0.2 M NaHCO₃ and analyzed by gel filtration chromatography on a Superdex™ Peptide HR column (10 × 300 mm; Amersham Biosciences), which was eluted with 0.2 M NaHCO₃ at a flow rate of 0.4 ml/min, being monitored with a RF-10A Shimadzu fluorescent detector.

**RESULTS**

**Preparation of the CS/DS Fractions from Shark Liver**—GAGs were extracted from shark liver by protease digestion and alkali treatment, recovered by ethanol precipitation, and purified by anion exchange chromatography on a DEAE-Sephadex column. The GAG preparation thus obtained was treated with nitrous acid followed by gel filtration to remove heparin/HS (~50% of all GAG). No significant hyaluronic acid-derived oligosaccharides were found by anion exchange HPLC in the digests of the CS/DS preparations treated with CSase ABC or hyaluronidase (data not shown). This preparation was further fractionated by anion exchange chromatography using an Accel™ Plus QMA cartridge, which was eluted stepwise with buffers containing 0.2, 1.0, 1.5, and 2.0 M NaCl. Only trace amounts of CS/DS were detected in the fractions eluted with buffers containing 0.2 and 2.0 M NaCl. 80 and 20% of the total CS/DS were detected in the fractions eluted with buffers containing 1.0 and 1.5 M NaCl and designated as SL-CS/DS (1.0 m) and SL-CS/DS (1.5 m), respectively. These preparations were passed through a C₁₈ cartridge to remove peptides.

**Determination of the Molecular Mass of Shark Liver CS/DS**—The molecular mass of SL-CS/DS preparations was determined by gel filtration (Fig. 1). Using the calibration curve generated with standard polysaccharides, the average molecular mass of SL-CS/DS (1.5 m) was estimated to be 75.7 kDa, whereas SL-CS/DS (1.0 m) gave a broader peak with a molecular mass ranging from 3.8 to 38.9 kDa. The distinct sizes of these two preparations may suggest different structures and functions. Interestingly, the mass of SL-CS/DS (1.5 m) was comparable with that of shark skin CS/DS (SS-CS/DS) preparations (28), and the large molecular mass may be characteristic of shark CS/DS.

**Analysis of the Disaccharide Composition**—The disaccharide composition of SL-CS/DS fractions was determined by digestion with CSases differing in specificity, followed by anion
exchange HPLC. The two SL-CS/DS preparations were individually digested with CSase ABC, a mixture of CSases AC-I and AC-II, or CSase B. Each digest was labeled with a fluorophore, 2AB, for high sensitivity and resolution and analyzed by anion exchange HPLC. The analysis revealed a unique and heterogeneous disaccharide composition with diverse sulfation patterns for both SL-CS/DS (1.0 M) and SL-CS/DS (1.5 M) as shown in Fig. 2 and Table 1. Both fractions showed $\Delta^{4,5}$HexUAα1–3GalNAc (ΔO), $\Delta^{4,5}$HexUAα1–3GalNAc(6S) (ΔC), $\Delta^{4,5}$HexUAα1–3GalNAc(4S) (ΔΔ), $\Delta^{4,5}$HexUA(2S)α1–3GalNAc(6S) (ΔD), $\Delta^{4,5}$HexUA(2S)α1–3GalNAc(4S) (ΔB), and $\Delta^{4,5}$HexUAα1–3GalNAc(45,6S) (ΔE) in varying proportions (Table 1), where 2S, 4S, and 6S stand for 2-, 4-, and 6-O-sulfate and 4S, 6S stands for 4,6-sulfate, respectively. Note that a small yet appreciable proportion of the $\Delta^{4,5}$HexUA(2S)α1–3GalNAc(45,6S) (ΔT) unit was detected in SL-CS/DS (1.5 M). SL-CS/DS (1.0 M) was relatively low sulfated due to significant proportions of nonsulfated disaccharides, ΔC (31.8%) and ΔA (33.1%), with a sulfate/disaccharide unit ratio of 1.18, whereas SL-CS/DS (1.5 M) was enriched with oversulfated disaccharides, ΔB (18%), ΔE (22.8%), and ΔT (1.8%), with a sulfate/disaccharide unit ratio of 1.43 (Table 1). Thus, an unique composition was revealed for SL-CS/DS (1.0 M) with three kinds of disulfated disaccharide units (ΔB, ΔD, and ΔE) for SL-CS/DS (1.5 M) with small proportions of ΔD and ΔT in addition to significant proportions of ΔB and ΔE.

To discriminate GlcUA- or IdoUA-containing disaccharides in CS/DS chains, an analysis was also carried out using a mixture of CSases AC-I and AC-II, which specifically digest GalNAc-GlcUA linkages in the CS structure (42), and CSase B, which specifically attacks GalNAc-IdoUA linkages in the DS structure (43). Both digestions yielded most of the unsaturated disaccharide units except for ΔO, ΔC, and ΔT. Nonsulfated units appear to exist as O units rather than iO units. However, the fact that no ΔO was observed in the CSase B digest of the two preparations may be partially due to the resistant nature of the iO unit to CSase B (44) and may not necessarily indicate the absence of this unit. Interestingly, the rare B unit was demonstrated for both preparations, as in the case of SS-CS/DS (28), and may be a unique feature of shark CS/DS. Another interesting feature is the obvious presence of iC units in the 1.0 M fraction but not in the 1.5 M fraction, although no C units were found in either. It is interesting that the ΔT unit for SL-CS/DS (1.5 M) was almost completely recovered in the CSase B digest and not in the digest obtained with a mixture of CSases AC-I and AC-II, suggesting that ΔT was derived exclusively from IdoUA(2S)α1–3GalNAc(45,6S) (iT unit), not from GlcUA(2S)α1–3GalNAc(45,6S). Thus, the composition of both preparations, although highly heterogenous, is distinct.

These results also clearly revealed a significantly higher proportion of GlcUA than IdoUA in SL-CS/DS (1.0 M) with a molar ratio of 1.56:1 (GlcUA/IdoUA), whereas SL-CS/DS (1.5 M) showed a higher proportion of IdoUA with a molar ratio of 3.35:1 (IdoUA/GlcUA). In this context, these two SL-CS/DS
Inhibitory effects of SL-CS/DS and commercial CS and DS preparations on the binding of PTN to the immobilized embryonic pig brain CS/DS fraction with high PTN binding activity. A fixed concentration of PTN (100 ng) was mixed with SL-CS/DS (1.0 M), SL-CS/DS (1.5 M), CS-E, or E as an inhibitor at a final concentration of 0.5 μg/ml and incubated for 15 min. The interaction of PTN with the immobilized embryonic pig brain CS/DS fraction, which has high affinity for PTN (E-CS/DS-H), was analyzed using a BIAcore J system as described under “Experimental Procedures.” Results are expressed as relative percentages of inhibition compared with the binding of PTN to E-CS/DS-H in the absence of inhibitors.

TABLE 1
Disaccharide composition of the SL-CS/DS preparations

| Unsaturated disaccharide | SL-CS/DS (1.0 M) pmol (mol %) | SL-CS/DS (1.5 M) pmol (mol %) |
|--------------------------|-------------------------------|-------------------------------|
| ΔDi-OS                   | 15.5 (6.3)                    | 6.9 (2.6)                     |
| ΔDi-6S                   | 78.1 (31.8)                   | 11.7 (4.3)                    |
| ΔDi-4S                   | 81.4 (33.1)                   | 132.7 (49.4)                  |
| ΔDi-diS                  | 10.5 (4.3)                    | 2.8 (1.0)                     |
| ΔDi-diSB                 | 16.7 (6.8)                    | 48.2 (18.0)                   |
| ΔDi-diSE                 | 45.4 (17.7)                   | 61.2 (22.8)                   |
| ΔDi-triS                 | 245.6                         | 4.9 (1.8)                     |
| Total                    | 268.3                         | 118.9                         |

*S/unit, a molar ratio of sulfate to disaccharide.

fractions have a unique GlcUA/IdoUA composition and might have distinct functional properties. In view of the importance of A/iA, B/iB, and E/iE units for the growth-factor-binding and NOP activities of E-CS/DS-H (32), SL-CS/DS preparations rich in iA, iB, and iE units may exert strong biological activities in growth-factor-binding and neurotogenesis.

Demonstration of Strong Binding of PTN to SL-CS/DS—To investigate biological activities of the purified SL-CS/DS preparations, their possible inhibitory activity was evaluated against the binding of PTN to the PTN-high affinity fraction of embryonic pig brain-derived CS/DS (E-CS/DS-H) using a BIAcore system (Fig. 3). This combination of PTN and CS/DS was used to evaluate biological activities, because E-CS/DS-H interacts strongly with PTN and also promotes the outgrowth of neurites in hippocampal neurons in vitro (32). Compared with various commercial CS and DS preparations, such as CS-E (sulfate/ disaccharide unit ratio = 1.53), SL-CS/DS (1.5 M) showed stronger inhibition of the PTN binding to E-CS/DS-H (Fig. 3). In strong contrast, SL-CS/DS (1.0 M) showed no significant inhibition. These results have suggested the presence of a PTN-binding domain in the SL-CS/DS (1.5 M) but not in the SL-CS/DS (1.0 M) chains.

Demonstration of the Binding Activity of SL-CS/DS (1.5 M) toward Various Growth Factors Expressed during Brain Development—Based on the finding that SL-CS/DS (1.5 M) strongly binds PTN, we speculated that this SL-CS/DS preparation might interact with other growth factors involved in the brain’s development. Hence, an analysis was carried out using the BIAcore system. The purified SL-CS/DS (1.5 M) preparation was biotinylated and immobilized on the streptavidin-precoated sensor chip. To determine the association and dissociation rate constants (k_a and k_d) as well as the dissociation equilibrium constants (K_d), various heparin-binding growth factors (PTN, MK, HB-EGF, VEGF_165, HGF, FGF-7, or FGF-1) were injected at different concentrations individually onto the surface of a sensor chip coated with SL-CS/DS (1.5 M). Overlaid sensograms are shown in Fig. 4. These sensograms were analyzed collectively by using “the 1:1 Langmuir binding model with mass transfer” of the BiAevaluation 3.1 software to calculate the kinetic parameters. The kinetic parameters are summarized in Table 2 for all the growth factors except FGF-1, which exhibited only a weak binding response.

The tested growth factors varied in their ability to bind SL-CS/DS (1.5 M). HGF, FGF-2, MK, and PTN displayed quick binding and a slow dissociation, giving K_d values in the low nanomolar range and signifying their strong affinity for SL-CS/DS (1.5 M) (Table 2). In contrast, HB-EGF, VEGF_165, and FGF-7 showed weaker affinity for SL-CS/DS (1.5 M) as reflected in the K_d values listed in Table 2. These differences in affinity support the specificity of the interactions between SL-CS/DS (1.5 M) chains and various heparin-binding growth factors, suggesting a biological significance of these interactions.

NOP Activity of the SL-CS/DS Preparation—That SL-CS/DS (1.5 M) chains specifically interacted with some of the growth factors involved in the brain’s development suggests that they may possess NOP activity. To evaluate the NOP activity of SL-CS/DS preparations, embryonic day 16 mouse hippocampal...
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neuronal cells were utilized. The cells were cultured on a substrate coated with SL-CS/DS (1.5 M), SL-CS/DS (1.0 M), CS-E (a positive control), or P-ORN alone (a negative control). The length of the longest neurite of each of 100 randomly selected cells cultured on each substrate was measured. The neuronal cells cultured on the P-ORN substrate had some short neurites; however, their length was not significant (Fig. 5, A and bottom). In contrast, the neuronal cells cultured on coverslips precoated with SL-CS/DS (1.5 M) exhibited striking NOP activity (Fig. 5, D and bottom), showing neurites axonic in nature with stronger activity than a positive control CS-E (Fig. 5, B and bottom). It is interesting that although SL-CS/DS (1.5 M) and CS-E have comparable sulfate/unit ratios, 1.43 and 1.53, respectively, the former displayed stronger activity, suggesting that the types and sequential arrangement of oversulfated disaccharides are important. In contrast, SL-CS/DS (1.0 M) (Fig. 5, C and bottom) showed weak yet significant NOP activity stronger than the negative control of P-ORN (Fig. 5, A and bottom). These results suggest that the two preparations from SL-CS/DS exert stimulatory effects on hippocampal neurons to different extents.

Investigation of the Distribution of GlcUA and IdoUA in the SL-CS/DS (1.5 M) Chains—Previously, our group showed IdoUA-containing CS/DS hybrid structures for E-CS/DS (26), hagfish notochord CS-H (27), and SS-CS/DS (28) as a key func-

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**Figure 6.** Analysis of the distribution of CS and DS domains in SL-CS/DS (1.5 M) chains on a Superdex peptide column. The purified SL-CS/DS (1.5 M) (1 μg as GAG) was digested with CSase ABC (A), a mixture of CSases AC-I and AC-II (B), or CSase B (C), and digests were individually labeled with ZAB and analyzed on a Superdex peptide column as described under “Experimental Procedures.” The elution positions of ZAB-labeled authentic unsaturated CS-derived standard disaccharides/oligosaccharides described in the legend to Fig. 1. The elution positions of ZAB-labeled authentic unsaturated CS-derived standard disaccharides/oligosaccharides determined earlier (22) are indicated by arrows as follows: 1, CS-decasaccharides; 2, CS-octasaccharides; 3, CS-hexasaccharides; 4, CS-tetrasaccharides; 5, trisulfated CS-disaccharides; 6, disulfated CS-disaccharides; 7, monosulfated CS-disaccharides; 8, nonsulfated CS-disaccharides.

**Table 3.** The sulfation pattern of CS/DS is crucial to the growth factor-binding and NOP activities of SL-CS/DS (1.5 M). The sequence of CS/DS (1.5 M) was analyzed by ZAB labeling, and the resultant preparation was used for assay of growth factor-binding and NOP activities of SL-CS/DS (1.5 M).

**Figure 7.** Fluorescence intensity at 530 nm, plotted against time (min), for various digests of SL-CS/DS (1.5 M) with CSases AC-I and AC-II, or CSase B were further evaluated, first by assaying inhibition of the binding of growth factors to the SL-CS/DS-immobilized sensor chip using a BIACore system as shown in Fig. 6A. Digestion of SL-CS/DS (1.5 M) with CSase ABC or B almost completely abolished its inhibitory activity against most of the growth factors binding to SL-CS/DS. In contrast, only 10–30% inhibition was observed with a CSase AC-I/AC-II digest for all of the ligands used. Subsequently, NOP assays were carried out to confirm the different contributions of the CS and DS moieties in SL-CS/DS (1.5 M). Enzyme digests were individually coated on P-ORN-precoated coverslips, and then hippocampal neuronal cells were cultured (Fig. 7, top). As expected, the NOP activity of SL-CS/DS (1.5 M) was completely eliminated by digestion with CSase ABC or B, and the activity was dramatically reduced to the basal level, comparable with that for P-ORN alone, whereas digestion with a mixture of CSases AC-I and AC-II resulted in only a partial loss of the activity (Fig. 7, bottom). The results confirmed the key role of CSase ABC-containing DS domains in the growth factor-binding and NOP activities of SL-CS/DS (1.5 M).

**Figure 8.** Analysis of the distribution of the DS-like structure in the NOP Activity of SL-CS/DS (1.5 M). The sulfation pattern of CS/DS is crucial to the growth factor-binding and NOP activities of SL-CS/DS (1.5 M). To clarify this viewpoint, the bioactivities of digests of SL-CS/DS (1.5 M) obtained with CSase ABC, a mixture of CSases AC-I and AC-II, or CSase B were further evaluated, first by assaying inhibition of the binding of growth factors to the SL-CS/DS-immobilized sensor chip using a BIACore system. As shown in Fig. 7, top, digestion of SL-CS/DS (1.5 M) with CSase ABC or B almost completely abolished its inhibitory activity against most of the growth factors binding to SL-CS/DS. In contrast, only 10–30% inhibition was observed with a CSase AC-I/AC-II digest for all of the ligands used. Subsequently, NOP assays were carried out to confirm the different contributions of the CS and DS moieties in SL-CS/DS (1.5 M). Enzyme digests were individually coated on P-ORN-precoated coverslips, and then hippocampal neuronal cells were cultured (Fig. 7, top). As expected, the NOP activity of SL-CS/DS (1.5 M) was completely eliminated by digestion with CSase ABC or B, and the activity was dramatically reduced to the basal level, comparable with that for P-ORN alone, whereas digestion with a mixture of CSases AC-I and AC-II resulted in only a partial loss of the activity (Fig. 7, bottom). The results confirmed the key role of CSase ABC-containing DS domains in the growth factor-binding and NOP activities of SL-CS/DS (1.5 M).
Taken together, the DS and/or DS/CS domains containing oversulfated disaccharides, iB and/or iE/E, might be involved in forming functional sequences for the NOP activity of SL-CS/DS (1.5 M).

Demonstration of the HGF and PTN Signaling Pathways in the NOP Activity of SL-CS/DS (1.5 M)—SL-CS/DS (1.5 M) specifically interacts with HGF, MK, PTN, and bFGF, which have neurotrophic activity and are widely expressed as autocrine and paracrine pleiotropic factors in the distinct neuronal cell populations of the developing and adult brain, including hippocampal neuronal cells (45–49). Hence, these endogenous heparin-binding growth factors may be involved in the mechanism of the expression of the NOP activity of SL-CS/DS (1.5 M). To examine this possibility, antibodies against HGF, PTN, MK, or bFGF were individually added to the system for the neutralization assay of the NOP activity of SL-CS/DS (1.5 M) toward hippocampal neurons from an embryonic mouse brain. The addition of anti-HGF or anti-PTN antibody markedly suppressed the NOP activity of SL-CS/DS (1.5 M) to the basal level of P-ORN (Fig. 9A). In strong contrast, the anti-MK or bFGF antibody showed no significant inhibition. These results suggest
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FIGURE 9. Effects of antibodies against growth factors and their receptors on the NOP activity of SL-CS/DS (1.5 m) and E-CS/DS. E16 mouse hippocampal neuronal cells were seeded on P-ORN-coated coverslips precoated with SL-CS/DS (1.5 m) (A) or E-CS/DS (B). 2 h after the seeding, anti-HGF (3 µg/ml), anti-PTN (10 µg/ml), anti-MK (20 µg/ml), or anti-bFGF (10 µg/ml) was added to the culture, and the mean length of the longest neurite was evaluated under each set of conditions as described in the legend to Fig. 5. Since antibodies against HGF and PTN inhibited the NOP activity of the SL-CS/DS (1.5 m) or E-CS/DS preparation, anti-HGF receptor and anti-PTN receptor (ALK) antibodies were also tested separately. In parallel, the same amount of a IgG fraction purified from the corresponding host animal (mouse, goat, or rabbit) was used as a control, which showed no significant inhibition in the mean length of the longest neurite measured for SL-CS/DS (1.5 m) or E-CS/DS without the antibodies (data not shown). **, p < 0.01; ***, p < 0.001 (For the significance of differences, see the legend to Fig. 5).

that the NOP activity of SL-CS/DS (1.5 m) is selectively mediated by HGF and PTN, whereas MK and bFGF do not mediate the NOP activity of SL-CS/DS and may not be directly involved in the neuritogenesis of hippocampal neuronal cells through interactions with brain CS/DS chains either.

To prove the involvement of the signaling pathways of HGF and PTN in the NOP activity of the CS/DS chains, effects of the antibodies against the receptors for HGF (c-Met) and for PTN (ALK) were investigated. The anti-c-Met and anti-ALK antibodies inhibited the NOP activity of SL-CS/DS (1.5 m) by 60 and 56%, respectively, revealing the involvement of the HGF and PTN signaling pathways in the NOP activity of the sugar chains (Fig. 9A).

To investigate the in vivo mechanism of the NOP activity of brain CS/DS, SL-CS/DS (1.5 m) was replaced by E-CS/DS, which was prepared from embryonic pig brain (26), in the above mentioned inhibition assay using the antibodies against the growth factors and receptors. The NOP activity of E-CS/DS was also strongly suppressed by the antibody against HGF, PTN, c-Met, and ALK by 58, 45, 47, and 38%, respectively, but barely by the antibody against MK or bFGF (Fig. 9B). These results suggest the involvement of the signaling pathways of HGF and PTN in the expression of the NOP activity of the embryonic brain CS/DS in vivo.

DISCUSSION

The growth factor-binding activities of CS/DS chains exhibit a positive correlation with their NOP activity. Some oversulfated CS and DS, which can interact with various brain-derived growth factors and neurotrophic factors, show significant NOP activities (24, 25, 27, 28). In this study, it was demonstrated for the first time that both the signaling pathways of PTN-ALK and HGF-cMet are involved in the NOP activities of CS/DS hybrid chains isolated from E16 embryonic mouse brain and of SL-CS/DS (1.5 m). Pleiotrophin uses protein-tyrosine phosphatase ζ and ALK as its receptors. Although the former, which is a CS proteoglycan, has been shown to be involved in neuritogenesis through the CS chains (31), ALK has also been demonstrated here to be involved in the NOP activity of E-CS/DS. Notably, E-CS/DS and SL-CS/DS chains immobilized on the P-ORN-coated substrate served as a scaffold to recruit endogenous PTN and HGF in the culture system and to stimulate neuronal cells, promoting neuritogenesis.

PTN, MK, HGF, and bFGF, examined in this study, are broadly expressed in the brain, including hippocampal neuronal progenitor cells, and implicated in the development of the brain (45–49). The NOP activity of SL-CS/DS (1.5 m) and E-CS/DS was significantly suppressed by antibodies against PTN or HGF and also by antibodies against their respective receptors (ALK and cMet) but not by anti-MK or anti-bFGF antibody. These results suggested that endogenous PTN and HGF were recruited by E-CS/DS and SL-CS/DS and mediated their NOP activities. Although ALK has been identified as a common receptor for PTN (50) and MK (51), it appears to be involved in the NOP activity of CS/DS through PTN signaling. MK and bFGF may be important for the survival or adhesion of neuronal cells. Anti-HGF antibody showed much stronger inhibitory activity than anti-PTN antibody, suggesting that HGF plays a more crucial role in the CS/DS-generated signaling to promote neuritogenesis.

HGF is a pleiotropic factor. It binds to and activates the tyrosine kinase receptor cMet and is also an axonal chemoattractant and neurotrophic factor for motor neurons (52, 53). It is required for the axonal growth of dorsal root ganglia sensory neurons (54) and elicits multiple functions in sympathetic neurons (55–57). HGF signaling potentiates the response of different neurons to specific signals (52). GAGs are essential co-re-
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59% iC but only less than 1% iA, whereas SL-CS/DS (1.5 m) contains 42.6–49.4 mol % of iA. In addition, ΔA was observed with increasing proportions of ΔB and ΔE in the high PTN affinity fraction of E-CS/DS and SS-CS/DS and with a decrease in ΔC. The greater NOP activity of SL-CS/DS (1.5 m) than CS-E (Fig. 5) suggests that iB, iE, and iA units are preferred for the PTN binding activity and also for the NOP activity of CS/DS chains.

Previously, it was demonstrated that anti-PTN antibody strongly suppressed the endogenous PTN-mediated NOP activity of E-CS/DS chains with low affinity for PTN (E-CS/DS-L) but did not significantly inhibit that of E-CS/DS with high affinity for PTN (E-CS/DS-H) (32). Hence, it seems that PTN mediates the NOP activity of E-CS/DS-L but not E-CS/DS-H. A series of octasaccharides containing at least one D unit have been isolated from E-CS/DS-L (32). Although a signaling molecule responsible for the NOP activity of E-CS/DS-H remains to be identified, the possibility exists that PTN and other growth factors share the binding sites in E-CS/DS-H chains. The binding of other growth factors to the putative overlapping binding sites on E-CS/DS-H chains may be involved in the neuritogenesis. The specific interaction of SL-CS/DS (1.5 m) with PTN, MK, HGF, and bFGF implies the existence of such overlapping binding sites for these growth factors. HGF is a strong candidate for a factor involved in the NOP activity of E-CS/DS-H.

SL-CS/DS (1.5 m) is a potential candidate for a non-mammal-derived therapeutic agent. Further investigation of the functional domains of SL-CS/DS (1.5 m) involved in PTN and HGF signaling should provide a basis for developing specific oligosaccharide drugs with fewer side effects than the parental polysaccharides.

Acknowledgment—We thank Dr. Toshikazu Nakamura (Osaka University) for the anti-rat HGF rabbit IgG and technical advice.

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