Novel 70-kDa Chondroitin Sulfate/Dermatan Sulfate Hybrid Chains with a Unique Heterogenous Sulfation Pattern from Shark Skin, Which Exhibit Neuritogenic Activity and Binding Activities for Growth Factors and Neurotrophic Factors*

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Chondroitin sulfate (CS) and dermatan sulfate (DS) hybrid chains of proteoglycans are critical in growth factor binding, neuritogenesis, and brain development. Here we isolated CS/DS hybrid chains from shark skin aiming to develop therapeutic agents. Digestion with various chondroitinases showed that both GlcUA- and IdoUA-containing disaccharides are scattered along the polysaccharide chains with an unusually large average molecular mass of 70 kDa. The CS/DS chains were separated into major (80%) and minor (20%) fractions by anion-exchange chromatography. Both fractions had relatively low degrees of sulfation (sulfate/disaccharide molar ratio = 1.17 versus 0.87), showing a unique feature compared with the marine CS and DS isolated to date, most of which are oversulfated. They were highly heterogeneous and characterized by multiple disaccharides including GlcUA-GalNAc, GlcUA-GalNAc(6S), GlcUA-GalNAc(4S), IdoUA-GalNAc(4S), GlcUA-GalNAc(4S,6S), IdoUA-GalNAc(4S,6S), GlcUA(2S)-GalNAc(6S), and/or IdoUA(2S)-GalNAc(6S), IdoUA(2S)-GalNAc(4S) and novel GlcUA(2S)-GalNAc(4S), where 2S, 4S, and 6S represent 2-O-, 4-O- and 6-O-sulfate, respectively. The CS/DS chains bound two neurotrophic factors and various growth factors expressed in the brain with high affinity as evaluated for the major fraction by kinetic analysis using a surface plasmon resonance detector, and also promoted the outgrowth of neurites of both an axonic and a dendritic nature. The neuritogenic activity was abolished completely by digestion with chondroitinase ABC, AC-I, or B, suggesting the importance of both GlcUA- and IdoUA-containing moieties. It also showed anti-heparin cofactor II activity comparable to that exhibited by DS from porcine skin. Thus, by virtue of its unique structure and biological activities, DS will find a potential use in therapeutics.

Dermatan sulfate (DS) proteoglycans are widely distributed in most tissues including skin and have been implicated in various biological processes including cell adhesion, proliferation, interactions with various growth factors, and wound repair. DS glycosaminoglycan (GAG) is particularly recognized for its antithrombotic activity, exhibited through a mechanism different from that of heparin (Hep) (5). DS often occurs in co-polymers with chondroitin sulfate (CS) consisting of varying proportions of -4GlcUA-3GalNAcβ1- and -4idoUAα1-3GalNAcβ1- variably sulfated on both hexuronic acid and GalNAc residues, thus forming a hybrid structure (6). CS and DS have the potential to display enormous structural diversity, comparable to that of HS, thereby embedding multiple overlapping sequences constructed with distinct disaccharide units modified by different patterns of sulfation (7). This heterogeneity is the structural basis for the diverse biological functions of DS (8).

Our laboratory has been elucidating the structural and functional aspects of CS isoforms, showing the importance of CS GAGs from a simple molecule such as chondroitin in the cell division of a nematode (9) to differentially oversulfated CS chains (10–12) and DS chains (13) in neural development, thus highlighting the importance of the rare oversulfated disaccharide units, D (GlcUA(2S)-GalNAc(6S)), iD (IdoUA(2S)-GalNAc(6S)), E (GlcUA-GalNAc(4S,6S)), and iE (IdoUA-GalNAc(4S,6S)), of CS and DS in various biological functions, where 2S, 4S, and 6S represent 2-O-, 4-O-, and 6-O-sulfate, respectively, and the i in iE stands for IdoUA. The neuritogenic properties exhibited by CS and DS chains are controversial with both inhibitory (14, 15) and promotive effects (16, 17) observed for various neurons. The discrepancies are most likely caused by their spatiotemporal distributions (18, 19) and developmental changes in structure and function (21, 65). The critical importance of IdoUA in neuritogenesis and growth factor binding and the developmental change of its expression in the brain have recently been demonstrated for CS/DS hybrid chains of pig embryos (22).

It has also been our endeavor to look for sources of GAGs with unique structures and prominent activity, which have potential as a therapeutic agent. This pursuit revealed the ability of structurally characterized oversulfated DS, named CS-H, from hagfish notochord (12, 23), which promotes neuritogenesis and growth factor binding, as well as the essentiality of both GlcUA- and IdoUA-containing moieties for these activ-

FGF, fibroblast growth factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; PTN, pleiotrophin; MK, midkine; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; HexUA, hexuronic acid; 3,4,5-HexUA, 4,5-unsaturated hexuronic acid; ZAB, 2-aminobenzamide; CPC, cetyl pyridinium chloride; DMBB, 1,9-dimethylmethylene blue; P-ORN, poly-DL-ornithine; HPLC, high performance liquid chromatography.
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Ities (25). It is also noteworthy that DS is being used for other applications such as the preparation of artificial tissues (26).

In furthering the understanding of DS in relation to its structure and function, especially in light of the recent finding that relatively low sulfated CS/DS hybrids (the sulfate/disaccharide molar ratio (S/unit) = 0.83–0.84) with a considerable proportion (23–25%) of non-sulfated units and small proportions (1–2%) of oversulfated disaccharide units could promote growth factor binding and neurite outgrowth (22), we looked for other possible sources of CS/DS chains that are less sulfated than CS-H. Toward that end, DS chains with unique structural features and multiple biological activities were isolated from shark skin, which is an industrial waste with an immense potential to be exploited for pharmaceutical purposes. While a classical preliminary work on GAGs from the skin of blue shark and sandbar shark showed oversulfated DS with an S/unit of 1.42–1.62 and 1.26–1.37, respectively, in addition to hyaluronic (HA) and CS-C by conventional analyses of amino sugars, hexuronic acid, infrared spectrum, rotation etc. (27), no detailed structure or biological activities have been reported.

EXPERIMENTAL PROCEDURES

Materials—Chondroitinase ABC (EC 4.2.2.14), chondroitin AC-I (EC 4.2.2.5), chondroitin B (EC 4.2.2), chondroitin-4-sulfatase (EC 3.1.6.9), chondroitin-6-sulfatase (EC 3.1.6.10), hyaluronidase (EC 4.2.2.1) from Streptomyces hyalurolyticus, unsaturated disaccharides and bovine serum albumin were obtained from Seikagaku Corp. (Tokyo, Japan). Chondroitin B preparations were sometimes contaminated with chondroitin-4-sulfatase and hence each lot was examined for contamination. In addition, chondroitin B was also obtained from Sigma. Actinase E was from Kaken Pharmaceutical Co. (Tokyo, Japan). 2-Aminobenzamidine (2AB) was purchased from Nacalai Tesque (Kyoto, Japan). EZ-Link™ biotin-LC-hydrazide was obtained from Pierce. Prepacked acetate and left at 4 °C overnight, after which the precipitate was precipitated by adding 4 volumes of 80% ethanol containing 5% sodium acetate. The precipitate was removed by extraction with diethyl ether, and the GAGs were resuspended in 5% trichloroacetic acid, and centrifuged again. The digestion continued. After a total of 95 h of digestion, proteins were respectively, and subjected to digestion with a protease (actinase) (2%)

Disaccharide Composition Analysis—The disaccharide composition was analyzed by digesting with chondroitinase ABC (34), B (35), or AC-I (36). Briefly, 1 μg or 3.6 μg of the SS-DS preparation was digested with either 10 milli-international units of chondroitinase ABC, 5 milli-international units of chondroitinase B, and then each digest was individually labeled with 2AB according to the method of Kinoshita and Sugahara (37), except that excess 2AB was removed by repeated extraction with a water/chloroform mixture (1:1, v/v) (38). The 2AB-labeled disaccharides were diluted to 400 μl with 16 mM NaH2PO4 and an aliquot analyzed by anion-exchange HPLC on a PA-03 silica column (YMC-Pack PA, Kyoto, Japan) using a solvent system of 16 and 530 mM NaH2PO4 over a period of 1 h by fluorescent detection. The analysis using 2AB labeling is superior to the conventional method for the following reasons. First, the separation of all three disaccharides was (1–3)-GalNAc(4S,6S), Δ3HexUA(2S)1–3GalNAc(4S,6S) and Δ3-HexUA1–3GalNAc(4S,6S) was achieved only after labeling with 2AB (Fig. 1), while unlabeled counterparts, Δ3HexUA(2S)1–3GalNAc(4S) and Δ3-HexUA1–3GalNAc(4S,6S), were not separated from each other as monitored by measuring absorbance at 232 nm. Second, 2AB-derivatives of Δ3-HexUA1–3GalNAc and Δ3-HexUA1–3GalNAc derived from HA and CS, respectively, could be separated (Fig. 1) unlike the non-derivatized counterparts.

For digestion with 4- or 6-sulfatases, 1.6 μg of the SS-DS preparation was first digested with 10 milli-international units of chondroitinase ABC in a total volume of 10 μl. A one-third portion of the chondroitin ABC digest was subjected to digestion with 10 milli-international units of chondroitinase AC-I followed by chondroitin-4-sulfatase, and the digests were labeled with 2AB and analyzed by HPLC employing conditions mentioned above.

Gel Filtration Analysis of the Chondroitinase Digests of SS-DS on a Superdex Peptide Column—The SS-DS (Native) preparation (1.0 g) was digested with chondroitinase AC-I or B. An equal amount was also sequentially digested with either chondroitinase AC-I followed by B or chondroitinase B followed by AC-I. All the digests were individually digested with a water/chloroform mixture as above. The digests were made up with 0.2 m ammonium bicarbonate containing 7% 1-propyl alcohol and analyzed on a Superdex peptide column using the same buffer as eluent at a flow rate of 0.4 ml/min using fluorescence detection.

Molecular Mass Determination—The molecular mass of DS preparations was determined by gel filtration using a column of Superdex 200 (10 x 300 mm) calibrated with molecular mass markers including...
**RESULTS**

\[ \text{Fraction} \times \text{Yield} \times \text{Uronic acid content} \]

| Fraction | Yield (g) | Uronic acid content (%) |
|----------|-----------|-------------------------|
| Ethanol precipitate | 3.5 (4.2) | 12 |
| 1st CPC precipitate | 1.6 (1.9) | 21 |
| 2nd CPC precipitate | 0.4 (0.5) | 53 |
| Hyaluronidase digest | 0.17 (0.2) | 39 |
| Nitrous acid digest | 0.044 (0.05) | 33 |
| C18 fraction | 0.036 (0.04) | 33 |
| Anion-exchange | 0.007 (0.008) | 33 |

**Isolation and Purification of DS from Shark Skin**—GAGs were isolated from shark skin by exhaustive protease digestion followed by ethanol precipitation. The precipitate comprising a crude mixture of GAGs consisted of HA, DS, and a small amount of HS as examined by electrophoresis on a cellulose acetate membrane and disaccharide analysis after digestion with various GAG lyases (data not shown). It was purified further by CPC precipitation followed by digestion with *Streptomyces* hyaluronidase to remove the bulk of the HA, and nitrous acid treatment to degrade the HS. Since the preparative still showed small amounts of tetra- and hexasaccharides characteristic of HA on HPLC analysis of the hyaluronidase digest, HA was eventually removed by anion-exchange chromatography (see "Experimental Procedures"). Fractions obtained during each step of purification were monitored by electro-}

**Neurite Outgrowth Promotion Assays of the Purified SS-DS**—This was done as reported earlier (13, 18). Briefly, the coverslips were coated with 600 μl of 1.5 mg/ml poly-DL-ornithine (P-ORN) for 2 h at 37 °C and then incubated with 2 μg/ml of the SS-DS preparations or equivalent amounts of chondroitin ABC, AC-1, or B digested-SS-DS preparations and left at 37 °C overnight. The hippocampal neuronal cells freshly isolated from E16 mouse embryos were seeded in Eagle’s minimum essential medium at a density of 10,000 cells/mm² and allowed to grow for 24 h at 37 °C, 5% CO₂. Thereafter the cells were fixed and subjected to immunohistochemistry staining using monoclonal antibodies directed against neurofilament and microtubule-associated protein-2.

The immunostained cells on each coverslip were scanned and digitized with a ×20 objective lens on an optical microscope (BH-2, Olympus, Tokyo, Japan) equipped with a digital camera. The length of the longest neurite and the number of primary neurites of cells chosen at random was calculated using a morphological analysis software (Mac SCOPE, Mitani Corp., Tokyo, Japan).
respectively) including serine, aspartic acid, glutamic acid, glycine, alanine, and arginine at a ratio of 1.0:0.4:0.7:1.7:0.3:0.3. All the three SS-DS preparations were free of HS/Hep, which was confirmed by HPLC analysis of the heparan/heparitinase digest (data not shown).

**Disaccharide Composition Analysis of SS-DS—**The disaccharide composition of the purified SS-DS preparations was determined by digestion with chondroitinases followed by anion-exchange HPLC. The DS preparations were individually digested with chondroitinase ABC, AC-I, or B, and each digest was labeled with a fluorescent 2AB for high sensitivity analysis and high resolution (see “Experimental Procedures”).

The disaccharide analysis after digestion with chondroitinase ABC revealed that the SS-DS preparations are highly complex and heterogeneous. The enzyme digest of SS-DS (Native) is shown in Fig. 1A as a representative. All three preparations showed the presence of $\Delta^4$-HexUA(1-3)GalNAc, $\Delta^4$-HexUA(2S)(1-3)GalNAc(4S), $\Delta^4$-HexUA(1-3)GalNAc(4S,6S), $\Delta^4$-HexUA(2S)(1-3)GalNAc(4S,6S), and $\Delta^4$-HexUA(1-3)GalNAc(4S,6S) in varying proportions (Table II) with quantitative recoveries of disaccharides. The identity of all three disulfated disaccharides was confirmed by sequential digestion of chondroitin ABC digests with chondroitinase ABC or -6-sulfatase: $\Delta^4$-HexUA(1-3)GalNAc(4S,6S), and $\Delta^4$-HexUA(1-3)GalNAc(4S,6S) were sensitive to 6-sulfatase, whereas $\Delta^4$-HexUA(1-3)GalNAc(4S,6S) was sensitive to 4-sulfatase (data not shown) (47). The first and second major components in all the SS-DS preparations were $\Delta^4$-HexUA(1-3)GalNAc(4S) and $\Delta^4$-HexUA(1-3)GalNAc(4S) accounting for 48.8 and 21.3%, 43.5 and 21.3%, and 55.0 and 16.6% of the disaccharides in SS-DS (Native), SS-DS (1.0 M), and SS-DS (1.5 M), respectively (Table II). The SS-DS (1.0 M) fraction had a higher proportion of $\Delta^4$-HexUA(1-3)GalNAc (23.8%) than SS-DS (Native) (10.5%) and SS-DS (1.5 M) (5.8%). In contrast, SS-DS (1.5 M) contained more (22.6%) disulfated disaccharides than SS-DS (Native) (19.3%) and SS-DS (1.0 M) (11.4%). Consequently, SS-DS (1.0 M) was significantly undersulfated, whereas SS-DS (1.5 M) was modestly yet significantly oversulfated compared with SS-DS (Native), with an S/unit ratio of 1.17 for SS-DS (1.5 M) and 1.08 for SS-DS (Native) compared with 0.87 for SS-DS (1.0 M). Thus, SS-DS (Native) comprises a wide variety of DS chains with distinct degrees of sulfation. Most of the marine DS preparations isolated so far, including those from shark cartilage, squid cartilage, ascidians, sea urchin, and hagfish, are oversulfated (a molar ratio of sulfate to disaccharide (S/unit) = 1.2–1.9) with a large proportion of one of the disulfated disaccharides of E, I, D, I, or I(13, 44, 48, 49). In this context, SS-DS is rather unique in that it contains multiple disulfated disaccharide units in appreciable amounts although the S/unit ratio was 0.87–1.17 (Table I).

Dissociation of the SS-DS preparations with chondroitinase B resulted in $\Delta^4$-HexUA(1-3)GalNAc(4S) and $\Delta^4$-HexUA(2S)(1-3)GalNAc(4S) along with oligosaccharides. The enzyme digest of SS-DS (Native) is shown in Fig. 1B as an example. The disaccharides accounted for only 2% of the total amount of disaccharides obtained by chondroitinase ABC digestion of the equivalent amounts for all three SS-DS preparations (Table II). The release of only small proportions of $\Delta^4$-HexUA(1-3)GalNAc(4S) (2–7%) and $\Delta^4$-HexUA(2S)(1-3)GalNAc(4S) (11–23%) probably suggests that a majority of their parent units are present as GlcUA-GalNAc(4S) (A unit) and GlcUA(2S)- GalNAc(4S) (B unit) rather than IdoUA-GalNAc(4S) (Ia unit) or IdoUA(2S)-GalNAc(4S) (Ib unit), which are present only in smaller proportions (For the naming of the units, see Refs. 7 and 8). It is also assumed that a majority of the existing A units and B units do not form a cluster, and hence are resistant to digestion with chondroitinase B. Chondroitinase B did not release $\Delta^4$-HexUA(1-3)GalNAc(6S), suggesting either that all 6-O-sulfated units exist as GlcUA-GalNAc(6S) (C units) as some were detected after digestion with chondroitinase AC-I (see below) or that they exist as IdoUA-GalNAc(6S) (C units), which may be resistant to the enzyme action or embedded in the resistant sequences. In addition to disaccharides, major oligosaccharides were observed at 29 and 55 min along with a number of minor oligosaccharides (Fig. 1B), probably indicating the hybrid nature of the SS-DS chains. The non-sulfated disaccharide observed in the chondroitinase B digest was apparently generated by the action of 4-sulfatase contaminating the enzyme preparation on $\Delta^4$-HexUA(1-3)GalNAc(4S) units, which was confirmed using the chondroitinase B preparation devoid of 4-sulfatase (Table II).

Digestion with chondroitinase AC-I of SS-DS (Native) gave little or no disulfated disaccharides, but yielded non-sulfated and monosulfated disaccharides including $\Delta^4$-HexUA(1-3)GalNAc, $\Delta^4$-HexUA(1-3)GalNAc(6S), and $\Delta^4$-HexUA(1-3)GalNAc(4S) (Fig. 1C), which again accounted for only 10% of all the disaccharides in the SS-DS preparations (Table II). The
Oligosaccharides corresponding to hexa-, octa-, and decasaccharides, which were obtained from the digestion with chondroitinase ABC, B, or AC-I, and each digest was analyzed by gel filtration chromatography, the fraction corresponding to the disulfated disaccharides of the chondroitinase AC-I digest, which is marked by a horizontal bar in Fig. 2A, showed the presence of \( \Delta^4,5\text{HexUA}_{1-3}\text{GalNAc(4S,6S)} \) as a major and \( \Delta^4,5\text{HexUA}_{1-3}\text{GalNAc(4S)} \) as a minor constituent, respectively (Fig. 2A, inset), whereas the disulfated disaccharide fraction in the chondroitinase B digest (marked by a horizontal bar in Fig. 2B) had \( \Delta^4,5\text{HexUA}_{2S(1-3)}\text{GalNAc(4S)} \) as a major and \( \Delta^4,5\text{HexUA}_{1-3}\text{GalNAc(4S,6S)} \) as a minor component (Fig. 2B, inset). The identity of \( \Delta^4,5\text{HexUA}_{2S(1-3)}\text{GalNAc(4S)} \) and \( \Delta^4,5\text{HexUA}_{1-3}\text{GalNAc(4S,6S)} \) units in these enzyme digests was confirmed by co-chromatography with the authentic standards (data not shown). Although the disulfated disaccharides were not unambiguously detected in the chondroitinase AC-I digest of SS-DS (Native) by anion-exchange HPLC (Fig. 1C) apparently because of the smaller proportions compared with other disaccharides (Table II), they were clearly revealed when fractionated first on a Superdex Peptide column then on an anion-exchange column. These results suggest that at least some of the 4-O-sulfate and 6-O-sulfated units exist as A and C units. As in the case of the chondroitinase B digest, differences in the proportion of disaccharides were dependent on the SS-DS preparations. Along with the above mentioned disaccharides, a number of oligosaccharides were also observed, supporting the notion of the hybrid nature of SS-DS with a mixed sequential arrangement of GlcUA- and IdoUA-containing disaccharide units. Although it was assumed that more GlcUA is present than IdoUA in view of the greater susceptibility to chondroitinase AC-I (10%) than to chondroitinase B (2%), the precise molar ratio of IdoUA to GlcUA remains to be clarified.

**Table II**

| Disaccharide composition of the SS-DS preparations | SS-DS (1.0 M) | SS-DS (1.5 M) |
|-------------------------------------------------|--------------|---------------|
| SS-DS (Native) chondroitinasises | ABC | AC-I | ABC | AC-I | ABC | AC-I |
| **Disaccharides** | | | | | | |
| \( \Delta\text{Di-0S} \) | 31.5 | — | 2.5 | — | 71.5 | — | 4.2 | — | 17.4 | — | 1.3 |
| \( \Delta\text{Di-6S} \) | (10.5) | (8.1) | (15.0) | (23.5) | (15.0) | (23.5) | (15.0) | (23.5) | (15.0) | (23.5) |
| \( \Delta\text{Di-4S} \) | 146.5 | 3.3 | 12.5 | 130.5 | 4.2 | 16.7 | 165.0 | 3.4 | 14.0 |
| \( \Delta\text{Di-diS}_d \) | (48.9) | (51.6) | (40.6) | (43.5) | (70.0) | (55.7) | (55.0) | (46.7) |
| \( \Delta\text{Di-diS}_b \) | 9.0 | ND | ND | 6.3 | ND | ND | ND | ND |
| \( \Delta\text{Di-diSE} \) | 17.7 | 2.7 | 0.12 | 8.0 | 1.8 | nd | 24.1 | 2.6 | nd |
| \( \Delta\text{Di-diSE} \) | (5.9) | (42.3) | (0.40) | (2.7) | (30.0) | (8.0) | (43.3) |
| \( \Delta\text{Di-diSB} \) | 31.3 | 0.39 | 0.69 | 19.8 | nd | nd | 33.6 | nd | nd |
| \( \Delta\text{Di-diSE} \) | (10.4) | (6.1) | (2.2) | (6.6) | | | (11.2) | | |
| Total | 300 | 6.59 | 30.8 | 300 | 6.0 | 30.0 | 300 | 6.0 | 30.0 |
| pmol (mol%)a | — | — | 0.87 | — | — | 1.17 | — | — |
| a \( \Delta\text{Di-0S}, \Delta^4,5\text{HexUA}_{1-3}\text{GalNAc(4S)} \); \( \Delta\text{Di-4S}, \Delta^4,5\text{HexUA}_{1-3}\text{GalNAc(4S)} \); \( \Delta\text{Di-6S}, \Delta^4,5\text{HexUA}_{1-3}\text{GalNAc(4S)} \); \( \Delta\text{Di-diS}_d, \Delta^4,5\text{HexUA}_{1-3}\text{GalNAc(4S)} \); \( \Delta\text{Di-diS}_b, \Delta^4,5\text{HexUA}_{1-3}\text{GalNAc(4S)} \) |

The amounts of disaccharides obtained are given in picomoles, and the values in parentheses give molar proportions of the respective disaccharides obtained by integrating the peak areas.

b The amounts of disaccharides obtained are given in picomoles, and the values in parentheses give molar proportions of the respective disaccharides obtained by integrating the peak areas.

c ND, not detected.

d ND, not detected.

e The peaks were not detected clearly in Fig. 1, B and C, but were identified in Fig. 2, A and B.

f ND, not detected.

g ND, not detected.
h S/unit, a molar ratio of sulfate to disaccharide.
the elution positions of the above oligosaccharides were determined earlier (22). The column was developed at a flow rate of 0.4 ml/min and monitored by absorbance at 280 nm. The elution positions of standard disaccharides and oligosaccharides indicated by arrows represent the following: 1, CS-decascaccharides; 2, CS-octasaccharides; 3, CS-hexasaccharides; 4, CS-tetrasaccharides; 5, disulfated CS-disaccharides; 6, monosulfated CS-disaccharides; 7, unsulfated CS-disaccharides. The elution positions of the above oligosaccharides were determined earlier (22). The column was developed at a flow rate of 0.4 ml/min as described under “Experimental Procedures.” V₀ represents the void volume and the total volume, Vₜ, was 24 ml. The peaks formed represent the molar ratios of the respective oligosaccharides labeled with 2AB and analyzed on a column of Superdex 200, which had been calibrated using markers of known molecular mass as detailed under “Experimental Procedures.” All three preparations included in the column as monitored using a metachromatic dye, DMMB, with absorbance at 525 nm. V₀ and Vₜ were determined using dextran (average mass: 170–200 kDa) and NaCl, respectively. The diamonds, squares, and triangles show the elution profiles of SS-DS (Native), SS-DS (1.0 M), and SS-DS (1.5 M), respectively. The average molecular masses were estimated using the calibration curve (inset).

Molecular Size Determination of SS-DS—The average molecular masses of the purified SS-DS preparations were determined by gel filtration using a column of Superdex 200, which had been calibrated using markers of known molecular mass as detailed under “Experimental Procedures.” All three preparations were included in the column as monitored using a metachromatic dye DMMB (Fig. 3) to give a similar average molecular mass of 70 kDa. SS-DS (Native) and SS-DS (1.5 M) showed a fairly symmetrical peak compared with SS-DS (1.0 M), which showed a broader distribution of the molecular mass. The molecular masses of the SS-DS preparations are high compared with those of DS from hagfish notochord (18 kDa) (25), porcine skin (19 kDa), eel skin (14 kDa) (44), endocan of endothelial cells (30 kDa) (52), and pig brain (40 kDa) (22). Since CS-C chains from shark cartilage are also rather large (43–70 kDa) (53), the large molecular mass of SS-DS may be characteristic of CS and DS chains of certain tissues of shark.

Interaction of SS-DS with Various Hep-binding Growth Factors—Recently, it was demonstrated that IdoUA-containing units in CS/DS hybrid chains isolated from the pig brain were critical for binding growth factors (22), and oversulfated CS-E from squid cartilage interacted with high affinity with various Hep-binding growth factors in a comparable fashion to that of Hep (41). The CS-H preparation purified from hagfish notochord, which had a high proportion of IdoUA-containing iE units/GlcUA-containing E units, also interacted with multiple growth factors with high affinity (25). In light of these findings, we investigated using an IAAsys system whether the SS-DS preparation, which contains substantial proportions of IdoUA-

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2 X. Bao and K. Sugahara, unpublished results.
compared with Hep, SS-DS bound a larger number of most growth factors per polysaccharide chain. The specific binding capacity, which is expressed by the number of growth factor molecules bound to the same length of each GAG chain (equivalent to for example 30 disaccharide units), was still severalfold higher for SS-DS than for CS-H (the right column in Table III). Despite the lower values for the binding of these growth factors to SS-DS as compared with Hep, the highly specific binding capacity of SS-DS is indicative of the high sequence specificity considering the lower sulfation degree of SS-DS (S/unit = 1.17) than Hep (S/unit = 2.55).

To determine the binding affinity, growth factors at varying concentrations were allowed to interact with the immobilized SS-DS (1.5 nM) preparation, which represented the major fraction (80%) and was completely free of other GAGs such as HA or HS. The sensorgrams obtained with various growth factors are shown in Fig. 5, except for FGF-1 with a low binding response (see above). All the growth factors tested exhibited binding patterns typical of a growth factor. A plot of on-rate association (\(k_a\)) versus ligate concentration was obtained, from which \(K_{a,SS-DS}\) and \(K_{a,Hep}\) were calculated, and showed the high affinity binding of all the growth factors with SS-DS (Table IV). The highest affinity was exhibited toward FGF-18 (\(K_{a,SS-DS} = 4.4\) nM) and HB-EGF (\(K_{a,Hep} = 4.5\) nM) followed by PTN (\(K_{a,Hep} = 7.6\) nM) and FGF-10 (\(K_{a,SS-DS} = 8.6\) nM), reflecting higher association and lower dissociation rates, which resulted in lower equilibrium dissociation constants. MK showed the lowest association rate among the growth factors tested but the dissociation rate was comparable to that exhibited by FGF-10, PTN and HB-EGF, resulting in a somewhat lower affinity (\(K_d = 58.5\) nM). Interaction of PTN with DS (porcine skin), which contains exclusively IdoUA, has been reported, wherein a \(K_d\) of 51 nM was obtained in an IAsys system (54), whereas we have obtained a \(K_d,SS-DS\) of 7.6 nM for SS-DS (Table IV). The differences could be due to the structural differences in the DS preparations used and suggest the importance of the hybrid nature of SS-DS for the binding of PTN, being consistent with the recent observation about the CS/DS chains from embryonic pig brain (22).

Interestingly, the affinity of SS-DS (1.5 nM) for various growth factors was higher than that exhibited by Hep (Table IV) except for FGF-2 and HB-EGF, which showed a stronger or comparable affinity for Hep than for SS-DS, respectively. The high capacity, affinity and specificity of the binding of SS-DS toward these growth factors strongly suggest the importance of the hybrid nature of SS/DS.
CS-H presumably due largely to the CS/DS hybrid nature and partly to the larger chain size (Table III).

Kinetic analysis was done after overlaying the sensorgrams obtained for BDNF and GDNF (Fig. 6, B and C, respectively) by injecting at varying concentrations and fitting the sensorgrams globally as mentioned in “Experimental Procedures” to obtain the kinetic parameters, which are presented in Table V, revealing the high affinity binding of both factors to SS-DS. Their binding to SS-DS was characterized by higher association and dissociation rates compared with the binding to CS-H, which may indicate that SS-DS is a better binding partner than CS-H as described below in the “Discussion.” BDNF exhibited higher association and dissociation rates than GDNF.

Neurite Outgrowth-promoting Activity—Oversulfated CS

| Protein factors | Average molecular mass | Maximum binding to | Binding capacity |
|-----------------|------------------------|--------------------|-----------------|
|                 | kDa                    | SS-DS mmol/mol     | SS-DS mmol/30 disaccharides |
| FGF-2           | 17.5                   | 9.6                | 2.1             |
| FGF-10          | 24.0                   | 6.2                | 1.4             |
| FGF-18          | 28.0                   | 8.6                | 1.9             |
| HB-EGF          | 12.0                   | 13.8               | 3.1             |
| MK              | 13.4                   | 14.4               | 3.2             |
| PTN             | 15.4                   | 13.2               | 2.9             |
| BDNF            | 13.6                   | 11.1               | 2.4             |
| GDNF            | 20.0                   | 11.4               | 2.5             |

**TABLE III**

| Protein factors | Average molecular mass | Maximum binding to | Binding capacity |
|-----------------|------------------------|--------------------|-----------------|
|                 | kDa                    | SS-DS mmol/mol     | SS-DS mmol/30 disaccharides |
| FGFR-2          | 17.5                   | 9.6                | 2.1             |
| FGFR-10         | 24.0                   | 6.2                | 1.4             |
| FGFR-18         | 28.0                   | 8.6                | 1.9             |
| HB-EGF          | 12.0                   | 13.8               | 3.1             |
| MK              | 13.4                   | 14.4               | 3.2             |
| PTN             | 15.4                   | 13.2               | 2.9             |
| BDNF            | 13.6                   | 11.1               | 2.4             |
| GDNF            | 20.0                   | 11.4               | 2.5             |

For comparison of the binding capacity of the SS-DS, CS-H and Hep chains, the number of each growth factor molecule bound to each GAG chain equivalent to 30 disaccharide units was calculated. Note that sodium salts of SS-DS (S/unit = 1.17), CS-H (S/unit = 1.43) and Hep (S/unit = 2.55) chains with respective average molecular masses of 70, 18, and 15 kDa correspond to 135, 33, and 23 disaccharide units.

* Taken from Ref. 25.
* Taken from Ref. 41.
* Not determined.

**FIG. 5.** Overlaid sensorgrams of SS-DS binding to various concentrations of growth factors. Various concentrations of growth factors were tested for their binding to SS-DS (1.5 μM), and kinetic analyses of the binding were carried out with the FASTfit software. Growth factors tested included FGF-2 (A), FGF-10 (B), FGF-18 (C), HB-EGF (D), MK (E), and PTN (F). Short and long arrows indicate the beginning of the association and dissociation phases, respectively.
and DS variants (S/unit = 1.41 ± 1.90) exhibited neuritogenic activity toward mouse hippocampal neurons in contrast to DS from porcine skin (S/unit = 1.09), which did not exhibit significant activity (13). On the other hand, undersulfated CS/DS chains from embryonic pig brain (S/unit = 0.83–0.84) promoted the outgrowth of dendrite-like neurites (22). Since the SS-DS preparations showed degrees of sulfation ranging from S/unit = 0.87 for SS-DS (1.0 m) to S/unit = 1.17 for SS-DS (1.5 m), and have unique CS/DS hybrid structures unlike other DS preparations reported so far, the effects of such preparations on neuritogenesis were evaluated.

The preparations were immobilized on coverslips precoated with P-ORN. The hippocampal neuronal cells were separated from E16 mouse embryos and seeded. After 24 h incubation at 37 °C, the cells were fixed and stained to visualize the neurites as described in “Experimental Procedures.” CS-E, derived from squid cartilage, was used as a positive control. All three SS-DS preparations promoted neurite outgrowth (Fig. 7). Neurite outgrowth-promoting activity was stronger for SS-DS (Native) than SS-DS (1.0 M). The neuronal cells cultured on SS-DS (Native)-coated coverslips exhibited outgrowth of a long axon-like neurite along with dendrite-like neurites (Fig. 7D) in contrast to cells cultured on P-ORN-coated control coverslips (Fig. 7E). On average, there were more than 3 primary neurites per cell in the case of SS-DS (Fig. 7C). The neurite outgrowth-promoting activity, in terms of the formation of the longest neurite, was stronger than that exhibited by CS-E (Fig. 7A). This is intriguing because, though not oversulfated to the extent of CS-E, SS-DS is still better able to promote neurite outgrowth than CS-E, suggesting that it is not due to the oversulfation alone but to the sequential arrangement of the disaccharide constituents, and that the IdoUA content might play an equally important role as recently suggested for embryonic pig brain CS/DS (22).

To investigate the neuritogenic activity due to the SS-DS preparations and their structures, neurite outgrowth-promoting activity was evaluated after digestion with chondroitinase ABC, AC-I, or B. All three enzymatic digestions resulted in an abolishment of the neuritogenic activities (Fig. 7B), suggesting that CS/DS chains were responsible for the activity and that both CS- and DS-like structures were required for promotion of the neurite outgrowth.

**AntiHC-II Activity—** DS from porcine skin along with Hep exhibit potent anti-coagulation activity (57). The anti-coagulation activity is due to DS acting as an antithrombotic agent by binding to HC-II (58). There are reports of an increase in anti-coagulation activity with oversulfation (48, 59). On the other hand, there are also reports that oversulfated DS chains are not necessarily good promoters of anti-coagulation (44, 48). In light of these findings, anti-coagulation activity was evaluated as a measure of inhibition of thrombin via SS-DS binding to HC-II, which was estimated colorimetrically as the rate of change of absorbance using an artificial substrate, chromozym TH. All the three SS-DS preparations inhibited inhibition of thrombin activity, which was comparable to that exhibited by CS-B (porcine skin). SS-DS (Native) showed slightly higher antiHC-II activity at lower concentrations than the other two preparations (Fig. 8). Hep exhibited higher antiHC-II activities even at one-tenth the concentrations used for DS (porcine skin) and SS-DS. There was no inhibition of thrombin when GAGs were not added.

**DISCUSSION**

In this study, DS purified from shark skin was structurally and functionally characterized. The isolated DS preparations had a copolymeric structure comprising CS- and DS-like moieties. SS-DS (Native) had an S/unit ratio of 1.08, and similar to that (S/Di ratio of 1.08) exemplified by the CS/DS-proteoglycan named endocan secreted from human endothelial cells and circulating in the human bloodstream (52), suggesting that CS/DS hybrid chains with a degree of sulfation comparable to SS-DS are present in the mammalian systems despite some differences in composition from SS-DS as described below. The existence of similar structures in mammals has wide implica-

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**Table IV**  
Kinetic parameters for the interaction of growth factors with immobilized SS-DS (1.5 m), Hep and CS-H

| Growth factors | $k_{a}$ | $k_{d}$ | $r^{a}$ | $K_{d}^{b}$ |
|----------------|--------|---------|---------|-----------|
| SS-DS (1.5 m)-immobilized |          |          |         |           |
| FGF-2          | (2.2 ± 1.5) × 10⁵ | (0.1 ± 0.04) × 10⁻² | 0.980 | 13.8 ± 11.1 |
| FGF-10         | (1.9 ± 0.2) × 10⁵ | (0.2 ± 0.03) × 10⁻² | 0.980 | 8.6 ± 2.3  |
| FGF-18         | (31.3 ± 4.8) × 10⁴ | (1.2 ± 0.90) × 10⁻² | 0.995 | 4.4 ± 3.6  |
| HB-EGF         | (4.7 ± 0.6) × 10⁵ | (0.2 ± 0.10) × 10⁻² | 0.963 | 4.5 ± 2.5  |
| MK             | (1.0 ± 0.8) × 10⁵ | (0.2 ± 0.04) × 10⁻² | 0.972 | 58.5 ± 21.5 |
| PTN            | (2.9 ± 1.5) × 10⁵ | (0.2 ± 0.09) × 10⁻² | 0.990 | 7.6 ± 5.5  |
| CS-H-immobilized |          |          |         |           |
| FGF-2          | (2.4 ± 0.2) × 10⁵ | (0.2 ± 0.06) × 10⁻² | 0.991 | 8.6 ± 3.2  |
| FGF-10         | (1.4 ± 0.3) × 10⁵ | (0.2 ± 0.15) × 10⁻² | 0.966 | 17.4 ± 14.4 |
| FGF-18         | (6.3 ± 2.9) × 10⁴ | (0.4 ± 0.30) × 10⁻² | 0.833 | 10.8 ± 9.7  |
| HB-EGF         | (6.6 ± 0.4) × 10⁵ | (0.2 ± 0.10) × 10⁻² | 0.996 | 4.7 ± 3.7  |
| MK             | (3.5 ± 1.5) × 10⁵ | (0.6 ± 0.08) × 10⁻² | 0.853 | 204.0 ± 100.0 |
| PTN            | (2.6 ± 0.9) × 10⁵ | (0.3 ± 0.20) × 10⁻² | 0.902 | 16.1 ± 13.3 |

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$^{a}$ The correlation coefficient of the linear regression through the $k_{a}$ values.

$^{b}$ The $K_{d}$ value was calculated from the ratio of $k_{d}/k_{a}$, and the S.E. is the combined S.E. of the two kinetic parameters.

$^{c}$ The kinetic parameters for Hep were taken from Ref. 41.

$^{d}$ Taken from Ref. 25.
tions in terms of developing therapeutics using SS-DS. The disaccharide analysis revealed a unique picture of SS-DS being a hybrid structure with a higher proportion of GlcUA than IdOuA and appreciable proportions of multiple oversulfated disaccharide units including B, iB, D (or iD), E, and iE with A (GlcUA-GalNAc(4S)) plus iA (IdOuA-GalNAc(4S)) as the major units and C (GlcUA-GalNAc(6S)) and/or iC (IdOuA-GalNAc(6S)) as the second major units as in the case of the CS/DS chain of endocan. Notably, this is probably the first report of the existence of a GlcUA-containing B unit in CS or DS chains. It remains to be determined in what proportions the E and iE units, B and iB units, or D and iD units are present, which would further add to the complexity of the heterogeneous structure of SS-DS. DS preparations, which consist of rare building blocks comprising iE units from hagfish notochord (23) and sea

### TABLE V

Kinetic parameters for interaction of SS-DS (1.5 m) with BDNF and GDNF

Neurotrophic factors, BDNF and GDNF, were injected at various concentrations and the overlaid sensorgrams globally fitted the 1:1 Langmuir binding with mass transfer model using the BIAevaluation 3.1 software.

|          | $k_a$   | $k_d$   | $K_d$   |
|----------|---------|---------|---------|
|          | $\text{m}^{-1} \cdot \text{s}^{-1}$ | $\text{s}^{-1}$ | $\text{nm}$ |
| SS-DS (1.5 m)-immobilized sensor surface | | | |
| BDNF     | $1.7 \times 10^8$ | 61.0 | 36 |
| GDNF     | $2.5 \times 10^7$ | $2.4 \times 10^{-2}$ | 9.6 |
| CS-H-immobilized sensor surface$^b$ | | | |
| BDNF     | $4.4 \times 10^7$ | $3.0 \times 10^{-2}$ | 0.7 |
| GDNF     | $2.0 \times 10^6$ | $5.2 \times 10^{-3}$ | 2.5 |

$^aK_d$ was obtained from the ratio of $k_d/k_a$.

$^b$ Taken from Ref. 25.
and SS-DS (1.5M), respectively. Neuritogenic activities are not solely dependent on the charge density but are dependent on the sequential arrangement of the disaccharide units. Although the molecular mechanism of the action of CS/DS chains in neurogenesis is not well understood, accumulating evidence suggests that the neuroregulatory effects of these chains may be attributable at least in part to their binding of the growth factors and regulating of their signaling (8, 20, 65; also see “Discussion” in Ref. 25). The abolition of neurite outgrowth-promoting activity by digestion with chondroitinases suggests that for such activity, the CS/DS hybrid structure has functional domains consisting of both IdoUA and GlcUA, which are equally responsible.

DS shows anti-coagulation and anti-thrombotic activities (66, 67) and displays less hemorrhagic effects than unfractionated Hep (57, 66). SS-DS showed anti-HC-II activity comparable to that exhibited by porcine skin DS. It has been reported that the HC-II-binding domain of DS contains contiguous sequences of at least three iB units (57). DS from ascidians, Holocynthia pyriformis and Styela plicata, which were oversulfated containing a high proportion (66−70%) of iB showed good anti-HC-II activity compared with mammalian DS (48). On the other hand, DS from Ascidian nigra characterized by a high proportion (80%) of oversulfated iD units showed no discernible anti-HC-II activity, indicating that sulfation patterns play an important role (48). The structural basis for the observed antithrombotic activity of SS-DS remains to be investigated to clarify whether consecutive iB units or any other sequential combinations of oversulfated units such as iE units (61) are involved in the activity.

In recent years therapeutics from non-mammalian sources, which reduce the risk of contamination with pathogenic agents, have attracted attention. SS-DS with its unique structure, potent biological activities and high binding capacity toward various growth factors and neurotrophic factors may well serve as a good candidate for therapeutic application. Notably, co-administration of insulin-like growth factor-1 and GAGs greatly delays motor neuron disease and affects expression of insulin-like growth factor-1 in the wobbler mouse (68). On the other hand, neurotrophic factors exert various trophic effects on neurons. BDNF levels are decreased in neurodegenerative diseases like Alzheimer’s disease (69), implying a role in the prevention of neurodegeneration. A neuropoietic cytokine, GDNF, has been successfully tested in the prevention of neurodegeneration in Parkinson’s disease (70). It is of interest to test a possible preventive role and/or cooperative role of SS-DS for neurotrophic factors. Further studies on DS varying in sulfation profiles, structural motifs required for growth factor/neurotrophic factor binding and neurotogenesis would pave the way for developing therapeutics for neuronal disease in view of the emerging concept of neurogenesis in adult hippocampus (24).

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FIG. 8. AntiHC-II assay of the SS-DS preparations. AntiHC-II activities were tested by the ability of various concentrations of the SS-DS preparations to inhibit thrombin, and the residual activity of thrombin was monitored by hydrolysis of the substrate, chromozym, as the rate of change of absorbance at 405 nm. Hep (closed circle) and DS from pig skin (closed square) were used as positive controls. Closed triangles, triangles, and X represent SS-DS (Native), SS-DS (1.0 s), and SS-DS (1.5 s), respectively.
Novel 70-kDa Chondroitin Sulfate/Dermatan Sulfate Hybrid Chains with a Unique Heterogenous Sulfation Pattern from Shark Skin, Which Exhibit Neuritogenic Activity and Binding Activities for Growth Factors and Neurotrophic Factors
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