Structural and Functional Characterization of Oversulfated Chondroitin Sulfate/Dermatan Sulfate Hybrid Chains from the Notochord of Hagfish

NEURITOGENIC AND BINDING ACTIVITIES FOR GROWTH FACTORS AND NEUROTROPHIC FACTORS*

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Oversulfated chondroitin sulfate (CS)/dermatan sulfate (DS) hybrid chains were purified from the notochord of hagfish. The chains (previously named CS-H for hagfish) have an average molecular mass of 18 kDa. Composition analysis using various chondroitinases demonstrated a variety of GlcUA- and IdoUA-containing disaccharides variably sulfated with a higher proportion of GlcUA/IdoUA-GalNAc 4,6-O-disulfate, revealing complex CS/DS hybrid features. The hybrid chains showed neurite outgrowth-promoting activity of an axonic nature, which resembled the activity of squid cartilage CS-E and growth-promoting activity of an axonic nature, which variably sulfated with a higher proportion of GlcUA/IdoUA and partial digestion, which was abolished fully by chondroitinase ABC digestion, indicating the involvement of both GlcUA and IdoUA. Competition inhibition revealed the involvement of both GlcUA and IdoUA in neuritogenic activity. Purified CS-H exhibited interactions in a BIAcore system with various heparin-binding proteins and neurotrophic factors (viz. fibroblast growth factor-2, -10, -16, and -18; midkine; pleiotrophin; heparin-binding epidermal growth factor-like growth factor; vascular endothelial growth factor; brain-derived neurotrophic factor; and glial cell line-derived neurotrophic factor), most of which are expressed in the brain, although fibroblast growth factor-1 and ciliary neurotrophic factor showed no binding. Kinetic analysis revealed high affinity binding of these growth factors and, for the first time, of the neurotrophic factors. Competitive inhibition revealed the involvement of both GlcUA and IdoUA in the binding of these growth factors, suggesting the importance of the hybrid nature of CS-H for the efficient binding of these growth factors. These findings, together with those from the recent analysis of brain CS/DS chains from neonatal mouse and embryonic pig (Bao, X., Nishimura, S., Mikami, T., Yamada, S., Itoh, N., and Sugahara, K. (2004) J. Biol. Chem. 279, 9765–9776), suggest physiological roles of the hybrid chains in the development of the brain.

Pivotal functions of glycosaminoglycan (GAG)† side chains of proteoglycans (PGs), especially heparan sulfate (HS), have been implicated in biological processes such as cell adhesion, proliferation, and differentiation and tissue morphogenesis (1). Chondroitin sulfate (CS) and dermatan sulfate (DS) are also found ubiquitously in the extracellular matrices and at cell surfaces, being main components of cartilage and skin, respectively. CS consists of repeating disaccharide units of -4GlcUAβ1-3GalNAcβ1, whereas DS is an isomeric form of CS and is formed from precursor CS through the action of glucuronidase C epimerase, thus consisting of disaccharide units of -4GlcUAβ1-3GalNAcβ1- and -4IdoUAα1-3GalNAcβ1- in varying proportions (2). These disaccharide units are modified during chain elongation by specific sulfotransferases at C-2 of GlcUA and IdoUA, and/or C-4 and/or C-6 of GalNAc in various combinations, producing characteristic sulfation patterns critical for binding to various functional proteins displaying enormous structural diversity, compatible with that of HS, by embedding multiple overlapping functional sequences (3). Sulfation profiles of GAGs change during development (4, 5). Growing evidence indicates the involvement of CS and DS in the signaling of various heparin-binding growth factors and cytokines (6–8).

We and others have shown the importance of this class of molecule, from simple chondroitin involved in cell division of a nematode (9) to differentially oversulfated CS-D and CS-E involved in neuroregulatory functions (10–12) and the binding of growth factors in mammalian systems (13). While pursuing the critical structural elements in the CS variants, we became

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This article is dedicated to the memory of Prof. Nobuko Seno.

The abbreviations used are: GAG, glycosaminoglycan; PG, proteoglycan; HS, heparan sulfate; CS, chondroitin sulfate; DS, dermatan sulfate; GlcUA, L-glucuronic acid; IdoUA, L-iduronic acid; 2-AB, 2-aminoethylbenzenesulfonamide; DMMB, 1,9-dimethylmethylene blue; MK, midkine; FGF, fibroblast growth factor; PTN, pleiotrophin; VEGF, vascular endothelial growth factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; BDNF, brain-derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; MEF2, 1-(N-morpholino)ethanesulfonic acid; HPLC, high performance liquid chromatography; ΔHexUA, 4-deoxy-a-L-threo-hex-4-enepyranosyluronic acid; ΔDi-dis, ΔHexUAα1–3GalNAc(4S,6S); ΔDi-4S, ΔHexUAα1–3GalNAc(4S); ΔDi-6S, ΔHexUAα1–3GalNAc(6S); ΔDi-6S, ΔHexUAα1–3GalNAc; ΔDi-triS, ΔHexUAα2Sβ1–3GalNAc(4S,6S).

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 aware of vital contributions of IdoUA to biological activities. Neuritogenic activities were shown for various IdoUA-rich oversulfated DS chains from marine animals (14), and not only neuritogenic but also growth factor binding properties were revealed for the CS/DS hybrid chains attached to phosphacan/DSD-1-PG from neonatal mouse brains (14, 15), and for the free hybrid chains isolated from embryonic pig brains (16), both of which contain an appreciable proportion of functional IdoUA.

The neurite outgrowth-promoting activities of the CS/DS hybrid chains and oversulfated CS/DS chains are in contrast to the conventional concept that CS chains are intrinsic inhibitory components for axonal growth and path finding of various neurons (17, 18). The seemingly discrepant observations are most likely attributable to the structural changes during development as demonstrated for CS/DS hybrid chains of embryonic and adult pig brains (16). Enzymatic removal of CS chains permits axonal regeneration after nigrostriatal tract axotomy and spinal cord injury (19, 20) and reactivation of ocular dominance plasticity in the adult visual cortex (21) and is therefore a promising clinical strategy. We are searching for CS, DS, or hybrid chains with neuritogenic activities that have potential for therapeutic applications to neuronal diseases and injuries.

Here, we characterized GAGs from hagfish notochord, originally termed CS-H for hagfish (22). Notochord is a connective tissue that supports the neural tube and that induces the formation of the central nervous system during the development of vertebrates. CS-H contains the characteristic over sulfated units IdoUA1–3GalNAc4S,6S and IdoUA2S,3GalNAc4S,6S, where 2S, 4S, and 6S represent sulfate at C-2, C-4, and C-6, respectively (22, 23), hence regarded as DS, and exhibits axonic and dendritic neuritogenic activities for mouse hippocampal neurons (14). In view of the importance of the hybrid nature of the functional mammalian CS/DS chains in mouse (14, 15) and pig (16) brains, we searched for CS/DS hybrid chains in CS-H and demonstrated axonic neuritogenic and binding activities for a number of growth factors and a few major neurotrophic factors expressed in the brain.

**EXPERIMENTAL PROCEDURES**

Materials—GAG lyases and unsaturated disaccharides derived from CS were from Seikagaku Corp. (Tokyo, Japan). HS from bovine intestinal mucosa was purchased from Sigma. Actinase E was from Kaken Pharmaceutical Co. (Tokyo). 2-Aminobenzamidine (2-AB) was purchased from Nacalai Tesque (Kyoto, Japan). Sodium cyanoborohydride and 1.9-dimethyl-2,6-dihydroxyanthraquinone from Aldrich. Prepacked disposable PD-10 columns containing Sephadex G-25 (medium) were obtained from Amersham Biosciences (Tokyo). Sep-Pak Accell™ Plus QMA anion-exchange cartridges were from Waters Corp. (Milford, MA). EZ-Link™ biotin-LC-hydrazide was obtained from Pierce. Recombinant human midkine (MK) expressed in Escherichia coli and recombinant human fibroblast growth factor (FGF)-1 (or acidic FGF) expressed in E. coli were from PeproTech EC Ltd. (London, United Kingdom). Recombinant human FGF-2 (basic FGF) expressed in E. coli was from Genzyme TECHNE (Minneapolis, MN). Recombinant human pleiotrophin (PTN) expressed in E. coli and recombinant human vascular endothelial growth factor-165 (VEGF165) expressed in insect cells were from RELIA Tech GmbH (Braunschweig, Germany). Recombinant human heparin-binding epidermal growth factor-like growth factor (HB-EGF) expressed in S212 insect cells, recombinant human brain-derived neurotrophic factor (BDNF), and recombinant human glial cell line-derived neurotrophic factor (GDNF) were obtained from R&D Systems. Recombinant human FGF-10 expressed in E. coli was provided by Takashi Katsumata (Sumitomo Pharmaceutical Research Center, Osaka, Japan). Recombinant rat FGF-16 and recombinant mouse FGF-18 (24) and recombinant rat ciliary neurotrophic factor (CNTF) (25) were prepared as described previously.

**Extraction and Purification of CS-H—CS-H was isolated from the notochord of hagfish (Eptatretus burgeri) as detailed previously (22). Briefly, acetone-dried notochord was subjected to Pronase digestion. The proteins were removed by precipitation with 20% trichloroacetic acid, and GAGs were precipitated with 2 volumes of ethanol containing 2.5% calcium acetate and 0.25 M acetic acid. The GAG mixture was fractionated on a Dowex 1-Cl− column and eluted stepwise by increasing the NaCl concentration of the eluents. The fraction obtained by elution with 2.0 M NaCl was used for further characterization and subjected to treatment with freshly prepared nitric acid (pH 1.5) according to the procedure of Shively and Conrad (26) to remove HS. After the treatment, nitric acid was neutralized by addition of 0.5 M Na2CO3, and the resultant HS fragments were separated from CS-H by passing the treated sample through a Sephadex G-50 column (56 × 1 cm) with 50 ml pyridine acetate buffer (pH 5.0) as eluent at a flow rate of 0.6 ml/min. Finally, the CS-H preparation was freed of hydrophilic impurities by passing it through a Sep-Pak Accell™ Plus QMA cartridge.

**Molecular Mass Determination—**Molecular mass was determined using a Superdex 200 column (10 × 300 mm) calibrated with known molecular mass markers, including dextran preparations (average molecular masses of 65.5, 37.5, and 18.1 kDa), HS from bovine intestinal mucosa (average molecular mass of 7.5 kDa), and heparin from porcine intestinal mucosa (average molecular mass of 6 kDa) (27). V1 and V2 were determined using dextran (molecular mass of 170–200 kDa) and NaCl, respectively. Dextran were monitored by the orcinol method for neutral sugars (28). The purified CS-H preparation (13 μg as uronic acid) was loaded onto the column and eluted with 0.2 M ammonium acetate at a flow rate of 0.3 ml/min; the fractions were collected at 3-min intervals, evaporated to dryness, and reconstituted in 100 μl of water. Neutral sugar content was measured after estimating GAG using DMMB according to the procedure of Chandrasekhar et al. (29), except that the absorbance was measured at 525 nm.

**Determination of the Digestibility of the Purified CS-H Preparation by Various Chondroitinases—**The purified CS-H preparation (1.3 μg as uronic acid) was digested with 10 mIU of chondroitinase ABC (30), 5 mIU of chondroitinase AC-IV (31), 5 mIU of chondroitinase AC-II, or 2 mIU of chondroitinase B (32). After each enzymatic treatment, the digest was reconstituted in 20 or 50 μl of distilled water, and an aliquot was taken to estimate the resistant structure of CS-H by complexation with the metachromatic dye DMMB, which complexes with sulfated GAGs and long oligosaccharides, but not with short oligosaccharides. Briefly, 35 μl of 0.05 M acetate buffer (pH 6.8) and 200 μl of DMMB solution were added to a 5–10-μl aliquot of the above digest, and the absorption was measured at 525 nm within 1 min. Absorbance values were corrected by subtracting the absorbance values of 1- and 2-mU digests. The digestive capacity of the purified CS-H preparation was determined by estimating the uronic acid content of the digested samples after each digestion.

**Neurite Outgrowth Promotion Assay—**The neurite outgrowth-promoting activity of the purified CS-H preparation was assayed as reported previously (14). Briefly, the CS-H preparation (0.7 μg as uronic acid) or an equivalent amount of its digest with chondroitinase ABC, B, or AC-1 diluted with phosphate-buffered saline was coated on plastic coverslips, which had been precoated with poly-2-histidine at 37 °C overnight. Hippocampal neuronal cells established from embryonic day 18 rat brain were plated at a density of 10,000 cells/cm2 on the coverslips. Coverslip cultures were maintained in a humidified atmosphere at 37 °C with 5% CO2 for 24 h, after which the cells were fixed using 4% (w/v) paraformaldehyde and the neurites were visualized by immunochemical staining using anti-neurofilament antibody and FITC-conjugated secondary antibody. The immunostained cells on each coverslip were scanned and digitalized with a ×20 objective lens on an Olympus BX 51 optical microscope equipped with an Olympus HC-300Z/OL digital camera. The photographs were analyzed using morphological analysis software (Mac SCOPE, Mitany Corp., Tokyo). The length of the longest neurite, with at least one process chosen at random being longer than the cell body, was determined for 100 cells. At least three independent experiments per parameter or condition were carried out.

**Biotinylation of the Purified CS-H Preparation and HS—**Purified CS-H or HS was dissolved in 100 mM MES (pH 5.5) at a concentration of 2 mg/ml. To this solution, were added 50 mM biotin-LC-hydrazide freshly dissolved in dimethylsulfoxide and 0.5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Pierce) dissolved in MES. The mixture was incubated overnight at room temperature with continuous shaking. Excess biotinylating reagents were removed by dialysis in Spectra/por molecular porous membrane tubing (molecular mass cutoff of 3.5 kDa; Spectrum Medical Industries, Inc., Laguna Hills, CA) against several changes of phosphate-buffered saline.

**Immobilization of Purified CS-H and HS on a Sensor Chip—**A streptavidin-coated sensor chip (BIACore AB) was conditioned using 1 M
NaCl and 50 mM NaOH for 1 min three times according to the manufacturer’s instructions. Biotinylated CS-H or HS (3.3 μg as uronic acid) in phosphate-buffered saline was perfused and allowed to interact with the sensor’s surface for 3 min in phosphate-buffered saline. Immobilization was confirmed by the increase in response units.

**Interaction Analysis**—An interaction analysis was carried out in the BIAcore J system using the CS-H-immobilized sensor chip. Growth factors (200 ng each) were checked individually for binding to immobilized CS-H in running buffer (10 mM HEPES-NaOH (pH 7.4), 0.15 M NaCl, 3 mM EDTA, and 0.085% Tween 20). The flow rate was kept at a medium pace of 30 μl/min according to the manufacturer’s protocol. Each growth factor was allowed to interact with immobilized CS-H for 3 min, which constituted the association phase. Dissociation of the growth factor, constituting the dissociation phase, was allowed to go on for an additional 2 min, after which the sensor chip was regenerated by injecting 1 mM NaCl for 2 min. Neurotrophic factors (100 ng each) were tested for binding to immobilized CS-H or HS at a high flow rate of 60 μl/min, and the time allowed for the association and dissociation phases was 3 and 2 min, respectively.

To determine the kinetics of the binding of various growth factors to CS-H, each growth factor in varying concentrations was allowed to interact with immobilized CS-H. Kinetic parameters (k_on, k_off, and K_d) were determined by collectively fitting the overlaid sensorgrams locally using a Langmuir binding model with mass transfer of the BIAevaluation 3.1 software. Interaction analyses of CS-H and HS with BDNF and GDNF for determination of kinetic parameters were carried out by global fitting of the sensorgrams obtained by employing high flow rates using the model mentioned above. High flow rates were used because of the high mass transfer effects observed at medium flow rates, due to which overlaid sensorgrams could not fit with the 1:1 Langmuir binding model with mass transfer.

**Disaccharide Composition Analysis**—A composition analysis of purified CS-H was carried out after digesting 0.5 μg (as uronic acid) with 10 μl of chondroitinase ABC or 1.0 μg (as uronic acid) with 5 mIU of chondroitinase AC-1 or 1 mIU of chondroitinase B for 1 hr at 37 or 30 °C (for chondroitinase B) as described above. The digested products were then derivatized with 2-AB (33). Excess 2-AB was removed by repeated extraction with a 1:1 (v/v) water/chloroform mixture, and the water phase was dried. The residue was then reconstituted in 400 μl of 16 mM NaHPO_4 solution, and a 200-μl aliquot was analyzed by anion-exchange HPLC on an anion-bound silica PA-03 column (33).

**Gel Filtration Analysis of the Chondroitinase Digests of the CS-H Preparation on a Superdex Peptide Column**—The purified CS-H preparation (0.17 μg as uronic acid) was digested with chondroitinase ABC, AC-1, or B. An equal amount of each digest was digested sequentially with chondroitinase B followed by chondroitinase AC-I as described above. The digests were individually labeled with the fluorophore 2-AB and processed as described above. Each digest was made up with 400 μl of 0.2 M ammonium bicarbonate containing 7% 1-propanol, and a 200-μl aliquot was analyzed by gel filtration on a Superdex peptide column using the above-mentioned solvent as eluent at a flow rate of 0.4 ml/min.

**Effects of Digestion of CS-H on Its Ability to Bind Growth Factors**—The binding of various growth factors to immobilized CS-H was evaluated based on the ability of exogenously added CS-H or its digested products to competitively inhibit the binding. Purified CS-H (1.7 μg as uronic acid) was digested with chondroitinase ABC (10 mIU), AC-I (5.0 mIU), or B (2 mIU), and an equal amount was digested sequentially with chondroitinase B followed by chondroitinase AC-I as described above. The digests were individually labeled with the fluorophore 2-AB and processed as described above. Each digest was made up with 400 μl of 0.2 M ammonium bicarbonate containing 7% 1-propanol, and a 200-μl aliquot was analyzed by gel filtration on a Superdex peptide column using the above-mentioned solvent as eluent at a flow rate of 0.4 ml/min.

**RESULTS**

**Isolation and Purification of CS-H from Hagfish Notochord**—In vertebrates, the notochordal primordium plays a central role in morphogenetic movement during gastrulation to induce the central nervous system. In cyclostomes, to which hagfish belong, the notochord is not atrophied during evolution and is available in a sufficient amount for preparing GAG chains, although PGs in notochord have not been well characterized in terms of the core proteins. CS-H from hagfish notochord was isolated as described (22). Briefly, it was obtained by Pronase digestion of the acetone-dried notochord, followed by trichloroacetic acid precipitation for removal of proteins and ethanol precipitation of GAGs. It was then fractionated on a Dowex 1-Cl column by stepwise elution at varying concentrations of NaCl. A unique predominant disaccharide unit (IdoUA1–3GalNAc4S,6S), representing 68% of all disaccharides, was previously revealed in the major fraction eluted at 3.0 M NaCl from a Dowex column, in addition to the minor units IdoUA1–3GalNAc6S and IdoUA1–3GalNAc4S (22), and was named the H or iE unit (where “i” stands for iduronic acid) (7). In this study, the 2 mM NaCl-eluted fraction, representing ~35% of total CS-H, was characterized as having less sulfated CS/DS hybrid chains. Because the preparation showed trace amounts of HS disaccharides upon HPLC analysis after heparitinase digestion (data not shown), it was purified further by subjecting it to nitrous acid treatment (26) to remove HS. Peptides were removed by passing through a Sep-Pak C18 cartridge with water as eluent. The purified final preparation gave a single band between pig skin CS-B and shark cartilage CS-C on electrophoresis on a cellulose acetate membrane (data not shown), which disappeared completely after digestion with chondroitinase ABC, but only partially after digestion with chondroitinase AC-I or B, suggesting the presence of both CS and DS chains or CS/DS hybrid chains in the CS-H preparation.

**Determination of the Molecular Mass of CS-H**—The molecular size of purified CS-H was determined by gel filtration HPLC using a calibrated Superdex 200 column (27). The CS-H preparation eluted as a single fairly symmetrical peak when monitored using the dye DMMB (Fig. 1), giving an average molecular mass of ~18 kDa. This is similar to the molecular masses of porcine skin DS (19 kDa) and porcine intestine DS (21 kDa), but slightly larger than the molecular mass of DS from eel skin (14 kDa) (34).

**The CS/DS Hybrid Nature of CS-H**—The digestibility of the purified CS-H fraction was evaluated using bacterial chondroitinases ABC, AC-I, AC-II, and B by taking advantage of the ability of DMMB to form a complex with the GAGs (29). DMMB reportedly forms a complex with intact GAGs or with sulfated oligosaccharides long enough to be recognized by the dye, but not with shorter oligosaccharides. The digestibility of GAGs was therefore measured in terms of the elimination of reactivity with DMMB as a result of fragmentation of CS-H by the action of the respective chondroitinases (35). Chondroitinases

![Image](333x574 to 547x738)
AC-I and AC-II specifically cleave N-acetylgalactosaminidic linkages with GlcUA, whereas chondroitinase B cleaves N-acetylgalactosaminidic linkages with IdoUA. Chondroitinase ABC splits both types of linkages. It should be noted that, unlike the others, chondroitinase AC-II exhibits an exolytic action (36). As shown in Fig. 2, the CS-H preparation was fragmented almost completely by chondroitinase ABC and partially by chondroitinase AC-I or B (58 and 75%, respectively). It is likely that chondroitinase B produces disaccharides and non-retainable short oligosaccharides to a greater extent than chondroitinase AC-I. The oligosaccharides produced by the former and latter enzymes likely contain internal GlcUA and IdoUA residue(s) as a predominant uronic acid, respectively. Treatment with chondroitinase AC-II resulted in only 15% digestion, probably reflecting its exolytic action.

Neurite Outgrowth-promoting Activity of the CS-H Preparation—To evaluate the biological activity of the purified CS/DS hybrid CS-H preparation, the neurite outgrowth-promoting activity was tested in embryonic day 18 rat hippocampal neuronal cells (37). The CS-H preparation exhibited activity that was principally axonic in nature (Fig. 3A) and weaker than that shown by CS-E derived from squid cartilage (Fig. 3C). Control cells (cultured on coverslips coated with poly-DL-ornithine alone) showed no significant promotion of neurite outgrowth (Fig. 3B). Digestion of the CS-H preparation with chondroitinase ABC abolished the neurite outgrowth-promoting activity by 97%, whereas digestion with chondroitinase B or AC-I resulted in a loss of the neuritogenic activity of ~65 and 69%, respectively, as calculated based on the residual activity remaining after subtraction of the background activity due to poly-DL-ornithine, with the mean length of the neurites obtained using the undigested CS-H preparation taken as 100% (Fig. 3C). Analysis of the data was also carried out by enumerating the number of primary neurites/cell for 100 randomly selected cells, and the results show that the neuronal cells cultured on the CS-H substratum had, on average, 1.5 neurites/cell compared with 3 or 1.9 neurites observed for cells grown on the substratum coated with poly-DL-ornithine alone or CS-E, respectively (Fig. 3D), which is in contrast to the formation of multiple dendritic neurites in addition to a single axonic neurite observed for the CS-H preparation eluted at 3.0 M NaCl from a Dowex column (14), which contained almost exclusively IdoUA and little GlcUA (23).

Specific Interactions of CS-H with Various Heparin-binding Growth Factors—Molecular interaction experiments were carried out using the BIAcore system. The purified CS-H preparation was biotinylated using the carboxyl groups of the uronic acid moieties. Approximately 2% of the uronic acid residues
were derivatized with the biotin tag as evaluated by the modified protocol of Green (38). Biotinylated CS-H was immobilized onto a streptavidin-coated sensor chip. The level of immobilization was 736 response units, which corresponds to ~0.92 ng/1.25 mm² CS-H immobilized on the sensor chip. Binding was first tested by perfusion with 200 ng each of growth factors FGF-1, FGF-2, FGF-10, FGF-16, FGF-18, MK, PTN, HB-EGF, and VEGF₁₆₅ and allowing the growth factor to interact with immobilized CS-H for 3 min (see “Experimental Procedures”) using running buffer in the BIAcore system. The association phase obtained as a result of binding was taken as the response measured in terms of response units (RU). The values represent the average of two separate determinations.

A kinetic analysis to determine association, dissociation, and dissociation equilibrium constants (kₐ, k₈, and Kₐ) was carried out for all growth factors except FGF-1. Growth factors in varying concentrations were perfused onto the CS-H-immobilized sensor chip, and analysis was carried out by fitting the overlaid sensorgrams with the 1:1 Langmuir binding model with mass transfer of the BIAevaluation 3.1 software (Fig. 5). All growth factors tested bound to the immobilized CS-H preparation to some extent, except FGF-1, which did not show any significant binding. Among the growth factors, FGF-18 exhibited the greatest response, followed by FGF-16 and PTN.

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To characterize the structure of the functional CS-H preparation, the disaccharide composition was first analyzed by anion-exchange HPLC after digestion with chondroitinase ABC, AC-I, or B. The chondroitinase ABC digest showed the presence of large proportions of ΔHexUA₁–3GalNAc₄S,₆S (ΔDi-diS) (39.6%) and ΔHexUA₁–3GalNAc₄S,₆S (ΔDi-4S) (38.3%) and smaller proportions of ΔHexUA₁–3GalNAc₄S,₆S (ΔDi-6S), ΔHexUA₁–3GalNAc₄S,₆S (ΔDi-OS), and ΔHexUA₂₅,₁–3GalNAc₄S,₆S (ΔDi-tSₙₙ) in this order (Fig. 8A and Table III). Thus, the CS-H preparation used in this and a previous study (14), which was eluted from a Dowex column with 2.0 and 3.0 M NaCl, had a sulfate/disaccharide ratio of 1.43 and 1.68, respectively.

To identify the isomer (GlcUA or IdUA) of the hexuronic acid of the parent disaccharides from which the ΔDi-diS_{ₙₙ} unit was derived, a composition analysis was carried out by anion-exchange HPLC after digestion with either chondroitinase AC-I or B, which specifically targets the N-acetylgalactosaminic linkage with GlcUA or IdUA, respectively. Digestion by both enzymes gave rise to ΔDi-diS_{ₙₙ}, suggesting the presence of both disaccharide units, GlcUAₙ₁–3GalNAc₄S,₆S (E unit) and IdUAₙ₁–3GalNAc₄S,₆S (iE unit), revealing the intrinsic microheterogeneity in this complex molecule. Digestion by chondroitinase AC-I or B resulted in ΔDi-OS, ΔDi-4S, and ΔDi-6S and a number of oligosaccharides as well (Fig. 8, B and C), indicating the existence of GlcUA-GalNAc (O unit), GlcUA-GalNAc₄S (A unit), IdUA-GalNAc₄S (iA unit), GlcUA-GalNAc₆S (C unit), and IdUA-GalNAc₆S (iC unit), respectively (3, 7, 14). The presence of ΔDi-OS derived from the iO unit in the chondroitinase B digest was unexpected because the enzyme has been considered to recognize certain sulfation patterns (32). It apparently originated from ΔDi-4S by the action of
4-sulfatase contaminating the enzyme preparation, as was determined later using another chondroitinase B preparation free of 4-sulfatase (see the legend to Fig. 8). The multiple oligosaccharides formed by digestion with chondroitinase AC-I or B indicates the distribution of both GlcUA and IdoUA along the chain, which displays the complex CS/DS hybrid structure. The oligosaccharides formed as a result of chondroitinase AC-I digestion would represent the IdoUA-containing moieties, whereas those formed by chondroitinase B digestion would contain GlcUA to a larger extent and IdoUA to a smaller extent, reflecting the specificity of chondroitinase B, which appears to require hitherto uncharacterized sulfation patterns of GalNAc and IdoUA residues adjacent to the cleavage site (32). The exact IdoUA content in the CS-H polymer chains remains to be determined.

To determine the distribution of the IdoUA- and GlcUA-
containing moieties in the purified CS-H preparation, the oligosaccharides formed as a result of digestion with chondroitinase AC-I or B or by sequential digestion with chondroitinases B and AC-I were individually labeled with 2-AB and analyzed by gel filtration on a Superdex peptide column. Digestion with either one of the above-mentioned enzymes resulted in disaccharides and oligosaccharides (Fig. 9, A and B). Sequential digestion by chondroitinase B followed by chondroitinase AC-I resulted in cleavage of many (but not all) oligosaccharides to disaccharides (Fig. 9C; note that the vertical axis is different from those in Fig. 9, A and B), giving credence to the proposal that the digested oligosaccharides harbor GlcUA-containing disaccharide units. Some hexa- and octasaccharides could not, however, be broken down through sequential digestion, in contrast to the complete digestion achieved with chondroitinase ABC (Fig. 9D), presumably reflecting the resistant structure of certain uniquely sulfated domains. It should be noted that, because the oligosaccharides were labeled at the reducing ends with 2-AB, the peak areas reflect the molar ratios of the oligosaccharides formed, but do not correspond to their amounts in terms of weight, with larger ones appearing smaller.

**Inhibition of the Binding of Growth Factors to Immobilized CS-H Using Chondroitinase-digested Products of CS-H**—Because the purified CS-H preparation showed significant digestibility by chondroitinase AC-I or B (Fig. 2), each enzyme digest was used to examine the effects of the digestion of CS-H on its growth factor binding ability, which would suggest whether either the CS- or DS-like moiety or both are responsible for efficient binding of the growth factors. This was carried out by investigating the binding of each growth factor in the presence of exogenously added CS-H or CS-H products digested with various chondroitinases. Preliminary experiments were first performed to determine the amount of exogenous CS-H required for achieving 20–50% inhibition of the binding of a given growth factor; 0.12 μg of the purified CS-H preparation (as uronic acid) was found to be suitable (data not shown). The same amount of the CS-H preparation and growth factors was used for comparative inhibition studies. Thus, binding to immobilized CS-H was tested for the growth factors listed in Fig. 4 in the absence and presence of undigested soluble CS-H or an equivalent amount of CS-H digested with chondroitinase ABC, AC-I, or B. FGF-1 and VEGF165 were not tested because of comparatively weak binding (Fig. 4). The results from representative binding assays with HB-EGF on immobilized CS-H in the presence of soluble CS-H or its digested products are given in Fig. 10. The cumulative results for all growth factors tested are shown as bar graphs in Fig. 11. The result of the inhibition assay with FGF-16 was excluded from Fig. 11, but the pattern was similar to that of FGF-18.

Exogenous intact CS-H inhibited various growth factors from binding to immobilized CS-H to some extent, presumably reflecting a difference in the stoichiometry of the binding of soluble CS-H to growth factors in this assay system (see “Dis-
chondroitin/dermatan sulfate hybrid chains of fish notochord

In this study, CS-H from hagfish notochord was structurally and functionally characterized. The CS/DS hybrid chains of CS-H exhibited marked yet somewhat weaker neuritogenic activity than a more highly sulfated CS-H preparation exclusive of GlcUA (14), supporting the structure-function relationship. Digestions with various chondroitinases revealed the involvement of both GlcUA and IdoUA. CS/DS chains bind heparin-binding growth factors (13, 16) and may act as receptors or coreceptors in addition to conventional HS chains, and such growth factors may, in turn, be involved in the neuritogenic mechanism of CS/DS chains in vivo. Indeed, Bao et al. recently identified PTN as an endogenous ligand for embryonic pig brain CS/DS chains and observed stimulation of the neuritogenic activity of these chains by exogenously added PTN. However, no such synergistic effects were observed for CS-H with PTN or MK, which was added at different concentrations in both soluble and immobilized forms to the CS-H-coated substrate. In addition, anti-PTN antibody inhibited the activity of the brain CS/DS chains, but not that of CS-H or CS-E (data not shown). Notably, antibody 473HD, raised against the CS/DS chains of phosphacan/DSD-1-PG, neutralizes the dendritic neuritogenic activity of DSD-1-PG and CS-D, but not the axonic neuritogenic activity of CS-E (11). These findings together suggest that the mechanism of neuritogenesis by CS-H and CS-E is distinct from that by CS-D or pig brain CS/DS. The possible involvement of growth factors other than PTN and MK as well as neurotrophic factors remains to be examined.

CS-H bound various growth factors and two major neurotrophic factors with high affinity, all of which (except for FGF-16) are expressed in the brain. The binding of growth factors (including FGF-2) to HS and DS requires IdoUA residues (43, 44). However, CS-E exclusive of IdoUA also exhibits specific interactions of high affinity with various heparin-binding growth factors (13), implying the importance of the E unit and the sulfation patterns as well. CS/DS hybrid chains from embryonic pig brain bind various growth factors (16), and hybrid chains of endothelial DS-PG on the endothelial surface are also instrumental in bringing about hepatocyte growth factor-me-

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**TABLE III**

Disaccharide composition of purified CS-H

| Disaccharides          | Chondroitinase ABC | Chondroitinase AC-I | Chondroitinase B |
|------------------------|--------------------|---------------------|------------------|
| ΔDi-0S                 | 68.3 (4.7)         | 4.0 (8.7)           | ND               |
| ΔDi-6S                 | 197.6 (13.5)       | 13.8 (30.0)         | 2.7 (7.8)        |
| ΔDi-4S                 | 558.0 (38.3)       | 10.3 (22.4)         | 25.9 (74.6)      |
| ΔDi-diSE              | 577.5 (39.6)       | 17.9 (38.9)         | 6.1 (17.6)       |
| ΔDi-triS              | 56.4 (3.9)         | ND                  | ND               |
| Total                  | 1425 (100)         | 46.0 (5)            | 34.7 (21.4)      |

*The amounts were obtained from 0.25 μg of purified CS-H (as uronic acid).

**DISCUSSION**

Chondroitin/dermatan sulfate hybrid chains of fish notochord

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**FIG. 8.** Anion-exchange HPLC analysis of the purified CS-H preparation. Three aliquots of the purified CS-H preparation (0.5–1.0 μg as uronic acid) were digested extensively with chondroitinase ABC (A), AC-I (B), or B (C), and the individual digests were labeled with the fluorophore 2-AB and analyzed by anion-exchange HPLC on a PA-03 column as described under "Experimental Procedures." Fractions were monitored by fluorescence detection. The elution positions of the 2-AB derivatives of authentic unsaturated disaccharides are shown as follows: arrow 1, 2-AB-Di-0S; arrow 2, 2-AB-Di-6S; arrow 3, 2-AB-Di-4S; arrow 4, 2-AB-Di(di)S; arrow 5, 2-AB-Di(di)S; arrow 6, 2-AB-Di-triS. The major peak marked by the asterisks is derived from the 2-AB reagent. The peak corresponding to 2-AB-Di-0S (arrow 1) in C is likely to have originated from ΔDi-4S by the action of 4-sulfatase contaminating the chondroitinase B preparation, which was confirmed by digesting CS-H with a chondroitinase B preparation free of 4-sulfatase.

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Disaccharide composition of purified CS-H

| Disaccharides | Chondroitinase ABC | Chondroitinase AC-I | Chondroitinase B |
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**DISCUSSION**

In this study, CS-H from hagfish notochord was structurally and functionally characterized. The CS/DS hybrid chains of CS-H exhibited marked yet somewhat weaker neuritogenic activity than a more highly sulfated CS-H preparation exclusive of GlcUA (14), supporting the structure-function relationship. Digestions with various chondroitinases revealed the involvement of both GlcUA and IdoUA. CS/DS chains bind heparin-binding growth factors (13, 16) and may act as receptors or coreceptors in addition to conventional HS chains, and such growth factors may, in turn, be involved in the neuritogenic mechanism of CS/DS chains in vivo. Indeed, Bao et al. recently identified PTN as an endogenous ligand for embryonic pig brain CS/DS chains and observed stimulation of the neuritogenic activity of these chains by exogenously added PTN. However, no such synergistic effects were observed for CS-H with PTN or MK, which was added at different concentrations in both soluble and immobilized forms to the CS-H-coated substrate. In addition, anti-PTN antibody inhibited the activity of the brain CS/DS chains, but not that of CS-H or CS-E (data not shown). Notably, antibody 473HD, raised against the CS/DS chains of phosphacan/DSD-1-PG, neutralizes the dendritic neuritogenic activity of DSD-1-PG and CS-D, but not the axonic neuritogenic activity of CS-E (11). These findings together suggest that the mechanism of neuritogenesis by CS-H and CS-E is distinct from that by CS-D or pig brain CS/DS. The possible involvement of growth factors other than PTN and MK as well as neurotrophic factors remains to be examined.

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needed to investigate the GlcUA/IdoUA distribution pattern. The purified CS-H preparation contained substantial proportions of both E and iE units, the importance of which has been suggested in the growth factor-binding (13, 45) and neurotogenic (11, 14, 46) activities of CS and DS and which needs to be proven in such activities of CS-H. The iE unit has been identified in DS from mammalian liver (47), bovine kidney (48), and embryonic and adult pig brains (16).

All growth factors tested (except FGF-1) bound to CS-H with high affinity (Table 1). Among them, MK requires the DS domain for the migration of MK-induced osteoblast-like cells (49), but does not bind to DS from porcine skin (12), probably due to differences in the sulfation pattern. A high affinity interaction \(K_d = 94\) nM has been shown between immobilized PTN and soluble DS using the BIACore system (15). The interaction between soluble PTN and immobilized DS \(K_d = 111\) nM has also been evaluated using the IAsys system (39), with the result contrasting with the \(K_d\) of 0.17 nM obtained in this study using immobilized CS-H and soluble PTN. The differences could be attributed to differences in the methodology or system as well as the sulfation patterns of the glycan chains. The high affinity of MK and PTN for CS-H and the negligible dissociation from CS-H prompt us to speculate that they are not released as free signaling molecules, but rather are enzymatically released as growth factor-oligosaccharide complexes from a parent PG molecule by endoglycosidases such as hyaluronidase and endo-\(\beta\)-glucuronidase (50) and transferred to the cell-surface receptor. Alternatively, CS/DS-PGs may keep holding these growth factors when presenting them as coreceptors to the cell-surface receptors or may act as signal transducing receptors. Such possibilities may also be applicable to FGF-10 and FGF-18, which had sensorgrams similar to those of MK and PTN (Fig. 5).

VEGF_{165} is a heparin-binding angiogenic growth factor highly specific for endothelial cells (51). VEGF is also expressed

FIG. 9. Analysis of chondroitinase digests of the CS-H preparation on a Superdex peptide column. The purified CS-H preparation (0.17 \(\mu\)g as uronic acid) was digested with 5, 1, or 10 mIU of chondroitinase AC-1 (A), B (B), or ABC (D), respectively. The same amount of CS-H was also sequentially digested with chondroitinase B followed by chondroitinase AC-1 (C). All digests were individually labeled with 2-AB (for details, see “Experimental Procedures”) and analyzed by gel filtration on a Superdex peptide column using 0.2 M ammonium bicarbonate containing 7% 1-propanol as eluent. The elution positions of the authentic unsaturated CS-derived disaccharides/oligosaccharides are indicated as follows: arrow 1, deca-saccharides; arrow 2, octasaccharides; arrow 3, hexasaccharides; arrow 4, tetrascaccharides; arrow 5, disulfated disaccharides; arrow 6, monosulfated disaccharides; arrow 7, unsulfated disaccharides. \(V_o\) represents the void volume, and the total volume \(V_f\) was 24 ml. The peaks formed represent the molar ratios of the respective oligosaccharides labeled with 2-AB.

FIG. 10. Effects of digestions with chondroitinases on the binding activity of CS-H for HB-EGF. Competitive inhibition experiments were carried out on the binding of HB-EGF as a representative growth factor to immobilized CS-H using various enzyme digests of the CS-H preparation as competitive inhibitors to investigate the inhibitory domains in CS-H. The CS-H preparation (1.7 \(\mu\)g as uronic acid) was digested with chondroitinase ABC, AC-I, or B. Each digest (0.12 \(\mu\)g as uronic acid) or an equivalent amount of intact CS-H was mixed with HB-EGF (50 ng) and then perfused onto a sensor chip on which CS-H (0.9 ng as GAG) had been immobilized (for details, see “Experimental Procedures”). HB-EGF binding was monitored in the BIAcore system as described in the legend to Fig. 4, and overlaid sensorgrams are shown. Trace a, intact CS-H; trace b, chondroitinase AC-I digest; trace c, chondroitinase B digest; trace d, chondroitinase ABC digest; trace e, no inhibitor. RU, response units.
in subpopulations of neurons in the developing and mature central nervous systems (52). Extracellular matrix-associated HS-PGs appear to serve as an extracellular storage reservoir for the heparin-binding VEGF types (53). Interestingly, such VEGF types bind to cell-surface and extracellular matrix-associated HS-PGs, releasing angiogenic factors such as FGF-2, which are stored on the heparin-like molecules in the extracellular matrix (54). Notably, DS released after injury is a potent promoter of FGF-2 activity (43). The sensorgrams obtained in this study for the binding of FGF-2, FGF-16, HB-EGF, and VEGF165 to CS-H were similar to each other, showing a faster association and dissociation compared with the other four growth factors tested (Fig. 5 and Table I). These binding features, together with the high affinity for CS-H as evidenced by the $K_d$ values (Table I), would facilitate the efficient association of these growth factors with CS/DS chains and their transfer to the respective cell-surface receptors. This study has demonstrated, for the first time, the high affinity binding of HB-EGF and VEGF to CS/DS chains. The possibility that these factors indeed use CS/DS in addition to HS as a physiological coreceptor in vivo remains to be explored.

BDNF influences myelin formation during nerve regeneration (55), and GDNF prevents neurodegeneration in Parkinson's disease (56). Recombinant human GDNF binds to GAGs showing high affinity for heparin, which is dependent on 2-O-sulfate and inhibited by DS (57). Our interaction analysis of BDNF and GDNF with HS and CS-H revealed that the affinity exhibited by CS-H was 360- and 10-fold higher, respectively. The high affinity for CS-H and the negligible dissociation from CS-H are in contrast to their interactions with HS (Fig. 7 and Table II) and are similar to the interactions of FGF-10, FGF-18, MK, and PTN with CS-H. These findings suggest, for the first time, that not only HS but also CS/DS may be involved in regulating the functions of these neurotrophic factors and that HS and CS/DS act in different ways. HS may transmit the factors as coreceptors to the cell-surface receptors (57, 58), whereas CS/DS may keep holding the factors when presenting them to these receptors or may be enzymatically released as a functional neurotrophic factor-oligosaccharide complex from a parent PG. The domains responsible for high affinity binding to the growth factors and neurotrophic factors remain to be investigated.

Based on the binding of the growth factors to immobilized CS-H, the amounts of growth factors that bound to 1 mol of CS-H (18 kDa) were calculated as 0.5, 0.2, 1.1, 1.3, 1, 0.6, 0.7, and 0.07 mol for FGF-2, FGF-10, FGF-16, FGF-18, PTN, MK, HB-EGF, and VEGF165, respectively. The stoichiometry of the binding of BDNF and GDNF was also calculated to be 1.4 and 0.6 mol/mol of CS-H, using molecular masses of 13.6 and 20 kDa, respectively. These values indicate that the amounts of bound protein factors are not the same, but vary from one factor to another, thereby indirectly reflecting the differences in structure and average number of respective binding sequences on the GAG chains, only some of which appear to have binding sites. The possible in vivo implications of the observed interactions between the CS/DS chains and growth factors or neurotrophic factors remain to be explored to develop therapeutic agents for neuronal diseases and brain injury.

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