Chondroitin sulfate K (CS-K) from king crab cartilage rich in rare 3-O-sulfated glucuronic acid (GlcUA(3S)) displayed neuritogenic activity and affinity toward various growth factors like CS-E from squid cartilage. CS-K-mediated neuritogenesis of mouse hippocampal neurons in culture was abolished by digestion with chondroitinase (CSase) ABC, indicating the possible involvement of GlcUA(3S). However, identification of GlcUA(3S) in CS chains by conventional high performance liquid chromatography has been hampered by its CSase ABC-mediated degradation. To investigate the degradation process, an authentic CS-E tetrasaccharide, GlcUA(3S)-GalNAc(4S)-GlcUA(3S)-GalNAc(4S), was digested with CSase ABC, and the end product was identified as GalNAc(4S) by electrospray ionization mass spectrometry (ESI-MS). Putative GalNAc(6S) and GalNAc(4S,6S), derived presumably from GlcUA(3S)-GalNAc(4S) and GlcUA(3S)-GalNAc(4S,6S), respectively, were also detected by ESI-MS in the CSase ABC digest of a CS-E oligosaccharide fraction resistant to CSases AC-I and AC-II. Intermediates during the CSase ABC-mediated degradation of Δ^3HexUA(3S)-GalNAc(4S) to GalNAc(4S) were identified through ESI-MS of a partial CSase ABC digest of a CS-K tetrasaccharide, GlcUA(3S)-GalNAc(4S)-GlcUA(3S)-GalNAc(4S), and the conceivable mechanism behind the degradation of the GlcUA(3S) moiety was elucidated. Although a fucose branch was also identified in CS-K, defucosylated CS-K exhibited greater neuritogenic activity than the native CS-K, excluding the possibility of the involvement of fucose in the activity. Rather, (3S)-containing disaccharides are likely involved. These findings will enable us to detect GlcUA(3S)-containing disaccharides in CS chains to better understand CSase-mediated biological processes.

Chondroitin sulfate (CS) is a ubiquitous component of the cell surface and extracellular matrix, belongs to the glycosaminoglycan (GAG) family, and participates in diverse biological processes such as cell growth, neuronal development, and viral invasion (1–4). The ability of CS to regulate these processes is attributed to its complex structure, which arises from extensive modifications of a nonsulfated precursor with the structure (-4GlcUAβ1–3GalNAcβ1–), by specific modifying enzymes such as sulfotransferases and epimerases (for review, see Ref. 5). The resulting sulfated chains show enormous structural diversity depending on the type of cell and tissue.

The regulated expression of CS chains during the development of the brain suggests that these changes reflect neuroregulatory functions of CS chains (6). Structural features of CS chains involved in neuroregulatory events have been studied to a considerable extent by using oversulfated CS variants, CS-D
from shark cartilage and CS-E from squid cartilage, characterized by GlcUA(2S)-GalNAc(6S) (D-unit) and GlcUA-GalNAc(4S,6S) (E-unit), respectively, (7,8). CS-E in addition to heparin (Hep) inhibits neuronal cell adhesion mediated by Hep-binding neuroregulatory factor midkine (8). CS-E specifically interacts with various Hep-binding growth factors involved in brain development (9), and GlcUA(3S)-containing disaccharides GlcUA(3S)β1–3GalNAc(4S) (K-unit), GlcUA(3S)β1–3GalNAc(6S) (L-unit), and GlcUA(3S)β1–3GalNAc(4S,6S) (M-unit), where 3S, 4S, and 6S stand for 3-O-sulfate, 4-O-sulfate, and 6-O-sulfate, respectively, have been demonstrated in CS-E (10). Hence, these rare structures may play regulatory roles in the biological functions of CS-E.

To evaluate the biological significance of GlcUA(3S)-containing structures, oligosaccharides containing GlcUA(3S) structures were previously isolated from CS-K derived from king crab cartilage after digestion with hyaluronidase (11), because preliminary studies by Seno et al. (12) showed that a CS-K preparation from king crab cartilage was rich in GlcUA(3S)-containing disaccharides. However, GlcUA(3S)-containing disaccharides cannot be quantified using conventional high performance liquid chromatography (HPLC) because of their unexpected degradation following digestion with chondroitinase (Csase) A/C (11). Thus, GlcUA(3S)-containing disaccharides might have been overlooked in CS chains derived from various tissues including mammalian samples after Csase ABC treatment. Therefore, it is essential to re-evaluate the disaccharide composition of oversulfated CS chains, which is estimated currently by means of Csase ABC treatment. With the growing interest in the fine structure of CS chains (4), there is a need to understand the fate of GlcUA(3S)-containing disaccharides upon digestion with Csase ABC. This will enable us to decipher the domain structures of CS and to understand their interactions with growth/neurotrophic factors by which CS coordinates the diverse aspects of biological events and could reveal new therapeutic opportunities.

In this study, we showed the binding activity toward growth/neurotrophic factors and neurite outgrowth-promoting (NOP) activity of CS-K. In addition, we developed a method of detecting the GlcUA(3S)-containing disaccharides of CS-E and CS-K rich in GlcUA(3S) by monitoring intermediates after Csase ABC digestion and elucidated the possible mechanism by which the disaccharides are degraded. Preliminary results have been reported in an abstract form (13).

**EXPERIMENTAL PROCEDURES**

**Materials**—Gill cartilage of king crab (*Tachypleus tridentatus*) was obtained from Maruha Corp. (Tokyo, Japan). CS-K was isolated from the gill cartilage as reported previously (14). Briefly, a GAG fraction was obtained by Pronase digestion of the gill cartilage, followed by ethanol precipitation, and purified by anion exchange and reverse phase chromatographies. The fraction, which was eluted from an anion exchange column with 2.0 M NaCl and accounted for 89% of all GAG, was used in the analysis. By contrast, the 0.15 and 0.5 M NaCl-eluted fractions accounted for less than 10% of GAG. The following materials and enzymes were purchased from Seikagaku Corp. (Tokyo, Japan): six unsaturated CS-disaccharide standards, CS-E from squid cartilage, CS-K and CS-E preparations were individually coated on to coverslips precoated with poly-d-arginine (P-ORN) (Sigma) at 4 °C overnight. To investigate the structural characteristics of CS-K responsible for the neuritogenic activity, an aliquot (10 µg as GAG) was digested with 10 mIU of Csase ABC, a mixture of Csases AC-I and AC-II, or α-L-fucosidase, and a 2-µg aliquot of each digest was coated onto the coverslips precoated with P-ORN. Although the binding efficiency of oligosaccharides in the enzyme digests to P-ORN has not been evaluated, a recent study by Sotogaku et al. (18) showed the efficient binding of disaccharides to P-ORN. Control experiments were carried out using inactivated enzymes. The hippocampal neuronal cells freshly isolated from E16 mouse embryos were suspended in Eagle’s minimum essential medium containing supplements described previously (16, 17). Subsequently, the cells were seeded on coverslips at a density of 20,000 cells/cm² and allowed to grow in a humidified atmosphere for 24 h at 37 °C, 5% CO₂. Thereafter, the cells were fixed using 4% (w/v) paraformaldehyde for 30 min at room temperature, and the neurites were visualized by immunochromic staining using anti-microtubule-associated protein-2 (Liecio Technologies Inc., St. Louis, MO) and anti-neurofilament (Sigma) as described previously (19). The antibodies were then detected using a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA) with 3’,3’-diaminobenzidine as a chromogen. The stained cells on each coverslip were scanned and digitalized with a ×20 objective lens on an optical microscope (BH-2; Olympus, Tokyo, Japan) equipped with a digital camera (HC-300Z/OL; Olympus). One hundred cells with at least one neurite longer than the cell body were chosen at random to determine the length of the longest neurite using morphological analysis software (MacSCOPE; Mitani Corp., Tokyo, Japan). At least three independent experiments per parameter or condition were carried out.

**Interaction Analysis**—The interaction of various growth/neurotrophic factors with CS-K was examined using a BIAcore
Enzyme Digestion and Fluorophore Labeling—CSase ABC digestion was carried out using 1 μg of GAG or 1 nmol of tetrasaccharide and 5 mU of the enzyme in a total volume of 30 μl of the appropriate buffer at 37 °C for 60 min as described (21) unless otherwise specified. To generate the oligosaccharides, which are resistant to CSases AC-I and AC-II, CS-E was subjected to digestion with a mixture of CSases AC-I and AC-II in a 50 mM Tris-HCl buffer, pH 7.3, at 37 °C for 60 min as described previously (15). α-L-Fucosidase treatment of CS-K (50 μg as GAG) was performed using 50 mU of the enzyme in a total volume of 500 μl of 0.05 M citrate buffer, pH 4.5, for 120 min at 37 °C (22). After incubation, each reaction mixture was boiled at 100 °C for 1 min, cooled to room temperature, vacuum-dried, and derivatized with 2AB as described previously (21). These products were used for the structural analysis.

HPLC—The analysis of monosaccharides/disaccharides/oligosaccharides was carried out by HPLC on an amine-bound silica PA-03 column (YMC Co., Kyoto, Japan) using a linear gradient of NaH₂PO₄ from 16 to 798 mM at a flow rate of 1 ml/min at room temperature. Because the flow rate was kept at a moderate speed (30 μl/min) as the manufacturer recommended. Each growth/neurotrophic factor was allowed to interact with the CS-K-immobilized sensor chip for 2 min of association and dissociation. Before each injection, the base-line stability was achieved by injecting 1 mM NaCl for 2 min. The kinetic parameters were evaluated with BIA evaluation software 3.1 (BIAcore AB) using a 1:1 binding model with mass transfer.

Results

NOP Activity of the CS-K Preparations—CS-E derived from squid cartilage and containing a GlcUA(3S)-disaccharide is involved in several intriguing biological events such as the binding of growth factors and differentiation of neurons (8–10, 26). To understand the possible involvement of GlcUA(3S)-containing disaccharides in NOP activity, a CS-K preparation derived from king crab cartilage rich in K-unit was assessed for NOP activity. Hippocampal neuronal cells from E16 mouse embryos were cultured on CS-K or CS-E (a positive control) immobilized onto coverslips precoated with P-ORN (negative control) (20,000 cells/cm²) were grown for 24 h on various substrates coated on P-ORN, fixed, and immunostained with antibodies for microtubule-associated protein 2 and neurofilament (see “Experimental Procedures”). Representative morphological features of E16 hippocampal neurons were cultured on P-ORN (negative control) (A), CS-E (positive control) (B), CS-K (C), and CS-K treated with α-L-fucosidase (D). Note that the neuronal cells cultured on CS-E, CS-K, or CS-K treated with α-L-fucosidase showing prominent elongated neurite(s) compared with the cells cultured on P-ORN (scale bar, 50 μm). In E, the mean length of the longest neurite was measured for 100 randomly selected neurons cultured on various substrates (see “Experimental Procedures”). The values obtained from two independent experiments are expressed as the means ± S.E. Mann-Whitney’s U test was used to evaluate the significance of differences between means (**, p < 0.01).

**500-MHz 1H NMR Spectroscopy**—An aliquot of an unidentified fraction (40 nmol as disaccharides) derived from CS-K after CSase ABC digestion was repeatedly exchanged in D₂O with intermediate lyophilization. 1H NMR spectra were measured on a Varian VNMRS-500 (1H: 499.7 MHz) with a Nano gHX probe (sample volume 40 μl) at a spinning rate of 2000 Hz. One-dimensional spectrum with presaturation of the HOD signal, correlation spectroscopy, and two-dimensional homonuclear Hartmann-Hahn spectroscopy were measured at 26 and 55 °C. Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly relative to acetone (δ 2.225) in D₂O (23–25).
3-O-Sulfated Glucuronic Acid in CS-E and CS-K

**TABLE 1**

Kinetic parameters for the interaction of growth or neurotrophic factors with immobilized CS-K

The $k_a$, $k_d$, and $K_d$ values were determined using a 1:1 Langmuir binding model with mass transfer as described under "Experimental Procedures." The value for each growth factor is expressed as the mean ± S.E. of five different concentrations.

| Growth or neurotrophic factors | $k_a$ | $k_d$ | $K_d$ | $K_a$a |
|--------------------------------|-------|-------|-------|--------|
| HGF                           | $(4.8 \pm 1.3) \times 10^4$ | $(5.8 \pm 3.6) \times 10^{-4}$ | 12.0  |       |
| FGF18                         | $(1.8 \pm 0.2) \times 10^5$ | $(8.4 \pm 1.9) \times 10^{-5}$ | 0.5   |       |
| PTN                           | $(1.4 \pm 0.6) \times 10^5$ | $(1.1 \pm 0.6) \times 10^{-3}$ | 8.0   |       |
| MK                            | $(6.5 \pm 2.4) \times 10^4$ | $(3.2 \pm 0.8) \times 10^{-3}$ | 20.4  |       |
| BDNF                          | $(1.4 \pm 0.08) \times 10^4$ | $(8.8 \pm 1.9) \times 10^{-3}$ | 542.3 |       |
| GDNF                          | $(1.6 \pm 0.2) \times 10^4$ | $(8.8 \pm 1.9) \times 10^{-3}$ | 542.3 |       |

The values were taken from Ref. 9 and were determined in an IAsys system.

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**FIGURE 2.** Binding of various growth factors to immobilized CS-K. Various concentrations of HGF (A), FGF-18 (B), PTN (C), MK (D), BDNF (E), or GDNF (F) were injected onto the surface of a CS-K-immobilized sensor chip. Sensograms obtained with various concentrations of each growth/neurotrophic factor were overlaid using a BIA evaluation software (version 3.1). RU, resonance units. Long and short arrows indicate the beginning of the association and dissociation phases, respectively.

**CS-K Interacts with Various Growth Factors and Neurotrophic Factors**—CS chains used as the culture substrate recruit endogeneous Hep-binding growth factors such as PTN and HGF to promote the outgrowth of neurites in hippocampal neurons (20, 27). Thus, signaling of various growth/neurotrophic factors appears to be involved in the NOP activity of CS-K. Hence, we analyzed the interaction using the BIAcore system to determine the association and dissociation rate constants ($k_a$ and $k_d$) as well as the dissociation equilibrium constants ($K_d$), various growth/neurotrophic factors expressed in the brain during embryonic development. The purified CS-K preparation was biotinylated and immobilized on the streptavidin-coated 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 growth/neurotrophic factors expressed in the brain during embryonic development.

**Identification of the End Product of the Digestion of a GlcUA(3S)-containing Disaccharide**—The above results clearly showed the functional properties of CS-K. However, the quantification of GlcUA(3S)-containing disaccharides is not feasible using conventional anion exchange HPLC because of degradation upon digestion with CSase ABC (11). Therefore, we have isolated oligosaccharides containing GlcUA(3S) from CS-K after digestion with testicular hyaluronidase as models and identified them using $^1$H NMR spectroscopy (11). Here we investigated the probable intermediates/end products because of the degradation of GlcUA(3S)-containing disaccharides upon CSase ABC digestion. The structurally defined ΔA–K tetrasaccharide ΔHexUA-GalNAc(4S)–GlcUA(3S)–GalNAc(4S) isolated from CS-E (15) was used. It was subjected to CSase ABC digestion and labeled with the fluorophore 2AB. HPLC of the CSase ABC digest gave rise to ΔA (ΔHexUAα1–3GalNAc(4S)) in addition to an unidentified peak eluting shortly after ΔO (ΔHexUAα1–3GalNAc) in a molar ratio of 46:54 (Fig. 3A). To identify this peak, an
3-O-Sulfated Glucuronic Acid in CS-E and CS-K

spectrum of fraction A showed a major signal at m/z 578, consistent with the molecular mass of a monosulfated disaccharide ΔA-unit (data not shown). Interestingly, fraction B afforded a molecular ion at m/z 420 corresponding to the molecular mass of a monosulfated N-acetylgalactosamine labeled with 2AB (Fig. 3C). Thus, it is reasonable to assign this product as GalNAc(4S), resulting from the degradation of K-unit (GlcUA(3S)β1–3GalNAc(4S)) by the action of CSE ABC.

Identification of Intermediates during the CSE ABC-mediated Degradation of K-unit—To elucidate the mechanism by which K-unit is degraded to GalNAc(4S) after CSE ABC treatment and to identify the intermediates during the digestion, a structurally defined K-K tetrasaccharide, GlcUA(3S)β1–3GalNAc(4S)β1–4GlcUA(3S)β1–3GalNAc(4S), which was isolated from king crab cartilage (11), was partially digested with CSE ABC and subjected to ESI-MS. As expected, the ESI-MS profile in the Q1 mode of the CSE ABC digest showed no molecular ion signal corresponding to ΔK (ΔHexUA(3S)α1–3GalNAc(4S)) (molecular weight, 539) derived from the reducing end (data not shown). To evaluate possible intermediates generated from the degradation of ΔK, a precursor ion mode was used. Results with ΔA–K showed GalNAc(4S) as an end product after the degradation of ΔHexUA(3S)α1–3GalNAc(4S) followed by CSE ABC digestion. Because GalNAc(4S) contains a sulfate group, the precursor ions for the sulfate group at m/z 97 were measured in the negative ion mode (Fig. 4A). The ESI-MS analysis afforded a signal predominantly at m/z 300.3, corresponding to the molecular mass of GalNAc(4S). To detect signals of the intermediates generated after CSE ABC digestion, the precursor ions for the GalNAc(4S) signal at m/z 300 were measured. Four major signals at m/z 357.9, 382.0, 416.0, and 439.8 were detected as possible intermediates (Fig. 4B). For their identification, the product ion MS/MS mode was used, and particular ions were selected for collision-activated dissociation. The product ion m/z 382 displayed a major signal at m/z 300.3 corresponding to GalNAc(4S) and a signal at m/z 358.4 (Fig. 4C). The signal at m/z 382.0 was assigned to 1-hydroxy-1-[GalNAc(4S)-3-oxo]-but-3-yn-2-one (54) (Scheme 1). The signal at m/z 439.8 can be assigned to 5,6-dihydroxy-6-[GalNAc(4S)-3-oxo]-hex-1-yn-3,4-dione (52), whose product ion gave a major signal at m/z 300.1 and detectable peaks at m/z 381.9 and 49.8 (Fig. 4D). Based on these results, we propose a possible mechanism for the degradation of K-unit to GalNAc(4S) via ΔHexUA(3S)α1–3GalNAc(4S) as depicted in Scheme 1.

Analysis of the CS-E Oligosaccharide Fraction Resistant to CSE ABC—The above approach was applied to CS-E from squid cartilage, from which unique GlcUA(3S)-GalNAc(6S) (L-unit) and GlcUA(3S)-GalNAc(4S,6S) (M-unit) structures had been isolated in addition to K-unit after digestion with CSE AC-II as the following tetrascarharides (15): ΔA–L (ΔHexUAα1–3GalNAc(4S)β1–4GlcUA(3S)β1–3GalNAc(6S)), ΔA–K (ΔHexUAα1–3GalNAc(4S,6S)GlucUA(3S)β1–3GalNAc(4S)), ΔA–M (ΔHexUAα1–3GalNAc(4S,6S)β1–4GlcUA(3S)β1–3GalNAc(4S,6S)), ΔE–K (ΔHexUAα1–3GalNAc(4S,6S)β1–4GlcUA(3S)β1–3GalNAc(4S)), and ΔE–M (ΔHexUAα1–3GalNAc(4S,6S)β1–4GlcUA(3S)β1–3GalNAc(4S,6S)). To detect L-unit and M-unit along with K-unit in the aliquot of a 2AB-labeled CSE ABC digest of ΔA–K was fractionated on a Superdex peptide column, yielding fractions A and B as shown in Fig. 3A. Each fraction was collected, desalted, and subjected to ESI-MS in negative ion mode. The ESI-MS
CS-E sample, an oligosaccharide fraction, which is supposed to contain GlcUA(3S), was first prepared by digesting CS-E exhaustively with a mixture of CSases AC-I and AC-II, because N-acetylgalactosaminidic linkages to GlcUA(3S) are resistant to these enzymes. This oligosaccharide fraction was subjected to digestion with CSase ABC, labeled with 2AB, and fractionated on a Superdex peptide column as shown in Fig. 5B. Fractions E1–E5 were collected separately, and each fraction was subjected to ESI-MS, which showed an unidentified molecular ion signal for fraction E1 in the negative mode, whereas fractions E2 and E3 showed signals at m/z 658 and 578 corresponding to the molecular masses of disulfated and monosulfated unsaturated CS disaccharides labeled with 2AB, respectively (data not shown). In contrast, fraction E4 eluted at the position of a 2AB-labeled nonsulfated disaccharide showed a molecular ion signal at m/z 522 (Fig. 5C) instead of the one expected at m/z 498. This molecular mass corresponds to the monosulfated disulfated GalNAc labeled with 2AB presumably derived from M-unit. Although the disulfated GalNAc and ΔO disaccharide labeled with 2AB have similar masses, 501 and 499, respectively, the signal at m/z 522 can be explained by GalNAc(4S,6S), which is stabilized by a sodium ion via two sulfate groups. Based on this observation, it is reasonable to assign this product as GalNAc(4S,6S) resulting from the degradation of O-1 of M or E after CSase ABC digestion (Fig. 5C). Fraction E5 showed a major signal at m/z 420 (data not shown), which correspond to the molecular mass of a monosulfated N-acetylhexosamine labeled with 2AB, indicating that the product is either GalNAc(4S) or GalNAc(6S) or both, resulting from the degradation of ΔA–K and ΔE–K or ΔA–L. Thus, ESI-MS allowed us to detect a putative GalNAc(6S) derived from ΔA–L and GalNAc(4S,6S) from ΔA–M and ΔE–M in addition to
GalNAc(4S) from ΔA–K and ΔE–K after digestion with CSase ABC.

Identification of GalNAc Derivatives by Anion Exchange Chromatography—To quantify the amount of GlcUA(3S)-containing disaccharide in CS-E, the elution positions of mono- and disulfated GalNAc residues were next determined under conventional HPLC conditions. An aliquot of fraction E4 or E5 (Fig. 5B) was analyzed by anion exchange HPLC. The HPLC profile of fraction E4 showed a single major peak X near the elution positions of ΔC and ΔA as shown in Fig. 6A. Co-chromatographic analysis with a mixture of standard 2AB-labeled ΔCS disaccharides and GalNAc(4S) showed fraction E4 eluting distinctly between ΔC and ΔA as shown in Fig. 6B. The molar percentage of this peak corresponded to 29% of all disaccharides in the oligosaccharide fraction resistant to a mixture of CSases AC-I and AC-II, although the value is arbitrary because no authentic GalNAc(4S,6S) is available. The detection of GalNAc(4S,6S) derived presumably from ΔA–M and ΔE–M units is reported here for the first time. In contrast, the anion exchange HPLC profile of fraction E5 showed two peaks (data not shown). On co-chromatography with a mixture of standard unsaturated disaccharides and GalNAc(4S), one peak eluted prior to ΔO and the other peak co-eluted with GalNAc(4S) (data not shown). They most likely correspond to GalNAc(6S) and GalNAc(4S), respectively, because an N-acetylgalactosaminidic linkage to GlcUA sulfated at either C2 or C3 is resistant to CSases AC-I and AC-II. This assumption was sup-
to the molecular mass of a 2AB-labeled monosulfated disaccharide \( \Delta A \) (data not shown). Peak \( a \), which was eluted at the position of the authentic GalNAc(4S) and was derived from K-unit, accounted for 33% of the sum of peaks \( a-c \), corresponding to peak K3 on gel filtration (Fig. 7B). The ESI-MS profile of this peak showed a lone molecular ion at \( m/z \) 420 (data not shown), consistent with the previous finding that CS-K predominantly consisted of K-units (11). Because mono- or di-sulfated GalNAc residues have been reported as nonreducing terminal modifications in CS chains (28–30), the possibility exists that GalNAc(4S) was released from the nonreducing terminal because of the action of CSase ABC. Hence, the CS-K preparation was digested with a mixture of CSases AC-I and AC-II, which gave no significant peak at the position where GalNAc(4S) was eluted upon anion exchange HPLC (data not shown), indicating that the CS-K preparation contains no significant amount of GalNAc(4S) at the nonreducing ends of the polysaccharide chains. The unidentified peak \( b \) (Fig. 7A), which accounted for 18.6% of the sum of peaks \( a-c \), was eluted between the positions of \( \Delta C \) and \( \Delta A \). To characterize the compound in this peak, an aliquot of the 2AB derivative of the CSase ABC digest was fractionated on a Superdex peptide\( ^{TM} \) column into peaks K1–K3 as shown in Fig. 7B. Peak K1, corresponding to peak \( b \) in Fig. 7A, was collected and further subjected to ESI-MS, which showed a molecular ion at \( m/z \) 724.2 (data not shown), corresponding to a 2AB derivative of monosulfated unsaturated CS disaccharide substituted with a methyl pentose (data not shown). This prompted us to characterize the structure of this compound by \( ^{1}H \) NMR spectroscopy.

**Identification of a Fucosylated Trisaccharide in CS-K**—To characterize peak K1 (Fig. 7B), an aliquot of the CSase ABC digest of CS-K was fractionated on a Superdex peptide\( ^{TM} \) column (data not shown), and the fraction corresponding to the peak was subjected to \( ^{1}H \) NMR spectroscopy, which allowed us to identify this component as a monosulfated CS disaccharide fucosylated at the C3 position of GlcUA, namely Fuc(\( \alpha 1-3 \))HexUA(\( \alpha 1-3 \))GalNAc(4-O-sulfate), as shown in supplemental Table S1 and supplemental Fig. S1. Although the trisaccharide structure Fuc(\( \alpha 1-3 \))HexUA(\( \alpha 1-3 \))GalNAc(4-O-sulfate) was previously detected in the CSase ABC digest of the pentasaccharide, GlcUA(3S)-GalNAc(4S)-(Fuc-)GlcUA-GalNAc(4S) isolated from CS-K (24), the NMR data for the fucosylated trisaccharide was obtained here for the first time. Based on the peak area, 18% of the disaccharide units appear to be modified by fucosylation in the CS-K preparation.

**Investigation of the Involvement of Fucosylation in CS-K-mediated Neuritogenesis**—Because CS-K contained a significant proportion of Fuc, we investigated the possible involvement of fucosylation in the NOP activity of CS-K. The CS-K was subjected to \( \alpha 1 \)-fucosidase treatment. The disaccharide analysis of the \( \alpha 1 \)-fucosidase-treated CS-K after digestion with CSase ABC and 2AB labeling showed a 43% decrease in Fuc content (data not shown). The partially deucosylated CS-K showed greater NOP activity toward hippocampal neurons than native CS-K (Fig. 1, D and E). These results suggest that GlcUA(3S)-containing K-units rather than Fuc residues in CS-K contribute to the NOP activity.
DISCUSSION

In this study, we demonstrated NOP activity and strong affinity toward growth/neurotrophic factors expressed in the brain during development for a CS-K preparation rich in GlcUA(3S)-containing disaccharides. Both CS-K and CS-E preparations are characterized by GlcUA(3S)-containing disaccharides (10, 11). Although CS-E contains more heterogeneous GlcUA(3S)-containing disaccharides than CS-K (11), the two exhibited similar neuronal cell morphological features: a relatively small cell soma and a few prominent long neurites. Most of the CS variants derived from marine animals, which are neuritogenic in vitro, contain oversulfated disaccharide units such as D or E. However, CS-K, which is devoid of D or E units, still exhibited potent neuritogenic activity, signifying the role of GlcUA(3S)-containing disaccharides. Furthermore, the complete abolishment of NOP activity upon digestion with CSase ABC or with a mixture of CSases AC-I and AC-II indicates that an intact GlcUA(3S)-containing structure of sufficient length is essential for the neuritogenic activity of CS-K chains.

Morphometric analysis of primary neurons grown on the CS-K substrata showed a good correlation between the neuritogenic property and growth/neurotrophic factor binding ability of CS-K. HGF, FGF-18, PTN, MK, BDNF, and GDNF examined here are broadly expressed in the brain and implicated in its development (31–35). The greater affinity of CS-K, particularly for HGF, FGF-18, PTN, and MK, may indicate that the NOP activity of CS-K involves at least one of these growth factors. Earlier, we demonstrated the involvement of PTN and HGF, through their receptors anaplastic lymphoma kinase and cMet, in the NOP activity of CS/DS hybrid chains isolated from embryonic pig brain and shark liver (27). The strong affinity of CS-K for PTN, HGF, FGF-18, or MK, which is greater than that of CS-E or Hep (9), implies that GlcUA(3S)-containing structures may be present on cell surfaces or in extracellular matrices in the embryonic brain and play a critical role in the regulation of biological functions of various Hep-binding growth factors. However, BDNF and GDNF showed low affinity toward CS-K, indicating that GlcUA(3S)-containing structures may not be involved in the binding of these neurotrophic factors to CS/DS. Earlier, Nandini et al. (36, 37) reported the strong affinity of BDNF and GDNF for shark skin DS and CS-H from hagfish notochord containing a significant proportion of the DS structure, which in turn implies that iduronic acid-containing structures may be involved in the CS/DS-mediated BDNF and GDNF signaling pathways. However, precisely how CS/DS chains are involved in BDNF/GDNF signaling remains to be clarified.

In this study, we also demonstrated the degradation of GlcUA(3S)-containing disaccharides by CSase ABC to produce either mono- or disulfated GalNAc residues and proposed a pathway for the degradation process. Previously, we observed that digestion with CSase ABC of tetrasaccharides isolated from CS-K with GlcUA(3S) at the internal position unexpectedly destroyed the disaccharide unit containing GlcUA(3S) (11). This might explain why this structure has never been reported in any CS samples or in mammalian tissues. Therefore, here we developed a method to detect GlcUA(3S)-containing structures in CS samples. To understand the mechanism of the degradation of biologically active GlcUA(3S)-containing structures after CSase ABC digestion, we used CS-K- or CS-E-derived oligosaccharides as model compounds generated by either sheep testicular hyaluronidase or a mixture of CSases AC-I and AC-II (10, 15, 24). HPLC and ESI-MS revealed that digestion of the structurally defined tetrasaccharide ΔA−K, isolated from CS-E, with CSase ABC resulted in the generation of a GalNAc(4S) residue from the GlcUA(3S)-containing disaccharide (K-unit) in addition to the ΔA-unit from the nonreducing end (Fig. 3). Therefore, it is essential to reevaluate the disaccharide composition of CS chains from various biological sources, which can be achieved by anion exchange HPLC after CSase ABC treatment as described here or by fluorescence-assisted carbohydrate electrophoresis (38). In fact, fluorescence-assisted carbohydrate electrophoresis should be a good alternative to HPLC, because the separation of mono or di-sulfated GalNAc residues can be clearly achieved (38).

Furthermore, a possible mechanism of degradation of K-units by CSase ABC was revealed. A series of events during the degradation process are depicted in Scheme 1. CSase ABC digestion of the K-K tetrasaccharide GlcUA(3S)β1−3GalNAc(4S)β1–4GlcUA(3S)β1–3GalNAc(4S) leads to an unstable ΔHexUA(3S)α1−3GalNAc(4S) (ΔK-unit) from the reducing side of the tetrasaccharide. This unstable ΔK is degraded because of the influence of the 3-O-sulfate group, which attacks the adjacent α,β-unsaturated carbonylic acid resulting in cleavage of the ΔHexUA ring with the elimination of a water molecule and a SO2 molecule in succession to attain 5,6-di-hydroxy-6-[GalNAc(4S)-3-oxyl]-hex-1-yn-3,4-dione (S2). The intermediate (S2) is also thermally unstable and forms 3,4-dihydroxy-2-oxo-4-[GalNAc(4S)-3-oxyl]-butan-1-ol (S3), by immediately losing the acetylene molecule. Generally, the adjacent α-keto carbaldehydes are unstable, so the intermediate (S3) eliminates a water molecule to form an intermediate, 1-hydroxy-1-[GalNAc(4S)-3-oxyl]-but-3-yn-2-one (S4), which was detected in the ESI-MS spectrum at m/z 382 along with the GalNAc(4S) (S6) obtained in the precursor ion mode (Fig. 4A). The intermediate (S4) liberates an acetylene molecule to give the intermediate 2-hydroxy-2-[GalNAc(4S)-3-oxyl]-acetaldehyde (S5), which immediately loses a water molecule and an acetylene molecule to form the stable compound GalNAc(4S) (S6) as the end product. We found that the ESI-MS spectrum of S4 in the product ion mode yielded GalNAc(4S) as the major product and its immediate precursor (S5) as a detectable intermediate along with a sulfate group (Fig. 4C), implying that the above mechanism takes place during the degradation.

GlcUA(3S) has been detected in glycolipids isolated from human peripheral nerves using the antibody HNK-1 (39, 40). The terminal GlcUA(3S) residue is essential for immunoreactivity with HNK-1, and this carbohydrate epitope is also expressed on glycoproteins (41). The HNK-1 epitope associates with neural crest cell migration (42), neuron-to-glial cell adhesion (43), outgrowth of astrocytic processes and migration of cell body (44), and the outgrowth of neurites from motor neurons (45). It has been proposed to serve as a ligand for selectins.
3-O-Sulfated Glucuronic Acid in CS-E and CS-K

which are leukocyte-endothelial cell adhesion molecules (46). Interestingly, a similar epitopic structure has been reported for CS-proteoglycans of mammalian tissues (47). Although its association with CS chains has not been elucidated, these findings suggest that GlcUA(3S) residues may occur on CS chains of proteoglycans in the mammalian nervous system and are potentially involved in the functions of CS polysaccharides. This possibility remains to be investigated.

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