Structural Determination of Five Novel Tetrasaccharides Containing 3-*O*-Sulfated D-Glucuronic Acid and Two Rare Oligosaccharides Containing a β-D-Glucose Branch Isolated from Squid Cartilage Chondroitin Sulfate E†

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ABSTRACT: Oversulfated chondroitin sulfate E (CS-E) derived from squid cartilage exhibits intriguing biological activities, which appear to reflect the biological activities of mammalian CS chains containing the so-called E disaccharide unit [GlcAβ1-3GalNAc(4,6-*O*-disulfate)]. Previously, we isolated novel tetra- and hexasaccharides containing a rare GlcA(3-*O*-sulfate) at the nonreducing end after digestion of squid cartilage CS-E with testicular hyaluronidase. In this study, squid cartilage CS-E was extensively digested with chondroitinase AC-II, which yielded five highly sulfated novel tetrasaccharides and two odd-numbered oligosaccharides (tri- and pentasaccharides) containing D-Glc. Their structures were determined by fast atom bombardment mass spectrometry and ^{1}H NMR spectroscopy. The results revealed an internal GlcA-(3-*O*-sulfate) residue for all the novel tetrasaccharide sequences, which rendered the oligosaccharides resistant to the enzyme. The results suggest that GlcA(3-*O*-sulfate) units are not clustered but rather interspersed in the CS-E polysaccahride chains, being preferentially located in the highly sulfated sequences. The predominant structure on the nearest nonreducing side of a GlcA(3-*O*-sulfate) residue was GalNAc-(4-*O*-sulfate) (80%), whereas that on the reducing side was GalNAc(4,6-*O*-disulfate) (59%). The structural variety in the vicinity of the GlcA(3-*O*-sulfate) residue might represent the substrate specificity of the unidentified chondroitin GlcA 3-*O*-sulfotransferase. The results also revealed a trisaccharide and a pentasaccahride sequence, both of which contained a β-D-Glc branch at the C6 position of the constituent GalNAc residue. Approximately 5 mol % of all disaccharide units were substituted by Glc in the CS-E preparation used.

Chondroitin sulfate proteoglycans (CS-PGs)[1] are ubiquitous components of the extracellular matrix of connective tissues and are also found at the surface of many cell types. They exhibit a developmentally regulated expression in various tissues of the rodent fetus (*1*) and also in embryonic chick brains (*2*) and are involved in the regulation of various biological processes such as cell proliferation, differentiation, and migration, cell−cell recognition, extracellular matrix deposition, and tissue morphogenesis (for reviews, see refs *3*−*5*). Since their functions are often associated with the CS glycosaminoglycan (GAG) side chains, the structures of the CS chains are of particular interest. CS chains have a linear polymer structure composed of the repeating disaccharide unit -4GlcAβ1-3GalNAcβ1-, where GlcA and GalNAc represent D-glucuronic acid and *N*-acetyl-D-galactosamine, which are sulfated at different positions with various combinations. GlcA is sulfated at C2 and/or C3 and GalNAc at C4 and/or C6. Although it is assumed by analogy to heparan sulfate (HS) GAG chains that sulfation profiles are the structural bases of a variety of biological functions of CS chains, even chondroitin chains with no sulfate group were recently revealed to play a crucial role in cytokinesis and cell division during the early embryogenesis of *C. elegans* (*6*, *7*). On the other hand, the sulfation of CS chains is essential to the structural integrity of the cartilage tissue and defects in the synthesis of active sulfate and the sulfate transporter cause genetic defects in skeletal tissues (*8*−*10*).

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[1] Abbreviations: 1D, one-dimensional; 2D, two-dimensional; COSY, correlation spectroscopy; DQF-COSY, double-quantum filtered-COSY HOHAHA, homonuclear Hartmann−Hahn; ROESY, rotating frame Overhauser effect spectroscopy; FAB-MS, fast atom bombardment-mass spectrometry; CS, chondroitin sulfate; GAG, glycosaminoglycan; PG, proteoglycan; GlcA, D-glucuronic acid; HexA, hexuronic acid; ΔHexA, 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid; 3S, 3-*O*-sulfate; 4S, 4-*O*-sulfate; 6S, 6-*O*-sulfate.

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More importantly, the CS chains of a variety of CS-PGs present in extracellular matrices and at the cell surface appear to interact specifically with various protein ligands to regulate their functions (see below). It is also noteworthy that a variety of CS-PGs are found in the central nervous system (*5*, *11*−*15*), and have been implicated in the development of the brain through the regulation of cell adhesion, differentiation, migration, pathfinding, etc. (*13*, *15*−*17*).

Oversulfated CS structures are particularly intriguing because oversulfated disaccharide constituents such as the D unit [GlcA(2S)-GalNAc(6S)] and E unit [GlcA-GalNAc-(4S,6S)] (2S, 4S, and 6S represent 2-*O*-, 4-*O*-, and 6-*O*-sulfate, respectively) may form unique functional motifs, which interact specifically with protein ligands to regulate protein functions (*18*). CS-D and CS-E preparations rich in these units, obtained from cartilage of shark and squid, exhibit activity promoting neurite outgrowth in vitro (*19*−*21*), the former mimicking the functions of phosphacan CS−PG (DSD-1-PG) isolated from neonatal mouse brains (*22*). Squid CS-E inhibits neuronal migration (*23*) and neuronal cell adhesion, mediated by the heparin (Hep)-binding neuroregulatory growth factor midkine through direct molecular interaction with this factor (*24*), which forms along with pleitrophin a scaffold for neuronal migration (*23*). Squid CS-E shows specific high-affinity interactions with multiple Hep-binding growth/differentiation factors expressed in the brain (*25*), including midkine, pleiotrophin, Hep-binding epidermal growth factor-like growth factor, and several fibroblast growth factors. Squid CS-E specifically inhibits, through the E unit-containing tetrasaccharide motif, the binding of proinflammatory molecules such as L-selectin, *P*-selectin, and various chemokines to PG-M/versican (*26*), which is expressed in various mammalian tissues and carries CS chains with the E unit (*26*, *27*). In addition, squid CS-E is a potent inhibitor of infection of herpes simplex virus 1 through glycoprotein C binding to the CS chains on the host mammalian cells (*28*, *29*). These findings suggest that similar oversulfated CS-E structures are likely expressed in mammals, as has been demonstrated in various vertebrate tissues, including mammalian brains (*2*, *16*, *23*, *30*−*32*), and squid cartilage CS-E may mimic the structure and functions of the mammalian CS-E.

In view of these biological activities, we have been characterizing the structure of various kinds of oversulfated CS preparations from marine animals, including squid cartilage CS-E (*18*). Several oligosaccharide sequences have been isolated from CS-E and some contained a novel GlcA-(3S) residue, where 3S represents 3-*O*-sulfate (*33*, *34*). The overall content of GlcA(3S) was approximately 10% (w/w) in the CS-E chains (*34*), with significant variation among different preparations. Although GlcA(3S), which is also present in CS chains of invertebrates such as king crab (*35*) and sea cucumber (*36*), has not been reported in mammalian CS chains, this structure is present in the HNK-1 carbohydrate antigen in glycolipids and glycoproteins isolated from mammalian nervous tissues (*37*, *38*). Its expression is temporally and spatially regulated during the development of the nervous system (*39*), being implicated in cell−cell adhesion as well as the recognition of neurons and astrocytes (*40*). Although no evidence has been obtained for the involvement of the GlcA(3S) structure in CS-E's functions (*32*), it is of interest to determine the structural context around the GlcA(3S) in the CS-E chains and to clarify the relation of the structure to the variety of biological activities.

The distribution of GlcA(3S) and sequences adjacent to GlcA(3S) in the CS polymer chain are not well understood. Oligosaccharides containing GlcA(3S) at the nonreducing terminus previously isolated after digestion of squid cartilage CS-E and CS−K from king crab cartilage with testicular hyaluronidase gave information about the sequences on the reducing side of GlcA(3S) (*33*, *34*, *41*, *42*). In this study, oligosaccharides were isolated from squid cartilage CS-E chains to extract sequence information on the nonreducing side of GlcA(3S), taking advantage of the resistant nature of the linkage in GalNAcβ1−4GlcA(3S) to the action of chondroitinase AC-II.

EXPERIMENTAL PROCEDURES

Materials. Squid cartilage CS-E, six unsaturated standard CS disaccharides, and chondroitinases ABC (EC 4.2.2.4) and AC-II (EC 4.2.2.5) were purchased from Seikagaku Corp., Tokyo, Japan. Bio-Gel P-10 resin was obtained from Bio-Rad.

Preparation of Chondroitinase AC-II-resistant Tetrasaccharides. A commercial squid cartilage CS-E (100 mg) was exhaustively digested with 1 IU of chondroitinase AC-II (*43*), and the digest was separated into fractions I−IV by gel filtration on a Bio-Gel P-10 column (1.6 × 95 cm). Fraction II, which is presumed to contain tetrasaccharides, was subfractionated by HPLC on an amine-bound silica PA03 column (4.6 × 250 mm) (YMC Co., Kyoto, Japan) using a linear NaH_2PO_4 gradient from 16 to 798 mM over a 90 min period at a flow rate of 1.0 mL/min at room temperature. The eluates were monitored by measuring absorbance at 232 nm. Major peaks were purified by rechromatography under the same conditions as the first step and desalted through a column (1.5 × 46 cm) of Sephadex G-25 (fine) using distilled water as the eluent. Each peak was quantified by the carbazole method, using GlcA as a standard (*44*).

Fast Atom Bombardment-Mass Spectrometry (*FAB-MS*). The sugar and sulfate compositions of oligosaccharides were determined by FAB-MS. FAB mass spectra of the oligosaccharide samples were obtained using a VG Analytical ZAB-2SE 2FPD mass spectrometer fitted with a cesium ion gun operated at 20−25 kV, as described previously (*33*). Data were obtained and processed using VG Analytical Opus software. Monothioglycerol was used as the matrix.

Sugar Composition Analysis. Fraction 6 (1 nmol) was acid-hydrolyzed, *N*-acetylated, pyridylaminated, and analyzed by HPLC, as reported previously (*45*, *46*) for determination of the sugar composition.

^{1}H NMR Spectroscopy. Oligosaccharides for NMR analysis were repeatedly exchanged in ^2H_2O with intermediate lyophilization. One-dimensional (1D) and two-dimensional (2D) ^{1}H NMR spectra were obtained on Varian INOVA-600 (599.89 MHz) and VXR-500 (499.99 MHz) at 26 or 60 °C with a Nano-probe containing 40 μL of the sample solution (*47*−*51*). 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 ^2H_2O (*52*).

Double-quantum filtered correlation spectroscopy (DQF-COSY), 2D homonuclear Hartmann−Hahn (HOHAHA) and

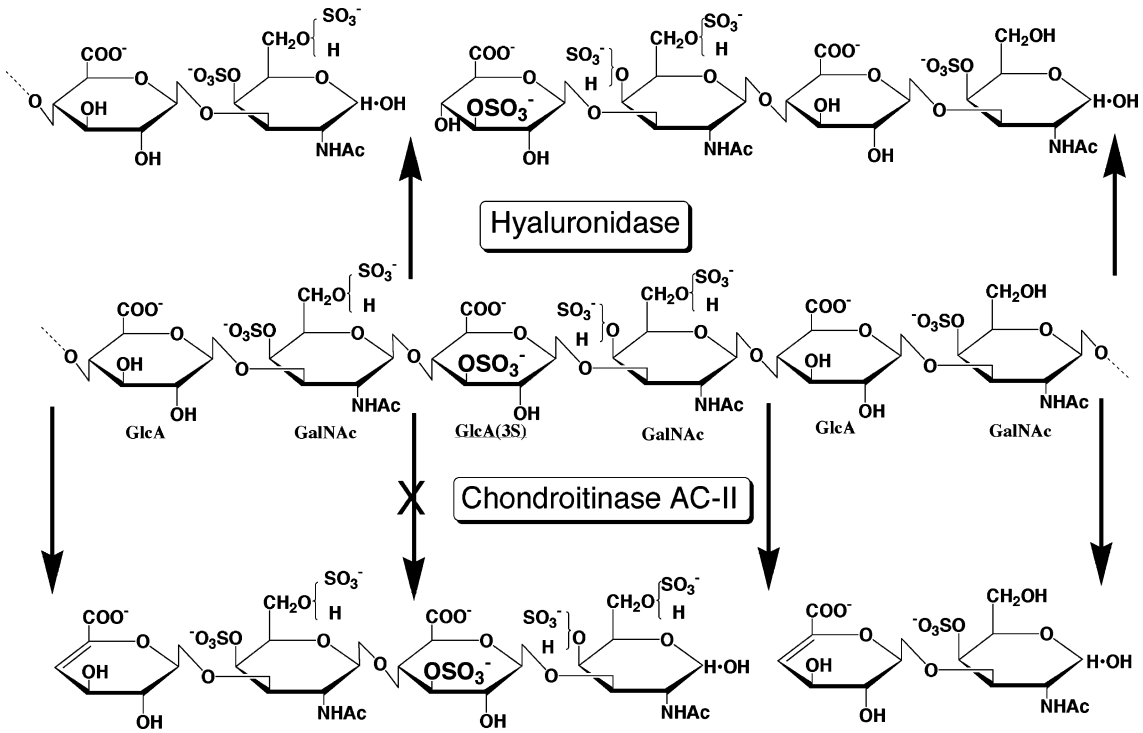

FIGURE 1: Substrate specificity of testicular hyaluronidase and chondroitinase AC-II. Testicular hyaluronidase cleaves the *N*-acetyl-D-galactosaminidic linkages in CS chains in a hydrolytic fashion to yield tetra- and hexasaccharides with GlcA at the nonreducing ends as major products. In contrast, bacterial chondroitinase AC-II is a lyase that cleaves *N*-acetyl-D-galactosaminidic linkages in an eliminative fashion to give unsaturated disaccharides as major products, but 3-*O*-sulfation of GlcA renders the resultant GalNAc-GlcA(3S) linkage resistant to the enzyme giving rise to tetra- or larger oligosaccharides with unsaturated hexuronic acid at the nonreducing ends. Note that chondroitinase ABC cleaves the GalNAc-GlcA(3S) linkage, but the resultant unsaturated form derived from GlcA(3S) decomposes (*41*).

rotating frame Overhauser effect spectroscopy (ROESY) spectra were acquired in the phase-sensitive mode using the standard pulse sequences. Mixing time for HOHAHA spectra was 80 ms, and that for ROESY spectrum was 500 ms at 600 MHz. The spin-lock power and spin-lock offset from the center of the spectrum width for HOHAHA were 8.4 kHz and zero Hz, respectively, whereas those for ROESY were 5.0 kHz and zero Hz, respectively. The time-domain matrix consisted of 256 × 2048 complex data points and was zero-filled to obtain a frequency domain matrix of 2048 × 2048 real data points.

Other Analytical Methods. Unsaturated uronic acid was spectrophotometrically quantified based upon an average millimolar absorption coefficient of 5.5 at 232 nm (*53*). Capillary electrophoresis was carried out to examine the purity of each isolated fraction in a Waters capillary ion analyzer, as reported (*47*).

RESULTS

Isolation of the Oligosaccharides. The *N*-acetylgalactosaminidic linkage in the GalNAcβ1−4GlcA(3S) sequence differs in sensitivity to the actions of testicular hyaluronidase and chondroitinases ABC and AC-II (*42*). Testicular hyaluronidase efficiently hydrolyses the linkage to generate saturated oligosaccharides (*41*), whereas chondroitinase AC-II cannot cleave it (Figure 1). In contrast, chondroitinase ABC breaks down the GlcA(3S) structure during the eliminative cleavage of the *N*-acetylgalactosaminidic linkage (*42*). Therefore, in this study, a commercial CS-E preparation derived from squid cartilage was extensively digested with chondroitinase AC-II to obtain oligosaccharides containing the sequences on the nonreducing side of GlcA(3S) residues. The digest was fractionated into fractions I−IV by gel filtration on a column of Bio-Gel P-10 (Figure 2A). The major fraction III contained disaccharides, and the chondroitinase AC-II-resistant oligosaccharide fraction II, which represents approximately 4.1% (w/w) of the initial CS-E preparation and most likely contains GlcA(3S) residues, was characterized in this study. Fraction II was subfractionated by HPLC on an amine-bound silica column into fractions II-1−II-11, as indicated in Figure 2B. The amounts of the fractions isolated from 100 mg of the CS-E preparation as well as their purity were examined by both HPLC and capillary electrophoresis and are summarized in Table 1. Fractions 2−4, and 6−11 were first characterized by mass spectrometry. Fraction I, which represented 16.9% (w/w) of the initial CS-E preparation, was susceptible to both chondroitinases ABC and AC-II, indicating that the preparative chondroitinase AC-II digestion was incomplete. Upon chondroitinase ABC digestion, fraction I yielded ΔDi-0S, ΔDi-6S, ΔDi-4S, and ΔDi-diS_E in a molar ratio of 4:7:25:64, which is similar to the disaccharide composition of the initial CS-E preparations (*41*). Upon chondroitinase AC-II digestion, it was also extensively digested to give rise to three disaccharides ΔDi-6S, ΔDi-4S, and ΔDi-diS_E in a molar ratio of 12:23:65, and therefore fraction I was judged to have resulted from incomplete digestion and was not analyzed further.

FAB-MS Analysis. The molecular weights of the isolated samples were determined by FAB-MS spectrometry in the negative ion mode. The sugar composition and the maximum number of *O*-sulfate groups present in each fraction were

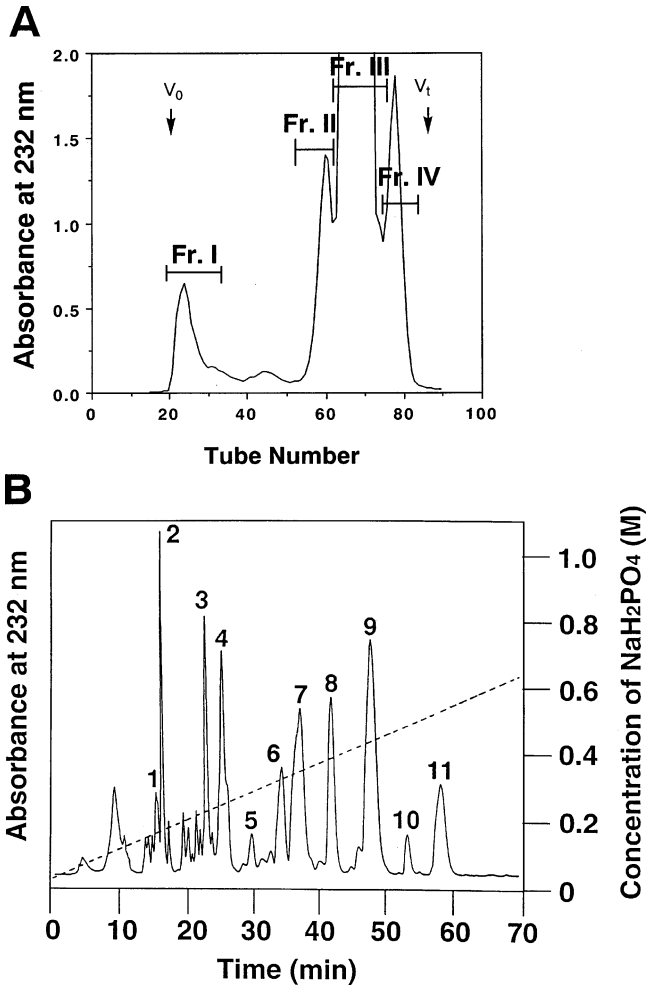

FIGURE 2: Isolation of tetrasaccahrides and rare oligosaccharides from CS-E. Commercial CS-E (100 mg) was exhaustively digested with chondroitinase AC-II, and the digest was fractionated by gel filtration on a column of Bio-Gel P-10, using 1.0 M NaCl/10% ethanol as eluent (A). Fractions (2 mL) were collected and monitored by measuring absorbance at 232 nm. Fractions I−IV were pooled as indicated. The presumable tetrasaccharide fraction (Fraction II) was separated into subfractions 1−11 by anion-exchange HPLC on an amine-bound silica PA-03 column, using an NaH_2PO_4 gradient (indicated by the dashed line) (B). Each isolated fraction was desalted by gel filtration on Sephadex G-25 under the conditions described in Experimental Procedures then subjected to structural analysis.

Table 1: Quantity and Purity of Each Tetrasaccharide Fraction

fraction	yield[a] (nmol)	HPLC (%)	capillary electrophoresis (%)
1	51	68	100
2	82	100	100
3	110	100	100
4	236	55	69
5	45	60	50
6	186	96	100
7	285	100	75
8	243	100	100
9	508	100	100
10	48	100	100
11	195	100	100

[a] Yield is given in nmol obtained from 100 mg CS-E.

inferred from the mass number. In the negative ion mode, FAB-MS spectra of sulfated oligosaccharides, alkali-metal-attached molecular ions of the type $[M + xNa - (x + 1)H]^-$ (M represents the fully protonated acid form of an oligosaccharide), were preferentially observed. The assignments of the molecular ion signals afforded by each of the analyzed fractions are tabulated in Table 2.

The spectra of fractions 6 and 11 are depicted in Figure S1, Supporting Information, as two distinct representatives. The molecular ion signals at *m*/*z* 1245, 1267, and 1289 afforded by fraction 11 (Figure S1A, Supporting Information) corresponded, respectively, to tetra-, penta- and hexa-sodiated molecular ions of a pentasulfated tetrasaccharide $\Delta HexA_1$-$HexA_1HexNAc_2(OSO_3H)_5$ ($[M + 4Na - 5H]^-$, $[M + 5Na - 6H]^-$, and $[M + 6Na - 7H]^-$, respectively), where HexA, ΔHexA, and HexNAc represent hexuronic acid, unsaturated hexuronic acid, and *N*-acetylhexosamine, respectively. Likewise, fractions 3, 4, and 7−11, gave molecular ion signals characteristic of the sulfated unsaturated tetrasaccharides (Table 2), and the molecular compositions were estimated from the *m*/*z* values as listed in the right column.

Fraction 6 was clearly different from the above factions in that it contained a hexose component. The spectrum afforded molecular ions $[M + 2Na - 3H]^-$, $[M + 3Na - 4H]^-$, and $[M + 4Na - 5H]^-$ at *m*/*z* 1203, 1225, and 1247, respectively (Figure S1B, Supporting Information). Compared to the signals of fraction 7, which contains a trisulfated tetrasaccharide, those of fraction 6 were detected at 162 mass units higher, respectively, indicating that the compound in fraction 6 contains an additional hexose. Thus, the observed signals corresponded to tetra-, penta- and hexa-sodiated molecular ions of $\Delta HexA_1HexA_1HexNAc_2Hex_1(OSO_3H)_3$, respectively. Analogously, fraction 2 gave molecular ion signals characteristic of the unsaturated trisaccharide $\Delta HexA_1$-$HexNAc_1Hex_1(OSO_3H)_1$, as shown in Table 2.

Sugar Composition Analysis of Fraction 6. On the basis of the FAB-MS analysis, fractions 2 and 6 contained an unsaturated di- and tetrasaccharide backbone with a hexose branch, respectively. To identify the neutral sugar components, sugar composition analysis was performed for the more abundant compound fraction 6, which was subjected to acid hydrolysis followed by *N*-acetylation and subsequent pyridylamination. The resulting pyridylaminated sugars were quantitatively analyzed using a PALPAK Type A column (Takara Shuzo Co., Kyoto, Japan) (Figure S2, Supporting Information), which revealed that the compound in fraction 6 contained 1 mol of Glc per mol of ΔHexA. The hexose in fraction 2 was identified by ^{1}H NMR, as described below.

^{1}H NMR Analysis of the Isolated Oligosaccharides. The structure of the major compound in each isolated oligosaccharide fraction was analyzed by ^{1}H NMR spectroscopy, and the individual monosaccharide units were identified based on the chemical shifts of the proton signals, which were assigned by 2D HOHAHA and COSY analyses. Although fraction 7 was a mixture of at least two components as revealed by capillary electrophoresis, it was possible to extract sequence information about the major compound in this fraction by taking advantage of ^{1}H NMR spectroscopy. Since peak heights of resonances reflect molar ratios of the components, signals for the major compound in the mixture could be easily distinguished from those of the minor compound. The internal uronic acid residue of the major tetrasaccharide component in fractions 7, 8, 9, 10, and 11 was identified as 3-*O*-sulfated GlcA unambiguously based

Table 2: FAB-MS Analysis of the Nine Oligosaccharides Isolated from CS-E of Squid Cartilage

fraction	MW	*m/z* for $[M-H]^-$	*m/z* for $[M+Na-2H]^-$	*m/z* for $[M+2Na-3H]^-$	*m/z* for $[M+3Na-4H]^-$	*m/z* for $[M+4Na-5H]^-$	*m/z* for $[M+5Na-6H]^-$	*m/z* for $[M+6Na-7H]^-$	assignment
2	621	620	642						ΔHexAHexNAcHexOSO$_3$H
3	918	917							ΔHexAHexAHexNAc$_2$(OSO$_3$H)$_2$
4	998		1019						ΔHexAHexAHexNAc$_2$(OSO$_3$H)$_3$
6	1160			1203	1225	1247			ΔHexAHexAHexNAc$_2$Hex(OSO$_3$H)$_3$
7	998			1041	1063	1085			ΔHexAHexAHexNAc$_2$(OSO$_3$H)$_3$
8	998			1041	1063	1085			ΔHexAHexAHexNAc$_2$(OSO$_3$H)$_3$
9	1078				1143	1165	1187		ΔHexAHexAHexNAc$_2$(OSO$_3$H)$_4$
10	1078				1143	1165	1187		ΔHexAHexAHexNAc$_2$(OSO$_3$H)$_4$
11	1158					1245	1267	1289	ΔHexAHexAHexNAc$_2$(OSO$_3$H)$_5$

Table 3: ^{1}H Chemical Shifts of the Constituent Monosaccharides of the Tetrasaccharides Isolated from CS-E[a]

		Fr. 7[b]		Fr. 8		Fr. 9		Fr. 10		Fr. 11	
		ΔU-G-U-G (6S; 4S 3S)		ΔU-G-U-G (4S 3S 4S)		ΔU-G-U-G (6S; 4S 3S 4S)		ΔU-G-U-G (6S; 4S 3S 4S)		ΔU-G-U-G (6S 6S; 4S 3S 4S)	
		α[c]	β	α	β	α	β	α	β	α	β
GalNAc-1	H-1	5.233	4.70	5.222	4.737[d]	5.228	4.752[d]	5.221	4.74	5.228	4.74[d]
	H-2	4.27	4.01	4.337	4.01[d]	4.350	4.05[d]	4.343	4.03	4.359	4.04[d]
	H-3	4.18	N.D.[e]	4.182	4.06[d]	4.196	4.09[d]	4.167	N.D.	4.198	N.D.
	H-4	4.22	N.D.	4.846[d]	4.772[d]	4.877	4.809[d]	4.856	N.D.	4.895	4.815[d]
	H-5	4.355	N.D.	4.259	N.D.	4.498	N.D.	4.244	N.D.	4.498	N.D.
	H-6	4.21	N.D.	3.78	N.D.	4.27	N.D.	3.78	N.D.	4.27	N.D.
	H-6'	4.14	N.D.	3.71	N.D.	4.14	N.D.	3.72	N.D.	4.14	N.D.
	NAc	2.021		2.033		2.032		2.040		2.043	
GlcA-2	H-1	4.691	4.635	4.631	4.579	4.634	4.585	4.663	4.611	4.669	4.618
	H-2	3.688		3.667		3.667		3.651		3.643	
	H-3	4.422		4.402		4.400		4.464		4.456	
	H-4	4.076		4.06		4.047		4.077		4.10	
	H-5	3.88		3.79		3.790		3.93		3.93	
GalNAc-3	H-1	4.67		4.675		4.674		4.74		4.77[d]	
	H-2	4.04		4.07		4.064		4.03		4.05	
	H-3	4.12		4.12		4.11		4.13		4.15	
	H-4	4.606		4.602		4.602		4.703		4.707	
	H-5	N.D.		N.D.		N.D.		4.06		4.07	
	H-6	N.D.		N.D.		N.D.		4.24		4.23	
	H-6'	N.D.		N.D.		N.D.		4.16		4.14	
	NAc	2.106		2.106		2.107		2.108		2.111	
ΔHexA-4	H-1	5.272		5.272		5.270		5.279		5.280	
	H-2	3.830		3.829		3.824		3.821		3.827	
	H-3	3.947		3.948		3.946		3.946		3.948	
	H-4	5.960		5.958		5.956		5.956		5.957	

[a] Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly in reference to acetone (δ 2.225 ppm) in ^2H_2O at 26 °C. The estimated error for the values to two decimal places was only ± 0.01 ppm because of partial overlap of signals. That for the values to three decimal places was ± 0.002 ppm. [b] G, U, ΔU, 3S, 4S, and 6S represent GalNAc, GlcA, ΔHexA, 3-*O*-sulfate, 4-*O*-sulfate, and 6-*O*-sulfate, respectively. [c] The symbols α and β represent the α- and β-form of each oligosaccharide, respectively, which are in equilibrium in water (see also the text). Consequently, anomeric effects were observed for H2 −H6 protons of each GalNAc-**1** residue and the H-1 proton of each GlcA-**2** residue. [d] Values determined at 60 °C. [e] N.D.: not determined.

upon the chemical shifts of the anomeric proton and H-3 signals (*41*, *42*). In contrast, fractions 2 and 6 gave spectra with no 3-*O*-sulfated GlcA signals, but with additional proton signals characteristic of a β-D-glucopyranosyl residue, as described below. The components in fractions 3 and 4 remain to be characterized due to difficulty in completely assigning the signals. Although the internal uronic acid residue of the major compound in fractions 3 and 4 was identified as a nonsulfated GlcA residue (data not shown), it remained undigested by the action of chondroitinase AC-II and appeared to be less sensitive to this enzyme. The NMR data for the major oligosaccharide in each fraction, which was successfully sequenced, are summarized in Tables 3 and 4.

The major compound in each fraction except for fractions 2 and 6 has the tetrasaccharide structure ΔHexA−GalNAc−HexA−GalNAc, with *O*-sulfate groups. The types of uronic acids and sulfation sites were determined by ^{1}H NMR analysis. The 1D and 2D HOHAHA spectra of fraction 11 are shown in Figures 3A and 4A. The internal uronic acid residue in each compound was identified as GlcA rather than IdoA based upon the chemical shift of the anomeric proton signal. The anomeric proton signals of αIdoA and βGlcA in CS oligosaccharides are observed at around δ 5.0−5.2 and 4.5−4.8 ppm, respectively (*54*, *55*). Sulfated positions on the saccharide residues of the compound in each fraction were determined by comparison with the proton signals of nonsulfated saccharide residues, based on the fact that *O*-sulfation causes downfield shifts of protons bound to the *O*-sulfated carbon atoms by approximately 0.4−0.8 ppm (*54*, *56*, *57*). Hence, the following structures of the indivi-

Table 4: [1]H Chemical Shifts of the Constituent Monosaccharides of the Oligosaccharides Isolated from CS-E

		Fr. 2		Fr. 6	
		Glc | ΔU - G | 4S		Glc 6S | | ΔU - G - U - G | | 4S 4S	
		α	β	α	β
GalNAc-1	H-1	5.227 ($J_{1,2}$ = 3.3)	4.792 ($J_{1,2}$ = 8.4)	5.218	4.74[d]
	H-2	4.379	4.073	4.341	4.04[d]
		($J_{2,1}$ = 3.3, $J_{2,3}$ = 11.2)	($J_{2,1}$ = 8.4, $J_{2,3}$ = 10.7)		
	H-3	4.316	4.159	4.214	4.07[d]
		($J_{3,2}$ = 11.2, $J_{3,4}$ = 2.6)	($J_{3,2}$ = 10.7, $J_{3,4}$ = 2.6)		
	H-4	4.718	4.647	4.847[d]	4.783[d]
		($J_{4,3}$ = 2.6)	($J_{4,3}$ = 2.6)		
	H-5	4.50	4.497	4.497	N.D.
	H-6	N.D.	4.094	4.27	N.D.
	H-6'	N.D.	3.879	4.17	N.D.
	NAc	2.089		2.021	
GlcA-2	H-1			4.531	4.490
	H-2			3.405	
	H-3			3.590	
	H-4			3.797	
	H-5			3.691	
GalNAc-3	H-1			4.643	
	H-2			4.086	
	H-3			4.140	
	H-4			4.656	
	H-5			4.07	
	H-6			4.27	
	H-6'			4.17	
	NAc			2.098	
ΔHexA-4	H-1	5.303 ($J_{1,2}$ = 2.6)	5.269 ($J_{1,2}$ = 2.0)	5.264	
(ΔHexA-2)	H-2	3.832	3.850	3.827	
	H-3	3.953 ($J_{3,4}$ = 4.0)	3.956	3.947	
	H-4	5.973 ($J_{4,3}$ = 4.0)	5.976	5.965	
Glc	H-1	4.518 ($J_{1,2}$ = 8.3)	4.504 ($J_{1,2}$ = 7.9)	4.553	
	H-2	3.298	3.294	3.314	
		($J_{2,1}$ = 8.3, $J_{2,3}$ = 8.9)	($J_{2,1}$ = 7.9, $J_{2,3}$ = 9.3)		
	H-3	3.496	3.493	3.510	
		($J_{3,2}$ = 8.9, $J_{3,4}$ = 9.2)	($J_{3,2}$ = 9.3, $J_{3,4}$ = 9.2)		
	H-4	3.391	3.388	3.391	
		($J_{4,3}$ = 9.2, $J_{4,5}$ = 9.6)	($J_{4,3}$ = 9.2, $J_{4,5}$ = 9.6)		
	H-5	3.461		3.450	
	H-6	3.905 ($J_{6,6'}$ = 12.4)		3.91	
	H-6'	3.722 ($J_{6',6}$ = 12.4, $J_{6',5}$ = 5.9)		3.72	

[a] Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly in reference to acetone (δ 2.225 ppm) in ^2H_2O at 26 °C. The estimated error for the values to two decimal places was only ± 0.01 ppm because of partial overlap of signals. That for the values to three decimal places was ± 0.002 ppm. [b] G, U, ΔU, 3S, 4S, and 6S represent GalNAc, GlcA, ΔHexA, 3-*O*-sulfate, 4-*O*-sulfate, and 6-*O*-sulfate, respectively. [c] The symbols α and β represent the α- and β-form of each oligosaccharide, respectively, which are in equilibrium in water (see also the text). Consequently, anomeric effects were observed for H2 −H6 protons of each GalNAc-**1** residue and the H-1 proton of each GlcA-**2** residue. [d] Values determined at 60 °C. [e] N.D.: not determined. Coupling constants (in Hz) are given in parentheses.

dual major compounds in fractions 7−11 are proposed: fraction 7, ΔHexAα1−3GalNAc(4S)β1−4GlcA(3S)β1−3GalNAc(6S); fraction 8, ΔHexAα1−3GalNAc(4S)β1−4GlcA(3S)β1−3GalNAc(4S); fraction 9, ΔHexAα1−3GalNAc(4S)β1−4GlcA(3S)β1−3GalNAc(4S,6S); fraction 10, ΔHexAα1−3GalNAc(4S,6S)β1−4GlcA(3S)β1−3GalNAc(4S); fraction 11, ΔHexAα1−3GalNAc(4S,6S)β1−4GlcA(3S)β1−3GalNAc(4S,6S).

The 1D and 2D HOHAHA spectra of fraction 6 and 2D ROESY spectrum of fraction 2 are shown in Figures 3B, 4B, and 5, respectively, and the NMR data are summarized in Table 4. The 1D spectrum of fraction 6 showed an additional H-1 signal at δ 4.553 in the anomeric region, which was compatible with the anomeric proton signal of a β-D-glucopyranosyl residue (*58*, *59*). The H-2 to H-4 resonances of the Glc residue were assigned based on the connectivity of these signals from the H-1 in the COSY (data not shown) and HOHAHA (Figure 4B) spectra. Likewise, most of the proton chemical shifts and coupling constants of the compound in fraction 2 were assigned as summarized in Table 4. The large coupling constants around the ring protons of the hexose confirmed that they are all trans diaxial, which is chracteristic of Glc. Since the chemical shifts of both H-4 and H-6 of the GalNAc-1 residue of fraction 2 were shifted to a lower magnetic field compared with those of the nonsulfated GalNAc residue of the CS oligosaccharides, the Glc residue was judged to be linked to a hydroxyl group of either C4 or C6 of the GalNAc-1 residue and the hydroxyl group of the other position was sulfated. To localize the position where the GalNAc residue is substituted by the Glc residue, the 2D ROESY spectrum of fraction 2, which is purer than fraction 6 (Table 1), was recorded. In the ROESY spectrum (Figure 5), cross-peaks were clearly detected between the anomeric proton signal of the Glc residue and the H-6/H-6′ signals of the GalNAc-1 residue, along with cross-peaks between the anomeric proton signals of the ΔHexA residue and the H-3 signal of the GalNAc-1 residue, revealing unambiguously that the compound in fraction 2 had the monosulfated disaccharide core ΔHexAα1−3GalNAc(4S) with an additional Glc branch bound to the C6 of GalNAc-1. On the basis of these NMR data, the structure of the major compound in fraction 2 is proposed as follows: fraction 2, ΔHexAα1−3(Glcβ1−6)GalNAc(4S).

When the NMR data of fraction 6 were compared with those of fraction 2, the chemical shifts of protons belonging to Glc, ΔHexA-4 and GalNAc-3 in fraction 6 were found very similar to those of the trisaccharide in fraction 2. Furthermore, the enzymatic digestion of fraction 6 with chondroitinase AC-II yielded ΔDi-diS_E and a component that was eluted at the position of fraction 2 on HPLC (see below). On the basis of these findings, the following pentasaccharide structure is proposed for the major component in fraction 6: fraction 6, ΔHexAα1−3(Glcβ1−6)-GalNAc(4S)β1−4GlcAβ1−3GalNAc(4S,6S).

Chondroitinase Digestion of the Isolated Oligosaccharides. As reported previously (*42*), the *N*-acetylgalactosaminidic linkage bound to the 3-*O*-sulfated GlcA residue is resistant to chondroitinase AC-II, whereas it is sensitive to and decomposed by chondroitinase ABC. Therefore, the susceptibility of the isolated oligosaccharides to chondroitinase AC-II was examined. Upon chondroitinase AC-II digestion, components in fractions 7−11 were insusceptible (data not shown), confirming the resistant nature of the 3-*O*-sulfated GlcA residue. In contrast, the internal GalNAc linkage of the component in fraction 6 was cleaved by chondroitinase AC-II, as well as chondroitinase ABC, and on HPLC the two resultant products were eluted at the same position as the component in fraction 2 and authentic ΔHexA−GalNAc-(4S,6S), respectively (data not shown). Thus, the C6-glucosylation on GalNAc residues seems to render the *N*-acetylgalactosaminidic linkages less sensitive to chondroitinase AC-II, because the oligosaccharides were prepared in this study using chondroitinase AC-II, and a portion of the fraction 6 oligosaccharide was not completely digested to give rise to the trisaccharide found in fraction 2 and had remained undigested as the pentasaccharide in fraction 6.

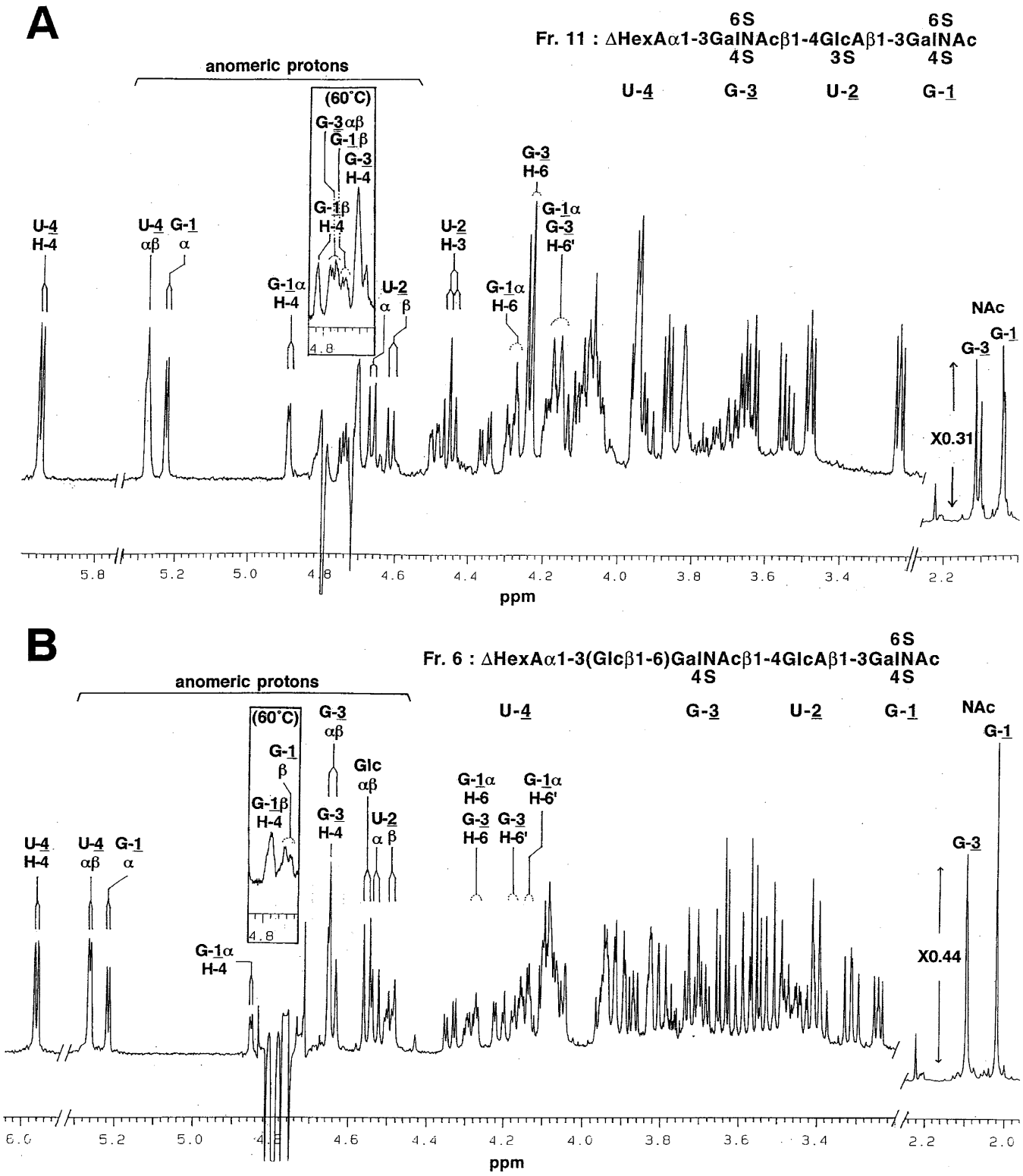

FIGURE 3: 1D 500 MHz ^1H NMR spectra of fractions 11 (A) and 6 (B) recorded in ^2H_2O at 26 °C. The numbers and letters in the spectra refer to the corresponding sugar residues in the structures. The *insets* are the spectra recorded at 60 °C to suppress a disturbance by the HOD line. U and G represent GlcA and GalNAc, respectively.

DISCUSSION

Oversulfated CS-E exhibits differentiation-associated expression as has been demonstrated for embryonic chick chondrocytes (*60*), glomeruli (*61*), mesengial cells (*62*), and hemopoietic cells (for a review, see ref *63*), and is likely to be associated with specific functions of these cells (see Introduction in ref *33*). Kawashima et al. (*26*) recently demonstrated that some proinflammatory molecules such as selectins and chemokines bound specifically to the oversulfated CS/DS structure containing the E disaccharide unit, which suggested that such oversulfated CS/DS are likely involved in leukocyte trafficking and may function as a reservoir for chemokines in vivo. The entry of herpes simplex virus type 1 (HSV-1) into cells involves a cascade of events

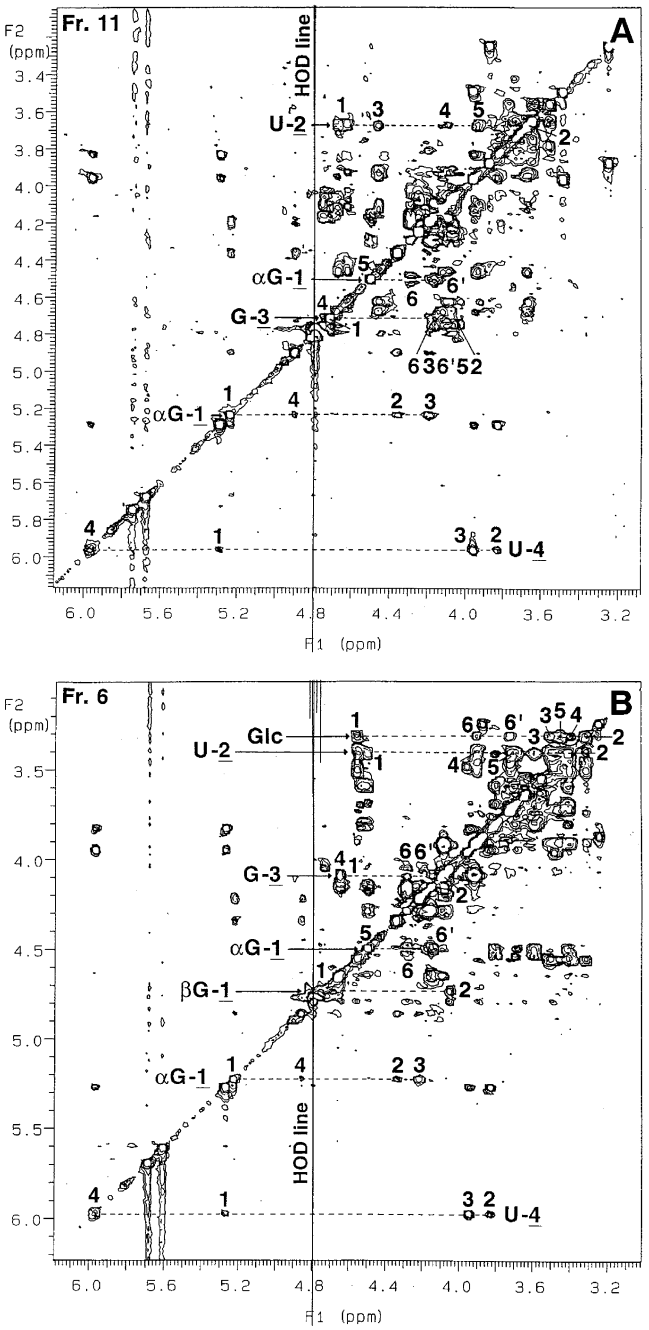

FIGURE 4: 2D HOHAHA spectra of fractions 11 (A) and 6 (B) recorded in ^2H_2O at 26 °C. The numbers and letters in the spectra refer to the corresponding sugar residues in the structures. U and G represent GlcA and GalNAc, respectively.

that are initiated by the attachment of the virus glycoprotein C to GAG molecules in the form of HS (*28*). Recently, it was reported that HSV-1 glycoprotein C bound to the cell surface CS of gro2C cells, which are deficient in HS expression (*28*), and the binding was specifically and more strongly inhibited by squid cartilage CS-E than with Hep (*29*), suggesting that HSV-1 requires the same structure as that in the CS-E chains for infection. The sequence recognized by the virus remains to be investigated and will be useful for developing efficient inhibitors of the infection. Despite the important roles of the CS-E structure in various biological processes, reports on the mammalian CS−PGs containing the oversulfated E unit are limited. Although small yet appreciable amounts of E disaccharide units have been found in mammalian tissues, including the brain (see above), specific PG molecules, which have been demonstrated to bear the E unit, are limited to β-thrombomodulin (*64*), appican (*31*) and PG-M/versican (*27*). Hence, a highly purified CS-E preparation derived from squid cartilage was used for the present structural investigation.

Here, we isolated five unsaturated tetrasaccharides containing a GlcA(3S) residue and two odd-numbered unsaturated oligosaccharides containing a β-D-Glc residue, which had never been isolated as discrete structures. The structures and yields of GlcA(3S)-containing oligosaccharides identified in the previous and present studies are summarized in Table 5. The yield of each oligosaccharide obtained from 100 mg of CS-E was utilized for calculations of the frequencies of the sequences located on the reducing and nonreducing sides of GlcA(3S) residues (Figure 6). It should be noted that medium-sized oligosaccharide sequences with consecutive GlcA(3S)-containing disaccharide units are not taken into account, because such sequences are minor, if not nonexistent, in squid cartilage CS-E. In this study, gel filtration chromatography of the chondroitinase AC-II digest of CS-E yielded distinct di- and tetrasaccharide products in addition to larger fragments in fraction I, which were produced by incomplete digestion (see above) but negligible amounts of medium-sized oligosaccharides (Figure 1), indicating that the GlcA(3S) structure is not or is seldom clustered but rather interspersed in squid cartilage CS-E chains. This is consistent with the structural feature of the GlcA(3S)-containing oligosaccharides previously isolated from this source (*33*, *34*), most of which contained only a single 3-*O*-sulfate group in each of these oligosaccharide structures. Oligosaccharides with consecutive GlcA(3S)-containing disaccharide units (fractions Mb and O in ref *34*) have been isolated only in a small proportion (12 mol % of the GlcA(3S)-containing tetra- and hexasaccharide sequences) from this source, whereas such oligosaccharides with consecutive GlcA(3S)-containing disaccharide units were isolated from king crab cartilage CS−K in a larger proportion (at least 55 mol % of the GlcA-(3S)-containing tetra- and hexasaccharide sequences) (*41*, *42*). These differences may reflect the distinct substrate specificities of the unidentified chondroitin GlcA 3-*O*-sulfotransferases in squid and king crab.

The reducing side structures of GlcA(3S) residues in the CS-E and CS−K chains have been reported based on the systematic structural study of the oligosaccharides isolated after digestion with testicular hyaluronidase (*33*, *34*, *41*, *42*), since GlcA(3S) is frequently found at the nonreducing end of the isolated oligosacharides, reflecting the cleavage specificity of the hyaluronidase. The nonreducing side structures of GlcA(3S) residues in squid cartilage CS-E chains were revealed for the first time in this study. The unsaturated tetrasaccharides obtained after the chondroitinase AC-II digestion contained a GlcA(3S)-bearing disaccharide unit at the reducing ends. Since chondroitinase AC-II acts on the GalNAc linkage bound to a nonsulfated GlcA residue, the unsaturated hexuronic acids in the isolated tetrasaccharides are derived from GlcA but not from IdoA, as shown in Figure 6. On the nonreducing side of the GlcA(3S) residue, either GlcA−GalNAc(4S) [A unit] or GlcA−GalNAc(4S,6S) [E unit] but no GlcA−GalNAc(6S) [C unit] was found (Table 5, Figure 6). By contrast, on the reducing side of the GlcA-(3S)-containing disaccharide units (K, L, and M units) or

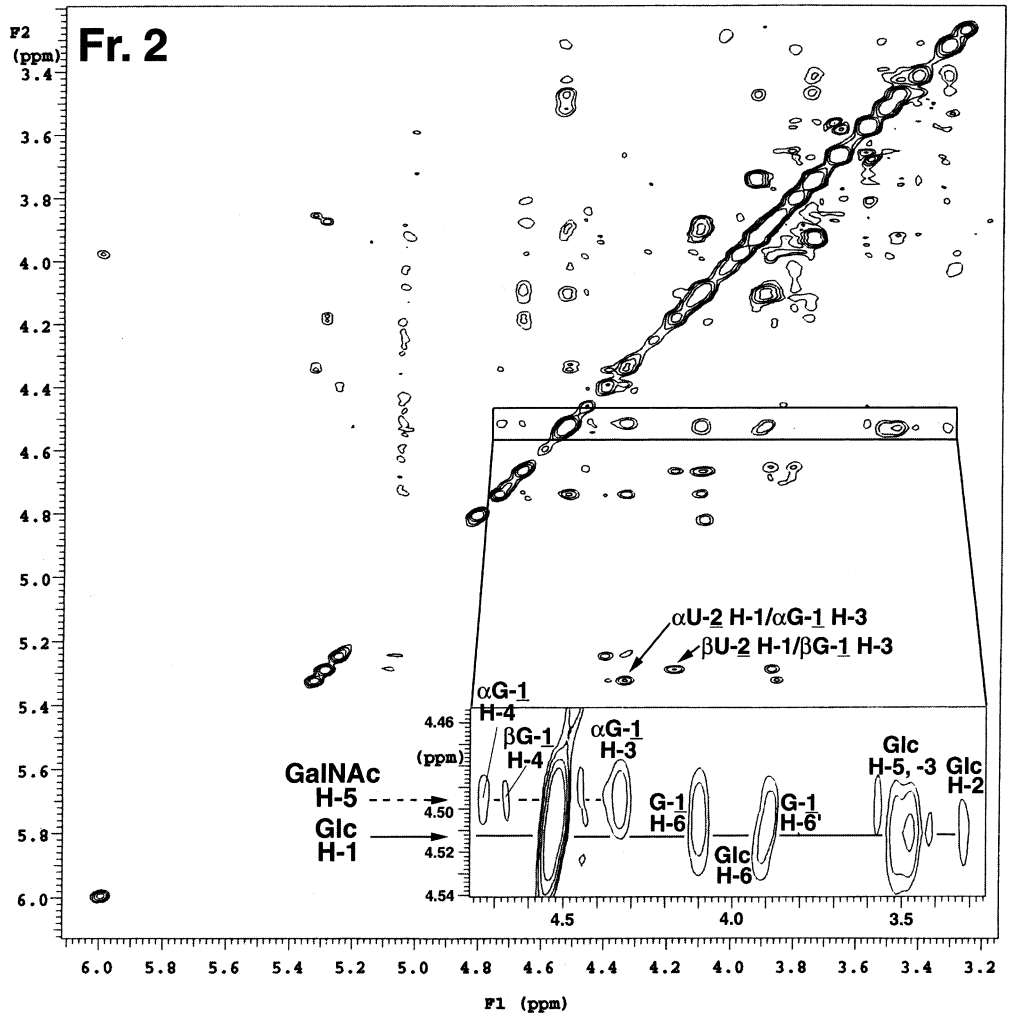

FIGURE 5: 2D ROESY spectrum of fraction 2 recorded in ^2H_2O at 26 °C. An enlargement of a part of the spectrum including the Glc H-1 line (solid) and the GalNAc H-5 line (dotted) is shown in the *inset*. The cross-peaks were identified between Glc H-1 and the H-6/H-6′ of the GalNAc-1 residue.

two consecutive such units (K−L and K−K), an A-unit was always found as indicated by underlines in the symbolic presentation of oligosaccharide structures in Table 5, although marked structural diversity was found in the sulfation profile of the GalNAc residue immediately adjacent to the GlcA(3S) residue on the reducing side. At least one of the C4 and C6 positions is always sulfated, and approximately 60% of the GalNAc are 4,6-*O*-disulfated (Figure 6). The predominant structure on the reducing side is GalNAc(4S,6S) (59%), whereas that on the nonreducing side is GalNAc-(4S) (80%). The structural variety found in the vicinity of the GlcA(3S) residue might represent the substrate specificity of the unidentified biosynthetic enzyme chondroitin GlcA 3-*O*-sulfotransferase. The timing of 3-*O*-sulfation in biosynthesis remains to be investigated.

The unique Glc branch was previously reported for CS-E from squid cartilage based on analyses using enzymatic digestion and periodate oxidation. However, the position on the GalNAc residue substituted by the Glc residue was not firmly established (*65*). In this study, *β*-D-Glc-containing CS oligosaccharides (in fractions 2 and 6) were isolated for the first time as discrete structures and sequenced unambiguously through solid chemical analysis by FAB-MS and ^1H NMR. The yield of Glc-containing disaccharide units in squid cartilage CS-E was approximately 5 mol % of the total based on the results of the HPLC analysis of the chondroitinase digest after derivatization with 2-aminobenzamide (*66*) (data not shown). The biological significance of the rare *β*-D-glucosylation is unknown but may be the protection of the polysaccharide chains from enzymatic degradation. The possible involvement of the Glc residue in various in vitro biological activities observed for the parent CS-E polysaccharides, which include the anticoagulation, neuritogenesis, neuronal adhesion and migration, inhibition of virus infection, and growth factor binding, remains to be clarified. It should be noted that another branching structure, 3-*O*-fucosylation on GlcA residues, has been reported in king crab cartilage CS−K (*42*) and sea cucumber CS (*36*, *67*, *68*), and has been implicated in the protection of the CS chains from enzymatic degradation (*36*). The relation between the unique sugar branches and the 3-*O*-sulfation of GlcA remains to be investigated.

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Table 5: GlcA(3-*O*-sulfate)-Containing Oligosaccharides Isolated from Squid Cartilage CS-E

Structure[a]	Symbolic Presentation[b]	Yield[c] (nmol)	Ref.[d]
6S **GlcA**-GalNAc-GlcA-GalNAc **3S** 4S	[L-A]	280	Fr. l in Ref. 33
GlcA-GalNAc-GlcA-GalNAc **3S** 4S 4S	[K-A]	710	Fr. m in Ref. 33
6S **GlcA**-GalNAc-GlcA-GalNAc **3S** 4S 4S	[M-A]	930	Fr. q in Ref. 33
6S **GlcA**-GalNAc-GlcA-GalNAc-GlcA-GalNAc **3S** 4S 4S 4S	[M-A-A]	191	Fr. Ma in Ref. 34
6S **GlcA**-GalNAc-**GlcA**-GalNAc-GlcA-GalNAc **3S** 4S **3S** 4S	[K-L-A]	119	Fr. Mb in Ref. 34
GlcA-GalNAc-**GlcA**-GalNAc-GlcA-GalNAc **3S** 4S **3S** 4S 4S	[K-K-A]	220	Fr. O in Ref. 34
6S GlcA-GalNAc-**GlcA**-GalNAc-GlcA-GalNAc 4S **3S** 4S 4S	[A-M-A]	310	Fr. P in Ref. 34
6S ΔHexA-GalNAc-**GlcA**-GalNAc 4S **3S**	[ΔA-L]	214	Fr. 7
ΔHexA-GalNAc-**GlcA**-GalNAc 4S **3S** 4S	[ΔA-K]	243	Fr. 8
6S ΔHexA-GalNAc-**GlcA**-GalNAc 4S **3S** 4S	[ΔA-M]	508	Fr. 9
6S ΔHexA-GalNAc-**GlcA**-GalNAc 4S **3S** 4S	[ΔE-K]	48	Fr. 10
6S 6S ΔHexA-GalNAc-**GlcA**-GalNAc 4S **3S** 4S	[ΔE-M]	195	Fr. 11

[a] 3S, 4S, and 6S represent 3-*O*-sulfate, 4-*O*-sulfate, and 6-*O*-sulfate, respectively. The GlcA(3S) residues were highlighted in bold letters. [b] A, E, K, L, M, ΔA, and ΔE stand for the disaccharide units GlcA-GalNAc(4S), GlcA-GalNAc(4S,6S), GlcA(3S)-GalNAc(4S), GlcA(3S)-GalNAc(6S), GlcA(3S)-GalNAc(4S,6S), ΔHexA-GalNAc(4S), and ΔHexA-GalNAc(4S,6S), respectively. The disaccharide units located on the reducing side of the GlcA(3-*O*-sulfate)-containing disaccharide unit (K, L, or M unit) are underlined (see the text). [c] Yield is given in nanomoles obtained from 100 mg CS-E. [d] The fraction numbers refer to those designated in the original studies.

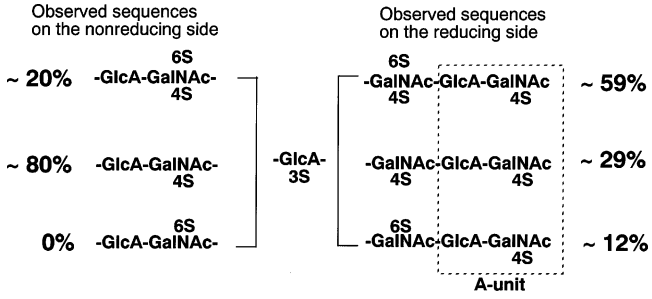

FIGURE 6: Summary of the sequences found on the reducing and nonreducing sides of the GlcA(3S) structure in the compounds isolated from squid cartilage CS-E. The frequencies of the sequences found on the nonreducing and reducing sides of the GlcA(3S) structure are shown. Minor sequences with consecutive GlcA(3S)-containing disaccharide units are not taken into account. The percentages shown are based on the data shown in Table 5, excluding those for fractions Mb and O. The disaccharide unit located on the reducing side of the GlcA(3S)-containing disaccharide unit is always -GlcA-GalNAc(4S)- [A unit] as indicated by the dotted box.

SUPPORTING INFORMATION AVAILABLE

The FAB-MS spectra of fractions 6 and 11 as two distinct representatives and the HPLC profile of the sugar composition analysis of fraction 6. This material is available free of charge via the Internet at http://pubs.acs.org.

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