

## Domain Structure of Chondroitin Sulfate E Octasaccharides Binding to Type V Collagen\*

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We demonstrated previously that chondroitin sulfate E (ChS-E) binds to type V collagen (Munakata, H., Takagaki, K., Majima, M., and Endo, M. (1999) *Glycobiology* 9, 1023–1027). In this study, we investigated the structure and binding of ChS-E oligosaccharides. Eleven oligosaccharides were isolated from ChS-E by gel filtration chromatography and anion-exchange high performance liquid chromatography after hydrolysis with testicular hyaluronidase. Separately, seven oligosaccharides were custom synthesized using the transglycosylation reaction of testicular hyaluronidase. Structural analysis was performed by enzymatic digestions in conjunction with high performance liquid chromatography and mass spectrometry. This library of 18 oligosaccharides was used as a source of model molecules to clarify the structural requirements for binding to type V collagen. Binding was analyzed by a biosensor based on surface plasmon resonance. The results indicated that to bind to type V collagen the oligosaccharides must have the following carbohydrate structures: 1) octasaccharide or larger in size; 2) a continuous sequence of three GlcA $\beta$ 1–3GalNAc(4S,6S) units; 3) a GlcA $\beta$ 1–3GalNAc(4S,6S) unit, GlcA $\beta$ 1–3GalNAc(4S) unit or GlcA $\beta$ 1–3GalNAc(6S) unit at the reducing terminal; 4) a GlcA $\beta$ 1–3GalNAc(4S,6S) unit at the nonreducing terminal. It is likely that these characteristic oligosaccharide sequences play key roles in cell adhesion and extracellular matrix assembly.

Proteoglycan exists in tissues of many mammalian species and is widely distributed in the cell surface and extracellular matrix. It is known that its carbohydrate chain, chondroitin sulfate (ChS),<sup>1</sup> has various important biological activities in

areas such as cell migration, recognition, and morphogenesis (1–5). Recently, greater attention has been directed toward the functions of ChS in the brain, optic nerves, and chondrocytes (6–8). ChS has many types of structural domains that are known to participate in specific physiological functions. However, the relationship between the biological function and structure of ChS domains is not yet fully understood.

Recently, we used a surface plasmon resonance (SPR) biosensor to investigate the interaction of glycosaminoglycans (GAGs) with collagens and glycoproteins from the extracellular matrix (9). It was found that chondroitin sulfate E (ChS-E) has specific affinity for type V collagen, and that a novel characteristic sequence included in ChS-E is probably involved in binding to type V collagen. ChS-E from squid cartilage consists mainly of the disaccharide units GlcA $\beta$ 1–3GalNAc(4S,6S), GlcA $\beta$ 1–3GalNAc(4S), GlcA $\beta$ 1–3GalNAc(6S), and GlcA $\beta$ 1–3GalNAc, where 4S and 6S represent 4-*O*- and 6-*O*-sulfate, respectively (10, 11). Thus, since ChS-E has a variety of structures, it is likely that some specific feature of its structure is necessary for binding to type V collagen. However, the structural features within ChS-E that bind to type V collagen remain unclear.

In the present work, we have prepared various oligosaccharides by testicular hyaluronidase digestion of squid cartilage ChS-E. However, not all of the expected oligosaccharides were obtained. Recently, the transglycosylation mechanism of testicular hyaluronidase, which is an endo- $\beta$ -*N*-acetylhexosaminidase, has been investigated with the aim of performing enzymatic synthesis of GAG oligosaccharides (12, 13). Using this enzymatic reconstruction system, it was possible to custom synthesize chimeric GAG oligosaccharides with sequences of nonsulfated and monosulfated disaccharide units, GlcA $\beta$ 1–3GalNAc, GlcA $\beta$ 1–3GlcNAc, IdoA $\alpha$ 1–3GalNAc, GlcA $\beta$ 1–3GalNAc4S, and GlcA $\beta$ 1–3GalNAc6S. Here, we newly synthesized various chimeric oligosaccharides with disulfated disaccharide units, GlcA $\beta$ 1–3GalNAc(4S,6S), at the internal position of the sugar chain and used them as model molecules to define the structural requirements needed to bind to type V collagen.

### EXPERIMENTAL PROCEDURES

**Materials**—Chondroitin 4-sulfate (Ch4S, from whale cartilage; average molecular weight 34,000), chondroitin 6-sulfate (Ch6S, from shark cartilage; average molecular weight 64,000), dermatan sulfate (DS, from pig skin; average molecular weight 32,000), chondroitin sulfate E (ChS-E, from squid cartilage; average molecular weight 70,000), hyaluronic acid (HA, from human umbilical cord; average molecular weight 900,000), chondroitinase AC-II (from *Arthrobacter aurescens*), chondroitinase ABC (from *Proteus vulgaris*), and six unsaturated di-

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<sup>1</sup> The abbreviations used are: ChS, chondroitin sulfate; GAG, glycosaminoglycan; Ch4S, chondroitin 4-sulfate; Ch6S, chondroitin 6-sulfate; ChS-E, chondroitin sulfate E; DS, dermatan sulfate; Ch, chondroitin; HA, hyaluronic acid; PA, 2-aminopyridine; GlcA, glucuronic acid; IdoA, L-iduronic acid; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine;  $\Delta$ GlcA, D-gluco-4-enopyranosyluronic acid; HexA, hexuronic acid; HexNAc, *N*-acetylhexosamine;  $\Delta$ Di-OS,  $\Delta$ GlcA- $\beta$ 1–3GalNAc;  $\Delta$ Di-4S,  $\Delta$ GlcA $\alpha$ 1–3GalNAc(4S);  $\Delta$ Di-6S,  $\Delta$ GlcA $\beta$ 1–3GalNAc(6S);  $\Delta$ Di-diS<sub>D</sub>,  $\Delta$ GlcA(2S) $\beta$ 1–3GalNAc(6S);  $\Delta$ Di-diS<sub>E</sub>,  $\Delta$ GlcA- $\beta$ 1–3GalNAc(4S, 6S);  $\Delta$ Di-triS,  $\Delta$ GlcA(2S) $\beta$ 1–3GalNAc(4S,6S); Di-OS, GlcA $\beta$ 1–3GalNAc; Di-4S, GlcA $\beta$ 1–3GalNAc(4S); Di-6S, GlcA $\beta$ 1–3GalNAc(6S); Di-diS<sub>E</sub>, GlcA $\beta$ 1–3GalNAc(4S,6S); 2S, 4S, and 6S, represent 2-*O*-sulfate, 4-*O*-sulfate, and 6-*O*-sulfate, respectively;

EDC, *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride; HPLC, high performance liquid chromatography. All sugars mentioned in this paper are of D-configuration except for iduronic acid.



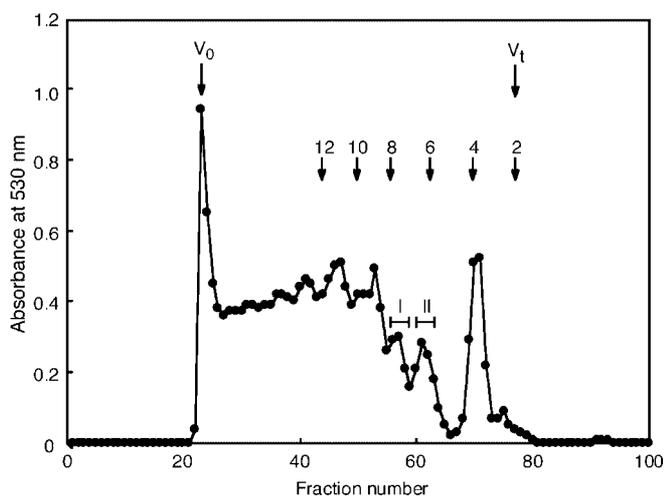


FIG. 1. Gel filtration column chromatography of the hyaluronidase digest of squid cartilage ChS-E on Bio-Gel P-4. Commercial squid cartilage ChS-E was digested with bovine testicular hyaluronidase as described under "Experimental Procedures." The digest was fractionated on a Bio-Gel P-4 column ( $1.6 \times 110$  cm) using  $0.5$  M pyridinium acetate buffer, pH 6.5, as the eluent. Fractions (3.0 ml) were collected and screened for hexuronic acid, and separated fractions were pooled as indicated. Arrows numbered 2–12 indicate the elution positions of the standards, disaccharide to dodecasaccharide, which were prepared by testicular hyaluronidase digestion of Ch6S.

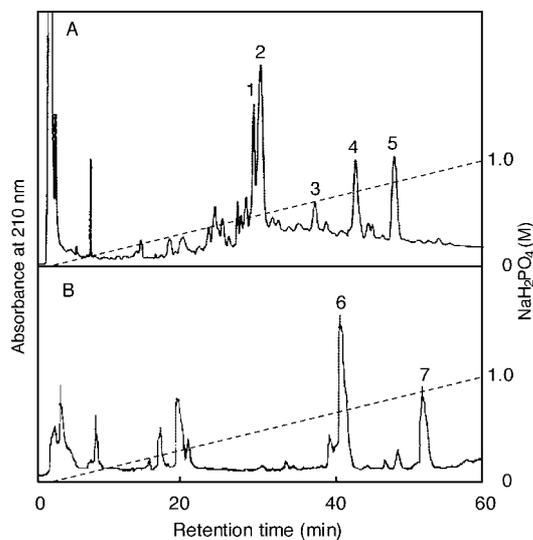


FIG. 2. Subfractionation of fraction I (A) and fraction II (B) by HPLC on a Polyamine II column. Fractions I and II obtained by size fractionation (Fig. 1) were separated into five oligosaccharides (numbers 1–5) (A) and two oligosaccharides (numbers 6 and 7) (B) on a Polyamine II column ( $4.6 \times 250$  mm) using a linear gradient of  $\text{NaH}_2\text{PO}_4$  from 16 to 1000 mM over a 60-min period at a flow rate of 1.0 ml/min at  $30^\circ\text{C}$ .

weight of oligosaccharide number 7 was computed to be  $1556.3 \pm 0.9$ . Therefore, oligosaccharide number 7 was presumed to be a hexasaccharide with five sulfate residues. The assignments of the multiply charged ions afforded by each of the oligosaccharides (numbers 1–7) are summarized in Table I.

**Enzymatic Characterization of Oligosaccharides**—The disaccharide composition of the ChS-E before hydrolysis into oligosaccharides was determined by digestion with chondroitinase ABC and AC-II in conjunction with HPLC (data not shown). As a result, the digestion of ChS-E gave four products,  $\Delta\text{Di-0S}$ ,  $\Delta\text{Di-4S}$ ,  $\Delta\text{Di-6S}$ , and  $\Delta\text{Di-diS}_E$ , in a molar ratio of 5.9:22.9:9.6:61.5. It was shown that ChS-E contains the structural disaccharide units  $\text{GlcA } \beta\text{-3GalNAc}$ ,  $\text{GlcA } \beta\text{1-3GalNAc(4S)}$ ,  $\text{GlcA } \beta\text{1-3GalNAc(6S)}$ , and  $\text{GlcA } \beta\text{1-3GalNAc(4S,6S)}$ .

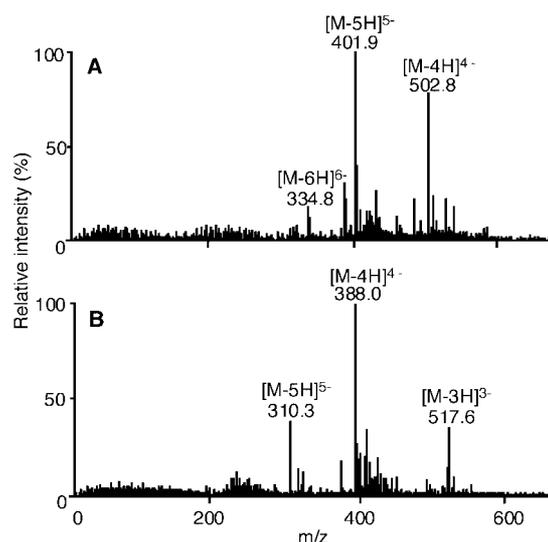


FIG. 3. Ion-spray mass spectra of oligosaccharides numbers 1 (A) and 7 (B) in the negative-ion mode. Oligosaccharides numbers 1 and 7 were subjected to ion-spray mass spectrometry in  $0.5$  mM ammonium acetate-acetonitrile (50:50 by volume) at  $2 \mu\text{l}/\text{min}$ . In the negative-ion mode, scanning was done from  $m/z$  0 to 700 during the 1-min scan (six cycles).

Disaccharide analysis of each oligosaccharide was then performed. To investigate the sequential arrangement of the representative disaccharide units in each oligosaccharide, aliquots of the oligosaccharides were used in two separate experiments. The first aliquot of the oligosaccharides was digested with chondroitinase AC-II, and then labeled with the fluorescent reagent PA. PA-labeled unsaturated and saturated disaccharides were analyzed by HPLC and detected by PA fluorescence at excitation and emission wavelengths of 320 and 400 nm, respectively. Chondroitinase AC-II, which is a bacterial eliminase, should degrade the oligosaccharide to a saturated disaccharide unit derived from the nonreducing terminal and a number of unsaturated disaccharide units derived from the internal region and the reducing terminal. The results are summarized as a composition analysis in Table II. The second aliquot was labeled with PA at the reducing terminal of the oligosaccharides, and then digested with chondroitinase AC-II. PA-labeled unsaturated disaccharide units derived from the reducing terminal were analyzed by HPLC. The results are summarized in Table III. This procedure not only gives the disaccharide composition but also gives information about the sequential arrangement of disaccharide units in the oligosaccharides.

The measured average molecular weight of oligosaccharide number 1 was 2015.2, which corresponded to an octasaccharide with six sulfate residues,  $(\text{HexA})_4(\text{HexNAc})_4(\text{OSO}_3\text{H})_6$ . HPLC analysis of the chondroitinase AC-II digest of number 1 after derivatization with PA showed PA derivatives of Di-diS<sub>E</sub>,  $\Delta\text{Di-6S}$ , and  $\Delta\text{Di-diS}_E$  in a molar ratio of 1.0:2.3:1.0 (Table II). We assume that the saturated disaccharide unit Di-diS<sub>E</sub> was derived from the nonreducing terminal. HPLC analysis of the chondroitinase AC-II digest of oligosaccharide number 1 labeled with PA before digestion showed  $\Delta\text{Di-6S}$  (Table III), which was derived from the reducing terminal. The sequential arrangement of the remaining two disaccharide units was determined by digestion with chondroitinase ABC (28). Chondroitinase ABC split the octasaccharide into one saturated disaccharide derived from the nonreducing terminal, one unsaturated disaccharide derived from the second disaccharide unit from the nonreducing terminal, and one unsaturated tetrasaccharide derived from the reducing terminal. The unsaturated disaccharide unit generated upon digestion with

TABLE I  
Ion-spray mass analysis of the seven oligosaccharides isolated from ChS-E

Oligosaccharide number	<i>m/z</i>				Molecular weight	Assignment
	[M-6H] <sup>6-</sup>	[M-5H] <sup>5-</sup>	[M-4H] <sup>4-</sup>	[M-3H] <sup>3-</sup>		
1	334.8	401.9	502.8		2015.2 ± 0.6	(HexA) <sub>4</sub> (HexNAc) <sub>4</sub> (OSO <sub>3</sub> H) <sub>6</sub>
2	334.1	401.5	503.9		2015.2 ± 0.6	(HexA) <sub>4</sub> (HexNAc) <sub>4</sub> (OSO <sub>3</sub> H) <sub>6</sub>
3	348.0	419.4	522.4		2094.9 ± 0.6	(HexA) <sub>4</sub> (HexNAc) <sub>4</sub> (OSO <sub>3</sub> H) <sub>7</sub>
4	348.0	418.7	525.7		2094.6 ± 0.4	(HexA) <sub>4</sub> (HexNAc) <sub>4</sub> (OSO <sub>3</sub> H) <sub>7</sub>
5	361.6	433.3	543.9		2176.5 ± 0.8	(HexA) <sub>4</sub> (HexNAc) <sub>4</sub> (OSO <sub>3</sub> H) <sub>8</sub>
6		310.4	388.1	517.5	1556.0 ± 0.6	(HexA) <sub>3</sub> (HexNAc) <sub>3</sub> (OSO <sub>3</sub> H) <sub>5</sub>
7		310.3	388.0	517.6	1556.3 ± 0.9	(HexA) <sub>3</sub> (HexNAc) <sub>3</sub> (OSO <sub>3</sub> H) <sub>5</sub>

TABLE II  
Disaccharide composition analysis of the isolated oligosaccharides

Each oligosaccharide was digested completely with chondroitinase AC-II, and then labeled with the fluorescent reagent, PA. PA-labeled saturated and unsaturated disaccharides were identified and quantified by HPLC as described under "Experimental Procedures."

Oligosaccharide number	Composition							
	PA-Di-0S	PA-Di-4S	PA-Di-6S	PA-Di-diS <sub>E</sub>	PA-ΔDi-0S	PA-ΔDi-4S	PA-ΔDi-6S	PA-ΔDi-diS <sub>E</sub>
	% molar proportion <sup>a</sup>							
1	ND <sup>b</sup>	ND	ND	21.7	2.4	4.5	50.3	21.1
2	ND	ND	1.1	23.2	20.2	7.3	4.3	43.9
3	ND	ND	ND	26.0	0.3	8.5	20.6	44.6
4	ND	0.3	0.3	25.1	ND	18.4	4.4	51.5
5	ND	ND	0.9	23.9	ND	1.7	0.9	72.6
6	ND	ND	0.2	32.7	0.2	1.1	32.6	33.2
7	ND	0.1	0.1	34.3	0.3	24.5	7.9	32.8

<sup>a</sup> Percent recoveries were calculated based on the peak area in HPLC and were expressed in molar proportions of the disaccharides produced by digestion.

<sup>b</sup> ND, not detected.

TABLE III  
Disaccharide analysis at the reducing terminal of the isolated oligosaccharides

Each oligosaccharide was fluorolabeled with PA and then digested with chondroitinase AC-II and the PA-labeled disaccharides were identified and quantified by HPLC as described under "Experimental Procedures."

Oligosaccharide number	Composition			
	PA-ΔDi-0S	PA-ΔDi-4S	PA-ΔDi-6S	PA-ΔDi-diS <sub>E</sub>
	% molar proportion <sup>a</sup>			
1	3.6	6.0	90.4	ND <sup>b</sup>
2	98.2	ND	1.8	ND
3	ND	8.1	86.4	5.5
4	ND	94.3	0.3	5.4
5	ND	1.5	4.4	94.1
6	ND	9.5	85.7	4.8
7	ND	90.9	6.1	3.0

<sup>a</sup> Percent recoveries were calculated based on the peak area in HPLC and were expressed in molar proportions of the PA-labeled disaccharides produced by digestion.

<sup>b</sup> ND, not detected.

chondroitinase ABC was ΔDi-6S (data not shown), suggesting that the structure of the second disaccharide unit from the nonreducing terminal of number 1 was GlcA β1-3GalNAc(6S). Hence, the following structure is proposed for oligosaccharide number 1: GlcA-GalNAc(4S,6S)-GlcA-GalNAc(6S)-GlcA-GalNAc(4S,6S)-GlcA-GalNAc(6S).

The average molecular weight of oligosaccharide number 2 was 2015.2, which corresponded to an octasaccharide with six sulfate residues, (HexA)<sub>4</sub>(HexNAc)<sub>4</sub>(OSO<sub>3</sub>H)<sub>6</sub>. HPLC analysis of the chondroitinase AC-II digest of number 2 after derivatization with PA showed PA derivatives of Di-diS<sub>E</sub>, ΔDi-0S, and ΔDi-diS<sub>E</sub> in a molar ratio of 1.0:0.9:1.9 (Table II). We assume that the saturated disaccharide unit Di-diS<sub>E</sub> was derived from the nonreducing terminal. HPLC analysis of the chondroitinase AC-II digest of oligosaccharide number 2 labeled with PA before digestion showed ΔDi-0S (Table III), which was derived from the reducing terminal. Therefore, the remaining two disaccharide units, ΔDi-diS<sub>E</sub>, were located in the internal region.

Hence, the following structure is proposed for oligosaccharide number 2: GlcA-GalNAc(4S,6S)-GlcA-GalNAc(4S,6S)-GlcA-GalNAc(4S,6S)-GlcA-GalNAc.

The average molecular weights of oligosaccharides 3 and 4 were 2094.9 and 2094.6, which corresponded to octasaccharides with seven sulfate residues, (HexA)<sub>4</sub>(HexNAc)<sub>4</sub>(OSO<sub>3</sub>H)<sub>7</sub>. HPLC analysis of the chondroitinase AC-II digests after derivatization with PA showed PA derivatives of Di-diS<sub>E</sub>, ΔDi-6S, and ΔDi-diS<sub>E</sub> in a molar ratio of 1.0:0.8:1.7 in number 3, and Di-diS<sub>E</sub>, ΔDi-4S, and ΔDi-diS<sub>E</sub> in a molar ratio of 1.0:0.7:2.1 in number 4 (Table II). HPLC analysis of chondroitinase AC-II digests labeled with PA before digestion showed ΔDi-6S in number 3 and ΔDi-4S in number 4 (Table III). Hence, the following structures are proposed for oligosaccharides numbers 3 and 4: GlcA-GalNAc(4S,6S)-GlcA-GalNAc(4S,6S)-GlcA-GalNAc(4S,6S)-GlcA-GalNAc(6S) and GlcA-GalNAc(4S,6S)-GlcA-GalNAc(4S,6S)-GlcA-GalNAc(4S,6S)-GlcA-GalNAc(4S), respectively.

The average molecular weight of oligosaccharide number 5 was 2176.5, which corresponded to an octasaccharide with eight sulfated residues, (HexA)<sub>4</sub>(HexNA)<sub>4</sub>(OSO<sub>3</sub>H)<sub>8</sub>. HPLC analysis of the chondroitinase AC-II digest of number 5 after derivatization with PA showed PA derivatives of Di-diS<sub>E</sub> and ΔDi-diS<sub>E</sub> in a molar ratio of 1.0:3.0 (Table II). HPLC analysis of a chondroitinase AC-II digest of number 5 labeled with PA before digestion showed ΔDi-diS<sub>E</sub> (Table III). Hence, the following structure is proposed for oligosaccharide number 5: GlcA-GalNAc(4S,6S)-GlcA-GalNAc(4S,6S)-GlcA-GalNAc(4S,6S)-GlcA-GalNAc(4S,6S).

The average molecular weights of oligosaccharide numbers 6 and 7 were 1556.0 and 1556.3, which corresponded to hexasaccharides with five sulfate residues, (HexA)<sub>3</sub>(HexNAc)<sub>3</sub>(OSO<sub>3</sub>H)<sub>5</sub>. HPLC analysis of chondroitinase AC-II digests after derivatization with PA showed PA derivatives of Di-diS<sub>E</sub>, ΔDi-6S, and ΔDi-diS<sub>E</sub> in a molar ratio of 1.0:1.0:1.0 in number 6, and Di-diS<sub>E</sub>, ΔDi-4S, and ΔDi-diS<sub>E</sub> in a molar ratio of 1.0:0.7:1.0 in number 7 (Table II). HPLC analysis of chondroitinase AC-II digests labeled with PA before digestion showed

TABLE IV  
 Structure of oligosaccharides and its affinity for collagen V

Oligosaccharide number	Structures	Affinity
1	(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)	-
2	$\begin{array}{cccc}   &   &   &   \\ 4S,6S & 6S & 4S,6S & 6S \\   &   &   &   \\ \text{(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)} \end{array}$	-
3	$\begin{array}{cccc}   &   &   &   \\ 4S,6S & 4S,6S & 4S,6S & 6S \\   &   &   &   \\ \text{(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)} \end{array}$	+
4	$\begin{array}{cccc}   &   &   &   \\ 4S,6S & 4S,6S & 4S,6S & 6S \\   &   &   &   \\ \text{(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)} \end{array}$	+
5	$\begin{array}{cccc}   &   &   &   \\ 4S,6S & 4S,6S & 4S,6S & 4S \\   &   &   &   \\ \text{(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)} \end{array}$	+
6	$\begin{array}{cccc}   &   &   &   \\ 4S,6S & 4S,6S & 4S,6S & 4S,6S \\   &   &   &   \\ \text{(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)} \end{array}$	-
7	$\begin{array}{cccc}   &   &   &   \\ 4S,6S & 4S,6S & 6S & 6S \\   &   &   &   \\ \text{(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)} \end{array}$	-
8	$\begin{array}{cccc}   &   &   &   \\ 4S,6S & 4S,6S & 4S & 4S \\   &   &   &   \\ \text{(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)} \end{array}$	-
9	(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)	-
10	$\begin{array}{cccc}   &   &   &   \\ 4S & 4S & 4S & 4S \\   &   &   &   \\ \text{(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)} \end{array}$	-
11	$\begin{array}{cccc}   &   &   &   \\ 6S & 6S & 6S & 6S \\   &   &   &   \\ \text{(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-GlcA} \end{array}$	-
12	$\begin{array}{cccc}   &   &   &   \\ 4S,6S & 4S,6S & 4S,6S & 6S \\   &   &   &   \\ \text{(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-PA} \end{array}$	-
13	$\begin{array}{cccc}   &   &   &   \\ 4S & 4S,6S & 4S,6S & 6S \\   &   &   &   \\ \text{(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-PA} \end{array}$	-
14	$\begin{array}{cccc}   &   &   &   \\ 6S & 4S,6S & 4S,6S & 6S \\   &   &   &   \\ \text{(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-PA} \end{array}$	-
15	$\begin{array}{cccc}   &   &   &   \\ 4S,6 & 4S,6S & 4S,6S & 6S \\   &   &   &   \\ \text{(GlcA-GlcNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-PA} \end{array}$	-
16	$\begin{array}{cccc}   &   &   &   \\ 4S,6S & 4S,6S & 4S,6S & 6S \\   &   &   &   \\ \text{(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-PA} \end{array}$	+
17	$\begin{array}{cccc}   &   &   &   \\ 4S,6S & 4S,6S & 4S,6S & 6S \\   &   &   &   \\ \text{GalNAc-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-PA} \end{array}$	-
18	$\begin{array}{cccc}   &   &   &   \\ 4S,6S & 4S,6S & 4S,6S & 6S \\   &   &   &   \\ \text{(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-(GlcA-GalNAc)-PA} \end{array}$	-

$\Delta$ Di-6S in number 6 and  $\Delta$ Di-4S in number 7 (Table III). Hence, the following structures are proposed for oligosaccharide numbers 6 and 7: GlcA-GalNAc(4S,6S)-GlcA-GalNAc(4S,6S)-GlcA-GalNAc(6S) and GlcA-GalNAc(4S,6S)-GlcA-GalNAc(4S,6S)-GlcA-GalNAc(4S), respectively. Five octasaccharides and two hexasaccharides were prepared from ChS-E after hydrolysis with testicular hyaluronidase and used as model oligosaccharides (Table IV).

**Binding of Oligosaccharides Containing E (GlcA $\beta$ 1-3GalNAc(4S,6S)) Units to Immobilized Type V Collagen**—To investigate the structural characteristics of oligosaccharides that bind to type V collagen, five types of octasaccharide (numbers 1–5) and two types of hexasaccharide (numbers 6 and 7), representing model oligosaccharides with GlcA $\beta$ 1-3GalNAc(4S,6S) units, were prepared from ChS-E after hydrolysis with testicular hyaluronidase. As model oligosaccharides without

GlcA $\beta$ 1-3GalNAc(4S,6S) units, three types of oligosaccharide (numbers 8–10) derived from Ch, Ch4S, and Ch6S were prepared according to the previous report (14). A heptasaccharide (number 11) was also obtained by  $\beta$ -elimination reaction of oligosaccharide number 3.

Oligosaccharide solutions were injected onto sensor surfaces bearing immobilized type V collagen and the binding of oligosaccharides to type V collagen was determined from the increased responses on the sensorgrams. Nonspecific binding and the change in refractive index were corrected for by subtracting the response of an albumin surface from that of type V collagen. The results are shown in Fig. 4. No significant binding of oligosaccharides numbers 1 and 2 to a sensor surface coated with type V collagen was detected (Fig. 4, A and B). On the other hand, the increase in resonance units from the initial baseline represents the binding of the injected oligosaccharide

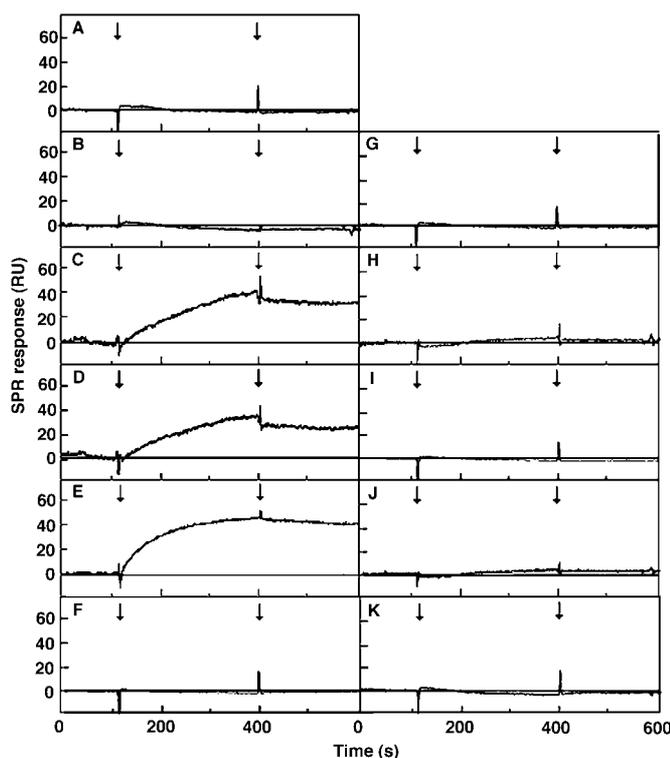


FIG. 4. Sensorgrams of binding of various oligosaccharides to type V collagen. Oligosaccharides 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F), 7 (G), 8 (H), 9 (I), 10 (J), and 11 (K) were injected onto immobilized type V collagen surface. Sensorgram data of albumin surface as the blank sensor surface was subtracted from that of type V collagen surface. All experiments were carried out at a flow rate of 10  $\mu$ l/min at 25  $^{\circ}$ C. The arrows indicate the beginning and the end of injections.

numbers 3–5 to the surface-bound type V collagen (Fig. 4, C–E). Judging from the change in response (data not shown), the binding of number 5 to type V collagen was higher than that of numbers 3 and 4. Neither the hexasaccharides (numbers 6 and 7) nor the heptasaccharide without a GalNAc(6S) residue at the reducing terminal of number 3 bound to type V collagen (Fig. 4, F, G, and K). Furthermore, the octasaccharides (numbers 8–10) without E units also did not bind to type V collagen (Fig. 4, H–J).

Judging from these results, it appears that the binding of oligosaccharides to type V collagen requires a sequence of repeating GlcA $\beta$ 1–3GalNAc(4S,6S) units. As for the structure at the reducing terminal of the oligosaccharide, octasaccharides with GlcA $\beta$ 1–3GalNAc(4S,6S), GlcA $\beta$ 1–3GalNAc(4S), or GlcA $\beta$ 1–3GalNAc(6S) units at the reducing terminal were found to bind to type V collagen, but those with a GlcA $\beta$ 1–3GalNAc unit did not. The heptasaccharide without a GalNAc(6S) residue at the reducing terminal did not bind to type V collagen.

**Custom Synthesis of New Model Oligosaccharides**—The above oligosaccharides, which were prepared by hydrolysis with testicular hyaluronidase, have mostly GlcA $\beta$ 1–3GalNAc(4S,6S) units at the nonreducing terminal. To study the effect of structural differences at the nonreducing terminal on binding to type V collagen, new model oligosaccharides were custom synthesized by using the transglycosylation reaction of testicular hyaluronidase, as described previously (22, 23). Ch, Ch4S, Ch6S, or desulfated DS as donors containing various disaccharide units, and a PA-hexasaccharide (PA-oligosaccharide number 6), GlcA-GalNAc(4S,6S)-GlcA-GalNAc(4S,6S)-GlcA-GalNAc(6S)-PA as acceptor, were incubated with hyaluronidase under optimal conditions (0.15 M Tris-HCl buffer, pH

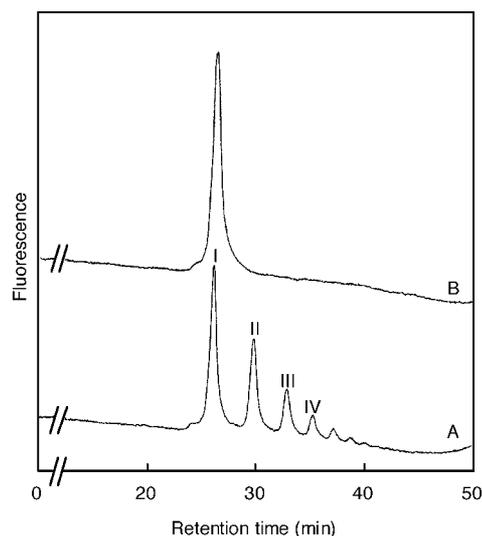


FIG. 5. HPLC chromatograms of the transglycosylation reaction products with a PA-hexasaccharide (PA-oligosaccharide number 6) as an acceptor and Ch as a donor. PA-hexasaccharide and Ch were incubated with testicular hyaluronidase under the conditions, pH 7.0, optimal for the transglycosylation reaction at 37  $^{\circ}$ C for 0 (A) and 1 h (B) and then subjected to HPLC (PALPAK Type S column; 4.6  $\times$  250 mm). The chromatographic conditions are described under “Experimental Procedures.”

7.0, in the absence of NaCl at 37  $^{\circ}$ C for 60 min). The reaction products were examined by HPLC by monitoring the fluorescence of the PA-oligosaccharides. A typical HPLC chromatogram with Ch used as a donor is shown in Fig. 5. Three peaks (II, III, and IV) of newly synthesized products were observed: PA-octasaccharide, PA-decasaccharide, and PA-dodecasaccharide elongated by GlcA $\beta$ 1–3GalNAc units derived from Ch added to the acceptor (peak I). Peak II was recovered and used as oligosaccharide number 12. The amounts of the other elongated PA-oligosaccharides as representative reaction products are shown in Table V. Overall, five new oligosaccharide types (PA-octasaccharides numbers 12–16) having different disaccharide units at the nonreducing terminal were custom synthesized. Number 17 was prepared from number 3 by fluorescence labeling with PA at the reducing terminal, and number 18 was obtained by  $\beta$ -glucuronidase digestion of number 17. The nonreducing terminal sugar residue of oligosaccharide number 15 was removed with  $\alpha$ -L-iduronidase but not with  $\beta$ -glucuronidase. In the same way, each oligosaccharide was structurally characterized by chemical analysis, enzymatic analysis, ion-spray mass analysis, and HPLC analysis as previously reported (9, 19) (data not shown).

**Binding of Newly Synthesized Oligosaccharides Containing GlcA $\beta$ 1–3GalNAc(4S,6S) Unit to Immobilized Type V Collagen**—Seven types of oligosaccharide (numbers 12–18) with different carbohydrate structures at the nonreducing terminals were synthesized enzymatically and used as model oligosaccharides in the binding reaction (Table IV). Solutions of these seven types of oligosaccharide were injected onto sensor surfaces bearing immobilized type V collagen, and the binding of the oligosaccharides to the type V collagen was determined from the increased responses on the sensorgrams. The results are summarized in Table IV. Oligosaccharide number 17, with an GlcA $\beta$ 1–3GalNAc(4S,6S) unit at the nonreducing terminal, was found to bind to type V collagen. A typical concentration-dependent binding is shown in Fig. 6. However, there was no significant binding of numbers 12–16, which have nonsulfated disaccharide units, GlcA $\beta$ 1–3GalNAc, IdoA $\alpha$ 1–3GalNAc, or GlcA $\beta$ 1–3GlcNAc, or monosulfated disaccharide units, GlcA $\beta$ 1–3GalNAc(4S) or GlcA $\beta$ 1–3GalNAc(6S), instead of a GlcA-

TABLE V  
Transglycosylation of various glycosaminoglycans to ChS-E  
hexasaccharide used as an acceptor

PA-ChS-E hexasaccharide was used as an acceptor. Values indicate percentages of total products.

Donor	Chain length of reaction products						
	6	8	10	12	14	16	18
				%			
Ch	46.9	23.5	12.3	10.1	3.9	2.2	1.1
Ch4S	78.3	12.5	5.7	2.5	1.0		
Ch6S	78.5	13.1	5.4	2.1	0.9		
Desulfated DS	95.6	3.1	1.3				
HA	35.0	26.3	18.2	11.8	4.9	2.5	1.3

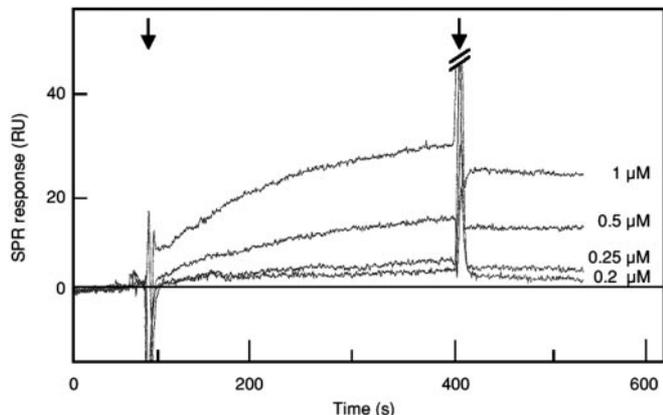


FIG. 6. Concentration dependence of the binding of oligosaccharide number 17 to immobilized type V collagen. *a*, 1  $\mu\text{M}$ ; *b*, 0.5  $\mu\text{M}$ ; *c*, 0.25  $\mu\text{M}$ ; *d*, 0.2  $\mu\text{M}$ . The arrows indicate the beginning and the end of injections.

$\beta$ 1-3GalNAc(4S,6S) unit at the nonreducing terminal. When the GlcA residue at the nonreducing terminal of oligosaccharide number 17 was removed, creating oligosaccharide number 18, there was a remarkable decrease in binding. These results show that three continuous GlcA $\beta$ 1-3GalNAc(4S,6S) units at the nonreducing terminal, and a minimum octasaccharide size, are important for binding to type V collagen.

#### DISCUSSION

In this study, a variety of artificial oligosaccharides containing GlcA $\beta$ 1-3GalNAc(4S,6S) units were synthesized as model oligosaccharides. The binding of the model oligosaccharides to type V collagen was analyzed using a surface plasmon resonance biosensor. The results revealed that to bind to type V collagen the oligosaccharides must have the following carbohydrate structures: 1) octasaccharide or larger size; 2) a continuous sequence of three GlcA $\beta$ 1-3GalNAc(4S,6S) units; 3) a GlcA $\beta$ 1-3GalNAc(4S,6S) unit, GlcA $\beta$ 1-3GalNAc(4S) unit, or GlcA $\beta$ 1-3GalNAc(6S) unit at the reducing terminal; 4) a GlcA $\beta$ 1-3GalNAc(4S,6S) unit at the nonreducing terminal.

Among the GAGs, only a few correlations between particular carbohydrate sequences and functions have been shown, such as the pentasaccharides with anticoagulant and antithrombotic activities (29–31), or the hexasaccharide having affinity for bFGF (32) in heparan sulfate and heparin, the hexasaccharides in the heparin cofactor II-binding domain of DS (33) and the decasaccharides of HA that bind to core proteins of proteoglycan (34). These examples have shown that the essential size of the oligosaccharide in active domains in GAG seems to be from pentasaccharide to decasaccharide.

Recently, squid cartilage ChS-E was demonstrated to possess neurite outgrowth promoting activity in embryonic rat hippocampal neurons (35). It was also demonstrated that cortical neuronal cell adhesion, mediated by heparin-binding neu-

roregulatory factor midkine, is inhibited by squid cartilage ChS-E (36). Binding of ChS-E to midkine was also demonstrated using a BIAcore system (37). Oversulfated ChS is not limited to invertebrates or marine vertebrates and has been found in various tissues and cells from higher land-dwelling vertebrates. ChS-E has been found in human rib cartilage (38), bovine brain (39), and chick brain (40). It is known that the GlcA $\beta$ 1-3GalNAc(4S,6S) unit is present in the GAGs of serglycin proteoglycan of mouse mast cells (36, 41) and also at the nonreducing terminal of GAGs of aggrecan proteoglycan of human knee cartilage (42). Moreover, it was demonstrated that the GalNAc(4S,6S) residue content of the nonreducing terminal region changes in relation to age (42). Therefore, it is likely that GlcA $\beta$ 1-3GalNAc(4S,6S) units also exist in higher animals, and affect the regulation of various biological phenomena such as adhesion, migration, and neurite outgrowth of neuronal cells.

Type V collagen is widely distributed as a minor component of the extracellular matrix in various tissues (43). It is thought to be an important component for connective tissue assembly, as type V collagen generally co-polymerizes with the more abundant type I collagen (44). Therefore, the binding of chondroitin sulfate E to type V collagen may affect the growth of collagen fibrils. Also, type V collagen has been shown to bind to biglycan (45) and NG2 (46). However, only the core protein part of the molecule responsible for binding to type V collagen has been characterized, not the glycosaminoglycan part. The type V collagen mainly consists of  $\alpha$ 1 (V),  $\alpha$ 2 (V), and  $\alpha$ 3 (V) chains. Although, the type V collagen used in this paper consisted of  $\alpha$ 1 (V) and  $\alpha$ 2 (V) chains (data not shown), no data is available at present concerning what part of the type V collagen binds to octasaccharides derived from chondroitin sulfate E.

GAGs have many types of structural domains, and are known to participate in physiological functions such as anticoagulation and antithrombotic activities. It is important to prepare different GAG oligosaccharides as investigational molecules to analyze the relationship between their structure and function. The chemical synthesis of oligosaccharides has been made possible by recent rapid advances in methodology (47, 48). However, chemical synthesis is not at present the best way to prepare these molecules, because of the many reaction steps, the time required and the necessity for special technology. In this respect, attention has been directed to the synthesis of oligosaccharides by using glycosidases, which catalyze transglycosylation as a reverse reaction of hydrolysis (49, 50).

The transglycosylation mechanism of testicular hyaluronidase, which is an endo- $\beta$ -N-acetylhexosaminidase, has been investigated with the aim of performing enzymatic synthesis of GAGs (9, 10). It was possible to custom synthesize GAG oligosaccharides having various arrangements of nonsulfated disaccharide units (GlcA  $\beta$ 1-3GalNAc, GlcA $\beta$ 1-3GlcNAc, and IdoA $\alpha$ 1-3GalNAc) and monosulfated disaccharide units (GlcA $\beta$ 1-3GalNAc(4S) and GlcA $\beta$ 1-3GalNA(6S)), by repeating the transglycosylation using suitable combinations of HA, Ch, desulfated DS, Ch4S, and Ch6S as acceptors and donors.

In this study, the possibility of using the disulfated disaccharide unit GlcA $\beta$ 1-3GalNAc(4S,6S) as a substrate for custom synthesis of GAG oligosaccharides by testicular hyaluronidase was examined. As a result, it was possible to transfer various disaccharide units to oligosaccharides having GlcA $\beta$ 1-3GalNAc(4S,6S) units at the nonreducing terminal, thereby synthesizing new ChS-E-related oligosaccharides each having a different disaccharide unit at the nonreducing terminal. Disulfated disaccharide domains are unique and are specifically recognized by other molecules and are involved in various biological processes. The GAG oligosaccharide libraries that

were custom synthesized in this report will facilitate the investigation of the relationship between the biological function and structure of GAG oligosaccharides.

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