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Involvement of Human Natural Killer-1 (HNK-1) Sulfotransferase in the Biosynthesis of the GlcUA(3-*O*-sulfate)-Gal-Gal-Xyl Tetrasaccharide Found in α-Thrombomodulin from Human Urine*[S]

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Thrombomodulin (TM) is an integral membrane glycoprotein, which occurs as both a chondroitin sulfate (CS) proteoglycan (PG) form (β-TM) and a non-PG form without a CS chain (α-TM) and hence is a part-time PG. An α-TM preparation isolated from human urine contained the glycosaminoglycan linkage region tetrasaccharide GlcUAβ1–3Galβ1–3Galβ1–4xylose, and the nonreducing terminal GlcUA residue is 3-*O*-sulfated. Because the human natural killer-1 sulfotransferase (HNK-1ST) transfers a sulfate group from 3′-phosphoadenosine 5′-phosphosulfate to the C-3 position of the nonreducing terminal GlcUA residue in the HNK-1 antigen precursor trisaccharide, GlcUAβ1–3Galβ1–4GlcNAc, the sulfotransferase activity toward the linkage region was investigated. In fact, the activity of HNK-1ST toward the linkage region was much higher than that toward the glucuronylneolactotetraosylceramide, the precursor of the HNK-1 epitope. HNK-1ST may be responsible for regulating the sorting of α- and β-TM. Furthermore, HNK-1ST also transferred a sulfate group from 3′-phosphoadenosine 5′-phosphosulfate to the C-3 position of the nonreducing terminal GlcUA residue of a chondroitin chain. Intriguingly, the HNK-1 antibody recognized CS chains and the linkage region if they contained GlcUA(3-*O*-sulfate), suggesting that HNK-1ST not only synthesizes the HNK-1 epitope but may also be involved in the generation of part-time PGs.

Thrombomodulin (TM)[3] is an integral membrane glycoprotein expressed on the vascular endothelial cell surface, which occurs as both a chondroitin sulfate (CS) proteoglycan (PG) form (β-TM) and a non-PG form without a CS chain (α-TM) and hence is a part-time PG (1). TM binds to thrombin with high affinity and acts as a mediator of endothelial anticoagulant defenses. Comparative studies on the anticoagulant effects of α-TM and β-TM revealed that the CS chain of β-TM plays an indispensable role in regulating the anticoagulation function (2). β-TM exhibits higher affinity for thrombin than α-TM and more effectively inhibits thrombin-mediated clotting activity. In addition, platelet factor 4, which prevents the formation of a complex between heparin and antithrombin III, leading to blood coagulation, binds to β-TM but not to α-TM (3). The platelet factor 4/β-TM complex displays anticoagulant activity by inactivating the clotting factors Va and VIIIa through limited proteolysis (3). Thus, the CS moiety of TM is essential for its biological functions. However, the control mechanism for the glycanation step has not been clarified.

CS-PGs are ubiquitous components of the cell surface and extracellular matrix and participate in diverse biological processes such as cell proliferation, cell differentiation, and the development of the nervous system (4–6). CS chains have a linear polymeric structure composed of the disaccharide units -4GlcUAβ1–3GalNAcβ1-. During the chain elongation step of CS biosynthesis, GlcUA residues are sulfated at C-2 and/or GalNAc residues are sulfated C-4 and/or C-6 in various combinations by multiple sulfotransferases, producing characteristic sulfation patterns that are critical for binding to a variety of functional proteins (6). This structural variability is the basis for the wide range of biological activities of CS chains.

CS chains are synthesized on Ser residues in core proteins through the so-called carbohydrate-protein linkage region, GlcUAβ1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser, where Xyl represents D-xylose. Even α-TM, a non-PG form of TM, contains this tetrasaccharide structure at least in certain circumstances (7), and the nonreducing terminal GlcUA residue in an α-TM preparation isolated from human urine was demonstrated to be 3-*O*-sulfated (8). Thus, 3-*O*-sulfation might be a key modification for the biosynthesis of part-time PGs. However, the GlcUA 3-*O*-sulfotransferase has not been identified although most of the sulfotransferases involved in the sulfation of CS chains have been cloned (9).

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[3] The abbreviations used are: TM, thrombomodulin; GAG, glycosaminoglycan; Chn, chondroitin; CS, chondroitin sulfate; PG, proteoglycan; GlcAT, glucuronyltransferase; HexUA, hexuronic acid; ΔHexUA, 4-deoxy-L-*threo*-hex-4-enepyranosyluronic acid; HNK-1, human natural killer-1; HNK-1ST, HNK-1 sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; CSase, chondroitinase; 2AB, 2-aminobenzamide; DE, delayed extraction.

The human natural killer-1 (HNK-1) carbohydrate epitope has a sulfated trisaccharide structure, GlcUA(3-*O*-sulfate)β1–3Galβ1–4GlcNAc (10), and is specifically recognized by the monoclonal antibody HNK-1 (11–13), which was originally reported as a specific antigen for human natural killer cells (14). Glucuronyltransferases, GlcAT-P and GlcAT-S, as well as HNK-1 sulfotransferase (HNK-1ST) are essential enzymes for the biosynthesis of the HNK-1 carbohydrate epitope (10). HNK-1ST transfers a sulfate group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to the C-3 position of the nonreducing terminal GlcUA residue in the HNK-1 antigen precursor trisaccharide, GlcUAβ1–3Galβ1–4GlcNAc (15, 16), and has significant homology in amino acid sequence with chondroitin sulfotransferases (17, 18).

Although the expression of GlcAT-P and GlcAT-S is restricted to HNK-1-positive cells, the expression pattern of HNK-1ST is wider than that of the HNK-1 epitope (15, 19). Because CS-PGs are also generated in all organs, HNK-1ST may be able to transfer a sulfate group from PAPS to CS.

In this study, we examined the sulfotransferase activity of HNK-1ST toward the GAG linkage region as well as various CS isoforms and demonstrated that HNK-1ST transferred a sulfate group from PAPS to the nonreducing terminal GlcUA residues in these substrates *in vitro*. HNK-1ST may be involved in regulating the biosynthesis of not only the HNK-1 epitope but also part-time PGs.

EXPERIMENTAL PROCEDURES

Materials—^{35}S-Labeled PAPS (1.59 mCi/mmol) was purchased from PerkinElmer Life Sciences. The pCMV-Sport6/human HNK-1ST vector (IMAGE Consortium cDNA clone, ID number 4158309) was obtained from Open Biosystems Inc. (Huntsville, AL). The anti-FLAG M2 affinity resin, p3×FLAG-CMV8 vector, monoclonal anti-HNK-1/N-CAM antibody (clone VC1.1), and unlabeled PAPS were purchased from Sigma. The following sugars and enzymes were from Seikagaku Corp. (Tokyo, Japan): CS-A from whale cartilage, CS-B from porcine skin, CS-C from shark cartilage, CS-D from shark cartilage, CS-E from squid cartilage, Chn, a chemically desulfated derivative of CS-A, standard unsaturated CS disaccharides, chondroitinase (CSase) ABC (EC 4.2.2.20) from *Proteus vulgaris*, CSase AC-II (EC 4.2.2.5) from *Arthrobacter aurescens*, and chondro-4-sulfatase (EC 3.1.6.9) and chondro-6-sulfatase (EC 3.1.6.10) from *P. vulgaris*. COS-7 cells were purchased from Japan Health Sciences Foundation (Tokyo, Japan). Glucuronylneolactotetraosylceramide (GlcUAβ1–3Galβ1-4GlcNAcβ1–3Galβ1–4Glcβ1–1ceramide) was obtained from Wako (Osaka, Japan). Sulfo-NHS-LC-Biotin and EZ-Link-Biotin hydrazide were purchased from Pierce. *KOD*-Plus DNA polymerase was purchased from Toyobo (Tokyo, Japan). The tetraosyl peptide GlcUAβ1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser-Gly-Asp-Asn-Gly (SGDNG) was chemically synthesized (20). CS-K from king crab cartilage was prepared as reported previously (21). The octa-, hexa-, and tetrasaccharides derived from Chn were prepared by treatment with sheep testicular hyaluronidase as described (22).The structurally defined tetrasaccharides K-A, GlcUA(3-*O*-sulfate)-GalNAc(4-*O*-sulfate)-GlcUA-GalNAc(4-*O*-sulfate); D-A, GlcUA(2-*O*-sulfate)-GalNAc(6-*O*-sulafte)-GlcUA-GalNAc(4-*O*-sulfate); C-A, GlcUA-GalNAc(6-*O*-sulfate)-GlcUA-GalNAc(4-*O*-sulfate); and A-A, (GlcUA-GalNAc (4-*O*-sulfate)-GlcUA-GalNAc(4-*O*-sulfate) were isolated from CS-K and CS-D as described (23, 24) (see Ref. 6 for abbreviations of the variously modified disaccharide GlcUA-GalNAc units by sulfation including the A, C, D, and K disaccharide units). The saturated disaccharide standard GlcUA(3-*O*-sulfate)-GalNAc was prepared by digestion of the K-A tetrasaccharide with CSase ABC (23) followed by chondro-4-sulfatase (25). The HNK-1 antigen preparation containing 3-*O*-sulfated glucuronylneolactotetraosylceramide was a gift from Dr. Hiroshi Kitagawa (Kobe Pharmaceutical University), which was purified from rat sciatic nerve according to the method described previously (26).

Construction of an Expression Vector Containing a Soluble Form of Human HNK-1ST—A truncated form of human HNK-1ST, lacking the first NH_2-terminal 31 amino acids of human HNK-1ST, was amplified by PCR using a 5′ primer containing an in-frame HindIII site (5′-GCAAGCTTCCAGATGTGTA-CAGTGCC-3′) and a 3′ primer containing an EcoRV site (5′-GCGATATCCTCAGATCTTCACCTGGT-3′) and pCMV-Sport6/human HNK-1ST as a template. The reaction was carried out with *KOD*-Plus DNA polymerase for 30 cycles of 94 °C for 30 s, 57 °C for 42 s, and 68 °C for 60 s. The amplified fragments were digested with HindIII and EcoRV and inserted into the p3×FLAG-CMV8 vector.

Expression of a Soluble Form of HNK-1ST—The expression plasmid (6.7 μg) was introduced into COS-7 cells using FuGENE 6 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. After 3 days of culture at 37 °C, 1 ml of the culture medium was collected and incubated with 10 μl of anti-FLAG M2 affinity resin (Sigma) at 4 °C overnight. The resin was washed with 50 mM Tris-buffered saline containing 0.05% Tween 20 (TBST) and then resuspended in the incubation buffer for the detection of enzymatic activity. The protein concentration was determined using a Micro BCA protein assay kit (Thermo Scientific).

Measurement of Sulfotransferase Activity—Sulfotransferase activity toward the tetraosyl peptide GlcUA-Gal-Gal-Xyl-SGDNG, various CS isoforms, octa-, hexa-, and tetrasaccharides from Chn, structurally defined sulfated tetrasaccharides from CS, and glucuronylneolactotetraosylceramide was assayed by a method described previously (15, 17, 27). The reaction mixture (60 μl) containing 10 μl of the resuspended resin, 50 mM imidazole-HCl, pH 6.8, 2 mM dithiothreitol, 10 μM [^{35}S]PAPS (2×10^5 dpm), and Chn, Chn-oligosaccharide, the tetraosyl peptide, or glucuronylneolactotetraosylceramide was incubated at 37 °C for 4 h. The reaction products of GlcUA-Gal-Gal-Xyl-SGDNG or Chn oligosaccharides were subjected to gel filtration chromatography on a HR10/330 Superdex peptide column (GE Healthcare, Uppsala, Sweden), which was eluted with 0.2 M ammonium bicarbonate at a flow rate of 0.4 ml/min. The reaction product of glucuronylneolactotetraosylceramide was subjected to hydrophobic chromatography on a Nova-Pak C18 column (Waters, Milford, MA) at a flow rate of 0.5 ml/min with the following program: 0–15 min, isocratic with H_2O; 15–20 min, 0–100% gradient with methanol; 20–60 min, isocratic with methanol. Fractions were collected at 1- or

2-min intervals, and radioactivity was quantified by liquid scintillation counting (LS6500, Beckman Coulter). The reaction products of CS isoforms were subjected to gel filtration chromatography using a syringe column packed with Sephadex G-25 (superfine) resin (28). The incorporation of [^{35}S]sulfate into polysaccharides was quantified by measuring the radioactivity in the flow-through fractions by liquid scintillation counting.

Structural Characterization of the HNK-1ST Reaction Products—Chn hexa- and tetrasaccharides (10 nmol as oligosaccharide) were incubated with 30 μl of the HNK-1ST-bound resin, 10 μM PAPS in 50 mM imidazole-HCl, pH 6.8, and 2 mM dithiothreitol, at 37 °C for 4 h. The oligosaccharides were purified by gel filtration chromatography on a Superdex peptide column eluted with 0.2 M ammonium bicarbonate at a flow rate of 0.4 ml/min and monitored by measuring absorption at 220 nm. The oligosaccharides were derivatized with a fluorophore 2-aminobenzamide (2AB) as described (29), and excess 2AB reagent was removed by paper chromatography. The 2AB-labeled reaction products were fractionated by anion-exchange HPLC on an amine-bound silica PA-03 column (YMC Co., Kyoto, Japan) (29), and the collected reaction products were desalted by gel filtration on a Superdex peptide column (30). After repeated lyophilization to completely remove ammonium bicarbonate, the 2AB-labeled reaction products of Chn hexa- and tetrasaccharides were digested with CSase AC-II and ABC (31), respectively, and the digests were analyzed by anion-exchange HPLC on a PA-03 column. An aliquot of the digests was labeled again with 2AB and analyzed by HPLC. The digests of the HNK-1ST reaction products with CSase were treated with chondro-4-sulfatase or chondro-6-sulfatase as described previously (25).

Delayed Extraction Matrix-assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry (DE MALDI-TOF MS)—The reaction products were analyzed by DE MALDI-TOF MS in the linear mode by Voyager-DE STR-H (Applied Biosystems, Foster, CA) at the Open Facility, Sousei Hall of Hokkaido University. 2,5-Dihydroxybenzoic acid was used as the matrix at a concentration of 1 mg/ml in water. Each sample was mixed with the matrix solution on a sample plate well, dried under an air stream, and analyzed by MS in the positive ion mode.

ELISA—The reactivity of the HNK-1 antibody with various CS isoforms as well as CS oligosaccharides was examined by ELISA as described (32). All steps of ELISA were performed at room temperature. The α-amino group of the peptide moiety of the HNK-1ST reaction product was biotinylated as follows. The reaction product of the tetraosyl peptide was purified by gel filtration chromatography and incubated with 10 mM Sulfo-NHS-LC-Biotin in PBS at a molar ratio of 1:20 at 4 °C overnight. The biotinylated glycopeptide was purified by gel filtration chromatography on a Superdex peptide column (30), and an aliquot was incubated in a streptavidin-coated 96-well plate (Nalge Nunc International, Rochester, NY) at room temperature for 1 h. CS isoforms were individually biotinylated and immobilized on the 96-well plates (33). The reducing terminal GalNAc residue of the structurally defined sulfated tetrasaccharides was biotinylated with EZ-Link hydrazide-LC-biotin as follows. A 5 mM biotin hydrazide reagent in dimethyl sulfoxide and 10 mM $NaCNBH_3$ in 0.1 M MES-NaOH (pH 5.5) were mixed with the sulfated tetrasaccharides at a molar ratio of (tetrasaccharide:biotin:$NaCNBH_3$) 1:5:10 and incubated at room temperature overnight. The biotinylated tetrasaccharides were purified by gel filtration chromatography on a Superdex peptide column (30), and an aliquot was incubated in a streptavidin-coated 96-well plate at room temperature for 1 h. The HNK-1 antigen preparation or glucuronylneolactotetraosylceramide was incubated in a Nunc-Immuno PolySorp plate (Nalge Nunc International) for immobilization at room temperature for 1 h.

After immobilization of tested compounds, each well was blocked with 100 μl of 3% bovine serum albumin in PBS for 1 h. The HNK-1 antibody in PBS (1:100) was added to the wells and incubated for 1 h at room temperature. Wells were washed with 200 μl of TBST three times and incubated with 50 μl of alkaline phosphatase-linked anti-mouse IgM (1:1,000) for 1 h at room temperature. Enzymatic activity was detected using *p*-nitrophenylphosphate, and the absorbance was measured at 415 nm after incubation for 30 min. In negative controls, no CS was immobilized.

RESULTS

HNK-1ST Activity toward the GAG-Core Protein Linkage Region—To facilitate the functional analysis of HNK-1ST, a soluble form of the protein was generated by replacing the putative signal sequence with a cleavable preprotrypsin leader sequence and with a 3×FLAG tag as described under "Experimental Procedures." The soluble protein was expressed in COS-7 cells at 37 °C as a recombinant protein fused with the 3×FLAG tag, which was secreted in the medium and adsorbed onto an anti-FLAG affinity resin. The protein-bound resin was purified by centrifugation and used as an enzyme source. The purity of the enzyme was confirmed by Western blotting (data not shown).

Because the rare 3-*O*-sulfated GlcUA residue has been demonstrated in α-TM (8) isolated from human urine, we hypothesized that HNK-1ST transfers a sulfate group from PAPS to the C-3 position of the nonreducing terminal GlcUA residue. The sulfotransferase activity of HNK-1ST toward GlcUA-Gal-Gal-Xyl-SGDNG was examined under the reaction conditions described under "Experimental Procedures." In fact, HNK-1ST transferred a [^{35}S]sulfate group to the linkage region tetraosyl peptide (Fig. 1 and Table 1). The structure of the nonradiolabeled reaction product was first characterized by DE MALDI-TOF MS to determine their molecular weights, from which the sugar composition and the number of *O*-sulfate groups present in the HNK-1ST reaction products were inferred. When the reaction product was analyzed, the molecular ion signal was observed as $[M - 3H + 4Na + SO_3]^+$ at *m*/*z* 1,272 (Fig. 1*B*), suggesting that a sulfate group was transferred to the linkage region. The predominant molecular signal of the substrate tetraosyl peptide was observed as $[M - 2H + 3Na]^+$ at *m*/*z* 1,170 (data not shown). The nonradiolabeled reaction product was further characterized by anion-exchange HPLC after treatment with LiOH to remove the peptide moiety followed by 2AB derivatization. The 2AB linkage tetrasaccharide was eluted at the position of monosulfated tetrasaccharide (Fig. 1*C*). Therefore, we concluded that a sulfate group was transferred to the

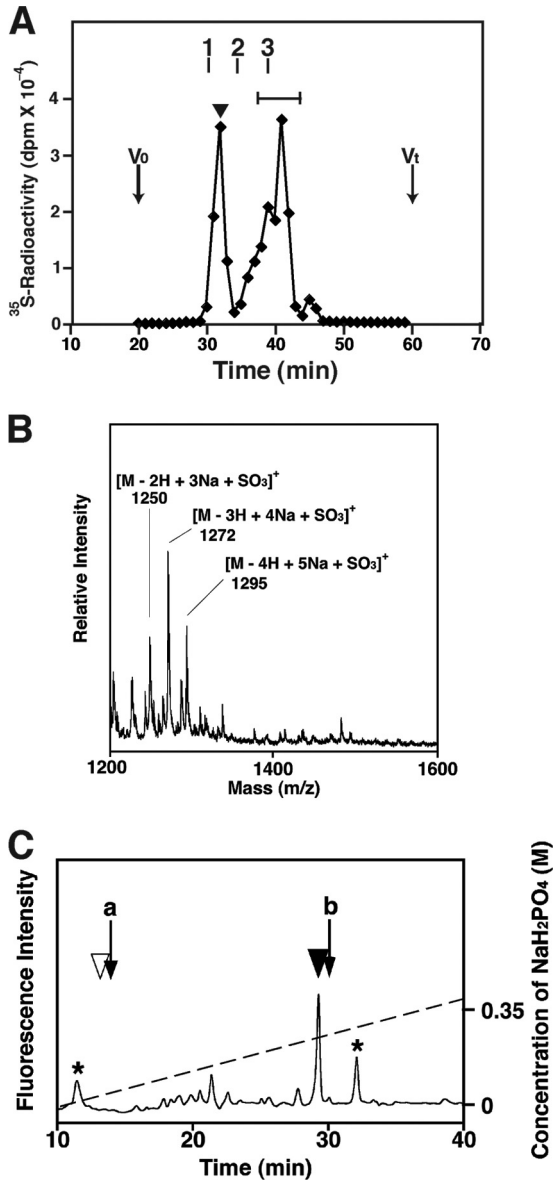


FIGURE 1. **Characterization of the HNK-1ST reaction product.** *A*, gel filtration chromatography of ^{35}S-labeled GlcUA-Gal-Gal-Xyl-SGDNG. The ^{35}S-labeled HNK-1ST reaction product (*arrowhead*) obtained using GlcUA-Gal-Gal-Xyl-SGDNG as an acceptor substrate was fractionated on a Superdex peptide column using 0.2 M ammonium bicarbonate as an effluent at a flow rate of 0.4 ml/min. Fractions were collected at 1-min intervals. V_0 and V_t represent the void and the total volume, respectively. The elution positions of 2AB-labeled oligosaccharides are indicated by *numbered arrows*: *1*, $(GlcUA\text{-}GalNAc)_4$; *2*, $(GlcUA\text{-}GalNAc)_3$; *3*, $(GlcUA\text{-}GalNAc)_2$. The peak marked by a *horizontal bar* is derived from [^{35}S]PAPS and its degradation products. *B*, DE MALDI-TOF mass spectrum. The spectrum was recorded in the positive ion mode with 2,5-dihydroxybenzoic acid as the matrix. Major molecular ion signals were assigned as indicated in the figure. *M* represents the fully protonated acid form of the substrate tetraosyl peptide. *C*, anion-exchange HPLC of the 2AB derivative of the tetrasaccharide derived from the reaction product. The HNK-1ST reaction product was treated with 0.5 M LiOH to liberate the oligosaccharide from the core peptide, and the liberated tetrasaccharide was derivatized with 2AB and analyzed by anion-exchange HPLC. A single predominant peak was observed (*closed arrowhead*). The product obtained using the mock transfectant (GlcUA-Gal-Gal-Xyl-2AB) was eluted at the position indicated by the *open arrowhead* (data not shown). The elution positions of 2AB-labeled linkage tetrasaccharide standards are shown by *arrows*: *a*, ΔHexUA-Gal-Gal-Xyl; *b*, ΔHexUA-Gal(4-*O*-sulfate)-Gal-Xyl. The peaks marked by *asterisks* were unknown substances and also detected in the chromatogram of a negative control run upon a high sensitivity analysis.

tetrasaccharide moiety of the linkage tetraosyl peptide by the enzymatic reaction. The structure of this reaction product was presumed to be GlcUA(3-*O*-sulfate)-Gal-Gal-Xyl-*O*-SGDNG, based on the specificity of the enzyme and the reactivity of the reaction product to the anti-HNK-1 antibody (see below), although no authentic standard is available for confirmation at present.

The initial reaction rates at various concentrations were measured for kinetic analyses, and the Michaelis-Menten constants K_m were determined (Table 2 and supplemental Fig. 1). Although sulfotransferase activity was higher toward the linkage region than glucuronylneolactotetraosylceramide, the precursor of the HNK-1 epitope (Table 1) under the same incubation conditions, it should be noted that the concentration of the latter in the reaction mixture might have been lowered because of its low solubility in water.

Assessment of the Reactivity of the HNK-1 Antibody toward the Sulfated Linkage Region—The reactivity of the HNK-1 antibody toward the HNK-1ST reaction products obtained using the tetraosyl peptide as a substrate was examined using ELISA. As shown in Fig. 2*A*, the antibody reacted with the HNK-1ST reaction product, presumably GlcUA(3-*O*-sulfate)-Gal-Gal-Xyl-*O*-SGDNG. The reactivity was higher than that obtained with the HNK-1 antigen (Fig. 2*B*), suggesting that the antibody recognizes the GlcUA(3-*O*-sulfate)-containing GAG-protein linkage region. However, it should be noted that the assay conditions were not identical because different 96-well plates had to be used for immobilization of the HNK-1ST reaction product and the HNK-1 antigen glycolipid.

HNK-1ST Activity toward Various CS Isoforms and Oligosaccharides—Although the expression of GlcATs is restricted to HNK-1-positive cells, HNK-1ST is more widely expressed than the HNK-1 carbohydrate epitope (15, 19). Because CS-PGs are also generated in most, if not all, tissues and HNK-1ST has significant homology in amino acid sequence to chondroitin sulfotransferases, HNK-1ST may also transfer a sulfate group from PAPS to the C-3 position of GlcUA residues in CS. Hence, the sulfotransferase activity of HNK-1ST toward various CS isoforms (Fig. 3), Chn oligosaccharides (Fig. 4), and sulfated tetrasaccharides, A-A and C-A, was analyzed using the recombinant HNK-1ST under the reaction conditions described in "Experimental Procedures." The reaction products were separated from [^{35}S]PAPS by gel filtration, and the radioactivity was measured by liquid scintillation counting. A soluble form of the recombinant HNK-1ST transferred sulfate to Chn as well as Chn tetra-, hexa-, and octasaccharides (Fig. 4), but not to the CS isoforms (Fig. 3) or the sulfated tetrasaccharides (data not shown). Sulfotransferase activity was weaker toward the oligosaccharides from Chn than toward the genuine substrate glucuronylneolactotetraosylceramide (Table 1). Kinetic analyses for these reactions were also performed to determine the K_m values for these substrates (Table 2 and supplemental Fig. 1). That the affinity of HNK-1ST for the Chn oligosaccharides is relatively weaker than for the glycolipid is not surprising. However, a possible biological significance of the observed moderate affinity to the Chn oligosaccharides will be discussed below.

TABLE 1

Comparison of the acceptor specificity of HNK-1ST

Sulfotransferase activity of the recombinant HNK-1ST was investigated using Chn (10 nmol as GlcUA equivalents), Chn oligosaccharides (10 nmol as oligosaccharide), glucuronylneolactotetraosylceramide (1 nmol), and GlcUA-Gal-Gal-Xyl-SGDNG (1 nmol) as acceptors under the reaction conditions described under "Experimental Procedures."

Acceptor	Enzymatic activity	
	HNK-1ST	Mock
	pmol/μg of protein/h	
Chn polysaccharide (GlcUA-(GalNAc-GlcUA)$_n$-GalNAc)	1.5 ± 0.05	NP[a]
Chn octasaccharide (GlcUA-(GalNAc-GlcUA)$_3$-GalNAc)	6.9 ± 0.4	NP
Chn hexasaccharide (GlcUA-(GalNAc-GlcUA)$_2$-GalNAc)	7.0 ± 0.6	NP
Chn tetrasaccharide (GlcUA-GalNAc-GlcUA-GalNAc)	6.3 ± 2.0	0.2 ± 0.05
Glucuronylneolactotetraosylceramide (GlcUA-Gal-GlcNAc-Gal-Glc-ceramide)	23.4 ± 2.4	ND[b]
GlcUA-Gal-Gal-Xyl-SGDNG	36.3 ± 0.3	0.2 ± 0.05

[a] NP, not performed.
[b] ND, not detected.

TABLE 2

Michaelis-Menten constants of the recombinant HNK-1ST toward various substrates

Michaelis-Menten constant K_m values of HNK-1ST were obtained from Lineweaver-Burk plots (supplemental Fig. 1), in which the reciprocals of initial velocities obtained by sulfotransferase activities of HNK-1ST were plotted against the reciprocals of varying concentrations of the substrates, Chn oligosaccharides, glucuronylneolactotetraosylceramide, and the linkage tetraosyl peptide. The sulfotransferase activities were quantified as described under "Experimental Procedures."

Substrate	K_m
	mM
Chn octasaccharide (GlcUA-(GalNAc-GlcUA)$_3$-GalNAc)	2.6
Chn hexasaccharide (GlcUA-(GalNAc-GlcUA)$_2$-GalNAc)	8.6
Chn tetrasaccharide (GlcUA-GalNAc-GlcUA-GalNAc)	2.3
Glucuronylneolactotetraosylceramide (GlcUA-Gal-GlcNAc-Gal-Glc-ceramide)	0.76
GlcUA-Gal-Gal-Xyl-SGDNG	0.024

Structural Characterization of the HNK-1ST Reaction Products—The position(s) of sulfation in the Chn oligosaccharides modified by the recombinant HNK-1ST was determined by the procedure outlined in Scheme 1. The reaction products of Chn tetra- and hexasaccharides were purified by anion-exchange HPLC after being derivatized with 2AB and analyzed by anion-exchange HPLC as shown in Fig. 5. The 2AB derivatives of HNK-1ST reaction products of Chn tetra- and hexasaccharides were eluted at the positions of presumed monosulfated tetra- (Fig. 5*A*) and hexasaccharides (Fig. 5*B*), respectively.

When the 2AB-derivatized tetrasaccharide fraction was analyzed by DE MALDI-TOF MS in the positive ion mode, the molecular ion signal was observed as $[M + H]^+$ at m/z 975 (Fig. 6*A*). Because the molecular mass of the 2AB-labeled monosulfated tetrasaccharide is 976 Da, the observed value was in reasonable agreement with the theoretical value. The molecular signals of the hexasaccharide fraction were observed as $[M + H]^+$ and $[M + Na]^+$ at m/z 1,355 and 1,377, respectively (Fig. 6*B*), suggesting that the component in this fraction was $HexUA_3HexNAc_3(OSO_3H)_1$-2AB (theoretical value, 1,355), where HexUA and HexNAc represent hexuronic acid and *N*-acetyl-hexosamine, respectively. These results demonstrated that HNK-1ST transferred a sulfate group from PAPS to oligosaccharides derived from Chn.

To characterize the reducing terminal structure of the HNK-1ST reaction products, reaction products obtained using Chn tetra- and hexasaccharides as substrates were labeled with 2AB, isolated by gel filtration, and digested with CSase AC-II and ABC, respectively. Each digest was analyzed by anion-exchange HPLC, and peaks of fluorescence were detected at the eluted positions of ΔHexUA-GalNAc-2AB and ΔHexUA-GalNAc-GlcUA-GalNAc-2AB, respectively, which were derived from the reducing side of the parent 2AB-labeled tetra- and hexasaccharides (Fig. 7, *A* and *C*), indicating that HNK-1ST transferred a sulfate group to the nonreducing terminal disaccharide unit. Unknown peaks marked in Fig. 7 by *asterisks* are presumably derived from the CSase preparations or the PA-03 resin because of a high sensitivity analysis since they were also detected in the chromatogram of a control run (supplemental Fig. 2). An aliquot of each digest of 2AB-labeled reaction products was further labeled with 2AB, isolated by gel filtration, and analyzed by HPLC to characterize the structure of the nonreducing terminal disaccharide unit of the parent 2AB-labeled tetra- and hexasaccharides. The disaccharide unit derived from the nonreducing end was eluted at the same position as GlcUA(3-*O*-sulfate)-GalNAc (Fig. 7, *B* and *D*). The peak was resistant to chondro-4- and -6-sulfatases (data not shown). Taken together, these results suggested that HNK-1ST transferred a sulfate group from PAPS to the C-3 position of the nonreducing terminal GlcUA residue in Chn oligosaccharides.

Assessment of the Reactivity of the HNK-1 Antibody toward Various CS Isoforms and Sulfated Oligosaccharides—The reactivity of the HNK-1 antibody toward CS isoforms and sulfated oligosaccharides was examined using ELISA, in which each biotinylated CS isoform and sulfated oligosaccharide was individually immobilized on a streptavidin-coated plate. As shown in Fig. 2*C*, the antibody reacted with CS-K, which contains the GlcUA(3-*O*-sulfate)-GalNAc(4-*O*-sulfate) unit (K unit) as the predominant disaccharide in a similar reactivity with the HNK-1 antigen, but not with other CS isoforms. To further investigate the specificity of the HNK-1 antibody toward the GlcUA(3-*O*-sulfate)-containing structure, ELISA was performed using three structurally defined sulfated tetrasaccharides, K-A, D-A, and A-A. The antibody moderately reacted with the K-A tetrasaccharide, but the binding to the other sulfated tetrasaccharides tested was not significant (Fig. 2*D*). These results indicate that the HNK-1 antibody recognizes GlcUA(3-*O*-sulfate)-containing CS structures and that the binding site of HNK-1 antibody does not tolerate GlcUA(2-*O*-sulfate) structure.

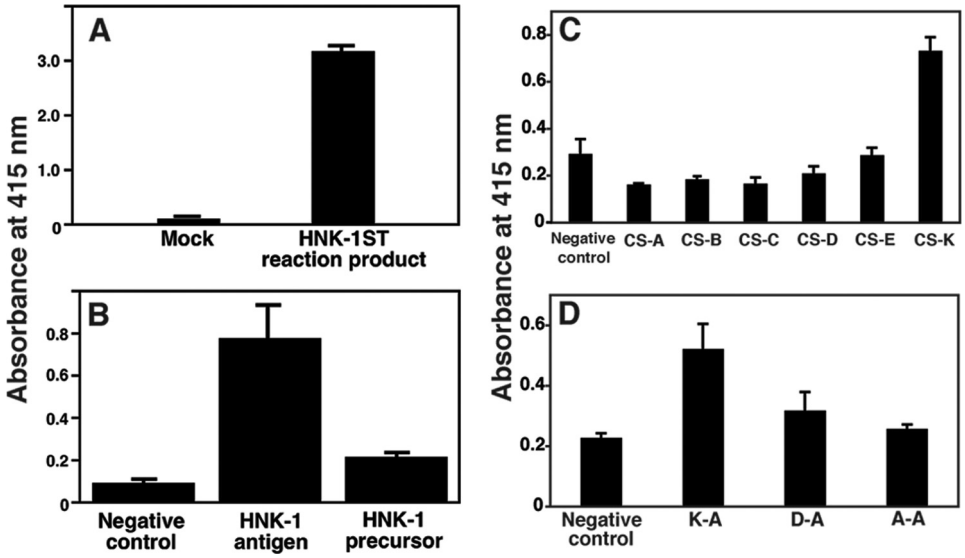


FIGURE 2. **Reactivity of the HNK-1 antibody toward the HNK-1ST reaction product as well as various CS isoforms and oligosaccharides.** *A*, *C*, and *D*, the biotinylated HNK-1ST reaction products obtained using GlcUA-Gal-Gal-Xyl-SGDNG as an acceptor substrate (*A*), CS isoforms (*C*) (0.5 μg each), and sulfated tetrasaccharides (*D*) were individually immobilized onto the wells of a streptavidin-coated plate. *B*, the HNK-1 antigen preparation and its precursor glucuronylneolactotetraosylceramide were immobilized onto the wells of a Nunc-Immuno PolySorp plate. Each plate was incubated with the HNK-1 antibody (1:100). Bound antibody was visualized by subsequent incubation with an alkaline phosphatase-linked goat anti-mouse Ig(G + M) (1:1,000). The enzymatic activity was detected using *p*-nitrophenylphosphate as a substrate, and absorbance was measured at 415 nm. *Mock* contained the nonsulfated acceptor tetraosyl peptide. Negative controls had no immobilized saccharides. Values represent the average ± S.E. for two independent experiments.

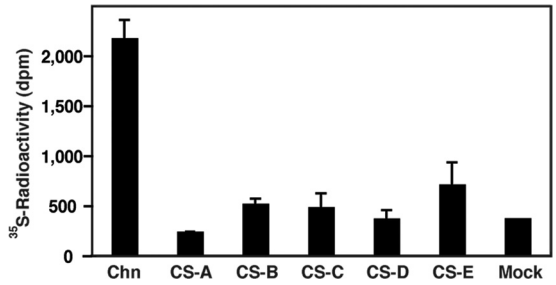


FIGURE 3. **Comparison of the acceptor specificity of HNK-1ST toward various CS isoforms.** The recombinant HNK-1ST was assayed using various CS isoforms as an acceptor (10 nmol as GlcUA) under the reaction conditions described under "Experimental Procedures." The reaction products were separated from [^{35}S]PAPS by gel filtration on a syringe column packed with Sephadex G-25 (superfine) resin. The radioactivity was measured by liquid scintillation counting. The vector control (*Mock*) was also assayed using Chn as the acceptor substrate. Values represent the average ± S.E. for two independent experiments.

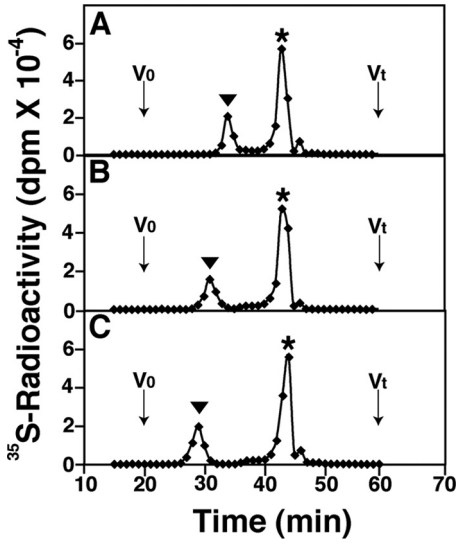
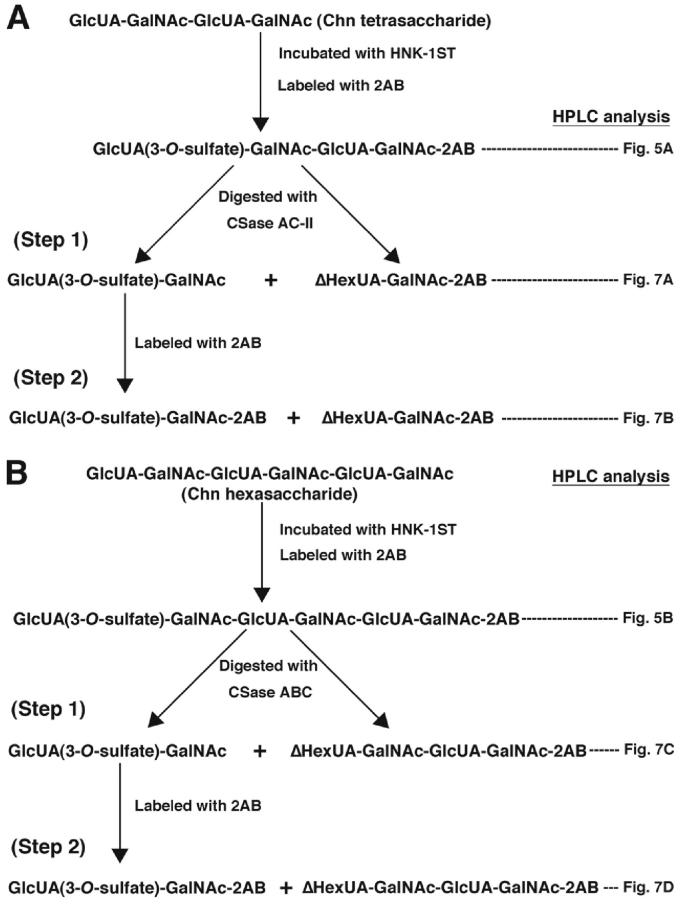


FIGURE 4. **Gel filtration chromatographic analysis of the ^{35}S-labeled sulfotransferase reaction products.** ^{35}S-Labeled HNK-1ST reaction products (*arrowheads*) obtained using Chn tetra- (*A*), hexa- (*B*), and octasaccharides (*C*) as acceptor substrates were fractionated on a Superdex peptide column using 0.2 M ammonium bicarbonate as an effluent at a flow rate of 0.4 ml/min. Fractions were collected at 1-min intervals. V_0 and V_t represent the void and the total volume, respectively. The peaks marked by *asterisks* are derived from [^{35}S]PAPS.

DISCUSSION

In this study, we found that HNK-1ST transfers a sulfate group from PAPS to the tetraosyl peptide of the GAG-core protein linkage region, presumably to the C-3 position of the nonreducing terminal GlcUA residue, because the reaction product was recognized by the HNK-1 antibody. This is the first identification of sulfotransferase activity that can catalyze 3-*O*-sulfation on a GlcUA residue in the GAG-core protein linkage region, the structure of which had been identified in an oligosaccharide synthesized on α-TM from human urine (8). To investigate the physiological significance of the GlcUA(3-*O*-sulfate) structure in α-TM, the presence or absence of the structure in blood-borne α-TM was analyzed. Human TM was purified from a serum sample of a normal subject by immunoprecipitation using anti-human TM antibody and protein G-Sepharose, and the precipitate was subjected to Western blotting under reducing conditions. A band having a molecular mass of ~100 kDa was detected by the monoclonal antibody HNK-1 (supplemental Fig. 3), suggesting that α-TM in human blood also bears a GlcUA(3-*O*-sulfate)-containing oligosaccharide.

The sulfotransferase activity of HNK-1ST toward the GAG-core protein linkage region was higher than that toward the glucuronylneolactotetraosylceramide (Table 1), although it should be noted that the latter is less water-soluble than the former. The biological significance of the sulfation in the link-



SCHEME 1. **Strategy for characterization of the NHK-1ST reaction products.** Chn tetra- (*A*) and hexasaccharide (*B*) were incubated with HNK-1ST, and the reaction products were derivatized with 2AB at the reducing termini. The 2AB-derivatized tetra- and hexasaccharide fractions were exhaustively digested with chondroitinase AC-II and ABC, respectively. Aliquots from these digests were analyzed to detect a 2AB-labeled unsaturated disaccharide or tetrasaccharide product, respectively, by anion-exchange HPLC to identify the reducing terminal structure (*Step 1*), whereas the rest of each digest was further labeled with 2AB and analyzed by anion-exchange HPLC to identify the nonreducing terminal disaccharide unit (*Step 2*).

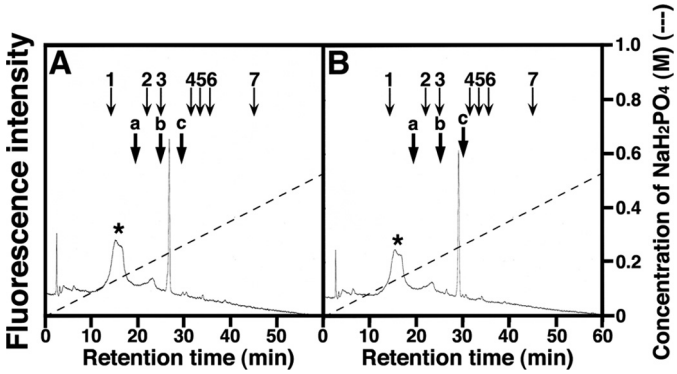


FIGURE 5. **Anion-exchange HPLC of the 2AB-labeled HNK-1ST reaction products.** HNK-1ST reaction products obtained using the Chn tetra- (*A*) or hexasaccharide (*B*) as the acceptor substrate were labeled with 2AB and analyzed by anion-exchange HPLC on a column of amine-bound silica PA-03 using a linear gradient of NaH_2PO_4 (indicated by the *dashed lines*). The eluted positions of 2AB-labeled authentic unsaturated disaccharides as well as Chn oligosaccharides are indicated by *numbered arrows*: *1*, ΔHexUA-GalNAc; *2*, ΔHexUA-GalNAc(6-*O*-sulfate); *3*, ΔHexUA-GalNAc(4-*O*-sulfate); *4*, ΔHexUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate); *5*, ΔHexUA(2-*O*-sulfate)-GalNAc(4-*O*-sulfate); *6*, ΔHexUA-GalNAc(4,6-*O*-disulfate); and *7*, ΔHexUA(2-*O*-sulfate)-GalNAc(4,6-*O*-disulfate). *Arrows a*, *b*, and *c* indicate the eluted positions of: $(GlcUA\text{-}GalNAc)_2$ (*a*); $(GlcUA\text{-}GalNAc)_3$ (*b*); and $(GlcUA\text{-}GalNAc)_4$ (*c*). The *asterisk* indicates the impurity derived from the PA-03 HPLC column.

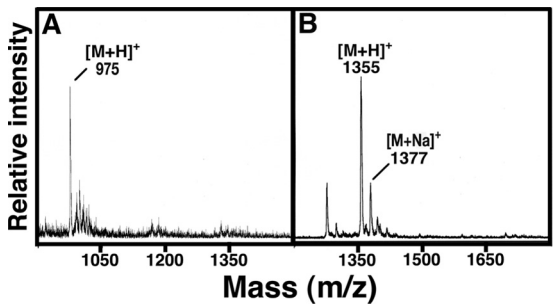


FIGURE 6. **DE MALDI-TOF mass spectrum of the HNK-1ST reaction products.** DE MALDI-TOF spectra of 2AB-labeled HNK-1ST reaction products, obtained using Chn tetra- (*A*) and hexasaccharides (*B*) as acceptor substrates in Fig. 4, were recorded in the positive ion mode with 2,5-dihydroxybenzoic acid as the matrix. Major molecular ion signals were assigned as indicated in the figure.

age region is unknown. However, the sulfated GlcUA residue may play an important role in the production of part-time CS-PGs. HNK-1ST may transfer a sulfate group to the nonreducing terminal GlcUA of the linkage region tetrasaccharide of α-TM to regulate the glycanation step during the synthesis of CS chains on TM. When a sulfate group is transferred to the nonreducing terminal GlcUA of the linkage region of TM, it may become a non-PG form, α-TM. Otherwise, the polymerization of CS chains may proceed by a default mechanism to generate a PG form, β-TM. Because the linkage region tetrasaccharide is common to CS and heparan sulfate, another interesting possibility cannot be excluded: that 3-*O*-sulfation of the GlcUA residue in the linkage region is also involved in the generation of part-time heparan sulfate PGs (1).

The HNK-1 antibody was demonstrated to recognize GlcUA(3-*O*-sulfate)-containing CS structures, in addition to the HNK-1 epitope oligosaccharide, GlcUA(3-*O*-sulfate)-Gal-GlcNAc. The expression of human GlcAT-P and GlcAT-S, which transfer a GlcUA residue to the precursor structure, Galβ1-4GlcNAc, and are responsible for the biosynthesis of the HNK-1 epitope, is restricted to the brain and liver and to the adrenal glands and trachea, respectively (34, 35). However, the staining by HNK-1 antibody is observed not only in the brain but also at endocrine cells in the gut and pancreas, where neither of the GlcATs is expressed (36). Expression of HNK-1ST is ubiquitous, and CS-PGs are widely produced by animal cells. Thus, the oligosaccharides recognized by the HNK-1 antibody in the organs where GlcATs are not expressed may contain GlcUA(3-*O*-sulfate)-bearing CS structures including non-PG forms of part-time PGs.

HNK-1ST also transferred a sulfate group to the C-3 position of the nonreducing terminal GlcUA residue in Chn or Chn oligosaccharides but not in CS isoforms (Figs. 1, 3, and 4). This is the first demonstration of sulfotransferase activity catalyzing 3-*O*-sulfation on a GlcUA residue in Chn. However, because the affinity of HNK-1ST for the Chn oligosaccharides was relatively low (Table 2), it is not clear whether this transfer reaction to Chn and Chn oligosaccharides plays a significant role in the regulation of the elongation of growing CS chains. Although Hiraoka *et al.* (18) previously analyzed the HNK-1ST activity

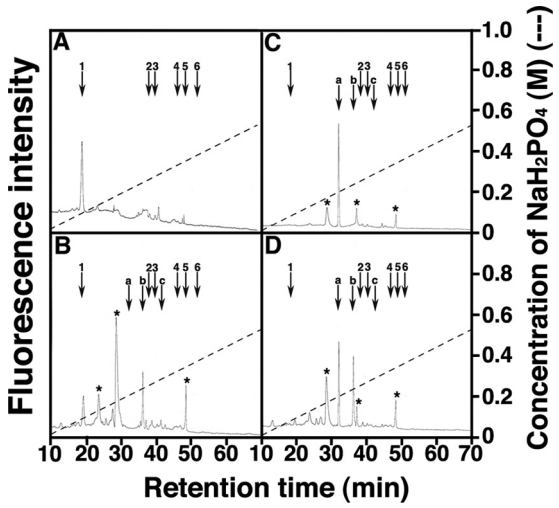


FIGURE 7. **Determination of the sulfated position in the HNK-1ST reaction products of Chn oligosaccharides.** *A* and *C*, HNK-1ST reaction products of the Chn tetra- and hexasaccharide were labeled with 2AB, isolated by gel filtration, and digested with CSase AC-II (*A*) and CSase ABC (*C*), respectively. The digests were analyzed by anion-exchange HPLC on a PA-03 column using a linear gradient of NaH_2PO_4 (indicated by the *dashed lines*) to characterize the reducing terminal di- and tetrasaccharide structure, respectively (*A* and *C*). *B* and *D*, to characterize the nonreducing terminal disaccharide structure, an aliquot of each digest of 2AB-labeled oligosaccharides from *A* and *C* was labeled with 2AB again and analyzed by HPLC. The eluted positions of 2AB-labeled authentic unsaturated disaccharides are indicated by *numbered arrows*: *1*, ΔHexUA-GalNAc; *2*, ΔHexUA-GalNAc(6-*O*-sulfate); *3*, ΔHexUA-GalNAc(4-*O*-sulfate); *4*, ΔHexUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate); *5*, ΔHexUA(2-*O*-sulfate)-GalNAc(4-*O*-sulfate); and *6*, ΔHexUA-GalNAc(4,6-*O*-disulfate). *Arrows a*, *b*, and *c* indicate the eluted positions of ΔHexUA-GalNAc-GlcUA-GalNAc-2AB (*a*), GlcUA(3-*O*-sulfate)-GalNAc-2AB (*b*), and GlcUA-GalNAc-GlcUA-GalNAc-GlcUA-GalNAc-2AB (*c*), respectively. The peaks marked by *asterisks* are unknown substances derived from the CSase preparation or the PA-03 resin upon a high sensitivity analysis.

toward Chn and CS isoforms, it was not detectable presumably because of much weaker reactivity to Chn when compared with the HNK-1 precursor oligosaccharide. 3-*O*-Sulfated GlcUA-containing disaccharide units have been found in CS preparations from sea cucumber as well as cartilages of squid and king crab (37–40), but not from mammals yet. Although CSase ABC is commonly used to depolymerize CS chains from various mammalian tissues and organs to analyze their disaccharide composition, the enzyme converts the GlcUA(3-*O*-sulfate)-containing disaccharide to a GalNAc monosaccharide (21, 23). This might explain why the GlcUA(3-*O*-sulfate) structure has not been reported in CS preparations from mammalian tissues. Whether mammals also synthesize GlcUA(3-*O*-sulfate)-containing CS remains to be investigated.

HNK-1ST transferred a sulfate group to the nonreducing terminal GlcUA in Chn but not to the internal GlcUA. Because the GalNAc transferase catalyzing CS chain elongation cannot transfer a GalNAc residue to the 3-*O*-sulfated GlcUA residue located at the nonreducing end of the growing CS chain (41), this specific sulfation observed in this study may serve as a termination signal for the CS chain elongation and regulate the chain length of CS during its biosynthesis. Although no GlcUA(3-*O*-sulfate) residue has ever been found at the nonreducing terminus of CS chains, the nonreducing terminal structure of CS has not been sufficiently characterized because of only 1 mol of the nonreducing disaccharide per single CS chain. However, Plaas *et al.* (42) noted that CS chains often have GalNAc(4,6-*O*-disulfate) at the nonreducing end, which may serve as a termination signal.

Among the oligosaccharides used as substrates in this study, HNK-1ST transferred a sulfate group to the nonreducing terminal GlcUA residue of nonsulfated Chn oligosaccharides, but not sulfated oligosaccharides from CS. The sulfate group(s) on GalNAc residue(s) appears to have an inhibitory effect on the sulfotransferase activity. However, disaccharide structures composed of a 3-*O*-sulfated GlcUA and sulfated GalNAc residue, GlcUA(3-*O*-sulfate)-GalNAc(6-*O*-sulfate) and GlcUA(3-*O*-sulfate)-GalNAc(4,6-*O*-disulfate), have been detected in CS preparations from squid cartilage (38). In addition, GlcUA(3-*O*-sulfate)-GalNAc(4-*O*-sulfate) has been demonstrated in CS from king crab cartilage (37). These disaccharide units may be formed by the transfer of a sulfate group from PAPS to the C-4 or C-6 position of the GalNAc residue in the GlcUA(3-*O*-sulfate)-containing precursor structure, GlcUA(3-*O*-sulfate)-GalNAc, by chondroitin 4-*O*-sulfotransferase and 6-*O*-sulfotransferase, respectively. Alternatively, 3-*O*-sulfotransferase from squid and king crab may transfer a sulfate group from PAPS to the C-3 position of the internal GlcUA residue flanked by sulfated GalNAc residues. Cloning and characterization of the HNK-1ST orthologs of squid and king crab will be of great interest to elucidate the mechanism of the biosynthesis of GlcUA(3-*O*-sulfate)-containing CS chains.

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