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

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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 GlcUA β1–3Gal β1–3Gal β1–4Xyl, 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, GlcUA β1–3Gal β1–4GlcNAc, the sulfotransferase activity toward the linkage region was investigated. In fact, the activity of HNK-1ST toward the linkage region was much higher than toward the glucurolyneolactotetraosylceramide, 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 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.

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§The abbreviations used are: TM, thrombomodulin; GAG, glycosaminoglycan; Chn, chondroitin; CS, chondroitin sulfate; PG, proteoglycan; GlcAT, glucuronyltransferase; HexUA, hexuronic acid; ΔHexUA, 4-deoxy-Δ-xylose; 4-xylitol; GlcNAcST, 2-acetamido-2-deoxy-β-D-glucosamine 2-epimerase; CSase, chondroitinase; 2AB, 2-aminobenzamide; DE, delayed extraction.
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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**—35S-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, p3FLAG-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).

Glucuronyleolectotaetraosylceramide (GlcUAβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1ceramide) was obtained from Wako (Osaka, Japan). Sulfo-NHS-LC-Biotin and EZ-Link-Biotin were purchased from Pierce.

**Measurement of Sulfotransferase Activity**—Sulfotransferase activity toward the tetraosyl peptide GlcUA-Gal-Gal-Xyl-SGDNG, various CS isoforms, octa- and hexa-, and tetrasaccharides from Chn, structurally defined sulfated tetrasaccharides from CS, and glucuronyleolectotaetraosylceramide 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 [35S]PAPS (2 × 105 dpm), and Chn, Chn-oligosaccharide, the tetraosyl peptide, or glucuronyleolectotaetraosylceramide 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 glucuronyleolectotaetraosylceramide 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 H2O; 15–20 min, 0–100% gradient with methanol; 20–60 min, isocratic with methanol. Fractions were collected at 1- or 2-min intervals.
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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 [35S]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 mM 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 modified 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 chondroitinase ABC and purified by gel filtration chromatography on a Superdex peptide column (30). 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 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.

**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 [35S]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 + SO₃]⁻ at m/z 1,272 (Fig. 1B), 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. 1C). Therefore, we concluded that a sulfate group was transferred to the
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-SDGNG, 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 glucuronylneolactotaetoasylceramide, 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. 2A, the antibody reacted with the HNK-1ST reaction product, presumably GlcUA(3-O-sulfate)-Gal-Gal-Xyl-O-SDGNG. The reactivity was higher than that obtained with the HNK-1 antigen (Fig. 2B), 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 glucuronylneolactotaetoasylceramide (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.
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**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), glucuronoylneolactotetraosylceramide (1 nmol), and GlcUA-Gal-Gal-Xyl-SDNNG (1 nmol) as acceptors under the reaction conditions described under "Experimental Procedures."  

| Acceptor | Enzymatic activity (pmol/µg of protein/h) |
|----------|-----------------------------------------|
| Chn polysaccharide (GlcUA-(GalNAc-GlcUA)₂-GalNAc) | HNK-1ST: 1.5 ± 0.05 | Mock: ND |
| Chn octasaccharide (GlcUA-(GalNAc-GlcUA)₃-(GlcUA(3-O-sulfate)-GalNAc) | HNK-1ST: 6.9 ± 0.4 | Mock: ND |
| Chn tetrasaccharide (GlcUA-GalNAc-GlcUA(3-O-sulfate)-GalNAc) | HNK-1ST: 7.0 ± 0.6 | Mock: ND |
| Chn hexasaccharide (GlcUA-(GalNAc-GlcUA)₂-GalNAc) | HNK-1ST: 6.3 ± 2.0 | Mock: 0.2 ± 0.05 |
| GlcUA-Gal-Gal-Xyl-SDNNG | HNK-1ST: 36.3 ± 0.3 | Mock: 0.2 ± 0.05 |
| GlcUA-Gal-Gal-Xyl-SDNG | HNK-1ST: 6.9 ± 0.3 | Mock: ND |

* ND, not detected.

**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. 5A) and hexasaccharides (Fig. 5B), 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. 6A). Because the molecular mass of the 2AB-labeled monosulfated tetrasaccharide is 976 Da, the observed mass 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. 6B), suggesting that the component in this fraction was HexUA₄HexNAc₅(OSO₃H)₂-2AB (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 Cβase AC-II and ABC, respectively. Each digest was analyzed by anion-exchange HPLC, and peaks of fluorescence were detected at the eluted positions of HexUA-GalNAc-2AB and HexUA-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 Cβase 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 immobilized on a streptavidin-coated plate. As shown in Fig. 2C, 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. 2D). 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.
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 H9251-TM from human urine (8). To investigate the physiological significance of the GlcUA(3-O-sulfate) structure in H9251-TM, the presence or absence of the structure in blood-borne H9251-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-
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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 NaH₂PO₄ (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-di-sulfate); and 7, ΔHexUA(2-O-sulfate)-GalNAc(4,6-O-di-sulfate). Arrows a, b, and c indicate the eluted positions of: (GlcUA-GalNAc)₂ (a); (GlcUA-GalNAc)₃ (b); and (GlcUA-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-GalNAc. 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...
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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 sulfated 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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