Recognition of Sulfation Pattern of Chondroitin Sulfate by Uronosyl 2-O-Sulfotransferase*

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We have shown previously that a highly sulfated sequence, GalNAc(4,6-SO4)-GlcA(2SO4)-GalNAc(6SO4), is present at the nonreducing terminal of chondroitin sulfate (CS), and this structure was synthesized from a unique sequence, GalNAc(4SO4)-GlcA(2SO4)-GalNAc(6SO4), by sulfation with N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase. Uronosyl 2-O-sulfotrasferase (2OST), which transfers sulfate from 3’-phosphoadenosine 5’-phosphosulfate (PAPS) to position 2 of the GlcA residue of CS, has been shown to be involved in synthesis of these structures; however, the specificity of 2OST concerning recognition of the sulfation pattern of the acceptor has largely remained unclear. In the present study, we examined the specificity of 2OST in terms of recognition of the sulfation pattern surrounding the targeting GlcA residue. The recombinant 2OST could sulfate CS-A, CS-C, and desulfated dermatan sulfate. When [35S]glycosaminoglycans formed from CS-A after the reaction with the recombinant 2OST and [35S]PAPS were subjected to limited digestion with chondroitinase ACII, a radioactive tetrasaccharide GalNAc(4SO4)-GlcA(2SO4)-GalNAc(6SO4) by enzymatic and chemical reactions. These observations indicate that 2OST transfers sulfate preferentially to the GlcA residue located in a unique sequence, -GalNAc(4SO4)-GlcA-GalNAc(6SO4)-. When oligosaccharides with different sulfation patterns were used as the acceptor, GalNAc(4SO4)-GlcA-GalNAc(6SO4) and GlcA-GalNAc(4SO4)-GlcA-GalNAc(6SO4) were the best acceptors for 2OST among trisaccharides and tetrasaccharides, respectively. These results suggest that 2OST may be involved in the synthesis of the highly sulfated structure found in CS-A.

Chondroitin sulfate (CS) chains attached to various proteoglycans are comprised of GlcA-GalNAc repeating units bearing sulfate groups

* This work was supported by Grants 14082206 and 16-4208 from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a special research cation, Culture, Sports, Science and Technology of Japan, and by a special research

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‡ The abbreviations used are: CS, chondroitin sulfate; 2OST, uronosyl 2-O-sulfotransferase; GalNAc(4SO4)-GlcA(2SO4)-GalNAc(6SO4); Tetra A was obtained as a sole intermediate product. The sequence of Tetra A was found to be DiHexA-GalNAc(4SO4)-GlcA(2SO4)-GalNAc(6SO4) by enzymatic and chemical reactions. These observations indicate that 2OST transfers sulfate preferentially to the GlcA residue located in a unique sequence, -GalNAc(4SO4)-GlcA-GalNAc(6SO4)-. When oligosaccharides with different sulfation patterns were used as the acceptor, GalNAc(4SO4)-GlcA-GalNAc(6SO4) and GlcA-GalNAc(4SO4)-GlcA-GalNAc(6SO4) were the best acceptors for 2OST among trisaccharides and tetrasaccharides, respectively. These results suggest that 2OST may be involved in the synthesis of the highly sulfated structure found in CS-A.

** This work was supported by Grants 14082206 and 16-4208 from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a special research fund from the Seikagaku Corporation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
**Sulfation of Chondroitin Sulfate by Uronosyl 2-O-Sulfotransferase**

**TABLE ONE**

| Glycosaminoglycans     | ΔDi-0S | ΔDi-4S | ΔDi-6S | ΔDi-diS_D | ΔDi-diS_E | ΔDi-diS_F |
|------------------------|--------|--------|--------|-----------|-----------|-----------|
| CS-A (whale cartilage) | 1.5    | 7.4    | 23.1   | 0.5       | 0.3       | ND*       |
| CS-A (sturgeon notochord) | 0.5    | 94.3   | 5.2    | ND        | ND        | ND        |
| CS-C                   | 4.2    | 26.9   | 56.0   | 10.8      | 2.1       | ND        |
| Chondroitin            | >99.8  | ND     | ND     | ND        | ND        | ND        |
| DS                     | 0.4    | 91.5   | 1.0    | ND        | ND        | 7.1       |
| Desulfated DS          | 92.2   | 7.8    | ND     | ND        | ND        | ND        |

* Not detected under the conditions used here.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following commercial materials were used: H$_2$SO$_4$, was from PerkinElmer Life Sciences; chondroitinase ACII, chondroitinase ABC, chondro-6-sulfatase, chondro-4-sulfatase, CS-A (whale cartilage), CS-C (shark cartilage), DS (pig skin), heparan sulfate (bovine liver), completely desulfated N-resulfated heparin, ΔDi-0S, ΔDi-4S, ΔDi-6S, ΔDi-4S, ΔDi-2S, ΔDi-diS$_D$, ΔDi-diS$_E$, and ΔDi-diS$_F$ were from Seikagaku Corporation, Tokyo; Partisil-10 SAX was from Whatman; CS-A (sturgeon notochord), unlabeled PAPS, N-acetylgalactosamine 4-sulfate, N-acetylgalactosamine 6-sulfate, hyaluronidase (sheep testis), β-glucuronidase (bovine liver, Type B-3), and 2-acetamido-2-deoxy-D-galacturonic acid-1,4-lactone from Sigma; N-glycosidase F was from Roche Molecular Biochemicals; Hǐlod Superdex 30 HR 16/60 and Fast Desalting Column HR 10/10 were from Amersham Bioscience.

[^35S]PAPS was prepared as described (24). Chondroitin (squid skin) was prepared as described (25). N-Acetylgalactosamine 4,6-bisulfate was prepared from ΔDi-diS$_D$, which was obtained from CS-E by chondroitinase AC digestion followed by Dowex 1 chromatography, by mercer analytic treatment as described below. After mercer analytic treatment, the monosaccharide was purified by Superdex 30 chromatography. Squid GalNAc4S-6ST was purified from squid cartilage as described (26). Keratan sulfate (bovine cornea) was a generous gift from Seikagaku Corporation. CS-E (squid cartilage), which was eluted with 1.5 M NaCl from DEAE-Sephadex A-50, was prepared as described (27). Desulfated DS was prepared from DS by Nagasawa et al. (28). Solvolytic with 90% (v/v) Me$_2$SO was performed at 100 °C for 60 min. Disaccharide compositions of glycosaminoglycans were determined by absorbance at 232 nm of unsaturated disaccharides separated by SAX-HPLC after chondroitinase ACII or ABC digestion. Analytical data of these glycosaminoglycans are shown in TABLE ONE. The tetrasaccharides GlcA-GalNAc(4SO$_4$)-GlcA-GalNAc(4SO$_4$) (Tetra-44), GlcA-GalNAc(4SO$_4$)-GlcA-GalNAc(6SO$_4$) (Tetra-46), GlcA-GalNAc(6SO$_4$)-GlcA-GalNAc(4SO$_4$) (Tetra-64), and GlcA-GalNAc(6SO$_4$)-GlcA-GalNAc(6SO$_4$) (Tetra-66), and trisaccharides GalNAc(4SO$_4$)-GlcA-GalNAc(4SO$_4$) (Tri-44), GalNAc(4SO$_4$)-GlcA-GalNAc(6SO$_4$) (Tri-46), GalNAc(6SO$_4$)-GlcA-GalNAc(4SO$_4$) (Tri-46), and GalNAc(6SO$_4$)-GlcA-GalNAc(6SO$_4$) (Tri-66) were prepared as described (22, 26).

**Preparation of the Recombinant Human 2OST**—A DNA fragment that codes for the open reading frame of 2OST (23) was amplified by PCR. The first PCR was carried out using oligonucleotides 2OST-F1, GGGTGACCTTCTTCTGGCAC, and 2OST-R1, GTCCCTTAGGTTTTACTCTCCCCAACA, which were synthesized according to the sequence of the 2OST cDNA clone (GenBank accession number AB020316), as primers, and the QUICK-Clone cDNA derived from human pancreas (Clontech) as a template. Amplification was carried out by 30 cycles of 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 3 min.

The second PCR was carried out using oligonucleotides 2OST-F2, CAGGAATTTGGAAGAGAACGAGCACGATC, and 2OST-R2, CGCGAGATTTCACTTTAATATATTCTCCAGCACA, as primers, and the first PCR mixture as the template. At the 5′-ends of oligonucleotides 2OST-F2 and 2OST-R2, restriction enzyme recognition sites were introduced; an EcoRI site for 2OST-F2 and a BglII site for 2OST-R2. Amplification was carried out by 30 cycles of 94 °C for 45 s, 52 °C for 1 min, and 72 °C for 2.5 min. The reaction products were subjected to agarose gel electrophoresis. The amplified DNA band was cut out and the DNA fragment was recovered from the gel, digested with EcoRI and BglII, and subcloned into these sites of pFLAG-CMV-2 plasmid (Eastman Kodak). Recombinant 2OST was expressed in COS-7 cells as a fusion protein with FLAG peptide and affinity purified (29). The purified protein was visualized with Western blot as described (30).

The affinity purified 2OST was precipitated with 2 volumes of ethanol containing 1.3% (w/v) potassium acetate and digested with recombinant N-glycosidase F (Roche Molecular Biochemicals) by the methods recommended by the manufacturer. After digestion, the samples were separated by SDS-polyacrylamide gel electrophoresis as described by Laemmli (30). The separated proteins were

FIGURE 1. Western blot of the affinity purified 2OST. The FLAG-2OST fusion protein was extracted from COS-7 cells that were transfected with the cDNA and purified with an anti-FLAG monoclonal antibody-conjugated column as described under "Experimental Procedures." The affinity purified protein was detected with anti-FLAG antibody before (lane 1) or after (lane 2) N-glycosidase F digestion. Molecular size standards were the following: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa).
Electrophoretically transferred to a Hybond ECL membrane (Amer- 
sham Biosciences), and stained with anti-FLAG M2 monoclonal anti-
body (Sigma). The blot was developed with polyclonal anti-mouse IgG 
antibody coupled to horseradish peroxidase using an ECL detection kit 
and a Hyperfilm ECL (Amerham Biosciences).

Assay of Sulfotransferase Activity—2OST activity was assayed by the 
method described previously (23). The standard reaction mixture con-
tained, in a final volume of 50 μl, 2.5 μmol of imidazole-HCl, pH 6.8, 2.6 
μg of proteamine chloride, 25 nmol (as galactosamine) of CS-A, 50 pmol of [35S]PAPS (about 5.0 × 10^5 cpm), and enzyme. The reaction mixtures 
were incubated at 37 °C for 30 min and the reaction was stopped by im-
mersing the reaction tubes in a boiling water bath for 1 min. 35S-
Labeled glycosaminoglycans were isolated by precipitation with ethanol 
followed by gel chromatography with a Fast Desalting Column as 
described previously (31), and radioactivity was determined. For deter-
mining the activity toward various glycosaminoglycans, CS-A was 
replaced with 25 nmol (as galactosamine for chondroitin, CS-C, DS, and 
oligosaccharides, or glucosamine for heparan sulfate, completely desul-
fated N-resulted heparin, and keratan sulfate) of glycosaminoglycans 
or oligosaccharides. When oligosaccharides were used as the acceptors, 
the reaction mixtures were applied directly to the Superdex 30 column 
as described below, and the 35S-labeled oligosaccharides were separated from 35SO4 and [35S]PAPS.

Digestion with Chondroitinase ACII, Chondroitinase ABC, Chondro-
sulfatase, Chondro-4-sulfatase, and β-Glucuronidase—Unless other-
wise stated, digestion with chondroitinase ACII or chondroitinase ABC 
under standard conditions was carried out for 4 h at 37 °C in the reac-
tion mixture containing, in a final volume of 25 μl, 1.25 μmol of Tris 
acetate buffer, pH 7.5, 2.5 μg of bovine serum albumin, and 30 milli-
units of chondroitinase ACII or chondroitinase ABC. For degrading 35S-la-
beled trisaccharides and tetrascarides with chondroitinase ABC, a 
strong condition was used under which digestion with chondroitinase 
ABC was carried out in the reaction mixtures described above three 
times successively; first with 120 milliunits of enzyme for 28 h, second 
with 100 milliunits of enzyme for 18 h, and finally with 100 milliunits of 
enzyme for 7 h. The new enzymes were added after heating the reaction 
mixtures at 100 °C for 1 min. Digestion with chondro-6-sulfatase of 
Tetra A or Tetra B was carried out for 5 h at 37 °C in the reaction 
mixture containing, in a final volume of 25 μl, 1.25 μmol of Tris acetate 
buffer, pH 7.5, 2.5 μg of bovine serum albumin, and 50 milliunits of 
chondro-6-sulfatase. After digestion of 35S-labeled glycosaminoglycans 
or oligosaccharides with chondroitinase ACII or chondroitinase ABC, 
digestion with chondro-6-sulfatase or chondro-4-sulfatase was carried 
out twice successively in the reaction mixtures described above; first 
with 100 milliunits of enzyme for 17 h and second with 100 milliunits of 
enzyme for 5 h. Digestion with β-glucuronidase was carried out for 4 h 
at 37 °C in a reaction mixture containing, in a final volume of 40 μl, 
35S-labeled tetrascaridase (~40 nmol as galactosamine), 2 μmol of 
sodium acetate buffer, pH 4.5, 20 nmol of 2-acetamido-2-deoxy-α-D-
galactonic acid-1,4-lactone, 0.8 μmol of sodium fluoride, and 18 units of 
β-glucuronidase. Under these conditions, removal of the nonreducing 
terminal GlcA was complete and no release of inorganic sulfate was 
observed.

Removal of Unsaturated Uronic Acid by Mercuric Acetate Treatment— 
Removal of unsaturated uronic acid was carried out as described (32). 
35S-Labeled or unlabeled tetrascaridase containing unsaturated uronic acid were dried and dissolved in 0.5 ml of 35 mM mercurate 
in 25 mM Tris with 25 mM sodium acetate, pH 5.0. The reaction was 
carried out for 1 h at room temperature. After the reaction was over, 
the samples were applied to a Dowex 50 (H+) column (bed volume of 1 ml).

| Glycosaminoglycans | Sulfotransferase activity (pmol/min/ml) |
|--------------------|----------------------------------------|
| CS-A (whale cartilage) | 12.27 |
| CS-A (sturgeon notochord) | 10.76 |
| CS-C | 3.40 |
| Chondroitin | 2.66 |
| CS-E | 0.40 |
| DS | 0.27 |
| DS (with 26.3 μg of protamine) | 5.83 |
| Desulfated DS | 21.68 |
| Keratan sulfate | 0.14 |
| Heparan sulfate | 0.07 |
| CDSNS*-heparin | 0.13 |

| Glycosaminoglycans | Sulfotransferase activity (pmol/min/ml) |
|--------------------|----------------------------------------|
| CDSNS | 0.13 |

The column was washed with 3 ml of water. The flow-through fractions 
and the washings were combined and lyophilized. The lyophilized 
materials were further purified with Superdex 30 and SAX-HPLC.

Sulfation of the Trisaccharide, Which Was Derived from Tetra A by 
Mercuric Acetate Treatment, with GalNAcα4S-6ST—The sulfotrans-
ferase reaction was carried out as described (26, 33). The reaction mix-
ture contained, in a final volume of 50 μl, 2.5 μmol of imidazole-HCl, 
pH 6.8, 1 μmol of CaCl2, 1 μmol of reduced glutathione, the 35S-labeled 
trisaccharide derived from Tetra A by mercuric acetate treatment (about 
6000 cpm), 1.35 nmol of Oligo 1 (final 27 μl), 100 nmol of 
unlabeled PAPS (final 2 ml), and 180 ng of the purified squi 
GalNAcα4S-6ST. The reaction mixture was incubated at 15 °C for 24 h 
and the reaction was stopped by immersing the reaction tube in a boil-
ing water bath for 1 min. After the reaction was stopped, the reaction 
mixture was applied to the Superdex 30 column. Fractions containing 
the radioactive oligosaccharides were collected, lyophilized, and sepa-
rated with SAX-HPLC.

Superdex 30 Chromatography and HPLC—A Superdex 30/16/60 column 
was equilibrated with 0.2 mM NH4HCO3, and run at a flow rate of 2 ml/min. One ml fractions were collected. Separation of the degradation 
products formed from 35S-labeled glycosaminoglycans and 35S-labeled 
oligosaccharides were carried out by HPLC using a Whatman Parti-
sil-10 SAX column (4.6 × 25 cm) equilibrated with 5 mM KH2PO4. 
The column was developed with a gradient (5 mM KH2PO4) for 10 min fol-
lowed by a linear gradient from 5 to 500 mM KH2PO4). Fractions (0.5 ml) 
were collected at a flow rate of 1 ml/min and a column temperature of 
40°C.

**RESULTS**

Sulfation of Various Glycosaminoglycans by the Recombinant 2OST—
The rates of transfer of sulfate to various glycosaminoglycans were 
determined (TABLE TWO). Among these glycosaminoglycans, desul-
fated DS was the best acceptor. CS-A from whale cartilage and sturgeon 
notochord were both better acceptors than CS-C. The rate of 
sulfation of chondroitin was much lower than the rate of sulfation of CS-A. 
Unlike a previous report (23), DS was a poor acceptor. The rate of sulfation 
of DS was increased when the concentration of protamine was 
increased as described previously in the activity of C45ST-1 (34). CS-E, 
keratan sulfate, heparan sulfate, and completely desulfated re-N-sul-
fated heparin hardly served as acceptor. To determine the position to 
which sulfate was transferred, the 35S-labeled glycosaminoglycans

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**Table Two**

Acceptor substrate specificity of the affinity purified 2OST

Sulfotransferase activities were assayed using various glycosaminoglycans as 
described under "Experimental Procedures.

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|--------------------|----------------------------------------|
| CS-A (whale cartilage) | 12.27 |
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| Chondroitin | 2.66 |
| CS-E | 0.40 |
| DS | 0.27 |
| DS (with 26.3 μg of protamine) | 5.83 |
| Desulfated DS | 21.68 |
| Keratan sulfate | 0.14 |
| Heparan sulfate | 0.07 |
| CDSNS*-heparin | 0.13 |

* CDSNS, completely desulfated N-resulted.
Sulfation of Chondroitin Sulfate by Uronosyl 2-O-Sulfotransferase

FIGURE 2. Chondroitinase ACII and chondro-6-sulfatase digestion of 35S-labeled glycosaminoglycans formed from CS-A and CS-C after the reaction with 2OST. 35S-Labeled glycosaminoglycans formed from whale cartilage CS-A (A and B) or CS-C (C and D) after the reaction with 2OST were digested with chondroitinase ACII (A and C) or chondroitinase ACII plus chondro-6-sulfatase (B and D), and separated with SAX-HPLC. The broken line depicts the concentration of KH2PO4. Arrows indicate the elution position of GalNAc(6SO4) (arrow 1), GalNAc(4SO4) (arrow 2), ΔDi-6S (arrow 3), ΔDi-2S (arrow 4), ΔDi-4S (arrow 5), GalNAc(4,6-SO4) (arrow 6), SO4 2− (arrow 7), ΔDi-diS4 (arrow 8), and ΔDi-diS6 (arrow 9).

FIGURE 3. Chondroitinase ABC and chondro-4-sulfatase digestion of 35S-labeled glycosaminoglycans formed from desulfated DS and DS after the reaction with 2OST. 35S-Labeled glycosaminoglycans formed from desulfated DS (A or DS (B and C)) after the reaction with 2OST were digested with chondroitinase ABC (A and B) or chondroitinase ACII plus chondro-4-sulfatase (C), and separated with SAX-HPLC. 2-O-Sulfation of DS was carried out in the presence of 26.3 μg of protamine. The broken line depicts the concentration of KH2PO4. The standards were the same as those described in the legend to Fig. 2.

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derived from whale cartilage CS-A and CS-C were digested with chondroitinase ACII and subjected to SAX-HPLC (Fig. 2, A and C). The radioactivity was exclusively detected at the position of ΔDi-dis. After chondro-6-sulfatase digestion, the radioactivity was shifted to the position of ΔDi-2S (Fig. 2, B and D). The same results were obtained for sturgeon notochord CS-A (data not shown), in which the GalNAc(6SO4) residue was less than 5% of the total GalNAc residues (TABLE ONE). The observation that 2OST transferred sulfate exclusively to position 2 of GlcA residues adjacent to the nonreducing side of GalNAc(6SO4) residue was almost below the detectable level under standard assay conditions.

Characterization of a Tetrasaccharide Obtained from 35S-Labeled Glycosaminoglycans Derived from CS-A by a Limited Digestion with Chondroitinase ACII—We have shown previously that GlcA(2SO4)-containing sequences in CS exhibited various degrees of resistance to chondroitinase ACII (22). To clarify the sequence around the 2-O-sulfated GlcA residue, we tried to prepare oligosaccharides derived from the 2-O-sulfated regions by limited digestion with chondroitinase ACII. When 35S-labeled glycosaminoglycans derived from chondroitin were digested with chondroitinase ACII, more than 90% of the radioactivity was recovered in ΔDi-diS4 (Fig. 3B). After chondro-4-sulfatase digestion, ΔDi-diS4 disappeared and shifted to ΔDi-2S (Fig. 3C). These observations indicate that, unlike the GlcA residue, IdoA residues adjacent to GalNAc residues could be sulfated efficiently by 2OST. In contrast, the rate of sulfation of the IdoA residue adjacent to the GalNAc(4SO4) residue was almost below the detectable level under standard assay conditions.

no disaccharide products were obtained (data not shown). These results indicate that 2OST has no preference to GlcA residue in the desulfated DS. When 35S-labeled glycosaminoglycans derived from DS in the presence of 26.3 μg of protamine were digested with chondroitinase ABC, the major radioactivity was detected at the position of ΔDi-diS4 (Fig. 3B). After chondro-4-sulfatase digestion, ΔDi-diS4 disappeared and shifted to ΔDi-2S (Fig. 3C). These observations indicate that, unlike the GlcA residue, IdoA residues adjacent to GalNAc residues could be sulfated efficiently by 2OST. In contrast, the rate of sulfation of the IdoA residue adjacent to the GalNAc(4SO4) residue was almost below the detectable level under standard assay conditions.

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no disaccharide products were obtained (data not shown). These results indicate that 2OST has no preference to GlcA residue in the desulfated DS. When 35S-labeled glycosaminoglycans derived from DS in the presence of 26.3 μg of protamine were digested with chondroitinase ABC, the major radioactivity was detected at the position of ΔDi-diS4 (Fig. 3B). After chondro-4-sulfatase digestion, ΔDi-diS4 disappeared and shifted to ΔDi-2S (Fig. 3C). These observations indicate that, unlike the GlcA residue, IdoA residues adjacent to GalNAc residues could be sulfated efficiently by 2OST. In contrast, the rate of sulfation of the IdoA residue adjacent to the GalNAc(4SO4) residue was almost below the detectable level under standard assay conditions.

Characterization of a Tetrasaccharide Obtained from 35S-Labeled Glycosaminoglycans Derived from CS-A by a Limited Digestion with Chondroitinase ACII—We have shown previously that GlcA(2SO4)-containing sequences in CS exhibited various degrees of resistance to chondroitinase ACII (22). To clarify the sequence around the 2-O-sulfated GlcA residue, we tried to prepare oligosaccharides derived from the 2-O-sulfated regions by limited digestion with chondroitinase ACII. When 35S-labeled glycosaminoglycans derived from CS-A was subjected to a limited digestion with chondroitinase ACII and separated with Superdex 30 chromatography, a radioactive peak appeared slightly before the position of the desulfated tetrasaccharide (Fig. 4A). As the conditions of the chondroitinase ACII digestion became stronger, the tetrasaccharide was converted to ΔDi-diS4 (Fig. 4, B–D). These observations indicate that 2OST transferred sulfate to a unique tetrasaccharide sequence in CS-A. When the tetrasaccharide fraction was separated with SAX-HPLC, a single radioactive peak was detected behind ΔDi-diS4 (Fig. 5A). This material was named Tetra A. After digestion with chondroitinase ACII under the standard conditions, radioactivity
of Tetra A was shifted to ΔDi-diS$_{O}$ (Fig. 5B). After further digestion with chondroitinase ACII, this peak was converted to ΔDi-diS$_{2}$ (Fig. 5C). When Tetra A was digested with chondroitinase ACII alone, a radioactive peak was detected slightly behind ΔDi-diS$_{O}$ (Fig. 5D). This radioactive peak was shifted to ΔDi-2S after further digestion with chondroitinase ACII (Fig. 5E). Because chondroitinase ACII could remove sulfate from the GalNAc(6SO$_{4}$)$_{-}$GalNAc(6SO$_{4}$)$_{-}$GlcA(2SO$_{4}$) residue located exclusively to the reducing end of the oligosaccharides (22, 36), these observations indicate that Tetra A should contain the GlcA(2SO$_{4}$)-GalNAc(6SO$_{4}$) unit at the reducing side. When Tetra A was treated with merccuric acetate, the resulting trisaccharide behaved identically with Oligo II in SAX-HPLC (Fig. 6B). When the trisaccharide was incubated with squid GalNAc4S-6ST in the presence of 2 mM nonradioactive PAPS, the radioactivity was quantitatively shifted to the position of Oligo II (Fig. 6C). We found previously that squid GalNAc4S-6ST could transfer sulfate to position 6 of both nonreducing terminal and reducing terminal GalNAc(4SO$_{4}$)$_{-}$residues of the 4-sulfated trisaccharide (26). Because the reducing terminal residue of Tetra A was found to be GalNAc(6SO$_{4}$)$_{-}$as described above, conversion of the trisaccharide to a material corresponding to Oligo II by the reaction with squid GalNAc4S-6ST indicates that the nonreducing terminal residue of the trisaccharide should be GalNAc(4SO$_{4}$)$_{-}$. The structural analysis of Tetra A is summarized in Scheme 1. Taken together, it is most probable that the trisaccharide is identical to Oligo I and thus Tetra A is ΔHexA-GalNAc(4SO$_{4}$)$_{-}$GlcA(2SO$_{4}$)$_{-}$GalNAc(6SO$_{4}$)$_{-}$.

**Isolation and Characterization of Tetrascarharides Containing GlcA(2SO$_{4}$) from CS-A**—To confirm the deduced structure of Tetra A, we tried to isolate a tetrascarharide from CS-A that behaved identically with Tetra A in SAX-HPLC. CS-A (550 µmol of galactosamine) was digested for 4 h at 37 °C with chondroitinase ACII in the reaction mixture containing, in a final volume of 10 ml, 500 µmol of Tris acetate buffer, pH 7.5, 1 mg of bovine serum albumin, and 2 units of chondroitinase ACII. The degradation products were separated with Superdex 30 chromatography (Fig. 7A). Tetrascarharide fractions were collected and further separated with SAX-HPLC (Fig. 7B). A peak was detected at the position of Tetra A. This tetrascarharide was further purified by Superdex 30 chromatography. About 0.4 µmol (as galactosamine) of the tetrascarharide was obtained from 550 µmol of whale cartilage CS-A. When the tetrascarharide was digested with chondroitinase ACII, ΔDi-diS$_{O}$ and ΔDi-4S were obtained (Fig. 8A). After further digestion with chondroitinase ACII, ΔDi-diS$_{2}$ was shifted to ΔDi-2S (Fig. 8C), whereas with further digestion with chondro-4-sulfatase, ΔDi-4S was shifted to ΔDi-0S (data not shown). When the tetrascarharide was digested with chondroitinase ACII, a peak was obtained slightly behind ΔDi-diS$_{O}$ (Fig. 8D); the retention time of this peak was strictly the same as the retention time of the radioactive product formed from Tetra A after chondroitinase ACII digestion (Fig. 5D). After further digestion with chondroitinase ACII, this peak was converted to ΔDi-4S and ΔDi-2S.
These observations clearly indicate that the tetrasaccharide is HexA-GalNAc(4SO₄)-GlcA(2SO₄)-GalNAc(6SO₄). The sequence of the tetrasaccharide was confirmed by another approach. When the tetrasaccharide was subjected to mercuric acetate treatment, a trisaccharide was obtained whose retention time was strictly the same as that of Oligo I. When the trisaccharide was digested with chondroitinase ABC and separated with SAX-HPLC, GalNAc(4SO₄) and Di-diSD were formed (data not shown). The structural analysis of the tetrasaccharide is summarized in Scheme 2. From the analytical data of Tetra A and the tetrasaccharide that behaved identically with Tetra A, we concluded that Tetra A is identical to the tetrasaccharide, and thus 2OST transferred sulfate to position 2 of the GlcA residue located in the unique sequence, GalNAc(4SO₄)-GlcA-GalNAc(6SO₄), present in CS-A.

**Sulfation of Various CS-derived Oligosaccharides by the Recombinant 2OST—**As described above, 2OST preferentially transferred sulfate to position 2 of the GlcA residue in the unique sequence GalNAc(4SO₄)-GlcA-GalNAc(6SO₄), present in CS-A.

**FIGURE 6.** HPLC separation of the trisaccharide derived from Tetra A by mercuric acetate treatment. The trisaccharide derived from Tetra A by mercuric acetate treatment was separated with SAX-HPLC before (A) or after the reaction with squid GalNAc4S-6ST in the presence of nonradioactive PAPS (C). Panel A shows the elution pattern of Oligos I and II detected by absorbance at 210 nm. The broken line depicts the concentration of KH₂PO₄. The standards were the same as those described in the legend to Fig. 2. Panel B shows the elution pattern of Oligos I and II detected by absorbance at 210 nm. The broken line depicts the concentration of KH₂PO₄. The standards were the same as those described in the legend to Fig. 2.

**FIGURE 7.** Preparation and separation of a tetrasaccharide from CS-A that behaved identically with Tetra A. A, CS-A was digested with chondroitinase ACII as described in the text. The digest was applied to the Superdex 30 column. The column was monitored at 232 nm. The fractions indicated by a horizontal bar were collected, concentrated, and lyophilized. The standards were the same as those described in the legend to Fig. 4. B, the lyophilized materials were separated with SAX-HPLC as described under “Experimental Procedures.” The column was monitored at 232 nm. The broken line depicts the concentration of KH₂PO₄. The standards were the same as those described in the legend to Fig. 2. The elution position of Tetra A was also indicated. The material eluted at the position of Tetra A was used for the following experiments.

**FIGURE 8.** Digestion of the tetrasaccharide that behaved identically with Tetra A with chondroitinase ACII and chondro-6-sulfatase. The isolated tetrasaccharide was separated with SAX-HPLC before (A) or after digestion with chondroitinase ACII (B), chondroitinase ACII and then chondro-6-sulfatase (C), chondro-6-sulfatase (D), or chondro-6-sulfatase and then chondroitinase ACII (E). The column was monitored at 232 nm. The broken line depicts the concentration of KH₂PO₄. The standards were the same as those described in the legend to Fig. 2.
GlcA-GalNAc(6SO_4). Thus, 2OST appears to recognize GalNAc(4SO_4) and GalNAc(6SO_4) residues neighboring the nonreducing side and reducing side, respectively, of the acceptor GlcA residue. This substrate specificity of 2OST was further confirmed by sulfation of various trisaccharides and tetrasaccharides with different sulfation patterns. As shown in TABLE THREE, Tetra-46 (GlcA-GalNAc(4SO_4)-GlcA-GalNAc(6SO_4)) and Tri-46 (GalNAc(4SO_4)-GlcA-GalNAc(6SO_4)) were the best acceptors among tetrasaccharides and trisaccharides, respectively. Tetra-46 was a better acceptor than Tri-46. No sulfate incorporation was detected under the same conditions when nonsulfated trisaccharide and tetrasaccharide were used as acceptors (data not shown). When 2-O-sulfated Tetra-46 was separated with SAX-HPLC, a single radioactive peak was detected at the retention time earlier than the retention time of Tetra A (Fig. 9A). This radioactive peak was shifted to the position of Oligo I after β-glucuronidase digestion (Fig. 9B), indicating that sulfate was transferred to the GlcA residue between GalNAc(4SO_4) and GalNAc(6SO_4) but not to the nonreducing terminal one. When 2-O-sulfated Tetra-46 was digested with chondroitinase ABC and subjected to SAX-HPLC, radioactivity was exclusively detected at the position of ΔDi-diS_0 (Fig. 9C), and shifted to the position to ΔDi-2S after chondro-6-sulfatase digestion (Fig. 9D). These observations are well consistent with the substrate specificity of 2OST revealed by the analysis of the 2-O-sulfated CS-A. The \( K_m \) and \( V_{max} \) for Tetra-46, Tetra-44 (GlcA-GalNAc(4SO_4)-GlcA-GalNAc(4SO_4)), and Tetra-66 (GlcA-GalNAc(6SO_4)-GlcA-GalNAc(6SO_4)) were compared (TABLE FOUR). It is evident that the sulfation pattern of the tetrasaccharides markedly affected their affinities to 2OST; Tetra-46 showed the highest affinity. These observations indicate that the selective sulfation of GlcA residues between GalNAc(4SO_4) and GalNAc(6SO_4) by 2OST seems to be due mainly to the high affinity of 2OST toward this sequence.

DISCUSSION

In this paper, we found that 2OST preferentially transferred sulfate to the GlcA residue located in the sequence, GalNAc(4SO_4)-GlcA-GalNAc(6SO_4), in CS-A. CS-D from shark cartilage was reported to
contain the GlcA(2SO₄)GalNAc(6SO₄) unit at the reducing side of the GlcA-GalNAc(4SO₄) unit (37–39). CS-D in the brain and cartilage disappeared in C6ST⁻/⁻ mice (40). A spondyloepiphyseal dysplasia, Omani type, was identified to be associated with a mutation in the C6ST-1 gene. In the cells and urine of a patient, the HexA(2S)-GalNAc(6S) unit was significantly reduced (41). These observations are consistent with the specificity of 2OST described here. Chondroitin was much less acceptor than CS-A, indicating that pre-existing sulfate consistent with the specificity of 2OST described here. Chondroitin was Omani type, was identified to be associated with a mutation in the gene stimulated 2OST activity. In contrast, desulfated DS was a much better acceptor than DS, suggesting that groups on the GalNAc residue stimulated 2OST activity. In contrast, much less acceptor than CS-A, indicating that pre-existing sulfate pre-existing sulfate 2-sulfotransferase (HS2ST, gene name HS2ST) (23, 42). 2-OST-sulfation of the nonreducing terminal may proceed much more slowly as that of glycosaminoglycans, the polysaccharide nature of the sequence of GalNAc(4SO₄)-GlcA-GalNAc(6SO₄)-might be very rare at the nonreducing terminal of CS-A.

In the present paper, we successfully prepared radioactive Oligo I through the sulfation of Tetra-46 with the recombinant 2OST followed by β-glucuronidase digestion. On the other hand, we described previously that radioactive Oligo II could be prepared from Oligo I by reaction with GalNAc4S-6ST (22). These radioactive oligosaccharides may be useful probes for elucidating the functional roles of these highly sulfated structures.

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