A Unique Nonreducing Terminal Modification of Chondroitin Sulfate by N-Acetylglalactosamine 4-Sulfate 6-O-Sulfotransferase*

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N-Acetylglactosamine 4-sulfate 6-O-sulfotransferase (GalNAc(4S-6ST)) transfers sulfate from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to position 6 of N-acetylglactosamine 4-sulfate (GalNAc(4SO₄)). We previously identified human GalNAc(4S-6ST) cDNA and showed that the recombinant GalNAc(4S-6ST) could transfer sulfate efficiently to the nonreducing terminal GalNAc(4SO₄) residues. We here present evidence that GalNAc(4S-6ST) should be involved in a unique nonreducing terminal modification of chondroitin sulfate A (CS-A). From the nonreducing terminal of CS-A, a GlcA-containing oligosaccharide (Oligo I) that could serve as an acceptor for GalNAc(4S-6ST) was obtained after chondroitinase ACII digestion. Oligo I was found to be GalNAc(4SO₄)-GlcA(2SO₄)-GalNAc(6SO₄) because GalNAc(4SO₄)-GlcA(2SO₄) and GalNAc(6SO₄) were formed after chondroitinase ABC digestion. When Oligo I was used as the acceptor for GalNAc(4S-6ST), sulfate was transferred to position 6 of GalNAc(4SO₄) located at the nonreducing end of Oligo I. Oligo I was much better acceptor for GalNAc(4S-6ST) than GalNAc(4SO₄)-GlcA-GalNAc(6SO₄). An oligosaccharide (Oligo II) whose structure is identical to that of the sulfated Oligo I was obtained from CS-A after chondroitinase ACII digestion, indicating that the terminal modification occurs under the physiological conditions. When CS-A was incubated with [35S]PAPS and GalNAc(4S-6ST) and the 35S-labeled product was digested with chondroitinase ACII, a 35S-labeled trisaccharide (Oligo III) containing [35S]GalNAc(4SO₄) residues was obtained. Oligo III behaved identically with the sulfated GalNAc(4SO₄)-GlcA-GalNAc(6SO₄); Tri-64, GalNAc(4SO₄)-GlcA-GalNAc(6SO₄); HPLC, high performance liquid chromatography.

Chondroitin sulfate (CS) chains attached to various proteoglycans undergo various structural modifications by sulfation of different positions of the sugar residues composing the repeating disaccharide units (1). The resulting sulfated chains show significant structural diversity depending on the type of tissues and cells or age of the animal from which CS was extracted. Among the sulfotransferases involved in the formation of the divergent structure, sulfotransferases belonging to the chondroitin 6-sulfotransferase family (2-4) and the chondroitin 4-sulfotransferase family (5-9) have been purified and cloned. Uronosyl 2-O-sulfotransferase was cloned as a sulfotransferase belonging to the heparan sulfate 2-sulfotransferase family (10). Chondroitin 6-sulfotransferase and chondroitin 4-sulfotransferase transfer sulfate to positions 6 and 4, respectively, of the GalNAc residue. On the other hand, uronosyl 2-O-sulfotransferase transfers sulfate to position 2 of GlcA or IdoA residues (10). GalNAc(4S-6ST) transfers sulfate to position 6 of GalNAc(4SO₄) residues. We previously purified GalNAc(4S-6ST) from squid cartilage (11) and identified human GalNAc(4S-6ST) cDNA on the basis of amino acid sequences of the squid GalNAc4S-6ST (12). Unlike squid GalNAc(4S-6ST), human GalNAc(4S-6ST) exhibited high activity toward the nonreducing terminal GalNAc(4SO₄) residue of CS. This specificity of human GalNAc(4S-6ST) suggests that human GalNAc(4S-6ST) may be involved in the modification of the nonreducing terminal of CS. The sulfotransferase activities capable of modifying the terminal end of CS-A have been found in quail oviduct (13) and human serum (14). It has been shown that, in CS of aggrecan obtained from various sources, GalNAc(4,6SO₄) residues are present at the nonreducing end much more abundantly than in the internal repeating units (15-18). The proportion of the nonreducing terminal GalNAc(4,6SO₄) contained in the aggrecan has been reported to decrease in osteoarthrosis (19). CS of thrombomodulin, which is involved in the antithrombin-dependent anticoagulant activity, has been reported to bear GalNAc(4SO₄)-GlcA-GalNAc(6SO₄) at the nonreducing end (20). These observations suggest that CS may participate in the various physiological processes through the nonreducing terminal structures containing GalNAc(4,6SO₄) residues; however, the structure and biosynthesis of the nonreducing terminal regions of CS have not been fully understood. In this report, we investigated the structure of the nonreducing ends that...
could be sulfated by GalNAc4S-6ST and found that GalNAc4S-6ST transfers sulfate to a unique nonreducing terminal sequence, GalNAc(4SO4)-GlcA(2SO4)-GalNAc(6SO4), to yield a highly sulfated structure similar to the structure found in thrombomodulin CS. Our findings suggest that GalNAc4S-6ST may be involved in the terminal modification of CS, through which a highly sulfated nonreducing terminal sequence is generated.

EXPERIMENTAL PROCEDURES

Materials—The following commercial materials were used. H235SO4 was from Perkin-Elmer; chondroitinase ACII, chondroitinase ABC, chondroitin-6-sulfatase, CS-A from whale cartilage, CS-C from shark cartilage, DS form pig skin, thrombomodulin CS, from Perkin-Elmer; chondroitinase ACII, chondroitinase ABC, 2-acetamido-2-deoxy-D-galactonic acid-1,4-sulfate, and 2-acetamido-2-deoxy-D-galactonic acid-1,3-sulfate, from Sigma.

Materials—The following commercial materials were used. H235SO4 was from Perkin-Elmer; chondroitinase ACII, chondroitinase ABC, chondroitin-6-sulfatase, CS-A from whale cartilage, CS-C from shark cartilage, DS form pig skin, thrombomodulin CS. Our findings suggest that GalNAc4S-6ST may be involved in the terminal modification of CS, through which a highly sulfated nonreducing terminal sequence is generated.

TABLE I

| Sources               | ΔΔ-Di-6S | ΔΔ-Di-4S | ΔΔ-Di-6S | ΔΔ-diS6 | ΔΔ-diS4 |
|-----------------------|----------|----------|----------|---------|---------|
|                       | %        | %        | %        | %       | %       |
| Whale cartilage       | 1.8      | 22.9     | 0.3      | ND*     | ND*     |
| Bovine cartilage      | 4.7      | 9.1      | ND       | ND      | ND      |
| Chick embryo cartilage| 17.8     | 23.5     | 58.5     | 0.2     | ND      |
| Sturgeon notochord    | 0.5      | 94.7     | 4.8      | ND      | ND      |

* ND, not detected under the conditions used here.

Each glycosaminoglycan (25 nmol as galactosamine) was digested with chondroitinase ACII and subjected to SAX-HPLC. Disaccharide compositions were determined by the absorbance at 232 nm of unsaturated disaccharides.

**FIG. 1. Chondroitinase ACII digestion of four trisaccharides prepared from chondroitin sulfate.** Trisaccharides Tri-44 (B), Tri-46 (C), Tri-64 (D), and Tri-66 (E) were digested with chondroitinase AC II as described under “Experimental Procedures,” and the degradation products were separated by SAX-HPLC as described under “Experimental Procedures.” The column was developed with gradient B and monitored at 210 nm. The elution profile of the standard materials is shown in A. The number above each peak in A indicates the elution position of the standard material. Peak 1, ΔΔ-Di-6S; peak 2, GalNAc(4SO4); peak 3, GalNAc(4SO4); peak 4, ΔΔ-Di-6S; peak 5, ΔΔ-Di-4S; peak 6, GalNAc(4SO4).

N-glycosidase F digestion (Fig. 2). After N-glycosidase F digestion, a single protein band was detected at the migration position of 66 kDa that agreed well with the molecular weight, 66,160, calculated from the cDNA.

Western Blot Analysis—The affinity-purified GalNAc4S-6ST was precipitated with 10% trichloroacetic acid. The precipitate was washed with acetone and digested with recombinant N-glycosidase F (Roche Applied Science) by the methods recommended by the manufacturer. After digestion, the samples were separated by SDS-polyacrylamide gel electrophoresis as described by Laemmli (25). The separated proteins were electrophoretically transferred to a Hybond ECL membrane (Amersham Biosciences) and stained with anti-FLAG M2 monoclonal antibody (Sigma). The blot was developed with polyclonal anti-mouse IgG antibody coupled to horseradish peroxidase using an ECL detection kit and a Hyperfilm ECL (Amersham Biosciences).

**ASSAY OF SULFATOTRANSFERASE ACTIVITY—GalNAc4S-6ST activity was assayed by a method described previously (12). The standard reaction mixture contained, in a final volume of 50 μl, 2.5 μmol of imidazole HCl, pH 6.8, 0.5 μmol of CaCl2, 1 μmol of reduced glutathione, 25 nmol (as galactosamine) of CS-A or trisaccharides, 50 pmol of [35S]PAPS (about 5.0 × 104 cpm), and enzyme. The reaction mixtures were incubated at 37 °C for 20 min, and the reaction was stopped by immersing the
TABLE II  
Analysis of trisaccharides having sulfate groups at the position 6 or position 4 of GalNAc residues

| Trisaccharides* | GalNAc(4SO₄) | GalNAc(6SO₄) | ΔD-4S | ΔD-6S |
|-----------------|-------------|-------------|-------|-------|
| Tri-44          | 1.00        | ND          | 1.25  | 0.02  |
| Tri-46          | 1.00        | ND          | ND    | ND    |
| Tri-64          | ND          | 1.00        | 1.17  | ND    |
| Tri-66          | ND          | 1.00        | ND    | 1.15  |

* Tri-44, Tri-46, Tri-64, and Tri-66 represent GalNAc(4SO₄), Glc-GalNAc(4SO₄), GalNAc(4SO₄)-Glc-GalNAc(6SO₄), GalNAc(6SO₄)-Glc-GalNAc(4SO₄), and GalNAc(6SO₄)-Glc-GalNAc(6SO₄), respectively.

ND, not detected

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The purified trisaccharides (25 nmol as galactosamine) were digested with chondroitinase ACII. The digested materials were subjected to SAX-HPLC. Monosaccharides and unsaturated disaccharides were monitored by the absorbance at 210 nm. The elution profiles shown in Fig. 1, composition of monosaccharides and unsaturated disaccharides were calculated on the basis of our previous observation that the ratio of (molecular absorption of monosaccharides)/(molecular absorption of unsaturated disaccharides) determined at 210 nm was 0.32 (11). The data represent molar ratios when the amount of monosaccharides was set at unity.

Fig. 2. Western blot of the affinity-purified GalNAc4S-6ST. The FLAG-GalNAc4S-6ST 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 as follows: 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).

reaction tubes in a boiling water bath for 1 min. After the reaction was stopped, ³⁵S-labeled glycosaminoglycans were isolated by precipitation with ethanol followed by gel chromatography with a fast desalting column as described previously (2), and the radioactivity was determined. When oligosaccharides were used as 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 Hyaluronidase, β-Glucuronidase, Chondroitinase ACII, Chondroitinase ABC, and Chondro-6-sulfatase—Digestion with hyaluronidase was carried out for 24 h at 37 °C in a reaction mixture containing, in a final volume of 0.7 ml, 35 mg of CS-C, 0.15 M NaCl in 0.1 M sodium acetate buffer, pH 5.0. Digestion with β-glucuronidase was carried out for 4 h at 37 °C in a reaction mixture containing, in a final volume of 200 μl, tetrasaccharide (∼1 μmol as galactosamine), 10 μmol of sodium acetate buffer, pH 4.5, 100 nmol of 2-acetamido-2-deoxy-β-D-glucuronic acid-1,4-lactone, 4 μmol of sodium fluoride, and 10 units of β-glucuronidase. Under these conditions, removal of the nonreducing terminal GlcA was complete, and no release of inorganic sulfate was observed. Unless otherwise stated, digestion with chondroitinase ACII or chondroitinase ABC under the standard conditions was carried out for 4 h at 37 °C in the reaction mixture containing, in a final volume of 25 μl, ³⁵S-labeled glycosaminoglycans or ³⁵S-labeled trisaccharides, 1.25 μmol of Tris acetate buffer, pH 7.5, 2.5 μg of bovine serum albumin, and 30 milliunits of chondroitinase ACII or chondroitinase ABC. For degrading oligosaccharides derived from the nonreducing terminal of CS-A with chondroitinase ABC or chondroitinase ACII, a strong condition was used under which digestion with chondroitinase ACII or chondroitinase ACII was carried out in the reaction mixtures described above three times successively; first with 120 milliunits enzyme for 28 h, second with 100 milliunits enzyme for 18 h, and finally with 100 milliunits 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 under the standard conditions was carried out for 5 h at 37 °C in the reaction mixture containing, in a final volume of 25 μl, trisaccharides or GalNAc(4,6-SO₄), 1.25 μmol of Tris acetate buffer, pH 7.5, 2.5 μg of bovine serum albumin, and 100 milliunits of chondro-6-sulfatase. After digestion of oligosaccharides with chondroitinase ABC or chondroitinase ACII under the strong conditions, digestion with chondro-6-sulfatase was carried out twice successively in the reaction mixtures described above; first with 100 milliunits enzyme for 17 h and second with 100 milliunits enzyme for 5 h.

Removal of Unsaturated Uronic Acid by Mercuric Acetate—Removal of unsaturated uronic acid was carried out as described (26). Oligosaccharides containing unsaturated uronic acid were dried and dissolved in 1 ml of 35 mM mercuric acetate in 25 mM Tris with 25 mM sodium acetate, pH 5.0. The reaction was carried out for 2 h at room temperature. After the reaction was over, the samples were applied to Dowex 50 (H⁺) column (bed volume, 1 ml). 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.

Identification of Uronic Acid—Glycosaminoglycans or oligosaccharides (100 nmol as galactosamine) were hydrolyzed with 2 M trifluoroacetic acid at 100 °C for 4 h. The hydrolysates were dried in a vacuum desicator on P₂O₅ and NaOH. The dried materials were dissolved in distilled water and applied to a column of Dowex 50 (H⁺) (bed volume, 1 ml). 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.

Superdex 30 Chromatography and HPLC—A Superdex 30 16/60 column was equilibrated with 0.2 M 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 Partisil-10 SAX column (4.6 mm × 25 cm) equilibrated with 8 or 5 mM KH₂PO₄. The column was developed with gradient A (8 mM KH₂PO₄ for 10 min followed by a linear gradient from 8 to 720 mM KH₂PO₄) or gradient B (5 mM KH₂PO₄ for 10 min followed by a linear gradient from 5 to 500 mM KH₂PO₄) depending of the lot of the column; the gradient used is indicated in the legend for each figure. The fractions (0.5 ml) were collected at a flow rate of 1 ml/min and a column temperature of 40 °C. The conditions used for HPLC using a YMC-Pack Polymide II column (4.6 mm × 25 cm) were the same as those for SAX-HPLC, except that the column was developed with 10 mM KH₂PO₄ for 10 min followed by a linear gradient from 10 to 500 mM KH₂PO₄

RESULTS

Isolation of an Oligosaccharide with the Acceptor Activity for GalNAc4S-6ST from the Nonreducing Terminal of Chondroitin Sulfate—We previously showed that GalNAc4S-6ST efficiently transferred sulfate to the nonreducing terminal GalNAc4S-6ST residue of CS-A to yield GalNAc(4,6-SO₄) (12). On the other hand, a highly sulfated trisaccharide bearing GalNAc(4,6-SO₄) at the nonreducing end was obtained from the nonreducing terminal of thrombomodulin CS by a partial digestion with chondroitinase ABC (20). GalNAc4S-6ST may possibly be involved in the synthesis of the highly sulfated trisaccharide structure from a corresponding precursor. To determine whether commercially available CS-A also contains a unique nonreducing terminal structure that could serve as the acceptor for GalNAc4S-6ST, we tried to isolate oligosaccharides from the nonreducing end of CS-A after chondroitinase ACII digestion. The strategy for detection of oligosaccharides released from the nonreducing end is based on the fact that oligosaccharides derived from the internal repeating units have unsaturated uronic acid and hence show the absorption at 232 nm, but those
derived from the nonreducing end exhibit no absorption at this wavelength. Instead, oligosaccharides released from the nonreducing end of CS are able to be detected by the absorbance at 210 nm but not at 232 nm. Of these two peaks, we analyzed the absorbance at 210 nm (Fig. 3B) was compared with the elution profile detected at 232 nm (Fig. 3C). Two peaks eluted around 29 and 33.5 min were found to have much higher absorption at 210 nm than at 232 nm. Of these two peaks, we analyzed the peak eluted at 33.5 min (indicated by arrowhead X in Fig. 3B), because the peak at 29 min was not obtained reproducibly. The materials eluted at 33.5 min were designated as Oligo I and were purified by the second SAX-HPLC and Superdex 30 chromatography. About 0.57 µmol (as galactosamine) of Oligo I was obtained from 550 µmol of whale cartilage CS-A.

Oligo I showed absorption at 210 nm but not at 232 nm (Fig. 4, A and B). Oligo I was not degraded completely by chondroitinase ABC under the standard conditions but degraded completely under the strong conditions. When the completely degraded Oligo I was separated with SAX-HPLC, two peaks corresponding to GalNAc(4SO₄) and ΔDi-diS₉ were detected (Fig. 4C). When Oligo I was digested with chondro-6-sulfatase after digestion with chondroitinase ABC, the second peak disappeared and shifted to the position of ΔDi-2S (Fig. 4D). These observations clearly indicate that Oligo I is a trisaccharide with three sulfate groups, GalNAc(4SO₄)-HexA(2SO₄)-GalNAc(6SO₄). When Oligo I was digested with chondroitinase ACII under the standard conditions, no peaks corresponding to GalNAc(4SO₄) and ΔDi-diS₉ were detected (data not shown). Even under the strong conditions, only 59% of Oligo I was degraded with chondroitinase ACII to yield GalNAc(4SO₄) and ΔDi-diS₉ (Fig. 4E). To determine whether the observed resistance of Oligo I against chondroitinase ACII digestion might be...
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To determine whether Oligo I could serve as the acceptor for GalNAc4S-6ST and be converted to an oligosaccharide with the nonreducing end by GalNAc4S-6ST was observed in the in vitro reaction. To investigate whether such modification of the non-reducing terminal structure of CS occurs in the physiological conditions, we tried to isolate an oligosaccharide from CS-A, the structure of which is identical to that of the sulfated Oligo I. When Fig. 3 (B and C) was examined, a peak was observed at the position of the sulfated Oligo I (indicated by arrowhead Y in Fig. 3B except that arrow 7 indicates the elution position of SO4²⁻).

Presence of the Nonreducing Terminal Structure Corresponding to the Sulfated Oligo I—Conversion of Oligo I to the sulfated product by GalNAc4S-6ST was observed in the in vitro reaction. To investigate whether such modification of the non-reducing terminal structure of CS occurs in the physiological conditions, we tried to isolate an oligosaccharide from CS-A, the structure of which is identical to that of the sulfated Oligo I. When Fig. 3 (B and C) was examined, a peak was observed at the position of the sulfated Oligo I (indicated by arrowhead Y in Fig. 3B except that arrow 7 indicates the elution position of SO4²⁻).

Experimental Procedures

Experimental Procedures. The standards were the same as those described in the legend to Fig. 3A, B and C, the isolated sulfated Oligo I was separated with SAX-HPLC before (B) or after digestion with chondroitinase ABC under the strong conditions. The column was developed with gradient A. The standards were the same as those described in the legend to Fig. 3B except that arrow 7 indicates the elution position of SO4²⁻.

Fig. 6. Isolation of the sulfated Oligo I and digestion with chondroitinase ABC. A, Oligo I was incubated with [35S]PAPS and GalNAc4S-6ST as described under “Experimental Procedures.” The sulfated Oligo I was isolated by Superdex 30 chromatography. The peak fractions (indicated by a horizontal bar) were pooled, concentrated, and lyophilized. The standards were the same as those described in the legend to Fig. 3A, B and C, the isolated sulfated Oligo I was separated with SAX-HPLC before (B) or after digestion with chondroitinase ABC under the strong conditions. The column was developed with gradient A. The standards were the same as those described in the legend to Fig. 3B except that arrow 7 indicates the elution position of SO4²⁻.

Fig. 5. Thin layer chromatography of the hydrolysates of CS-A, DS, Tri-44, and Oligo I. CS-A (lane 2), DS (lane 3), Tri-44 (lane 4), and Oligo I (lanes 5 and 6) were hydrolyzed with 2 M trifluoroacetic acid and separated by thin layer chromatography as described under “Experimental Procedures” before (lanes 2–5) or after (lane 6) treatment with NaOH. Under the hydrolysis conditions, GlcA was released from CS-A (lane 2) and Tri-44 (lane 4), and IdoA was released from DS (lane 3). IdoA was not detected in the hydrolysate of Oligo I (lanes 5 and 6). After 2-O-desulfation with NaOH, GlcA was clearly detected in the hydrolysate of Oligo I (lane 6). Spots migrating more slowly than GlcA represent partially degraded materials. The spot migrating behind IdoA detected in lanes 4 and 5 represents galactosamine that was not completely trapped by Dowex 50. Another spot migrating between Gal and Glc observed in lane 5 was not identified. Standard sugars were spotted on lanes 1 and 7. The migration positions of the standard sugars are indicated on the right.
II is a trisaccharide with four sulfate groups, GalNAc(4,6-SO4)-
tected (Fig. 8). These observations clearly indicate that Oligo
containing unsaturated oligosaccharides, we treated the
materials recovered in the peak Y with mercuric acetate as
described under “Experimental Procedures,” and the mercuric
acetate-resistant component was further purified with Super-
dex 30 chromatography and SAX-HPLC. About 0.16 μmol (as
galactosamine) of the purified oligosaccharide (designated as
Oligo II) was obtained from 1650 μmol of whale cartilage CS-A.
Oligo II was eluted at the same position as that of the sulfated
Oligo I in the SAX-HPLC (Fig. 8A) and Superdex 30 chromato-
graphy (data not shown). The purified Oligo II showed no
absorption at 232 nm (Fig. 8B). When Oligo II was digested
with chondroitinase ABC under the strong conditions and sub-
jected to SAX-HPLC, two peaks corresponding to GalNac(4,6-
SO4) and ADi-diS4 were detected (Fig. 8C). When Oligo II was
digested with chondro-6-sulfatase after digestion with chon-
droitinase ABC, the two peaks disappeared and shifted to the
position of GalNAc(4SO4) and ΔDi-2S (Fig. 8D), respectively.
When Oligo II was digested with chondroitinase ACII under
the strong conditions, GalNAc(6,8-SO4) and ΔDi-diS4 were
detected (Fig. 8E). These observations clearly indicate that Oligo
II is a triasaccharide with four sulfate groups, GalNAc(4,6-SO4)–
GlcA(2SO4)–GalNAc(6SO4). These observations strongly sug-
ject that the terminal modification catalyzed by GalNAc4S-
6ST should occur in physiological conditions.

Fig. 7. Effect of the concentration of Oligo I, Tri-44, and Tri-46 on the activity of GalNAc4S-6ST. The GalNAc4S-6ST activity was determined as described under “Experimental Procedures” except that the concentrations of Oligo I (closed circles), Tri-44 (open circles), and Tri-46 (closed triangles) were varied.

Formation and Characterization of an Oligosaccharide from 35S-Labeled Glycosaminoglycans Synthesized from CS-A after Incubation with 35S/PAPS and Human GalNAc4S-6ST—As shown above, the terminal modification of Oligo I occurred when the sulfated oligosaccharide was used as the acceptor for GalNAc4S-6ST. To demonstrate that such a terminal modification could occur in polysaccharide level, we analyzed the sulfated products formed from CS-A. When the 35S-labeled glycosaminoglycans derived from CS-A after incubation with 35S/PAPS and the recombinant human GalNAc4S-6ST were digested with chondroitinase ACII under the standard conditions, three radioactive peaks were obtained in SAX-HPLC (Fig. 9A). The peaks at 29 and 38.5 min corresponded to GalNAc(4,6-SO4) and ΔDi-diS4, respectively. The elution position of the third peak was exactly the same as that of the sulfated Oligo I. The third peak was not obtained when the 35S-labeled glycosaminoglycan was digested with chondroitinase ABC (data not shown). The materials eluted at the position of the sulfated Oligo I (designated as Oligo III) were purified by Superdex 30 chromatography and SAX-HPLC. To determine the structure of Oligo III, Oligo III was digested with chondroitinase ABC and applied to SAX-HPLC (Fig. 10A). The radioactivity appeared at the position of GalNAc(4,6-SO4). To establish the position to which 35SO4 was transferred, we digested Oligo III with chondro-6-sulfatase after digestion with chondroitinase ABC and after being subjected to SAX-HPLC. The radioactivity of GalNAc(4,6-SO4) disappeared and was shifted to the position of inorganic sulfate (Fig. 10B). These results indicate that Oligo III contained GalNAc(4,6-SO4) residue at the non-
reducing end. When Oligo III was digested with chondro-6-
sulfatase alone, the 35S radioactivity was detected at the posi-
tion of ΔDi-diS4 (Fig. 10C). However, this material was not ΔDi-diS4 but an oligosaccharide containing GalNAc(4,6-SO4) at its nonreducing end, because GalNAc(4,6-SO4) was formed...
Chondro-6-sulfatase could remove sulfate from GalNAc(6SO₄) and Tri-46, respectively. These results clearly indicate that the elution position of SO₄²⁻, S-I indicates the position of the sulfated Oligo I. B, the radioactive peak at the position of the sulfated Oligo I in A was collected and separated with the Superdex 30 column. The standards were the same as those described in the legend to Fig. 3B except that arrow 7 indicates the elution position of SO₄²⁻. S-I indicates the elution position of the sulfated Oligo I.

After further digestion with chondroitinase ABC (Fig. 10D). Chondro-6-sulfatase was reported to remove sulfate groups attached to position 6 of GalNAc residue located at the reducing end of hexasaccharides containing unsaturated hexuronic acid at their nonreducing end (29). To determine whether chondro-6-sulfatase could act on oligosaccharides containing GalNAc(4SO₄) or GalNAc(6SO₄) at their nonreducing terminal in the same manner, we digested three trisaccharides, Tri-66, Tri-64, and Tri-46, with chondro-6-sulfatase and analyzed the reaction products by SAX-HPLC after chondroitinase ACII digestion (Fig. 11). After chondro-6-sulfatase digestion followed by chondroitinase ACII digestion, GalNAc(6SO₄) and ΔDi-0S (Fig. 11B), GalNAc(6SO₄) and ΔDi-4S (Fig. 11C), and GalNAc(4SO₄) and ΔDi-0S (Fig. 11D) were formed from Tri-66, Tri-64, and Tri-46, respectively. These results clearly indicate that chondro-6-sulfatase could remove sulfate from GalNAc(6SO₄) residue located exclusively at the reducing end of these trisaccharides. On the basis of the specificity of chondro-6-sulfatase indicated above, Oligo III should bear nonradioactive sulfate at position 6 of GalNAc residue located at the reducing end. When the isolated Oligo III was digested with chondroitinase ACII under the strong conditions, Oligo III was degraded to give rise to [³⁵S]GalNAc(4,6-SO₄) (data not shown), indicating that Oligo III contains GlcA.

Susceptibility of Oligosaccharides to Chondroitinase ACII—The susceptibility of Oligo I, the sulfated Oligo I, Oligo II, Oligo III, and Tri-46 to chondroitinase ACII under the standard conditions or strong conditions was summarized in Table III. Under the standard conditions, Oligo I was hardly degraded, but Tri-46 was completely degraded, indicating that the resistance of Oligo I against chondroitinase ACII digestion is attributable to the presence of 2-O-sulfate attached to the GlcA residue. Because Oligo II was more sensitive than Oligo I to chondroitinase ACII, the presence of nonreducing terminal GalNAc(4,6-SO₄) should promote the rate of reaction with chondroitinase ACII. These results indicate that the rate of degradation with chondroitinase ACII is markedly affected by the sulfation pattern of these oligosaccharides. The susceptibility of Oligo III was nearly the same as those of Oligo II and sulfated Oligo I. Because Oligo III was indistinguishable from the sulfated Oligo I in the chromatographic behaviors, the position to which sulfate was transferred, the existence of 6-sulfate on the reducing terminal GalNAc residue, and the susceptibility to chondroitinase ACII, the structure of Oligo III is most probably identical to that of the sulfated Oligo I. These results strongly suggest that the terminal modification could occur at the polysaccharide level. However, at present the possibility that Oligo III may contain GalNAc(4,6-SO₄) residues at the reducing end could not be excluded.

Formation of Oligo III from Various Chondroitin Sulfate Preparations Derived from Different Sources—Oligo III was initially found in the chondroitin ACII digests of the [³⁵S]-glycosaminoglycan formed from whale cartilage CS-A after the reaction with GalNAc4S-6ST. To determine whether Oligo III could be formed from other CS preparations obtained from different sources, bovine cartilage CS, chick embryo cartilage CS, or sturgeon notochord CS were incubated with [³⁵S]PAPS and GalNAc4S-6ST. The [³⁵S]-glycosaminoglycans formed from these CS preparations were digested with chondroitinase ACII and analyzed by SAX-HPLC (Fig. 12). Disaccharide compositions of these CS preparations are shown in Table I. The relative rates of incorporation of sulfate into CS from whale cartilage, bovine cartilage, chick embryo cartilage, and sturgeon notochord were 1.00, 1.81, 0.46, and 0.88, respectively. The proportion of the radioactivity recovered in the peak at the

**Fig. 9.** Separation of Oligo III by SAX-HPLC and Superdex 30 chromatography. A, the [³⁵S]-labeled glycosaminoglycan formed from CS-A after the reaction with GalNAc4S-6ST was digested with chondroitinase ACII, and the degradation products were separated by SAX-HPLC as described under “Experimental Procedures.” The column was developed with gradient A. The standards were the same as those described in the legend to Fig. 3B except that arrow 7 indicates the elution position of SO₄²⁻. S-I indicates the position of the sulfated Oligo I. B, the radioactive peak at the position of the sulfated Oligo I in A was collected and separated with the Superdex 30 column. The standards were the same as those described in the legend to Fig. 3A.

**Fig. 10.** Digestion of Oligo III with chondroitinase ABC, chondro-6-sulfatase, and chondroitinase ACII. Oligo III was purified with SAX-HPLC and Superdex 30 as indicated in Fig. 9. The isolated Oligo III was separated with SAX-HPLC after digestion with chondroitinase ABC (A), chondroitinase ACII and then chondro-6-sulfatase (B), chondro-6-sulfatase (C), or chondro-6-sulfatase and then chondroitinase ACII (D). The column was developed with gradient A. The standards were the same as those described in the legend to Fig. 3B except that arrow 7 indicates the elution position of SO₄²⁻. S-I indicates the elution position of the sulfated Oligo I.
position of the sulfated Oligo I was highest when CS from chick embryo cartilage was used as the acceptor (Fig. 12C). In contrast, no peak was observed at the position of the sulfated Oligo I when sturgeon CS was used as the acceptor, although [35S]GalNAc(4,6-SO4) was formed (Fig. 12D). These observations indicate that the terminal structure from which Oligo III was produced is present at least in avian and mammalian CS. The ratio of Oligo III to the sum of Oligo III and GalNAc(4,6-SO4) was found to be related to the contents of Di-6S in each CS (Table I); the higher the content of Di-6S was, the higher the ratio of Oligo III was.

**DISCUSSION**

In this report, we presented data that GalNAc4S-6ST could transfer sulfate to the unique nonreducing terminal sequence and catalyzed the formation of the highly sulfated structure. The highly sulfated nonreducing terminal structure produced by the reaction with GalNAc4S-6ST is present in native CS-A because Oligo II was obtained from CS-A. These observations suggest that the terminal modification catalyzed by GalNAc4S-6ST may occur in the physiological conditions. The 2-O-sulfation of the GlcA residue adjacent to GalNAc(6SO4) may stimulate 6-sulfation of the nonreducing terminal GalNAc(4SO4) residue, because Oligo I was much better acceptor for GalNAc4S-6ST than Tri-46.

At present the physiological role of the highly sulfated nonreducing terminal sequence is not known. Thrombomodulin with anticoagulant activity was reported to have CS as an essential functional domain (30). CS attached to thrombomodulin contained GalNAc(di-SO4) at the nonreducing end (20). When thrombomodulin CS was partially digested with chondroitinase ABC, an oligosaccharide was obtained. After chondroitinase AC digestion, GalNAc(di-SO4) and HexA-GalNAc(di-SO4) were formed from the oligosaccharide. HexA-GalNAc(di-SO4) migrated to the position of Di-diS2 on paper electrophoresis at pH 1.7. However, under the conditions for the paper electrophoresis, it is not yet known whether the 2-O-sulfation of the GlcA residue adjacent to GalNAc(6SO4) may stimulate 6-sulfation of the nonreducing terminal GalNAc(4SO4) residue, because Oligo I was much better acceptor for GalNAc4S-6ST than Tri-46.

**TABLE III**

**Susceptibility of the oligosaccharides derived from the nonreducing terminal of CS-A to chondroitinase ACII**

The standard and strong conditions for chondroitinase ACII digestion were described under “Experimental Procedures.” 15 nmol (as galactosamine) of Oligo I, Oligo II, and Tri-46, or 5,000 cpm of the sulfated Oligo I and Oligo III were used for the substrate. After chondroitinase ACII digestion, each sample was separated with SAX-HPLC. The percentages degradation of Oligo I, Oligo II, and Tri-46 were calculated from the absorbance at 210 nm of each oligosaccharide remaining after chondroitinase ACII digestion. The percentages degradation of the sulfated Oligo I and Oligo III were determined from the 35S radioactivity of each oligosaccharide remaining after chondroitinase ACII digestion.

| Oligosaccharides | Degradation (% Strong conditions) | Degradation (% Standard conditions) |
|------------------|----------------------------------|-------------------------------------|
| Oligo I          | 59                               | <2                                  |
| Oligo II         | 100                              | 67                                  |
| Sulfated Oligo I | 100                              | 51                                  |
| Oligo III        | 100                              | 74                                  |
| Tri-46           | 100                              | 100                                 |

FIG. 11. Digestion of trisaccharides having sulfate groups at position 6 or 4 of GalNAc residues by chondroitinase ACII after treatment with chondro-6-sulfatase. Trisaccharides Tri-66 (B), Tri-64 (C), and Tri-46 (D) were digested with chondro-6-sulfatase. The digests were heated at 100 °C for 1 min and then further digested with chondroitinase ACII. After digestion with chondroitinase ACII, the degradation products were separated by SAX-HPLC as described under “Experimental Procedures.” The column was developed with gradient B. The elution profile of the standard materials are shown in A. The number above each peak in A was the same as those described in the legend to Fig. 1. The broad peaks observed around 20 min represent materials derived from the column.

FIG. 12. Chondroitinase ACII digestion of 35S-labeled glycosaminoglycans formed from various chondroitin sulfate preparations after the reaction with GalNAc4S-6ST. 35S-Labeled glycosaminoglycans formed from whale cartilage CS-A (A), bovine nasal cartilage CS (B), 12-day-old chick embryo cartilage CS (C), and sturgeon notochord CS (D) after the reaction with GalNAc4S-6ST were digested with chondroitinase ACII, and the digests were separated with SAX-HPLC. The column was developed with gradient A. The standards were the same as those described in the legend to Fig. 3B except that arrow 7 indicates the elution position of SO42-. S-I indicates the elution position of the sulfated Oligo I.
phoresis, ΔDi-di₃S₄ could not be separated from ΔDi-di₃SO₄; therefore, it remains possible that the nonreducing terminal structure of thrombomodulin CS may be the same as that of Oligo II. Midkine, chemokines, and fibroblast growth factor family proteins have been reported to interact with squid cartilage CS-E (31–34). The highly sulfated nonreducing terminal sequence generated by the enzymatic reaction with GalNAc4S-6ST might interact with such molecules. Approximately 30% of the CS chains of proteochondroitin sulfate extracted from the cell matrix pool of the cultured chick embryo chondrocytes was found to have nonreducing terminal GalNAc4,6-SO₄ residues, but none of the CS chains in the proteochondroitin sulfate recovered from the culture medium pools were terminated with these residues (16). These observations raise the possibility that the cell matrix proteoglycans might interact with some extracellular matrix or cell surface components through nonreducing terminal GalNAc(4,6-SO₄) residues of CS chains.

Under the standard conditions for chondroitinase ACII digestion, Tri-46 was degraded completely, but Oligo II was hardly affected. In contrast, Oligo II was more sensitive to chondroitinase ACII digestion than Oligo I. These observations suggest that chondroitinase ACII may recognize not only the kind of uronic acid but also sulfation pattern of the component sugar residues; the presence of 2-O-sulfate on GlcA residue may make the trisaccharide resistant to chondroitinase ACII, and the presence of nonreducing terminal GalNAc(4,6-SO₄) may relieve the inhibitory effect of the 2-O-sulfate. The observed resistance of Oligo I to chondroitinase ACII might be due to the presence of 3-O-sulfated GlcA (36); however, this possibility is not the case, because GalNAc4SO₄ and ΔDi-di₃SO₄ were formed from Oligo I on chondroitin ABC digestion, whereas the GlcA3SO₄ residue has been reported to be degraded by chondroitinase ABC digestion (36). Because Oligo II was rather sensitive to chondroitinase ACII digestion, the amount of Oligo II obtained from CS-A after digestion with chondroitinase ACII may not necessarily reflect the amount of the nonreducing terminal sequence from which Oligo II was derived. Instead, the nonreducing terminal highly sulfated sequence may be present more abundantly than the yield of Oligo II. In this report, chondroitinase ACII digestion was carried out at pH 7.5. In contrast, the reaction with chondroitinase ACII was originally performed in acetate buffer, pH 6.0 (35). It may be possible that specificity of chondroitinase ACII might be altered by the pH of the reaction mixture, but this possibility is not the case because the same results were obtained when the reaction of chondroitinase ACII was carried out at pH 6.0 (data not shown). It might be possible that Oligos I and II were formed from the respective unsaturated tetrasaccharides by the reaction with a hypothetical unsaturated uronate-specific glycuronidase that might be included in chondroitinase ACII. However, this possibility is unlikely, because neither ΔDi-di₄S (ΔHexA-GalNAc4SO₄) nor ΔDi-di₄SE (ΔHexA-GalNAc4,6-SO₄) was degraded by chondroitinase ACII even under the strong conditions (data not shown).

The molecular weight of CS-A from whale cartilage is within 25,000–50,000 according to the manufacturer's data. It is thus assumed that CS-A from whale cartilage contains 50–100 repeating disaccharide units. The recovery of Oligo I was about 0.1% on the basis of the content of galactosamine. We found previously that the content of the nonreducing terminal GalNAc4SO₄ of the same CS-A preparation was about 0.8% of the total repeating units (12). From these data, the contents of the nonreducing terminal structures from which GalNAc4SO₄ and Oligo I were generated after chondroitinase ACII digestion could be roughly estimated to be 40–80 and 2.5–5%, respectively, of the total nonreducing terminal.

Because the loss of Oligo I during the purification was not included in this consideration, the content of the nonreducing terminal structure from which Oligo I was generated may be higher than the calculated value. On the other hand, the content of the nonreducing terminal structure from which Oligo II was generated could not be determined from the recovery of Oligo II, because Oligo II was rather sensitive to chondroitinase ACII.

When trisaccharides were used as the acceptor for GalNAc4S-6ST, the sulfation pattern of the trisaccharides affected the rates of the sulfation of GalNAc4SO₄ residues at the nonreducing terminal. The rate of sulfation of Tri-46 was much lower than that of Tri-44, indicating that the presence of the GalNAc4SO₄ residue at the reducing side inhibits the sulfation of position 6 of the GalNAc4SO₄ residue at the nonreducing end. On the other hand, the rate of sulfation of Oligo I was much higher than that of Tri-46. The Kₘ for Tri-46 was 60-fold of the Kₘ for Oligo I, indicating that 2-O-sulfate on the GlcA residue markedly augmented the affinity for the acceptor substrate. Thus, the 2-O-sulfation of the penultimate GlcA residue appears to forward the production of the highly sulfated terminal sequence.

We determined substrate specificity of chondro-6-sulfatase using the defined trisaccharide substrates. As observed previously in hexasaccharides with unsaturated uronate at the nonreducing end (29), chondro-6-sulfatase could release sulfate from the reducing terminal GalNAc6SO₄ but not from the nonreducing terminal GalNAc6SO₄.

When CS from various origins except for the sturgeon notochord were used as the acceptors for GalNAc4S-6ST, and the ³⁵S-labeled products formed were digested with chondroitinase ACII, the ³⁵S radioactivity was detected at the position of the sulfated Oligo I. These results suggest that the nonreducing terminal sequence from which Oligo I was derived is present in CS from avian and mammalian tissues. Among the CS used here, CS from chick embryo cartilage had the highest content of ΔDi-6S, and CS from sturgeon notochord had the lowest one (Table 1). On the other hand, the proportion of the radioactive peak detected at the position of the sulfated Oligo I was also highest when chick embryo CS was used as the acceptor. Taken together, the synthesis of the nonreducing terminal structure from which Oligo I was released by chondroitinase ACII digestion may depend on the synthesis of the GalNAc4SO₄ residue adjacent to the reducing GlcA. Uronosyl 2-O-sulfotransferase has been reported to transfer sulfate to position 2 of GlcA residue adjacent to GalNAc6SO₄ residue (10). Such specificity of uronosyl 2-O-sulfotransferase seems to agree with the hypothetical requirement for the GalNAc6SO₄ residue.

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**Sulfation of Nonreducing Terminal Sequence by GalNAc4S-6ST**