Purification and Characterization of Chondroitin 4-Sulfotransferase from the Culture Medium of a Rat Chondrosarcoma Cell Line*

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Chondroitin 4-sulfotransferase, which transfers sulfate from 3'-phosphoadenosine 5'-phosphosulfate to position 4 of N-acetylgalactosamine in chondroitin, was purified 1900-fold to apparent homogeneity with 61% yield from the serum-free culture medium of rat chondrosarcoma cells by affinity chromatography on heparin-Sepharose CL-6B, Matrex gel red A-agarose, 3',5'-ADP-agarose, and the second heparin-Sepharose CL-6B. SDS-polyacrylamide gel electrophoresis of the purified enzyme showed two protein bands. Molecular masses of these protein were 60 and 64 kDa under reducing conditions and 50 and 54 kDa under nonreducing conditions. Both the protein bands coeluted with chondroitin 4-sulfotransferase activity from Toyopearl HW-55 around the position of 50 kDa, indicating that the active form of chondroitin 4-sulfotransferase is a monomer. Dithiothreitol activated the purified chondroitin 4-sulfotransferase. The purified enzyme transferred sulfate to chondroitin and desulfated dermatan sulfate. Chondroitin sulfate A and chondroitin sulfate C were poor acceptors. Chondroitin sulfate E from squid cartilage, dermatan sulfate, heparan sulfate, and completely desulfated N-resulfated heparin hardly served as acceptors of the sulfotransferase. The transfer of sulfate to the desulfated dermatan sulfate occurred preferentially at position 4 of the N-acetylgalactosamine residues flanked with glucuronic acid residues on both reducing and nonreducing sides.

Chondroitin sulfate proteoglycan is found in various tissues and thought to play important roles in various cellular interactions involving cell adhesion (1, 2), regulation of neurite outgrowth (3–6), migration of neural crest cells (7), binding of phospholipase A2 (8), and an adherence receptor for chondrocytes, rat chondrosarcoma cells were reported to synthesize chondroitin 4-sulfate as a major component (21–23). The Swarm rat chondrosarcoma cell line RCS-LTC was shown to actively secret chondroitin 4-sulfate from the culture medium of chick chondrocytes (20), but the purification of C4ST from the culture medium of chick chondrocytes has been hampered by the presence of an excess amount of C6ST. Unlike chick chondrocytes, rat chondrosarcoma cells were reported to synthesize chondroitin 4-sulfate in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. Charges of the sulfotransferases and molecular cloning of these cDNAs are basically important to reveal the functional roles of these chondroitin sulfate isomers. We have purified C6ST from the culture medium of chick chondrocytes (16) and cloned the cDNA (17). Unexpectedly, the purified C6ST was found to catalyze not only chondroitin but also keratan sulfate (18) and sialyl N-acetyllactosamine oligosaccharides (19). We previously observed that C4ST was also secreted to the culture medium of chick chondrocytes (20), but the purification of C4ST from the culture medium of chick chondrocytes has been hampered by the presence of an excess amount of C6ST. Unlike chick chondrocytes, rat chondrosarcoma cells were reportedly synthesized chondroitin 4-sulfate as a major component (21–23). The Swarm rat chondrosarcoma cell line RCS-LTC was shown to display a stable, differentiated, chondrocyte-like phenotype (24). We investigated whether C4ST was secreted from the chondrosarcoma cells, and found that C4ST, but not C6ST, was actively secreted from these cells. We report here the purification and characterization of C4ST from the serum-free culture medium of the chondrosarcoma cells.

EXPERIMENTAL PROCEDURES

Materials

The following commercial materials were used: H235SO4 was from DuPont NEN; chondroitinase ACII, chondroitinase ABC, chondroitin sulfate A (whale cartilage), chondroitin sulfate C (shark cartilage), dermatan sulfate (pig skin), heparan sulfate (bovine kidney), completely desulfated N-resulfated heparin (CDNSNS-heparin), ΔI-ΔS, ΔI-ΔSΔb, ΔI-ΔsΔb, and ΔI-ΔsΔb were from Seikagaku Corp. (Tokyo, Japan); Partisil SAX-10 was from Whatman; Dulbecco's modified Eagle's medium and fetal bovine serum were from Life Technologies, Inc. (Life Tech Oriental, Tokyo, Japan); Cosmemed-001 (a commercial serum-free culture medium developed for the culture of hybridoma, which contains human transferrin and bovine insulin as growth factors) was from Cosmo-Bio (Tokyo, Japan); Hank's solution was from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan); trypsin (from bovine pancreas, type III), penicillin G, streptomycin sulfate, 3',5'-ADP, unlabeled PAPS, 3',5'-ADP-agarose, and molecular weight standards for SDS-PAGE and gel chromatography were from Sigma-Aldrich Ja-

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1 The abbreviations used are: C4ST, chondroitin 4-sulfotransferase; C6ST, chondroitin 6-sulfotransferase; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; CDSNS-heparin, completely desulfated N-resulfated heparin; ΔI-ΔS, 2-acetamide-2-deoxy-3-(β-D-glucopyranosyluronic acid)-6-O-sulfogalactose; ΔI-ΔSΔb, 2-acetamide-2-deoxy-3-O(β-D-glucopyranosyluronic acid)-6-O-sulfogalactose; ΔI-ΔsΔb, 2-acetamide-2-deoxy-3-O(β-D-glucopyranosyluronic acid)-4,6-bis-O-sulfogalactose.
pan (Tokyo, Japan); recombinant N-glycanase was from Genzyme (Cambridge, MA); HiloLoad Superdex 30 HR 16/60, fast desalting column HR 10/10, and heparin-Sepharose CL-6B were from Amersham Pharmacia Biotech; Toyopearl HW-55 Superfine was from Tosoh (Tokyo, Japan); Matrex gel red A (cross-linked 6% agarose with covalently coupled dye from Amicon Inc.) was generously gifted from Seikagaku. Partially desulfated dermatan sulfate was prepared from pig skin dermatan sulfate according to the method of Nagasawa et al. (27). Solvolytic with dimethyl sulfoxide was carried out at 100 °C for 60 min. The densitometer of the dialyzed solution was calculated as 83% from the proportion of \( \Delta \Phi \)-OS to the total unsaturated disaccharides formed after chondroitinase ABC digestion. When the desulfated dermatan sulfate was digested with chondroitinase ACII, the yield of \( \Delta \Phi \)-OS was only 5% of the total unsaturated disaccharides formed after chondroitinase ABC digestion (see Fig. 10, B and C). Chondroitin sulfate E (quid cartridge), which was eluted with 1.5 m NaCl from DEAE-Sephadex A-50, was prepared as described (28).

**Culture of Chondrosarcoma Cells and Preparation of the Medium Fraction**

Rat chondrosarcoma cells (frozen cell line LTC) (23) were plated in 10-cm culture dishes (Falcon) at a density of 2 \( \times 10^5 \) cells/dish. Volume of the medium was 10 ml. The medium in which the cells were plated consisted of Dulbecco’s modified Eagle’s medium adjusted to pH 7.4 containing penicillin (100 units/ml), streptomycin (50 \( \mu \)g/ml), 5% fetal bovine serum, and 5 \( \mu \)g/ml insulin, and cells were grown at 37 °C in 5% CO\(_2\), 95% air. The medium was changed every other day. When the cells grew to confluence, they were treated with Hank’s solution containing 2%.

The desulfated dermatan sulfate was digested with chondroitinase ACII, the yield of \( \Delta \Phi \)-OS was only 5% of the total unsaturated disaccharides formed after chondroitinase ABC digestion (see Fig. 10, B and C). Chondroitin sulfate E (quid cartridge), which was eluted with 1.5 m NaCl from DEAE-Sephadex A-50, was prepared as described (28).

**Purification of Chondroitin 4-Sulfotransferase**

All operations were performed at 4 °C.

**Step 1: Chromatography on Heparin-Sepharose CL-6B**—The buffered medium fraction prepared as above (4.7 liters obtained from 200 \( \times 10^6 \) cells/dishes) was applied to a column of heparin-Sepharose CL-6B (2.2 × 28 cm) equilibrated with 0.15 m NaCl in buffer A (10 mm Tris-HCl, pH 7.2, 2.0 mM MgCl\(_2\), 10 mM 2-mercaptoethanol, 20% glycerol, and 3 \( \mu \)M pepstatin A). The pooled medium containing these additives was designated the buffered medium fraction and stored at 4 °C until purification was started.

**Step 2: Chromatography on Matrex Gel Red A**—Half of the combined fraction from Step 1 was applied to a Matrex gel red A column (2.2 × 9.5 cm) equilibrated with 0.4 m NaCl in buffer A. The column was eluted stepwise with 200 ml of buffer A containing 0.4 m NaCl, 200 ml of buffer A containing 0.75 m NaCl, and 200 ml of buffer A containing 1 m guanidine hydrochloride. All C4ST activity was eluted in the 1 m guanidine hydrochloride fraction. The fractions containing C4ST activity were combined, dialyzed against 0.05 m NaCl in buffer A. Chromatography on Matrex gel red A was repeated once more, and dialyzed solution was combined. To the combined dialyzed solution 10% Triton X-100 was added to a final concentration of 2%.

**Step 3: Chromatography on 3′,5′-ADP-Agarose**—The dialyzed fraction from Step 2 containing 2% Triton X-100 was applied to a 3′,5′-ADP-agarose column (1.2 × 11 cm) equilibrated with modified buffer A containing 0.05 m NaCl. The composition of modified buffer A was the same as that of buffer A, except that the concentration of Triton X-100 was increased to 2%. The column was washed with 150 ml of the modified buffer A containing 0.05 m NaCl. The sulfotransferase activity was eluted with 150 ml of a modified buffer A containing 0.1 m 3′,5′-ADP. The fractions containing sulfotransferase activity were pooled and applied to the second heparin-Sepharose CL-6B column.

**Step 4: Second Heparin-Sepharose CL-6B Chromatography**—The fraction containing the sulfotransferase activity from Step 3 was applied to a column of heparin-Sepharose CL-6B (0.9 × 6.7 cm) equilibrated with 0.05 m NaCl in buffer A containing 2% Triton X-100. The column was washed with 50 ml of 0.05 m NaCl in buffer A. The absorbed sulfotransferase was eluted with 25 ml of buffer A containing 0.4 m NaCl. The collected fraction was dialyzed against buffer A containing 0.05 m NaCl. The purified enzyme was stored at -20 °C.

**Assay of Sulfotransferase Activity**

C4ST and C6ST activity were assayed by the method described previously (18). The standard reaction mixture contained 2.5 \( \mu \)mol of imidazole-HCl, pH 6.8, 1.25 \( \mu \)M of protamine chloride, 0.1 \( \mu \)mol of dithiorthreitol, 25 \( \mu \)mol (as glucuronic acid) of chondroitin, 50 \( \mu \)mol of \( ^{35} \)S-labeled glucosaminoglycans, and enzyme in a final volume of 50 \( \mu \)l. For determining the activity for various glycosaminoglycans, chondroitin was replaced with 25 \( \mu \)mol (as galactosamine for chondroitin sulfate, dermatan sulfate, and desulfated dermatan sulfate or as glucuronic acid of heparan sulfate, CDSNS-heparin) of glycosaminoglycans. The reaction mixtures were incubated at 37 °C for 20 min, and the reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 min. After the reaction was stopped, \( ^{35} \)S-labeled glucosaminoglycans were isolated by the precipitation with ethanol followed by gel chromatography with a fast desalting column as described previously, and radioactivity was determined. For determining C6ST and C4ST activity, \( ^{35} \)S-labeled chondroitinase was digested with C6ST and C4ST activity, and the radioactivity of unsaturated disaccharides (\( \Delta \Phi \)-4S and \( \Delta \Phi \)-6S) separated with paper chromatography was counted. The chondroitinase ACII-digested materials were also analyzed with HPLC using a Whatman Partisil 10-SAX column as described below. 1 unit of enzyme activity is defined as the amount required to catalyze the transfer of 1 pmol of sulfate/min.

**SDS-Polyacrylamide Gel Electrophoresis**

Polyacrylamide gel electrophoresis of proteins in SDS was carried out on 10% polyacrylamide gels under reducing or nonreducing conditions as described (29). Protein bands were detected by silver stain or Coomassie Brilliant Blue.

**Gel Chromatography of the Sulfotransferase on Toyopearl HW-55 A**

A Toyopearl HW-55 column (1.4 × 99 cm) was equilibrated with a buffer containing 2 m NaCl, 10 mm Tris-HCl, pH 7.2, 20 mM MgCl\(_2\), 2 mM CaCl\(_2\), 0.1% Triton X-100, and 20% glycerol (buffer B). 0.8 m of sample was applied to the column and eluted with buffer B. 1.2-ml fractions were collected.

**Superdex 30 Chromatography, Paper Chromatography, and HPLC**

A Superdex 30 16/60 column was equilibrated with 0.2 m NH\(_4\)HCO\(_3\). The flow rate was 1 ml/min. 1-ml fractions were collected. On the analysis of the desulfated dermatan sulfate and chondroitinase ACII-digested desulfated dermatan sulfate, the eluates were monitored with absorption at 232 nm and with orcinol color reaction using glucuronic acid as a standard (30). Paper chromatography was performed on Toyo 5A paper using a solvent system of 1-butanol-methanol-concentrated acetic acid in NH\(_3\) (2:3:1, by volume). Regions containing unsaturated disaccharides on the paper chromatogram were visualized under a UV lamp and excised, and the radioactivity was determined. Separation of the degradation products formed from \( ^{35} \)S-labeled chondroitin and \( ^{35} \)S-labeled desulfated dermatan sulfate after digestion with chondroitinase ACII or chondroitinase ABC was carried out by HPLC using a Whatman Partisil 10-SAX column (4.5 mm × 25 cm) equilibrated with 35 mM KH\(_2\)PO\(_4\). The column was developed with gradient elution as shown in Fig. 7. The flow rate was 1 ml/min, and the column temperature was 40 °C. 0.5-ml fractions were collected.

**Assay of Protein**

Protein was determined by the method of Bradford using bovine serum albumin as a standard (31). Protein assay reagent was obtained...
Secreion of chondroitin 4-sulfotransferase from the cultured chondrosarcoma cells to the serum-free medium. The culture of chondrosarcoma cells was carried out as described under “Experimental Procedures,” except that the cells were plated in 5-cm dishes at 1 × 10⁶ cells/dish. On day 6 and thereafter, culture medium was replaced every other day with fresh Cosmedium-001. In this figure, day 6 from the start of the culture was set as day 0. The spent Cosmedium-001 recovered every other day was stored separately. The cell layer was scraped, homogenized with a buffer containing 0.5% Triton X-100, and centrifuged as described under “Experimental Procedures.” C4ST activity in the spent medium (closed circle) and in the cell layer (open circle) and protein content of the cell layer (open square) were determined.

RESULTS

Secretion of Chondroitin 4-Sulfotransferase into the Serum-free Culture Medium—Fig. 1 shows sulfotransferase activity contained in the spent culture medium and cell layer when chondrosarcoma cells were cultured in Cosmedium-001 for 10 days. The level of C4ST activity secreted to the serum-free medium was decreased rapidly. On day 4 in the serum-free medium, the activity of C4ST in the medium dropped to 48% of the initial activity. In contrast, C4ST activity retained in the cell layer was nearly constant during the whole culture period. The decrease in C4ST activity in the medium during the culture in the serum-free medium seems not to be caused by cell death or cell detachment from the dish, because the cellular protein increased gradually during 10-day culture in the serum-free conditions as described under “Experimental Procedures.”

Purification of Chondroitin 4-Sulfotransferase—C4ST was purified to apparent homogeneity—1900-fold over the specific activity of the pooled spent culture medium. Table I summarizes the purification of the sulfotransferases from 14.2 liters of the buffered medium fraction.

As observed in most of glycosaminoglycan sulfotransferase,

C4ST was also absorbed to heparin-Sepharose CL-6B. A large volume of the buffered medium fraction was efficiently concentrated with the heparin-Sepharose CL-6B step. C4ST bound to Matrex gel red A was not eluted with 0.75 M NaCl and eluted with 1 M guanidine hydrochloride. At this step, C4ST was still stable in 1 M guanidine hydrochloride, however, decreased as the purity of the enzyme increased. Fig. 3 shows the elution pattern of sulfotransferase from 3',5'-ADP-agarose. Unlike C6ST from the culture medium of chick chondrocytes, efficient absorption of C4ST to 3',5'-ADP-agarose was achieved only in the presence of 2% Triton X-100. Because the high concentration of Triton X-100 disturbed further analysis of the purified C4ST, the concentration of the detergent was decreased to 0.1% during the second heparin-Sepharose CL-6B chromatography. 3',5'-ADP, which is a strong inhibitor of the sulfotransferase, was also removed from the purified sample after the second heparin-Sepharose CL-6B chromatography. The final fraction was used for the later experiments as the purified C4ST fraction.
Purification of Chondroitin 4-Sulfotransferase

**Purity of the Chondroitin 4-Sulfotransferase**—The different purified fractions of C4ST were analyzed by SDS-PAGE under nonreducing conditions and stained with silver nitrate stain (Fig. 4, lanes 1–4). For the routine analysis of the purified C4ST with SDS-PAGE, we removed 2-mercaptoethanol from the sample buffer to avoid an artifact of silver staining. A broad protein band of 50 kDa was predominantly stained in the second heparin Sepharose CL-6B fraction, and a weaker protein band of 54 kDa was also detected (Fig. 4, lane 4). When the purified C4ST was treated with sample buffer containing 2-mercaptoethanol and stained with Coomassie Brilliant Blue, two protein bands of 60 and 64 kDa were detected (Fig. 4, lane 5). The protein bands of 50 and 54 kDa disappeared after N-glycanase digestion, and protein bands of 33 kDa as a major band and 35 kDa as a minor band appeared (Fig. 4, lane 7), indicating that the purified protein contained N-linked oligosaccharides. A protein band of 70 kDa also appeared after N-glycanase digestion (Fig. 4, lane 7). This protein band seems to be a dimer formed from the deglycosylated 35 kDa protein. To confirm that the protein bands observed in SDS-PAGE corresponded to C4ST, the purified C4ST was applied to a Toyopearl HW-55 column, and elution profiles of the C4ST activity and protein were determined (Fig. 5). The protein bands of 50 and 54 kDa appeared almost exclusively when the peak fractions (tubes 43 and 44 in Fig. 5A) were subjected to SDS-PAGE (Fig. 5B). The molecular mass of the peak fraction determined from the elution position in the Toyopearl HW-55 chromatography was 50 kDa, which agreed well with molecular mass determined by SDS-PAGE. These results suggest that C4ST may behave as a monomer. A small part of C4ST activity was also observed around tubes 31–38 in the Toyopearl HW-55 chromatography (Fig. 5A), but only the protein bands of 50 and 54 kDa were faintly detected in SDS-PAGE (data not shown). These observations suggest that C4ST may tend to aggregate as observed previously in chick C6ST (16).

**Properties of the Purified Chondroitin 4-Sulfotransferase**—Dithiothreitol was found to stimulate C4ST activity (Fig. 6A). Other sulfhydryl compounds such as 2-mercaptoethanol and reduced glutathione also showed a stimulatory effect on C4ST activity (data not shown). Optimum pH was ~7.2 (Fig. 6B). The

**FIG. 3.** Affinity chromatography on 3',5'-ADP-agarose of the fraction eluted from Matrex gel red A. The fractions eluted from the Matrex gel red A with 1 mM guanidine hydrochloride were pooled and dialyzed. After the concentration of Triton X-100 was adjusted to 2%, the pooled fraction was applied to a 3',5'-ADP-agarose column as described under “Experimental Procedures.” After the column was washed with modified buffer A containing 0.05 M NaCl, C4ST was eluted stepwise with modified buffer A containing 0.1 mM 3',5'-ADP (arrow). C4ST activity (closed circle), and protein concentration (open circle) of each fraction were assayed.

**FIG. 4.** SDS-polyacrylamide gel electrophoresis of C4ST. Lanes 1–4, different purified fractions of C4ST were analyzed under nonreducing conditions: lane 1, buffered medium fraction; lane 2, first heparin-Sepharose CL-6B fraction; lane 3, Matrex gel red A fraction; and lane 4, second heparin-Sepharose CL-6B fraction. Lane 5, the second heparin-Sepharose CL-6B fraction was analyzed under reducing conditions. Lanes 6–8, the second heparin-Sepharose CL-6B fraction was analyzed under nonreducing conditions before (lane 6) or after (lane 7) N-glycanase digestion. Lane 8, control with N-glycanase alone. Proteins were visualized with silver nitrate stain (lanes 1–4, 6, and 7) or Coomassie Brilliant Blue (lane 5). 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).

**FIG. 5.** Toyopearl HW-55 gel chromatography of chondroitin 4-sulfotransferase. A, C4ST eluted from the second heparin-Sepharose CL-6B column (1 μg as protein) was applied to a Toyopearl HW-55 column and eluted with buffer B as described under “Experimental Procedures.” C4ST activity was assayed after each fraction was dialyzed against buffer A containing 0.05 M NaCl. The arrows indicate the elution position of β-amylase (200 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). B, protein contained in each fraction was precipitated with 10% trichloroacetic acid. The precipitates were washed with acetone and analyzed by SDS-PAGE under nonreducing conditions. Proteins were visualized with silver nitrate stain. Molecular size standards are the same as used in Fig. 3.

For PAPS was 2.7 × 10^{-7} M (Fig. 6C), C4ST was activated not only with protamine but also with various metal ions such as Ca^{2+}, Fe^{2+}, Mn^{2+}, Ba^{2+}, and Sr^{2+}. When added at 5 μM, Ca^{2+} was most effective among these ions. Mg^{2+} was less effective. Co^{2+}, which was activated C6ST (18), was inhibitory. The purified C4ST was less stable than the purified C6ST from the culture medium of chick chondrocytes; after 4 months at
The concentration of [35S]PAPS was varied. Values of the enzymes was determined as described under “Experimental Procedures,” except that the concentration of dithiothreitol was varied. Chondroitin (filled circle), desulfated dermatan sulfate (open circle), chondroitin sulfate A (filled triangle), chondroitin sulfate C (open triangle), and dermatan sulfate (filled square) were used as acceptors. Incorporation into keratan sulfate, heparan sulfate, CDSNS-heparin, and chondroitin sulfate E was below the incorporation into dermatan sulfate.

−20 °C, the activity of the purified C4ST was decreased to −20% of the original activity.

Acceptor Substrate Specificity of Chondroitin 4-Sulfotransferase—For determining acceptor specificity, the purified C4ST was incubated with different glycosaminoglycans in the presence of varying amounts of protamine chloride. Fig. 7 shows that the purified C4ST was able to transfer sulfate to chondroitin and desulfated dermatan sulfate. Slight activity was observed when chondroitin sulfate A and chondroitin sulfate C were used as acceptors. Dermatan sulfate, keratan sulfate, chondroitin sulfate E, heparan sulfate, and CDSNS-heparin did not serve as acceptors. Optimum protamine concentration varied with the kind of glycosaminoglycans used as acceptors. The lowest optimum concentration was observed for chondroitin (Fig. 7).

To determine the position of the sulfate group transferred to chondroitin and desulfated dermatan sulfate, we digested [35S]-labeled glycosaminoglycans with chondroitinase ACII or chondroitinase ABC and analyzed the digestion products with SAX HPLC (Fig. 8). When the [35S]-labeled chondroitin and the [35S]-labeled desulfated dermatan sulfate were digested with chondroitinase ACII and chondroitinase ABC, respectively, radioactivity was detected only at the position of ΔDi-4S (Fig. 8, A and B). When [35S]-labeled desulfated dermatan sulfate was digested with chondroitinase ACII, −50% of the total radioactivity was recovered in ΔDi-4S, and the remainder appeared in minor peaks with higher retention time (Fig. 8C). The digestion products derived from the [35S]-labeled desulfated dermatan sulfate were also separated with Superdex 30 gel chromatography (Fig. 9). After digestion with chondroitinase ABC, radioactivity was only detected in ΔDi-4S (Fig. 9B). Approximately 50% of the total radioactivity was recovered in ΔDi-4S after chondroitinase ACII as observed in the SAX HPLC. Oligosaccharides observed in Fig. 9C appeared to correspond to the minor peaks observed in Fig. 8C. One oligosaccharide with retention time of 76 min (Fig. 9C, peak 6) was eluted between chondroitin sulfate hexasaccharide and chondroitin hexasaccharide, and another oligosaccharide with retention time of 82 min (Fig. 9C, peak 7) was eluted between chondroitin sulfate tetrasaccharide and chondroitin tetrasaccharide. Comparison of the retention times of these oligosaccharides with those of standard oligosaccharides allowed us to deduce that peaks 6 and 7 may be a monosulfated hexasaccharide and a monosulfated tetrasaccharide, respectively. To clear the sensitivity to chondroitinase ACII of the partially desulfated dermatan sulfate used as acceptor, we digested it with chondroitinase ACII and separated with Superdex 30 (Fig. 10). Desulfated dermatan sulfate was slightly depolymerized with chondroitinase ACII (Fig. 10E), but the UV-absorbing materials were detected only at the position of ΔDi-0S. The proportion of ΔDi-0S to the total unsaturated disaccharide formed after chondroitinase ABC digestion was only 5%. After digestion with chondroitinase ABC, no UV-absorbing materials other than ΔDi-4S and ΔDi-0S were formed from the partially desulfated dermatan sulfate (Fig. 10B), suggesting that structural changes in the polymer, which would make the polymer resistant to chondroitinase ABC, were not likely to be introduced after chemical desulfation.

**DISCUSSION**

In the present study, we have purified chondroitin 4-sulfotransferase from the culture medium of rat chondrosarcoma cells. The purified preparation showed broad protein bands of 50 kDa as a major component and 54 kDa as a minor component in SDS-PAGE under nonreducing conditions. Under reducing conditions, these protein bands shifted to the slower moving bands of 60 and 64 kDa, suggesting that these proteins may contain intramolecular disulfide bonds. Both protein bands disappear after N-glycanase digestion, and protein bands of 33 and 35 kDa appeared, indicating that these proteins contain N-linked oligosaccharides. From the decrease in the molecular mass observed after N-glycanase digestion, the
contents of N-linked oligosaccharides could be estimated as ~35%. Although the relation of the two protein bands is not clear at present, it may be possible that both protein bands correspond to C4ST, because both protein bands coeluted with C4ST activity from Toyopearl HW-55. We have previously found that the purified preparations of C6ST (17), heparan sulfate 6-sulfotransferase (32), and heparan sulfate 2-sulfotransferase (33) were composed of two protein bands, and that amino-terminal amino acid sequences of the two protein bands were identical in the case of C6ST (17) and heparan sulfate 6-sulfotransferase (34). The GalNAc-4-sulfotransferase responsible for the sulfation of GalNAcβ1-4GlcNAc-bearing oligosaccharides was purified from submaxillary gland, but this enzyme seems to be quite distinct from C4ST, because the molecular mass of the GalNAc-4-sulfotransferase was 128 kDa (35).

Before starting the purification of C4ST from the culture medium of chondrosarcoma cells, we tried to purify the enzyme from the culture medium of chick embryo chondrocytes. After the 3′,5′-ADP agarose chromatography, however, the C4ST fraction from the culture medium of chick chondrocytes was still contaminated by a large amount of C6ST, and it was difficult to remove C6ST activity from the C4ST preparation. In contrast, the culture medium of rat chondrosarcoma cells contained C4ST exclusively and, therefore, was an excellent source for the purification of C4ST. A major difference in the purification procedures between C4ST and C6ST was in the composition of the buffer used for the 3′,5′-ADP agarose chromatography; the concentration of Triton X-100 was 2% for C4ST and 0.1% for C6ST. In the absence of the high concentration of Triton X-100, efficient absorption of C4ST could not be achieved.

Desulfated dermatan sulfate was efficiently sulfated by the purified C4ST. After digestion with chondroitinase ABC, only ΔDi-4S was detected as the degradation product, indicating that desulfated dermatan sulfate was also sulfated at position 4 of GalNAc residues. The 35S-labeled, desulfated dermatan sulfate was applied to the Superdex 30 column before (A) or after digestion with chondroitinase ABC plus chondroitinase ACII (B) or chondroitinase ACII alone (C). Arrows indicate the elution position of: 1, ΔDi-0S; 2, ΔDi-6S; 3, ΔDi-4S; 4, ΔDi-diSM; 5, ΔDi-diSE; and 6, ΔDi-diSC. The sulfotransferase reaction was carried out as described under “Experimental Procedures,” except that 25 nmol (as galactosamine) of desulfated dermatan sulfate instead of chondroitin was added and that the amount of protamine chloride was increased to 10 μg when desulfated dermatan sulfate was used as acceptor. Conditions of HPLC were as described under “Experimental Procedures.” The broken line depicts concentration of KH2PO4. The arrows indicate the elution position of: 1, ΔDi-0S; 2, ΔDi-6S; 3, ΔDi-4S; 4, ΔDi-diSM; 5, ΔDi-diSE; and 6, ΔDi-diSC.

The sulfotransferase reaction was carried out as described under “Experimental Procedures,” except that the amount of protamine chloride was increased to 10 μg when desulfated dermatan sulfate was used as acceptor. Conditions of HPLC were as described under “Experimental Procedures.” The broken line depicts concentration of KH2PO4. The arrows indicate the elution position of: 1, ΔDi-0S; 2, ΔDi-6S; 3, ΔDi-4S; 4, ΔDi-diSM; 5, ΔDi-diSE; and 6, ΔDi-diSC.
in glycoprotein hormones, was reported to be stimulated by 2-mercaptoethanol (37). The intracellular level of sulphydryl compound, especially reduced form of glutathione, might contribute to the regulation of sulfation of glycosaminoglycans.

C4ST has been obtained from chick embryo cartilage (15), microsomal fraction of chick chondrocyte (42), and the culture medium of chick chondrocytes (20). C4ST purified from the rat chondrosarcoma cells in the present study shares several common features with chick cartilage C4ST (15); both preparations were stimulated with protamine and sulphydryl compounds and had similar optimum pH and \( K_m \) values for PAPS. These observations suggest that the protein nature of C4ST obtained from the rat chondrosarcoma cells may be similar to that of C4ST from chick cartilage. A marked difference between the purified C4ST and chick cartilage C4ST was solubility in buffers; the chick cartilage C4ST was almost insoluble in buffers unless guanidine hydrochloride was included in the buffer. We have previously found that C6ST secreted from chick chondrocytes appeared to be truncated at the transmembrane domain (17). C4ST from the culture medium of the rat chondrosarcoma cells may also be cleaved during secretion and may have lost its putative hydrophobic transmembrane domain and, as a result, may become a soluble form. The microsomal C4ST required the presence of detergents in the reaction mixture for the maximum activity (42), but C4ST obtained from the culture medium did not (20). C4ST secreted to the culture medium as a soluble form might be derived from C4ST that was bound to the Golgi membrane. We have achieved purification of C4ST to apparent homogeneity for the first time. By using homogeneous preparation, we found that C4ST could also sulfate desulfated dermatan sulfate. If molecular cloning of C4ST is attained from the amino acid sequence of the purified C4ST, not only strict substrate specificity of C4ST but also a relationship of C4ST between the secreted and Golgi forms and differences and similarities among different species will be revealed.

Several reports have suggested that the functional roles of chondroitin 4-sulfate may be different from those of chondroitin 6-sulfate. Chondroitin 4-sulfate inhibited the cytoadherence of \( P. falciparum \)-infected erythrocytes to melanoma cells, whereas chondroitin 6-sulfate had little or no effect (38). Chondroitin 4-sulfate, but not chondroitin 6-sulfate, exhibited an inhibitory effect during \( Cu^{2+} \)-mediated low density lipoprotein oxidation (39). Chondroitin 6-sulfate inhibited neurite growth from rat cerebellar and dorsal root ganglion neurons, whereas chondroitin 4-sulfate stimulated elongation of dorsal root ganglion neurites (40). Chondroitin 6-sulfate was shown to inhibit moderately the binding of 6B4 proteoglycan, an extracellular variant of a receptor-like protein-tyrosine phosphatase (PTP/ RPTP) \( b \), to pleiotrophin, but chondroitin 4-sulfate scarcely influenced the binding (41). Molecular cloning of C4ST would offer new approaches for investigating the function of chondroitin 4-sulfate.

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Purification of Chondroitin 4-Sulfotransferase 2463