The Putative Heparin-specific N-Acetylglucosaminyl N-Deacetylase/
N-Sulfotransferase Also Occurs in Non-heparin-producing Cells*

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Leny Toma‡, Patricia Berninsone, and Carlos B. Hirschberg§
From the Department of Molecular and Cell Biology, Boston University Goldman School of Dental Medicine, Boston, Massachusetts 02118

N-Deacetylation and N-sulfation of N-acetylglucosamine of heparin and heparan sulfate are hypothesized to be mediated by different tissue-specific N-acetylglucosaminyl N-deacetylases/N-sulfotransferases, which in turn lead to the higher L-iduronic acid and sulfate content of heparin versus heparan sulfate. Furthermore, the putative heparin-specific N-acetylglucosaminyl N-deacetylase/N-sulfotransferase has been reported to require auxiliary proteins for its N-acetylglucosaminyl N-deacetylase activity in vitro based on its requirement of polycations in vitro. We have now found that cells derived from embryonic bovine trachea, a tissue that does not synthesize heparin, has a N-acetylglucosaminyl N-deacetylase/N-sulfotransferase, which has 95% amino acid sequence identity to the above enzyme postulated to be involved in the biosynthesis of heparin. Both enzymes also have very similar affinity for their substrates. The trachea enzyme does not require additional effectors for its N-acetylglucosaminyl N-deacetylase activity in vitro even though its biochemical characteristics are virtually the same as the enzyme previously isolated from cells of a heparin-producing mastocytoma tumor. The trachea enzyme, which is encoded by an abundant 4.6-kilobase mRNA, like mastocytoma cells, has 70% amino acid sequence identity with the corresponding enzyme from rat liver postulated to participate in the biosynthesis of heparan sulfate. Heparan sulfate synthesized by trachea cells has a higher content of sulfated iduronic acid than from other tissues. Together, the above results strongly suggest that the above enzymes from mastocytoma, liver, and trachea, per se, are not solely responsible for the selective tissue-specific synthesis of heparin or heparan sulfate; more likely cellular factors, additional enzymes, and availability of substrates in the Golgi lumen also play important roles in the differential synthesis of the above proteoglycans.

Heparan sulfate and heparin are proteoglycans with sulfated glycosaminoglycans of alternating glucosamine and uronic acid; heparin has a higher content of iduronic acid and sulfate than heparan sulfate. The latter plays biological roles in cell acid; heparin has a higher content of iduronic acid and sulfate than heparan sulfate. The latter plays biological roles in cell...
MATERIALS AND METHODS

Cell Culture—Embryonic bovine trachea cells (CCL 44) were obtained from ATCC. They were grown at 37 °C with 5% CO₂ in Eagle’s minimum essential medium containing non-essential amino acids and 10% fetal bovine serum.

Glycosaminoglycans and Glycosaminoglycan Degradative Enzymes—Chondroitin 4-sulfate (from whale cartilage) and dermatan sulfate (from pig skin) were purchased from Seikagaku America. Heparin from bovine intestinal mucosa and heparan sulfate from bovine pancreas were gifts from Dr. P. Bianchini (Ospedale Pediatrico Bambino Gesù, Rome, Italy). Chondroitinase ABC was purchased from Sigma. Heparinase I, heparitinase II were prepared from inducted P. fluorescens (33).

Radial Labeled of Embryonic Bovine Trachea Cells with [35S]Sulfate—25-mm² confluent embryonic bovine trachea cells (CCL 44), containing 5 ml of growth medium, were labeled for 48 h with 150 μCi/ml H₂[35SO₄]. The medium was removed and cells were washed twice with phosphate-buffered saline (PBS). Cells were scraped with 1 ml of 3.5× urea in PBS. After the addition of 100 μg of dermatan sulfate from pig skin as carrier, proteoglycans from cells and medium were precipitated with 2 volumes of ethanol. Cells and medium pellets were subjected to proteolysis with Pronase (6 mg/ml, final concentration) in a final volume of 1 ml of 0.05 M Tris-HCl, pH 8.0, at 37 °C for 24 h. The incubation mixture was boiled at 100 °C for 10 min, and the radiolabeled GAGs were precipitated with 2 volumes of ethanol at −20 °C. The pellet was then suspended in water, and the GAGs were analyzed with specific enzymes. A portion was left untreated, and samples were digested with heparitinases I and II (33) (0.01 unit each) or chondroitinase ABC (0.02 unit). Aliquots of 5 ml of each incubation mixture were analyzed by agarose gel electrophoresis in 1,3-diaminepropane acetate buffer, pH 9.0. GAGs were precipitated in the gel with 0.2% of cetyltrimethylammonium bromide for 1 h; the gel was dried, and standard GAGs were stained with toluidine blue. Radioactive GAGs were visualized by autoradiography.

Preparation of [3H]Heparan Sulfate and Fractionation of Heparitinases and Heparinase Products by Size Exclusion—Eight flask (25 cm² each) of CCL 44 cells were labeled with H₂[35SO₄] and radiolabeled GAG, prepared as described above. Intracellular GAGs were digested with chondroitinase ABC at 37 °C under conditions suggested by the manufacturer. The incubation mixture was heat-inactivated at 100 °C for 10 min, dialyzed exhaustively against distilled water, and concentrated under vacuum. Following resuspension in water, samples of 100,000 cpm of isolated [3H]heparan sulfate were incubated with buffer alone (control), heparitinases I and II (0.1 unit each), or heparanase (0.1 unit in the presence of 10 mM calcium acetate) at 30 °C in 0.05 M ethylene-diamine acetate (33) (pH 7.0). The incubation mixtures were then applied to Sephadex G-50 columns (114 × 1 cm) previously equilibrated with 0.5 M acetic acid and calibrated with standard GAGs and their derivatives. Fractions of 1 ml were collected. Aliquots of 5 μl of each incubation mixture were analyzed by agarase gel electrophoresis in 1,3-diaminepropane acetate buffer, pH 9.0. GAGs were precipitated in the gel with 0.2% of cetyltrimethylammonium bromide for 1 h; the gel was dried, and standard GAGs were stained with toluidine blue. Radioactive GAGs were visualized by autoradiography.

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DNA Sequencing—DNA sequencing of both strands was done with dye primers using the dideoxy chain termination method (24). An Applied Biosystems DNA sequencer was used at the Molecular Genetics Facility of the University of Georgia, Athens, GA. DNA sequence results were assembled into a contiguous sequence data base with a SEQUENASE program (DNA Star, Madison, WI).

Analysis of Genomic DNA from CCL 44 Cells—Genomic DNA was prepared from embryonic bovine trachea cells by the method of Gross-Bellard et al. (25). 10 μg of genomic DNA were digested with restriction enzymes and resolved by electrophoresis on a 0.8% agarose gel in TEAN buffer. Fragments were transferred to nylon membranes and hybridized with two different probes at high stringency. CCL 44 cells’ cDNA probe and rat liver N-deacetylase/N-sulfotransferase probe were prepared as described previously. Both membranes were washed at high stringency using the same conditions for the Northern analysis and then exposed to x-ray film at −70 °C.

Northern Blots and RNA Hybridization—RNA samples were denatured at 65 °C for 10 min in 34% (v/v) formamide, 4% formaldehyde, 13.5 mM MOPS, pH 7.0, 67 μg/ml ethidium bromide and resolved on a 1.2% agarose gel containing 6% (v/v) formaldehyde, 20 mM MOPS, pH 7.0, 0.5% SDS, 1% formaldehyde, and 1× EDTA. The gel was treated with 50 mM NaOH and blotted to a Hybond N+ nylon membrane for 18 h. RNA was cross-linked to the membrane in a UV Stratalinker oven and incubated at 42 °C for at least 3 h in a prehybridization solution of 6× SSPE (0.9 M NaCl, 60 mM sodium phosphate, 6 mM EDTA, pH 7.4), 5× Denhardt’s solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. For RNA hybridization at low stringency, a single-stranded probe was prepared according to Church and Gilbert (22), using as template cDNA from M1ST cells. The probe was added to the prehybridization solution and incubated at 42 °C for 18 h. The membrane was washed twice in 2× SSC, 0.1% SDS for 15 min at room temperature and then exposed to x-ray film at −70 °C. For RNA hybridization at high stringency, Northern blots with poly(A)+ RNA from CCL 44 cells and rat liver were probed using cDNA from CCL 44 cells. The 4.1-kb insert was isolated and labeled by random hexamer using an oligolabeling kit. Hybridization was done in high stringency conditions using 50% formamide, 6× SSPE, 5× Denhardt’s solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. The membrane was washed also at high stringency with 2× SSC, 0.1% SDS twice at room temperature for 15 min and then twice with 0.1× SSC, 0.1% SDS at 65 °C for 15 min. The membrane was exposed at −70 °C.

Construction and Screening of a 9gt10 Library—The library was constructed with 7 μg of poly(A)+ RNA from CCL 44 cells using the Super Script Choice System (Life Technologies, Inc.), according to the manufacturer’s instructions. The cDNA was ligated to 9gt10 arms (Life Technologies, Inc.) and packaged in vitro using a DNA packaging kit from Stratagene.

Phages without amplification were used to infect Escherichia coli C600 hfl and plated at 1× 10⁷ plaque-forming units/150-mm plate. 9.0 × 10⁶ plaques were screened using a single-stranded probe obtained as described previously (21). The primer used to synthesize the probe had the following sequence: 5’CGTGGTCGACCTCAGCCCGAACTG3’.

Construction of pCMV5 Plasmids—DNA from λ clones containing the 3.9-kb insert was excised with SauI and ligated into the SalI site of pCMV5 expression vector (23). DH-5α cells were transformed with the ligation mixture and plated on LB ampicillin plates. Recombinant plasmids were selected by restriction mapping. This construction generated pCMV5(−), which was cut with BamHI and EcoRI and ligated into the pBluescript.

DNA Sequencing—DNA sequencing of both strands was done with dye primers using the dideoxy chain termination method (24). An Applied Biosystems DNA sequencer was used at the Molecular Genetics Facility of the University of Georgia, Athens, GA. DNA sequence results were assembled into a contiguous sequence data base with a SEQUENASE program (DNA Star, Madison, WI).

Results—In view of previous reports that N-deacetylation and N-sulfation of N-acetylglucosamine in heparin and heparan sulfate were mediated by different heparin/heparan sulfate N-deacetylases/N-sulfotransferases, we determined whether a cell line which did not synthesize heparin, such as from embryonic bovine trachea, expressed an mRNA that encoded the putative heparin N-deacetylase/N-sulfotransferase.

Embryonic bovine trachea cells were grown in the presence

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[35S]Glycosaminoglycan Synthesis by CCL 44 Embryonic Bovine Trachea Cells—In view of previous reports that N-deacetylation and N-sulfation of N-acetylglucosamine in heparin and heparan sulfate were mediated by different heparin/heparan sulfate N-deacetylases/N-sulfotransferases, we determined whether a cell line which did not synthesize heparin, such as from embryonic bovine trachea, expressed an mRNA that encoded the putative heparin N-deacetylase/N-sulfotransferase.

Embryonic bovine trachea cells were grown in the presence
of \(^{35}\text{S}\) sulfate, and GAG chains were isolated and separated on agarose gel electrophoresis as described under “Materials and Methods.” Chondroitin sulfate and dergmatan sulfate were the main GAGs isolated from the culture medium and the intracellular compartment, whereas heparan sulfate was only 6% of the total radioactive GAGs (Fig. 1). The identity of these GAGs was confirmed by their sensitivity to heparitinase I and II (lanes 2) and chondroitinase ABC (lanes 3).

### Northern Analyses of Embryonic Bovine Trachea Cells—Poly(A\(^+\)) RNA from CCL 44 cells was obtained and analyzed by Northern blots at low stringency using a single-stranded probe with an average size of 300 nucleotides directed to a DNA sequence encoding amino acids 517–617 of the MST cells’ heparin/heparan sulfate membrane domain, type II protein. When compared with other heparin/heparan sulfate N-deacetylases/N-sulfotransferases, a 95% amino acid identity was found with the mastocytoma and mastocytema cell line (MST) heparin sulfate N-deacetylase/N-sulfotransferase cDNA.

A cDNA library was then constructed from CCL 44 cells in \(\lambda\)gt10 and screened at low stringency. Three positive clones were identified containing a full-length cDNA as confirmed by sequence analysis. One of these clones was used as template, and using random hexamers as primers, a radioactive probe was obtained. This probe was then used in Northern blots and recognized a 4.6-kb messenger RNA from CCL 44 cells (not shown). The same probe constructed with this cDNA was used in Southern blots of genomic DNA from CCL 44 cells and was compared with the Southern blots probed with rat liver heparan sulfate N-deacetylase/N-sulfotransferase cDNA. A completely different pattern of restriction sites between both Southern blots was obtained demonstrating that the rat liver heparin sulfate N-deacetylase/N-sulfotransferase cDNA and the CCL 44 cDNA clone are coded by different genes (Fig. 4).

### Nucleic Acid Sequence Analysis and Comparison with Other Heparin/Heparan Sulfate N-Deacytles/N-Sulfotransferases—The nucleic acid sequence (GenBank™ AF064825) revealed a single open reading frame, predicting a protein of 884 amino acids with a molecular mass of 101,665 Da. Hydrophobicity plots of the protein revealed a single putative transmembrane domain, type II protein. When compared with other heparin/heparan sulfate N-deacetylases/N-sulfotransferases, the CCL 44 cDNA clone was also not expected to survive approximately 20 passages in culture that CCL 44 cells had undergone.

### Expression of CCL 44 Heparin/Heparan Sulfate N-Acetylglucosaminyl N-Deacytles/N-Sulfotransferase in COS-1 Cells—In view of the high amino acid identity of the CCL 44 cells’ heparin/heparan sulfate N-acetylglucosaminyl N-deacetylase/N-sulfotransferase with the corresponding mastocytema enzyme, a soluble, secreted protein was constructed containing the putative luminal portion of the CCL 44 protein, fused at its amino terminus, with protein A, analogous to the fusions made for the rat liver and mastocytema heparin/heparan sulfate N-deacetylases/N-sulfotransferases. COS-1 cells were transfected with an expression vector containing the above chimera as well as the corresponding chimeras of rat liver and mastocytema heparin/heparan sulfate N-deacytles/N-sulfotransferases. Both enzyme activities were assayed 72 h after transfection using the purified, secreted, soluble
proteins on IgG beads. As seen in Table I, both activities were expressed by the CCL 44 protein chimera. The N-acetylglucosaminyl N-deacetylase/N-sulfotransferase activity ratio was comparable with that of the mastocytoma protein and was approximately 8-fold higher than that of the rat liver protein. A more detailed kinetic analysis of both activities of the trachea and MST enzymes was also done. Fig. 6 and Table II show that the $K_m$(app) for both activities was comparable, again demonstrating the biochemical similarity between these enzymes.

Structure of Heparan Sulfate Synthesized by CCL 44 Cells—In view of the similarity between the trachea and mastocytoma N-deacetylase/N-sulfotransferase, the structure of heparan sulfate synthesized by CCL 44 cells was investigated using F. heparinum enzymes. Heparinase (EC 4.2.2.7) acts on glucosaminido-iduronic acid linkages where the iduronic acid and the glucosamine are obligatorily sulfated at the 2-position. Heparitinase I is specific for N-acetyl or N-sulfated glucosaminido-glucuronic acid linkages. The enzyme acts only on heparan sulfate regions where the N-acetyl or N-sulfated glucosamine is not sulfated at the 6-position. Heparitinase II (EC 4.2.2.8) acts upon glucosaminido-iduronic acid linkages; it also cleaves glucosaminido-glucuronic acid linkages, but the glucosamine must be sulfated at the 6-position.

We determined the regions of heparan sulfate from CCL 44 cells susceptible to the action of the above enzymes. [35S]Heparan sulfate was incubated as described under “Materials and Methods.”
Methods,” and the products were separated on a Sephadex G-50 column (Fig. 7). The combined action of heparitinase I and heparitinase II resulted mainly in disaccharides, eluted at $V_t$ (Fig. 7). Fractions were pooled, and the radioactive products were identified by paper chromatography as described under “Materials and Methods.” Heparitinase I and II resulted in formation of four radioactive products (Fig. 8), each comigrating with degradation products from standard heparan sulfate.
and previously identified as (in slower to faster migrating order) ΔUA,2S-GlcNS,6S (Pool I), ΔUA-GlcNS,6S, ΔUA-GlcNS, and ΔUA-GlcNAc,6S (Pool II). Heparinase acted on trachea heparan sulfate producing several products after fractionation on Sephadex G-50 (Fig. 7). Pools III and IV have elution volumes corresponding to oligosaccharides of \( M_r \sim 6000 \) and \( M_r \sim 3000 \). When identified on paper chromatography most of both products remain at the origin (Fig. 8). Pool V has an elution volume consistent with being tetrasaccharides, in agreement with the pattern seen by paper chromatography. Pool VI has an elution value consistent with being disaccharides. Indeed, on paper chromatography of this pool, two products can be seen, ΔGlcA-GlcNS,6S and GlcNS. These were probably released from the non-reducing end of the heparan sulfate chain after the action of heparinase (35).

Because heparinase specifically cleaves at sulfated iduronic acid residues, its action allows one to determine the arrangement and distribution of this sugar on the polymer. Heparinase cleaves standard heparin from bovine intestine, resulting in tetrasaccharides and trisulfated disaccharides (pools IX and X). This same enzyme acts on standard heparan sulfate from bovine pancreas releasing the few residues of trisulfate disaccharide and 2,6-disulfated glucosamine from the non-reducing end (35). The results obtained with trachea heparan sulfate imply that the iduronic acid residues are located in different regions along the molecule; this pattern of distribution differs from known standards. The same type of profile of heparinase products was found previously in rabbit aorta endothelial cells (36).

**DISCUSSION**

We have unexpectedly found in cells from a tissue that does not synthesize heparin a very abundant message encoding a protein that has 95% sequence identity with the putative heparan N-acetylgalactosaminyl N-deacetylase/N-sulfotransferase. The protein has biochemical characteristics very similar to the mastocytoma and not the rat liver N-acetylgalactosaminyl N-deacetylase/N-sulfotransferase (15), the latter postulated to be involved in the biosynthesis of heparan sulfate (12, 16–20).

Previously, our laboratory (15), as well as Lindahl and co-workers (16), cloned from mastocytoma tumor-derived cells and the tumor itself a protein that has 95% amino acid sequence identity to the above described from embryonic bovine trachea cells and had 70% amino acid sequence identity to the putative heparan sulfate N-acetylgalactosaminyl N-deacetylase/N-sulfotransferase from rat liver. We (15) also showed that a full-length and a secreted recombinant MST cell-derived protein expressed in vitro both N-acetylgalactosaminyl N-deacetylase and N-sulfotransferase activities and that a single polypeptide expressed both these activities. Lindahl and co-workers (12, 16–19), on the other hand, reported that the mastocytoma-derived enzyme requires polycation cofactors for expression of the N-deacetylase activity in vitro. These investigators hypothesized that heparin acting as a polycation cofactor is probably involved in the biosynthesis of heparan sulfate (12, 18–20).

**TABLE I**

| Sample                  | N-Deacetylase activity | N-Sulfotransferase activity | NID/NS ratio |
|-------------------------|------------------------|-----------------------------|--------------|
| Rat liver chimera       | 140 ± 13               | 1,030 ± 57                  | 0.14         |
| Reverse orientation     | 20 ± 7                 | 13 ± 4                      | 1.60         |
| Mast cells chimera      | 240 ± 17               | 150 ± 12                    | 1.57         |
| Reverse orientation     | 10 ± 3                 | 15 ± 3                      | 1.44         |
| Trachea CCL 44 chimera  | 137 ± 10               | 90 ± 12                     | 1.20         |
| Reverse orientation     | 20 ± 7                 | 12 ± 4                      | 1.44         |

* Nanograms of \(^{3}H\)-acetylated K5 deacetylated/h/10-cm plate. Results are mean ± S.E. of three independent determinations.

**TABLE II**

| Activity                           | \( K_{\text{m(app)}} \) (M) | \( V_{\text{max}} \) (picomoles/min/10-cm plate) |
|------------------------------------|------------------------------|-----------------------------------------------|
| N-Deacetylase (\(^{3}H\)-acetylated K5, variable substrate) | 1.6                          | 212                                           |
| N-Sulfotransferase                 | 9.0                          | 18.6                                          |

* N-Deacetylase, picomoles of acetyl units removed from K5 in 15 min/10-cm plate; N-sulfotransferase, picomoles of sulfate transferred to deacetylated K5 in 15 min/10-cm plate.
esized that the mouse mastocytoma enzyme was associated with the biosynthesis of heparin (12, 16–20), whereas the one from rat liver was implicated in the biosynthesis of heparan sulfate (12, 16–20, 37). They further hypothesized that endogenous polycations in mast cells, probably polypeptides, are required for the N-acetylglucosaminyl N-deacetylase activity in vivo.

We differ in the interpretation of the above results. We believe that the purified enzyme from mastocytoma (17), in a manner analogous to the purified one from rat liver (27), has lost during the many purification steps its ability to N-deacetylate N-acetylglucosamine of heparin or heparan sulfate in vitro; in the presence of polycations, this activity is regained in assays in vitro. When a soluble chimera of the heparin/heparan sulfate N-acetylglucosaminyl N-deacetylase/N-sulfotransferase of either MST cells or rat liver with protein A is expressed and secreted, thus allowing a rapid, one-step purification of the enzyme by its binding to IgG beads, both proteins have both catalytic activities in assays in vitro without need of polycations (14, 15). The same result was obtained with the above described trachea cell-derived enzyme.

The above results describing that bovine trachea-derived cells contain a N-acetylglucosaminyl N-deacetylase/N-sulfotransferase, resembling the mastocytoma enzyme and not the rat liver enzyme, require a re-examination of the hypothesis that the mastocytoma enzyme is necessary for the synthesis of heparin and not heparan sulfate, whereas the liver one is required for heparan sulfate and not heparin. It would appear more likely that the decision as to whether a cell synthesizes heparin or heparan sulfate may depend on the presence of specific heparin or heparan sulfate precursor intermediates and additional cellular factors rather than solely specific N-acetylglucosaminyl N-deacetylase/N-sulfotransferase. Among such factors may be the availability of PAPS (28) or nucleotide sugars in the Golgi lumen; the Km of the different acceptors for these nucleotide derivatives and other possible effectors that are not yet characterized. Because current approaches in vitro with non-physiological substrates such as K5 are subject to equivocal interpretation, definite answers to this important question will have to await gene disruption studies of these enzymes and replacement of one enzyme for the other in different cells.
We have recently shown that availability of nucleotide sugars in the Golgi lumen greatly influences which glycosaminoglycan chains are synthesized; thus, in MDCK cells having limited availability of UDP-galactose in the Golgi lumen, the biosynthesis of heparan sulfate and chondroitin sulfate proceeds normally, whereas that of keratan sulfate is abolished (29). We had previously shown in biochemical and genetic experiments with *Saccharomyces cerevisiae* that the nucleotide sugar/nucleoside phosphate antiporter system, which provides for the Golgi membranes (32) and the above results with MDCK cells (29) raise the possibility that such an antiporter system plays a significant role in the biosynthesis of either heparin or heparan sulfate. Furthermore, in the above mutant MDCK cells, chondroitin 4-sulfate became hypersulfated in the 6-O-position (29), perhaps because of a lack of absolute substrate specificity of a putative keratan sulfate 6-O-sulfotransferase.

In conclusion, we believe that it is unlikely that the heparin/heparan sulfate N-acetylgalcosaminyl N-deacetylase/N-sulfotransferase per se is solely responsible for the selective synthesis of heparin or heparan sulfate and that cellular factors including availability of substrates in the Golgi lumen may also play important roles in the differential synthesis of the above proteoglycans.

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