The Effect of Precursor Structures on the Action of Glucosaminyl 3-O-Sulfotransferase-1 and the Biosynthesis of Anticoagulant Heparan Sulfate*

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To understand how 2-O-sulfation of uronic acid residues influences the biosynthesis of anticoagulant heparan sulfate, the cDNA encoding glucosaminyl 3-O-sulfotransferase-1 (3-OST-1) was introduced into wild-type Chinese hamster ovary cells and mutant pgsF-17 cells, which are defective in 2-O-sulfation. 3-OST-1-transduced cells gained the ability to bind to antithrombin. Structural analysis of the heparan sulfate chains showed that 3-OST-1 generates sequences containing GlcUA-GlcN(SO3)3(SO3)2 and GlcUA-GlcN(SO3)3(SO3)6(SO3)i n both wild-type and mutant cells. In addition, IdoUA-GlcN(SO3)3(SO3)2 and IdoUA-GlcN(SO3)3(SO3)6(SO3)i accumulate in the mutant chain. These disaccharides were also observed by tagging [6-3H]GlcN-labeled pgsF-17 heparan sulfate in vitro with [35S]PAPS and purified 3-OST-1. Heparan sulfate derived from the transduced mutant also had ~2-fold higher affinity for antithrombin than heparan sulfate derived from the transduced wild-type cells, and it inactivated factor Xa more efficiently. This study demonstrates for the first time that (i) 3-O-sulfation by 3-OST-1 can occur independently of the 2-O-sulfation of uronic acids, (ii) 2-O-sulfation usually occurs before 3-O-sulfation, (iii) 2-O-sulfation blocks the action of 3-OST-1 at glucosamine residues located to the reducing side of IdoUA units, and (iv) that alternative antithrombin-binding structures can be made in the absence of 2-O-sulfation.

Heparan sulfate (HS)† is a linear polymer covalently attached to the protein cores of proteoglycans, which are abundant and ubiquitously expressed in almost all animal cells as integral membrane proteins, glycosylphosphatidylinositol-linked membrane proteins, and proteins of the extracellular matrix. HS assembles by the action of a large family of enzymes that catalyzes chain polymerization (alternating the addition of GlcNAc and GlcUA residues), GlcNac N-deacetylation and N-sulfation, glucuronidase (GlcUA) epimerization to L-iduronic acid (IdoUA), 2-O-sulfation of uronic acid residues, and 3-O- and 6-O-sulfation of glucosamine residues. Tissue-specific and developmentally regulated expression of enzyme isoforms (e.g. at least four N-deacetylation/N-sulfotransferases (1–5), three 6-O-sulfotransferases (6, 7), and five 3-O-sulfotransferases (8, 9)) produces HS chains with distinct sequences. These different sequences enable interactions to occur with a broad array of protein ligands that modulate a wide range of biological functions in development, differentiation, homeostasis, and bacterial/viral entry (reviewed in Refs. 10–16).

The specificity of HS-protein interactions is largely dictated by arrangements of sulfate groups and uronic acid epimers along the chain. For example, the pentasaccharide sequence GlcNAc/NSS6S-GlcUA-GlcNAcSSS6S±6S-IdoUA2S-GlcNS6S represents the minimum sequence for antithrombin (AT) binding, whereas the 3S and 6S groups constitute the most critical elements involved in the interaction (17–19). However, the NS, 2S, 6S, GlcUA, and IdoUA units are usually present in the AT-binding pentasaccharides prepared from both heparin and heparan sulfate (20, 21). To delineate the biosynthetic pathway that regulates anticoagulantly active heparan sulfate (Hsact) biosynthesis, our laboratory has purified and molecularly cloned 3-OST-1 (8, 22). 3-OST-1, usually present in limiting amounts, acts on precursor oligosaccharides to produce HSact oligosaccharides (22, 23).

The overall structure of Hsact and Hsinact differs at the disaccharide level (21). In general, the AT binding oligosaccharides always have a 2-O-sulfated IdoUA adjacent to the 3-O-sulfated glucosamine unit. To understand how 2-O-sulfation contributes to HSact biosynthesis and AT binding, we used a 2-O-sulfation-defective Chinese hamster ovary (CHO) mutant designated pgsF-17 (24). This mutant does not express mRNA for 2-OST and therefore synthesizes HS chains without 2-O-sulfated IdoUA or GlcUA residues. Interestingly, this mutant has higher amounts of GlcNS residues compared with parental wild-type cells, suggesting that 2-O-sulfation suppresses the action of the GlcN.N-sulfotransferases (24). We now report that 3-OST-1 can act in the absence of 2-O-sulfation and still generate AT-binding sequences. One of the functions of 2-O-sulfation is apparently to restrict the action of 3-OST-1 at certain sites along the chain.
EXPERIMENTAL PROCEDURES

Cell Culture—Wild-type CHO cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61; ATCC, Manassas, VA). Wild-type cells and the 2-O-sulfotransferase-deficient mutant pgsF-17 (24) were maintained in Ham’s F-12 medium supplemented with 10% fetal bovine serum (HyClone), penicillin G (100 units/ml), and streptomycin sulfate (100 μg/ml) at 37 °C under an atmosphere of 5% CO₂ in air and 100% relative humidity. The cells were passaged every 3–4 days with 0.125% (w/v) trypsin and 1 mM EDTA, and after 10–15 cycles, fresh cells were revived from stocks stored under liquid nitrogen. Low sulfate medium was composed of Ham’s F-12 medium supplemented with penicillin G (100 units/ml) and fetal bovine serum that had been dialyzed 200-fold against phosphate-buffered saline (PBS) (25). All tissue culture media and reagents were purchased from Life Technologies, Inc. unless otherwise indicated.

The PHOENIX ecotropic retroviral packaging cell line (ATCC no. SD 3444) was a generous gift from Dr. Gary Nolan (Stanford University Medical Center). PHOENIX ecotropic cells were maintained as described (26). Wild-type or mutant pgsF-17 cells (1 × 10⁶) were transfected with 10 μg of pcB7-ECOTROPIC (a generous gift from Dr. Harvey Lodish) using calcium phosphate precipitation (27). The plasmid contains MCAT1, an ecotropic retrovirus receptor, and hygromycin resistance genes from separate constitutive promoters. The transfected cells were selected for resistance to 200 μg/ml hygromycin B (Life Technologies, Inc.). Each stable hygromycin-resistant clone was assayed for its ability to be infected by the reporter virus MSCVPLAP, containing alkaline phosphatase (28).

Virion Production—The retrovirus plasmid pMSCVpac was a generous gift of Dr. Robert Hawley (University of Toronto) (29). pCMV3-OST-1 was digested with BglII and XhoI to release the wild-type murine 3-OST-1 cDNA (8). The cDNA fragment (1,623 base pairs) was cloned into the BglII + XhoI sites in pMSCVpac. All plasmid DNA

FIG. 1. Dual color flow cytometric analysis of cells. Wild-type control (A and B, solid line), mutant control (C and D, solid line), a wild-type CHO cell clone with three copies of 3-OST-1 (A and B, dotted line), and mutant cell pools stably transduced with the 3-OST-1 (C and D, dotted line) were double-labeled with Alexa 594-FGF-2 (A and C) and fluorescein-AT (B and D) and subjected to dual color FACS (see “Experimental Procedures”). Each panel represents the analysis of 10,000 cells. Mutant cells that exhibited high AT fluorescence were single-cell-sorted into a 96-well plate.

FIG. 2. Determination of 3-OST-1 gene copy numbers in 12 3-OST-1-transduced mutant clones. EcoRI-digested genomic DNA was hybridized to a 3-OST-1 probe (see “Experimental Procedures”). 0, mutant control; 1–12, 3-OST-1-transduced mutant clones.
FIG. 3. IPRP-HPLC of [\text{35S}]HS disaccharides. Mutant metabolically labeled \text{[\text{35S}]HS} and in vitro 3-OST-1 and cold PAPS modified mutant \text{[\text{35S}]HS} samples were depolymerized completely by hydrazinolysis and nitrous acid digestion, the products were purified by gel-filtration chromatography, and the disaccharides were analyzed by IPRP-HPLC (see “Experimental Procedures”). Solid line, mutant \text{[\text{35S}]HS}; dotted line, in vitro 3-OST-1 and cold PAPS modified \text{[\text{35S}]HS}; peak 1, free \text{35S} sulfate; peak 2, GlcUA-[6-O-3\text{5S}]aManR; peak 3, IdoUA-[6-O-3\text{5S}]aManR; peak 4, IdoUA-[6-O-3\text{5S}]aManR; peak 5, GlcUA-[6-O-3\text{5S}]aManR. The broken line indicates the gradient of acetonitrile.

FIG. 4. IPRP-HPLC of \text{[\text{3H}]HS} disaccharides. \text{[\text{3H}]HS} samples were depolymerized completely by hydrazinolysis and nitrous acid digestion, the products were purified by gel-filtration chromatography, and the disaccharides were analyzed by IPRP-HPLC (see “Experimental Procedures”). A, pgsF-17 control; B, wild-type control; C, pgsF-17 with three copies of 3-OST-1; D, wild type with three copies of 3-OST-1; E, pgsF-17 HS modified in vitro with pure 3-OST-1; F, wild-type HS modified in vitro with pure 3-OST-1. Peak 1, aManR6S; GlcUA-aManR, and IdoUA-aManR6S; peak 2, GlcUA-aManR3S; peak 3, IdoUA28-aManR6S; peak 4, GlcUA-aManR6S; peak 5, IdoUA-aManR6S; peak 6, GlcUA-aManR3S; peak 7, IdoUA28-aManR6S; peak 8, IdoUA-aManR3S; peak 9, IdoUA-aManR3S. The broken line indicates the gradient of acetonitrile.

prepared for transfection was made with the Invitrogen SNAP-MIDI kit according to manufacturer directions.

Infectious virions were produced by programming ecotropic PHOENIX packaging cells with recombinant provirus plasmids using the calcium phosphate transfection technique as described previously (26). After the precipitation step, the cells were re-fed with 2 ml/well of fresh Dulbecco’s modified Eagle’s medium and incubated overnight. Viral supernatants were collected, either flash-frozen in liquid nitrogen, and stored at -80 °C or used directly after low speed centrifugation.

Wild-type and mutant cells containing ecotropic receptors were treated with trypsin and then plated at 150,000 cells/well in a 6-well dish. One day later, target cells (~70% confluent) were incubated overnight with viral supernatants containing 5 ml/ml Polybrene surfactant. More than 90% of mutant cell populations express ManR3S. The metabolically labeled disaccharides were analyzed by IPRP-HPLC (see “Experimental Procedures”).

Antithrombin and FGF-2 Labeling—The standard reaction mixture for preparing fluorescent AT contained 20 mM NaH2PO4 (pH 7.0), 0.3 mM CaCl2, 25 μM of PBS-dialyzed AT (GlycoMed), 4 milliunits neuraminidase (Worthington), 4 milliunits galactose oxidase (Worthington), and 125 μM/mL fluoroscence hydrazide (C-356, Molecular Probes) in a final volume of 280 μl. The mixtures were incubated at 37 °C for 1 h. PBS (1 ml) and a 50% slurry of heparin-Sepharose in PBS (100 μl) was added and mixed end-over-end for 20 min. After centrifugation, the heparin-Sepharose beads were washed four times with PBS (1 ml). Labeled AT was eluted with four 0.25-ml aliquots of 10× concentrated PBS and desalted by centrifugation for 35 min at 14,000 rpm through two Microcon-10 columns (Millipore). The concentrated AT was diluted with 0.5 ml of 10% FBS in PBS containing 2 mM EDTA and used directly for cell labeling studies.

Fluorescent FGF-2 was prepared by mixing 50 μl of 1 mM sodium bicarbonate to 0.5 ml of PBS containing 2 mg/ml bovine serum albumin and 3 μg of FGF-2. The mixture was then transferred to a vial of reactive dye (Alexa 594, Molecular Probes) and stirred at room temperature for 1 h. The isolation of the labeled FGF-2 was identical to that described above for labeled AT.

Cell Sorting—Nearly confluent monolayers of cells were detached by adding 10 ml of 2 mM EDTA in PBS containing 10% FBS and centrifuged. The cell pellets were placed on ice, and 50 μl each of fluorescein-in-AT and Alexa 594-PEG-2 was added. After 30 min, the cells were washed once and resuspended in 1 ml of 10% FBS in PBS containing 2 mM EDTA. Flow cytometry and cell sorting was performed on FACScan and FACStar instruments (Becton Dickinson) using dual color detection filters. Double-positive cells were subsequently single-cell-sorted into a 96-well plate. The single cell clones were expanded and frozen for further analysis.

Twelve F17 clones were obtained as described above. The number of copies of 3-OST-1 in the individual clones was determined by Southern
blot analysis. Genomic DNA (10 μg) was digested with 40 units of EcoRI overnight at 37 °C, electrophoresed on a 0.7% (w/v) agarose gel, transferred to GeneScreen Plus (PerkinElmer Life Sciences), and probed with 3-OST-1 cDNA labeled with the Megaprimer labeling kit (Amer- sham Pharmacia Biotech). The blots were hybridized with ExpressHyb solution (CLONTECH) containing the 3-OST-1 probe (2 × 10^6 cpm/ml), followed by autoradiography.

**HS Preparation and Analysis**—Cell monolayers were labeled overnight with 100 μCi/ml sodium [35S]sulfate (carrier free, ICN) in sulfate-deficient Dulbecco’s modified Eagle’s medium, supplemented with penicillin G (100 units/ml), and 10% (v/v) dialyzed FBS. Metabolic labeling with [6-3H]glucosamine was done by incubating cells overnight in Dulbecco’s modified Eagle’s medium containing 1 mM glucose, 10% dialyzed FBS, and 100 μCi/ml [6-3H]glucosamine (40 Ci/mmol, ICN). The proteoglycan fraction was isolated by DEAE-Sepharose chromatography (30) and beta-eliminated in 0.5 M NaBH4 in 0.4 M NaOH at 4 °C overnight. The samples were neutralized with 5 M acetic acid until bubble formation ceased, and the released chains were purified by another round of DEAE-Sepharose chromatography followed by ethanol precipitation. The pellet from centrifugation was washed with 75% ethanol and resuspended in water. The glycosaminoglycans were digested with 20 milliunits of chondroitinase ABC (Seikagaku, Inc.) in buffer containing 50 mM Tris-HCl and 50 mM sodium acetate (pH 8.0). Complete digestion of chondroitin sulfate by chondroitinase ABC was assured by monitoring the extent of conversion of the carrier to disaccharides (100 μg = 1.14 absorbance units at 232 nm). HS was purified from chondroitinase-degraded products by phenol/chloroform (1:1 (v/v)) extraction and ethanol precipitation. After washing the pellets with 0.5 ml of 75% ethanol, the HS was dissolved in water for further analysis.

HS chains were analyzed by gel filtration HPLC (TSK G3000SW, 60 cm × 7.5 mm inner diameter, Tosohaas, Inc.). The column was equilibrated in 100 mM KH2PO4 buffer (pH 6.0) containing 0.2% CHAPS and 0.5 M NaCl and run at a flow rate of 0.5 ml/min. Blue dextran (Sigma) and [35S]sulfate were used to determine the V_o and V_t of the column, respectively. Radioactivity in the effluent was determined by in-line liquid scintillation spectrometry (Packard) with a 12-s sampling rate, and the data were averaged over 1-min intervals.

The absolute levels of HS were determined by a modified Alcian blue staining assay. A dye stock containing 0.5% Alcian blue, 0.018 M H2SO4, and 0.4 M guanidine HCl was centrifuged and filtered (0.2 μm). The working dye solution contained 0.027 M H2SO4, 0.375% Triton X-100, and 4 M guanidine HCl was added to samples or standards in water (10 μl) along with 100 μl of working
dye solution. After centrifugation for 10 min at 10,000 × g in a micro-
centrifuge the supernatant was removed, and the pellet was dissolved in
8 μl guanidine HCl. The absorbance at 600 nm was measured, and the HS
concentration was determined from a standard curve.

AT Inactivation of Thrombin and Factor Xα—Anticoagulant Heparan Sulfate Biosynthesis
The concentration of thrombin in the assay was calculated based on the activity measured in the
presence of inhibitor (34).

RESULTS

3-OST-1 Transduction Impairs Binding to AT but not to FGF-2—CHO wild-type and pgsF-17 cells do not express
3-OST-1 and therefore do not make detectable 3-O-sulfate con-
taining saccharides or bind to AT. The lack of uronyl 2-O-
sulfotransferase activity in the mutant also prevents binding to
FGF-2 (24). To study the effect of 3-OST-1 on ligand binding,
mature and wild-type cells were stably transduced with mul-
tiple copies of 3-OST-1 cDNA. As shown by flow cytometry, the
expression of 3-OST-1 gave rise to wild-type and mutant cells
that bind AT, but the binding of FGF-2 was unchanged (Fig.
1). The wild-type transductant contained three copies of
3-OST-1 (B, broken line), whereas the population of transduced
mutant cells (D, broken line) contained one or more copies of the
gene based on the observation that 90% of the cells exhib-
ited binding to AT (D, broken line).

The mutant cells that exhibited high AT binding were single-
cell-sorted into a 96-well plate, and 12 clones were expanded for
further analysis. Genomic DNA from the clones was digested with EcoRI and probed for 3-OST-1 by Southern blot (Fig. 2).
All the clones, including wild-type cells and the original mu-
tant, have an endogenous copy of 3-OST-1. No enzyme activity
was found in nontransduced wild-type and mutant cells, indi-
cating that the endogenous gene was inactive. The 12 clones
have 1–5 inserted copies of the gene. 3-OST-1 activity roughly
paralleled the number of extra copies of the gene (coefficient
of correlation = 0.948; data not shown).

Heparan Sulfate from Mutant Cells Transduced with 3-OST-1 Accumulate Unusual Disaccharides—We have shown
previously that 3-OST-1 generally acts on glucosamine units

Table I

| Fraction no. | HSact percentage |
|-------------|-----------------|
| Mutant      | Wild type       |
| 7           | 45              | 42 |
| 8           | 45              | 41 |
| 9           | 42              | 39 |
| 10          | 37              | 39 |
| 11          | 30              | 34 |
| 12          | 28              | 35 |
| 13          | 21              | 30 |
| 14          | 22              | 25 |
| 15          | 16              | 20 |
| 16          | 15              | 17 |
| 17          | 13              | 18 |
| 18          | 13              | 17 |
| 19          | 13              | 13 |
| 20          | 17              | 12 |
| 21          | 19              | 9  |
| 22          | 16              | 8  |
| 23          | 11              | 6  |
| 24          | 6               | 3  |
| 25          | 4               | 2  |
| 26          | 4               | 2  |
| 27          | 3               | 2  |
| 28          | 3               | 2  |
| 29          | 3               | 2  |
| 30          | 1.1             | 0.1|

Anticoagulant activity of HS oligosaccharides

[6-3H]GlcN-labeled HS samples were 3-O-sulfated in vitro with pure
3-OST-1 and [35S]PAP and digested with 1 milliunit of heparitinase I.
The disaccharides were analyzed by Bio-Gel P6 chromatography (Fig. 6).
Individual fractions representing oligosaccharides of different lengths
were analyzed for AT binding. The HSact percentages are presented.

| Anticoagulant Heparan Sulfate Biosynthesis |
|------------------------------------------|
| dyes | 3-O-Act | 4 units/ml | 15 units/ml |
| 1% (w/v) Triton X-100 | 5 mM MnCl₂ | 5 mM MgCl₂ | 25 mM CaCl₂ |
| 0.075 mg/ml proteamine chloride | 1.5 mg/ml | 70 ng of purified baculovirus-expressed murine 3-OST-1 |
| metabolically labeled [3H]HS | [35S]HS | nonradioactive HS | chains | 7.5 ml of ammonium bicarbonate | 0.0004% Triton X-100 |
| 100 μM of HS | 0.002% | 0.0004% | 0.0004% | 0.0004% |

Disaccharide Analysis of HS—Radiolabeled HS samples were mixed
with 10 μg of bovine kidney HS (ICN) and depolymerized by hydrazi-
nolysis and nitrous acid degradation (22). Disaccharides were purified
by Bio-Gel P2 chromatography and resolved by ion-pairing reverse-
phase HPLC with appropriate disaccharide standards. Bio-Gel P2 col-
omns (0.75 × 200 cm) were equilibrated with 100 mM ammonium
bicarbonate. Radiolabeled samples (200 μl) were mixed with dextran
blue (5 μg) and phenol red (5 μg) and loaded onto the column. The
samples were eluted at a flow rate of 4 ml/h, collecting 0.5-ml fractions.
The desired fractions were either pooled or dried individually under a
vacuum to remove ammonium bicarbonate. HS was digested at 37 °C
for 4 h in 10 mM sodium acetate (pH 7.0) containing 1 mM
CaCl₂ and 1 milliunit of heparitinase I (EC 4.2.2.8, Seikagaku Corp.)
and analyzed on a P6 column (0.75 × 200 cm) as described above. For
iduronidase digestion, 10% of the total radiolabeled disaccharides col-
lected from Bio-Gel P2 chromatography was dissolved in 400 μl of 1×
incubation buffer (glycosaminoglycan-5006, Oxford GlycoSciences) with
0.5 U-iduronidase (glycosaminoglycan-5006) at 37 °C for 16 h. After adding
1 ml of a 1:333 tetraethylammonium dihydrogen phosphate, the
digested sample was injected into ion-pairing reverse-phase HPLC.

Affinity co-electrophoresis was done as described (33). HSact from
both wild type and mutants was electrophoresed in an agarose gel
through zones containing AT at different concentrations. The migration of HSact was retarded by the presence of AT, with the degree of retar-
dation dependent on AT concentration. A plot of R/[AT] versus R (where
R = [M] - [M]/[M]c, M is the migration of [3H]HS in the absence of AT,
and M is the observed migration of HS in the presence of AT) was used
to determine the apparent Kᵣ values (33).

AT Inactivation of Thrombin and Factor Xα—Human α-thrombin (4 mg/ml 50% glycerol, 3,000 units/ml) and factor Xa (10.4 mg/ml 50% glycerol, 820 units/ml) were from Hematologic Technologies. Factor Xa and α-thrombin were diluted 1:200 with PBS containing 1 mg of bovine
serum albumin (4 units/ml and 15 units/ml, respectively). AT (2.5 mg/ml) was diluted 1:200 to give a 2 × 10⁻⁷ M stock solution. The chromogenic substrates, S-2765 (factor Xa assays) and S-2238 (thrombin
assays), were from Chromogenix. Both substrates were made up at
1 mM with 1 mg/ml Polybrene in water. Heparin (174 international
unit/mg, Sigma) was used as a standard. HSact was used for factor Xa
(25 μg/ml) and α-thrombin assays (50 μg/ml). The protocol involved adding
25 μl of AT (2 × 10⁻⁷ M) to 25 μl of a serial dilution of heparin standards or
HSact from wild-type or mutant cells in Tris-EDTA (50 mM Tris, 7.5
mM EDTA, and 175 mM NaCl (pH 8.4)) buffer in a 96-well plate. The
reaction was incubated at 37 °C for 75 s. Factor Xa (25 μl, 4 units/ml) or thrombin (25 μl, 6 units/ml check concentration) was added. After incubating at 37 °C for 190 s, 25 μl of S-2765 or S-2238 was added. The
absorbance at 405 nm was read every minute for 10 min in a Spectro-
MAX Plus plate reader (Molecular Devices, Inc.).
located to the reducing side of GlcUA to generate products containing GlcUA-GlcNS3S and GlcUA-GlcNS3S6S (8). To determine whether removing uronyl 2-O-sulfation affects the specificity of the 3-OST-1, metabolically labeled $[^{35}S]HS$ from the mutant was 3-O-sulfated in vitro with purified 3-OST-1 and cold PAPS. $[^{35}H]HS$ chains were purified and depolymerized completely by hydrazinolysis and nitrous acid, and the resultant disaccharides were analyzed by IPRP-HPLC (see “Experimental Procedures”). The predominant sulfated disaccharides in the mutant cells (Fig. 3, solid line) consist of GlcUA-$\alpha$ManR6S (peak 2) and IdoUA-$\alpha$ManR6S (peak 3). In vitro treatment of mutant HS with pure 3-OST-1 and cold PAPS yielded two new disulfated disaccharides (Fig. 3, dotted line). The first unknown disaccharide (peak 4) has the same retention time as an IdoUA-$[3-O-^{35}S]\alpha$ManR6S standard on IPRP-HPLC (36). The second disaccharide (peak 5) has the same retention time as a GlcUA-$[3-O-^{35}S]\alpha$ManR6S standard. This result suggests that the addition of 3-O-sulfate to an IdoUA-$[6-O-^{35}S]GlcNS$ residue occurs in the mutant HS chain in vitro.

Several lines of evidence demonstrated that X and Y were IdoUA-$\alpha$ManR3S and IdoUA-$\alpha$ManR3S6S, respectively. First, $^3$H-labeled peak Y co-elutes with an IdoUA-$[3-O-^{35}S]\alpha$ManR6S standard on IPRP-HPLC (36). Second, when the disaccharides were treated with iduronidase, IdoUA residues were removed from IdoUA-$\alpha$ManR, IdoUA-$\alpha$ManR6S, IdoUA-$\alpha$ManR3S, and IdoUA-$\alpha$ManR3S6S, whereas GlcUA-$\alpha$ManR, GlcUA-$\alpha$ManR3S, GlcUA-$\alpha$ManR6S, and GlcUA-$\alpha$ManR3S6S were not affected (Fig. 5). Third, a precursor-product relationship was observed in the IPRP-HPLC profiles (Fig. 3) when metabolic $[^{35}S]HS$ from the mutant was 3-O-sulfated in vitro with purified 3-OST-1. The appearance of GlcUA-$[6-O-^{35}S]\alpha$ManR3S (2.2%) and IdoUA-$[6-O-^{35}S]\alpha$ManR (3.7%) roughly correlates with the decrease of GlcUA-$\alpha$ManR6S (from 5.8 to 4.2%) and IdoUA-$[6-O-^{35}S]\alpha$ManR (from 9.9 to 7.6%) disaccharides. Thus, the 2-O-sulfotransferase-deficient mutant contains a precursor sequence not present in the wild-type CHO cells.
Quantitation of the disaccharide composition of HS from the counts recovered in each peak shown in Fig. 4 revealed that HS from wild-type cells transduced with 3-OST-1 possesses two 3-O-sulfate sites/100 disaccharides. In contrast, HS from the transduced mutant possessed eight 3-O-sulfate sites/100 disaccharides. When 3-O-sulfation was performed in vitro, wild-type HS was found to contain six 3-O-sulfation sites/100 disaccharides, whereas HS from the mutant contained 14 3-O-sulfate sites/100 disaccharides. An increased amount of 3-O-sulfate containing disaccharides in the 3-OST-1 expression mutant suggests that 2-O-sulfation blocks the action of 3-OST-1. To prove it, we have transfected 2-OST into the 3-OST-1 expression mutant clone. Disaccharide analysis of 2-OST expression mutant clones revealed that 2-O-sulfation restores the wild-type disaccharide profile and leads to the disappearance of IdoA-aManR3S and IdoA-[6-3H]aManR3S disaccharides (data not shown). This observation confirms that 2-O-sulfation occurs before 3-O-sulfation.

To examine how the unusual disaccharides might affect the binding of HS to AT, [6-3H]GlcN-labeled HS chains from F17 cells were 3-O-sulfated in vitro and digested with heparitinase I, which cleaves the chains in regions poor in sulfate, generating oligosaccharides from domains rich in sulfated residues. The products were analyzed by Bio-Gel P6 chromatography (Fig. 6, A and B), and individual fractions representing oligosaccharides of different lengths were analyzed for AT binding. Only oligosaccharides larger than hexamers bound AT regardless of the source of the material (Table I). The AT-binding oligosaccharides in the wild-type oligosaccharides had a 3H/35S ratio of 1:(0.9–1.1), whereas that of the mutant had a ratio of 1:(1.7–2.1) depending on the chain length. This result suggested that the mutant had twice as many 3-O-sulfated disaccharides, which was consistent with the disaccharide analysis shown in Fig. 4.

HSact Chains from Mutant and Wild Type Are the Same Size—Previous studies of F17 HS showed that chains were much longer than in wild-type cells because of the lack of heparanase cleavage sites (37). To determine whether chain length affected sulfation by 3-OST-1, we prepared [6-3H]GlcNH2-labeled chains from mutant and wild-type cells and introduced 3-O-sulfate groups in vitro with PAPS and pure 3-OST-1. The chains were affinity-purified and then compared by gel filtration HPLC to chains that were not affinity-fractionated (Fig. 7). HS from the wild type consisted of three populations of chains that differed in size as reported (37). However, the affinity-fractionated HS consisted of only relatively large chains of the same overall size in both mutant and wild-type preparations. Similar results were obtained when the affinity chains were analyzed from cells transduced with 3-OST-1 (data not shown), indicating that the larger chains preferentially contain the precursor sequence recognized by 3-OST-1.

HS from 3-OST-1 Containing Mutant Cells Has Higher Affinity for AT—To compare the AT-binding affinity of HS from 3-OST-1-transduced wild-type and mutant cells, [35S]HS was purified from the transduced cell lines. HSact was purified by AT affinity chromatography and analyzed by affinity co-electrophoresis using agarose gels containing different amounts of AT (Fig. 8). Using the midpoint of the bands to determine relative mobilities, the data for the mutant indicated a Kd value of 17 nM, whereas the wild-type HSact gave a value of 32 nM (linear coefficient values = 0.98 and 0.97, respectively). This finding is consistent with earlier studies of synthetic oligosaccharides that showed that pentasaccharides containing two 3-O-sulfate groups (GlcNS3S-GlcUA-GlcNS3S6S-IdoUA2S-GlcNS3S6S-OCH3) had a higher affinity for AT compared with mono-3-O-sulfated pentasaccharide (GlcNS6S-GlcUA-GlcNS3S6S-IdoUA2S-GlcNS6S-OCH3) (38). However, we have not determined whether the additional 3-O-sulfate groups in the transduced mutant are present on adjacent glucosamine units as they are in the synthetic oligosaccharides.

**AT Inhibition of Xa and Thrombin**—Based on the higher affinity of the HS from the transduced mutant, we predicted that the HSact fraction should display a greater rate of AT-mediated factor Xa inactivation (Fig. 8). It was unknown whether the mutant HSact would mediate AT-dependent thrombin inactivation, because thrombin binding to HS might require 2-O-sulfation of the uronic acids. To examine this question, HS was purified from the mutant and wild-type cells and modified by 3-OST-1 and PAPS in vitro. HSact was fractionated by AT affinity assay, quantitated chemically, and tested for its ability to activate AT-dependent inhibition (see “Experimental Procedures”). HSact from the mutant showed a 2.2-fold increase in k, value for factor Xa inhibition but only a 1.2-fold increase in thrombin inhibition compared with the values obtained for the HSact derived from wild-type cells transduced with 3-OST-1 (Table II). Thus, the lack of 2-O-sulfate groups does not diminish factor Xa and thrombin inhibition by AT and in fact may accelerate the reactions directly through an enhanced affinity of the HS chains caused by the increase in 3-O-sulfate groups.

**DISCUSSION**

The unexpected finding presented in this report is that in the absence of 2-O-sulfation of uronic acid residues, 3-OST-1 will generate disaccharide units containing IdoUa to the nonreducing side of the 3-O-sulfated glucosamine unit. This specificity is different from that originally described for 3-OST-1 (8), in which GlcUA was the preferred unit in this position. We cannot tell if the change in substrate specificity is directly the effect of reduced 2-O-sulfation of IdoUA units or if the higher levels of N-sulfated glucosamine residues in the mutant might play a role (24). However, the extent of N-sulfation increases by 50% (~40% GlcNS in the wild type to ~60% in the mutant). In contrast, the change in 2-O-sulfation is quantitative and therefore more likely the cause of altered specificity of 3-OST-1. Previous work showed that HSact had a lower content of IdoUA2S-aManR compared with HSinact, which is consistent with the idea that 2-O-sulfated substrates inhibit 3-O-sulfation (32). Thus, we imagine an exclusion mechanism catalyzed by uronyl 2-O-sulfotransferase, which normally prevents glucosa-
mine residues to the reducing side of IdouA from acting as substrates for 3-OST-1.

Because in vitro 3-O-sulfation can impart anticoagulant activity to inactive HS chains, it has been assumed that 3-O-sulfation is the final modification step during HS biosynthesis. This study provides direct evidence that 2-O-sulfation preferentially occurs before 3-O-sulfation catalyzed by 3-OST-1. The facts are: 1) the 2-OST transfectant of the 3-OST-1 expression mutant cells does not form the new 3-O-containing disaccharides, and 2) 2-O-sulfation results in less sites recognizable by the enzyme (two in wild-type HS versus eight in mutant 3-OST-1 transduced cells and six in wild type versus 14 in the mutant after in vitro sulfation; Fig. 4). The order of reactions for other isoforms of the 3-O-sulfotransferases is unknown, but the presence of IdouA-GlcNS3S and IdouA2S-GlcNS3S units in bovine glomerular basement membrane HS suggests that the reaction sequence may differ (39).

Multiple sulfotransferases have been cloned and shown to be expressed in a tissue-specific and developmentally regulated pattern. Furthermore, the individual isozymes seem to differ in substrate specificity (2, 6, 40). The results shown here suggest another layer of control determined by the level of alternate precursor structures on the action of HS biosynthetic enzymes. Taking advantage of this approach, a series of CHO mutants generating specific HS can be sought after chemical mutagenesis of this arrangement is that other genes responsible for the sequence for AT.

This study provides direct evidence that 2-O-sulfation is the final modification step during HS biosynthesis. Further, the individual isozymes seem to differ in substrate specificity (2, 6, 40). The results shown here suggest another layer of control determined by the level of alternate precursor structures on the action of HS biosynthetic enzymes. Taking advantage of this approach, a series of CHO mutants generating specific HS can be sought after chemical mutagenesis of this arrangement is that other genes responsible for the sequence for AT.

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The Effect of Precursor Structures on the Action of Glucosaminyl 3-O-
-Sulfotransferase-1 and the Biosynthesis of Anticoagulant Heparan Sulfate
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