Enzymatically Active N-Deacetylase/N-Sulfotransferase-2 Is Present in Liver but Does Not Contribute to Heparan Sulfate N-Sulfation*

Received for publication, May 1, 2006, and in revised form, August 17, 2006 Published, JBC Papers in Press, September 19, 2006, DOI 10.1074/jbc.M604113200

Johan Ledin, Maria Ringvall, Maria Thuveson, Inger Eriksson, Maria Wilén, Marion Kusche-Gullberg, Erik Forsberg, and Lena Kjellen

From the Department of Medical Biochemistry and Microbiology, Uppsala University, SE-751 23 Uppsala, Sweden

Heparan sulfate (HS) proteoglycans influence embryonic development through interactions with growth factors and morphogens. The interactions depend on HS structure, which is largely determined during biosynthesis by Golgi enzymes. NDST (glucosaminyl N-deacetylase/N-sulfotransferase), responsible for HS N-sulfation, is a key enzyme directing further modifications including O-sulfation. To elucidate the role of the different NDST isoforms in HS biosynthesis, we took advantage of mice with targeted mutations in NDST1 and NDST2 and used liver as our model organ. Of the four NDST isoforms, only NDST1 and NDST2 transcripts were shown to be expressed in control liver. The absence of NDST1 or NDST2 in the knock-out mice did not affect transcript levels of other HS isoforms, only NDST1 and NDST2 transcripts were shown to be present in similar amounts. In embryonic day 18.5 liver (≈40%), its presence did not appear to affect the contribution of NDST2 to total NDST enzyme activity in embryonic day 18.5 embryos. Enzymatically active NDST2 was shown to be present in similar amounts in wild-type, NDST1−/− mice and NDST1−/− embryonic day 18.5 liver. Despite the substantial contribution of NDST2 to total NDST enzyme activity in embryonic day 18.5 liver (≈40%), its presence did not appear to affect HS structure as long as NDST1 was also present. In NDST1−/− embryonic day 18.5 liver, in contrast, NDST2 was responsible for N-sulfation of the low sulfated HS. A tentative model to explain these results is presented.

Heparan sulfate (HS) proteoglycans consist of sulfated polysaccharide chains covalently attached to different core proteins. HS proteoglycans are expressed by most cell types in the mammalian body and are predominantly found on the surface of cells and in the extracellular matrix (1). HS chains interact with a large number of proteins, influencing a variety of important biological functions including organogenesis, growth factor and cytokine action, angiogenesis, and cell adhesion (1–3). Binding of proteins depends on charge and/or fine structure of the HS chain (4). HS structure varies distinctly between different cell types and during different developmental stages (5–7) but appears to be largely independent of the HS proteoglycan core protein (8, 9).

HS biosynthesis is a complex process with many different endoplasmic reticulum and Golgi-resident enzymes involved (4). The HS polymerases EXT1 and EXT2 synthesize a polysaccharide backbone consisting of repeating units of glucuronic acid (GlcA) and GlcNAc. The first modifying event is the N-deacetylase/N-sulfotransferase (NDST) reaction, where N-acetyl groups of selected GlcNAc residues are removed and replaced by sulfate groups (10). N-Sulfated glucosamine (GlcNS) units are typically found in contiguous stretches (NS domains) separated by non-modified N-acetylated regions (NA domains). Often, regions of alternating GlcNS and GlcNAc units (NA/NS domains) surround the NS domains (11). After N-sulfation, the C5 epimerase converts most GlcA residues within the NS domain and many GlcA residues in the NA/NS domain to iduronic acid (IdoA). Sulfation of the 2-O position on IdoA residues is carried out by the 2-O-sulfotransferase and occurs almost exclusively in NS domains (12). In contrast, GlcN 6-O-sulfation occurs both in NS and in NA/NS domains (13).

Four mammalian NDST isoforms have been recognized (NDST1–4) (10). On the transcript level, NDST1 and NDST2 are widely expressed (10). However, recent results demonstrate that the expression of the NDSTs may be largely translationally controlled (14), and little data on NDST protein expression are so far available. It is not known how different patterns of NA and NS domains are created. We have previously shown that overexpression of NDST1 and NDST2 in HEK293 cells results in an increased N-sulfation of HS produced by the cells (15). The level of N-sulfation was higher in cells transfected with NDST2 (80%) than with NDST1 (60%), indicating that the

* This work was funded by the Swedish Research Council, the programme “Glycoconjugates in Biological Systems” sponsored by the Swedish Foundation for Strategic Research, Polysackaridforskning AB, and Gustaf V:s 80-årsfond. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Medical Biochemistry and Microbiology, The Biomedical Ctr., Box 582, SE-751 23 Uppsala, Sweden. Tel.: 46-18-471-4217; Fax: 46-18-471-4244; E-mail: lena.kjellen@imbim.uu.se.

2 The abbreviations used are: HS, heparan sulfate; GlcA, glucuronic acid; GlcNS, N-sulfated glucosamine; IdoA, iduronic acid; NA domains, non-modified N-acetylated regions; NA/NS domains, regions of alternating GlcNAc and GlcNS units; NDST, glucosaminyl N-deacetylase/N-sulfotransferase; NS domains, contiguous stretches of GlcNS-containing disaccharides; RIP, reverse phase ion pair; PBS, phosphate-buffered saline; nt, nucleotide(s); HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid.
Liver Heparan Sulfate Biosynthesis

N-sulfation pattern may be, to some extent, isoform-dependent. Tentatively, altering the relative numbers/concentrations of the various NDST isoforms in a cell could result in distinct N-sulfation patterns. When it comes to downstream HS modification patterns, a large part of these can be explained by substrate specificity of the enzymes (4). The C5 epimerase requires a GlcNS residue on its non-reducing side, whereas 2-O-sulfotransferase needs a contiguous (IdoA-GlcNS), structure for substrate recognition. For the three known 6-O-sulfotransferases (6-O-sulfotransferase-1–3), all positions in the close vicinity of a GlcNS residue constitute acceptable substrates (13) and 6-O-sulfation can even occur in the absence of N-sulfation (16). However, not all potential sites are modified, creating the great diversity in HS structure. How this “lack of modification” is regulated is not known.

As mentioned, the NDST2 transcript is widely expressed in both mice and humans (17, 18). Despite its abundance in most tissues, no alteration in HS structure has been found in mice with a targeted mutation in this gene (19). Heparin biosynthesis in connective tissue-type mast cells is, however, severely disturbed, resulting in a non-sulfated polysaccharide (20). Despite its abundance in most tissues, no alteration in HS structure has been found in mice (17, 18). The C5 epimerase was purified with the Oligotex mini kit (Qiagen). 10 μg of each mRNA sample was separated and transferred to Hybond-N+ nylon membrane (Amersham Biosciences). The following cDNA probes with the translation start codon defined as nt 1–3 were radioactively labeled with the Strip-EZ-Kit (Ambion); mNDST1, nt 1–435 (AF074926); mNDST2, nt −157 to 780 (X75885); mNDST3, 1-kb fragment starting with nt 1959 (AF221095); mNDST4, nt −97 to 459 (AB036838); bovine C5 epimerase, nt 1028–1851(AF 003927); m2-O-sulfotransferase, nt 112–544 (AF169243); m6-O-sulfotransferase1, nt 114–945 AB024566; m6-O-sulfotransferase2, nt 1–1520 (AB024565); and m6-O-sulfotransferase3, nt 1175–1523 (AB024567). The filter was hybridized with the 32P-labeled probe at a concentration of 1 × 106 counts/min/ml for 15 h at 42 °C using UltraHyb (Ambion) and then washed according to the Northern Max kit (Ambion) recommendations. The filter was exposed to film (Fuji) for 1–4 days at −70 °C. Radioactive bands were also detected with phosphor screens (FujiFilm, BAS-2500), and the relative amounts of radioactivity in the mRNA bands were determined with the program Image Gauge version 3.3 (FujiFilm Science Laboratory). The 32P-labeled probe was removed before the next hybridization with the Strip-EZ kit (Ambion) according to the manual.

Metabolic Labeling of Liver Glysosaminoglycans—Embryonic day 18.5 embryos were collected from pregnant mice injected intraperitoneal with 250 μCi [3H]GlcN (PerkinElmer Life Sciences) 2 h prior to decapitation. The embryonic livers were dissected out and snap-frozen in liquid nitrogen. Embryos from heterozygous crossings were genotyped, and livers from five embryos of the same genotype were pooled. Livers from adult mice were dissected out 2 h after intraperitoneal injection of 250 μCi [3H]GlcN and snap-frozen in liquid nitrogen.

Isolation of 3H-Labeled Liver Heparan Sulfate—Liver tissue (from 3–5 embryonic day 18.5 embryos) was homogenized in 4 ml of 0.05 M Tris-HCl buffer, pH 8, 1% Triton X-100 and incubated for 30 min at 4 °C. After centrifugation at 16,000 × g for 30 min, the supernatant was collected, adjusted to 0.1 M with regard to NaCl, and applied to a 1.4-ml column of DEAE-Sephaloc (Pharmacia Corp.), equilibrated with 0.05 M Tris-HCl, pH 8, 0.10 M NaCl, and 0.1% Triton X-100. The column was first washed with equilibration buffer and subsequently with 0.05 M acetate buffer, pH 4.0, containing 0.10 M NaCl and 0.1% Triton X-100. The proteoglycans were eluted with 0.05 M acetate buffer, pH 5.5, containing 0.1% Triton X-100 and 2 M NaCl followed by digestion with papain as described previously (24). After desalting in water on PD10 columns (Amersham Biosciences) followed by lyophilization, the 3H-labeled glycosam
inoglycans were subjected to digestion with 0.1 unit of chondroitinase ABC (Seikagaku) as described previously (24). The resulting 3H-labeled HS chains were recovered in the void volume after gel filtration on a column (100 × 0.5 cm) of Sephadex G-50 (superfine grade; Amersham Biosciences) in 0.2 m NH₄HCO₃. The resultant disaccharides, containing N-sulfated GlcN residues by deamination at pH 1.5 (25). The resulting 3H-labeled oligosaccharides were separated by gel chromatography on a column (1 × 140 cm) of Bio-Gel P-10 (fine grade; Bio-Rad) eluted in 0.2 m NH₄HCO₃. Estimation of the percentage of GlcN residues carrying N-sulfate groups was made using weighted integration of the oligosaccharide peak areas.

For total disaccharide composition, isolated 3H-labeled HS was chemically N-deacetylated by hydrazinolysis for 5 h followed by combined deamination at pH 1.5 and 3.9 (26) and reduction with NaBH₄. The resultant disaccharides were subjected to digestion with 0.1 unit of chondroitinase ABC (Seikagaku) as described previously (24). The generated HS disaccharides were subjected to RPIP-HPLC analysis followed by post-column derivatization with cyanoacetamide and detection in a fluorescence detector (5).

**RESULTS**

We have previously demonstrated a structural alteration of HS in most basement membranes of NDST1−/− embryos using HS-recognizing phage display antibodies (21). However, judged from the staining of liver sections from embryonic day 18.5 NDST-1−/− and NDST-1−/− embryos with antibodies against perlecan and nidogen, the altered HS structure does not affect basement membrane distribution in the liver (data not shown). Furthermore, staining with the endothelial marker platelet endothelial cell adhesion molecule-1 and von Willebrand factor demonstrated no obvious changes in endothelial structures or in the number of megakaryocytes as a result of NDST1 or NDST2 deficiency (data not shown). We conclude that neither NDST-1−/− nor NDST-2−/− mice display any obvious liver phenotype and that this has provided us with a unique in vivo system to elucidate the roles of the different NDST isoforms in HS biosynthesis.

**Does Ablation of One NDST Gene Influence Transcription of Other NDST Genes?**—To investigate whether deficiency of NDST2 would lead to increased transcription of NDST1, -3, or -4 and whether the lack of NDST1 influenced NDST2 transcription, Northern blots were performed. Liver mRNA from NDST1−/−, NDST2−/−, and control mice were blotted to nylon membranes and hybridized with radioactive NDST probes (Fig. 1A). No transcriptional up-regulation of NDST1 could be seen in liver from NDST2−/− adult mice or embryonic day 18.5 embryos. Likewise, the level of NDST2 expression was the same in wild-type and NDST1-deficient embryonic day 18.5 embryos. It has previously been shown, using reverse transcription-PCR, that no NDST3 or NDST4 is expressed in adult mouse liver (18). This result was confirmed (Fig. 1A), and it could be demonstrated also that embryonic day 18.5 embryonic liver of all tested genotypes lacked expression of these NDST isoforms. Thus, no up-regulation of any of the NDSTs occurred as a response to the loss of either NDST1 or NDST2. Expression levels of the O-sulfotransferases and the C5 epimerase also appeared to be unaltered (Fig. 1B), although the expression levels of 6-O-sulfotransferase-3 was difficult to judge due to hybridization of the probe with multiple transcripts. However, the difference in labeling intensity of the same transcript in control and knock-out mice never exceeded 30% (as determined by phosphorimaging).
NDST2 Can Synthesize HS-like Structures—Because NDST1 is the only isoform expressed in NDST1−/− liver, whereas NDST1−/− liver only contains NDST2, a detailed picture of the sulfation properties of each of the enzymes could be achieved by determining HS structure in the two knock-out strains. Previous structural characterization of HS isolated from NDST1−/− embryonic liver, brain, and lung have shown that both N- and O-sulfation are reduced when NDST1 is knocked out (19). In that study, total HS was isolated and analyzed by RPIP-HPLC. If instead, newly synthesized metabolically [3H]GlcN-labeled HS is analyzed, the possible contribution of NDST3 and NDST4 to N-sulfation of any HS present in NDST1−/− liver at the time of analysis but synthesized earlier during development can be avoided. [3H]-Labeled liver HS was therefore isolated from embryonic day 18.5 embryos (originating from heterozygous crossings) 2 h after injection of their mother with [3H]GlcN. Information regarding the amounts and distribution of N-sulfate groups was obtained by cleaving the polysaccharide at N-sulfated glucosamine units with nitrous acid at pH 1.5 followed by gel chromatography on Bio-gel P10. Metabolically [3H]GlcN-labeled liver HS from embryonic day 18.5 NDST1−/− and wild-type embryos contained 17 and 45% N-sulfate groups, respectively (Fig. 2A). Because all N-sulfate groups in the NDST1−/− HS sample are a result of NDST2 activity, the HNO2 cleavage pattern shows that NDST2 is capable of synthesizing HS-like structures, including both NS domains (present as disaccharides in Fig. 2A) and NA/NS domains (present as tetrasaccharides). Furthermore, as judged by the large proportion of material eluting in the void volume (and hence devoid of N-sulfate groups), NDST2 has acted on a restricted part of the polysaccharide. The corresponding experiment with NDST2−/− embryonic day 18.5 embryos (data not shown) and adult mice (Fig. 2B) shows that the N-sulfation performed by NDST1 alone is identical to that in control animals. Samples of liver HS from embryonic day 18.5 NDST1−/− were similar to control mice (data not shown), demonstrating that one gene copy of NDST1 is sufficient to attain normal HS N-sulfation.

Compositional disaccharide analysis previously performed with the RPIP-HPLC method (19) shows that all of the species of 2-O-sulfated disaccharides were reduced in NDST1−/− HS, whereas the overall 6-O-sulfation was lowered mainly through the reduction of the trisulfated disaccharide containing N-sulfate, 2-O-sulfate, and 6-O-sulfate groups. With the RPIP-HPLC method, GlcA-containing disaccharides cannot be distinguished from those containing IdoA. This information can, however, be obtained by ion exchange chromatography in combination with descending paper chromatography. In applying this method to [3H]GlcN-labeled disaccharides from NDST1−/− HS, a dramatic decrease in IdoA-containing disaccharides could also be demonstrated (Fig. 3). Interestingly, 6-O-sulfation of GlcA-containing disaccharides was increased in NDST1−/− HS.

FIGURE 1. Transcript levels of HS biosynthetic enzymes. mRNA isolated from livers of embryonic day 18.5 control, NDST1−/−, and NDST2−/− embryos from livers of adult control and NDST2−/− mice and from embryonic day 13.5 control embryos were separated on a 0.8% agarose gel and transferred to Hybond-N+ nylon membrane. The membrane was hybridized with probes specific for the four NDST isoforms (A) and the CS epimerase (EPI) (B), the 2-O-sulfotransferase (HS2ST), and the three 6-O-sulfotransferases (6OST1, 6OST2, and 6OST3). Hybridization with a probe specific for β-actin was also performed to allow for comparison of the amounts of mRNA loaded. The successful removal of each probe was assured before the next probe was added. E, embryonic day.
whereas those containing IdoA was decreased (Fig. 3A). As expected, comparison of liver HS from adult NDST-2−/− and control mice did not reveal any significant differences in disaccharide composition (data not shown).

Is NDST2 Translated?—The identical structure of HS in control and NDST2−/− liver may suggest that NDST1 alone is active in HS N-sulfation in wild-type animals. Results by Grobe and Esko (14) have demonstrated that NDST expression may be controlled also at the translational level. If NDST2 translation for some reason is repressed in wild-type liver and no protein is produced, the lack of effect of NDST2 gene ablation on liver HS structure could be explained. To look for the presence of NDST2, an antibody recognizing the cytoplasmic tail of NDST2 not interfering with the catalytic parts of the protein was used in immunoprecipitation followed by measurement of enzyme activity. To investigate how much NDST2 contributed to total liver NDST activity, livers were isolated from embryonic day 18.5 embryos of different genotypes. Both total N-deacetylase activity and N-deacetylase activity contributed by NDST2, determined after immunoprecipitation with the NDST2 antibody, was measured. On analyzing total N-deacetylase activity, it was found that NDST1 deficiency resulted in a 60% reduction in enzyme activity, whereas the lack of one allele gave half the reduction (Fig. 4A). NDST2−/− embryonic day 18.5 livers retained close to 60% of the enzyme activity, whereas NDST1+/−NDST2−/− livers only contained a little less than 30% of control enzyme activity. As shown in Fig. 4B, no N-deacetylase activity could be detected in immunoprecipitates from NDST2−/− livers, confirming the NDST2 selectivity of the antibodies. However, liver from control, NDST1−/−, and NDST1+/− embryos contained the same level of NDST2 activity. These results indicate that, in the control embryonic day 18.5 liver, NDST1 contributes with 60% and NDST2 with 40% of total activity and that absence of one isoform does not result in any compensatory increase in activity of the other isoform. Similar experiments with liver from adult control and NDST-2−/− mice demonstrated that the contribution of NDST2 to total N-deacetylase activity in this case was lower (≈15% reduction, p < 0.007). Taken together, our results demonstrate that enzymatically active
NDST2 is present in liver but that its presence does not affect HS structure.

Normal HS Structure in NDST1+/−/NDST2−/− Mice—Because the enzyme activity in NDST1+/−/NDST2−/− liver was lower than that in NDST1−/− liver, it was interesting to analyze HS structure in these mice. In contrast to the NDST1−/− mice, the NDST1+/−/NDST2−/− mice are viable and fertile and have been used in breeding to generate NDST1/NDST2 double knock-outs (16). Structural analyses of liver HS from the NDST1+/−/NDST2−/− mice using the RPIP-HPLC method (19) show that it had the same composition as HS from wild-type mice (Fig. 5). We conclude that one allele of the NDST1 gene is sufficient to generate normal HS structure and that a higher level of NDST2 activity is not able to compensate for a complete loss of NDST1.

DISSCUSSION

HS biosynthesis is a complex process that results in HS structures important for a large number of biological processes. How is the biosynthesis regulated? N-Deacetylation/N-sulfation of GlcNAc residues is the first modification step but usually only half of the GlcNAc residues in the uniform (GlcA-GlcNAc)n polymer is N-sulfated. Thus, although other HS-modifying enzymes are restricted by the availability of allowed substrates (1, 4, 11), factors that regulate N-sulfation have remained unidentified. If N-sulfation simply relied on the concentration of NDST enzymes in the Golgi compartment, a direct relationship between N-sulfation and NDST enzyme activity would be expected. Indeed, cells stably transfected with NDST1 or NDST2 synthesize HS with an increased level of N-sulfation compared with untransfected cells (15), arguing that enzyme concentration may influence the outcome of biosynthesis. Thus, if the number of N-sulfation events corresponds to the concentration of NDST enzymes, the levels of active NDST enzymes must be regulated at some level. Because overexpression of NDST2 in HEK293 cells results in 80% N-sulfation, whereas HS produced in NDST1 overexpressing cells only reach 60% N-sulfation (15), the relative concentration of the different isoforms may also be subject to regulation.

We know from previous investigations that both N-sulfation as well as further modifications are dramatically lowered in liver HS from NDST1−/− embryos (19, 21), whereas the HS structure of liver from NDST2−/− mice, both with regard to composition and domain structure (19), was similar to control HS. We first concluded that the lack of effect of NDST2 deficiency on HS structures cannot be explained by the increased expression of other NDST isoforms. Analyzing by Northern blotting the expression of the four NDSTs in control and NDST-deficient liver, we found that NDST3 and -4 are not expressed in control liver either at embryonic day 18.5 or in the adult animal. Likewise, the absence of NDST1 or NDST2 did not trigger any transcription of NDST3 or NDST4. We could also demonstrate that the level of expression of the NDST1 or NDST2 transcript was not influenced by the absence of the other NDST transcript (Fig. 1). This means that, in NDST1−/−...
liver, the only NDST transcript present is NDST2, and its level of expression is the same as that in control liver.

Also, we can conclude that NDST2, in addition to producing highly sulfated heparin in mast cells (20), is capable of synthesizing HS in vivo. The polysaccharide produced in NDST1−/− embryonic day 18.5 liver contains spaced blocks of N-sulfated residues characteristic of HS (Fig. 2) further modified by the 2-O- and 6-O-sulfotransferases and the C5 epimerase (Fig. 3). Similar results have also been reported for HS synthesized by endothelial cells deficient in NDST1 (29) as well as in various tissues obtained from NDST1−/− embryonic day 18.5 embryos (19). However, any compensatory expression of other NDSTs was not analyzed in these studies. Notably, because also the transcripts of the other biosynthesis enzymes were expressed at the same level in control and NDST1−/− embryo liver (Fig. 1B), we conclude that the absence of NDST1 or lowered levels of N-, 2-O-, and 6-O-sulfation or IdoA in the HS produced (Figs. 2 and 3) do not trigger compensating expression of HS-modifying enzymes in liver.

The finding that NDST2 is active in HS biosynthesis in the absence of NDST1 does not necessarily imply that it is present in active form in control animals. Results from Grobe and Esko (14) have shown that the expression of NDSTs as well as other HS biosynthesis enzymes may be largely controlled at the translational level. Maybe NDST2 mRNA is just translated in the absence of NDST1. However, as shown in Fig. 4, enzymatically active similar amounts of NDST2 activity were present in control, NDST1−/−, and NDST1+/− liver extracts, indicating that no compensatory increase in the level of enzymatically active NDST2 occurs when NDST1 is absent. From the obtained data, it is apparent that the two alleles of NDST1 contribute with apparently similar but differed from the embryonic samples by lacking the 1.6-kb transcript and containing more of the 4-kb band.

This was the case also in the blot presented in Fig. 1B, where four major hybridizing transcripts could be identified. Comparing the hybridization patterns, it was apparent that the three samples from embryonic day 18.5 liver looked the same. The two samples from adult tissue were also similarly but differed from the embryonic samples by lacking the 1.6-kb transcript and containing more of the 4-kb band.

It has previously been shown that 6-O-sulfotransferase-3 probes hybridize with several transcripts of different sizes (1.6, 2.0, 2.8, 4.0, and 6.5 kb; (38)). This was the case also in the blot presented in Fig. 1B, where four major hybridizing transcripts could be identified. Comparing the hybridization patterns, it was apparent that the three samples from embryonic day 18.5 liver looked the same. The two samples from adult tissue were also similarly but differed from the embryonic samples by lacking the 1.6-kb transcript and containing more of the 4-kb band.

3 It has previously been shown that 6-O-sulfotransferase-3 probes hybridize with several transcripts of different sizes (1.6, 2.0, 2.8, 4.0, and 6.5 kb; (38)). This was the case also in the blot presented in Fig. 1B, where four major hybridizing transcripts could be identified. Comparing the hybridization patterns, it was apparent that the three samples from embryonic day 18.5 liver looked the same. The two samples from adult tissue were also similarly but differed from the embryonic samples by lacking the 1.6-kb transcript and containing more of the 4-kb band.

So what limits the level of HS N-sulfation if not the total amount of active NDST enzymes? Characterization of HS produced by mice deficient in the 2-O-sulfotransferase and the C5 epimerase, respectively, has shown that HS N-sulfation is increased in these mice (32, 33). These observations do not agree with the accepted model for HS biosynthesis where NDSTs, through N-sulfation, create the substrate for subsequent modifications (4). The presence of 6-O-sulfate groups in HS devoid of N-sulfate groups (16) also indicates that the order in which the reactions occur sometimes may be different. The presence of a GAGosome (1), an enzyme complex composed of the different biosynthesis enzymes, could facilitate a more flexible modification where the NDST enzyme could act on the same region of the polysaccharide substrate both before and after modification by the C5 epimerase and the O-sulfotransferases. In this manner, also the substrate specificity of the NDST enzymes will be important for the outcome of biosynthesis. Previous results have indicated that HS is a better substrate than the unsulfated bacterial polysaccharide K5 for both NDST1 and NDST2 (34), supporting the notion that NDSTs may act on previously modified substrates. This would also be possible, as previously suggested, if different NDST isoforms localized to different Golgi compartments. However, the presence of a GAGosome with a higher affinity for NDST1 could tentatively explain why the lack of NDST2 in livers from knockout mice does not result in any structural changes in the HS produced. Previous work has shown that EXT1 and EXT2 form a complex that is the active polymerase in HS biosynthesis (35, 36). The 2-O-sulfotransferase and the C5 epimerase have also been shown to interact (37). Preliminary results in our laboratory further indicate that NDST1 can
Liver Heparan Sulfate Biosynthesis

...occur bound to EXT2. Here we hypothesize that incorporation of NDST enzymes into the tentative GAGosome with a limited number of sites for NDST enzymes will determine whether the enzyme takes part in HS modification or not (Fig. 6). In the control mice, NDST2 is "frozen out" of the enzyme complex by NDST1, which more readily takes the NDST position. In the absence of NDST1, NDST2 is incorporated into the complex and takes on the NDST position. In the absence of NDST1, NDST2 is incorporated into the tentative GAGosome with a...  

Acknowledgments—We thank Emanuel Smeds and Jin-ping Li for cDNA-probes and Mats Olsson for helpful discussions.

REFERENCES

1. Esko, J. D., and Selleck, S. B. (2002) Annu. Rev. Biochem. 71, 435–471  
2. Salminvirta, M., Lidholt, K., and Lindahl, U. (1996) FASEB J. 10, 1270–1279  
3. Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. J., and Zhang, L. (1997) J. Clin. Investig. 99, 2062–2070  
4. Lindahl, U., Kusche-Gullberg, M., and Kjellen, L. (1998) J. Biol. Chem. 273, 24979–24982  
5. SAFAIYAN, F., Lindahl, U., and Salminvirta, M. (2000) Biochemistry 39, 10823–10830  
6. van Kuppevelt, T. H., Dennissen, M. A., van Venrooij, W. J., Hoet, R. M., and Veerkamp, J. H. (1998) J. Biol. Chem. 273, 12960–12966  
7. Brickman, Y. G., Ford, M. D., Gallagher, J. T., and Hjertson, E. (1998) J. Biol. Chem. 273, 4350–4359  
8. Kato, M., Wang, H., Bernfield, M., Gallagher, J. T., and Turnbull, J. E. (1994) J. Biol. Chem. 269, 18881–18890  
9. Zhao, M., Dong, J., Goldberger, O., Bernfield, M., Gallagher, J. T., and Deakin, J. A. (2003) J. Biol. Chem. 278, 13561–13569  
10. Grobe, K., Ledin, J., Ringvall, M., Holmborn, K., Forsberg, E., Esko, J. D., and Kjellen, L. (2000) Biochim. Biophys. Acta 1573, 209–215  
11. Esko, J. D., and Lindahl, U. (2001) J. Clin. Investig. 108, 169–173  
12. Rong, J., Habuchi, H., Kimata, K., Lindahl, U., and Kusche-Gullberg, M. (2001) Biochemistry 40, 5548–5555  
13. Smeds, E., Habuchi, H., Do, A. T., Hjertson, E., Grundberg, H., Kimata, K., Lindahl, U., and Kusche-Gullberg, M. (2003) Biochem. J. 372, 371–380  
14. Grobe, K., and Esko, J. D. (2002) J. Biol. Chem. 277, 30699–30706  
15. Pikas, D. S., Eriksson, I., and Kjellen, L. (2000) Biochemistry 39, 4522–4558  
16. Holmborn, K., Ledin, J., Smeds, E., Eriksson, I., Kusche-Gullberg, M., and Kjellen, L. (2004) J. Biol. Chem. 279, 42355–42358  
17. Kusche-Gullberg, M., Eriksson, I., Pikas, D. S., and Kjellen, L. (1998) J. Biol. Chem. 273, 11902–11907  
18. Aikawa, J., Grobe, K., Tsujimoto, M., and Esko, J. D. (2001) J. Biol. Chem. 276, 5876–5882  
19. Ledin, J., Staatz, W., Li, J. P., Gotte, M., Selleck, S., Kjellen, L., and Spillmann, D. (2004) J. Biol. Chem. 279, 42732–42741  
20. Forsberg, E., Pejer, G., Ringvall, M., Lunderius, C., Tomasini-Johansson, B., Kusche-Gullberg, M., Eriksson, I., Ledin, J., Hellman, L., and Kjellen, L. (1999) Nature 400, 773–776  
21. Ringvall, M., Ledin, J., Holmborn, K., van Kuppevelt, T., Ellin, F., Eriksson, I., Olsson, A. M., Kjellen, L., and Forsberg, E. (2000) J. Biol. Chem. 275, 25926–25930  
22. Schulze, B., Mann, K., Battistutta, R., Wiedemann, H., and Timpl, R. (1995) Eur. J. Biochem. 231, 551–556  
23. Vecchi, A., Garlanda, C., Lampugnani, M. G., Resnati, M., Matteucci, C., Stoppacciaro, A., Schnurch, H., Risau, W., Rojo, L., Mantovani, A., and Dejana, E. (1994) Eur. J. Cell Biol. 63, 247–254  
24. Cheung, W. F., Eriksson, I., Kusche-Gullberg, M., Lindahl, U., and Kjellen, L. (1996) Biochemistry 35, 5250–5256  
25. Shively, J. E., and Conrad, H. E. (1976) Biochemistry 15, 3932–3942  
26. Riesenfeld, I., Hozok, M., and Lindahl, U. (1982) J. Biol. Chem. 257, 7050–7055  
27. Maccarana, M., Casu, B., and Lindahl, U. (1993) J. Biol. Chem. 268, 23898–23905  
28. Pettersson, I., Kusche, M., Unger, E., Wlad, H., Nylund, L., Lindahl, U., and Kjellen, L. (1991) J. Biol. Chem. 266, 8044–8049  
29. Wang, L., Fuster, M., Sriaramarao, P., and Esko, J. D. (2005) Nat. Immunol. 6, 902–910  
30. Grobe, K., Inatani, M., Pallerla, S. R., Castagnola, J., Yamaguchi, Y., and Esko, J. D. (2005) Development (Camb.) 132, 3777–3786  
31. Humphries, D. E., Sullivan, B. M., Aleixo, M. D., and Stow, J. L. (1997) Biochem. J. 325, 351–357  
32. Merry, C. L., Bullock, S. L., Swan, D. C., Backen, A. C., Lyon, M., Beddington, R. S., Wilson, V. A., and Gallagher, J. T. (2001) J. Biol. Chem. 276, 35429–35434  
33. Li, J. P., Gong, F., Hagner-McWhirter, A., Forsberg, E., Abrink, M., Kishlevsky, R., Zhang, X., and Lindahl, U. (2003) J. Biol. Chem. 278, 28363–28366  
34. Van Den Born, J., Pikas, D. S., Pisa, B. J., Eriksson, I., Kjellen, L., and Berden, J. H. (2003) Glycobiology 13, 1–10  
35. McCormick, C., Duncan, G., Goutsos, K. T., and Tufaro, F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 668–673  
36. Senay, C., Lind, T., Muguruma, K., Tone, Y., Kitagawa, H., Sugahara, K., Lidholt, K., Lindahl, U., and Kusche-Gullberg, M. (2000) EMBO Rep. 1, 282–286  
37. Pinhal, M. A., Smith, B., Olson, S., Aikawa, J., Kimata, K., and Esko, J. D. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12984–12989  
38. Habuchi, H., Tanaka, M., Habuchi, O., Yoshida, K., Suzuki, H., Ban, K., and Kimata, K. (2000) J. Biol. Chem. 275, 2859–2868

4. J. Presto, M. Thuveson, I. Eriksson, M. Busse, M. Kusche-Gullberg, and L. Kjellen, manuscript in preparation.  
5. M. Rahmanian, M. Thuveson, J. Presto, and L. Kjellen, unpublished data.