Targeted Disruption of a Murine Glucuronyl C5-epimerase Gene Results in Heparan Sulfate Lacking 1-Iduronic Acid and in Neonatal Lethality*

Received for publication, May 23, 2003, and in revised form, June 4, 2003
Published, JBC Papers in Press, June 4, 2003, DOI 10.1074/jbc.C300219200

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The glycosaminoglycan, heparan sulfate (HS), binds proteins to modulate signaling events in embryogenesis. All identified protein-binding HS epitopes contain 1-iduronic acid (IdoA). We report that targeted disruption of the murine β-glucuronyl C5-epimerase gene results in a structurally altered HS lacking IdoA. The corresponding phenotype is lethal, with renal agenesis, lung defects, and skeletal malformations. Unexpectedly, major organ systems, including the brain, liver, gastrointestinal tract, skin, and heart, appeared normal. We find that IdoA units are essential for normal kidney, lung, and skeletal development, albeit with different requirement for 2-O-sulfation. By contrast, major early developmental events known to critically depend on heparan sulfate apparently proceed normally even in the absence of IdoA.

The sulfated polysaccharide, heparan sulfate (HS), is a ubiquitous component of proteoglycans on cell surfaces and in the extracellular matrix. HS chains interact with a variety of proteins, including growth factors/morphogens, cytokines, enzymes, extracellular matrix proteins, and thus modulate important processes in development and homeostasis (1, 2). Selective protein binding is mediated by saccharide domains containing sulfate groups in specific patterns, along with l-iduronic acid (IdoA) units that promote ligand apposition through their conformational flexibility (3). The biosynthesis of HS is initiated by polymerization of D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) residues in alternating sequence and is pursued through a series of modification reactions that include N-deacetylation/N-sulfation of GlcNAc, C5-epimerization of GlcA to the C5-epimer, IdoA, and, finally O-sulfation at various positions (4). Mutational studies in Drosophiila and targeted disruption of murine genes involved in HS homologous recombination have demonstrated a critical role for HS proteoglycans in developmental processes (5–7). The observed phenotypes vary dramatically in severity, from failure to gastrulate (8) to selective disturbance of mast cell function in otherwise seemingly healthy mice (9). Some of the biosynthetic enzymes occur in multiple isoforms with potentially different functions and thus different deletion phenotypes (9–11). IdoA occurs in all protein-binding HS domains so far identified. Here we show that mouse embryos lacking GlcA C5-epimerase synthesize an abnormal HS, with GlcA but no IdoA residues and a highly distorted sulfation pattern. The resulting phenotype is lethal, with defects that can be differentially ascribed to HS structural alterations. However, it also shows apparently normal organ development, believed to depend on selective HS-protein interactions. These findings raise intriguing questions as to the need for regulation in HS biosynthesis.

EXPERIMENTAL PROCEDURES

Gene Targeting Construct—A 19-kb genomic clone containing 3’-untranslated region and 3 exons of the GlcA C5-epimerase gene (Hsepi) was isolated from a bacteriophage mouse (strain 129/Sv) genomic library (Stratagene). Three SacI fragments of this genomic clone were separately cloned into Bluescript plasmid (12). A 2-kb fragment immediately upstream of exon 3 was inserted downstream of a PGK-neo cassette as a short arm homologous sequence to the endogenous gene, and a 4-kb fragment downstream of exon 3 was inserted upstream of the PGK-neo cassette as a long arm of homology to the endogenous gene. The targeting vector construct had a total size of 11 kb.

Homologous Recombination in ES Cells and Generation of Hsepi-deficient Mice—The targeting vector was linearized by restriction enzyme NotI and electroprotoplotted into R1 embryonic stem (ES) cells. Clones expressing the neo-resistant gene were selected by including G418 (350 µg/ml; Invitrogen) in the cell cultures and analyzed for target gene homologous recombination. The clone showing homologous recombination was detected by Southern blot analysis using an external 700-bp fragment as probe (Fig. 1A). The positive clone was injected into C57BL/6 blastocysts, and chimeric male founder mice were crossed with C57BL/6 females. The offspring was genotyped for the mutation by tail biopsies or by PCR. Genomic DNA from ES cells and tail biopsies were isolated as described previously (14) and digested with BglIII and SaI. The resulting fragments were separated in agarose gel and subsequently blotted onto a nylon membrane, followed by hybridization with a 700-bp external probe as shown in Fig. 1A. The PCR amplification was performed using the following primers: 5’-AGTGT-
C-terminal amino acid residues of the 618-residue protein. Following electroporation, homologous recombination in the Hsepi gene was found in one ES cell clone, identified by Southern blot analysis, with no additional sites of integration (not shown). Microinjection of this clone into C57BL/6 blastocysts yielded chimeric animals and germ line transmission. No overt defects were seen in heterozygous animals.

Genotype analysis of offspring from intercrosses between heterozygous mice (Fig. 1B) showed essentially Mendelian heritance (22% wild type, 57% heterozygous, 21% homozygous; n = 168), indicating no early embryonic death. The offspring of heterozygous intercrosses was born on d.p.c. 18.5. Wild type (Hsepi+/−) and heterozygous (Hsepi+/−) littermates showed no aberrant phenotype and were fertile, with a lifespan of more than one year. By contrast, Hsepi−/− pups died immediately after birth, apparently from respiratory failure. Analysis of embryo (d.p.c. 18.5) extracts demonstrated that Hsepi−/− pups lacked detectable GlcA C5-epimerase activity, whereas heterozygotes showed decreased activity compared with wild type animals (Fig. 1C).

HS from Hsepi−/− Mice Lacks IdoA—HS from Hsepi+/+, Hsepi+/−, and Hsepi−/− embryos (d.p.c. 17.5–18.5) was analyzed by composition, with particular regard to the occurrence of IdoA. Samples were cleaved to disaccharides by exhaustive deamination with nitrous acid, and the products were reduced with NaBH4 and analyzed by anion-exchange HPLC (Fig. 2A). Notably, all three samples showed the same overall proportions of non-O-sulfated versus O-sulfated 3H-labeled disaccharide units (Table I). The Hsepi+/+ and Hsepi−/− patterns of O-sulfated disaccharides were indistinguishable and typical of normal HS, with a predominance of mono-O- and di-O-sulfated, IdoA-containing species. By contrast, these disaccharides were completely absent from the Hsepi−/− samples, which instead showed increased proportions of GlcA-containing species, including a disaccharide with 2-O-sulfated GlcA (Fig. 2A; Table 1). Gel chromatography of metabolically [3H]GlcN-labeled HS indicated similar chain length for the various samples (not shown), whereas the Hsepi−/− polysaccharide emerged more retarded than the corresponding Hsepi+/+ and Hsepi−/− products on anion-exchange chromatography (Fig. 2B). This difference in charge density was reflected by changes in N-substitution pattern, as demonstrated by size analysis of fragments obtained by selective deaminative cleavage of the HS chains at N-sulfated glucosamine residues (Fig. 2C). The proportion of disaccharides, derived from contiguous N-sulfated domains in mutant HS, was increased relative to wild type HS, whereas that of intermediate sized (4- to 14-mer) fragments was decreased. Total N-sulfation was calculated to 51 and 43% of disaccharide units in Hsepi−/− and Hsepi+/+ HS, respectively. These findings indicate a change in HS structure, from a largely intermixed distribution of N-substituents to a pattern of extended N-sulfated domains, along with a switch from -IdoA(2-OSO3)GlcN(-SO3)- in wild type HS, to -GlcA-GlcNOSO3(6-OSO3)- in mutant HS.

RESULTS AND DISCUSSION

Targeted Disruption of Hsepi Results in Neonatal Lethality with Respiratory Failure—The GlcA C5-epimerase is encoded by a single Hsepi gene with three exons and accounts for the formation of IdoA units in HS biosynthesis (12, 13). To assess the functional significance of IdoA residues in HS, we have generated Hsepi-deficient mice by gene targeting in ES cells. A targeting vector was constructed to create a functional mutation by deletion of exon 3 (Fig. 1A) corresponding to the 341
cage and sternum and often cleft palate (not shown). However, the brain, heart, liver, gastrointestinal tract, pancreas, and skin all appeared normal. Given the recognized importance of HS in early embryonic patterning and morphogenesis (5, 6), the selective nature of the IdoA-deficient phenotype, albeit severe, was unexpected. The requirements for defined HS structure in several functionally important interactions thus would not seem to include the presence of IdoA units.

Comparison of the numerous defects in the Hsepi<sup>−/−</sup> mice with phenotypes due to elimination of other enzymes involved in HS biosynthesis shows similarities as well as differences. HS synthesized by mice deficient in N-deacetylase/N-sulfotransferase isoform 1 (NDST-1) is low in N-sulfate, hence in IdoA (sulfated and non-sulfated) and total O-sulfate compared with

![Fig. 2. Structural analysis of HS from embryonic mice.](image)

**A**, HS samples were N-deacetylated by hydrazinolysis and treated with HNO<sub>2</sub> at pH 1.5 and 3.9 to convert the chains to disaccharides, which were subsequently reduced with NaB<sub>3</sub>H<sub>4</sub>. The 3H-labeled (metabolically and chemically) disaccharides were analyzed by anion-exchange HPLC on a Partisil-10 SAX column. Peaks represent: 1, GlcA-aMan<sub>R</sub> and IdoA-aMan<sub>R</sub> (separately resolved by paper chromatography); 2, GlcA(2-OSO<sub>3</sub>)-aMan<sub>R</sub>; 3, GlcA-aMan<sub>R</sub>(6-OSO<sub>3</sub>); 4, IdoA-aMan<sub>R</sub>(6-OSO<sub>3</sub>); 5, IdoA(2-OSO<sub>3</sub>)-aMan<sub>R</sub>; 6, IdoA(2-OSO<sub>3</sub>)-aMan<sub>R</sub>(6-OSO<sub>3</sub>). The aMan<sub>R</sub> ([1-3H]anhydromannitol) residues are derived from GlcN units (N-acetylated or N-sulfated) in intact HS. Upper panel (+/+), HS from wild type embryos; lower panel (+/−), HS from mutant embryos.

**B**, anion-exchange chromatography (DEAE-Sephacel, linear salt gradient) of metabolically 3H-labeled HS from Hsepi<sup>−/−</sup> (open circles), Hsepi<sup>+/−</sup> (closed circles), and Hsepi<sup>+/+</sup> (open triangles) embryos. C, gel chromatography on Bio-Gel P-10 (1.3 × 185 cm column) of products obtained on deamination at pH 1.5 (selective cleavage at N-sulfated GlcN units) of metabolically 3H-labeled HS from Hsepi<sup>−/−</sup> (upper panel) and Hsepi<sup>+/−</sup> (lower panel) embryos. The sizes of even-numbered oligomers are indicated above the peaks in the upper panel. N-Acetyl/N-sulfate ratios were calculated based on peak areas. The material in the peaks corresponding to the excluded volume (42–45 ml) is resistant to heparitinase I, thus presumably not related to HS.

**D**, structures of the predominant N- and O-sulfated disaccharide units found in wild type (Hsepi<sup>+/+</sup>) (upper structure) and in mutant (Hsepi<sup>−/−</sup>) (lower structure) HS.

**Table I**

Composition of disaccharides obtained upon deaminative cleavage of HS isolated from embryonic (d.p.c 18.5) animals

Disaccharides were analyzed as described in the legend to Fig. 2. Results are expressed to account for the hexuronic acid composition of -GlcA/IdoA-2R-GlcNR<sub>1</sub>6R-disaccharide units, in which R<sub>1</sub> is –H or –SO<sub>3</sub> and R<sub>6</sub> is –SO<sub>3</sub> or –COCH<sub>3</sub>.

| Hsepi genotype | Disaccharide units | Non-O-sulfated | 2-O-Sulfated | 6-O-Sulfated | 2,6-O-Sulfated |
|----------------|--------------------|----------------|--------------|--------------|---------------|
|                | GlcA        | IdoA         | GlcA        | IdoA         | GlcA        | IdoA         | GlcA        | IdoA         | GlcA        | IdoA         |
| +/+            | 61          | 9.9          | 1.0         | 11           | 5.8         | 4.5          | ND          | 6.5          |
| +/−            | 60          | 9.6          | 0.4         | 13           | 5.7         | 4.7          | ND          | 6.6          |
| −/−            | 69          | 9.6          | 3.3         | ND           | 28          | ND           | ND          | ND           |

* Not determined.
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Fig. 3. Phenotype display of Hsepi−/− and Hsepi+/+ embryos born at d.p.c. 18.5. A, dissected urgenital systems of wild type (+/+), and homozygous mutant (−/−) male embryos. a, adrenal gland; b, bladder; k, kidney; t, testis. Note complete absence of kidneys in the mutant embryo. B, lungs from −/− mutants are immature and poorly inflated, with thickened, cell-rich interalveolar septa, compared with wild type +/+ lungs. Tissues were fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin. C, iris coloboma of mutant mice. D, skeleton samples were stained with alcin blue-alizarin red to distinguish ossified tissue (red) from cartilage (blue). Multiple abnormalities are seen, as described under "Results and Discussion." E and F, all examined Hsepi−/− embryos (n = 4) lacked the two proximal phalanges of the fore paws (E) and the inner phalanx of hind paws (F; arrow indicates tarsal bones). All Hsepi−/− embryos showed twisted tail (G) and polydactyly on one or both forelimbs (H).

The present study was supported by grants to M.K.-G. from the Swedish Research Council, the Swedish Cancer Society, and the Swedish Governmental Agency for Work Environment and Working Life. We thank Marion Kusche-Gullberg, Dorothe Spillmann, Per Lindahl, and Alan McWhiter for critical reading of the manuscript. The technical assistance of Lena Nylund is acknowledged.

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* M. Kusche-Gullberg, personal communication.
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J. Biol. Chem. 2003, 278:28363-28366.
doi: 10.1074/jbc.C300219200 originally published online June 4, 2003

Access the most updated version of this article at doi: 10.1074/jbc.C300219200

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