Embryonic Fibroblasts with a Gene Trap Mutation in Ext1 Produce Short Heparan Sulfate Chains*

Received for publication, November 19, 2003, and in revised form, April 14, 2004
Published, JBC Papers in Press, May 25, 2004, DOI 10.1074/jbc.M312624200

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Mutational defects in either EXT1 or EXT2 genes cause multiple exostoses, an autosomal hereditary human disorder. The EXT1 and EXT2 genes encode glycosyltransferases that play an essential role in heparan sulfate chain elongation. In this study, we have analyzed heparan sulfate synthesized by primary fibroblast cell cultures established from mice with a gene trap mutation in Ext1. The gene trap mutation results in embryonic lethality, and homozygous mice die around embryonic day 14. Metabolic labeling and immunohistochemistry revealed that Ext1 mutant fibroblasts still produced small amounts of heparan sulfate. The domain structure of the mutant heparan sulfate was conserved, and the disaccharide composition was similar to that of wild type heparan sulfate. However, a dramatic difference was seen in the polysaccharide chain length. The average molecular sizes of the heparan sulfate chains from wild type and Ext1 mutant embryonic fibroblasts were estimated to be around 70 and 20 kDa, respectively. These data suggest that not only the sulfation pattern but also the length of the heparan sulfate chains is a critical determinant of normal mouse development.

Heparan sulfate proteoglycans (HSPGs) are composed of one or more linear HS polysaccharide chains covalently bound to selected serine residues in a protein core. HSPGs are ubiquitously present at cell surfaces and in the extracellular matrix, affecting a variety of biological processes, including specific signaling pathways (1–5). The constituent sulfated HS polysaccharide chains exert their biological functions by interacting with a multitude of proteins in differential and specific fashion. The critical roles of HS structure in normal growth and development have become evident using model animals including *Drosophila* and mouse (4, 5).

The biosynthesis of HSPG is a multistep process involving the concerted action of several enzymes and includes (a) formation of the polypeptide core; (b) assembly of the polysaccharide-protein linkage region; (c) generation of a polymer consisting of alternating GlcA and GlcNAc residues; and (d) a series of modification reactions by which N- and O-sulfate groups are introduced and a fraction of the GlcA residues are C5-epimerized to L-iduronic acid (IdoUA) units. The HS-protein linkage region consists of the tetrasccharide: glucuronic acid-galactose-galactose-xylose (GlcA1–3Gal1–3Gal1–4Xyl), where xylose is linked to a serine residue in the protein core. Separate enzymes catalyze the incorporation of the different sugar units (6). Transfer of a single GlcNAc unit to the tetrasccharide linkage region initiates HS assembly. The alternating addition of GlcA and GlcNAc, from their respective UDP-derivatives, to the nonreducing terminus of the growing polymer thus forms the (GlcA-GlcNAc)n HS chain, which is modified through sulfation and GlcA C5-epimerization reactions (reviewed in Refs. 7–9). The regulation of this process, as required to generate specific saccharide structures, is poorly understood but apparently involves arrays of enzyme isoforms that differ with regard to substrate specificities and kinetic properties.

Hereditary multiple exostoses is an autosomal dominant skeletal disorder characterized by the presence of cartilage capped bony outgrowths mainly located at the juxtaepiphyseal region of the long bones (10). Genetic linkage studies in families with hereditary multiple exostoses disclosed two main loci, EXT1 on chromosome 8q24.1 and EXT2 on chromosome 11p11-p12 (11, 12). Although linkage to another locus, EXT3, on chromosome 19p has been described (13), the EXT2 gene has not yet been isolated, and genetic linkage to this locus has only been detected in a few pedigrees to date. Together with EXT1 and EXT2, three additional members, designated EXT1L1, EXT1L2, and EXT1L3, form the exostin (EXT) gene family (14–16). The EXT as well as EXT1L proteins show sequence homology especially in the C-terminal regions. However, there is no evidence that defects in the EXT1L genes result in hereditary multiple exostoses. Despite extensive genetic characterization, the function of the EXT proteins remained unknown until 1998, when two independent studies revealed the connection between HS-synthesizing glycosyltransferases and the EXT gene family (17, 18). An ~70-kDa enzyme committed to HS polymer formation was isolated from bovine serum, and analysis of the corresponding cDNA showed that the isolated protein was identical to EXT2 (17). Transfection of EXT1 into an HS-deficient L-cell mutant restored the ability to synthesize

* This work was supported by Swedish Medical Research Council, Konung Gustav V 80-års Fond Grant 13401, the Human Frontier Science Program, Polysaccharidforskning AB (Uppsala, Sweden), the Science Research Promotion Fund from the Japan Private School Promotion Foundation, and Grants-in-aid for Encouragement of Young Scientists. The abbreviations used are: HSPG, heparan sulfate proteoglycan; HS, heparan sulfate; Ext, hereditary multiple exostosis gene; Ext1 (Ext1/2), mice homozygous for a gene trap mutation in Ext1; IdoUA, L-iduronic acid; α-Man2, 2,5-anhydro-α-mannitol (formed by reduction of terminal 2,5-anhydromannose residues with NaBH4); αHexA, 4,5-unsaturated hexorionate; nt, nucleotide; RT, reverse transcriptase; HPLC, high performance liquid chromatography.

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1 The abbreviations used are: HSPG, heparan sulfate proteoglycan; HS, heparan sulfate; Ext, hereditary multiple exostosis gene; Ext1/2, mice homozygous for a gene trap mutation in Ext1; IdoUA, L-iduronic acid; α-Man2, 2,5-anhydro-α-mannitol (formed by reduction of terminal 2,5-anhydromannose residues with NaBH4); αHexA, 4,5-unsaturated hexorionate; nt, nucleotide; RT, reverse transcriptase; HPLC, high performance liquid chromatography.
HS, suggesting that chain elongation may involve both EXT1 and EXT2 (18).

Recently, more detailed functional data on the role of the EXT protein family members in HS biosynthesis has been provided by several studies. EXT1 and EXT2 form a hetero-oligomeric complex in vivo that is accumulated in the Golgi apparatus (19, 20). The Golgi-localized EXT1-EXT2 protein complex possesses substantially higher glycosyltransferase activity than EXT1 alone, suggesting that this complex represents the biologically relevant form of the HS polymer modification unit (20, 21). Mutational analysis of CHO cells defective in EXT1 demonstrated that the GlcA-transferase catalytic domain is localized to the N-terminal region of the EXT1 protein (22). No mutations in EXT1 affecting only GlcNAc-transferase activity have yet been found. Less is known about the domain organization of EXT2 that has been shown to have both GlcA- and GlcNAc transferase activities when expressed in COS-7 cells and in yeast (17, 21). Interestingly, the three other members of the EXT gene family, EXT1L1, EXT2L2, and EXT3 also encode glycosyltransferases, which are likely to be involved in HS biosynthesis (23, 24). Furthermore, mutations in the Drosophila orthologs of EXT2 and EXT3 (sister of tout-velu [sotv] and brother of tout velu [botv], respectively) seriously diminish Drosophila growth factor signaling.

These findings may have important implications for our understanding of the role of the EXT protein family members in HS biosynthesis has been suggested by the observations that HS synthesis is abolished in Ext1-deficient embryonic stem cells (27) and in Drosophila bearing a mutation in tout-velu, the orthologue of human EXT1 (28). Ext1-deficient mice generated by gene targeting fail to gastrulate, lack HS, and die by embryonic day 8.5 (27). In contrast, Ext1 mutant mice generated by the gene trap method (Ext1Gt/GtEXTMox; G68Wv, ExtGtyo) survive to embryonic day 14.5 and have a much less severe phenotype (29). The longer survival time of the ExtGtyo mice suggested that the gene trap insertion created a hypomorphic allele of Ext1. To study the effect of the gene trap disruption of Ext1 on HS polymerization, we have studied the structural properties of HS produced by fibroblasts from ExtGtyo embryos. Our results show that sulfated HS chains are still produced, albeit much less than normally. The decrease in HS was mainly due to reduced chain length. The overall HS sulfation pattern was moderately affected by the mutation. HS is required for growth factor signaling and diffusion, and the embryonic lethal phenotype of ExtGtyo may reflect the inability of the mutant HS to mediate some of the HS-dependent steps in embryogenesis. These findings may have important implications for our understanding of the structural requirements of HS in regulating growth factor signaling.

EXPERIMENTAL PROCEDURES

Materials—Heparinase (EC 4.2.2.8), heparitinase (EC 4.2.2.7), and chondroitinase ABC (EC 4.2.2.4) were obtained from Seikagaku Corp., Tokyo. n-Bromo-5-[3H]GlCN (24 Ci/mmol) and Na2[35S]O4 were from PerkinElmer Life Sciences, t-Bromo-4-[3H]Galactose (0.1 Ci/mmol), [a-32P]dCTP, DEAE-Sepharose, PD-10 columns containing Sephadex G-25 (medium), and Superdex 30 columns were from Amersham Biosciences. HexA-D-[14C]ManP, disaccharides, with and without O-sulfate groups in different positions, used as reference compounds were as described (30). Polysaccharide standards for estimations of molecular mass values were derived from heparin (3.8 and 8.6 kDa) and from hyaluronan (19, 30, 43, and 210 kDa) as described (31). The generation of Ext1-deficient mice was performed as described (29).

Northern Blot Analysis—Total RNA from mouse embryonic fibroblasts was obtained using the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. Denatured RNA was electrophoresed on 1.2% agarose-formaldehyde, transferred to a nylon membrane (Hybond-N; Amersham Pharmacia Biotech), and hybridized at three occa-

sions with probes labeled with [a-32P]dCTP using Ready-To-Go DNA labeling beads (Amersham Biosciences). Each lane of the blot contained 10 µg of total RNA. The probes used were a 0.55-kb fragment (nt 862–1407) of the mouse Ext1 cDNA, a 0.53-kb fragment (nt 1938–2464) of the mouse Ext1 cDNA, and a 0.39-kb fragment (nt 777–1163) of the mouse Ext2 cDNA. Unincorporated [a-32P]dCTP was removed with a Microspin S-300 HR column (Amersham Biosciences). The filters were hybridized at 68 °C in ExpressHyb solution (Clontech) first with the 550-bp Ext1 fragment, subsequently with the 530 bp Ext1 fragment, and then with the mouse Ext2 cDNA probe.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis—Total RNA from mouse embryonic fibroblasts was isolated as described above, and 0.8 µg of each preparation were used for RT-PCR (32). The RT-PCR was performed using primers specific for exons 4 and 5 with following forward primer, 5’-TCTGGAGGATTGTTCGTC-3’ (nt 1939–1957); reverse primer, 5’-TAGCAGCTCTGTCAACAC-3’ (nt 2450–2468). The products were analyzed by electrophoresis in 1% (w/v) agarose gel. Assay of GlcNAc- and GlcA-transferase Activities—Oligosaccharide acceptors were prepared from a purified capsaicin polysaccharide of Echechichis coli K5, with the structure (GlcA[12]GlcNAc[14]). The polysaccharide was partially N-deacetylated by incubation in 2 µM NaOH for 40 min at 60 °C. The incubation mixture was cooled to room temperature, the pH was adjusted to 7.0, and the modified polysaccharide was desalted, lyophilized, and finally depolymerized by deaminative cleavage (pH 3.9 (32)). The generated fragments were reduced with NaBH4 yielding reducing 2,5-anhydromannitol (2,5-ManR) acceptors. The resulting oligosaccharides (GlcA-[GlcNAc-GlcA]-nManR) were size-separated on a column (2.5 × 140 cm) of Sephadex-G50 (superfine grade) in 0.5 M NH4HCO3. Fractions containing oligosaccharides larger than hexasaccharides were pooled and lyophilized. [GlcNAc-GlcA]-nManR acceptors were prepared by digestion of GlcA-[GlcNAc-GlcA]-nManR with bovine liver f-glucosidase (Sigma). To assay GlcNAc and GlcA transferase activity, protein preparations from wild type or Ext1Gt/Gt fibroblast cultures were incubated for 1 or 2 h at 37 °C with [3H]-labeled UDP-GlcA and [GlcNAc-GlcA]-nManR oligosaccharide acceptors (measuring GlcA-transferase activity) or with [3H]-labeled UDP-GlcNAc and GlcA-[GlcNAc-GlcA]-nManR acceptors (measuring GlcNAc-transferase activity). Labeled acceptors were isolated by gel chromatography on a Sephadex G-25 superfine column (1 × 22 cm) eluted with 0.2 M NH4HCO3, and quantified by scintillation counting. The protein concentration of cell homogenate was quantified using the BCA Protein Assay Reagent (Pierce) with bovine serum albumin as a standard.

Isolation of Metabolically Labeled HS—Fibroblast cells obtained from wild type and homozygous Ext1Gt/Gt animals (embryonic day 11.5) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 20% fetal calf serum and 1% penicillin G-streptomycin. At passage 3 or 4, cells were labeled with 200 µCi/ml Na2[35SO4], 50 µCi/ml [3H]galactose, or 50 µCi/ml [3H]glucosamine for 48 h. The medium was exchanged every 24 h and the cells were cultured at 37 °C with ice-cold PBS. The cell layer was solubilized with 50 ml Tris-HCl, pH 8.0, containing 150 mM NaCl and 1% Triton X-100 under gentle rocking at 4 °C for 4 h and centrifuged. To release the O-linked sugars from the protein core, the supernatants were treated with 0.5 % NaBH4, 0.5 µM NaOH at 4 °C overnight. The reactions were terminated by the addition of 4 M acetic acid to decompose excess NaBH4 and neutralized with 1 M NaOH. After centrifugation at 13,000 rpm for 10 min, the supernatants were applied to a Sephadex G-50 superfine column (1 × 145 cm) eluted with 0.2 µM NH4HCO3. Fractions eluting at the void volume were recovered, galactosaminoglycans were eliminated by chondroitinase ABC digestion, and resistant HS chains were recovered by phenol extraction (32). Alternatively, after precipitation, the supernatants were collected, treated with 0.5 µM NaOH at 4 °C overnight, and neutralized, and labeled glycosaminoglycans were isolated by anion exchange chromatography using DEAE-Sepharose chromato-

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a 10-cm dish were incubated with 2 ml of the culture medium containing 0.2 ml of the viral supernatant at 37 °C for 2 h, and then 8 ml of the growth medium was added. Colonies of transformed cells started to appear after 1.5–2 weeks. Immortalized cells were labeled with 50 μCi/ml [3H]glucosamine or 200 μCi/ml Na35SO4 for 48 h, and the resultant labeled HS was isolated as described above.

Labeled HSPGs present at the cell surface and in the extracellular matrix were isolated after labeling of immortalized cells with 50 μCi/ml [3H]glucosamine. After 24 h of labeling, the culture medium was removed and frozen. The cells were washed twice with PBS and then treated with 1 mg/ml trypsin (Sigma) for 10 min at 37 °C. The trypsin was then neutralized with 2 mg/ml trypsin inhibitor (Sigma). Cells were then centrifuged at 1,000 rpm for 10 min at 4 °C. The cell pellets were frozen for future use. The supernatants were treated with 0.5 × NaOH, and labeled HS chains were recovered by gel filtration as described above.

Enzymatic Digestions—Enzymatic digestion with a mixture of heparinase and heparitinase or chondroitinase ABC was carried out in 100 mM Tris-HCl buffer, pH 7.4, containing 3.3 mM CaCl2 and 0.1 mM bovine serum albumin or 50 mM Tris-HCl buffer, pH 8.0, containing 30 mM sodium acetate and 0.1 mg/ml bovine serum albumin, respectively. The digestion products were analyzed by gel filtration chromatography on a column of Sephadex G-50 superfine (0.55 × 95 cm) using 0.5 M NH4HCO3 as the eluent.

Structural Analysis of HS—The size of labeled HS chains prepared from wild type and Ext1Gt/Gt mouse embryonic fibroblasts was analyzed by gel chromatography on a Superose 6 column (Amersham Biosciences) eluted with 50 mM Tris-HCl, pH 8.0, containing 1.0 M NaCl, at a flow rate of 0.5 ml/min. Fractions were collected at 1-min intervals, and the radioactivity was monitored by liquid scintillation counting.

Nitrous acid treatment at pH 1.5 was conducted with 0.5 M HNO2 followed by reduction of the products with NaBH4, yielding an AmMan residue at the reducing end (32). Under these conditions, N-sulfated glucosamine units are selectively attacked, but N-acetylated glucosamine residues are not affected. The degradation products were size-fractionated by gel chromatography on a column of Sephadex G-150 (1 × 180 cm) eluted with 0.2 M NH4HCO3 or on Superdex 30 in 0.2 M NH4HCO3. For essentially complete depolymerization to disaccharides, labeled HS was chemically N-desacetylated by treatment with 70% (w/v) aqueous hydrazine (Fluka) containing 1% (w/v) hydrazine sulfate at 96 °C for 4 h, and the product was treated with nitrous acid at pH 1.5 and at pH 3.9 (35). Recovered labeled disaccharides were analyzed on a Whatman Partisil-10 SAX column eluted at a rate of 1 ml/min with K2HPO4 solutions of stepwise increasing concentration as described in the legend to Fig. 6.

Alternatively, the disaccharide composition of [3H]glucosamine-labeled HS was determined after depolymerization with a mixture of heparinase and heparitinase and separation on an amino-bound silica PA-03 HPLC column. Individual disaccharides were identified by co-chromatography with disaccharide standards as described previously (36).

RESULTS

HS Formation in Ext1 Mutant Embryos—It has been demonstrated that EXT1 plays an essential role in the HS polymerization process and that the formation of HS is abolished in mice with a targeted mutation in Ext1 (27). However, it has never been clarified whether the HS chain elongation process stops after the formation of the HS protein linkage region (GlcNAc-GlcA-Gal-Gal-Xyl) or at some later stage of HS synthesis. We have generated mutant mice with a lethal insertion in the gene encoding Ext1 (Ext1Gt/Gt) (29) using a gene trap strategy and generated fibroblasts from embryonic day 11.5 wild type and gene trap mutated Ext1Gt/Gt embryos.2 To examine the effect of the gene trap mutant Ext1 on the sequential addition of GlcA and GlcNAc contiguous to the HS-protein linkage region, wild type fibroblasts and Ext1Gt/Gt fibroblasts were metabolically labeled with [3H]galactose. During glycosaminoglycan biosynthesis, galactose is primarily incorporated into the linkage region. Galactose is also converted to UDP-GlcA and incorporated into the uronic acid units of glycosaminoglycans although less efficiently. Cell extracts were treated with alkali to liberate O-glycans including glycosaminoglycan-related polysaccharides from core proteins, and samples were subjected to gel filtration chromatography on a Sephadex G-50 column, to separate di-, tetra-, hexa-, and octasaccharide from large polysaccharides (data not shown). Both wild type and Ext1Gt/Gt fibroblasts incorporated very small amounts of [3H]galactose (less than 1% of isolated labeled O-linked sugars) at the elution position of a linkage pentasaccharide. Instead, the majority of the radioactivity was incorporated into larger polysaccharides containing material susceptible to specific HS-degrading enzymes, indicating that HS chain elongation had occurred also in the mutant cells.

To determine whether the Ext1Gt/Gt mice indeed synthesize HS chains, embryonic fibroblasts were metabolically labeled with [35S]sulfate or [3H]glucosamine. The yields of [35S]sulfate-labeled glycosaminoglycans were comparable between wild type cells (2.3 × 105 cpm/mg protein) and Ext1Gt/Gt cells (2.5 × 105 cpm/mg protein). Isolated 35S-labeled polysaccharides from wild type and Ext1Gt/Gt fibroblasts were subjected to selective degradation of chondroitin sulfate (chondroitinase ABC digestion) or HS (nitrous acid deamination), and the respective degradation products were analyzed by gel chromatography (Fig. 1). The mutant sample contained material susceptible to nitrous acid treatment, showing that Ext1Gt/Gt embryonic fibroblasts synthesize sulfated HS chains. The proportion of HS (i.e. material resistant to digestion by chondroitinase ABC but susceptible to nitrous acid deamination) decreased from ~65% of the 35S-labeled glycosaminoglycans in the wild type cells to ~35% in the Ext1Gt/Gt cells. When wild type and Ext1Gt/Gt cells were labeled with [3H]glucosamine, the incorporation of label into glycosaminoglycans was slightly reduced in Ext1Gt/Gt fibroblasts (2.65 × 105 cpm/mg protein) as compared with the wild type cells (3.1 × 105 cpm/mg protein). The proportion of HS in Ext1Gt/Gt and wild type cells was determined as 12 and 55%, respectively, of the 3H-labeled glycosaminoglycans (data not shown). Thus, the amount of 3H-labeled HS synthesized by Ext1Gt/Gt cells was ~18% of the HS produced by wild type cells.

The presence of HS in Ext1Gt/Gt cells was confirmed by indirect immunofluorescent staining using the HepSS-1 antibody that recognizes sulfated HS chains (37) and subsequent confocal microscopic examination. A dense staining for HS was observed (data not shown).

Analysis of Ext1 Gene Disruption—To investigate whether the gene trap insertion leads to a deficiency of the normal Ext1 transcript, the mRNA level was analyzed by Northern blotting. The gene trap mutation is inserted in the intron between exons 1 and 2 in the Ext1 gene (Fig. 2). As a result of the insertion, a fusion transcript is generated containing part of the Ext1 mRNA (exon 1) fused to the gene trap vector sequence (Fig. 2).
To characterize the Ext1 mRNA expression, two different probes A and B (corresponding to nucleotides 862–1407 and 1939–2464 in the mouse Ext1 cDNA sequence) were hybridized to RNA from wild type and Ext1/Gt/Gt cells (Fig. 3). A band of ~3.5 kb was detected in wild type fibroblasts (Fig. 3, A and B), consistent with the previously reported size of the Ext1 mRNA (38). In the Ext1/Gt/Gt cells, the putative fusion transcript (>9 kb) was detected by probe A (Fig. 3A). However, no transcript was detected using probe B, indicating that the gene was disrupted prior to the sequence corresponding to probe B (Fig. 3B). No differences in the expression levels of Ext2 were observed between wild type and Ext1/Gt/Gt fibroblasts. To use a more sensitive method, an RT-PCR amplification experiment was performed using primers F1 and R1 shown in Fig. 2. The expected 500-bp product, corresponding to nucleotides 1939–2468, was generated in the wild type but initially not in the Ext1/Gt/Gt sample (data not shown). However, using the PCR product mixture from the initial PCR as a template in an additional PCR resulted in the amplification of a 500-bp product also from the Ext1/Gt/Gt sample, suggesting that the mutant cells still produced small amounts of wild type transcript.

HS GlcNAc- and GlcA-transferase Activities—The alternating addition of GlcNAc and GlcA from their respective UDP-sugars generates HS chains. The two transferase activities were separately assayd at several different occasions using freshly prepared cell extracts from wild-type and Ext1/Gt/Gt embryonic fibroblast cell cultures (see “Experimental Procedures”). The specific GlcNAc- and GlcA-transferase activities, expressed on a cellular protein basis, varied between different cell extracts. However, even with variations between single extracts, the ratio of the activities in Ext1/Gt/Gt versus wild type cells was repeatedly ~0.15 for GlcNAc transfer and 0.1 for GlcA transfer (Table I).

Effect of Ext1 Gene Trap Mutation on HS Structure—The molecular size of the HS chains was analyzed by gel chromatography on Superox 6. A clearly reduced chain length of HS in Ext1/Gt/Gt fibroblasts was evident, with a complete lack of longer chains (Fig 4, A and B). Compared with the elution positions of size-defined hyaluronan and heparin standard polysaccharides, the peak elution positions of the rather broad peaks correspond to ~50–100 kDa for wild type HS and ~20 kDa for Ext1/Gt/Gt HS.

To obtain a stable source of embryonic fibroblasts, wild type and Ext1/Gt/Gt fibroblasts were immortalized by transformation with recombinant retroviruses encoding simian virus 40 large T antigen (see “Experimental Procedures”) (34). Analysis of [35S]sulfate- (data not shown) or [3H]glucosamine-labeled glycosaminoglycans from immortalized wild type and Ext1/Gt/Gt fibroblasts gave results virtually identical with those from the primary cells both regarding the level of HS synthesis and chain length (Fig. 4C). During development, numerous secreted factors bind HS, and HS is suggested to play a vital role in morphogen signaling and/or transport. Since these molecules encounter HS associated with the cell membrane or the extracellular matrix, we analyzed trypsin-released HS from the surface of wild type and Ext1/Gt/Gt fibroblasts. Similar to the results obtained with HS isolated from whole cell extracts, the Ext1/Gt/Gt HS chains lacked the longer HS chains present on wild type cells (Fig. 4D).

The N-substitution patterns of HS from wild type and Ext1/Gt/Gt cells were analyzed by treatment of [3H]glucosamine-labeled polysaccharides with nitrous acid at pH 1.5 that results in cleavage of the chains at the sites of N-sulfated glucosamine units. Under these conditions, N-acetylated units remain intact. Contiguous N-sulfated sequences will be degraded to disaccharides, whereas alternating N-sulfated and N-acetylated glucosamine residues will give rise to tetrasaccharides. Spaced sequences with solitary N-sulfate groups will yield oligosaccharides of at least hexasaccharide size. The gel chromatography profiles of the products revealed very similar disposition of the sulfated regions (Fig. 5). Calculations based on the distribution of radiolabel between oligosaccharides of different sizes indicated that ~40% of the glucosamine units in HS from Ext1/Gt/Gt cells and from wild type cells were N-sulfated. Similar analysis using labeled immortalized cells gave comparable results. Thus, the gene trap mutation did not significantly change the N-sulfation content of the polysaccharide.

Detailed information regarding the distribution of sulfate groups was obtained by analysis of the disaccharides generated by low pH HNO2/NaBH4 treatment of [35S]labeled HS fractions.
The resultant disaccharides were separated by anion exchange HPLC (Fig. 6) into three different mono-O-sulfated and one di-O-sulfated species. The HS from wild type and Ext1Gt/Gt embryos yielded similar patterns (Fig. 6, Table II). There was, however, a small increase in mono-6-O-sulfated disaccharides in the mutant HS compared with the wild-type.

Table I

| Cell line          | GlcNAc transferase activity | GlcA transferase activity |
|--------------------|-----------------------------|---------------------------|
|                    | 1^a | 2            | 3            | 1^a | 2            | 3            |
| Wild type          | 460 | 281          | 334          | 50  | 22           | 15.5         |
| Ext1Gt/Gt          | 85  | 43           | 48           | 10  | 2.5          | 1.5          |
| Ext1Gt/Gt/wild type| 0.18 | 0.15        | 0.14         | 0.2 | 0.11         | 0.10         |

^a Cell extracts from primary cells.

^b Similar amounts of proteins from mutant and wild-type cells were used for the assays. The GlcA transferase activities of the mutant cells are tentative since they were very low.
TABLE II

| Deamination products     | Cell type |  |  |
|--------------------------|-----------|---|---|
|                          | Wild type | Ext1Gt/Gt | Ext1Gt/Gt |
| % of total disaccharides |           |           |           |
| aMan₆, the 2,5-anhydromannitol deamination products of GlcNS residues. |           |           |           |
| GlcA-aMan₆(6S)           | 12        | 20        |           |
| IdoUA-aMan₆(6S)          | 8         | 11        |           |
| IdoUA(2S)-aMan₆          | 48        | 42        |           |
| IdoUA(2S)-aMan₆(6S)      | 32        | 27        |           |

The overall disaccharide composition of HS from wild type and Ext1Gt/Gt cells was determined following N-desacytelation and complete deaminative cleavage of [35S]glucosamine-labeled HS to disaccharides (see “Experimental Procedures”). Separation of the products by anion exchange HPLC again revealed increased 6-O-sulfation in HS from Ext1Gt/Gt cells (Table III). To confirm the sulfation patterns, we determined the disaccharide composition of medium-derived [3H]glucosamine-labeled HS after digestion with a mixture of heparinase and heparitinase. The resultant disaccharides were analyzed by HPLC on an anion-bound silica PA-03 column. The composition of the medium-derived mutant HS was very similar to that of the wild type, and contrary to the analysis of cell-derived HS, no differences in 6-O-sulfation were observed (Table III). Although the 6-O-sulfation of Ext1Gt/Gt HS may differ to some extent, taken together, our results indicated that the overall domain organization and disaccharide pattern is similar to the wild type HS.

DISCUSSION

Targeted mutagenesis or gene trap mutations of genes encoding enzymes essential for normal HS biosynthesis result in severe developmental abnormalities (27, 39–43). The most severe effect was obtained by the targeted deletion of Ext1, which results in failure of the embryos to gastrulate, demonstrating the importance of HS for early signaling events (27).

In this study, we describe the molecular properties of HS in an Ext1Gt/Gt gene trap mutant mouse. The Ext1Gt/Gt mice die around embryonic day 14, and in contrast to the previous report of the complete loss of HS in embryonic stem cells from Ext1-deficient mice generated by the gene targeting event (27), our gene trap mice still produce some HS. The longer survival time for the Ext1 gene trap mouse may reflect the ability of its short but apparently normally modified HS chains to mediate some HS-dependent signaling events.

We are intrigued by the fact that the gene trap mice still produce HS. If the hetero-oligomeric complex of EXT1 and EXT2 is the unique enzyme complex responsible for chain elongation, the complete disruption of the Ext1 or Ext2 gene would result in the interruption of HS biosynthesis after the formation of the linkage pentasaccharide. We did detect very small amounts of normal Ext1 transcript in the Ext1Gt/Gt fibroblasts by PCR. The presence of HS in the gene trap mouse could be a result of residual transferase activity in the N-terminal part of the protein or indicate that splicing around the large gene trap vector has generated a small amount of normal Ext1 transcript.³ The mutation mainly affected HS chain length, although some reduction in the number of chains may also have occurred. Our findings raise intriguing questions regarding the regulation of chain elongation. By what mechanism does the mutation in Ext1 result in shorter chains and not in fewer chains of normal length? How does HS formation due to defective Ext1 influence HS-protein interactions?

³ Quantitative real-time-PCR on limbs from Ext1Gt/Gt mice shows that 1–3% of Ext1 wild-type transcripts is produced (A. Vortkamp, personal communication).
The HS polysaccharide chains are constructed on the HS-protein linkage region by the alternating addition of GlcNAc and GlcA to the nonreducing end of the growing chain. Coexpression studies of the Ext1 and Ext2 proteins have been shown to result in increased glycosyl transferase activities, and the polymerase reaction has been ascribed to a hetero-oligomeric complex of Ext1 and Ext2 (20, 21). Homozygous null embryos of Ext2 have similar developmental abnormalities as the Ext1-deficient mice, and diminished Ext2 expression in cultured mammalian cells blocks HS synthesis, further impli- cating the requirement of both proteins in the chain elongation process (9, 44).

The two exons of the exostosin family, the EXT-like proteins (EXTL1–3) have all been demonstrated to have GlcNAc transferase activity (23, 24). Ext2 possibly catalyzes the addition of the first GlcNAc unit onto the GlcA–Gal–Xyl–Ser linkage region, thus committing the pathway toward generation of an HS polymer. If instead an N-acetyl-D-galactosaminase is first added to the GlcA residue, the same linkage structure will serve to initiate chondroitin sulfate formation (6, 45). Ext1 transfers GlcNAc to growing HS chains, whereas Ext3 seems to have dual functions catalyzing the transfer of GlcNAc both to the HS-protein linkage region and to the growing chain (24). GlcA-transferase activity has so far only been demonstrated for Ext1 and Ext2 proteins.

The gene trap cassette is inserted between exons 1 and 2 (Fig. 2). exon 1 encodes 43% of total amino acids (i.e. 320 amino acids of 746), indicating that the mutant EXT1 protein still contains almost half of the N-terminal side of the protein. This part of the protein has been proposed to contain the GlcA-transferase activity (22). The C-terminal half of the Ext1 protein is conserved among the EXT family members and is postulated to harbor the catalytic site of the GlcNAc-transferase activity. However, so far it has not been shown that the two activities constitute two independent sites. In the Ext1/Gt/Gt fibroblasts, small but detectable levels of both GlcNAc and GlcA transferase activities were observed. The GlcA transferase activity may remain in the chimeric protein of Ext1/β-Gal/neo and/or Ext2, whereas the GlcNAc transferase activity, which was significantly higher than the GlcA transferase activity, may be a result of the Ext2, Ext1/1, and/or Ext3 activities.

Several signaling molecules, including members of the fibroblast growth factor, Wingless/Int, and Hedgehog families, require HSPG at the cell surface for optimal signaling (1). Whereas biochemical studies using cell culture systems and in vitro binding assays have provided data concerning the minimal growth factor binding epitope on HS, the influence of HS chain length on growth factor binding and signaling probably provides insight into the structural requirements of HS in regulating growth factor binding and signaling.

Acknowledgments—We thank Eva Hjortson (Upstate University) for excellent technical assistance, Maria Thuvesson (Upstate University) for providing the Ws conditionend medium, and Ulf Lindahl (Upstate University) for critical reading of the manuscript.

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Embryonic Fibroblasts with a Gene Trap Mutation in *Ext1* Produce Short Heparan Sulfate Chains

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*J. Biol. Chem. 2004, 279:32134-32141.*
doi: 10.1074/jbc.M312624200 originally published online May 25, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M312624200

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