Contribution of EXT1, EXT2, and EXTL3 to Heparan Sulfate Chain Elongation*

Received for publication, April 30, 2007, and in revised form, August 9, 2007. Published, JBC Papers in Press, August 29, 2007, DOI 10.1074/jbc.M703560200

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The exostosin (EXT) family of genes encodes glycosyltransferases involved in heparan sulfate biosynthesis. Five human members of this family have been cloned to date: EXT1, EXT2, EXTL1, EXTL2, and EXTL3. EXT1 and EXT2 are believed to form a Golgi-located hetero-oligomeric complex that catalyzes the chain elongation step in heparan sulfate biosynthesis, whereas the EXTL proteins exhibit overlapping glycosyltransferase activities in vitro, so that it is not apparent what reactions they catalyze in vivo. We used gene-silencing strategies to investigate the roles of EXT1, EXT2, and EXTL3 in heparan sulfate chain elongation. Small interfering RNAs (siRNAs) directed against the human EXT1, EXT2, or EXTL3 mRNAs were introduced into human embryonic kidney 293 cells. Compared with cells transfected with control siRNA, those transfected with EXT1 or EXT2 siRNA synthesized shorter heparan sulfate chains, and those transfected with EXTL3 siRNA synthesized longer chains. We also generated human cell lines overexpressing the EXT proteins. Overexpression of EXT1 resulted in increased HS chain length, which was even more pronounced in cells coexpressing EXT2, whereas overexpression of EXT2 alone had no detectable effect on heparan sulfate chain elongation. Mutations in either EXT1 or EXT2 are associated with hereditary multiple exostoses, a human disorder characterized by the formation of cartilage-capped bony outgrowths at the epiphyseal growth plates. To further investigate the role of EXT2, we generated human cell lines overexpressing mutant EXT2. One of the mutations, EXT2-Y419X, resulted in a truncated protein. Interestingly, the capacity of wild type EXT2 to enhance HS chain length together with EXT1 was not shared by the EXT2-Y419X mutant.

Heparan sulfates (HSs) are sulfated glycosaminoglycans (GAGs) distributed on the cell surfaces and in the extracellular matrices of most tissues. HS chains are synthesized covalently linked to various proteins in a proteoglycan structure. HS proteoglycans have been implicated as regulators in a diverse number of biological events related to intracellular signaling, cell-cell interactions, and tissue morphogenesis (1). Most of these activities depend on the binding of the negatively charged HS chains to a variety of molecules including members of the fibroblast growth factor family, serine protease inhibitors, and extracellular matrix proteins (2, 3).

The biosynthesis of HS is a complex process (reviewed in Refs. 2–4). Chain elongation is initiated by the formation of a tetrasccharide linkage region composed of glucuronic acid-galactose-galactose-xylene (GlcAβ1–3Galβ1–3Galβ1–4Xylβ1–), where Xyl is attached to a serine residue in the core protein (5). After the addition of a single GlcNAc residue by GlcNAc transferase I (GlcNAc-TI), elongation proceeds by the action of glycosyltransferases (GlcNAc-TII and GlcA-TII), which add β1,4-GlcA and α1,4-GlcNAc units in alternating sequence to the nonreducing end of the growing polymer (6). The polymerization reactions are carried out by the exostosins proteins EXT1 and EXT2 (7–9). Concomitantly with chain elongation modification of the chain occurs, initiated by N-deacetylation and N-sulfation of glucosamine units, carried out by the bifunctional enzyme N-deacetylasel/N-sulfotransferase (NDST). These reactions occur in a more or less blockwise fashion and create stretches of N-sulfated regions and nonmodified N-acetylated domains, interspersed with short mixed regions (N-sulfated/N-acetylated domains) (10, 11). The N-deacetylation/N-sulfation reaction has been regarded as a key regulatory step because all subsequent modifications, epimerization of GlcA to iduronic acid and various sulfation reactions, only occur in the vicinity of N-sulfate groups. However, NDST1+/−/NDST2−/− embryonic stem cells that completely lack N-sulfation are still able to produce 6-O-sulfated HS (12). Subsequent modifications include C-5 epimerization of GlcA to iduronic acid, 2-O-sulfation of iduronic acid, and 6-O-sulfation of N-acetylated and N-sulfated GlcN residues. In addition, a small number of N-sulfated α-glucosamine residues become O-sulfated at C-3, and a few GlcA units become O-sulfated at C-2. Five members of the EXT family of glycosyltransferases are known in humans: EXT1, EXT2, EXT11 (for EXT-like 1), EXT12, and EXT13. Members of the EXT gene family encode...
glycosyltransferases that are suggested to be involved in HS biosynthesis, and most of the members seem to possess more than one in vitro glycosyltransferase activity (5, 9).

EXT1 and EXT2 appear to have dual enzyme activities, GlcA-TII and GlcNAC-TII, although those of EXT2 are weak and have been questioned. Even though EXT1 alone is able to polymerize the HS backbone structure in vitro, all evidence suggests that both EXT1 and EXT2 are necessary for chain elongation (13, 14). All three EXTs catalyze GlcNAC transferase reactions. EXT1 adds GlcNAC to the nonreducing end of the growing polysaccharide (GlcNAC-TII activity); EXT3 adds GlcNAC to the GAG protein linkage region (GlcNAC-TI activity) and to the growing chain (GlcNAC-TII activity), thus harboring enzyme activities involved in both the initiation and elongation of HS chains (15); and EXT2, the shortest member of the EXT family, exhibits GlcNAC-TI activity (16). The biological roles of EXT1 and EXT2 in HS biosynthesis have not yet been demonstrated, and in contrast to the other members of the EXT family, orthologs of the mammalian EXT1 and EXT2 are absent in Drosophila, suggesting that they are not essential for HS biosynthesis.

Hereditary multiple exostoses (HME), an autosomal dominant hereditary disorder, is one of the most common benign skeletal conditions affecting 1:100,000, with a risk for malignant transformation. The clinical signs are shorter stature and skeletal deformities caused by cartilage capped bony outgrowths at the epiphyseal end of the long bones (17). The mechanism by which exostosis develops is poorly understood, but mutations in either EXT1 or EXT2 and the resulting reduction or absence of HS in the exostosis cartilage cap has been implicated in disturbed signaling response in exostosis chondrocytes (18, 19). A number of mutations in EXT1 and EXT2 have been reported in HME patients, and the majority of cases involve mutations in EXT1 (60–70% of the patients) (9, 20). The mutations are randomly distributed over the entire EXT1 gene, whereas EXT2 mutations appear to be concentrated toward the N-terminal part of the protein. Most mutations result in premature termination of translation and loss of function. Less common are missense mutations affecting single amino acids. The amino acid substitutions are believed to alter the functional properties of the EXT proteins. However, although it is generally believed that the mutations result in abnormal HS synthesis, this is not always the case. Some of the reported EXT1 mutant proteins retain their ability to synthesize HS (21).

Using the human embryonic kidney (HEK) 293 cells, we evaluated the contribution of EXT1, EXT2, and EXT3 to HS chain elongation by up-regulation of their expression by overexpression and down-regulation of their expression by small interfering RNAs (siRNA). We also analyzed the effect on HS synthesis of EXT2 with two different mutations found in HME patients.

**EXPERIMENTAL PROCEDURES**

**Gene Silencing with siRNA**—Predesigned siRNAs for human EXT1 (5′-GGAAUACCCAGGACAGGA-3′; 5′-GGAAUCCAGCGUGCUCCACAUU-3′) and EXT2 (5′-GGGUGUAUAUCUAGUCUG-3′, 5′-GGGUGUUUUGAUGUCUG-3′, and 5′-GGAGACAGGACAAGCGCAUG), EXT3 (5′-GGCAGGCAAGGCGCAGUU-3′; 5′-GGCAAGCGGAUUUUGGUC-3′ and 5′-GGU-CUCUUCUACCCUUU-3′), and complement C1r (control siRNA, 5′-GGCUGCUUCAUGUAUAG-3′) were purchased from Ambion. HEK 293 cells were transfected with the siRNAs (50 nM of each) using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). After 24 h the transfection was repeated, and the cells were cultured at 37 °C for another 24 h in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum with 200 µCi/ml Na₂¹³⁵SO₄ (PerkinElmer Life Sciences) or 50 µCi/ml [6-³H]glucosamine HCl (Amersham Biosciences). Radiolabeled GAGs were isolated after trypsin treatment as described below.

**Quantitative Real Time PCR**—Total RNA was isolated from cells transiently transfected with siEXT1, siEXT2, siEXT3, or control siRNA using RNeasy Mini prep kit (Qiagen). cDNA was generated by reverse transcription using random primers (iScript cDNA synthesis kit; Bio-Rad). Quantification of mRNA expression was performed using LightCycler FastStart DNA Master SYBR Green kit (Roche Applied Science) and LightCycler instrument (Roche Applied Science). The primers were selected using PrimerBank (pga.mgh.harvard.edu/primerbank/) (22). The primers used were: EXT1 forward, 5′-GCTCCTGGTCTCGGCCCTTTTGT-3′ and reverse 5′-GTTGCAAGGCATACTTACC-3′; EXT2 forward, 5′-AAGGACAGGCTTCTGATTACC-3′ and reverse, 5′-GAAGTACCGTCCAGGAGAACCA-3′; and EXT3 forward, 5′-CTGCTATCGGCC-3′ and reverse, 5′-GTTCCTGCTGTGGC-3′. cDNAs were normalized against transcript levels of glyceraldehyde-3-phosphate dehydrogenase (forward primer, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and reverse, 5′-CATGTGGGCCATGAGGTCCACCAC-3′). Each primer/cDNA set was performed in duplicate with cDNAs from each different transfection. Data analysis was performed using the ΔΔCt method (23).

**Construction of Expression Plasmids and Transfection of HEK 293 Cells**—A cDNA corresponding to the full-length mouse EXT1 or EXT2 open reading frame was amplified from a mouse brain Quick-clone cDNA library (Clontech) using the following primers for EXT1: sense primer, 5′-CTTCTGACCCCGGAGACGCAG-3′ and antisense primer, 5′-GGTTTCTCTCTGACC-3′; and for EXT2: sense primer, 5′-GGAGACAGGAGCACCTGTTTTT-3′ and antisense primer, 5′-GGAGAAGGCTGCAAGCCACACC-3′. The amplified product was cloned into pCRII-TOPO (Invitrogen) and subsequently sequenced. The EXT1 and EXT2 cDNAs were inserted, either separately or together, into the pBudCE4.1 expression vector (Invitrogen), adopted for double insertions.

Mutant bases were introduced into EXT2 cDNA using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. EXT2-D227N point mutation was constructed using forward primer 5′-CTTCTGACCCCGGAGACGCAG-3′ and complementary reverse primer 5′-CTTTCTGACCGTCTTCTCACC-3′ and mutant C1r was generated using forward primer 5′-CTTCTGACCCCGGAGACGCAG-3′ and complementary reverse primer 5′-CTTTCTGACCGGCACTTCTCACC-3′ (mutated nucleotides are underlined and
EXT1 and Heparan Sulfate Chain Elongation

in bold). The wild type and mutated inserts were excised with EcoRI and subcloned into the corresponding site of the pcDNA-3 expression vector (Invitrogen). Plasmids with inserts containing the coding region in 5' to 3' direction were selected by sequencing.

A truncated, soluble form of human EXT3, lacking the first 51 amino acids, was amplified using a human placenta cDNA (Clontech) with the primers 5'-ATATGGGCGGCAGACCCAC-TCTGGATGAG (forward) and 5'-GGTCTCAGAGATGAACTTTGAAGCACTTGGT (reverse), and the PCR products were cloned in-frame with the preprotrypsin leader sequence and the N-terminal 3-FLAG tag p3XFLAGCMV9 expression vector (Sigma). The truncated forms of EXT1 and EXT2 were as described (13).

Wild type and mutant expression constructs were stably or transiently transfected into HEK cells using Lipofectamine 2000 (Invitrogen). Stable clones expressing the EXT1, EXT2, and EXT1/EXT2 expression constructs and control clones transfected with vector alone (pcDNA-3 or pBudCE4.1) were selected as described (24). Selected cellular clones were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) complemented with 10% (v/v) fetal calf serum (Invitrogen), 1% penicillin G-streptomycin, 1% Fungizone (2.5 IU/ml), and 1% sodium bicarbonate. Selected clones were transiently transfected into HEK 293 cells using Lipofectamine 2000 (Invitrogen). Stable clones expressing the EXT1, EXT2, EXT3, and EXT5 expression constructs and control clones transfected with vector alone (pcDNA-3 or pBudCE4.1) were selected as described (24). Selected cellular clones were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) complemented with 10% (v/v) fetal calf serum (Invitrogen), 1% penicillin G-streptomycin, 1% Fungizone (2.5 IU/ml), and 1% sodium bicarbonate (the reducing terminal sugar is modified to a 2,5-anhydro-
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fated species. There was a small increase of 6-O-sulfated disaccharides in siEXT2 and siEXTL3 HS compared with the control (Table 1).

Effect of Overexpression of EXT1, EXT2, and EXTL3 on HS Chain Length—We next investigated the effect of overexpression of EXT1 and EXT2 on HS chain length. HEK 293 cells were stably transfected with the vector alone (mock), EXT1, EXT2, or coexpressed EXT1 and EXT2 (EXT1/EXT2). The specific GlcNAc and GlcA transferase activities of the EXT1 overexpressing cells expressed on a cellular protein basis were increased compared with the activities obtained for mock transfected cells (Fig. 4, A and B). Similar to previous observations (8) coexpression of EXT1 and EXT2 further augmented the two glycosyltransferase reactions. Mock transfected, EXT1, EXT2, and EXT1/EXT2 overexpressing cells were metabolically [35S]sulfate-labeled for 60 min, and cell-associated35S-labeled HS proteoglycans were isolated by gel chromatography on Superose 6, and the HS chains were released from the core protein by alkali treatment (28). As shown in Fig. 5A, EXT1 overexpression resulted in a significant increase in chain length, which was even more enhanced in EXT1/EXT2-overexpressing cells. HS from EXT2 or EXTL3 overexpressing cells showed no striking differences in chain length from control cells, even though some variations in chain length were observed between different labeling experiments. The effect on chain elongation was not affected by the labeling time because the effect on HS chain elongation was similar also when cells were labeled for 6 h or overnight. That the chondroitinase ABC-resistant chains indeed represent HS was ascertained by the complete degradation of labeled material to di- and smaller oligosaccharides by digestion with a combination of chondroitinase ABC and heparitinase I and II (data not shown).

The EXT proteins are type II membrane-bound proteins, and to determine whether the observed effects on HS chain elongation were dependent on the membrane-bound status of the enzymes, we next investigated the effect on HS chain length of overexpression of soluble EXT1, EXT2, and EXTL3. HEK 293 cells were stably transfected with 3-FLAG-tagged human EXT1, 3-FLAG-tagged EXTL3, and 3-FLAG-tagged EXT2, or cotransfected with 3-FLAG-EXT1/MycHis-EXT2 (EXT1/EXT2) constructs. All of the constructs lacked the transmembrane domains, thus yielding soluble fusion proteins that were released into the culture medium. The expressed proteins were affinity captured on anti-FLAG-agarose, and the bound fusion proteins were analyzed for glycosyltransferase activities. The immunopurified EXT1, EXT2, and EXT1/EXT2 constructs have been described previously (13). The immunopurified EXTL3 exhibited weak GlcNAc-TII activity and no detectable GlcA-TII activity (data not shown). The weak

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TABLE 1

| Disaccharide composition within the N-sulfated domains of [3H]GlcN-labeled HS from siControl, siEXT1, siEXT2, and siEXTL3 transfected cells |
|---|---|---|---|---|
| Deamination products | Cell type | % of total disaccharides |
| | siControl | siEXT1 | siEXT2 | siEXTL3 |
| GlcA/IdoA-aMan(6S) | 10 | 11 | 8 | 10 |
| GlcA(2S)-aMan(6S) | 11 | 1 | 1 | 1 |
| GlcA-aMan(6S) | 10 | 11 | 18 | 13 |
| IdoA-aMan(6S) | 4 | 4 | 7 | 6 |
| IdoA(2S)-aMan(6S) | 57 | 56 | 47 | 47 |
| IdoA(2S)-aMan(6S) | 18 | 16 | 19 | 23 |

*aMan(6S), the 2,5-anhydromannitol deamination products of N-sulfated 0-glucosamine residues; IdoA, iduronic acid.
activity may be explained by the fact that, although EXT3 has been shown to efficiently transfer GlcNAc to GlcA-[GlcNAc-GlcA]-αManα acceptors (measuring GlcNAc-TII), its activity toward acceptors mimicking the HS protein linkage region (measuring GlcNAc-TI) is much higher (15).

Overexpression of secreted EXT1 and EXT1/EXT2 again resulted in a significant increase in chain length, whereas overexpression of EXT2 or EXT3 did not significantly affect HS chain elongation (Fig. 5B). However, in contrast to the result observed with the full-length constructs, coexpression of soluble EXT2 and soluble EXT1 did not enhance HS chain length more than EXT1 alone. These findings suggest that the catalytic activities of EXT1 do not depend on the transmembrane region of the expressed EXT1 protein. Western blotting of transfected cells showed that the expressed soluble proteins were also present in the cell extracts. Thus, soluble EXT1 protein apparently is able to interact with the biosynthetic machinery in the Golgi apparatus.

Effect of Overexpression of Mutated EXT on HS Chain Length—To evaluate the effect of EXT2 mutations in this system, we generated HEK 293 cell clones stably transfected with EXT2-D227N and EXT2-Y419X cDNAs. Previously, HME patients have been grouped according to several clinical parameters such as onset of exostoses growth, number of exostoses, stature, and functional rating (20). One of the mutations that we introduced, EXT2-D227N, is considered to give a mild phenotype. The other mutation, EXT2-Y419X, a nonsense mutation resulting in a truncated protein, gives a moderate severe phenotype.

Lysates of transfected cells were analyzed for GlcA and GlcNAc transferase activities. The enzyme activities of the different cell lines expressing the mutated EXT2 were similar to those of mock transfected cells (data not shown), indicating that overexpression of mutated or wild type EXT2 did not affect the in vitro glycosyltransferase activities. Stable clones expressing high levels of EXT2, EXT2-D227N, and EXT2-Y419X proteins, respectively, were selected by Western blotting and used to investigate the effect of mutated EXT2 expression on HS synthesis. The HEK 293 cell clones were metabolically labeled for 24 h with [35S]sulfate and radiolabeled GAGs were isolated from solubilized cells and from the culture medium and quantified. Expression of wild type or mutated forms of EXT2 did not change the total amount of [35S]GAGs produced nor the HS/CS ratio as compared with mock transfected cells (data not shown). Similar to the EXT2 overexpressing cells, the cells overexpressing mutated forms of EXT2 displayed no extensive changes in chain length compared with HS in mock transfected cells (Fig. 5C), indicating that the mutated EXT2 proteins did not exhibit a dominant negative effect on HS synthesis.

We next considered whether the EXT2 mutation would affect the enhancing capacity of EXT2 on HS chain elongation when coexpressed with EXT1. HEK 293 cells were transiently transfected with EXT1 or EXT2-Y419X alone or cotransfected with EXT1 and EXT2 or with EXT1 and EXT2-Y419X (EXT1/EXT2-Y419X). Analysis of 35S-labeled HS from cells transiently transfected with EXT1 and EXT1/EXT2 again showed that the most extended HS chains were made in cells coexpressing EXT1 and EXT2 (Fig. 5D). In contrast, EXT1/EXT2-Y419X coexpression did not stimulate chain elongation more than EXT1 alone (Fig. 5D). Similar results were obtained after transient transfection of EXT2-Y419X into stably transfected EXT1 cell clones (data not shown). Western blotting of the transfected cells demonstrated that similar amounts of EXT2 and EXT2-Y419X, respectively, were expressed in the transiently transfected cells (data not shown). Thus, the lack of effect of the
EXT2 mutant was not due to less EXT2 protein available. Analogous to the effect on chain elongation, transient expression of EXT1/EXT2-Y419X did not influence the transferase activities (Fig. 4, C and D).

Golgi Localization of the Overexpressed EXT Proteins—In an attempt to identify the mechanism responsible for this differential response of HS chain elongation of HEK 293 cells transfected with EXT1/EXT2 versus EXT1/EXT2-Y419X, we stained the transfected cells with an antibody against EXT2 to determine the cellular localization of the overexpressed proteins. When expressed in HEK 293, both EXT2 and EXT2-Y419X proteins colocalized with the Golgi marker GM130 (Fig. 6). Therefore, the lack of effect after transfection of EXT1/EXT2-Y419X is not caused by retention of the mutated protein in the ER.

DISCUSSION

Our knowledge of how the synthesis of HS is regulated is surprisingly limited. The mechanism responsible for regulation of HS chain elongation is not known, and the exact role of each particular EXT and EXTL protein is still an enigma. All of them have been shown to possess in vitro glycosyltransferase activities relevant for HS chain initiation or elongation (5, 9). A prerequisite to understand the cellular functions of these proteins is to know the molecular mechanism of their actions. Thus, we addressed here the role of EXT1, EXT2, and EXTL3 in HS biosynthesis by means of overexpression studies and gene silencing. The most striking results were the effects of decreased amounts of the EXT proteins on HS chain elongation.

We have shown previously that embryonic fibroblasts isolated from mice carrying a hypomorphic mutation in Ext1 synthesize significantly shorter HS chains than do wild type fibroblasts (27). Consistent with this, silencing of EXT1 by EXT1-targeted siRNA in human cells resulted in lower transferase activities (Fig. 1) and the formation of shorter HS chains (Fig. 2). Interestingly, similar inhibition of EXT2 also resulted in the synthesis of shorter chains, confirming that EXT2 is indeed a partner of EXT1 in chain elongation. To gain more insight into the role of EXT2 in HS synthesis, we also compared the effect on HS chain elongation of cell lines overexpressing wild type and mutated forms of EXT2 alone or coexpressed with EXT1.

Mutational defects in either EXT1 or EXT2 cause HME. Because all evidence suggests that both EXT1 and EXT2 are essential for HS polymerization, it is generally believed that defects in HS synthesis cause the abnormalities related to HME. Both EXT1 and EXT2 are ubiquitously and abundantly expressed in mammalian tissues. However, the effects of mutations seem to be limited to the growing bone. Despite the relatively high expression of the mutated EXT2 constructs, they did not alter HS synthesis, indicating that the mutated EXT2 constructs (EXT2-D227N with an Asp to Asn exchange, and EXT2-
Y419X, encoding a 419-amino acid protein lacking the 300 most C-terminal amino acid residues) did not exhibit a dominant negative effect. Interestingly, introduction of mutant EXT1 into Chinese hamster ovary cells also did not suppress HS synthesis (29). Taken together, these results indicate that the function of endogenous EXT proteins is not affected by the presence of increased levels of mutant forms of EXT1 or EXT2.

Contrary to the results obtained with the mutated EXT2, coexpression of the wild type EXT2 with EXT1 in mamalian cells had a dramatic effect on both enzyme activity and HS chain length (Figs. 4 and 5). The effect of EXT2 on chain elongation is intriguing. EXT2 is necessary for HS polymerization as evident from mutational analysis in Drosophila, mouse, and zebrafish orthologs of EXT2 (30–34). Previous in vitro data indicate that EXT1 has readily detectable GlcA and GlcNAc transferase activities, whereas the enzyme activities of EXT2 are less manifest (7, 8, 13). The lack of effect of EXT2 overexpression could be due to the fact that EXT2 may have a different function in HS polymerization from EXT1. If both EXT1 and EXT2 were catalytically active glycosyltransferases, one would expect gene silencing and overexpression to give similar results for both proteins. EXT2 forms a complex with EXT1, which facilitates the transfer of both proteins to the Golgi apparatus and modulates the activity of the latter protein (7, 8, 13); therefore, the EXT1/EXT2 hetero-complex is believed to be the functional HS polymerase. Because of the low transferase activities of EXT2, it is tempting to speculate that EXT2 is not involved in the actual elongation of the HS backbone but instead the function of EXT2 could be in assisting the folding and transport of EXT1 to the Golgi complex. One hypothetical explanation of our results could be that an excess of EXT2 protein is normally synthesized in the cell. Thus, increased levels of EXT2 will be without effect and will not affect polymerization. If instead the amounts of EXT1 are increased, more EXT1/EXT2 heterocomplexes will form and thus affect chain elongation. Overexpression of both EXT1 and EXT2 will generate even more complexes that can participate in chain elongation. It is not known how the complex of EXT1 and EXT2 is formed. Although the mutant localized to the Golgi (Fig. 6), it is possible the mutant EXT2 failed to associate with EXT1 and thus was not able to further promote chain elongation. Further studies are needed to establish the role of EXT2 in HS polymerization. From our results we can conclude that chain length rather than the initiation of new HS chains is affected by the amounts of EXT1 and EXT1/EXT2 complex. Interestingly, NDST1−/−/NDST2−/− embryonic stem cells, which lack N-sulfation, showed increased HS chain length without affecting the amount of HS produced by the cells, indicating a more complex regulation of chain elongation (12).

In contrast to the silencing of EXT1 or EXT2, silencing of EXT3 resulted in the synthesis of longer HS chains. This protein has been shown to be involved in HS chain initiation (GlcNAc-TI activity), catalyzing the incorporation of the first GlcNAc onto the polysaccharide protein linkage region (15). The increase in HS chain length after siRNA-mediated EXT3 silencing could be explained by the fact that the reduction in EXT3 results in fewer linkage regions that contain the first GlcNAc necessary to start the HS elongation process (-GlcNAc-GlcA-Gal-Gal-Xyl-O-Ser, where the first GlcNAc is indicated in bold). With less acceptor substrates available, more extensive polymerization may occur in the chains that are being synthesized. The observed lack of effect of EXT3 overexpression could be due to the fact that the untransfected cells produce saturating levels of EXT3, which is enough to catalyze the incorporation of GlcNAc residues in all of the available linkage regions.

It has also been proposed that EXT3 may be involved in chain elongation or chain termination (15). Reducing the amounts of a chain terminator would naturally lead to longer HS chains, but overexpression of EXT3 did not significantly alter the HS chain length as would have been expected if EXT3 was a terminator of HS chains. In addition, Chinese hamster ovary cells lacking GlcNAc-TII activity still display GlcNAc-TI activity but lack HS chains (35) Moreover, this mutant accumulates a pentasaccharide intermediate with the structure GlcNAc-GlcA-Gal-Gal-Xyl (36), favoring the concept that EXT3 catalyzes the initiation rather than the termination of HS chain elongation. It has been proposed in a recent report that a GlcA residue is present at the nonreducing end of the HS chains (37). EXT3 harbors no GlcA-T activities (15), so it would be unlikely to catalyze chain termination.

In summary, our results show that siRNA silencing of EXT1, EXT2, or EXT3 will affect HS chain elongation, which may be one reason behind the formation of exostoses, because the shorter chains produced by the hypomorphic EXT1 mouse affect HS-dependent growth factor signaling. Furthermore, these three proteins may play specific and distinct roles in HS biosynthesis in human cells. EXT1 and EXT2 are together responsible for chain elongation, and the levels of the individual proteins affect the polymerization process. The level of EXT3 also affects chain elongation, but the changes indicate that or EXT3 must be an initiator of HS chains.

Acknowledgment—We thank Eva Hjertson for excellent technical assistance.

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EXTs and Heparan Sulfate Chain Elongation

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