The Putative Tumor Suppressors EXT1 and EXT2 Are Glycosyltransferases Required for the Biosynthesis of Heparan Sulfate*

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Hereditary multiple exostoses, characterized by multiple cartilaginous tumors, is ascribed to mutations at three distinct loci, denoted EXT1–3. Here, we report the purification of a protein from bovine serum that harbored the δ-glucuronyl (GlcA) and N-acetyl-δ-glucosaminyl (GlcNAc) transferase activities required for biosynthesis of the glycosaminoglycan, heparan sulfate (HS). This protein was identified as EXT2. Expression of EXT2 yielded a protein with both glycosyltransferase activities. Moreover, EXT1, previously found to rescue defective HS biosynthesis (McCormick, C., Leduc, Y., Martin-dale, D., Mattison, K., Esford, L. E., Dyer, A. P., and Tufaro, F. (1998) Nat. Genet. 19, 158–161), was shown to elevate the low GlcA and GlcNAc transferase levels of mutant cells. Thus at least two members of the EXT family of tumor suppressors encode glycosyltransferases involved in the chain elongation step of HS biosynthesis.

Heparan sulfate (HS) proteoglycans, ubiquitously distributed on cell surfaces and in the extracellular matrix, consist of sulfated glycosaminoglycan chains that are covalently bound to various core proteins. HS polysaccharide, increasingly implicated in physiological processes such as cell adhesion, cytokine action, and regulation of enzymic catalysis, owes its biological properties to interactions with various proteins, mediated by specific saccharide sequences. Biosynthesis of HS chains involves the formation of an initial, simple polysaccharide, composed of alternating δ-glucuronic acid (GlcA) and N-acetyl-δ-glucosamine (GlcNAc) units, joined by 1→4 linkages. This polymer is subsequently modified through a series of reactions, which involves partial N-deacetylation and N-sulfation of GlcNAc units, C-5 epimerization of GlcA to l-iduronic acid residues, and O-sulfation at various positions (1). The GlcA transferase (GlcA-T) and GlcNAc transferase (GlcNAc-T) reactions required to generate the initial HS polysaccharide precursor have been associated with a single protein (2), hereafter referred to as “HS-polymerase” (HS-POL). Partial purification of proteins from bovine serum revealed a ~70-kDa component with both activities (3). We now report the molecular cloning of this protein and demonstrate that it is 94% identical to human EXT2, a member of the EXT family of tumor suppressors. Also EXT1, another member of the same family (4–7), is implicated with similar catalytic activities. Mutations of EXT genes have been associated with the development of hereditary multiple exostoses (HME), the most frequent of all skeletal dysplasias. These findings suggest that alterations in the formation of the HS precursor polysaccharide may be involved in tumor formation and further point to an important role for HS in control of bone growth.

MATERIALS AND METHODS

Isolation of HS-POL—The polymerase was isolated from bovine serum using an extension of the protocol described previously (3). Briefly, the procedure involved successive chromatographies through the following matrices: Red-Sepharose, concanavalin A-Sepharose connected to Red-Sepharose (recirculation for 48 h), phenyl-Sepharose, Superdex 200 (gel chromatography), UDP-Sepharose, Mono Q (anion-exchange chromatography), and Mono P (chromatofocusing). The product was finally separated by preparative SDS-PAGE and stained with Coomassie Blue. The ~70-kDa component was digested with trypsin in the gel, and the resultant peptides were separated and sequenced as described (8).

cDNA Library Screening and DNA Sequencing—The cDNA probe used for screening was derived from a human EST clone (826 bp) from Soares fetal liver spleen library (GenBank accession no. U13869 (IMAGE Consortium)). The clone was excised from vector pT7T3D (Amer-sham Pharmacia Biotech) with PacI and EcoRI and was labeled with [α-32P]dCTP, using a random-priming kit (Boehringer Mannheim). Nitrocellulose replicas of plaques from the bacteriophage gt10 bovine kidney cDNA library (catalog no. BL3001a; CLONTECH) were hybridized with the labeled probe according to the instructions of the manufacturer.

The nucleotide sequences of cDNAs were determined by repeated sequencing of both strands of alkaline-denatured plasmid DNA using the Cy5 AutoRead sequencing kit (Amer-sham Pharmacia Biotech). Nucleotide sequences were labeled using a Cy5-dATP labeling mix, and the sequencing reactions were performed using T7 DNA polymerase. Sequences were determined on an ALFexpress system (Amer-sham Pharmacia Biotech) and analyzed using the DNA-Star (DNASTAR Inc., Wisconsin) program. The nucleotide and protein sequences were applied to data base screening using BLAST search, NCBI (Internet address: http://www.ncbi.nlm.nih.gov/).

Transient Expression of HS-POL in COS-7 Cells—The 2884 bp cDNA insert recovered from the bovine kidney cDNA library was cleaved with restriction enzyme BsrRI to generate a 2256-bp fragment (corresponding to nucleotides 185–2441), which was then treated with Klenow fragment to generate blunt ends. This product was ligated into a pC3NA3 expression vector (Invitrogen), modified to introduce a His/FLAG (MGGSHHHHHHHHHHHDDDDK-) tag at the N terminus.

COS-7 cells were cultured in Dulbecco's modified Eagle's medium-F12 (catalog no. 31300-038, Life Technologies, Inc.) supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% (v/v) heat-inactivated (56 °C, 30 min) fetal calf serum at 37 °C and 7.5% CO2. For electrotransfection 70% confluent cells in a 175-cm2 flask were trypsinized and washed with PBS supplemented with 10 μl Heps, 2 µl (PBS, phosphate-buffered saline; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

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§ The abbreviations used are: HS, heparan sulfate; GlcA-T, GlcA transferase; GlcNAc-T, GlcNAc transferase; HS-POL, HS-polymerase; HME, hereditary multiple exostoses; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); PBS, phosphate-buffered saline; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

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containing 2% Me2SO, transferred to a 10-cm culture dish, left at room temperature for 20 min, and finally incubated at 37 °C for 72 h.

Briefly, GlcA-T activity was measured by incubating lysates with UDP-[14C]GlcA and a GlcNAc-[GlcA-GlcNAc] oligosaccharide acceptor (non-reducing terminal GlcNAc unit), whereas GlcNAc-T was assayed by incubation with UDP-[3H]GlcNAc and a [GlcA-GlcNAc] oligosaccharide acceptor (non-reducing terminal GlcA unit). Labeled oligosaccharides were isolated and quantified by scintillation counting.

**RESULTS**

Cloning of HS-POL—The putative HS-POL isolated previously from bovine serum (3) was subjected to further purification through a series of chromatography steps (see “Materials and Methods”). The GlcA- and GlcNAc-T activities remained associated throughout this procedure. Final separation by SDS-PAGE yielded a 70-kDa protein, which was isolated, and four tryptic peptides were sequenced. One of the peptides, residues 129–147 in Fig. 1, matched a human EST cDNA clone (EBI/GenBank™ accession no. AF089748).

mm MgCl2, pH 7.2. The cells were resuspended in 500 µl of washing buffer, and 30 µg of plasmid cDNA was added along with 50 µg of fish sperm carrier DNA (Boehringer Mannheim). Electroporation was carried out in a 0.4-cm cuvette (BTX) at 360 V and 500 microfarads. Following transfection the cells were resuspended in culture medium containing 2% MeSO2, transferred to a 10-cm culture dish, and left at room temperature for 20 min, and finally incubated at 37 °C for 72 h.

**Assay of Cellular Glycosyltransferase Activities—**Cell lines analyzed for GlcA-T and GlcNAc-T activities included clone 1D from Lmtk mouse fibroblasts, mutant gro2C derived from the same cells (9), COS-7 cells, and after transfection with EXT1. Both activities were 5–10-fold elevated, respectively, compared with mock-transfected controls (Fig. 3). The transferase activities displayed by control cells were probably because of endogenous enzymes committed to HS biosynthesis. A recent report shows that defective HS biosynthesis in a mutant mouse fibroblast cell line (gro2C) could be partially rescued by transfection with EXT1 (11). We therefore decided to analyze the gro2C cells as well as the corresponding wild-type L cells for the two glycosyltransferase activities, before and after transfection with EXT1. Both activities were 5–10-fold lower in the mutant than in the wild-type cell lysates (Table 1), indicating that the abrogated HS biosynthesis in gro2C cells is probably caused by defects in HS-POL. Transfection of gro2C cells with EXT1 showed a 2-fold increase in both enzymatic activities (Table 1), which suggested that EXT1, like EXT2, harbors HS-POL activities. By contrast, transfection of control L cells with EXT1 led to a decrease in HS-POL activities (Table 1). A similar decrease in polymerase activities was noted after transfection of COS-7 cells with bovine HS-POL/EXT2, thus explaining why expression of recombinant enzyme was detectable in terms of catalytic activity only after recovery of fusion protein by immunabsorption. These results suggested that overexpression of either EXT1 or EXT2 proteins interfered with the apparent activity in normal cells. The HS produced by such transfected cells showed a somewhat lower apparent negative charge density than corresponding control HS, as demonstrated by ion-ex-
temperature for 1 h and eluted with 0.14 mM FLAG octapeptide (DYKD-DDDK) in PBS, 0.1% Triton X-100 by batch incubation at room temperature. Glycosyltransferase assays were performed as described under "Materials and Methods." The bars indicate values derived from two independent experiments.

**TABLE I**

GlcNAc-T and GlcA-T activities in gro2C cells and L cells, with or without overexpression of EXT1

| Cell          | GlcNAc-T activity<sup>a</sup> (cpm) | GlcA-T activity<sup>a</sup> (cpm) |
|---------------|------------------------------------|----------------------------------|
| HS mutant     |                                    |                                  |
| gro2C         | 180 ± 20                            | 200 ± 40                          |
| gro2C EXT1    | 310 ± 40                            | 370 ± 110                         |
| Wild type     |                                    |                                  |
| L             | 890 ± 80                            | 1900 ± 200                        |
| L EXT1        | 570 ± 100                           | 1100 ± 100                        |

<sup>a</sup> Cells were grown and lysed as described under "Materials and Methods," and GlcA-T and GlcNAc-T activities were determined. Values indicate counts/min of [3H]GlcNAc and [14C]GlcA incorporated per mg of protein per min and represent means ± S.D. of four independent assays.

change chromatography of HS from control and EXT1-transfected L cells (Fig. 2 in Ref. 11) and HS from control and HS-POL-transfected human kidney epithelial 293 cells (Fig. 4).

**FIG. 3.** Assay of GlcNAc-T and GlcA-T activities following transfection of COS-7 cells with bovine HS-POL-His/FLAG fusion protein (see "Materials and Methods"). Expressed protein was bound to anti-FLAG M2 monoclonal antibody immobilized to agarose (Kodak) in PBS, 0.1% Triton X-100 by batch incubation at room temperature for 1 h and eluted with 0.14 mM FLAG octapeptide (DYKD-DDDK) in the same buffer. Glycosyltransferase assays were performed as described under "Materials and Methods." The bars indicate values derived from two independent experiments.

**FIG. 4.** DEAE-Sephacel chromatography of [35S]sulfate-labeled HS produced by 293 cells (open circles) and by bovine HS-POL/EXT2-transfected 293 cells (closed circles). Cells were transfected with bovine HS-POL (in pcDNA3 without the His/FLAG-tag), and stable expression was generated by selection with Geneticin (G418, Life Technologies, Inc.) as described (34). Proteoglycans were extracted from cell lysates and HS chains (free from galactosaminoglycans) were purified as described (35) except that after digestion with chondroitinase ABC, HS chains were recovered from Superose-12 (Amersham Pharmacia Biotech).

ly saccharide is converted into the mature, sulfated product (1, 22). A mutation in the appropriate EXT protein might affect such interaction as well as the initial polymerization reaction itself, with presently unpredictable effects on the structure of the final product. Indeed, many different types of tumors are associated with distinct changes in glycosaminoglycan, particularly HS, structure (23–27). Although the mechanisms behind these changes are generally unknown, the alterations may be expected to affect functional interactions with a variety of proteins that are potentially involved in neoplastic transformation. Examples of such proteins include a variety of growth factors that may be functionally dependent on HS fine structure (28), growth factor receptors, extracellular matrix macromolecules (29), and HS-degrading endoglycosidase(s) (heparinase) (30). Interestingly, a Drosophila homologue of EXT1 was recently implicated with the diffusion of Hedgehog, a presumably glycosaminoglycan-dependent process in embryonic development (31).

The present findings raise intriguing questions regarding the number of EXT type HS-POLs and the functional relation between these enzymes. Notably, deletion of either EXT1 or EXT2 causes disease, suggesting that these enzymes are not able to substitute for each other. We know that several of the polymer-modifying enzymes, acting further downstream in the process, have also recently been found to occur in genetically distinct isoforms (32). It has been proposed that HS chains with specifically tailored structure (designed for interactions with defined proteins) may be generated through the appropriate combination of such isoforms in biosynthetic assembly systems (32, 33). Interactions involving EXT proteins at the cellular level are inferred from the consistent down-regulation of overexpression of EXT1 and EXT2 causes disease, suggesting that these enzymes are not able to substitute for each other. We know that several of the polymer-modifying enzymes, acting further downstream in the process, have also recently been found to occur in genetically distinct isoforms (32). It has been proposed that HS chains with specifically tailored structure (designed for interactions with defined proteins) may be generated through the appropriate combination of such isoforms in biosynthetic assembly systems (32, 33). Interactions involving EXT proteins at the cellular level are inferred from the consistent down-regulation of over expression of EXT1 or EXT2. Understanding the role of HS biosynthesis in relation to HME, and possibly other types of neoplastic disease, will require detailed analysis of the expression of EXT/HS-POLs in different cells and tissues, as well as of their interaction with other components of the HS biosynthetic machinery.
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