Mutation in the Heparan Sulfate Biosynthesis Enzyme EXT1 Influences Growth Factor Signaling and Fibroblast Interactions with the Extracellular Matrix*

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Heparan sulfate (HS) chains bind and modulate the signaling efficiency of many ligands, including members of the fibroblast growth factor (FGF) and platelet-derived growth factor families. We previously reported the structure of HS synthesized by embryonic fibroblasts from mice with a gene trap mutation of Ext1 that encodes a glycosyltransferase involved in HS chain elongation. The gene trap mutation results in low expression of Ext1 mutant fibroblasts, whereas neither PDGF-BB nor FGF10 signaling was significantly affected. Furthermore, Ext1 mutants displayed reduced ability to attach to collagen I and to contract collagen lattices, even though no differences in the expression of collagen-binding integrins were observed. Reintroduction of Ext1 in the Ext1 mutant fibroblasts rescued HS chain length, FGF2 signaling, and the ability of the fibroblasts to contract collagen. These data suggest that the length of the HS chains is a critical determinant of HS-protein interactions and emphasize the essential role of Ext1 in providing specific binding sites for growth factors and extracellular matrix proteins.

Heparan sulfate proteoglycans (HSPGs), composed of a core protein and one or more negatively charged heparan sulfate chains, are ubiquitous components of the extracellular matrix and cell surfaces. As co-receptors for numerous growth factors and cytokines, HS proteoglycans modify cell behavior and regulate cell growth and thus play vital roles in various biological processes, such as cell proliferation and tissue morphogenesis.

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2 The abbreviations used are: HSPG, heparan sulfate proteoglycans; FGF, fibroblast growth factor; FGFR, FGF receptor; MAPK, mitogen-activated protein kinase; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; MCF, median channel fluorescence; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor.

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Several growth factors, including the fibroblast growth factors (FGFs), depend on HSPGs for cell-signaling activity (21). FGFs comprise a family of 23 structurally related members and are essential for normal embryonic development. FGFs act through tyrosine kinase receptors (FGFRs) on a wide range of cell types, such as fibroblasts, endothelial cells, and other cell types. The formation of FGF-HSPG-FGFR ternary complexes at the cell surface leads to activation and phosphorylation of the receptor tyrosine kinase that triggers various intracellular signaling cascades, including the MAPK pathway (22–24). Studies of ternary complex formation at different stages of mouse embryo development suggest that different FGF-FGFR pairs require distinct HS structures for complex formation (25). The binding of FGF2 to HS is the best characterized of the different members of the FGF family. FGF2 binds to HS on the cell surface, and this binding facilitates and stabilizes FGF2 binding to its cell surface receptor (26). HS also mediates binding to various extracellular matrix proteins, including fibronectin, laminin, and different types of collagen including the fibrillar collagen type I. HS interaction with collagen I promote cell attachment (27) and angiogenesis (28). In this study, we investigated how the Ext1 gene trap mutation affects fibroblast growth factor signaling and interaction with collagen I.

EXPERIMENTAL PROCEDURES

Cell Culture—SV40-immortalized mouse embryonic cell lines derived from wild-type and Ext1 gene-trapped (Ext1<sup>Gt/Gt</sup>, <em>Ext1<sup>Gt/Gt</sup></em>; Ref. 17) embryonic day 11.5 mouse embryos were prepared as described previously (20). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin (PAA Laboratories). For re-expression of Ext1 in Ext1<sup>Gt/Gt</sup> fibroblasts, a full-length mouse Ext1 cDNA cloned into the pBudCE4.1 vector (29) was transfected into Ext1<sup>Gt/Gt</sup> fibroblasts using the Nucleofector electroporation kit for adherent cells (Amaxa). Cell clones stably expressing the Ext1 expression constructs were selected using 0.5 mg/ml of Zeocin (Invitrogen).

Isolation of Metabolically Labeled HS—Ext1 gene trap (Ext1<sup>Gt/Gt</sup>) and wild-type immortalized embryonic fibroblasts were cultured as described above. 80% confluent cells were cultured in 24-well plates with serum-deprived DMEM and starved for 18 h in the same medium. Growth factors (FGF2, PDGF-BB, and FGF10; all from R&D Systems) were added at different concentrations (ranging from 0.2–100 ng/ml). After incubation for 10 min at 37 °C, the medium was discarded, and the cells were solubilized by addition of 200 μl of boiling nonreducing SDS sample buffer. Equal amounts of cell extracts were separated on two separate SDS–10% polyacrylamide gels under nonreducing conditions and electrotransferred onto nitrocellulose membranes (Amersham Biosciences). The membranes were blocked in 5% skim milk in Tris-buffered saline, 0.5% Tween 20 for 1 h at room temperature. One of the membranes was incubated with mouse anti-phospho-ERK 1/2 (1:2,000, Cell Signaling Technology, Inc.) recognizing the activated, phosphorylated kinase, and the other membrane was incubated with mouse anti-total ERK-2 (1:5,000, Santa Cruz) recognizing both the unphosphorylated and phosphorylated kinase. The membranes were developed using ECL reagent (Pierce), and the bands were visualized and quantified with the Biolmage (Bio-Rad) using Quantity One software (Bio-Rad).

Glycosyl Transferase Activity Assays—GlcNAc and GlcA transferase activities were measured by incubating enzyme protein preparations with 14C-labeled UDP sugars (62.5 μCi/μmol; prepared by mixing radiolabeled and unlabeled UDP sugars)
and oligosaccharide acceptors as described (31). Protein concentrations were determined using the BCA protein assay (Pierce).

**Cell Attachment**—Cells were trypsinized, washed three times in Puck’s saline containing Mg²⁺/Ca²⁺, seeded at a concentration of 10⁵ cells/well in 48-well plates precoated overnight with varying concentrations of collagen I. The cells were allowed to attach for 55 min at 37 °C. The plates were then washed three times in Puck’s saline. The cells were fixed with 96% EtOH and stained with 0.1% crystal violet for 30 min. After washing under running tap water, cells were lysed by incubation for 30 min with 100 µl of 1% Triton X-100/well. Cell attachment was measured colorimetrically in a Versamax microplate reader (Molecular Devices) at 595 nm. In heparin blocking experiments, wells were precoated with 5 µg/ml of collagen, and the cells were incubated with heparin (50 µg/ml) on ice for 15 min before plating; cell attachment to collagen was analyzed as described above.

**Cell Proliferation Assay**—Cell proliferation was determined by [³H]thymidine incorporation. 50,000 cells/well were seeded in a 24-well plate. After 24 h in culture, cells were subject to starvation for 24 h in serum-free DMEM. Then, FGF2 or FGF10 was added to cells at a final concentration of 10 ng/ml for another 24 h. Cells incubated with serum-free DMEM alone served as a negative control. During the last 4 h of growth factor stimulation, [³H]thymidine (1 µCi/ml; specific activity, 6.7 Ci/mmol; Perkin Elmer Life Sciences) was added to the cells. Cells were harvested by trypsinization and washed three times in cold PBS. After precipitation with 5% trichloroacetic acid, the cell pellets were dissolved in 0.5 M NaOH containing 0.5% SDS. The amount of incorporated [³H]thymidine was determined by scintillation counting.

**Collagen Gel Contraction Assay**—Wild-type and Ext¹Gt/Gt fibroblasts were trypsinized, washed, and resuspended in serum-free DMEM containing 2 mM glutamine and antibiotics. The cell suspension was mixed with a collagen I solution (1.2 mg/ml in DMEM with 20 mM Hepes, pH 8.0) to a final concentration of 10⁵ cells/ml. 100 µl aliquots of the resulting solution were transferred to a 96-well plate precoated with 2% bovine serum albumin and incubated at 37 °C for 1 h to allow collagen polymerization. Collagen gels were detached by the addition of 100 µl DMEM or 100 µl DMEM containing 10 ng/ml of FGF2. Gels were further incubated at 37 °C, and gel diameters were measured microscopically at successive time points up to 10 h. In a separate series of experiments, 100 µl DMEM or DMEM containing 50 µg/ml heparin or a 5 µg/ml of a blocking antibody (HA2/5, BD Biosciences) directed against the integrin β₁-subunit, were added to the gels at the time of detachment, and the gels were further incubated at 37 °C; gel diameters were measured as above.
RESULTS

FGF2- but Not PDGF-BB- or FGF10-induced Activation of ERK1/2 Is Reduced in Ext1<sup>Gt/Gt</sup> Fibroblasts—Previously, we have shown that the HS chains produced by Ext1<sup>Gt/Gt</sup> mouse embryonic fibroblasts are shorter than those of the wild-type fibroblasts (20). HS acts as an important co-receptor for several growth factors and to determine whether the reduction in chain length on the Ext1<sup>Gt/Gt</sup> cells had a biological effect, we examined the ability of wild-type and mutant cells to respond to various growth factors. FGF2 activated both wild-type and Ext1<sup>Gt/Gt</sup> mutant cells in a dose-dependent manner, as determined by the levels of phosphorylated p44/42 (ERK1/2) (Fig. 1A). Comparisons at the highest levels of phosphorylation in the wild-type cells, showed that the relative values of pERK1/2 were decreased by ~75% in the Ext1<sup>Gt/Gt</sup> mutant cells. In contrast, Ext1<sup>Gt/Gt</sup> mutant and wild-type cells showed no striking difference in FGF10- or PDGF-BB-stimulated ERK1/2 phosphorylation (Fig. 1, B and C), even though some variations in the response to high concentrations of PDGF-BB (100 ng/ml) were observed in the mutant cells between different experiments.

Reduced Binding of HS-specific Antibodies to Ext1<sup>Gt/Gt</sup> Fibroblasts—To directly test whether the gene trap mutation in Ext1 alters ligand binding to cell surface HS, we used flow cytometry to quantify the binding of the HepSS1 and 10E4, two antibodies that recognize sulfation patterns within HS chains (32, 33). Both 10E4 and HepSS1 antibodies showed a markedly reduced binding to Ext1<sup>Gt/Gt</sup> cells relative to wild-type cells. The median channel fluorescence (MCF) values after staining with 10E4 were 270 for the mutant and 731 for the wild-type cells (Fig. 2A). In contrast, no significant difference in the binding of the 3G10 antibody could be observed between the wild-type and mutant cells after digestion with a combination of heparitinase I and II (Fig. 2C), MCF<sub>Ext1</sub> = 433 and MCF<sub>wild-type</sub> = 448. The 3G10 antibody does not react with intact HS but recognizes the unsaturated hexuronic acid-containing oligosaccharide stubs that remain associated with core proteins after heparitinase digestion (32). Therefore, 3G10 serves as a marker of the amount of HS chains without providing information about their structure. Thus, our results indicate that the wild-type and the mutant cells have similar numbers of HS chains exposed at the cell surface but that the HS chains on the mutant cells contain less binding epitopes for the HepSS1 and 10E4 antibodies.

Attachment to Collagen and Collagen Gel Contraction Is Attenuated in Ext1<sup>Gt/Gt</sup> Fibroblasts—Normal tissue repair involves migration of fibroblasts into the wound followed by tissue remodeling and repair. Cell surface HSPGs are important in mediating the adhesion to the extracellular matrix. To analyze the impact of the Ext1 mutation on cell-matrix interactions, we compared the attachment of wild-type and mutant fibroblasts to collagen I. At low collagen concentrations, the attachment of Ext1<sup>Gt/Gt</sup> mutant cells to collagen was reduced ~20–30% as compared with wild-type cells. At higher collagen concentrations, there was an increased attachment of wild-type cells, which was not observed to the same extent for Ext1<sup>Gt/Gt</sup> fibroblasts (Fig. 3).
Because the attachment of Ext1\textsuperscript{Gt/Gt} cells to collagen was reduced, we next investigated the contractile capacity of wild-type and Ext1\textsuperscript{Gt/Gt} cells to determine whether the mutation in Ext1 influenced cell-mediated remodeling of three-dimensional collagen matrices. Wild-type and Ext1\textsuperscript{Gt/Gt} mutant fibroblasts were embedded in collagen gels, and the diameter of the contracted collagen gel was compared with the initial gel diameter. Wild-type cells contracted the collagen gel within hours and reached a maximum contraction toward the end of the observation period at 10 h (Fig. 4A). In contrast, Ext1\textsuperscript{Gt/Gt} fibroblasts showed a considerable decrease in both the initial and overall resultant contraction of collagen matrix as compared with wild-type controls (Fig. 4A). Wild-type cells contracted the gels to ~35% of the initial size, whereas the corresponding value for the mutant cells was ~75%. Addition of FGF2 stimulated the contraction of collagen gels by both wild-type and Ext1\textsuperscript{Gt/Gt} cells (Fig. 4B). However, although gel contraction by Ext1\textsuperscript{Gt/Gt} cells was increased by the presence of FGF2, it was not comparable with that of wild-type cells.

Because integrins are the major collagen receptors on mouse fibroblasts, we analyzed the expression levels of the collagen binding integrins α1, α2, α11, and β1 by flow cytometry (Fig. 5). Except for a slight tendency to increased expression of α1 in the mutant cells, there were no significant differences in integrin expression between wild-type and Ext1\textsuperscript{Gt/Gt} cells. To further examine the effect of the Ext1 mutation on the ability of the mouse embryonic fibroblasts to interact with collagen I, we analyzed the effect of heparin on cell attachment to collagen I and on cell-mediated collagen contraction. In cell attachment assays, heparin reduced the attachment of wild-type and Ext1\textsuperscript{Gt/Gt} cells to collagen I by 16 and 32%, respectively (Fig. 6A). Heparin also reduced the contractile capacity of both the wild-type (Fig. 6B) and the Ext1 mutant cells (Fig. 6C), and, similar to the attachment to collagen, the reduction was more pronounced in the Ext1\textsuperscript{Gt/Gt} cells. Finally, incubation with an antibody to β1 (Ha2/5) inhibited collagen contraction completely. Similar results were obtained with the 9EG7 antibody (data not shown).

**Cell Proliferation Induced by FGF2 and FGF10 Is Reduced in Ext1\textsuperscript{Gt/Gt} Fibroblasts—As ERK phosphorylation induced by transient stimulation with FGF2 was reduced in Ext1\textsuperscript{Gt/Gt} cells as compared with wild-type cells, we wanted to investigate whether there was a functional effect on mitogenic activity following sustained growth factor stimulation. Addition of FGF2 stimulated proliferation of both cell types (Fig. 7). Despite the significantly weaker signaling response of the mutant compared with the wild-type cells after stimulation with FGF2 (Fig. 1A), the proliferation of Ext1\textsuperscript{Gt/Gt} cells after

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**FIGURE 3.** Ext1\textsuperscript{Gt/Gt} fibroblasts exhibit reduced attachment to collagen I. Ext1\textsuperscript{Gt/Gt} and wild-type fibroblasts were allowed to adhere to different concentrations of immobilized collagen and the number of adherent cells determined as described under “Experimental Procedures.” The absorbance reflects the number of attached cells. The numbers of attached Ext1\textsuperscript{Gt/Gt} fibroblasts (white bars) were reduced compared with wild-type cells (black bars), both at high and low collagen concentrations. The graph shows one of five representative experiments, and each experimental condition was measured in duplicate.

**FIGURE 4.** Ext1\textsuperscript{Gt/Gt} fibroblasts show impaired ability to contract collagen gels. Wild-type and Ext1\textsuperscript{Gt/Gt} fibroblasts were embedded in type I collagen as described under “Experimental Procedures.” Contraction of collagen gels was monitored at the indicated time points, in the absence (DMEM) or presence of FGF2 (10 ng/ml). A, Ext1\textsuperscript{Gt/Gt} fibroblasts displayed a reduced capacity to contract floating collagen gels, as compared with wild-type fibroblasts. The decrease was evident after 2 h and remained consistent throughout the measurements. Re-expression of Ext1 by the mutant cells increased contractility to near wild-type levels (shown for clone 4.7). B, addition of FGF2 caused a moderate increase in contractility for all three cell types. The degree of contraction is presented as the gel area in percentage of the original area ± S.D. (n = 12). wt, wild-type; Gt/Gt, Ext1\textsuperscript{Gt/Gt}, cl. 4.7, clone 4.7 (Ext1\textsuperscript{Gt/Gt}×Ext).

**FIGURE 5.** Integrin expression by wild-type and Ext1\textsuperscript{Gt/Gt} fibroblasts. Ext1\textsuperscript{Gt/Gt} and wild-type cells were examined for expression of collagen binding integrins (α1, α2, α11, and β1) by flow cytometry using integrin-specific antibodies as described under “Experimental Procedures.” The expression levels of these integrins were similar for both cell types. Black line, α1, α2, α11, or β1; filled gray profile, control (using secondary antibody only).
EXT1 Influences Fibroblast Matrix Interactions

**Growth Factor Interactions**—The polymerization of HS is generated by alternating additions of GlcNAc and GlcA. To corroborate that the changes in HS chain length correlated with GlcNAc and GlcA transferase activities, we assayed the in vitro glycosyltransferase activities in cellular extracts. As shown in Fig. 8, A and B, the in vitro enzyme activities of Ext1Gt/Gt cells were dramatically reduced. To confirm that this and the above-described results were specifically caused by the mutation in the Ext1 gene, Ext1Gt/Gt fibroblasts were stably transfected with Ext1, generating cellular clones expressing Ext1Gt/Gt+Ext1. Transfection of Ext1 into Ext1Gt/Gt fibroblasts restored enzyme activities to approximately the same levels as in the wild-type cells (Fig. 8, A and B). Re-expression of Ext1 also restored HS chain length to that of wild type cells (Fig. 8C). The longer HS chains also resulted in more binding epitopes for the 10E4 antibody (Fig. 8D) and an increased FGF2 signaling response (Fig. 8E). In addition, collagen gel contraction by Ext1Gt/Gt+Ext1 fibroblasts was similar to that of the wild-type cells (Fig. 4, A and B).

Taken together, we have demonstrated that reduction in Ext1 enzyme activity in mouse embryonic fibroblasts results in decreased MAPK signaling induced by FGF2 but not FGF10 or PDGF-BB and a lower proliferation rate. Another consequence of reduced Ext1 expression is a decreased interaction with collagen I, resulting in impaired cell adhesion and ability of the cells to reorganize collagen gels.

**DISCUSSION**

In this study, we examined how Ext1 levels affect fibroblast growth factor signaling and interaction with the extracellular matrix. Several signaling molecules, including members of the FGF, Wingless/Wnt, and Hh families, require HSPG at the cell surface for optimal signaling activity. Whereas biochemical studies, using structurally defined HS oligosaccharides in 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 intact cell-associated proteoglycans for growth factor binding is not much studied. We have previously observed that HS chain length is substantially reduced in Ext1Gt/Gt mutant fibroblasts, without significantly affecting the sulfation (20). Our present results demonstrate that the reduced HS chain length has a dramatic effect on fibroblast response to FGF2 and interaction with the extracellular matrix. The interaction of FGF2 with HS has received much attention because of the essential role of HS for FGF2-induced biological cellular responses. A range of size-defined heparin oligosaccharides has been tested in different studies for their ability to bind to FGF2 and to form FGF2-HS-FGFR ternary complexes. These studies show that the N-and O-sulfate groups play essential roles for the FGF2-HS interaction and suggest that although tetra-, penta-, and hexasaccharides are able to bind FGF2, at least an HS octasaccharide with sufficient sulfation is required for binding of HS to both FGF2 and its receptor (34–37). We observed that FGF2-induced MAPK signaling and proliferation was clearly reduced in the Ext1Gt/Gt fibroblasts indicating that the short HS chains synthesized by Ext1Gt/Gt fibroblasts have a

FGF2 stimulation was ~65% of the wild-type cells. FGF10 induced a more modest proliferation response of wild-type cells and had virtually no effect on the Ext1Gt/Gt cells (Fig. 7).

**Re-expression of Ext1 in Ext1Gt/Gt Fibroblasts Increases HS Chain Length and Rescues HS-dependent Cell Matrix and
decreased capacity to respond to FGF2. However, although the FGF2-mediated ERK phosphorylation was reduced in the mutant cells, it was not completely abolished, which could explain their proliferation response during sustained FGF2 stimulation. The shorter chain length may also influence the intracellular signaling by FGF2. The band intensity of phosphorylated ERK1/2 (shown for clone 1.4, triangles) is decreased in Ext1Gt/Gt cells with Ext1 (clones 1.4, 4.7, and 4.8) as compared with wild-type and mutant fibroblasts; E) increased intracellular signaling by FGF2. The band intensity of phosphorylated versus total ERK was quantified and presented in the bar graph as relative activation of the receptor. The figure shows one representative experiment of three independent experiments. Black bars, wild-type cells; white bars, Ext1Gt/Gt cells; gray bars, clone 4.8; cl., clone.

PDGF-BB-induced PDGFRα receptor activation (26, 39).

Collagens are abundant constituents of the extracellular matrix, where they provide important functions as protective support during tensile force and as scaffolds to help cells organizing the matrix. In a recent study, utilizing dermal fibroblasts from patients with systemic sclerosis, it was shown that HS-dependent ERK signaling contributes to the increased contractility seen in the lesions developing during this condition (40). This suggests that the reduced contractility of the Ext1Gt/Gt fibroblasts observed in the present study may rely on a reduced signaling response to FGF2; however, this remains to be confirmed. FGF2-induced contraction of floating collagen lattices was previously shown to involve phosphatidylinositol 3-kinase, Rac, Rho, and also Rho kinase (41), implying that the intracell-
collar signaling mechanisms that drive collagen gel contraction are complex and that several different signaling pathways act in parallel. HS binds to collagen I via a single binding site near the NH₂ terminus on the collagen monomer (42). The corresponding sequence on the HS chain is less characterized, but our results from the cell attachment assay and also the collagen gel contraction analysis suggest that this domain is absent or less prevalent in the HS chains expressed by Ext₁/Gt/Gt cells. Delayed collagen gel contraction was recently reported for fibroblasts deficient in the major cell surface HSPG syndecan-4 (43), supporting the role for HS in remodeling of collagen gels. Previous work has shown that the expression of syndecan-4 is not decreased in Ext₁/Gt/Gt mutant cells (44), and also that although these cells are able to adhere to fibronectin, they are unable to form focal adhesion (45). The ability of fibroblasts to contract free-floating collagen gels rely on functional β1-integrins (46, 47) and HS proteoglycans could possibly act both as primary receptors for collagen or as co-receptors in the interaction between collagen and integrins (42). In the present study, heparin reduced the contractile capacity of the mouse embryonic fibroblasts, emphasizing the important role of HS in this context. Recently, cell surface HS chains on syndecan-1 were shown to be essential for αβ1-mediated adhesion of Chinese hamster ovary cells to collagen and for α2β1-mediated cell spreading and actin organization on collagen (48). One of the most prevalently expressed integrins in cultured immortalized mouse embryonic fibroblasts has previously been implicated to be α1β1 (30), and this was also the case in the present study. However, any potential cooperation between HS and α1β1 remains to be established.

It has been shown that there is a reduction, but not a total loss, of HS in the chondrocytes of exostoses, suggesting that exostoses formation is a result of reduced HS (49). An intriguing issue is whether multiple exostoses-associated mutations in either Ext1 or Ext2 result in reduction of HS in affected growth plates, leading to shorter HS chains. This, in turn, could result in growth factor signaling defects in a subset of chondrocytes, as our results suggest that Ext1-dependent HS chain elongation makes a significant contribution to the specificity of HS-protein interactions.

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