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Etiological Point Mutations in the Hereditary Multiple Exostoses Gene *EXT1:* A Functional Analysis of Heparan Sulfate Polymerase Activity

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Hereditary multiple exostoses (HME), a dominantly inherited genetic disorder characterized by multiple cartilaginous tumors, is caused by mutations in members of the *EXT* gene family, *EXT1* or *EXT2*. The corresponding gene products, exostosin-1 (EXT1) and exostosin-2 (EXT2), are type II transmembrane glycoproteins which form a Golgi-localized heterooligomeric complex that catalyzes the polymerization of heparan sulfate (HS). Although the majority of the etiological mutations in EXT are splice-site, frameshift, or nonsense mutations that result in premature termination, 12 missense mutations have also been identified. Furthermore, two of the reported etiological missense mutations (G339D and R340C) have been previously shown to abrogate HS biosynthesis (McCormick et al. 1998). Here, a functional assay that detects HS expression on the cell surface of an EXT1-deficient cell line was used to test the remaining missense mutant exostosin proteins for their ability to rescue HS biosynthesis in vivo. Our results show that EXT1 mutants bearing six of these missense mutations (D164H, R280G/S, and R340S/H/L) are also defective in HS expression, but surprisingly, four (Q27K, N316S, A486V, and P496L) are phenotypically indistinguishable from wild-type EXT1. Three of these four "active" mutations affect amino acids that are not conserved among vertebrates and invertebrates, whereas all of the HS-biosynthesis null mutations affect only conserved amino acids. Further, substitution or deletion of each of these four residues does not abrogate HS biosynthesis. Taken together, these results indicate that several of the reported etiological mutant EXT forms retain the ability to synthesize and express HS on the cell surface. The corresponding missense mutations may therefore represent rare genetic polymorphisms in the *EXT1* gene or may interfere with as yet undefined functions of EXT1 that are involved in HME pathogenesis.

Introduction

Hereditary multiple exostoses (HME) (EXT [MIM 133700]) is an autosomal dominant bone disorder that results in the formation of benign cartilage-capped tumors, or exostoses, primarily on the long bones of affected individuals (Solomon 1963). Patients with HME are generally short in stature, with varying degrees of orthopedic deformity, and surgery may be required to alleviate secondary complications such as joint pain and restricted movement. Both hereditary and sporadic cases of exostoses are linked to two main loci, *EXT1* (EXT1 [MIM 133700]) on chromosome 8q24.1 (Cook et al. 1993; Lüdecke et al. 1995) and *EXT2* (EXT2 [MIM 133701]) on chromosome 11p11-p12 (Wu et al. 1994; Wuyts et al. 1995), although linkage to another locus, *EXT3* (EXT3 [MIM 600209]), on chromosome 19p has also been described (Le Merrer et al. 1994). Chondrosarcomas develop in 0.5%–3% of cases (Schmale et al. 1994; Luckert-Wicklund et al. 1995), apparently because of loss of heterozygosity (LOH) at one or several *EXT* loci, possibly in combination with additional LOH at other, nonrelated sites (Raskind et al. 1995). Insofar as mutation and, presumably, loss of function of either EXT1 or EXT2 appears to be sufficient for tumor formation, it has been suggested that these proteins are tumor suppressors (Hecht et al. 1995; Lüdecke et al. 1995; Raskind et al. 1995; Bovée et al. 1999).

Human *EXT1* and *EXT2* have both been cloned (Ahn et al. 1995; Stickens et al. 1996; Wuyts et al. 1996), as have homologs in rodents (Clines et al. 1997; Lin and Wells 1997; Lohmann et al. 1997; Stickens and Evans 1997; Wei et al. 2000), *Drosophila melanogaster* (Bellaiche et al. 1998) and *Caenorhabditis elegans* (Clines et al. 1997; Lohmann et al. 1997). Both *EXT1* and *EXT2* belong to a larger family of homologous genes,

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the *EXT* family of putative tumor-suppressor genes, for which three additional members have been cloned, the *EXT*-like genes, *EXTL1-3* (Wise et al. 1997; Wuyts et al. 1997; Saito et al. 1998; Van Hul et al. 1998). In humans, the corresponding EXT proteins, exostosin-1 (EXT1) and exostosin-2 (EXT2) are ubiquitously expressed glycoproteins of 746 and 718 amino acids, respectively (Ahn et al. 1995; Stickens et al. 1996; Wuyts et al. 1996). EXT1 and EXT2 have a predicted type II transmembrane glycoprotein structure, and both proteins localize predominantly to the endoplasmic reticulum when overexpressed in cells (Lin and Wells 1997; McCormick et al. 1998; McCormick et al. 2000).

Despite extensive genetic characterization, the function of the EXT proteins remained unknown until 1998, when the study of a HS-deficient cell line, sog9, revealed that EXT1 is involved in HS biosynthesis (McCormick et al. 1998). Biochemical studies have since confirmed that EXT1 and EXT2 both possess the *N*-acetylglucosamine (GlcNAc) and D-glucuronic acid (GlcA) transferase (T) activities representative of an HS-polymerase (HS-Pol) (Lind et al. 1998; Senay et al. 2000; Wei et al. 2000). Moreover, the related protein *EXTL2* encodes a functionally related enzyme, α1,4-*N*-acetylhexosaminyltransferase (Kitagawa et al. 1999). It is proposed that EXTL2 initiates HS-chain formation by transferring the first GlcNAc residue to a specific tetrasaccharide linker sequence on the HS proteoglycan core protein thereby providing the substrate for polymerization by EXT1 and EXT2. By contrast, a recent study of *C. elegans* EXT homologs suggests that a single protein, Rib-2, most closely related to the human *EXTL3* gene product, is able to carry out both the HS-chain initiation and polymerization steps, indicating that the biosynthetic mechanism of HS in *C. elegans* is distinct from that reported for the mammalian system (Kitagawa et al. 2001). Surprisingly, the human *EXTL3* gene encodes a molecule with a strikingly different function, the cell-surface receptor for Reg, which is a pancreatic β-cell regeneration factor (Kobayashi et al. 2000*a*). Further, most recently it has been suggested that EXTL3 may modulate TNF-α induced NF-κB activity (Mizuno et al. 2001).

Recent data indicates that EXT1 and EXT2 form a heterooligomeric complex in vivo that leads to an accumulation of both proteins in the Golgi apparatus (Kobayashi et al. 2000*b*; McCormick et al. 2000). In agreement with this, in situ hybridization studies in the mouse show identical expression patterns for *EXT1* and *EXT2* mRNA (Stickens et al. 2000). In vivo functional assays have shown that EXT2 cannot substitute for EXT1 in either sog9 (McCormick et al. 2000) or CHO cell systems (Wei et al. 2000). Moreover, the Golgi-localized EXT1/EXT2 complex was found to possess substantially higher glycosyltransferase activity than EXT1 alone, suggesting that this complex represents the biologically relevant form of the HS-Pol enzyme (McCormick et al. 2000). These findings have since been confirmed in a yeast cell system, which has the advantage of not exhibiting any endogenous HS-Pol activity (Senay et al. 2000). This heterooligomeric model of the HS-Pol provides a rationale to explain how inherited mutations in either of the two *EXT* genes can cause loss of HS biosynthesis activity, resulting in HME (McCormick et al. 2000). Recently, a study involving HS-deficient mutant CHO cells similarly rescued by human EXT1 expression, localized the GlcA-T catalytic domain to the N-terminal side of the central region of the EXT1 protein (Wei et al. 2000). The importance of HS for normal growth and development, and therefore the importance of EXT1, has also been demonstrated in several in vivo models, including *Drosophila* (Bellaiche et al. 1998; The et al. 1999; Toyoda et al. 2000), and the mouse (Lin et al. 2000), as well as in primary cell culture of HME diseased chondrocytes (Bernard et al. 2000; Legeai-Mallet et al. 2000). Moreover, the link between EXT and HME has been further affirmed by a study showing that exostosin expression was significantly reduced—and, in some cases, absent—in HME-derived chondrocytes (Bernard et al. 2001).

Extensive genetic analysis of HME patients' genomic DNA over the past few years has led to the identification of a number of mutations in both *EXT1* and *EXT2* that appear to be disease related, the majority of which result in premature termination. However, a small number of mutations have been identified—often repeatedly—affecting single amino acids, mainly in the N-terminal half of the EXT1 and EXT2 proteins (Wuyts and Van Hul 2000). Here, a functional assay that detects HS expression in *EXT1*-deficient cells, was used to test putative etiological mutant EXT1 proteins for their ability to rescue HS biosynthesis in vivo. Of the 12 reported etiological missense mutations, 4 exhibited EXT1 HS-Pol activity in this assay. Moreover, anion-exchange chromatography confirmed the presence of HS on the surfaces of cell lines stably expressing these mutant proteins, indicating that the herpes simplex virus (HSV)-infectivity assay is a reliable indicator of HS biosynthesis. Three of these four "active" mutations affect amino acids that are not conserved among vertebrates and invertebrates, whereas all of the HS-biosynthesis null mutations affect only conserved amino acids. Further, substitution or deletion of each of these four "non-essential" residues does not eliminate HS expression. Taken together, these results indicate that several of the reported etiological mutants retain the ability to synthesize and express HS on the cell surface. The corresponding missense mutations may therefore represent rare genetic polymorphisms in the *EXT1* gene or may

interfere with as yet undefined functions of EXT1 that are involved in HME pathogenesis.

Material and Methods

Cells, Virus, and DNA

The origin of the HS-deficient murine sog9 cell line has been described elsewhere (Banfield et al. 1995), as has their specific defect in the *EXT1* gene (McCormick et al. 2000). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) with 10% fetal bovine serum (Life Technologies). Stable cell lines were generated by transfection using LipofectAMINE PLUS reagent (Life Technologies), followed by selection in media supplemented with 700 μg/ml Geneticin (G-418 Sulfate) (Life Technologies). The HS-deficient mutant Chinese hamster ovary (CHO) cell line, pgsD-677, bearing a specific defect in the *EXT1* gene (Wei et al. 2000) has been described previously (Lidholt et al. 1992). These cells were maintained in Ham's F12 medium supplemented with 7.5% fetal bovine serum (Hyclone Laboratories). The three additional CHO cell lines were generously provided by Dr. Stephen H. Leppla (Gordon et al. 1995). The wild-type and furin-deficient (FD11) CHO cells were maintained in DMEM/F12 (1:1) (Life Technologies) supplemented with 5% fetal bovine serum. The stably transfected FD11+furin#5-1 CHO cells, were grown in the same medium supplemented with 400 μg/ml Geneticin. A mutant HSV-2 strain, L1BR1, which has a modified β-galactosidase gene inserted into the US3 protein kinase gene under the control of the HSV-1 beta 8 promoter (Nishiyama et al. 1992) was obtained as a generous gift from Y. Nishiyama. Two commercially available mammalian expression vectors were used for all EXT constructs: pcDNA3.1myc-his (Invitrogen) and pEGFP-N1 (Clontech). All DNA was amplified in *Escherichia coli* DH5α (Life Technologies) and was prepared for transfection using standard plasmid purification systems (Qiagen and Life Technologies).

Construction of EXT1 Etiological Mutant Proteins

All EXT mutants were constructed by site-directed PCR mutagenesis using the previously described wild-type EXT1 constructs, pEXT1myc-his and pEXT1gfp (McCormick et al. 1998; McCormick et al. 2000). It should be noted that the sequence of the human *EXT1* gene in pEXT1myc-his has been found to differ from the published sequence caused by both silent mutations ($672_{A\rightarrow G}$, $1062_{C\rightarrow T}$, $1326_{T\rightarrow C}$, $1431_{C\rightarrow T}$, $1650_{T\rightarrow C}$, and $1761_{G\rightarrow A}$) and missense mutations ($180_{G\rightarrow C}$→E60D, $181_{C\rightarrow G}$→P61A, and $1240_{G\rightarrow T}$→A413S). However, these mutations are phenotypically silent, as they are present in both wild-type and mutant constructs. All oligonucleotide primers (table 1), *Taq* DNA polymerase and restriction enzymes were obtained from Life Technologies, unless otherwise stated.

The Q27K, D164H, N316S, A486V, and P496L etiological mutants, as well as the corresponding conserved/severe change and deletion mutants, were constructed following Stratagene's QuikChange site-directed mutagenesis protocol. In brief, complementary oligonucleotides bearing the desired mutation were used to PCR amplify the entire pEXT1gfp plasmid, using *Pfu* Turbo DNA polymerase (Stratagene). The parental DNA template was then digested with *Dpn*I and the nicked vector DNA bearing the desired mutation was transformed directly into *E. coli* competent cells. The R280G, R280S, R340S, R340H, R340L, and repaired H340R mutants were all constructed by PCR amplification using mutagenic primers, followed by cloning into pEXT1myc-his, using appropriate restriction enzymes.

The single-codon deletion at position H627 was introduced by way of a two-step PCR mutagenesis strategy, using an overlapping and complementary primer set, bearing the 3-bp deletion, and two external primers. First, fragments were made with an ~40 bp overlap in the region that included the 3-bp deletion, then the two overlapping PCR fragments were denatured, were annealed together, were extended for 15 min with *Taq* polymerase, and were amplified by PCR, using the outermost primers to make a fragment which was cloned into the pEXT1myc-his vector.

To convert from myc epitope tags to green fluorescent protein tags, all EXT1 mutants were excised from the pEXT1myc-his vector, with *Bam*HI and *Sac*II, and were religated into the *Bgl*II and *Sac*II sites of the pEGFP-N1 vector, such that they were in frame for an EXT1-gfp fusion protein.

HSV-Infectivity Assay for EXT1 Function

The in vitro transfection/infection assay was performed in sog9 cells, as described elsewhere (McCormick et al. 1998), using the HSV-2 strain L1BR1.

FGF-2-Based Assay for HS Cell-Surface Expression in a CHO Cell System

Binding of biotinylated basic fibroblast growth factor (FGF-2) was measured by fluorescence-activated cell sorting, as described elsewhere (Bai et al. 1999).

Anion-Exchange Chromatography of Cell-Surface Glycosaminoglycans

Anion-exchange HPLC was performed, as described elsewhere (McCormick et al. 1998), using stably transfected cell lines.

Table 1

Oligonucleotide Primers Used for PCR Mutagenesis of EXT1[a]

Primer	DNA Sequence (5′→3′)
Q27K (forward)	TTTTATTTCGGAGGCCTGAAGTTTAGGGCATCG
Q27K (reverse)	CGATGCCCTAAACTTCAGGCCTCCGAAATAAAAC
Q27A (forward)	TTTTATTTCGGAGGCCTGGCGTTTAGGGCATCG
Q27A (reverse)	CGATGCCCTAAACGCCAGGCCTCCGAAATAAAAC
Q27del (forward)	TTTTATTTCGGAGGCCTGTTTAGGGCATCG
Q27del (reverse)	CGATGCCCTAAACAGGCCTCCGAAATAAAAC
D164H (forward)	CTGGATACTTTAGACAGACACCAGCTGTCACCTCAGTATGTG
D164H (reverse)	CACATACTGAGGTGACAGCTGGTGTCTGTCTAAAGTATCCAG
D164E (forward)	CTGGATACTTTAGACAGAGAACAGCTGTCACCTCAGTATGTG
D164E (reverse)	CACATACTGAGGTGACAGCTGTTCTCTGTCTAAAGTATCCAG
D164del (forward)	CTGGATACTTTAGACAGACAGCTGTCACCTCAGTATGTG
D164del (reverse)	CACATACTGAGGTGACAGCTGTCTGTCTAAAGTATCCAG
R280G (forward)	GGAAGAGGTACCTGACAGGGATAGGATCAGACACCGGGAATGCCTTATATC
R280S (forward)	GGAAGAGGTACCTGACAGGGATAGGATCAGACACCAGCAATGCCTTATATC
R280 (reverse)	GAGTTATCCCAGAAGTGGCTGCGCGCGGGGTACCCACAATCCTCTCAGG
N316S (forward)	CACAAGGATTCTCGCTGCGATCGAGACAGCACCGAGTATGAGAAG
N316S (reverse)	CTTCTCATACTCGGTGCTGTCTCGATCGCAGCGAGAATCCTTGTG
N316a (forward)	CACAAGGATTCTCGCTGCGATCGAGACGCCACCGAGTATGAGAAG
N316A (reverse)	CTTCTCATACTCGGTGGCGTCTCGATCGCAGCGAGAATCCTTGTG
N316del (forward)	CACAAGGATTCTCGCTGCGATCGAGACACCGAGTATGAGAAG
N316del (reverse)	CTTCTCATACTCGGTGTCTCGATCGCAGCGAGAATCCTTGTG
R340 (forward)	CGGGATCCCGCAGGACACATGCAGGCCAAAAAACGCTATTTCATCC
R340S (reverse)	CAAAGCCTCCAGGAATCTGAAGGACCCAAGCCTGCTACCACGAGGAACCAG
R340H (reverse)	CAAAGCCTCCAGGAATCTGAAGGACCCAAGCCTGTGACCACGAGGAACCAG
R340L (reverse)	CAAAGCCTCCAGGAATCTGAAGGACCCAAGCCTGAGACCACGAGGAACCAG
H340R (reverse)	CAAAGCCTCCAGGAATCTGAAGGACCCAAGCCTGCGACCACGAGGAACCAG
A486V (forward)	GCAGTCATCCATGTGGTGACGCCCCTGGTCTCTCAGTCC
A486V (reverse)	GGACTGAGAGACCAGGGGCGTCACCACATGGATGACTGC
A486H (forward)	GCAGTCATCCATCATGTGACGCCCCTGGTCTCTCAGTCC
A486H (reverse)	GGACTGAGAGACCAGGGGCGTCACATGATGGATGACTGC
A486del (forward)	GCAGTCATCCATGTGACGCCCCTGGTCTCTCAGTCC
A486del (reverse)	GGACTGAGAGACCAGGGGCGTCACATGGATGACTGC
P496L (forward)	CAGTCCCAGCTAGTGTTGAAGCTCCTCGTGGCTGCAGCC
P496L (reverse)	GGCTGCAGACACGAGGAGCTTCAACACTAGCTGGGACTG
P496H (forward)	CAGTCCCAGCACGTGTTGAAGCTCCTCGTGGCTGCAGCC
P496H (reverse)	GGCTGCAGACACGAGGAGCTTCAACACGTGCTGGGACTG
P496del (forward)	CAGTCCCAGGTGTTGAAGCTCCTCGTGGCTGCAGCC
P496del (reverse)	GGCTGCAGACACGAGGAGCTTCAACACCTGGGACTG
H627 (forward 1)	AGTATTCATCTTATCTGGGAG
H627 (reverse 1)	GGTAGTGATAATATTTAGTAAATAGCAG
H627 (forward 2)	TGACAGGAGCTGCTATTTACAAAATATT
H627 (reverse 2)	TTTTCCTTTTGCGGCCGCTTTTTTCCTTAAGTCGCTCAATGTCTCGGTA

[a] Mutated nucleotides are underlined.

Results

In Vivo Analysis of Mutant EXT1 Function

Twelve etiological missense mutations and one single codon deletion mutation have been identified in the EXT1 gene of HME patients (table 2). These mutations are found exclusively in the luminal portion of the protein in four of the eleven exons (fig. 1). To date, only two of these missense mutations, G339D and R340C, have been evaluated for HS biosynthetic activity in vivo (McCormick et al. 1998). In this study, the 10 remaining etiological missense mutants, affecting seven different residues (Q27, D164, R280, N316, R340, A486, and P496), were examined by way of the HSV-infectivity assay and representative results for each of the seven affected residues are shown in figure 2. Two types of mutant EXT1 proteins were identified (fig. 1): "active" mutants, which rescue HSV infectivity, and null mutants, which are inactive in the HSV-infectivity assay, suggesting that they have lost their ability to synthesize HS. Table 2 summarizes in vivo HS biosynthetic activity for all of the EXT1 missense mutants analyzed to date, as well as for the single–amino acid–deletion mutant.

Residues G339 and R340 are of particular interest, in that they are part of a R*G*R*R* motif (fig. 3), which is a classic recognition site for cleavage by furin, a cellular

Table 2

Functional Assay of EXT1 Proteins Bearing Mutations Implicated in HME

Etiological Mutation	Reference(s)	HS Biosynthesis[a]
wt EXT1	Ahn et al. (1995)	+[b]
Q27K	Wuyts and Van Hul (2000)	+
D164H	Bovée et al. (1999)	−
R280G	Raskind et al. (1998); Wuyts et al. (1998)	−
R280S	Raskind et al. (1998)	−
N316S	Bovée et al. (1999)	+
G339D	Philippe et al. (1997)	−[b]
R340C	Philippe et al. (1997); Dobson-Stone et al. (2000)	−[b]
R340S	Wuyts et al. (1998)	−
R340H	Raskind et al. (1998); Dobson-Stone et al. (2000)	−
R340L	Hecht et al. (1997); Dobson-Stone et al. (2000)	−
H340R[c]	…	+
A486V	Xu et al. (1999)	+
P496L	Xu et al. (1999)	+
H627del[d]	Raskind et al. (1998)	−

[a] As measured by the HSV-infectivity assay (McCormick et al. 1998), in which HS-deficient sog9 cells are transfected with EXT1 forms and then are challenged with a β-galactosidase–expressing HSV strain. + = wild-type EXT1 level of infectivity; − = background level of infectivity.

[b] As published elsewhere (McCormick et al. 1998).

[c] Repaired version of the R340H etiological mutant.

[d] del = deletion of the amino acid.

proprotein convertase (Lazure et al. 1998). To determine whether furin is essential for HS biosynthesis, furin-expressing and furin-deficient CHO cell lines were challenged with HSV. However, the furin-deficient cells were found to be as sensitive to HSV as wild-type cells (data not shown), indicating that a lack of furin does not inhibit HS biosynthesis and transduction to the cell surface. It is, therefore, unlikely that the etiological missense mutations affecting residues G339 and R340 inactivate EXT1 through loss of a furin-recognition site.

To determine whether EXT proteins are able to complement each other *in trans,* as has been observed for a bacterial β-glycosyltransferase (Jing and DeAngelis 2000), all possible combinations of the null mutants were cotransfected into sog9 cells and assayed for rescue of HSV infectivity. No complementation was observed (data not shown). Moreover, when sog9 cells were cotransfected with mutant and wild-type forms of EXT1, no measurable decrease in activity was observed, even when mutant and wild-type forms were coexpressed in a ratio of 10:1 (data not shown). These results indicate that wild-type EXT1 is able to polymerize HS in the presence of mutant forms of the EXT1 protein.

To further confirm that certain of the mutant EXT1 forms were indeed capable of inducing the cell-surface expression of HS, a second *in vivo* cell culture assay system was developed. In this assay, HS-deficient mutant CHO cells, pgsD-677, were transfected with gfp-tagged wild-type and mutant EXT1 proteins, and then tested for their ability to bind basic fibroblast growth factor (FGF2), a known ligand of HS (Bai et al. 1999). Results in this alternate cell system confirmed the respective "active" or null phenotypes observed with the HSV-infectivity assay (table 2; fig. 1) for each of the mutant constructs (data not shown).

Anion-Exchange Chromatography of Cell-Surface GAGs

To investigate GAG synthesis directly, in sog9 cells stably expressing mutant EXT1 proteins, anion-exchange chromatography of radiolabeled cell-surface GAGs was performed for each of the seven residues analyzed in figure 2. The results, illustrated in figure 4, show a single GAG peak corresponding to HS for each of the "active" mutants, suggesting that these mutants express HS to levels comparable to those of wild-type EXT1. In agreement with their null phenotype in the HSV-infectivity assay (fig. 1), none of the null mutant forms could catalyze the polymerization of HS.

Characterization of "Active" EXT1 Etiological Mutants

The results so far indicated that four of the putative etiological mutations retained significant HS biosynthesis activity. One possibility to explain these results is that these mutant forms of EXT1 possess reduced HS biosynthetic activity compared with wild-type forms. To test this possibility, the four codons in EXT1 were subjected to further alteration. As is summarized in table 3, substitution of the acidic Q27 or N316 residues by alanine, an unrelated aliphatic residue, did not inactivate HS-Pol activity. Likewise, substitution of the uncharged A486 and P496 residues by a basic, positively charged histidine residue did not abolish HS biosynthesis. Remarkably, HS expression was retained even when the affected

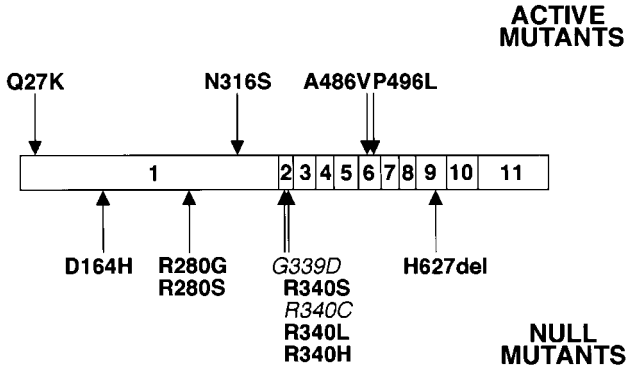

Figure 1 Two phenotypes for etiological EXT1 mutants. Schematic representation of the *EXT1* coding region, illustrating the 11 exons of the human *EXT1* gene with all 12 of the currently reported missense mutations from HME patients, and one single–amino acid deletion. The two missense mutations analyzed prior to the current study (McCormick et al. 1998) are indicated in italics. Mutant EXT1 forms can be divided into two categories: "active" mutants (*top arrows*), which were observed to synthesize HS, and null mutants (*bottom arrows*), which were observed to abrogate HS biosynthesis.

Figure 2 Cell-culture assay for EXT1 function. HSV-resistant, HS-deficient mouse fibroblast sog9 cells either were mock-transfected or were transfected with cDNAs corresponding to wild-type or mutant EXT1 constructs. Cells were then infected with a β-galactosidase expressing HSV and were stained blue with X-gal. For the two residues affected by multiple etiological missense mutations (R280 and R340) only one mutant form is shown.

amino acid was deleted from the EXT1 protein. Taken together, the data show that these four amino acid residues are not essential for the HS-Pol activity of EXT1.

An Aspartate Residue Required for HS-Pol Activity

The inactivating missense mutation D164H, located in a DXD sequence (fig. 3), was also subjected to further mutagenic analysis. DXD motifs, and aspartate residues in particular, have been reported to be important components of catalytic sites for glycosyltransferase activity (Busch et al. 1998; Wiggins and Munro 1998). The etiological change from aspartate to histidine represents a severe change from an acidic to a basic amino acid. To determine whether there was an absolute requirement for aspartate, as has been described for other glycosyltransferase enzymes (Busch et al. 1998; Griffiths et al. 1998; Wiggins and Munro 1998; Jing and DeAngelis 2000), the aspartate residue was replaced by the closely related amino acid, glutamate, in the otherwise wild-type EXT1 protein. This mutant form was also defective in HS polymerization, suggesting that EXT1 requires an aspartate residue at position 164 for HS-Pol function.

Discussion

Although the pathogenesis of HME was first described nearly 200 years ago (see Solomon 1963), it is only in the past few years that elegant genetic analyses (Ahn et al. 1995; Stickens et al. 1996; Wuyts et al. 1996) and intensive biochemical studies (Lind et al. 1998; McCormick et al. 1998) have permitted the identification and functional characterization of the exostosin family of putative tumor-suppressor proteins. The HSV assay employed in this study is useful for evaluating in vivo HS-Pol activity, as it measures the biosynthesis and cell-surface expression of HS—unlike in vitro enzyme assays, which measure the addition of a single sugar to an artificial substrate. It is highly specific, because it was used to show that EXT2 is distinct from EXT1 (McCormick et al. 2000), despite the fact that EXT2 has been shown to possess the same glycosyltransferase activities as EXT1 in vitro (Lind et al. 1998; Kitagawa et al. 2001; Senay et al. 2000). In the present study, we evaluated the effect on in vivo HS-Pol activity of HME etiological mutations identified in the *EXT1* locus of affected individuals, and we found that not all mutations were inactivating. These results raise the possibilities that these mutations are not related to disease pathogenesis and that EXT1 possesses additional functions not yet identified.

For other known glycosyltransferases, domain swaps and site-directed mutagenesis have often proven to be powerful tools for identification of key amino acids involved in enzyme-substrate specificity and catalytic-site activity (Xu et al. 1996; Seto et al. 1997; Hagen et al. 1999; Jing and DeAngelis 2000). In the case of the exostosin proteins, it is most likely that naturally occurring etiological missense and single-codon deletion mutations affect key functional domains, thereby providing us with a subset of candidates for elucidating enzyme function. Indeed, the results of the HSV-infectivity assay, published previously (McCormick et al. 1998) and presented here, have confirmed that eight previously reported HME-linked missense mutations and one single–amino acid–deletion mutation abolish enzyme function in vivo. All of these mutations affect amino acid residues that have been evolutionarily conserved, among both vertebrate and invertebrate species (fig. 3), suggesting that they are critical for EXT1 function. By contrast, three of the four remaining missense mutations—which do not inhibit HS expression in vivo—affect residues that are not conserved among distantly related eukaryotes. These amino acids are not essential for EXT1 function in polymerizing HS in cultured cells, as they can be deleted from the protein without incurring loss of HS-Pol function. The possibility that these mutants retain partial activity, producing an amount and/or type of HS sufficient for HSV entry but insuf-

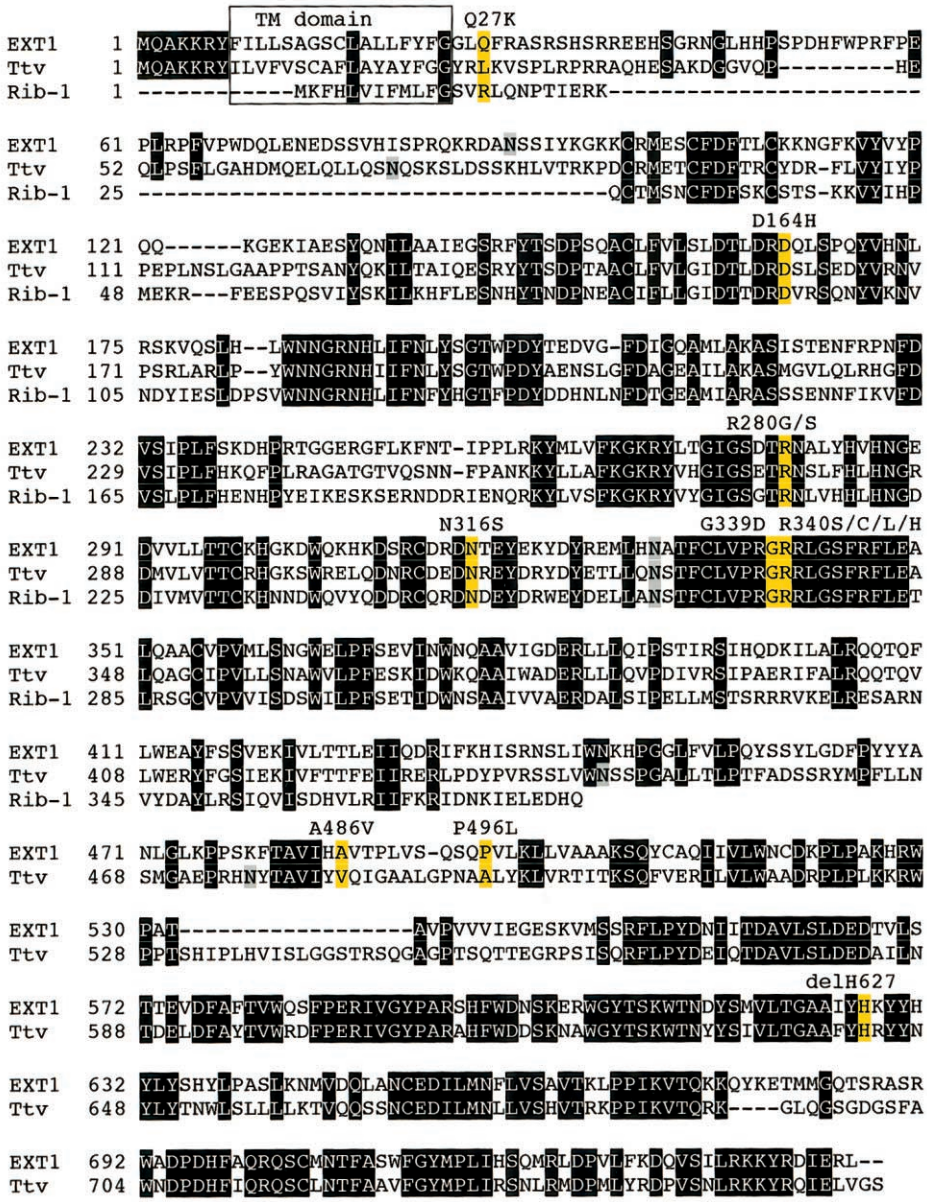

Figure 3 Amino acid–sequence alignment for three EXT1 homologs: human EXT1 (GenBank accession number S79639); Ttv, from *Drosophila melanogaster* (GenBank accession number AF083889) and Rib-1, from *Caenorhabditis elegans* (GenBank accession number U94834). The putative transmembrane (TM) domains are labeled, predicted N-linked glycosylation sites are shaded in gray, conserved amino acids are indicated by black boxes, and putative etiological EXT1 missense mutations are highlighted in yellow. Murine EXT1 (not shown) is 99% similar to hEXT1 at the amino acid level (Lohmann et al. 1997) and is identical to hEXT1 at all of the etiological mutation positions highlighted in yellow. Alignments were predicted and visualized using ClustalW 1.8 and Boxshade 3.21 software.

ficient for proper bone development, cannot be excluded.

The one exception, N316S, is conserved (fig. 3) and lies directly next to a putative glycosyltransferase catalytic site motif, DXD (Busch et al. 1998; Wiggins and Munro 1998), which is itself, however, not totally conserved (fig. 3). The fact that alteration and even deletion of N316 does not eliminate HS biosynthesis is intriguing, if this particular DXD motif is the catalytic site for GlcA-T activity, as has been proposed elsewhere (Wei et al. 2000). D164H also lies within a DXD motif (fig. 3). Furthermore, D164 appears to be highly sensitive to mutation, as even a very conserved amino acid substitution was observed to abrogate HS biosynthesis. It has been proposed that DXD motifs correspond to nucleotide and cation binding domains critical to catalysis (Busch

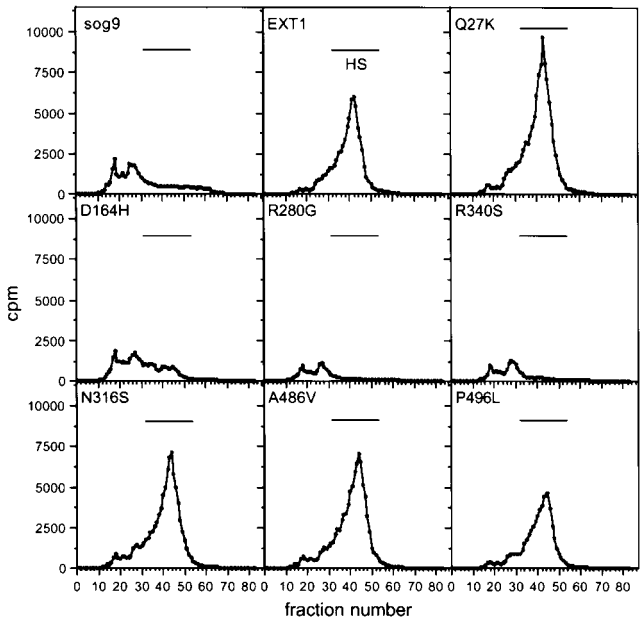

Figure 4 Anion-exchange chromatography of cell-surface GAGs. Sog9 cells, and sog9 cells stably transfected with the indicated EXT1 forms, were grown for 24 h in the presence of $^{35}SO_4$ (20 μCi/ml). GAGs were isolated and fractionated by HPLC. The elution position of HS is indicated by a horizontal bar. For the two residues affected by multiple etiological missense mutations (R280 and R340), only one mutant form is shown.

et al. 1998; Wiggins and Munro 1998), and it is therefore possible that D164 may play a key role in catalyzing HS polymerization. It will be interesting to determine whether mutation of aspartates 162, 164, 313, and/or 315 specifically abolishes either GlcA-T or GlcNAc-T activities.

The inactive H627 deletion mutant is of particular interest because this mutation is located in the C-terminus of the protein, which is highly conserved among exostosin family members and appears to interfere with specific protein-protein interactions in yeast and mammalian two-hybrid systems (Simmons et al. 1999). It is possible that this amino acid residue is involved in GlcNAc-T activity, although it does not complement specific defects in GlcA-T activity caused by mutations at residues 339 and 340. In vitro enzyme analysis and coimmunoprecipitation assays will be required to determine whether H627 affects HS synthesis directly, by eliminating GlcNAc-T activity, or whether it affects protein-protein interactions, such as oligomerization, or chaperone-mediated folding of EXT1.

Interestingly, 7 of the 12 reported missense mutations, all of which have the null phenotype, are found in two clusters, at position R280 and around positions G339 and R340 (fig. 1), suggesting that these regions may harbor key elements for EXT1 function. Indeed, in vitro enzyme-assay analysis of two of these missense mutants, G339D and R340C, has previously revealed that mutation of these residues selectively abrogates GlcA-T activity but not GlcNAc-T activity (McCormick et al. 2000). Moreover, missense mutations recently found in the same region, in hamster EXT1, also selectively alter GlcA-T activity, suggesting that this region of the protein contains an essential element of the GlcA-T catalytic domain (Wei et al. 2000).

The autosomal dominant–inherited pattern of HME would suggest that one mutant copy of the gene would be sufficient to cause disease. However, no dominant negative phenotype was observed, consistent with a previous report (Wei et al. 2000). This is a rather surprising result, in light of the ability of the EXT proteins—both wild-type and mutant—to form homooligomers and heterooligomers in vivo (Kobayashi et al. 2000*b*; McCormick et al. 2000). This implies that oligomer formation may require only one functional copy of EXT1 for the oligomer to retain its function. Alternatively, observed HS-Pol activity may be caused by a subset of totally functional heterooligomers, as it is likely that, in any cell, there are three types of oligomers: those containing only wild-type copies of EXT1, those containing copies of both wild-type and mutant EXT1 proteins, and those composed of only mutant proteins. Taken together, these results suggest that, at the cellular level, one functional copy of the EXT1 gene is sufficient for activity, an idea that is in agreement with the two-hit model of tumorigenesis proposed for both sporadic and HME-linked exostoses formation (Mertens et al. 1994; Hecht et al. 1995; Raskind et al. 1995; Bovée et al. 1999).

Studies with the *Drosophila* homolog of EXT1, Ttv, have shown that this protein is necessary for the diffusion of Hh, a cell-signaling molecule that plays a key role in fruit fly development (Bellaiche et al. 1998), and

Table 3

Functional Assay of EXT1 Proteins Bearing Selected Mutations

Etiological Mutation	New Mutation	Nature of Change	HS Biosynthesis[a]
Q27K	Q27A	Severe	+
Active	Q27P	Severe	+
	Q27del	Deletion	+
D164H	D164E	Conserved	−
Null	D164del	Deletion	−
N316S	N316A	Severe	+
Active	N316del	Deletion	+
A486V	A486H	Severe	+
Active	A486del	Deletion	+
P496L	P496H	Severe	+
Active	P496del	Deletion	+

[a] + = wild-type EXT1 level of infectivity; − = background level of infectivity.

that Ttv is responsible for the bulk of HS biosynthesis in this organism (The et al. 1999; Toyoda et al. 2000). This discovery has proven interesting, in light of the fact that the mammalian homolog of Hh, Indian Hedgehog (Ihh), appears to play a key regulatory role in chondrocyte differentiation (Lanske et al. 1996; Vortkamp et al. 1996). These results suggest a possible etiology for HME pathogenesis, in which a lack of HS expression would interfere with the Ihh-mediated regulation of chondrocyte maturation and, thus, with bone formation (Bellaiche et al. 1998; Lin et al. 2000; Stickens et al. 2000). It is likely that an examination of the HS expression of etiological mutation-bearing chondrocytes from HME patients will help to shed light on the molecular pathology of this disease.

In conclusion, we have shown that five of the amino acids previously identified in HME patients/families are critical for HS expression and that, remarkably, four putative etiological missense mutants retain the ability to synthesize HS. In fact, the four affected residues could each be deleted from the EXT1 protein without causing a detectable alteration in HS biosynthetic activity. These results raise an important question about the link between HS synthesis and HME, for which at least three possible explanations exist. First, EXT1 could harbor additional activities not detectable by the HSV-infectivity assay. Second, it is possible that these reported mutations may be rare genetic polymorphisms. In these patients, the HME phenotype could be caused by unidentified mutations present in the noncoding regions of the *EXT* genes, such as introns or enhancer and/or promoter regions, which could influence *EXT1* mRNA-expression levels. Alternatively, these patients could bear mutations in the *EXT3* or *EXTL* loci. Third, some of the mutations may partially suppress enzyme activity, but the residual catalytic capacity is sufficient to drive HS synthesis in vivo. The use of the HSV-infectivity assay to analyze possible roles for the 5′ and 3′ untranslated regions of HME-derived *EXT1* cDNAs, as well as in vitro enzyme assays and coimmunoprecipitation assays with the existing EXT1 etiological mutant proteins should help to distinguish among these possibilities. Taken together, the data suggest that the link between EXT activity and HME is more complex than first thought. Determination of the role of HS in bone formation—and, perhaps more importantly, of whether EXT proteins harbor additional functions—should help to further our understanding of the molecular pathogenesis of HME.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Baylor College of Medicine Human Genome Sequencing Center Search Launcher, http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html (for ClustalW and Boxshade software)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for HME, EXT1 [MIM 133700], EXT2 [MIM 133701], and EXT3 [MIM 600209])

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