The EXT1/EXT2 tumor suppressors: catalytic activities and role in heparan sulfate biosynthesis

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The D-glucuronyltransferase and N-acetyl-D-glucosaminyl-transferase reactions in heparan sulfate biosynthesis have been associated with two genes, *EXT1* and *EXT2*, which are also implicated in the inherited bone disorder, multiple exostoses. Since the cell systems used to express recombinant EXT proteins synthesize endogenous heparan sulfate, and the EXT proteins tend to associate, it has not been possible to define the functional roles of the individual protein species. We therefore expressed EXT1 and EXT2 in yeast, which does not synthesize heparan sulfate. The recombinant EXT1 and EXT2 were both found to catalyze both glycosyltransferase reactions *in vitro*. Coexpression of the two proteins, but not mixing of separately expressed recombinant EXT1 and EXT2, yields hetero-oligomeric complexes in yeast and mammalian cells, with augmented glycosyltransferase activities. This stimulation does not depend on the membrane-bound state of the proteins.

INTRODUCTION

Heparan sulfate (HS) proteoglycans, at cell surfaces and in the extracellular matrix of most tissues, consist of HS chains substituted on core proteins. HS binds many proteins and thus affects a variety of processes, including specific signaling pathways (Lander and Selleck, 2000). HS biosynthesis occurs by alternating addition of glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc) residues to the non-reducing end of a (GlcA-GlcNAc)_n polymer, which is subsequently modified through sulfation and GlcA C5-epimerization reactions (Lindahl *et al.*, 1998). Isolation and cloning of a protein associated with both GlcA-transferase (GlcA-T) and GlcNAc-T activities yielded

EXT2, a product of one of the exostosin gene loci (Lind *et al.*, 1998). Other members of this family include *EXT1* and *EXT3*, as well as three homologous EXT-like genes (*EXTL1–3*). Mutation of the *EXT1–3* loci leads to hereditary multiple exostoses, a human autosomal skeletal disorder characterized by the formation of cartilage-capped bony outgrowths at the ends of the long bones (Ahn *et al.*, 1995).

The relationship of the EXT and EXTL (see Kitagawa *et al.*, 1999) proteins to HS biosynthesis remains intriguing. EXT1 was identified as a factor capable of restoring HS synthesis in HS-deficient mutant L-cells (McCormick *et al.*, 1998), and was found to elevate the low levels of both the GlcA-T and GlcNAc-T activities in these cells (Lind *et al.*, 1998). Moreover, immunopurified recombinant EXT2 from transfected COS-7 cells displayed both transferase activities. However, interpretation of these experiments has been complicated by the general occurrence in the target cells of GlcA-T and GlcNAc-T enzyme(s) committed to endogenous HS biosynthesis. Thus, transfection of wild-type L-cells with EXT1 and of COS-7 cells with EXT2 followed by analysis of cell lysates consistently resulted in a paradoxical decrease in both glycosyl-T activities compared with mock-transfected cells (Lind *et al.*, 1998). These findings pointed to interactions between the recombinant EXT proteins and endogenous proteins, which could conceivably persist through immunopurification. Indeed, Simmons *et al.* (1999) suggested that observations linking EXT1 and EXT2 to the biosynthesis of HS would primarily reflect effects of copurified interacting proteins.

In view of these contradictory results, we considered it essential to define the catalytic activities of recombinant EXT1 and EXT2 proteins, following expression in cells that lack endogenous HS

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Fig. 1. Scheme of EXT constructs. **(A)** With transmembrane domain (TM, black box). For expression in yeast, EXT1 and EXT2 cDNA constructs were inserted into the pPICZB vector (Invitrogen). For expression in COS-1 cells, EXT1 and EXT2 were ligated into the pcDNA3.1 expression vector (Invitrogen). **(B)** Without transmembrane domain (SP, signal peptide, open box). EXT constructs (sEXT) lacking the first N-terminal 32 (sEXT1-GFP) or 48 (sEXT2myc-his) amino acid residues, respectively, were ligated into the pPICZαC vector (Invitrogen). sEXT1protA and sEXT2protA constructs lacking the first N-terminal 43 or 55 amino acid residues, respectively, were subcloned into pGIR201protA (Kitagawa and Paulson, 1994) and subsequently inserted into the expression vector pEF-BOS. N and C, N- and C-terminus, respectively.

biosynthesis. To this end we selected the yeast strain *Pichia pastoris*, which does not synthesize HS. Results reported here clearly demonstrate both GlcA-T and GlcNAc-T activities for both proteins. Moreover, both activities were strikingly elevated on coexpression of EXT1 and EXT2. Finally, actual complex formation between EXT1 and EXT2 proteins was demonstrated, in yeast as well as in mammalian cells.

RESULTS

Expression of membrane-bound EXT proteins

Yeast cells were transformed with epitope-tagged EXT1 or EXT2 (EXT1myc-his, EXT2myc-his; Figure 1A) and stable transformants were selected. One of the resultant EXT2-expressing clones was transformed with EXT1 for coexpression (EXT1/2) of the two proteins. Following induction of protein expression in selected integrants, lysates of yeast cells were analyzed for GlcA-T and GlcNAc-T activities, using the appropriate oligosaccharides as acceptors (Figure 2; Table I). Both transferase activities were detected in cells that had been transfected with either EXT1 or EXT2, whereas no significant activities were detected in mock-transfected cells. Coexpression of EXT1 and EXT2 dramatically augmented the two glycosyl-T reactions. The specific GlcNAc-T activity, expressed on a cellular protein basis, of the EXT1/2 cotransformed yeast cells, thus was almost 10-fold higher than

Table I. GlcNAc-T and GlcA-T activities of expressed EXT proteins

Cell	GlcNAc-T activity	GlcA-T activity
	pmol/mg/h ^a	pmol/mg/h ^a
<i>Pichia</i> (full-length)		
Mock	ND ^b	ND
EXT1	74 ± 32	8.0 ± 2.2
EXT2	78 ± 42	4.7 ± 0.1
EXT1/2	580 ± 13	99 ± 14
EXT1 + EXT2 ^c	64	not determined
	nmol/mg/h ^d	nmol/mg/h ^d
<i>Pichia</i> (truncated)		
Mock	ND	ND
EXT1	ND	not determined
EXT2	ND	not determined
EXT1/2	60 ± 4	14 ± 4
	pmol/mg/h ^a	pmol/mg/h ^a
COS-1 (full-length)		
Mock	1800	400
EXT1	3000	810
EXT2	1400	250
EXT1/2	8700	2800
EXT1 + EXT2 ^c	2000	430
	nmol/mg/h ^c	nmol/mg/h ^c
COS-1 (truncated)		
Mock	ND	ND
EXT1	230	420
EXT2	49	40
EXT1/2	440	1000

The values for *Pichia* represent means ±SD of three independent experiments and the values for the COS-1 cells represent averages of two analyses.

^aGlcNAc or GlcA incorporated per milligram of total cell protein per hour.

^bND, none detected.

^cValues obtained by mixing extracts from cells separately transformed/transfected with EXT1 or EXT2.

^dGlcNAc or GlcA incorporated per milligram of EXT protein per hour (see METHODS), assuming 50 µg EXT protein per milligram of total secreted protein (see text).

^eGlcNAc or GlcA incorporated per milligram of EXT protein (captured on IgG-Sepharose) per hour.

the corresponding activities determined for cells that had been separately transformed with either EXT1 or EXT2. A corresponding increase in specific activity was recorded for the GlcA-T reaction (Table I). Notably, these effects of cotransformation were not due to differences in the levels of protein expression, as assessed by western blot analysis (data not shown). Moreover, simply mixing lysates of separately EXT1- and EXT2-transformed yeast cells did not result in any increase in activity (Table I; only GlcNAc-T activity analyzed). These results indicate that whereas both the EXT1 and EXT2 proteins are GlcA/GlcNAc-cotransferases, they need to be simultaneously synthesized in the cell in order to attain full catalytic efficacy.

Fig. 2. Glycosyltransferase assays of yeast transformed with EXT proteins. The GlcNAc-T activities of yeast cell extracts were determined by incubating 70 μ g lysate protein with 0.125 μ Ci of UDP-[14 C]GlcNAc and 50 μ g of [GlcA-GlcNAc] $_n$ oligosaccharide (the reducing-terminal sugar is modified to an 2,5-anhydromannitol unit) under the conditions described (Lind *et al.*, 1993). After 1 h at 37°C, the mixtures were applied to columns (28 $\times$ 0.5 cm) of Sephadex G-25 in 0.5 M NaCl, 50 mM Tris-HCl, 0.1% Triton X-100, pH 7.4. Effluent fractions were analyzed by scintillation counting. The various samples were derived from yeast transformed with empty plasmid (mock, filled diamond), EXT1 (filled circle), EXT2 (open circle) and EXT1/2 (cotransformation, open diamond). GlcA-T activities were assayed in similar fashion, except that UDP-[3 H]GlcA was used as a sugar donor, and GlcNAc-[GlcA-GlcNAc] $_n$ oligosaccharides were used as acceptor. The resultant gel chromatograms (not shown) were highly similar to the GlcNAc-T assays shown in the figure.

Fig. 3. Endo H treatment of EXT-proteins expressed in *Pichia*. Membrane fractions from yeast transformed separately with myc-his tagged EXT1 or EXT2 or cotransformed with EXT1 and EXT2 (EXT1/2) were incubated in the absence or presence of Endo H and analyzed by 7.5% SDS-PAGE followed by western blotting with an antibody to the polyhistidine-tag. Transformed clones are identified at the top. The kilodalton values of protein molecular mass markers are indicated. Mock, transformed with vector alone.

Analysis of microsomal proteins by SDS-PAGE followed by western blotting with a monoclonal antibody to the his tag showed a predominant ~80 kDa band and a weaker ~90 kDa component in all cells that expressed EXT1 or EXT2 alone. In contrast, EXT1/2 coexpression consistently yielded the larger component only (Figure 3). Endo H treatment did not affect the ~80 kDa band but converted the ~90 kDa component into a product of intermediary size. These results indicate that the EXT1 and EXT2 proteins are appreciably *N*-glycosylated (each protein contains two potential *N*-glycosylation sites; Lind *et al.*, 1998;

Fig. 4. Hetero-oligomerization of EXT1 and EXT2. Proteins from EXT-transformed yeast or transfected COS-1 cells were analyzed by SDS-PAGE, either directly or following immunopurification as indicated (see Methods). (A) *Pichia* proteins. Microsomal proteins from yeast cells transformed with the indicated EXT constructs were immunoprecipitated with anti-GFP antibodies. (B) COS-1 cell proteins. Samples were immunopurified by chromatography on anti-flag antibody-agarose. All samples (A and B) were immunoblotted using anti-myc antibodies. Sample designation: mock, empty vector; EXT1 or EXT2 alone, single protein expression; EXT1/EXT2, coexpression; EXT1 + EXT2, proteins expressed in separate cultures, then mixed.

McCormick *et al.*, 1998) in yeast only when they are expressed together. The formation of Endo H susceptible *N*-linked mannose-containing oligosaccharide substituents on *Pichia* proteins occurs primarily in the Golgi (Gemmill and Trimble, 1999). The differential glycosylation patterns therefore suggest that the cotransformed EXT proteins reach the Golgi compartment, whereas the single-transformed forms are mainly retained in the endoplasmic reticulum.

The synergism between EXT1 and EXT2 observed in yeast cells applied also to mammalian cells. Cotransfection of COS-1 cells with full-length EXT-proteins (EXT1myc, EXT2flag; Figure 1A) resulted in appreciably increased GlcA-T and GlcNAc-T activities, although the effect was less pronounced than in the yeast system (Table I). As expected, significant GlcA-T and GlcNAc-T activities were detected already in mock-transfected cells, a reflection of endogenous HS biosynthesis. Transfection of EXT1 alone resulted in increased glycosyl-T activities whereas transfection of EXT2 had the opposite effect (Table I).

Association of full-length EXT1 and EXT2 was demonstrated by western blotting of immunopurified epitope-tagged complexes, following coexpression of the proteins in either the yeast (Figure 4A) or COS cells (Figure 4B). In contrast, no complex formation was found in either cell system upon mixing of cell lysates containing singly expressed EXT1 and EXT2.

Expression of soluble EXT proteins

EXT cDNA constructs were designed for generation of soluble proteins (Figure 1B). The products formed upon single- or cotransformation of yeast with EXT1 and EXT2 were recovered from culture media by adsorption to DEAE–Sephacel, and quantified by western blotting using anti-green fluorescent protein (GFP) and anti-his antibodies, respectively. Both proteins occurred in the range 20–50 µg/mg secreted protein, with no marked difference between single- and cotransformed species. The coexpressed (EXT1/2) proteins derived from yeast catalyzed the GlcA-T and GlcNAc-T reactions with high specific activities (Table I), whereas no glycosyl-T activities were detected following single expression of the soluble proteins. From COS-1 cells, soluble forms of EXT1 and EXT2 fused to protein A were captured on IgG–Sephacel beads and assayed for enzyme activities. The soluble EXT1 and EXT2 fusion proteins produced separately by the COS-1 cells appeared catalytically active, albeit less so than the cotransfected proteins (Table I).

DISCUSSION

Previous results in our and other laboratories have implicated members of the exostosin protein family with glycosyltransferase functions in the biosynthesis of HS (Lind *et al.*, 1998; Kitagawa *et al.*, 1999; McCormick *et al.*, 2000). In particular, EXT1 and EXT2 have been associated with the polymerization of the HS precursor chain, through GlcA-T and GlcNAc-T reactions (Lind *et al.*, 1998). However, experiments designed to define the catalytic activity of each individual protein have been inconclusive, due to the ability of these species to bind each other (McCormick *et al.*, 2000) as well as unrelated proteins (Simmons *et al.*, 1999). To clarify this problem we have expressed EXT1 and EXT2 in yeast, which lacks endogenous HS biosynthesis. The results of these experiments demonstrate clearly, for the first time, that EXT1 and EXT2 both catalyze the GlcA-T as well as the GlcNAc-T reactions (Figure 2).

Both the GlcA-T and the GlcNAc-T activities were dramatically augmented upon coexpression of EXT1 and EXT2 in the yeast cells (Figure 2; Table I). This finding could merely indicate that the heterodimer is the most active form of the enzyme. However, no stimulation of activity was noted on mixing the proteins expressed in separate cells. Moreover, western blotting in conjunction with Endo H treatment revealed differences in *N*-glycosylation, indicative of Golgi processing of the coexpressed but not of the separately expressed EXT proteins (Figure 3). These findings agree with recently published results of experiments with mammalian cells, in which cotransfected EXT1 and EXT2 were colocalized to the Golgi apparatus (Kobayashi *et al.*, 2000; McCormick *et al.*, 2000). It seems reasonable to conclude that both effects of coexpression, i.e. the Golgi location and the increased glycosyltransferase activities, were due to intracellular EXT1/EXT2 hetero-oligomer formation, possibly required for proper folding of the proteins. The promotion of glycosyltransferase activities was demonstrable also upon coexpression in yeast of EXT1 and EXT2 constructs lacking the transmembrane domains (Table I), suggesting that interaction between the intraluminal portions of the two proteins is sufficient to generate the hetero-oligomeric functional enzyme. The effects of coexpression of EXT1 and EXT2 were not restricted to yeast, but were also

seen, albeit less marked, with COS-1 cells (Table I). Direct physical interaction between the EXT1 and EXT2 proteins was demonstrated following expression both in yeast and COS-1 cells by specific immunopurification of one of the EXT proteins followed by detection of the reciprocal species in the purified material (Figure 4).

Notably, McCormick *et al.* (2000) was unable to detect any significant glycosyltransferase activity associated with EXT2 alone, but found appreciable GlcA-T and GlcNAc-T activities following cotransfection with EXT1. The present study, on the other hand, demonstrates that EXT1 and EXT2 are both GlcA/GlcNAc-cotransferases that need to interact to form an active HS ‘polymerase’. The available information suggests that neither of the two cotransferases can substitute for the other one. Thus, L-cells deficient in EXT1 were unable to synthesize HS even after transfection with EXT2 (McCormick *et al.*, 2000). Moreover, mutation of the EXT1 orthologue *tout-velu* (*ttv*) in *Drosophila* led to impairment of Hedgehog signaling along with selective, drastically reduced formation of HS, even though *Drosophila* also contains an EXT2 orthologue (The *et al.*, 1999). Additional work is needed to understand the roles of the EXT proteins in HS biosynthesis as well as in ‘tumor suppression’. Future experiments should be designed to clarify not only the interaction between the EXT proteins, but also the relation between the resultant complex and the other enzymes required for HS assembly.

METHODS

Expression of EXT1 and EXT2. EXT1 cDNA (McCormick *et al.*, 1998) was a gift from F. Tufaro, Vancouver, Canada, and EXT2 cDNA was as described previously (Lind *et al.*, 1998). The cDNA constructs used to express EXT proteins were as described in Figure 1. *Pichia pastoris* GS115 (his4) (American Type Culture Collection, USA) was transformed using the Easy Comp Kit (Invitrogen). Transformants were selected in YPDS-Zeocin plates. After 4 days at 30°C, 20 resistant colonies were picked, and the integration of each respective cDNA was verified by PCR analysis using oligonucleotide primers unique to EXT1 or EXT2 sequences. The clones with the highest expression of EXT1 or EXT2 were cultured from single colonies and protein expression was induced by methanol addition (described in the Manual Version D of the *Pichia* Expression Kit, Invitrogen). Cotransformation of *Pichia* with EXT1 and EXT2 was performed according to Higgins *et al.* (1998), based on the correlation between the expression level of a protein in *Pichia* and the level of Zeocin resistance. The integration of the constructs was verified by PCR. Transformed yeast cultures expressing full-length EXT proteins were harvested 48 h after methanol-induced protein expression. Cell lysates were prepared as described (Manual Version D of the *Pichia* Expression Kit; Invitrogen). Soluble forms of the EXT proteins were harvested after a 72 h methanol induction by adsorption of proteins from 5 ml culture media to 200 µl of DEAE–Sephacel (Amersham Pharmacia Biotech) in 20 mM Tris–HCl pH 8.0, followed by elution with 0.25 M NaCl in 20 mM Tris–HCl pH 7.4.

COS-1 cells transfected with full-length expression constructs using electroporation were as described (Lind *et al.*, 1998). COS-1 cells transfected with truncated cDNA using Lipofectamine 2000 (Life Technologies, Inc.) were cultured for 48 h, and

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protein A-containing fusion proteins were recovered after binding to IgG-Sepharose (Kitagawa *et al.*, 1999) and quantified using the BCA Protein Assay Reagent (enhanced protocol, Pierce).

Endo H treatment of expressed proteins was performed according to the instructions provided by the manufacturer (New England Biolabs). The various samples were analyzed for GlcA- and GlcNAc-T activities as described by Lind *et al.* (1993), and in the legend to Figure 2.

Immunoblots. For immunoprecipitation, membrane fractions (300 µg protein) from yeast cells transformed with GFP or myc-his tagged EXT-proteins were diluted in PBS, 1 mM CHAPS (final volume 200 µl), preincubated with 20 µl protein A-Sepharose (Pharmacia) for 30 min and then incubated with 3 µg of rabbit anti-GFP polyclonal antibody (Invitrogen) for 2 h. Another 20 µl portion of protein A-Sepharose was added, and incubation was continued for 1 h. After centrifugation the pellet was washed three times with PBS and finally suspended in SDS-PAGE sample buffer. Flag-tagged proteins expressed in COS-1 cells were affinity purified using anti-flag-M2 monoclonal antibodies immobilized on agarose (100 µl, Kodak) as described (Lind *et al.*, 1998), except for an additional 0.5 M NaCl washing step. Proteins were eluted with flag octapeptide (DYKDDDDK) and concentrated.

The protein samples were separated on 7.5% polyacrylamide gels and transferred to PVDF membranes (Millipore corp.) (Lind *et al.*, 1998). The membranes were incubated with a 1:5000 dilution of mouse monoclonal anti-polyhistidine-peroxidase conjugate antibody (Sigma), a 1:2000 dilution of mouse monoclonal anti-myc peroxidase conjugated antibody (Invitrogen), or a 1:500 dilution of rabbit anti-GFP peroxidase conjugated antibody. Immunoreactive proteins were visualized with an enhanced chemiluminescence system (ECL detection kit; Amersham Pharmacia Biotech) and, when applicable, quantified using the Molecular Analyst Computer Program (Bio-Rad).

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