FEBS Letters 584 (2010) 3962–3968

journal homepage: www.FEBSLetters.org

Molecular characterization of β1,4-galactosyltransferase 7 genetic mutations linked to the progeroid form of Ehlers–Danlos syndrome (EDS)

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ARTICLE INFO

Article history:
Received 3 June 2010
Revised 26 July 2010
Accepted 2 August 2010
Available online 6 August 2010

Edited by Takashi Gojobori

This work is dedicated to G.J. Dutton who died on the 1st June 2010 and who has been of great inspiration in our study of glucuronosyl- and other glycosyltransferases.

Keywords:
Ehlers–Danlos syndrome
Point mutation
Galactosyltransferase defect
Glycosaminoglycan synthesis
Congenital glycosyltransferase disorder

ABSTRACT

β1,4-Galactosyltransferase 7 (β4GalT7) is a key enzyme initiating glycosaminoglycan (GAG) synthesis. Based on in vitro and ex vivo kinetics studies and structure-based modelling, we molecularly characterized β4GalT7 mutants linked to the progeroid form of Ehlers–Danlos syndrome (EDS), a severe connective tissue disorder. Our results revealed that loss of activity upon L206P substitution due to altered protein folding is the primary cause for the GAG synthesis defect in patients carrying the compound A186D and L206P mutations. We showed that R270C substitution strongly reduced β4GalT7 affinity towards xyloside acceptor, thus affecting GAG chains formation. This study establishes the molecular basis for β4GalT7 defects associated with altered GAG synthesis in EDS.

1. Introduction

Amino acid exchanges in β1,4-galactosyltransferase 7 (β4GalT7, E.C. 2.4.1.133), which is involved in the synthesis of the glycosaminoglycan (GAG) linkage region of proteoglycans, are associated with the pathology of progeroid type Ehlers–Danlos syndrome (EDS) [1]. EDS forms a group of inherited connective tissue disorders characterized by defects in various extracellular matrix proteins including collagens and small leucine-rich proteoglycans, such as decorin. Patients harbouring mutations in the *B4GALT7* gene exhibit aged appearance, developmental delay, dwarfism, craniofacial disproportion and general osteopenia [1,2]. In addition, hypermobile joints, defects in wound healing and loose skin are observed. Aberrant GAG substitution of decorin was initially described in a progeroid EDS patient who carried compound heterozygous amino acid exchanges (A186D/L206P) in β4GalT7 enzyme [3,4]. More recently, two patients exhibiting typical progeroid EDS features including craniofacial appearance, skeletal abnormalities, and aged appearance were described [2]. Sequence analysis of the *B4GALT7* gene from these patients revealed a missense mutation causing the substitution R270C. This defect was found to be associated with reduced galactosyltransferase activity and aberrant GAG substitution of decorin and biglycan in cultured skin fibroblasts [5]. Interestingly, a recent report indicated that in addition to alterations of the dermatan-sulfate chain of decorin, changes in the structure of heparan-sulfate proteoglycans may also contribute to the phenotypic features observed in this β4GalT7-deficient form of EDS, such as altered cell migration and delayed wound repair [6].

Abbreviation: β4GalT7, β1,4-galactosyltransferase 7; EDS, Ehlers–Danlos syndrome; GAG, glycosaminoglycan; Gal, galactose; GlcA, glucuronic acid; 4-MUX, 4-methylumbelliferone-β-D-xylopyranoside; PBS, phosphate buffered saline; Xyl, xylose

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0014-5793/$36.00
doi:10.1016/j.febslet.2010.08.001

GAGs are extensively modified linear polysaccharide chains normally attached to core proteins to form proteoglycans. These complex molecules located on the cell surface and in extracellular matrices of virtually every tissue mediate highly diverse key cell events, ranging from mechanical support inside and outside cells to intricate effects on a wide variety of cellular and biochemical processes, such as blood clotting, cell adhesion, differentiation, proliferation and motility [7]. These functions depend on interactions of the GAG chains with a variety of molecules including growth factors, cytokines and their receptors, enzymes such as matrix proteases and coagulation factors, as well as extracellular matrix proteins [8]. The synthesis of GAG chains that govern these functions is initiated by the formation of a tetrasaccharide linkage region composed of glucuronic acid-galactose-galactose-xylose (GlcAβ1,3Galβ1,3Galβ1,4Xyl1-) followed by the elongation of the two main types of GAGs (*i.e.* heparin, heparan-sulfate and chondroitin-/dermatan-sulfate) [9]. The β4GalT7 enzyme catalyses the transfer of a galactose (Gal) residue provided by UDP-α-D-Gal (UDP-Gal) onto Xyl, a key step in the synthesis of the linkage region of GAG chains [10]. Since the formation of this linker tetrasaccharide is required prior to polymerization of both heparan-sulfate and dermatan-/chondroitin-sulfate chains, genetic defects of β4GalT7 strongly affect the biological functions mediated by GAGs, including tissue development and differentiation, leading to severe clinical features that are characteristic of the progeria-type variant forms of EDS [1,2].

Although the alterations in GAG synthesis have been well defined from studies performed in fibroblasts of patients affected by progeroid form of EDS, the consequences of these gene mutations on β4GalT7 properties have not yet been investigated at the molecular level. By site-directed mutagenesis and in vitro and ex vivo kinetic studies, combined to computer-aided modelling of the human protein structure, we deciphered the mechanisms by which the A186D, L206P and R270C mutations affect β4GalT7 function and disrupt GAG synthesis pathways in the EDS congenital glycosylation disorder.

2. Materials and methods

2.1. Materials

4-Methylumbelliferyl-β-D-xylopyranoside (4-MUX), UDP-α-D-galactose (UDP-Gal) were provided by Sigma–Aldrich, and UDP[^{14}C]Gal and $Na_2[S^{35}]SO_4$ was from Perkin–Elmer. The eukaryotic expression vector pcDNA3.1(+) and competent One Shot® Top10 *Escherichia coli* cells were from Invitrogen-Fisher Scientific, and pET-41a(+) vector and *E. coli* BL21(DE3) cells were from Novagen-EMD4Biosciences.

2.2. Expression vector construction

The human β4GalT7 sequence was cloned as previously described [11]. For expression of β4GalT7 in mammalian cells, the full-length cDNA was modified by PCR to include a KpnI site and a Kozak consensus sequence at the 5′ end, and a sequence encoding a myc tag (EQKLIEEDL) and a XbaI site at the 3′ end, prior subcloning into the pcDNA3.1(+) to produce pcDNA-β4GalT7. For bacterial expression, a truncated form of β4GalT7 was expressed as a fusion protein with glutathione-*S*-transferase (GST). The sequence lacking codons for the 60 N-terminal amino acids was amplified from full-length cDNA using the corresponding primers including NcoI and NotI sites and subcloned into the pET-41a(+) vector (Novagen, EMD4Biosciences) to produce plasmid pET-β4GalT7. Mutations were generated using the QuickChange II XL site-directed mutagenesis kit (Stratagene), employing pcDNA-β4GalT7 or pET-β4GalT7 plasmids as template, and sense and antisense primers listed in Table 1 (Supplementary data).

Table 1
Kinetic parameters of wild-type β4GalT7 and R270C EDS mutant for donor UDP-Gal and the acceptor 4-MUX.

Enzyme	K_A (mM) UDP-Gal	K_B (mM) 4-MUX	K_{ia} (mM)	k_{cat} (s^{-1})
β4GalT7	0.45 ± 0.09	0.61 ± 0.08	0.09 ± 0.03	1.14
R270C	0.46 ± 0.16	6.12 ± 0.96	0.29 ± 0.09	ND

Kinetic parameters towards donor and acceptor substrates were evaluated by double substrate kinetics using 0.2 μg purified GST-β4GalT7 protein at concentrations of UDP-Gal and of 4-MUX varying from 0 to 5 mM. Data were fitted to Eq. (1) described in Section 2 by non-linear least square regression analysis using the curve-fitter program of SigmaPlot 9.0. ND, not determined, *i.e.* no k_{cat} could not be accurately evaluated for R270C mutant since 4-MUX is poorly soluble at concentration higher than 10 mM required to achieve acceptor substrate saturation.

2.3. Heterologous expression of wild-type and mutated β4GalT7

CHO pgsB-618 cells defective in β4GalT7 activity established by Esko et al. [12] were purchased from the American Type Culture Collection (ATCC). Cultured cells were individually transfected at 80% confluency with wild-type and mutated pcDNA-β4GalT7 plasmids using ExGen 500 reagent (Euromedex, Souffelweyersheim, France), according to the manufacturer's recommendations. Cells were harvested in phosphate buffered saline (PBS) 48 h after transfection, pelleted by centrifugation at 5000×*g*, resuspended in 0.25 M sucrose, 5 mM HEPES buffer (pH 7.4) and sonicated three-fold 5 s. Protein concentration was determined by the method of Bradford [13] prior to SDS–PAGE or activity analyses. Alternatively, transfected cells were transferred to labelling medium in the presence of 4-MUX prior to GAG isolation, as described below.

To express wild-type and mutated GST-β4GalT7, *E. coli* BL21(DE3) cells harbouring pET-β4GalT7 expression vector were grown in Luria-Bertani (LB) medium and gene expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma–Aldrich) added to bacteria cultured at 20 °C for 16 h. GST-β4GalT7 proteins were purified by affinity chromatography using Glutathione Sepharose™ 4B columns (GE Healthcare Life Sciences).

2.4. Galactosyltransferase activity

The in vitro assay of β4GalT7 activity was performed as previously described [11]. Reactions were performed in 100 mM sodium cacodylate buffer (pH 7.4) containing 10 mM $MnCl_2$, 1 mM UDP-Gal, 0.05 μCi UDP[U^{14}C]Gal, 5 mM 4-MUX and 40 μg of total cell protein or 0.2 μg purified protein. Incubations were carried out at 37 °C for 30–60 min in a total volume of 50 μl, and reaction products were separated by thin layer chromatography [14,15]. For kinetic studies, reaction products were analyzed by high performance liquid chromatography (HPLC) using a reverse phase C_{18} column attached to a Waters e2695 instrument equipped with a Berthold FLowStar radioactivity monitor.

2.5. Kinetic analyses

The kinetic parameters for UDP-Gal and 4-MUX were determined using double substrate kinetics with concentrations of UDP-Gal and 4-MUX varying between 0 and 5 mM by non-linear regression using Sigma Plot™. Data were analyzed for a two-substrate system by fitting to an equation for sequential symmetrical initial velocity pattern, equation below (ordered or random equilibrium mechanism) [16,17]. In this equation V_{max} is the maximum velocity, [A] is the concentration of UDP-Gal, and [B] the concentration of 4-MUX. K_a and K_b are the true K_m for donor and acceptor substrates and K_{ia} is the dissociation constant for UDP-Gal.