

Chondroitin sulfate *N*-acetylgalactosaminyltransferase-1 (CSGalNAcT-1) involved in chondroitin sulfate initiation: Impact of sulfation on activity and specificity

Sandrine Gulberti^{1,2}, Jean-Claude Jacquinet³,
Matthieu Chabel², Nick Ramalanjaona²,
Jacques Magdalou², Patrick Netter², Michael W
H Coughtrie⁴, Mohamed Ouzzine², and
Sylvie Fournel-Gigleux^{1,2}

²UMR 7561 CNRS-Nancy Université-Nancy I, Faculté de Médecine, BP 184, 54505 Vandoeuvre-lès-Nancy Cedex, France; ³UMR 6005 CNRS, Institut de Chimie Organique et Analytique, UFR Sciences, 45067 Orléans, Cedex 02, France; and ⁴Medical Research Institute, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, UK

Received on August 31, 2011; revised on November 1, 2011; accepted on November 16, 2011

Glycosaminoglycan (GAG) assembly initiates through the formation of a linkage tetrasaccharide region serving as a primer for both chondroitin sulfate (CS) and heparan sulfate (HS) chain polymerization. A possible role for sulfation of the linkage structure and of the constitutive disaccharide unit of CS chains in the regulation of CS-GAG chain synthesis has been suggested. To investigate this, we determined whether sulfate substitution of galactose (Gal) residues of the linkage region or of *N*-acetylgalactosamine (GalNAc) of the disaccharide unit influences activity and specificity of chondroitin sulfate *N*-acetylgalactosaminyltransferase-1 (CSGalNAcT-1), a key glycosyltransferase of CS biosynthesis. We synthesized a series of sulfated and unsulfated analogs of the linkage oligosaccharide and of the constitutive unit of CS and tested these molecules as potential acceptor substrates for the recombinant human CSGalNAcT-1. We show here that sulfation at C4 or C6 of the Gal residues markedly influences CSGalNAcT-1 initiation activity and catalytic efficiency. Kinetic analysis indicates that CSGalNAcT-1 exhibited 3.6-, 1.6-, and 2.2-fold higher enzymatic efficiency due to lower K_m values toward monosulfated trisaccharides substituted at C4 or C6 position of Gal1, and at C6 of Gal2, respectively, compared with the unsulfated oligosaccharide. This highlights the critical influence of Gal substitution on both CSGalNAcT-1 activity and specificity. No GalNAcT activity was detected toward sulfated and unsulfated analogs of the CS

constitutive disaccharide (GlcA- β 1,3-GalNAc), indicating that CSGalNAcT-1 was involved in initiation but not in elongation of CS chains. Our results strongly suggest that sulfation of the linkage region acts as a regulatory signal in CS chain initiation.

Keywords: chondroitin sulfate / glycosaminoglycan synthesis / glycosyltransferase / linkage region / sulfation

Introduction

Glycosaminoglycans (GAGs) are essential effector molecules distributed as side chains of proteoglycans (PGs) located on the cell surface and in the extracellular matrix of virtually every tissue. Major GAGs include chondroitin sulfate (CS)/dermatan sulfate (DS) and heparin/heparan sulfate (HS) composed of repeating disaccharide units consisting of alternating uronic acid, and *N*-acetylgalactosamine (GalNAc) or *N*-acetylglucosamine (GlcNAc) residues, respectively. Although important roles for heparin/HS in developmental processes and specific signaling pathways have been established, CS has recently attracted increasing attention due to its critical implication in a number of physiological processes, such as cell adhesion, morphogenesis, cell division, and neural network formation (Maeda 2010). Moreover, defects in CS synthesis have been shown to impact several pathological conditions such as osteoarthritis, atherosclerosis, cancer (Kalathas et al. 2009), and neuropathies (Saigoh et al. 2011). Thus, there is considerable interest in understanding the CS synthesis pathway and its regulatory mechanism. The synthesis of both CS- and HS-GAG chains is initiated by the formation of a tetrasaccharide structure attached to specific serine residues of PG core proteins (GlcA- β 1,3-Gal- β 1,3-Gal- β 1,4-Xyl-*O*-) via the action of several glycosyltransferases, i.e. xylosyltransferases I/II (Götting et al. 2000), β 4GalT7 (Okajima et al. 1999), β 3GalT6 (Bai et al. 2001), and glucuronosyltransferase-1 (GlcAT-1; Kitagawa et al. 1998). Heparin/HS is synthesized once GlcNAc is transferred to the common linkage region, whereas CS is formed when a GalNAc residue is added. Therefore, the first hexosamine transfer is critical in determining whether HS or CS is selectively assembled on the common linkage region. Chondroitin sulfate *N*-acetylgalactosaminyltransferase-1 (CSGalNAcT-1) catalyzes the transfer of a GalNAc residue onto the nonreducing end of

¹To whom correspondence should be addressed: Tel: +33-383685408; Fax: +33-383685407; e-mail: sandrine.gulberti@medecine.uhp-nancy.fr, sfg@medecine.uhp-nancy.fr

GlcA and has been shown to play a key role in CS chain initiation, possibly in the elongation process (Gotoh et al. 2002). However, the molecular and regulatory mechanisms underlying the selective assembly of CS chains on the common tetrasaccharide linkage region remain to be elucidated.

Previous structural studies of the GAG–protein linkage region have revealed the presence of several modifications (Sugahara et al. 1992; Sugahara and Kitagawa 2000). Sulfation on C6 of both galactose (Gal) residues (Gal1 adjacent to xylose or Gal2 adjacent to GlcA) and on C4 of Gal2 was described in CS chains (Krishna and Agrawal 2000; Kitagawa et al. 2008). Sulfation of Gal residues was also present in the linkage region of DS-PG extracted from bovine aorta (Sugahara et al. 1995). So far, sulfated Gal residues have been identified for CS and DS, but have not yet been found in the linkage region of heparin or HS chains (Izumikawa et al. 2006). On the other hand, the presence of a phosphate group located on the C2 position of xylose linked to serine residues of PG core proteins has been found in both heparin/HS and CS/DS chains (Sugahara et al. 1992; Moses et al. 1997). Although the role of these substitutions is not fully understood, it has been suggested that phosphorylation and sulfation of the linkage region may regulate maturation and processing of growing nascent GAG chains (Sugahara and Kitagawa 2000). In agreement with this hypothesis, we showed that phosphorylated xylosides are not substrate for the human β 4GalT7 in contrast to their unsubstituted counterparts, reinforcing the idea that phosphorylation of xylose could represent a regulatory mechanism in GAG chain initiation (Gulberti et al. 2005). We and others also demonstrated that Gal sulfation critically influences GlcAT-1 activity, supporting the possibility that xylose phosphorylation and sulfation of the Gal residues control the biosynthetic machinery of the linkage tetrasaccharide sequence (Gulberti et al. 2005; Tone et al. 2008). Furthermore, it has been suggested that sulfation also affects enzyme activity for CS chain elongation and termination (Yamada et al. 2002).

Recent studies highlighted the key role of CSGalNAcT-1 in CS chain formation of aggrecan, the major PG of cartilage. Indeed, mice lacking the gene coding for the enzyme demonstrated that it has a major impact on endochondral ossification and aggrecan metabolism (Sato et al. 2011). Furthermore, it has been suggested that this glycosyltransferase could be a potential target for devising cartilage repair strategies in cartilage-degenerative diseases (Sakai et al. 2007). Since there is compelling *in vitro* and *in vivo* evidence for a critical role of CSGalNAcT-1 in CS chain synthesis, it is critical to gain further insights into the structure–function and regulation of this enzyme. Toward this aim, we investigated the influence (1) of sulfation of the linkage Gal residues and (2) of sulfation of the GalNAc residue of the CS constitutive disaccharide unit on activity and substrate specificity of the recombinant human CSGalNAcT-1. For this purpose, we generated a glycolibrary of sulfated and unsulfated oligosaccharide analogs of the GAG–protein linkage region and of CS disaccharide units by a stereo-controlled, high-yield synthesis approach. Determination of the apparent kinetic parameters of the recombinant CSGalNAcT-1 toward these oligosaccharides showed that sulfation of Gal residues of the linkage region could

markedly influence CSGalNAcT-1 activity and specificity, whereas sulfation of GalNAc did not trigger GalNAcT elongation activity. Our data indicate that modifications of the carbohydrate–protein linkage region of GAGs could enhance or reduce the transfer of the first GalNAc onto the terminal GlcA residue, thereby regulating the mechanism governing CS chain initiation.

Results

Functional characterization of CSGalNAcT-1

The recombinant human CSGalNAcT-1 was functionally expressed in HeLa cells as a membrane-bound enzyme with an apparent molecular mass of 55 kDa, in agreement with the predicted molecular mass of the protein. The recombinant enzyme was produced as a Myc-tagged protein to enable quantification of expression levels using a recombinant GST-Myc protein as standard (see Supplementary data, Figure S1). The GalNAcT activity of the recombinant enzyme was determined by high-performance liquid chromatography (HPLC) using a trisaccharide analog of the linkage region linked to a chromophoric aglycone, GlcA- β 1,3-Gal- β 1,3-Gal-*O*-methoxyphenyl (GlcA-Gal-Gal-OMP), as an acceptor substrate (Figure 1A; 1). A typical chromatogram of the HILIC–HPLC (hydrophilic interaction liquid chromatography–high-performance liquid chromatography) resolution of the acceptor substrate (peak 1) and the reaction product (peak 2) formed in the presence of a donor substrate (UDP-GalNAc, peak 3) is illustrated in Figure 2. The reaction product was identified and quantified by comparison of the retention time and absorbance with those of the chemically synthesized tetrasaccharide molecule (Figure 1B; 15), allowing us to perform kinetic analysis of the recombinant CSGalNAcT-1 (Figure 2, shown in inset). The values of the apparent kinetic parameters K_m and V_{max} of the recombinant enzyme toward GlcA-Gal-Gal-OMP were found to be 3.8 mM and 19.5 nmol·min⁻¹· μ g⁻¹ CSGalNAcT-1, respectively (Table I). As expected, mock-transfected HeLa cells did not show any detectable activity (see Supplementary data, Figure S2).

To assess whether the presence of a chromophoric aglycone linked to the trisaccharide structure may affect the kinetic properties of the recombinant CSGalNAcT-1, we next determined the activity of the enzyme toward GlcA- β 1,3-Gal- β 1,3-Gal (GlcA-Gal-Gal) (7) using the tetrasaccharide (GalNAc-GlcA-Gal-Gal) (18) as authentic standard. For this purpose, we employed a derivatization method based on the use of 7-amino-4-methylcoumarin (AMC) as chromophore to allow detection and quantification of the reaction product as described in *Materials and methods*. As shown in Table I, apparent K_m and V_{max} values of the recombinant enzyme towards GlcA-Gal-Gal were in the same range to those found for the trisaccharide linked to the methoxyphenyl (MP) moiety, indicating that the presence of the chromophoric aglycone does not affect substrate recognition or activity of the recombinant CSGalNAcT-1.

Thus, to further define the specificity of CSGalNAcT-1, we tested various structurally defined oligosaccharides linked to MP as a potential acceptor substrate. No GalNAcT activity was detected toward Gal-Gal-OMP (8),

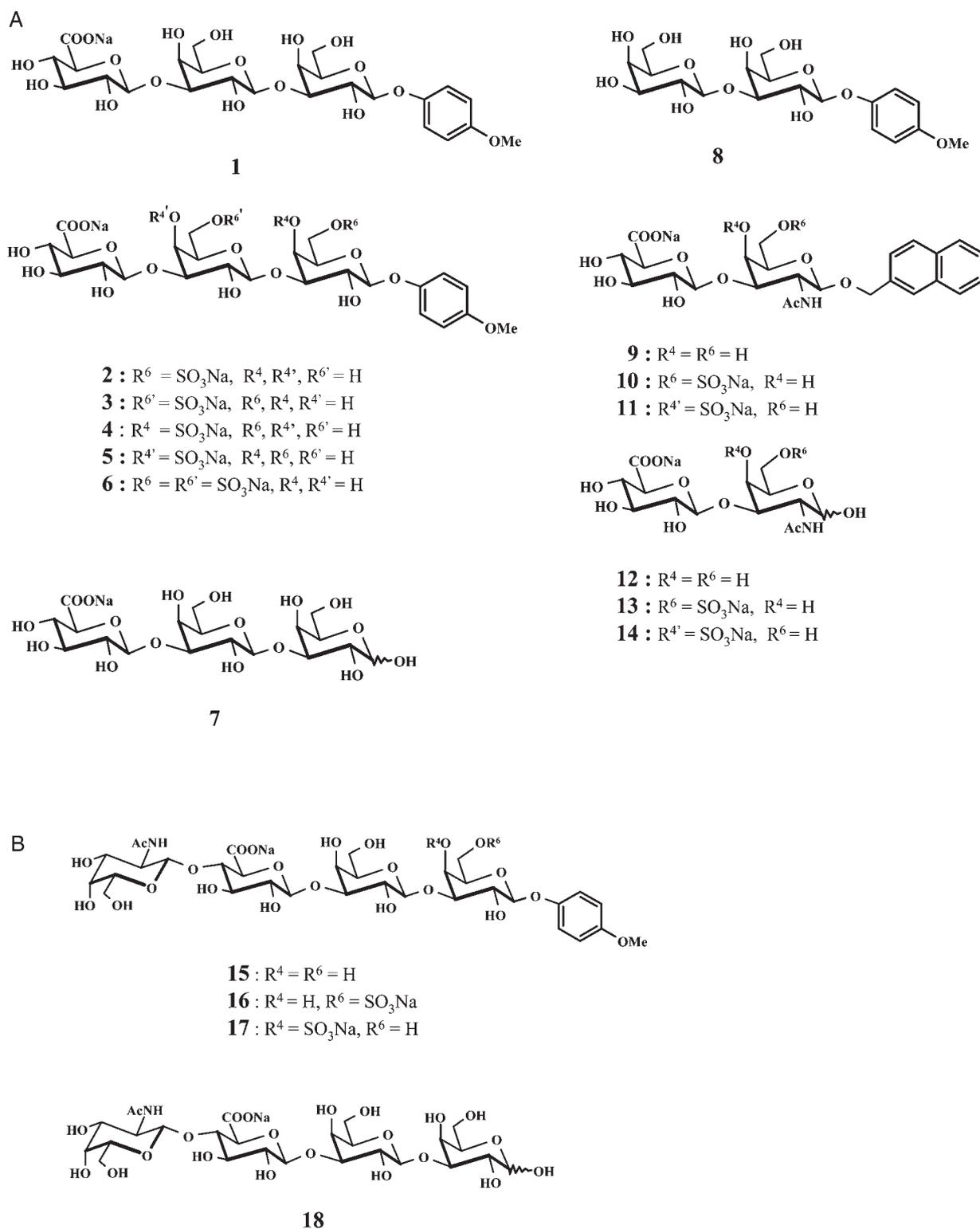


Fig. 1. Chemical structures of acceptor substrate analogs and transfer products. (A) Chemical structures of oligosaccharide analogs to the carbohydrate–protein linkage region of GAGs and constitutive disaccharide unit of CS chains. (B) Chemical structures of the reaction products used as authentic standards for enzyme assays.

indicating that the presence of the terminal GlcA residue is essential for enzymatic activity (Table I). However, CSGalNAcT-1 could not transfer GalNAc on shorter glucuronides coupled to a hydrophobic aglycone, like

4-nitrophenyl- or 4-methylumbelliferyl-glucuronide (data not shown). These results indicate that a trisaccharide motif including a terminal GlcA was the minimal structure that can be used as substrate for the GalNAcT reaction.

The substrate specificity of the recombinant human CSGalNAcT-1 toward the donor substrate was also investigated. No activity could be detected toward UDP-sugars (UDP-Glc, UDP-Gal, UDP-Man, UDP-GlcA) other than UDP-GalNAc, when tested as donor substrates, indicating a strict selectivity of CSGalNAcT-1 with regard to the nucleotide sugar recognition and GalNAcT activity (see Supplementary data, Figure S2).

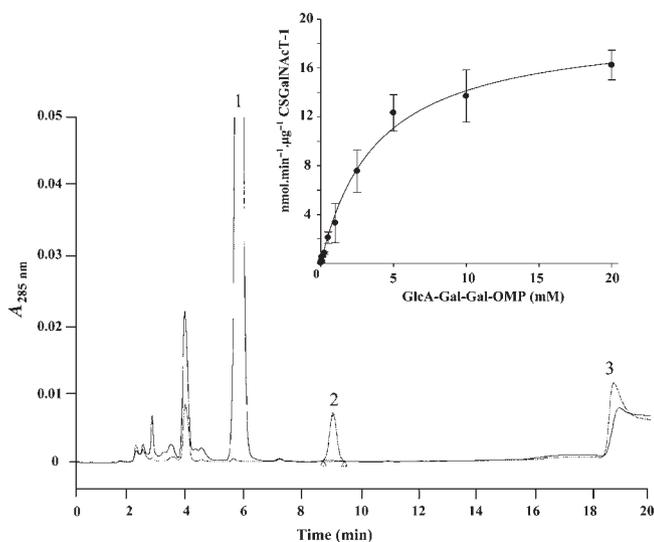


Fig. 2. HILIC–HPLC chromatogram for activity assay of the recombinant human CSGalNAcT-1 toward GlcA-Gal-Gal-OMP. Cell homogenates expressing recombinant human CSGalNAcT-1 were incubated in the presence of trisaccharide acceptor (1 mM) and donor UDP-GalNAc (2 mM), and then separated on a DIOL column, as described in *Materials and methods*. The elution peak positions of the authentic standards were compared with the HPLC separation of the reaction mixture and indicated as follows: peak 1, acceptor substrate GlcA-Gal-Gal-OMP; peak 2, reaction product GalNAc-GlcA-Gal-Gal-OMP; peak 3, donor substrate UDP-GalNAc. The control assay corresponds to an incubation in which acceptor substrate was omitted (dotted line). Inset: kinetic analysis of CSGalNAcT-1 using increasing acceptor substrate concentrations (0.01–10 mM) in the presence of a constant concentration of donor UDP-GalNAc (2 mM). K_m and V_{max} values were obtained by fitting the experimental data to the Michaelis–Menten equation by nonlinear least-squares regression analysis using SigmaPlot 9.0 Software.

Influence of Gal linkage sulfation on CSGalNAcT-1 activity

To analyze the influence of sulfation on Gal residues of the tetrasaccharide linkage region, we determined the activity of the recombinant CSGalNAcT-1 toward a series of sulfated analogs of GlcA-Gal-Gal-OMP substituted at either C4 or C6 position of Gal1 (adjacent to MP) and/or Gal2 (adjacent to GlcA), i.e. compounds 2–6 (Figure 1A). Interestingly, CSGalNAcT-1 was able to catalyze the transfer of GalNAc not only onto the unsulfated substrate, but also onto several monosulfated trisaccharide derivatives (Figure 3). The GalNAcT activity toward the trisaccharide analog sulfated onto C6 of Gal1 (GlcA-Gal-Gal(6S)-OMP) (2) was similar to its unsulfated counterpart (about $4 \text{ nmol}\cdot\text{min}^{-1}\cdot\mu\text{g}^{-1}$ CSGalNAcT-1) and up to $8 \text{ nmol}\cdot\text{min}^{-1}\cdot\mu\text{g}^{-1}$ CSGalNAcT-1 when Gal1 was sulfated on the C4 position (4). CSGalNAcT-1 also exhibited high activity toward the trisaccharide sulfated on the C6 position of Gal2 (GlcA-Gal(6S)-Gal-OMP) (3), whereas sulfation on the C4 position of this Gal residue (5) completely prevented GalNAcT activity (Figure 3). These results pointed out the critical influence of the position of sulfate substitution of Gal2 with regard to the substrate preference of CSGalNAcT-1. Moreover, the disulfated oligosaccharide substituted on the C6 position of each Gal of the linker region (GlcA-Gal(6S)-Gal(6S)-OMP) (6) was not a substrate for the recombinant CSGalNAcT-1, indicating that the active site of the enzyme could not accommodate sulfate substitution on both Gal residues.

To investigate the molecular basis underlying CSGalNAcT-1 activity toward the monosulfated trisaccharide derivatives, we determined the kinetic parameters of the recombinant enzyme toward this series of sulfated analogs (Table I). Our results showed that CSGalNAcT-1 exhibited higher enzymatic efficiency toward all monosulfated oligosaccharides compared with GlcA-Gal-Gal-OMP, except in the case of the trisaccharide substituted on the C4 position of Gal2. Interestingly, the increase in catalytic efficiency of CSGalNAcT-1 toward the trisaccharide monosulfated on the different positions of Gal1 or Gal2 was mainly due to reduced K_m values. For instance, the enzyme exhibited a 2-fold lower K_m toward GlcA-Gal(6S)-Gal-OMP with a similar V_{max} compared with the unsulfated counterpart. Lower K_m values were found in the case of the trisaccharides which were 4-*O*- and

Table I. Kinetic parameters of the recombinant human CSGalNAcT-1 toward various acceptor substrates

Acceptor substrates	K_m (mM)	V_{max} ($\text{nmol}\cdot\text{min}^{-1}\cdot\mu\text{g}^{-1}$ CSGalNAcT-1)	V_{max}/K_m ($\mu\text{L}\cdot\text{min}^{-1}\cdot\mu\text{g}^{-1}$ CSGalNAcT-1)
GlcA-Gal-Gal-OMP (1)	3.81 ± 0.45	19.52 ± 0.82	5.12
GlcA-Gal-Gal(6S)-OMP (2)	1.00 ± 0.06	8.14 ± 0.15	8.14
GlcA-Gal(6S)-Gal-OMP (3)	1.91 ± 0.19	21.56 ± 0.76	11.28
GlcA-Gal-Gal(4S)-OMP (4)	0.60 ± 0.01	11.01 ± 0.51	18.35
GlcA-Gal(4S)-Gal-OMP (5)	nd	nd	nd
GlcA-Gal(6S)-Gal(6S)-OMP (6)	nd	nd	nd
GlcA-Gal-Gal (7)	3.39 ± 0.51	28.32 ± 1.18	8.35
Gal-Gal-OMP (8)	nd	nd	nd

Standard reactions were performed using HeLa cell homogenates containing 2–5 ng of CSGalNAcT-1 in the presence of increasing concentrations of acceptor substrate (0.01–10 mM) at a constant concentration of UDP-GalNAc (2 mM) as described in *Materials and methods*. Apparent kinetic parameters (K_m and V_{max}) were determined using the nonlinear least-squares regression analysis of the data fitted to the Michaelis–Menten equation ($v = V_{max} \times [S]/K_m + [S]$) using the curve-fitting program SigmaPlot 9.0 (Systat Software, Inc., San Jose, CA). Each data point represents the mean \pm SE of three independent experiments (i.e. using three different batches of recombinant CSGalNAcT-1) carried out in triplicate. V_{max}/K_m ratio was determined for each acceptor substrate as a measure of catalytic efficiency. nd, not detectable.

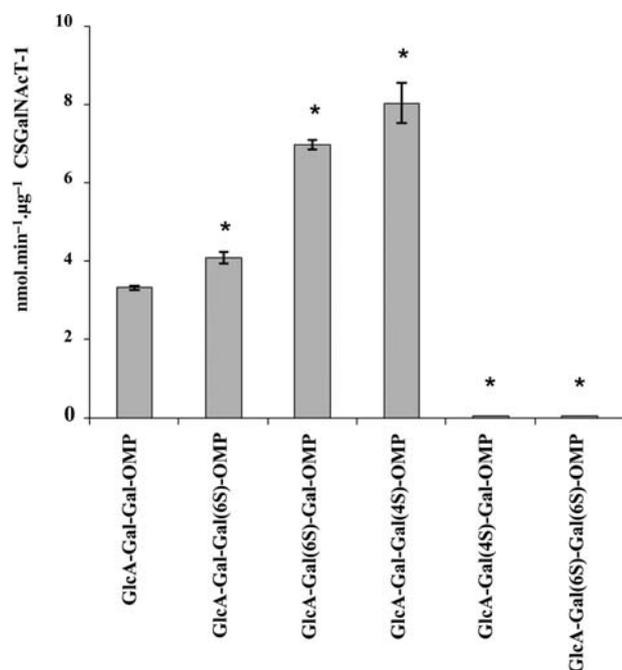


Fig. 3. GalNAcT activity of the recombinant CSGalNAcT-1 toward sulfated and unsulfated acceptor analogs of GlcA-Gal-Gal-OMP. The activity of CSGalNAcT-1 was evaluated toward sulfated and unsulfated analogs (1 mM) substituted on Gal1 at the C4 or C6 position, on Gal2 at the C4 or C6 position or on Gal1 and Gal2 at the C6 position, in the presence of 2 mM UDP-GalNAc as described in *Materials and methods*. Values are expressed in nmol.min⁻¹.μg⁻¹ CSGalNAcT-1. Mean ± SE of three independent experiments carried out in duplicate are shown. **P* < 0.05 versus activity towards GlcA-Gal-Gal-OMP.

6-*O*-sulfated on Gal1 (about 6- and 4-fold, respectively), compared with their unsulfated counterpart. With the exception of the trisaccharide sulfated on C4 of Gal2, our results indicate that the monosulfated oligosaccharides were better substrates compared with their unsulfated counterparts, due to a better apparent affinity of CSGalNAcT-1 toward these modified substrates. These results suggested that monosulfation of Gal1 linkage residue or Gal2 substitution at C6 position stimulates CS chain initiation activity catalyzed by the recombinant human CSGalNAcT-1. Taken together, these data clearly indicate that sulfation can markedly influence CSGalNAcT-1 activity and substrate recognition, strongly suggesting that specific sulfation of Gal residues can positively or negatively modulate the GalNAc transfer onto the linker region.

CSGalNAcT-1 is involved in CS initiation, but not in elongation

Two kinds of GalNAc-GlcA linkages are known in CS: one in the polymer structure (3GalNAc-β1,4-GlcA-β1)_n and the other between the CS chain and the linkage tetrasaccharide. Our results clearly indicated that the human recombinant CSGalNAcT-1 exhibits high activity toward the trisaccharide linkage oligosaccharide GlcA-Gal-Gal or its analog GlcA-Gal-Gal-OMP. Our next aim was to determine whether this enzyme is involved in CS chain elongation and the influence of sulfation on this process. To this objective, we synthesized a series of analogs of the CS constitutive disaccharide

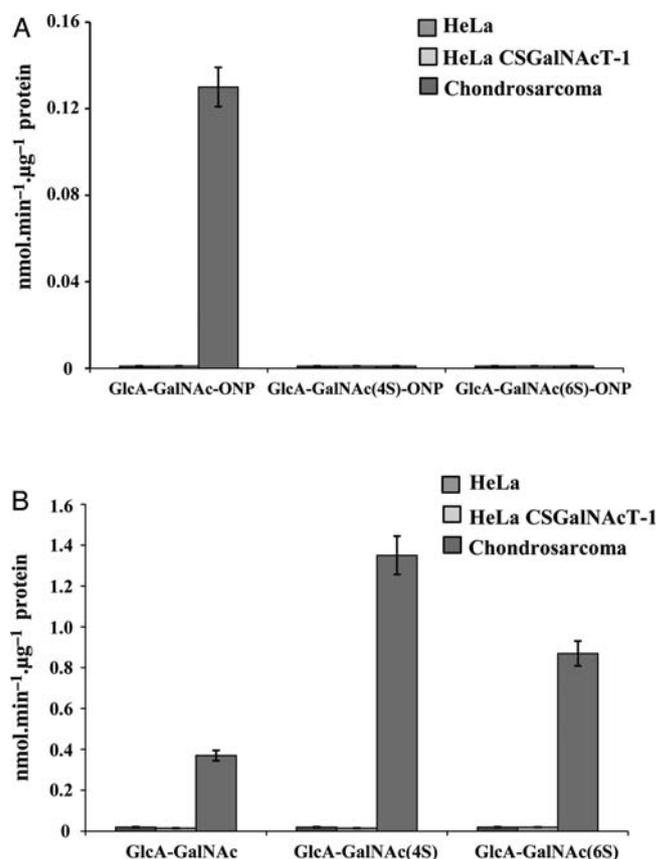


Fig. 4. CSGalNAcT activity of the recombinant CSGalNAcT-1 toward sulfated and unsulfated analogs of the disaccharide unit of CS chains. (A) Activity of CSGalNAcT-1 was evaluated toward sulfated and unsulfated analogs of GlcA-GalNAc-ONP (1 mM) substituted on GalNAc at the C4 or C6 position as described in *Materials and methods* using cell homogenates transfected from HeLa cells transfected with empty vector, HeLa cell homogenates expressing human recombinant CSGalNAcT-1 or chondrosarcoma cell homogenates. (B) Activity of CSGalNAcT-1 was evaluated toward analogs of the disaccharide unit of CS chains GlcA-GalNAc (1 mM) in the presence of UDP-GalNAc (2 mM), as described in *Materials and methods*. Values are expressed in nmol.min⁻¹.mg⁻¹ total protein. Results represent the mean ± SE from three independent experiments performed in duplicate.

unit (GlcA-β1,3-GalNAc-β1-*O*-naphthyl, GlcA-GalNAc-ONP; Figure 1A; 9–11). The GalNAcT activity of the recombinant enzyme was tested toward these molecules by HILIC–HPLC, as described in *Materials and methods*. Our results indicate that CSGalNAcT-1 was unable to transfer a GalNAc residue onto the terminal GlcA of unmodified CS disaccharides linked to NP (9), whereas enzyme activity could be detected in cell homogenates prepared from the SW1353 chondrosarcoma cell line taken as control (Figure 4A). This indicated that elongation activity could be evaluated toward GlcA-GalNAc-ONP in human cell homogenates, although this compound was not a substrate for the recombinant CSGalNAcT-1. This led us to investigate whether sulfation at C4 or C6 of GalNAc of the disaccharide unit, which are present in CS chains, may trigger CSGalNAcT-1 elongation activity. Our results showed that the recombinant glycosyltransferase did not exhibit detectable activity toward GlcA-GalNAc(6S)-ONP (10) or GlcA-GalNAc(4S)-ONP (11).

These findings support the idea that 4-*O*- or 6-*O*-sulfation of the GalNAc residues of the disaccharide unit did not promote CSGalNAcT-1 activity toward these disaccharide analogs.

However, since no activity could be detected toward the sulfated derivatives in chondrosarcoma cells, this led us to synthesize and test a second series of disaccharide analogs lacking the NP aglycone (Figure 1A; 12–14). GalNAcT activity was evaluated by HPLC after nonreductive amination of the anomeric carbon of GalNAc by AMC, as described in *Materials and methods*, and as described in the *Functional characterization of CSGalNAcT-1* section for the trisaccharide analogs. Cell homogenates prepared from SW1353 chondrosarcoma cells were tested for CS elongation activity as control in the same conditions. Results illustrated in Figure 4B showed that, as reported for the NP-linked analogs, GlcA- β 1,3-GalNAc (GlcA-GalNAc) (12), GlcA-GalNAc(6S) (13), and GlcA-GalNAc(4S) (14) were not substrates of the recombinant CSGalNAcT-1. On the other hand, high activity was detected toward both sulfated and unsulfated analogs in chondrosarcoma cells, indicating that the NP aglycone may impair GalNAcT activity toward the sulfated CS disaccharides in these cells. Furthermore, chondrosarcoma cell extracts were active when mixed with HeLa cell homogenates, ruling out the possibility that the presence of inhibitors, competing substrates, etc. in HeLa cell extracts may be responsible for the lack of activity of the recombinant enzyme toward the disaccharide unit (data not shown). Altogether, these results confirmed that CSGalNAcT-1 was not able to transfer GalNAc onto CS disaccharide fragments supporting the idea that this enzyme is involved in initiation but not elongation of CS chains. Moreover, 4-*O*- or 6-*O*-sulfation of the GalNAc residues of the disaccharide unit did not trigger GalNAcT activity of the recombinant enzyme toward the disaccharide analogs.

Discussion

Attempting to elucidate signal elements that regulate GAG initiation and elongation is a major issue in understanding the mechanisms underlying this complex process. Recent studies have focused on the influence of sulfation of the PG polysaccharide chain in this process. Indeed, in addition to its importance in fine-tuning CS chain structures governing their diverse biological functions, several lines of evidence point to a regulatory role of sulfation in the process of CS chain assembly and sorting. In this study, we investigated for the first time the impact of sulfation of the carbohydrate–protein linkage region of GAGs and of the CS constitutive disaccharide unit on the activity and kinetic parameters of CSGalNAcT-1, a key glycosyltransferase of the CS biosynthetic machinery. This goal was achieved by the design and synthesis of a glycolibrary containing series of sulfated and unsulfated oligosaccharide analogs that have been tested as potential acceptor substrates of the recombinant human CSGalNAcT-1 expressed in HeLa cells. We and others demonstrated that this strategy represents an effective way to probe the specificity of several glycosyltransferases involved in linkage GAG synthesis including β 4GalT7 and GlcAT-1 (Gulberti et al. 2005; Fondeur-Gelinotte et al. 2007; Tone et al. 2008; Talhaoui et al. 2010). A major finding of this

study was that several analogs monosulfated on the Gal residues of the linkage region were better substrates compared with their unsulfated counterparts mainly due to lower K_m values, suggesting that sulfation of Gal residues in the linker tetrasaccharide could stimulate CS chain initiation.

To further clarify the molecular mechanisms governing CS chain biosynthesis, it was essential to refine our understanding of the catalytic specificities of CSGalNAcT-1. We first analyzed the activity of this glycosyltransferase toward UDP-sugar donor substrates. Our results showed that the recombinant human CSGalNAcT-1 exhibits a strict specificity toward UDP-GalNAc. This finding was fully consistent with previous reports, indicating that this enzyme functions as a CS-synthase and shows no glucuronosyltransferase activity (Gotoh et al. 2002; Uyama et al. 2002). It is interesting to note that CSGalNAcT-1 exhibited no galactosyltransferase activity, although it possesses a conserved β 4GalT motif. The β 1,4-galactosyltransferases, β 4GalT1-7, contain a W/FGWGXEDDD/E sequence, whereas CSGalNAcT-1 contains a KGWGGEDVH motif. It would be interesting to determine whether amino acid changes within this motif account for differences in donor substrate specificity. We recently showed that the conserved Trp224 plays a central role in the organization of the human β 4GalT7 donor and acceptor substrate binding site but not in donor substrate specificity (Talhaoui et al. 2010). On the other hand, Ramakrishnan and Qasba (2002) reported that Tyr289 in bovine β 4GalT1 is essential for donor binding. Elimination of a hydrogen bond by mutating this residue to Leu, Ile, or Asn enhances GalNAcT in place of galactosyltransferase activity. Phylogenetic analysis and multiple sequence alignment of the GalNAcT family members indicate that in CSGalNAcT-1, the amino acid at the same position is an Ile residue (Oriol et al., unpublished data). Studies are underway to determine whether this sequence plays a role in governing donor substrate specificity.

On the other hand, two kinds of GalNAc-GlcA linkages are known in CS chains: one in its polymer structure (3GalNAc- β 1,4GlcA- β 1) $_n$ and the other between the CS polymer and the linkage tetrasaccharide (3GalNAc- β 1,4-GlcA- β 1,3-Gal- β 1,3-Gal- β 1,4-Xyl- β 1). Since the discovery of the CSGalNAcT-1 initiation activity in the medium of a human melanoma cell line using α -thrombomodulin containing a linkage region as acceptor substrate (Nadanaka et al. 1999), the role of CSGalNAcT-1 in the assembly of CS chains has not been clearly established yet. Initial cloning and activity studies suggested that CSGalNAcT-1 may be involved both in initiation and polymerization of CS chains (Gotoh et al. 2002; Uyama et al. 2002). In an in vitro assay system, using syndecan-4/fibroblast growth factor chimera protein as a substrate, Sato et al. (2003) demonstrated that this enzyme exerts effective initiation activity. Recent studies in chondrogenic cells and in gene a knock-out mouse model mice support the idea that CSGalNAcT-1 plays a critical role in the regulation of CS biosynthesis (Sakai et al. 2007; Sato et al. 2011).

To clarify this issue, we investigated in detail the substrate specificity of the recombinant human CSGalNAcT-1, taking advantage of the chemical synthesis of structurally defined analogs of the linkage region and of the CS constitutive disaccharide units. Since CS-synthases are thought to function as

multiple domain glycosyltransferases carrying both initiation (CSGalNAcT-I) and elongation (CSGalNAcT-II) activities or as enzyme complexes (Yada, Gotoh, et al. 2003; Yada, Sato, et al. 2003), we chose to express the full-length human CSGalNAcT-1 in HeLa cells. Our results showed that the human recombinant CSGalNAcT-1 exhibits marked activity toward the trisaccharide GlcA-Gal-Gal, an analog of the linkage region. The recombinant enzyme also showed high GalNAcT activity toward the trisaccharide derivative linked to a hydrophobic aglycone GlcA-Gal-Gal-OMP with a K_m value in the same range (3.4 and 3.8 mM, respectively), indicating that the presence of a hydrophobic aglycone does not affect the enzyme activity and affinity. This observation was similar to previous findings indicating that synthetic xylosides and digalactosides linked to a hydrophobic moiety act as substrates for β 4GalT7 and GlcAT-1, respectively (Gulberti et al. 2005). In contrast, a digalactoside derivative lacking a terminal GlcA (Gal-Gal-OMP) was not a substrate for CSGalNAcT-1, indicating that the presence of the uronic acid as a terminal acceptor saccharide residue was essential for GalNAc transfer. Furthermore, we observed that small glucuronides such as 4-nitrophenyl-GlcA or GlcA-Gal-Gal-Xyl-*O*-Ser-Gly, analog of the glycopeptide primer of PGs, were not substrates for CSGalNAcT-1. These results corroborate previous findings showing no detectable activity of a truncated form of CSGalNAcT-1 toward GlcA-Gal-Gal-Xyl-*O*-Ser (Uyama et al. 2002). Taken together, the specificity analysis carried out in this study reinforces the idea that CSGalNAcT-1 plays a major role in CS chain initiation and demonstrates that the minimal structure recognized by CSGalNAcT-1 is a GlcA-Gal-Gal trisaccharide motif. Whether the structure of the PG core protein influences CSGalNAcT-1 activity deserves further exploration.

To investigate the potential role of CSGalNAcT-1 in chondroitin polymer formation, we chemically synthesized the GlcA-GalNAc disaccharide and its analog linked to a hydrophobic aglycone (NP), as potential acceptor substrates for the recombinant enzyme. Our results showed no detectable activity toward these compounds, although it could be readily detected in chondrosarcoma cells taken as positive control. Two hypotheses may account for these findings: (1) the recombinant CSGalNAcT-1 possesses no elongation activity or (2) the enzyme may recognize longer (i.e. tetra- or hexasaccharide) CS oligosaccharide units. An important issue for this study was to determine whether sulfation affects the CS assembly process. Since we found no activity toward the constitutive disaccharide unit, we next investigated whether sulfation at various positions of the oligosaccharide may trigger CSGalNAcT-1 elongation activity. Indeed, sulfation of the GalNAc residue of the chondroitin disaccharide unit has been shown to profoundly influence CS maturation (Kitagawa et al. 1997; Uyama et al. 2006). Gene-trapped knock-out mice of *C4st1* (which encodes chondroitin-4-sulfotransferase 1) causes severe defects in aggrecan CS content and length, suggesting that 4-*O*-sulfation could regulate elongation of CS chains (Izumikawa et al. 2011). This corroborated early studies indicating that CSGalNAcT partially purified from bovine serum exhibited higher levels of activity when 6-*O*- or 4-*O*-sulfated GalNAc was utilized as an acceptor (Kitagawa

et al. 1995). On the other hand, the terminal 3-*O*-sulfated GlcA or penultimate 4,6-*O*-disulfated GalNAc inhibits the activity. The results of our study indicate that CSGalNAcT-1 showed no detectable activity toward the disaccharide units sulfated on the C4 or C6 position of the GalNAc residue. This indicated that sulfation does not trigger elongation activity, when a disaccharide was used as a substrate.

On the other hand, several structural modifications of the oligosaccharide linkage region of CS chains of PG isolated from various tissues, including cartilage, have been described (Sugahara et al. 1988, 1991, 1992). Interestingly, sulfation at C4 and/or C6 position of Gal1 and/or Gal2 occurs only in the case of CS/DS chains but not HS chains, leading to the proposal that sulfation of the linker region acts as a regulatory signal for galactosaminoglycan initiation. A major aim of this work was to investigate the impact of sulfation of the tetrasaccharide linkage region on CSGalNAcT-1 activity in order to determine whether this modification influences CS sorting. Our results clearly indicated that the trisaccharide analogs sulfated at C4 or C6 of Gal1, as well as at C6 of Gal2, were better substrates than their unsulfated counterparts. Interestingly, no activity was detected toward the trisaccharide sulfated on the C4 position of Gal2, suggesting that sulfation of this residue may facilitate the biosynthetic process when substitution occurred at C6 or prevents it when present at the C4 position. Interestingly, we and others previously showed that a monosulfated disaccharide Gal-Gal(6S)-OMP was a better substrate than the unsulfated compound for recombinant human GlcAT-1 (Gulberti et al. 2005; Tone et al. 2008). We also showed that phosphorylation of xylose at the C2 position prevents β 4GalT7 activity (Gulberti et al. 2005; Talhaoui et al. 2010).

In conclusion, this work demonstrated that sulfation of the Gal residues of the tetrasaccharide linkage region of GAGs could regulate human recombinant CSGalNAcT-1 activity. These findings together with previous reports support the concept that modifications (i.e. Gal sulfation and/or xylose phosphorylation) of the carbohydrate-protein linkage region of GAGs play an important role in CS biosynthesis and could act as a regulatory signal in the assembly of CS chains, facilitating or possibly arresting CS initiation according to the substitution position. From these data, we have gained an improved understanding of the substrate recognition of glycosyltransferases involved in GAG biosynthesis, especially CSGalNAcT-1, a key enzyme of CS chain initiation. On the basis of these studies, it would be possible to design molecules targeting glycosyltransferases to impact positively or negatively on GAG assembly and sorting. These compounds could represent promising approaches in the development of novel strategies toward GAG-based therapeutics.

Materials and methods

Chemical synthesis of oligosaccharide acceptors and reaction products

The preparation of different sets of various sulfoforms of oligosaccharide derivatives from the linkage region of GAG and CS disaccharide units was achieved as follows. The first series of

compounds was prepared as 4-methoxyphenyl-*O*-glycosides (MP-*O*-glycosides) in which the MP group was used for UV detection of the potential substrates and their reaction products. The trisaccharide acceptor GlcA-Gal-Gal-OMP (Figure 1A; **1**) and its various sulfoforms (**2–6**) were obtained by a stereo-controlled chemical synthesis, as previously reported (Thollas and Jacquinet 2004). The reducing trisaccharide GlcA-Gal-Gal (Figure 1A; **7**) was prepared by catalytic hydrogenation of the benzyl glycoside analog of **1**. The disaccharide Gal-Gal-OMP (**8**) was prepared from the common starting material 4-methoxyphenyl-4,6-*O*-benzylidene- β -D-galactopyranoside, as reported by Jacquinet (2004).

The CS disaccharide derivatives were isolated after a controlled acid hydrolysis of polymeric bovine CS and produced as 2-naphthyl-*O*-glycosides (NP-*O*-glycosides) in which the NP group was chosen for UV detection. GlcA-GalNAc-ONP (**9**) and its various sulfoforms (**10 and 11**) were produced by a stereo-controlled and highly divergent approach starting from a single precursor obtained by semisynthesis from the natural CS polymer, as previously reported (Jacquinet et al. 2009). Their corresponding reducing congeners (**12–14**) were prepared by catalytic hydrogenation of **9–11**, respectively.

The reaction products corresponding to the tetrasaccharide GalNAc-GlcA-Gal-Gal-OMP (Figure 1B; **15**) and its sulfoforms (**16 and 17**) were prepared by chemical synthesis using a similar strategy to that previously reported for the trisaccharide derivatives **1–6**. These compounds were used as authentic standards in HPLC analysis for the identification and quantification of the reaction products following enzymatic transfer of GalNAc on the trisaccharide acceptors. The reducing tetrasaccharide **18** was obtained by catalytic hydrogenation of the benzyl glycoside analog of **15**.

Cloning and expression of human CSGalNAcT-1

The wild-type human CSGalNAcT-1 cDNA sequence (GenBank accession number NM_018371) was cloned by polymerase chain reaction (PCR) amplification from a placenta cDNA library (Clontech, Palo Alto, CA) using a sense primer 5'-ATGATGATGGTTCGCCGGGGCTG-3' and an antisense primer 5'-GTTTCATGTTTTTGTCTACTTGTCTTCTG-3' corresponding to the 5'-end and 3'-end of the coding region of the human CSGalNAcT-1 (Uyama et al. 2002). The PCR fragment was subcloned into the pCR2.1-TOPO[®] vector (TOPO[®] TA Cloning Kit; Life Technologies, Van Allen Way Carlsbad, CA) and sequenced on both strands. The cDNA sequence was 100% identical to that previously described (Uyama et al. 2002). The full-length CSGalNAcT-1 cDNA sequence was modified by PCR to include an *Nhe*I site and a Kozak consensus sequence at the 5'-end, and a *Hind*III site and a sequence encoding a Myc tag (EQKLIIEDL) at the 3'-end using the appropriate oligonucleotides. The modified cDNA was then subcloned into the *Nhe*I-*Hind*III sites of the eukaryotic expression vector pcDNA3.1(+) to produce pcDNA-CSGalNAcT-1.

HeLa cells purchased from American Type Culture Collection (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-Invitrogen, Cergy-Pontoise, France), 2 mM glutamine (Gibco-Invitrogen),

and 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco-Invitrogen). The plasmid pcDNA-CSGalNAcT-1 was transfected into the cells grown at 80% confluence using ExGen-500 transfection reagent (Euromedex, Souffelweyersheim, France), according to the recommendations of the manufacturer. Cells were harvested 48 h after transfection, by scraping in phosphate-buffered saline (PBS) and centrifuged for 5 min at 3000 \times *g* at 4°C. Cell pellets were resuspended in saccharose-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (0.25 M saccharose, 5 mM HEPES, pH 7.4) and sonicated twice for 5 s on ice prior to further analysis.

Protein expression analysis

Protein concentration of cell homogenates was evaluated by the method of Bradford (1976) before analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions (Laemmli 1970). Protein samples from HeLa cells transfected with wild-type pcDNA-CSGalNAcT-1 or cells transfected with the empty vector were separated on 10% (w/v) polyacrylamide gel and electrotransferred to Immobilon-P[®] membrane (Millipore, Billerica, MA). The membrane was probed using monoclonal anti-Myc antibodies (Sigma, Saint-Louis, MO) and revealed with alkaline phosphatase-conjugated anti-mouse secondary antibodies (Sigma), as previously described (Fondeur-Gelinotte et al. 2007). The amount of recombinant wild-type human CSGalNAcT-1 expressed in HeLa cells was evaluated by scanning densitometry using a calibration curve established with 5–12 ng of recombinant GST-Myc protein (Pierce-Thermoscientific, Courtaboeuf, France) run on the same gel. Scanning densitometry was performed using Scion 1.63 Image Software.

CSGalNAcT-1 activity assay

The assay for activity of the recombinant human CSGalNAcT-1 is based on the transfer of GalNAc residue from the donor UDP-GalNAc to various acceptor substrates synthesized as described in the *Chemical synthesis of oligosaccharide acceptors* section. Individual stock solutions of oligosaccharides were prepared in water and stored at -20°C before use. For each substrate, assay conditions were optimized in terms of incubation time, protein amount, Mn²⁺ concentration, incubation buffer, and pH (data not shown). The standard reaction mixture contained 100 mM sodium cacodylate buffer (pH 7.0), 10 mM MnCl₂, 10–50 μ g HeLa cell homogenates expressing recombinant human CSGalNAcT-1, 2 mM donor substrate (UDP-GalNAc), and 0.1, 1, or 10 mM acceptor substrate in a total volume of 50 μ L at 37°C for 60 min. Control assays in which acceptor substrate or donor substrate was omitted, as well as control using cells transfected with empty vector (not shown) were systematically carried out for each set of experiments and analyzed under the same conditions. Quantification of the reaction products was performed with calibration curves obtained by using increasing concentrations of authentic standards resolved under same conditions. Enzymatic activities were expressed as nmol·min⁻¹· μ g⁻¹ CSGalNAcT-1 and are the mean \pm standard error (SE) of three independent experiments in duplicate.

For analysis of CSGalNAcT-1 activity toward sulfated or unsulfated GlcA-Gal-Gal-OMP trisaccharides or unsulfated Gal-Gal-OMP disaccharide analog, the reaction was stopped by the addition of 50 μ L (v/v) of acetonitrile (ACN). The samples were centrifuged for 10 min at 10,000 $\times g$ and an aliquot of the supernatant (40 μ L) was analyzed by a combination of HILIC and HPLC analyses on a 2695 Alliance module equipped with a 486 UV detector (Waters, Milford, MA), as follows: the oligosaccharides coupled to the MP group were separated on a DIOL analytical column (4.6 \times 250 mm, 6 μ m; Interchim, Montlucon, France) and detected at a wavelength of 285 nm. Samples were eluted over a 20 min linear gradient of 15–20% solvent B/solvent A at a flow rate of 1 mL/min (solvent A, 95% (v/v) ACN; solvent B: 0.02% (v/v) trifluoroacetic acid).

For determination of CSGalNAcT-1 activity toward GlcA-Gal-Gal trisaccharide, the samples were analyzed by reversed-phase HPLC after a reductive amination reaction using AMC, using a protocol adapted from Yodoshi et al. (2008). Briefly, 10 μ L of the labeling mixture (consisting of 5 μ mol AMC in 125 μ L of methanol, 17.5 mg of sodium cyanoborohydride (NaCNBH₃), and 20 μ L of acetic acid) was added to the reaction mixture and incubated 2 h at 80°C. The labeling reaction was stopped by cooling in ice and liquid extraction of the uncoupled fluorophore by 1 mL of chloroform. The AMC-labeled oligosaccharides were separated according to the number of residues on a reversed-phase C18 analytical column (X-Bridge, 4.6 \times 150 mm, 5 μ m; Waters, Milford, MA) at a detection wavelength of 365 nm. A linear gradient (10–50% over 20 min) of solvent C/solvent B was used to separate the AMC-labeled oligosaccharides at a constant flow rate of 1 mL/min (solvent C: 95% (v/v) methanol).

For analysis of CSGalNAcT-1 activity toward analogs of CS fragments GlcA-GalNAc-ONP, the reaction was stopped by the addition of 50 μ L (v/v) of ACN. The samples were centrifuged for 10 min at 10,000 $\times g$ before HPLC analysis of the supernatant. The oligosaccharides were analyzed on a reversed-phase C18 analytical column (X-Bridge, 4.6 \times 150 mm, 5 μ m; Waters, Milford, MA) at a detection wavelength of 275 nm and eluted over a 30 min linear gradient of 5–50% solvent C/solvent B at a constant flow rate of 1 mL/min.

For analysis of CSGalNAcT-1 activity toward CS disaccharides GlcA-GalNAc, the samples were analyzed by reversed-phase HPLC after a reductive amination reaction using AMC, using a procedure similar to that previously reported for the GlcA-Gal-Gal trisaccharide analog. The AMC-labeled oligosaccharides were separated according to the number of residues on a reversed-phase X-Bridge C18 analytical column at a detection wavelength of 365 nm. A linear gradient of solvent C/solvent B (5–50% in 30 min) was used to separate the AMC-labeled oligosaccharides at a constant flow rate of 1 mL/min.

CSGalNAcT activity assay using SW1353 chondrosarcoma cell homogenates

SW1353 chondrosarcoma cell line (purchased from ATCC) was cultured in DMEM/nutrient mixture F12 (DMEM/F12; Gibco-Invitrogen) supplemented with 10% (v/v) fetal bovine

serum, 2 mM glutamine, and 100 U/mL penicillin and 100 μ g/mL streptomycin. After cell harvest by scraping in PBS, cell homogenates were prepared in saccharose-HEPES buffer by sonication, as described in the *Cloning and expression of human CSGalNAcT-1* section for HeLa cells. CSGalNAcT activity toward unsulfated or sulfated analogs of disaccharide CS units GlcA-GalNAc-ONP and GlcA-GalNAc was determined in a reaction mixture containing 100 mM sodium cacodylate buffer (pH 7.0), 10 mM MnCl₂, 100 μ g cell homogenates, 2 mM donor substrate (UDP-GalNAc), and 1 or 5 mM acceptor substrate in a total volume of 50 μ L at 37°C for 60–120 min. Control assays in which acceptor or donor substrate was omitted were systematically prepared and run in the same analytical conditions. The samples were analyzed by reverse-phase HPLC as described above for HeLa cell homogenates expressing recombinant human CSGalNAcT-1.

Determination of kinetic constants

For the evaluation of kinetic parameters from initial velocity data, increasing concentrations of acceptor oligosaccharides (0–10 mM) were used in the presence of a constant concentration of UDP-GalNAc (2 mM). Apparent kinetic parameters (K_m , V_{max} , and V_{max}/K_m) were determined using the nonlinear least-squares regression analysis of the data fitted to the Michaelis–Menten equation $v = V_{max} \times [S]/K_m + [S]$, where v is the initial velocity, $[S]$ the substrate concentration, V_{max} the maximal velocity, K_m the Michaelis–Menten constant, and V_{max}/K_m corresponds to a measure of catalytic efficiency, using the curve-fitting program SigmaPlot 9.0 (Systat Software, Inc., San Jose, CA). Each data point represents the mean \pm SE of three independent experiments, with assays performed in triplicate.

Supplementary data

Supplementary data for this article is available online at <http://glycob.oxfordjournals.org/>.

Funding

This work was supported by Agence Nationale pour la Recherche (ANR) GAG-Network, grant ANR-08-PCVI-0023-01, INSERM University of Dundee International Collaboration Contract (C2I), Nancy-Dundee European Associated Laboratory (EAL, Sulfo- and GlycosylTransferase enzymes, *SFGEN*), the Royal Society International Joint Grant, and the Région Lorraine.

Conflict of interest

None declared.

Abbreviations

ACN, acetonitrile; AMC, 7-amino-4-methylcoumarin; ATCC, American Type Culture Collection; CS, chondroitin sulfate; CSGalNAcT-1, chondroitin sulfate *N*-acetylgalactosaminyltransferase-1; DMEM, Dulbecco's modified Eagle's medium;

DS, dermatan sulfate; GAG, glycosaminoglycan; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; GlcAT-1, glucuronosyltransferase-1; GlcNAc, glucosamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HS, heparan sulfate; MP, methoxyphenyl; NP, naphthyl; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PG, proteoglycan; SE, standard error.

References

- Bai X, Zhou D, Brown JR, Crawford BE, Hennet T, Esko JD. 2001. Biosynthesis of the linkage region of glycosaminoglycans: Cloning and activity of galactosyltransferase-II, the sixth member of the β 1,3-galactosyltransferase family (β 3GalT6). *J Biol Chem*. 276:48189–48195.
- Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 72:248–254.
- Fondeur-Gelinotte M, Lattard V, Gulberti S, Oriol R, Mulliert G, Coughtrie M, Magdalou J, Netter P, Ouzzine M, Fournel-Gigleux S. 2007. Molecular basis for acceptor substrate specificity of the human β 1,3-glucuronosyltransferases GlcAT-I and GlcAT-P involved in glycosaminoglycans and HNK-1 carbohydrate epitope biosynthesis, respectively. *Glycobiology*. 17:857–867.
- Gotoh M, Sato T, Akashima T, Iwasaki H, Kameyama A, Mochizuki H, Yada T, Inaba N, Zhang Y, Kikuchi N, et al. 2002. Enzymatic synthesis of chondroitin with a novel chondroitin sulfate *N*-acetylgalactosaminyltransferase that transfers *N*-acetylgalactosamine to glucuronic acid in initiation and elongation of chondroitin sulfate synthesis. *J Biol Chem*. 277:38189–38196.
- Götting C, Kuhn J, Zahn R, Brinkmann T, Kleesiek K. 2000. Molecular cloning and expression of human UDP-D-xylose-proteoglycan core protein β -D-xylosyltransferase and its first isoform XT-II. *J Mol Biol*. 304:517–528.
- Gulberti S, Lattard V, Fondeur M, Jacquinet JC, Mulliert G, Netter P, Magdalou J, Ouzzine M, Fournel-Gigleux S. 2005. Phosphorylation and sulfation of oligosaccharide substrates critically influence the activity of human β 1,4-galactosyltransferase 7 (GalT-1) and β 1,3-glucuronosyltransferase I (GlcAT-1) involved in the biosynthesis of the glycosaminoglycan-protein linkage region of proteoglycans. *J Biol Chem*. 280(2):1417–1425.
- Izumikawa T, Egusa N, Taniguchi F, Sugahara K, Kitagawa H. 2006. Heparan sulfate polymerization in *Drosophila*. *J Biol Chem*. 281(4):1929–1934.
- Izumikawa T, Okuura Y, Koike T, Sakoda N, Kitagawa H. 2011. Chondroitin 4-*O*-sulfotransferase-1 regulates the chain length of chondroitin sulfate in co-operation with chondroitin *N*-acetylgalactosaminyltransferase-2. *Biochem J*. 434(2):321–331.
- Jacquinet J-C. 2004. An expeditious preparation of various sulfoforms of the disaccharide β -D-Galp-(1 \rightarrow 3)- β -D-Galp, a partial structure of the linkage region of proteoglycans, as their 4-methoxyphenyl- β -D-glycosides. *Carbohydr Res*. 339(2):349–359.
- Jacquinet J-C, Lopin-Bon C, Vibert A. 2009. From polymer to size-defined oligomers: A highly divergent and stereocontrolled construction of chondroitin sulfate A, C, D, E, K, L and M oligomers from a single precursor. *Chem Eur J*. 15(37):9579–9595.
- Kalathas D, Theocharis DA, Bounias D, Kyriakopoulou D, Papageorgakopoulou N, Stavropoulos MS, Vynios DH. 2009. Alterations of glycosaminoglycan disaccharide content and composition in colorectal cancer: Structural and expression studies. *Oncol Rep*. 22(2):369–375.
- Kitagawa H, Tanaka Y, Tsuchida K, Goto F, Ogawa T, Lidholt K, Lindahl U, Sugahara K. 1995. *N*-Acetylgalactosamine (GalNAc) transfer to the common carbohydrate-protein linkage region of sulfated glycosaminoglycans: Identification of UDP-GalNAc:chondro-oligosaccharide α -*N*-acetylgalactosaminyltransferase in fetal bovine serum. *J Biol Chem*. 270(38):22190–22195.
- Kitagawa H, Tone Y, Tamura JI, Neumann KW, Ogawa T, Oka S, Kawasaki T, Sugahara K. 1998. Molecular cloning and expression of glucuronosyltransferase-1 involved in the biosynthesis of glycosaminoglycan-protein linkage region of proteoglycans. *J Biol Chem*. 273:6615–6618.
- Kitagawa H, Tsutsumi K, Ikegami-Kuzuhara A, Nadanaka S, Goto F, Ogawa T, Sugahara K. 2008. Sulfation of the galactose residues in the glycosaminoglycan-protein linkage region by recombinant human chondroitin 6-*O*-sulfotransferase-1. *J Biol Chem*. 283(41):27438–27443.
- Kitagawa H, Tsutsumi K, Tone Y, Sugahara K. 1997. Developmental regulation of the sulfation profile of chondroitin sulfate chains in the chicken embryo brain. *J Biol Chem*. 272(50):31377–31381.
- Krishna NR, Agrawal PK. 2000. Molecular structure of the carbohydrate-protein linkage region fragments from connective-tissue proteoglycans. *Adv Carbohydr Chem Biochem*. 56:201–234.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227:680–685.
- Maeda N. 2010. Structural variation of chondroitin sulfate and its roles in the central nervous system. *Cent Nerv Syst Agents Med Chem*. 10(1):22–31.
- Moses J, Oldberg A, Cheng F, Fransson LA. 1997. Biosynthesis of the proteoglycan decorin-transient 2-phosphorylation of xylose during formation of the trisaccharide linkage region. *Eur J Biochem*. 248(2):521–526.
- Nadanaka S, Kitagawa H, Goto F, Tamura JI, Neumann KW, Ogawa T. 1999. Involvement of the core protein in the first β -*N*-acetylgalactosamine transfer to the glycosaminoglycan-protein linkage region tetrasaccharide and in the subsequent polymerization: The critical determining step for the chondroitin sulfate biosynthesis. *Biochem J*. 340:353–357.
- Okajima T, Yoshida K, Kondo T, Furukawa K. 1999. Human homolog of *Caenorhabditis elegans* sqv-3 gene is galactosyltransferase-I involved in the glycosaminoglycan-protein linkage region of proteoglycans. *J Biol Chem*. 274:22915–22918.
- Ramakrishnan B, Qasba PK. 2002. Structure-based design of β 1,4-galactosyltransferase I (β 4Gal-T1) with equally efficient *N*-acetylgalactosaminyltransferase activity: Point mutation broadens beta 4Gal-T1 donor specificity. *J Biol Chem*. 277(23):20833–20839.
- Saigoh K, Izumikawa T, Koike T, Shimizu J, Kitagawa H, Kusunoki S. 2011. Chondroitin β -1,4-*N*-acetylgalactosaminyltransferase-1 missense mutations are associated with neuropathies. *J Hum Genet*. 56(2):143–146.
- Sakai K, Kimata K, Sato T, Gotoh M, Narimatsu H, Shinomiya K, Watanabe H. 2007. Chondroitin sulfate *N*-acetylgalactosaminyltransferase-1 plays a critical role in chondroitin sulfate synthesis in cartilage. *J Biol Chem*. 282(6):4152–4161.
- Sato T, Gotoh M, Kiyohara K, Akashima T, Iwasaki H, Kameyama A, Mochizuki H, Yada T, Inaba N, Togayachi A, et al. 2003. Differential roles of two *N*-acetylgalactosaminyltransferases, CSGalNAcT-1, and a novel enzyme, CSGalNAcT-2: Initiation and elongation in synthesis of chondroitin sulfate. *J Biol Chem*. 278(5):3063–3071.
- Sato T, Kudo T, Ikehara Y, Ogawa H, Hirano T, Kiyohara K, Hagiwara K, Togayachi A, Ema M, Takahashi S, et al. 2011. Chondroitin sulfate *N*-acetylgalactosaminyltransferase 1 is necessary for normal endochondral ossification and aggrecan metabolism. *J Biol Chem*. 286(7):5803–5812.
- Sugahara K, Kitagawa H. 2000. Recent advances in the study of the biosynthesis functions of sulfated glycosaminoglycans. *Curr Opin Struct Biol*. 10:518–527.
- Sugahara K, Masuda M, Harada T, Yamashina I, de Waard P, Vliegenthart JF. 1991. Structural studies on sulfated oligosaccharides derived from the carbohydrate-protein linkage region of chondroitin sulfate proteoglycans of whale cartilage. *Eur J Biochem*. 202(3):805–811.
- Sugahara K, Mizuno N, Okumura Y, Kawasaki T. 1992. The phosphorylated and/or sulfated structure of the carbohydrate-protein linkage region isolated from chondroitin sulfate in the hybrid proteoglycans of Engelbreth-Holm-Swarm mouse tumor. *Eur J Biochem*. 204:401–406.
- Sugahara K, Yamashina I, De Waard P, Van Halbeek H, Vliegenthart JF. 1995. Structural studies on the hexasaccharide alditols isolated from the carbohydrate-protein linkage region of dermatan sulfate proteoglycans of bovine aorta: Demonstration of iduronic acid-containing components. *J Biol Chem*. 270:7204–7212.
- Sugahara K, Yamashina I, De Waard P, Van Halbeek H, Vliegenthart JF. 1988. Structural studies on sulfated glycopeptides from the carbohydrate-protein linkage region of chondroitin 4-sulfate proteoglycans of swam rat chondrosarcoma: Demonstration of the structure Gal(4-*O*-sulfate) β 1-3Gal β 1-4Xyl β 1-*O*-Ser. *J Biol Chem*. 263(21):10168–10174.
- Talhaoui I, Bui C, Oriol R, Mulliert G, Gulberti S, Netter P, Coughtrie MW, Ouzzine M, Fournel-Gigleux S. 2010. Identification of key functional residues in the active site of human β 1,4-galactosyltransferase 7: A major enzyme in the glycosaminoglycan synthesis pathway. *J Biol Chem*. 285(48):37342–37358.
- Thollas B, Jacquinet J-C. 2004. Synthesis of various sulfoforms of the trisaccharide β -D-GlcpA-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow OMP) as probes for the study of the biosynthesis and sorting of proteoglycans. *Org Biomol Chem*. 2(3):434–442.

- Tone Y, Pedersen LC, Yamamoto T, Izumikawa T, Kitagawa H, Nishihara J, Tamura J, Negishi M, Sugahara K. 2008. 2-*O*-Phosphorylation of xylose and 6-*O*-sulfation of galactose in the protein linkage region of glycosaminoglycans influence the glucuronyltransferase-I activity involved in the linkage region synthesis. *J Biol Chem.* 283(24):16801–16807.
- Uyama T, Ishida M, Izumikawa T, Trybala E, Tufaro F, Bergström T, Sugahara K, Kitagawa H. 2006. Chondroitin 4-*O*-sulfotransferase-1 regulates E disaccharide expression of chondroitin sulfate required for herpes simplex virus infectivity. *J Biol Chem.* 281(50):38668–38674.
- Uyama T, Kitagawa H, Tamura JI, Sugahara K. 2002. Molecular cloning and expression of human chondroitin *N*-acetylgalactosaminyltransferase: The key enzyme for the chain initiation and elongation of chondroitin-dermatan sulfate on the protein linkage region tetrasaccharide shared by heparin-heparan sulphate. *J Biol Chem.* 277:8841–8846.
- Yada T, Gotoh M, Sato T, Shionyu M, Go M, Kaseyama H, Iwasaki H, Kikuchi N, Kwon YD, Togayachi A, et al. 2003. Chondroitin sulfate synthase-2: Molecular cloning and characterization of a novel human glycosyltransferase homologous to chondroitin sulfate glucuronyltransferase, which has dual enzymatic activities. *J Biol Chem.* 278(32):30235–30247.
- Yada T, Sato T, Kaseyama H, Gotoh M, Iwasaki H, Kikuchi N, Kwon YD, Togayachi A, Kudo T, Watanabe H, et al. 2003. Chondroitin sulfate synthase-3: Molecular cloning and characterization. *J Biol Chem.* 278(41):39711–39725.
- Yamada S, Okada Y, Ueno M, Iwata S, Deepa SS, Nishimura S, Fujita M, Van Die I, Hirabayashi Y, Sugahara K. 2002. Determination of the glycosaminoglycan–protein linkage region oligosaccharide structures of proteoglycans from *Drosophila melanogaster* and *Caenorhabditis elegans*. *J Biol Chem.* 277(35):31877–31886.
- Yodoshi M, Tani A, Ohta Y, Suzuki S. 2008. Optimized conditions for high-performance liquid chromatography analysis of oligosaccharides using 7-amino-4-methylcoumarin as a reductive amination reagent. *J Chromatogr A.* 1203(2):137–145.