RESEARCH COMMUNICATION

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 chondroitin sulphate biosynthesis

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α -Thrombomodulin (α -TM) with a truncated glycosaminoglycan–protein linkage tetrasaccharide, GlcA β 1-3Gal β 1-3Gal β 1-4Xyl, was tested as an acceptor together with a sugar donor, UDP-*N*-[³H]acetylgalactosamine, using a cell-free enzyme system prepared from the serum-free culture medium of a human melanoma cell line. The truncated tetrasaccharide on α -TM served as an acceptor, whereas the linkage tetrasaccharide–serine did not. Our characterization of the radioactively labelled product by enzymic digestion revealed that the *N*-[³H]acetylgalactosamine residue was transferred to α -TM through a β 1,4-linkage. The substrate competition experiments with the chondro-hexa-saccharide and α -TM reinforced our speculation that a common *N*-acetylgalactosaminyltransferase catalysed the transfer of *N*-acetylgalactosamine to both the linkage tetrasaccharide and the longer chondroitin oligosaccharides. Moreover, chondroitin

polymerization was demonstrated on the tetrasaccharide of α -TM using both UDP-glucuronic acid and UDP-*N*-acetylgalactosamine as sugar donors. Much longer chains were synthesized on α -TM than on the linkage penta- and hexa-saccharide–serines. Together, these results indicated that the core protein is required for the transfer of the first *N*-acetylgalactosamine residue through a β 1,4-linkage and also for subsequent efficient chain polymerization reactions, and that the critical determining step for chondroitin sulphate biosynthesis is the transfer of the first *N*-acetylgalactosamine residue.

Key words: *N*-acetylgalactosaminyltransferase, chondroitin polymerization, chondroitin sulphate, glycosaminoglycan, proteoglycan.

INTRODUCTION

Chondroitin sulphate (CS) biosynthesis is initiated by the addition of xylose to serine residues in the core protein, followed by the sequential addition of two galactose residues and a glucuronic acid residue to form the tetrasaccharide linkage structure, GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-*O*-Ser. Chondroitin polymerization with alternating *N*-acetylgalactosamine and glucuronic acid residues then takes place, forming the repeating disaccharide region. In a previous study, we demonstrated that α -thrombomodulin (α -TM), a so-called part-time proteoglycan, bore the truncated linkage tetrasaccharide, GlcA β 1-3Gal β 1-3Gal β 1-4Xyl, and we suggested that the critical determining step for proteoglycan biosynthesis may be the transfer of the fifth sugar residue, the first *N*-acetylgalactosamine [1]. The transferase for the first *N*-acetylgalactosamine residue has been postulated to be different from that which catalyses the elongation step by transferring *N*-acetylgalactosamine to the repeating disaccharide region [2]. However, despite extensive efforts, our search for the enzyme which catalyses the transfer of the first *N*-acetylgalactosamine residue in the β -anomeric configuration has been unsuccessful. Instead, the *N*-acetylgalactosamine residue transferred to the linkage tetrasaccharide–serine was found to be exclusively by an α -linkage [3,4], and several cultured cell lines incubated with 4-methylumbelliferyl β -xyloside produced an α -

N-acetylgalactosamine-capped pentasaccharide [5]. In addition, the linkage tetrasaccharide–serine was not utilized as an acceptor substrate for the β -*N*-acetylgalactosaminyltransferase (GalNAcT) responsible for chondroitin chain elongation [6]. We hypothesized, therefore, that a core protein portion might be required for the transfer of the first *N*-acetylgalactosamine residue to the linkage tetrasaccharide in a β 1,4-linkage.

In the present study, α -TM was used as an acceptor substrate for *N*-acetylgalactosamine transfer reactions in order to exploit the following possibilities: (1) the linkage tetrasaccharide on the core protein of α -TM but not the tetrasaccharide–serine serves as an acceptor for the first *N*-acetylgalactosamine transfer reaction; (2) the GalNAcT involved in the transfer of the first *N*-acetylgalactosamine residue to the tetrasaccharide linkage is the same as that involved in chondroitin polymerization; and (3) the transfer of the first *N*-acetylgalactosamine residue is the critical rate-limiting step for subsequent polymerization reactions.

EXPERIMENTAL

Materials

UDP-*N*-[³H]acetylgalactosamine (10 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Unlabelled

Abbreviations used: CS, chondroitin sulphate; GAG, glycosaminoglycan; α -TM, α -thrombomodulin; Δ HexA, 4-deoxy- α -threo-hex-4-enepyranosyluronic acid; GalNAcT, *N*-acetylgalactosaminyltransferase.

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UDP-*N*-acetylgalactosamine and UDP-glucuronic acid were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). The following tetra-, penta-, and hexa-saccharide-serines and a tetrasaccharide-peptide were chemically synthesized [7-9]: GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-*O*-Ser; GalNAc β 1-4GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-*O*-Ser; GlcA β 1-3GalNAc β 1-4GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-*O*-Ser; and GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-*O*-(Gly)Ser-(Gly-Glu). The purity of each of these compounds was confirmed by ^1H -NMR spectroscopy [7-9]. Purified α -TM [10] was provided by the Research Institute (Dai-ichi Pharmaceutical Co., Tokyo, Japan), and contained a linkage tetrasaccharide GlcA β 1-3Gal β 1-3Gal β 1-4Xyl [1]. Chondroitin ABC lyase (EC 4.2.2.4) and chondroitin AC lyase (EC 4.2.2.5), *Acremonium* sp. α -*N*-acetylgalactosaminidase and polymeric chondroitin were purchased from Seikagaku Corp. (Tokyo, Japan). Chondro-glycuronidase from *Flavobacterium heparinum* [11] was a gift from Dr. K. Yoshida (Seikagaku Corp., Tokyo, Japan). Even-numbered chondro-oligosaccharides (GlcA β 1-3GalNAc) $_{2-7}$ were prepared by hyaluronidase digestion of chondroitin, followed by gel filtration and anion-exchange HPLC as described previously [6]. 4-Deoxy- α -threo-hex-4-enepyranosyluronic acid (ΔHexA) α 1-3GalNAc β 1-4GlcA β 1-3Gal β 1-3Gal β 1-4Xyl was isolated by HPLC after digestion of whale cartilage CS-A with chondroitin ABC lyase followed by treatment with mild alkali (0.5 M LiOH), and was structurally identified by 500-MHz ^1H -NMR spectroscopy (M. Watanabe and K. Sugahara, unpublished work). It was then derivatized with a fluorophore, 2-aminobenzamide, and purified by HPLC as described previously [1]. GalNAc β 1-4GlcA β 1-3Gal β 1-3Gal β 1-4Xyl-2-aminobenzamide was prepared by the chondro-glycuronidase digestion of ΔHexA α 1-3GalNAc β 1-4GlcA β 1-3Gal β 1-3Gal β 1-4Xyl-2-aminobenzamide. Prepacked columns of HiTrap heparin (5 ml) and Superdex 30 or 75 (HiLoad 16/60, preparative grade) were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

Preparation of a cell-free chondroitin-synthesizing enzyme system from cultures of a human melanoma cell line (G361)

Human melanoma cells, G361 (A.T.C.C. CRL-1424), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-bovine serum. When the cells were confluent, they were incubated in serum-free COSMEDIUM-001 (COSMO BIO Co., Tokyo, Japan) for 2 days. The spent medium was pooled and centrifuged at 10000 *g* for 10 min to remove cell debris. The culture medium (390 ml) containing enzyme activities of both β -GalNAcT (0.10 m-unit/l) and β -glucuronic acid transferase (0.05 m-unit/l), which can catalyse chondroitin chain elongation, was applied to a column (5 ml) of HiTrap heparin equilibrated with 10 mM Hepes/NaOH, pH 7.4/0.15 M NaCl/10% (v/v) glycerin (Buffer A), washed with 125 ml of the starting buffer and then eluted with 50 ml of Buffer A containing 0.5 M NaCl. The eluate had high activities of β -GalNAcT and β -glucuronic acid transferase but no α -*N*-acetylgalactosamin-transferase activities, as reported previously for the corresponding serum enzymes [3,6,12]. The fractions were pooled, concentrated and dialysed twice against 15 ml of Buffer A containing 0.15 M NaCl (Centriplus-50 concentrator; Amicon, Beverly, MA, U.S.A.). The specific activities of β -GalNAcT (59.6 m-units/g) and β -glucuronic acid transferase (20.6 m-units/g) in the preparation were 1168- and 212-fold higher, respectively, than those of the corresponding enzymes in fetal-calf serum [6,12] and these enzymes were used in the present study (the chondroitin-synthesizing soluble-enzyme system).

Glycosyltransferase reactions

Assays for GalNAcT and glucuronic acid transferase were carried out using chondroitin as an acceptor and UDP-*N*-acetylgalactosamine or UDP-glucuronic acid as a sugar donor, as described previously [6,12]. GalNAcT reactions using linkage glycoserines or α -TM as acceptors were conducted in incubation mixtures containing the following in a total volume of 20 μl : 1 nmol linkage oligosaccharide-serine or α -TM, 0.25 mM UDP-*N*-[^3H]acetylgalactosamine (5.28×10^5 d.p.m.) in the absence (transfer reactions for a single *N*-acetylgalactosamine residue) or presence (polymerization reactions) of 0.25 mM UDP-glucuronic acid, 100 mM Mes buffer, pH 6.5, 10 mM MnCl_2 , 1 mM ATP and 10 μl of the enzyme preparation. The mixtures were incubated at 37 °C overnight and the ^3H -labelled products were separated by gel-filtration chromatography on a Superdex 30 column (1.6-cm $\times$ 60-cm) equilibrated and eluted with 0.2 M NH_4HCO_3 . Fractions (1 ml) were collected at a rate of 1 ml/min, and radioactivity was estimated by liquid scintillation spectroscopy.

Substrate competition experiments

Competition experiments for the β -GalNAcT between α -TM and the chondro-hexasaccharide (GlcA β 1-3GalNAc) $_3$ were performed at 37 °C for 2 h in the reaction mixture described above, except that the specific radioactivity of UDP-*N*-[^3H]acetylgalactosamine was 10 times greater. The mixtures contained 1 nmol of α -TM and various concentrations of (GlcA β 1-3GalNAc) $_3$. The radioactive products were analysed by gel filtration on Superdex 30, as described above.

Characterization of reaction products

Products of the polymerization reactions on α -TM were isolated by gel filtration on a Superdex 30 column (1.6-cm $\times$ 60-cm) with 0.2 M NH_4HCO_3 as the eluent. The *N*-[^3H]acetylgalactosamine-labelled oligosaccharide chains released from α -TM by mild alkaline treatment with 0.5 M LiOH were derivatized with 2-aminobenzamide [1] and then exhaustively digested with chondroitin ABC lyase (50 m-units for 1 h, as described previously [13]). The digest was subjected to gel filtration on Superdex 30 as described above. The radioactive peak of the 2-aminobenzamide-derivatized putative linkage hexasaccharide and its digest with chondro-glycuronidase were analysed by anion-exchange HPLC on an amine-bound silica column as described previously [14]. Identification of the reaction products was accomplished by co-chromatography with an authentic linkage hexa- and penta-saccharide labelled with 2-aminobenzamide.

RESULTS

Demonstration of the indispensable role of the core protein in the first β -*N*-acetylgalactosamine transfer to the tetrasaccharide-linkage region

Three tetrasaccharide linkage derivatives including GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-*O*-Ser, GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-(Gly)-*O*-Ser-(Gly-Glu) and α -TM bearing the tetrasaccharide GlcA β 1-3Gal β 1-3Gal β 1-4Xyl, were tested as acceptors for the first β -*N*-acetylgalactosamine transfer reaction using the chondroitin-synthesizing enzyme system as described in the Experimental section. The first two substrates showed no acceptor activity at all, as reported for serum chondroitin β -GalNAcT [6]. In sharp contrast, α -TM served as an acceptor. The reaction

Figure 1 Enzymic characterization of the anomeric configuration of *N*-acetylgalactosamine transfer reaction products obtained with α -TM as acceptor

The *N*-acetylgalactosamine transfer reaction was carried out using α -TM as an acceptor, and ^3H -labelled reaction products were isolated by gel filtration as described in the Experimental section. The isolated products were chromatographed on a Superdex 30 column before (○) and after (●) chondroitin AC lyase digestion as described previously [13]. Effluent fractions were analysed for radioactivity. The arrowhead indicates the elution position of free *N*-acetylgalactosamine. Vo, void volume; Vt, total volume.

products were treated with chondroitin AC lyase or α -*N*-acetylgalactosaminidase, and each digest was analysed by gel filtration on a Superdex 30 column. *N*-[^3H]Acetylgalactosamine transferred to α -TM was released as free *N*-[^3H]acetylgalactosamine by chondroitin AC lyase (Figure 1) but not by α -*N*-acetylgalactosaminidase (results not shown), indicating that *N*-[^3H]acetylgalactosamine had been β 1,4-linked to the non-reducing terminal glucuronic acid residue of the linkage tetrasaccharide on α -TM. These findings suggested that the core protein portion was an absolute requirement for the first *N*-acetylgalactosamine transfer through a β 1,4 linkage to the linkage tetrasaccharide.

Figure 2 Substrate competition experiments of *N*-acetylgalactosamine transfer reactions using a chondro-hexasaccharide as substrate and α -TM as an inhibitor

Lineweaver-Burk plots of chondro-hexasaccharide ($\text{GlcA}\beta 1\text{--}3\text{GalNAc}$)₃ with the chondroitin-synthesizing enzyme system in the presence (○) or absence (●) of α -TM (1 nmol) as described in the Experimental section.

Substrate competition experiments

Whether a common β -GalNAcT activity catalysed the transfer of the first *N*-acetylgalactosamine residue and the *N*-acetylgalactosamine residues subsequently added to the repeating disaccharide region of a growing chondroitin chain was investigated by conducting substrate-competition experiments using the chondro-hexasaccharide ($\text{GlcA}\beta 1\text{--}3\text{GalNAc}$)₃ as an acceptor and α -TM as an inhibitor. The Lineweaver-Burk plots showed competitive inhibition of the transfer of *N*-acetylgalactosamine to the chondro-hexasaccharide by α -TM (Figure 2). These findings implied that a common β -GalNAcT catalysed the transfer of *N*-acetylgalactosamine residues to both the tetrasaccharide linkage region and the disaccharide repeating region, which represented chain initiation and elongation respectively.

Demonstration of the first *N*-acetylgalactosamine transfer as a critical determining step for subsequent chondroitin polymerization

To investigate whether the addition of the first *N*-acetylgalactosamine residue was a commitment to subsequent polymerization reactions, the enzyme system containing chondroitin glucuronic acid transferase activity was used, as described in the Experimental section. Incubations of various acceptors with the enzyme system in the presence of UDP-*N*-[^3H]acetylgalactosamine and UDP-glucuronic acid yielded radioactively labelled polymerized chondroitin chains (Figure 3). The acceptors used were α -TM and the chemically synthesized penta- and hexa-saccharide-serines: $\text{GalNAc}\beta 1\text{--}4\text{GlcA}\beta 1\text{--}3\text{Gal}\beta 1\text{--}3\text{Gal}\beta 1\text{--}4\text{Xyl}\beta\text{--}O\text{--Ser}$ and $\text{GlcA}\beta 1\text{--}3\text{GalNAc}\beta 1\text{--}4\text{GlcA}\beta 1\text{--}3\text{Gal}\beta 1\text{--}3\text{Gal}\beta 1\text{--}4\text{Xyl}\beta\text{--}O\text{--Ser}$, and 7.7 %, 3.1 %, and 16.7 % of the total radioactivity of UDP-*N*-[^3H]acetylgalactosamine were incorporated into 1 nmol each of these three acceptors respectively. In contrast, no chain elongation was observed when the tetrasaccharide-serine was used as an acceptor (results not shown). The reaction products obtained with α -TM as an acceptor were subjected to reductive β -elimination using $\text{NaBH}_4/\text{NaOH}$ [15], and the released radioactively labelled oligosaccharide chains were analysed by gel chromatography (Superdex 30 or Superdex 75) (Figure 3). The saccharide chains synthesized on α -TM were much longer than those synthesized on the penta- and hexa-saccharide-serines (Figure 3A), but shorter than those of commercial polymer chondroitin chains (Figure 3B). Hence, it is likely that once the first *N*-acetylgalactosamine residue had been transferred to the linkage tetrasaccharide, polymerization reactions occurred. It should be emphasized that the core protein seemed to be required for synthesis of longer chondroitin chains.

Analysis of the chondroitin chains newly polymerized on the tetrasaccharide linkage region of α -TM

N-[^3H]Acetylgalactosamine-labelled chondroitin chains obtained from the polymerization reactions on α -TM were liberated by mild alkaline treatment with 0.5 M LiOH. The resulting radioactively-labelled chondroitin chains were derivatized with 2-aminobenzamide and isolated by gel filtration [1]. The isolated chains were digested with chondroitin ABC lyase and subjected to gel chromatography; fraction 1 contained the linkage hexasaccharides and fraction 2 contained both di- and monosaccharides in a molar ratio of 1:10 (Figure 4A). These results indicated that the average size of the synthesized chondroitin chains was approx. 20-mer. Fraction 1 was subjected to anion-exchange HPLC, and only one ^3H -labelled peak was found at the elution position of the authentic linkage hexasaccharide, $\Delta\text{HexA}\alpha 1\text{--}3\text{GalNAc}\beta 1\text{--}4\text{GlcA}\beta 1\text{--}3\text{Gal}\beta 1\text{--}3\text{Gal}\beta 1\text{--}4\text{Xyl}\text{--}2$

Figure 3 Comparison of the sizes of chondroitin polymerization reaction products obtained with various acceptors containing different linkage oligosaccharides

(A) The linkage pentasaccharide-serine, GalNAc β 1-4GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-*O*-Ser (○); the linkage hexasaccharide-serine, GlcA β 1-3GalNAc β 1-4GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-*O*-Ser (●) and α -TM (x) were each tested as an acceptor for β -GalNAcT reactions as described in the Experimental section. N -[3 H]Acetylglactosamine-labelled products isolated from each incubation were separately chromatographed on a Superdex 30 column (1.6-cm $\times$ 60-cm). In the case of α -TM, N -[3 H]Acetylglactosamine-labelled products were first isolated by gel filtration, subjected to reductive β -elimination using NaBH₄/NaOH, and then re-chromatographed. Effluent fractions were analysed for radioactivity. Numbered arrowheads (14–6) indicate the elution positions of chondroitin-derived tetradecasaccharides to hexasaccharides [6] respectively. Total volume was at around fraction 120 (not shown). (B) The 3 H-labelled oligosaccharide chains obtained from α -TM in (A) were re-chromatographed on Superdex 75. Effluent fractions were monitored by 3 H-radioactivity (○). Chondroitin (mean molecular mass approx. 30 kDa) was also chromatographed and monitored by UV absorbance at 210 nm (●).

aminobenzamide, which was shifted to the position of GalNAc β 1-4GlcA β 1-3Gal β 1-3Gal β 1-4Xyl-2-aminobenzamide by chondro-glycuronidase digestion (Figure 4B). These results clearly indicated that polymerization did take place on the truncated linkage tetrasaccharide of α -TM.

DISCUSSION

The present study provided the first evidence for the involvement of the core protein in the first *N*-acetylglactosamine transfer reaction through a β 1,4 linkage to the linkage tetrasaccharide of proteoglycans, based upon the finding that a *N*-acetylglactosamine residue was transferred through a β 1,4 linkage to the linkage tetrasaccharide on α -TM, but not to the tetrasaccharide-serine or -peptide. Although the peptide portion

Figure 4 Analysis of the protein-linkage region oligosaccharides derived from *N*-[3 H]acetylglactosamine-labelled chondroitin chains newly synthesized on α -TM

(A) N -[3 H]Acetylglactosamine-labelled polymerization reaction products were isolated by gel filtration, treated with alkali and derivatized with 2-aminobenzamide as described in the Experimental section. The 3 H-labelled products derivatized with 2-aminobenzamide were digested with chondroitin ABC lyase and co-chromatographed with authentic Δ^4 HexA α 1-3GalNAc on Superdex 30. Fractions (1 ml) were collected and monitored for 3 H-radioactivity (●) and UV absorbance at 232 nm (broken line). (B) Radioactively labelled fractions containing the linkage oligosaccharide (fraction 1 in A) were pooled and analysed before (○) and after glycuronidase digestion (●) by anion-exchange HPLC on an amine-bound silica column using a linear gradient of 16–530 mM NaH₂PO₄ over a 60-min period. The eluates were monitored for 3 H-radioactivity. Arrows indicate the elution positions of the 2-aminobenzamide-derivatized authentic linkage penta- and hexa-saccharides: 1, GalNAc β 1-4GlcA β 1-3Gal β 1-3Gal β 1-4Xyl-2AB; 2, Δ HexA α 1-3GalNAc β 1-4GlcA β 1-3Gal β 1-3Gal β 1-4Xyl-2AB.

of the tetrasaccharide-peptide GlcA β 1-3Gal β 1-3Gal β 1-4Xyl-(Gly)Ser-(Gly-Glu) contained the proposed xylosylation consensus sequence in α -TM (see [16] for a review), it seemed to be insufficient for the transfer of the first *N*-acetylglactosamine residue through a β 1,4 linkage. The core protein seems to be recognized by the putative β -GalNAcT. In this regard, it should be noted that animal cells utilize β -D-xylosides as primers for glycosaminoglycan (GAG) synthesis, and thus the core protein can be substituted by the mildly hydrophobic xylosides, such as 4-methylumbelliferyl- or *p*-nitrophenyl- β -D-xyloside [17]. In addition, Fernández and Warren [18] demonstrated CS synthesis using reconstituted Golgi in the presence of 4-methylumbelliferyl- β -D-xyloside. In fact, an *N*-acetylglactosamine residue was transferred through a β 1,4 linkage to GlcA β 1-3Gal β 1-*O*-naphthalenemethanol using the chondroitin-synthesizing enzyme

system described in the present paper, despite the relatively lower efficiency in comparison with α -TM (results not shown). Therefore it appears that hydrophobic aglycones mimic a determinant on core proteins and interact with β -GalNAcT. Further work should be aimed at better defining the acceptor recognition requirements of the β -GalNAcT.

On the basis of the present findings that *N*-acetylgalactosamine transfer to the chondro-hexasaccharide (GlcA β 1-3GalNAc)₃ was competitively inhibited by the linkage tetrasaccharide on α -TM (see Figure 2), it is likely that a common β -GalNAcT catalysed the transfer of the first *N*-acetylgalactosamine residue to the linkage tetrasaccharide and the subsequent *N*-acetylgalactosamine residues to the growing CS chains, although further purification and/or molecular cloning of the β -GalNAcT is required before a definitive conclusion can be drawn. These findings are in contrast with those of Rohrmann et al. [2], who reported that the alleged GalNAcT-I which catalysed the addition of an *N*-acetylgalactosamine to the linkage trisaccharide GlcA β 1-3Gal β 1-3Gal was different from the GalNAcT-II that was involved in the polymerization of a CS chain. This conclusion was based mainly on the differential thermostability of the two activities. However, considering the similarity in the terminal structures of the linkage region (GlcA β 1-3Gal β 1-) and the CS disaccharide repeating region (GlcA β 1-3GalNAc β 1-), there is a possibility that a single GalNAcT catalyses the transfer of *N*-acetylgalactosamine to both the linkage region and the repeating region. Although the existence of GalNAcT-I cannot be excluded, it may not be required for CS biosynthesis. In this context, the α -*N*-acetylgalactosaminyltransferase recently discovered should be reconsidered [3,4]. The enzyme transfers *N*-acetylgalactosamine to the tetrasaccharide-serine and -peptide exclusively through an α -linkage [3,4] but not to α -TM (H. Shimakawa, H. Kitagawa and K. Sugahara, unpublished work). In terms of thermostability and substrate specificity, α -*N*-acetylgalactosaminyltransferase is very similar to GalNAcT-I, despite their allegedly different linkage specificities [3]. It has been reported that GalNAcT-I catalysed *N*-acetylgalactosamine transfer to linkage oligosaccharides including GlcA β 1-3Gal, and α -*N*-acetylgalactosaminyltransferase transferred α -*N*-acetylgalactosamine to GlcA β 1-3GalNAc and GlcA β 1-3Gal β 1-*O*-naphthalenemethanol (H. Shimakawa, H. Kitagawa and K. Sugahara, unpublished work), in addition to the linkage oligosaccharide-serines [3]. Hence, GalNAcT-I can be proposed to be identical with α -*N*-acetylgalactosaminyltransferase. The properties of the partially purified β -*N*-acetylhexosaminidase preparation used by Rohrmann et al. [2] for identification of the GalNAcT-I reaction product were not described in detail and had not been checked for α -*N*-acetylgalactosaminidase activity. The anomeric configuration of the reaction products obtained with the GalNAcT-I preparation [2] should be re-examined in light of the presence of α -*N*-acetylgalactosaminyltransferase.

Interestingly, the CS chain polymerization reactions were also influenced by the core protein portion. The oligosaccharide chains synthesized on α -TM were much longer than those synthesized on the penta- and hexa-saccharide-serines (see Figure 3). In this regard, Oldberg et al. [19] reported that cells transformed with vectors encoding deletion variants of decorin synthesized proteoglycan with shorter GAG chains and suggested that the effect on the GAG chain length might be due to a lower affinity between the core protein and the glycosyltransferases. Thus in view of the present findings that the *N*-acetylgalactosamine transfer reactions catalysed by β -GalNAcT were

markedly stimulated by the core protein of the acceptor, it is possible that β -GalNAcT interacts with the core protein and regulates the GAG chain length. Furthermore, the penta- and hexa-saccharide-serines, but not the tetrasaccharide-serine, were utilized as an acceptor for the chain polymerization reactions although the chain length of chondroitin synthesized on the glycoserines was relatively shorter than that on α -TM (Figure 3), suggesting that the trigger for the chain-polymerization reaction was the transfer of the first *N*-acetylgalactosamine residue. In this context, we recently demonstrated the immature truncated GAG tetrasaccharide GlcA β 1-3Gal β 1-3Gal β 1-4Xyl on recombinant human α -TM, which is a so-called part-time proteoglycan, and we suggested that the fifth hexosamine transfer process played a critical role in the regulation of GAG biosynthesis [1]. Based on the present findings that the linkage tetrasaccharide of α -TM was a good acceptor substrate for β -GalNAcT, and that chondroitin polymerization occurred efficiently on α -TM, the first *N*-acetylgalactosamine transfer is indeed the rate-limiting step which determines whether chain elongation proceeds; the factor determining the generation of part-time proteoglycans seems to involve the level of β -GalNAcT activity expressed in a cell. Therefore it is likely that other naturally occurring proteoglycans, which are produced by cells with low β -GalNAcT activity relative to the synthesizing capacity of the core protein, have some immature GAG tetrasaccharides. In the future, if β -GalNAcT cDNA becomes available it will be of interest to use it to investigate how the expression of β -GalNAcT is controlled.

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