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β1,3-*N*-Acetylglucosaminyltransferase-7 (β3Gn-T7) acts efficiently on keratan sulfate-related glycans

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Abstract β1,3-*N*-Acetylglucosaminyltransferase-7 (β3Gn-T7) has been identified as an anti-migration factor for a lung cancer cell line but its enzymatic activity has not yet been characterized. Here we show that β3Gn-T7 efficiently acts on keratan sulfate-related glycans including Galβ1→4(SO_3^-→6)GlcNAcβ1→3Galβ1→4(SO_3^-→6)GlcNAc (L2L2), while lacto-*N*-tetraose and lacto-*N*-*neo*-tetraose were poor substrates. Moreover, we found that among the other five β3Gn-Ts and i antigen-producing β3Gn-T (iGn-T), β3Gn-T2 and iGn-T act well on L2L2, although these specific activities were lower than those for a tetraantennary *N*-glycan. These results indicate that β3Gn-T7 is the one that most efficiently elongates L2L2 and may be involved in the biosynthesis of keratan sulfate.

Key words: β1,3-*N*-Acetylglucosaminyltransferase; Keratan sulfate; Sulfated glycan; *N*-linked glycan

1. Introduction

β1,3-*N*-Acetylglucosaminyltransferase (β3Gn-T) catalyzes the transfer of a GlcNAc residue from UDP-GlcNAc to the C-3 position of non-reducing terminal Gal/GalNAc residues by β-linkage. To date, six β3Gn-Ts (-T2 to -T7) and the i antigen-producing β3Gn-T (iGn-T) have been cloned and in most cases, their substrate specificities have been extensively studied [1–6]. β3Gn-T2 and iGn-T have strong poly-*N*-acetyllactosamine-elongating activities [1,2], while β3Gn-T3 has a core 1 extension activity that leads to the synthesis of a 6-sulfosialyl Lewis X or 6-sulfo-*N*-acetyllactosamine antigen, MECA79, in lymph nodes [3]. β3Gn-T5 efficiently acts on lactosylceramide and plays a role in synthesizing lacto-series glycolipids [4], and β3Gn-T6 synthesizes the core 3 structure, GlcNAcβ1→3GalNAcα1→, which is one of the core units in *O*-linked glycans [5]. In contrast, the nature of the enzymatic activity of β3Gn-T7 is unclear. Kataoka and Huh have shown that transfection of lung cancer cells with an antisense oligonucleotide for β3Gn-T7 enhances the motility of the cells, which suggests that β3Gn-T7 may be involved in cancer cell migration and invasion [6]. Moreover, these observations imply that glycoconjugates synthesized by β3Gn-T7 may directly regulate cancer cell motility, invasion, and metastasis. Therefore, it is important to characterize the enzymatic activity and substrate specificity of β3Gn-T7.

Keratan sulfate is a linear polymer of *N*-acetyllactosamine, →3Galβ1→4GlcNAcβ1→, that bears a sulfate at the C-6 position of both monosaccharide moieties. Keratan sulfate is present in the brain, the cornea, and cartilage, where it is covalently conjugated to various proteins in the *O*- and *N*-linked forms (reviewed in [7]). In the cornea, keratan sulfate is linked to small leucine-rich core proteins, lumican, keratokan, and mimecan, and contributes to maintaining the hydration and transparency of the cornea. Akama et al. showed that GlcNAc6ST-5 (hCGn6ST or GST4β) bears GlcNAc 6-*O*-sulfotransferase activity and can synthesize keratan sulfate, and that several types of mutations in its gene cause macular corneal dystrophy, an autosomal recessive disorder that is characterized by corneal opacification [8–10]. Keratan sulfate is proposed to be produced by the following sequential reactions: 6-*O*-sulfation of the non-reducing terminal GlcNAc by GlcNAc6ST-5 [10], β1,4-galactosylation of the GlcNAc 6-*O*-sulfate by β4GalT-IV as shown by us [11], β1,3-*N*-acetylglucosaminylation of the Gal by β3Gn-T, and then 6-*O*-sulfation of internal Gal residues by Gal6ST(KS6ST) [12,13]. However, it remains unclear which of the β3Gn-Ts are involved in the elongation of keratan sulfate.

In this study, we characterized the enzymatic activity of β3Gn-T7 and found that β3Gn-T7 acts efficiently on Galβ1→4(SO_3^-→6)GlcNAcβ1→3Galβ1→4(SO_3^-→6)GlcNAc (L2L2) and Galβ1→4(SO_3^-→6)GlcNAcβ1→3-(SO_3^-→6)Galβ1→4(SO_3^-→6)GlcNAc (L2L4), both of which correspond to a partial structure of keratan sulfate. This suggests that β3Gn-T7 is associated with keratan sulfate biosynthesis.

2. Materials and methods

2.1. Materials

UDP-[6-^{3}H]GlcNAc (2.21 TBq/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). GalNAcα1-*O*-*p*-nitrophenyl (pNP) and Fucα1→3(Galβ1→4)GlcNAcβ1→3Galβ1→4Glc (lacto-*N*-fucopentaose-III, LNF-III) were purchased from Funakoshi (Tokyo, Japan). Bovine milk β1,4-galactosyltransferase, UDP-Gal, and UDP-GlcNAc were purchased from Sigma (St. Louis, MO, USA). Galβ1→3GlcNAcβ1→3Galβ1→4Glc (lacto-*N*-tetraose, abbreviated to LNT) and Galβ1→4GlcNAcβ1→3Galβ1→4Glc(lacto-*N*-*neo*-tetraose, abbreviated to LNnT) were prepared from human milk [14]. *Ricinus communis* agglutinin-I (RCA-I)-agarose (4 mg/ml

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Abbreviations: β3Gn-T, β1,3-*N*-acetylglucosaminyltransferase; pNP, *p*-nitrophenyl; PVL, *Psathyrella velutina* lectin; RCA-I, *Ricinus communis* agglutinin-I

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gel) was obtained from Hohnen Oil (Tokyo, Japan). *Psathyrella velutina* lectin (PVL) was prepared according to the method described by Kochibe and Matta [15], and the lectin was conjugated to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Galβ1→4GlcNAcβ1→2Manα1→3(Galβ1→4GlcNAcβ1→2Manα1→6)Manβ1→4GlcNAc (biGP) and Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→4)Manα1→3[Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→6)Manα1→6]Manβ1→4GlcNAc (tetraGP) were obtained from the urine of G_{M1} gangliosidosis patients [16]. *Streptococcus* 6646K β-galactosidase and keratanase II (*Bacillus* sp.) were purchased from Seikagaku (Tokyo, Japan). L2L2, L2L4, and SO_3^-→6Galβ1→4(SO_3^-→6)GlcNAcβ1→3(SO_3^-→6)Galβ1→4(SO_3^-→6)GlcNAc (L4L4) were kindly donated by Seikagaku. Authentic [^{3}H]GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc was prepared using the crude membrane of β3Gn-T2-transfected COS-7 cells described below.

2.2. cDNA cloning of β3Gn-Ts

The cDNAs encoding the full open reading frames of β3Gn-T3, -T6, and -T7 were amplified by polymerase chain reaction from BD Marathon-Ready human colon cDNA (Clontech, Palo Alto, CA, USA). In the case of β3Gn-T2, -T4, -T5, and iGn-T, the SuperScript human brain cDNA library (Invitrogen) was used as the template. The oligonucleotide primers used were 5′-tttaagcttGAGAAATGAGTGTTGGA-3′ (forward primer for β3Gn-T2), 5′-ttttctagaACACAACATGGGAAC-3′ (reverse primer for β3Gn-T2), 5′-tttaagcttGGCCAGGATGAAGTATC-3′ (forward primer for β3Gn-T3), 5′-ttttctagaGGAAGGTGTCGCCC-3′ (reverse primer for β3Gn-T3), 5′-tttaagcttCCAGCTGCCGTTCATG-3′ (forward primer for β3Gn-T4), 5′-ttttctagaGTCCACTCTCAGGCT-3′ (reverse primer for β3Gn-T4), 5′-tttaagcttGAGTGGATATGAGA-3′ (forward primer for β3Gn-T5), 5′-ttttctagaGGTTTGACTCAGTGACA-3′ (reverse primer for β3Gn-T5), 5′-tttaagcttATGGCTTTTCCCTGCCG-3′ (forward primer for β3Gn-T6), 5′-ttttctagaCCTCAGGAGACCCGGTG-3′ (reverse primer for β3Gn-T6), 5′-tttaagcttCCGCCATGTCGCTGTG-3′ (forward primer for β3Gn-T7), 5′-ttttctagaGCACCTGGAGCT-3′ (reverse primer for β3Gn-T7), 5′-tttaagcttGGCGTAGTCTGTGGCC-3′ (forward primer for iGn-T), and 5′-ttttctagaGCCAAGAGGCTGACT-3′ (reverse primer for iGn-T). The sequences shown by lowercase letters reveal appropriate restriction sites. The amplified cDNAs were digested with *Hin*dIII and *Xba*I and cloned into pcDNA3 (Invitrogen). The resulting plasmids were sequenced using an Applied Biosystems Prism 310 Genetic Analyzer (PE Biosystems). The nucleic acid sequences of cloned cDNAs for β3Gn-T2, -T3, -T4, -T5, -T6, and -T7, and iGn-T were equal to those for the accession numbers AB049584, AB049585, AB049586, AB045278, AB073740, AF502430, and AF029893, respectively. The transient expression of the β3Gn-Ts in COS-7 cells and the preparation of their crude membrane fractions were performed as described previously [17]. The crude membrane fractions were used to examine the enzymatic activities.

2.3. Assay of β3Gn-T activity

Twenty μl of reaction mixtures consisting of 50 mM HEPES-NaOH (pH 7.2), 10 mM $MnCl_2$, 0.5% (w/v) Triton X-100, 1 mM acceptor substrate, 2.5 μM UDP-[^{3}H]GlcNAc (6.7×10^6 dpm), 30 μM UDP-GlcNAc, 0.1 M GlcNAc, 1 mM AMP, and appropriately diluted membrane fractions were incubated at 37°C for 3 h. The ^{3}H-labeled products were purified by paper electrophoresis (pyridine:acetic acid:water, 3:1:387, pH 5.4) and then paper chromatography (pyridine:ethyl acetate:acetic acid:water, 5:5:1:3). In the case of L2L4 and L4L4, mild acid hydrolysis (0.01 N HCl, 100°C, 10 min) was performed to destroy residual UDP-[^{3}H]GlcNAc before paper electrophoresis since [^{3}H]GlcNAc→L2L4 and →L4L4 cannot be separated from UDP-[^{3}H]GlcNAc by paper electrophoresis. After drying, the paper was monitored for radioactivity with a radiochromatogram scanner and the ^{3}H-labeled products were extracted with water and counted. In the case of GalNAcα-*O*-pNP and Lac-*O*-pNP, the extracts were further applied onto a C18 Sep-Pak column (Waters, Milford, MA, USA) and the ^{3}H-labeled products were eluted with 50% MeOH and counted. The membrane fraction derived from empty pcDNA3-transfected COS-7 cells was used to control for intrinsic β3Gn-T activities. The protein concentrations of the membrane fractions were estimated using the Bio-Rad Protein Assay dye reagent with bovine serum albumin as a standard.

3. Results and discussion

3.1. Characterization of the ^{3}H-labeled compound produced by β3Gn-T7

We investigated the enzymatic activity of β3Gn-T7 by using the crude membrane fraction derived from β3Gn-T7-transfected COS-7 cells. When the reaction mixture containing L2L2 as the acceptor substrate was subjected to paper electrophoresis, a radioactive peak (fraction I) appeared (Fig. 1A). Other peaks (0, 6.6, and 9.8 cm) were also present when the reaction had been performed without L2L2 (data not shown) and thus are not L2L2-derived compounds. Fraction I was extracted with water and subjected to linkage analysis of [^{3}H]GlcNAc. After digesting fraction I with keratanase II, an endo-β-*N*-acetylglucosaminidase [18] that acts on the 6-*O*-sulfated GlcNAc in keratan sulfate, a radioactive peak appeared at a less quickly migrating position (Fig. 1B), which indicates that the [^{3}H]GlcNAc in fraction I was indeed incorporated into L2L2. However, fraction I was refractory to *Streptococcus* 6646K β-galactosidase digestion. Furthermore, fraction I could bind to a PVL-Sepharose column and was eluted with 0.3 M GlcNAc, while in contrast it flowed through an RCA-I-agarose column. Since PVL and RCA-I bind to the non-reducing terminal β-GlcNAc and Galβ1→4(R→6)GlcNAcβ1→, respectively [15,19], it appears that the [^{3}H]GlcNAc is attached to the distal Gal residue of L2L2. Fraction I was then galactosylated by bovine milk β1,4-galactosyltransferase. The product bound to a RCA-I-agarose column but not to a PVL column, which indicates that the

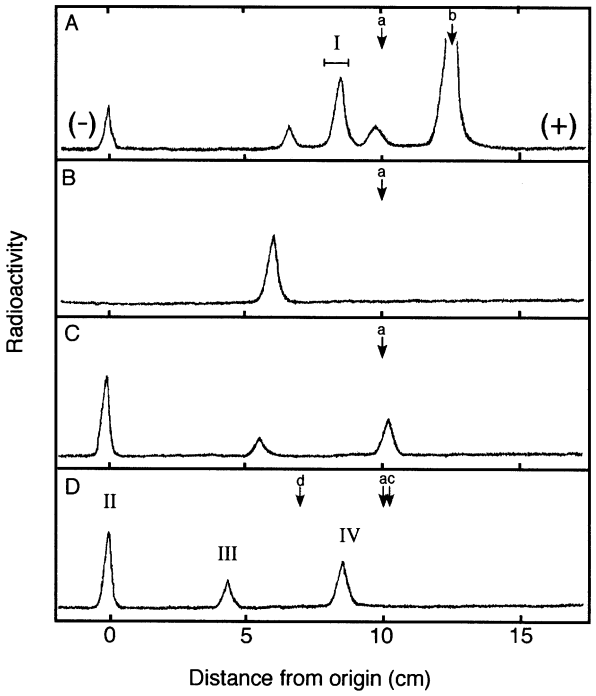

Fig. 1. Analysis of the [^{3}H]GlcNAc linkages of [^{3}H]GlcNAc→L2L2 synthesized by human β3Gn-T7 by paper electrophoresis at pH 5.4. A: The mixture after the enzymatic reaction. B: *Bacillus* sp. keratanase II digests of fraction I. C: The Smith degradation product of [^{3}H]L2L2 which was provided previously [11]. D: The periodate oxidation and the Smith degradation product of Galβ1→4[^{3}H]GlcNAc→L2L2. Arrows a–d indicate the positions of bromophenol blue, UDP-[^{3}H]GlcNAc, [^{3}H]L2L2, and Galβ1→4[^{3}H]GlcNAc→L2L2, respectively.

Table 1
Substrate specificity of β3Gn-T7

Substrate[a]	Relative activity (%)
Galβ1→4(SO_3^-→6)GlcNAcβ1→3Galβ1→4(SO_3^-→6)GlcNAc (L2L2)	100[b]
Galβ1→4(SO_3^-→6)GlcNAcβ1→3(SO_3^-→6)Galβ1→4(SO_3^-→6)GlcNAc (L2L4)	72
SO_3^-→6Galβ1→4(SO_3^-→6)GlcNAcβ1→3(SO_3^-→6)Galβ1→4(SO_3^-→6)GlcNAc (L4L4)	3
Gal_4·$GlcNAc_4$·Man_3·GlcNAc (tetraGP)	40
Gal_2·$GlcNAc_2$·Man_3·GlcNAc (biGP)	10
Galβ1→4GlcNAcβ1→3Galβ1→4Glc (LNnT)	5
Galβ1→3GlcNAcβ1→3Galβ1→4Glc (LNT)	<1
GalNAcα-*O*-pNP	<1
Galβ1→4Glcβ1-*O*-pNP (Lac-*O*-pNP)	3
Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4Glc (LNF-III)	<1

[a]The concentrations of the substrates were 1 mM.
[b]The relative values are taken from the value of L2L2 as 100. The enzymatic activities were the means of four independent experiments (S.D., less than 5%).

C-4 position of the [^{3}H]GlcNAc was completely β-galactosylated. The galactosylated fraction I was subjected to periodate oxidation and Smith degradation [17,20]. If [^{3}H]GlcNAc binds to the C-3 position of the distal Gal, [^{3}H]GlcNAcβ1→3Galβ1→4(SO_3^-→6)GlcNAcβ1→3Galβ1→4(SO_3^-→6)GlcNAc-ol and its partially desulfated derivatives should be produced by the treatment. The partial 6-*O*-desulfation caused by Smith degradation (0.05 N H_2SO_4, 80°C, 1 h) was confirmed by using [^{3}H]L2L2 [11] (Fig. 1C). In addition to a peak for [^{3}H]L2L2 (10.3 cm), two peaks for mono-sulfated (5.5 cm) and non-sulfated (origin) [^{3}H]L2L2 appeared. On the other hand, if [^{3}H]GlcNAc binds to the C-2, C-4, or C-6 position of the distal Gal, [^{3}H]GlcNAcβ1-*O*-glyceraldehyde (C-2), [^{3}H]GlcNAcβ1-*O*-glycerol (C-6), or [^{3}H]GlcNAcβ1-*O*-threitol (C-4) should be produced. After the oxidation and Smith degradation, the reaction mixture was subjected to paper electrophoresis (Fig. 1D). Three radioactive peaks (II, III, and IV) were detected. Peak IV migrated to the same position as fraction I on paper electrophoresis. This position was between [^{3}H]L2L2 (di-sulfated tetraose) (arrow c) and Galβ1→4[^{3}H]GlcNAc→L2L2 (di-sulfated hexaose) (arrow d), suggesting that peak IV consisted of pentaose with two sulfate residues. Peak IV migrated on silica gel thin layer chromatography (pyridine:ethyl acetate:acetic acid:water, 5:5:1:3) to the same position (R_f value, 0.20) as [^{3}H]GlcNAc→L2L2-ol which was prepared by $NaBH_4$ reduction of fraction I. These results indicated that peak IV is [^{3}H]GlcNAcβ1→3Galβ1→4(SO_3^-→6)GlcNAcβ1→3Galβ1→4(SO_3^-→6)GlcNAc-ol. When peak II was applied to thin layer chromatography, it moved to the same position as authentic [^{3}H]GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc, which indicates that it is a non-sulfated form. The migration position of peak III suggests it is the mono-sulfated form. These results reveal that [^{3}H]GlcNAc was attached to the C-3 position of the distal Gal of L2L2 and that β3Gn-T7 indeed has a β1,3-*N*-acetylglucosaminyltransferase activity. The specific activities of the crude membrane derived from β3Gn-T7-transfected COS-7 cells and empty-pcDNA3-transfected COS-7 cells were 4.87 and 1.08 pmol/min/mg protein, respectively. We estimated the specific activity of exogenous β3Gn-T7 as 3.79 pmol/min/mg protein by subtraction.

3.2. Enzymological properties of β3Gn-T7

The optimal pH of β3Gn-T7 was at 6.5–7.0. The addition of 10 mM EDTA to the reaction mixture instead of $MnCl_2$ decreased the enzymatic activity to 12%. Addition of $MgCl_2$ and $CaCl_2$ (10 mM) resulted in one-third of the activity that was observed in the presence of $MnCl_2$.

The substrate specificity of β3Gn-T7 is shown in Table 1. The keratan sulfate-related sulfated glycans L2L2 and L2L4 were much better substrates than LNnT, which indicates that β3Gn-T7 recognizes internal 6-*O*-sulfated *N*-acetylglucosamine moieties. However, 6-*O*-sulfation of the non-reducing terminal Gal hampered the enzymatic activity since L4L4 was a poor substrate, in contrast to L2L2 and L2L4. Moreover, β3Gn-T7 can act on tetraGP.

The kinetic parameters for L2L2, L2L4, and tetraGP are summarized in Table 2. The V_{max}/K_m values for L2L2 and L2L4 were higher than that for tetraGP, which indicates that β3Gn-T7 acts more efficiently on L2L2 and L2L4 than on tetraGP.

3.3. Substrate specificities of the seven β3Gn-Ts

Next, to assess whether L2L2 and L2L4 are good acceptors for other β3Gn-Ts as well, we transfected COS-7 cells with expression vectors containing cDNAs encoding each β3Gn-T and prepared crude membrane fractions, which served as the enzyme sources. As shown in Table 3, β3Gn-T2 and iGn-T act on L2L2 but L2L4 and L4L4 are not substrates for β3Gn-Ts other than β3Gn-T7. TetraGP was a good substrate for all the β3Gn-Ts, and β3Gn-T2 and iGn-T preferred tetraGP to L2L2, unlike the case of β3Gn-T7. These observations indicate that β3Gn-T7 is the most efficient enzyme for L2L2 and L2L4 of the β3Gn-Ts that have been identified to date.

3.4. General discussion

Based on these studies, we propose keratan sulfate is biosynthesized by the pathway shown in Fig. 2. First, GlcNAc6ST-5 transfers a sulfate to the C-6 position of the non-reducing terminal GlcNAc [10]. Next, galactosylation is performed by β4GalT-IV, which is the only β4GalT that is highly specific for GlcNAc 6-*O*-sulfate residues [11]. As shown in this study, Galβ1→4(SO_3^-→6)GlcNAc can be elongated by β3Gn-T7. Finally, internal Gal residues are 6-*O*-sulfated

Table 2
Kinetic analysis of β3Gn-T7 for several oligosaccharides

Substrate	L2L2	L2L4	TetraGP
K_m (mM)	0.36	0.10	0.23
V_{max} (pmol/min/mg protein)	16.7	6.5	2.9
V_{max}/K_m	46	65	13

Table 3
Substrate specificities of the seven β3Gn-Ts

Substrate[a]	β3Gn-T2 (pmol/min/mg protein)	β3Gn-T3 (pmol/min/mg protein)	β3Gn-T4 (pmol/min/mg protein)	β3Gn-T5 (pmol/min/mg protein)	β3Gn-T6 (pmol/min/mg protein)	β3Gn-T7 (pmol/min/mg protein)	iGn-T (pmol/min/mg protein)
L2L2	0.99	<0.1	<0.1	<0.1	<0.1	3.79	0.72
L2L4	<0.1	<0.1	<0.1	<0.1	<0.1	2.73	<0.1
L4L4	<0.1	<0.1	<0.1	<0.1	<0.1	0.11	<0.1
TetraGP	33.5	2.41	1.51	4.29	2.33	1.50	3.21
LNnT	4.93	0.19	0.22	0.70	ND[b]	0.19	0.28
Core 1-*O*-pNP	ND	0.72	ND	ND	ND	ND	ND
GalNAcα-*O*-pNP	ND	ND	ND	ND	0.17	<0.1	ND

The enzymatic activities shown are the means of four independent experiments (S.D. was less than 5%).
[a]The concentrations of the substrates were 1 mM.
[b]Not determined.

by Gal6ST [12,13]. It may thus be possible to synthesize keratan sulfate in vitro by using an enzyme mixture composed of GlcNAc6ST-5, β4GalT-IV, β3Gn-T7, and Gal6ST and a substrate mixture that includes an appropriate initiator acceptor glycan. Such in vitro synthesis of keratan sulfate is currently in progress.

Kataoka and Huh have shown that lung cancer KLN205-MUC1 cells become more migratory and invasive when they are transfected with the antisense oligonucleotide for β3Gn-T7 [6]. These observations suggest that glycoconjugates synthesized by β3Gn-T7 may serve to diminish the motility of the cancer cells. Our observations suggest that a probable candidate glycoconjugate may be keratan sulfate. Supporting this is that it has been shown that the expression of keratan sulfate is down-regulated in migrating corneal endothelial cells [21] and in fibroblasts that have been transdifferentiated from stromal keratocytes [22], as compared to the respective quiescent cells. Moreover, exogenous keratan sulfate inhibits matrix metalloproteinase-2 activation in corneal and skin explant cultures [23]. It is thus interesting to determine whether KLN205-MUC1 cells express keratan sulfate and whether this compound functions as an anti-migration factor.

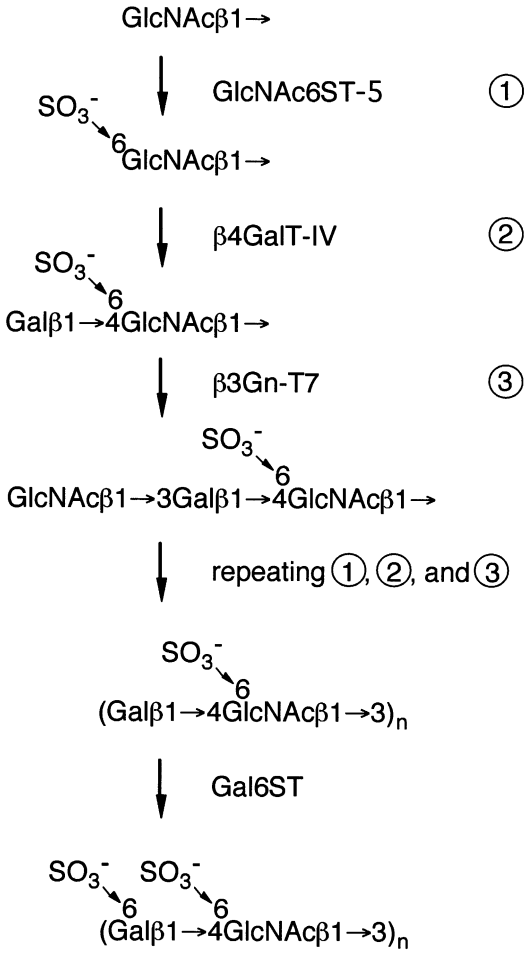

Fig. 2. Proposed scheme for the biosynthesis of keratan sulfate.

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