Identification and characterization of *N*-acetylglucosamine-6-*O*-sulfate-specific β1,4-galactosyltransferase in human colorectal mucosa

Akira Seko[a,b], Sayuri Hara-Kuge[a,b], Suguru Yonezawa[c], Koji Nagata[c], Katsuko Yamashita[a,b,*]

[a] *Department of Biochemistry, Sasaki Institute, Kanda-Surugadai 2-2, Chiyoda-ku, Tokyo 101-0062, Japan*
[b] *CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation (JST), Tokyo 101-0062, Japan*
[c] *Second Department of Pathology, Kagoshima University School of Medicine, Sakuragaoka 8-35-1, Kagoshima 890-8520, Japan*

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Abstract 6-Sulfo-sialyl Lewis X structure is attributable to recognition between lymphocytes and high endothelial venules. However, the biosynthetic pathway still remains unclear. We found that a β-galactosyltransferase (βGalT) in human colorectal mucosa preferentially acts on GlcNAc-6-*O*-sulfate (6S-GN). 6S-GN:β4GalT was partially purified by UDP-hexanolamine-Sepharose and asialo-agalacto-ovomucin-Sepharose chromatographies. The optimum pH of this enzyme was found to be 6.5–7.5 and the Michaelis constants for 6S-GN and UDP-Gal were 0.43 mM and 16 μM, respectively. The enzymatic activity was dependent on divalent cations and the substrate specificity was not affected by α-lactalbumin. This is the first demonstration of the occurrence of 6S-GN:β4GalT.

Key words: Galactosyltransferase; Colorectal mucosa; Sulfated glycan; Selectin; Sialyl Lewis X

1. Introduction

Sulfate residues at the C-6 position of GlcNAc have been found in various *N*-linked- and *O*-linked glycan chains. Although the biological roles of most sulfated glycans still remain unclear, it has been reported that the GlcNAc-6-*O*-sulfate moiety in the 6-sulfo-sialyl Lewis X structure is important for binding of lymphocytes to high endothelial venules in lymph nodes [1,2]. Sulfation at the C-6 of GlcNAc is catalyzed by GlcNAc:→6 sulfotransferase (SulT). The enzyme cannot act on internal GlcNAc residues such as Galβ1→4GlcNAcβ1→, but acts on non-reducing terminal GlcNAc [3–5]. The GlcNAc-6-*O*-sulfate is sequentially converted to Galβ1 → 4(SO_3^- → 6)GlcNAc by β1 → 4galactosyltransferase (GalT) [3–5]. However, the galactosylation has not yet been studied enzymologically and some ambiguities exist with respect to the biosynthesis of the Galβ1 → 4(SO_3^- → 6)GlcNAc structure.

In preliminary experiments, we noticed that β4GalT activity in human colorectal mucosa might be attributable to a mixture of several enzymes. We show in this paper that β4GalTs derived from crude microsomal fractions of colorectal mucosa can be separated into at least two components, one of which preferentially recognizes GlcNAc-6-*O*-sulfate (6S-GN) as a substrate. The demonstration that a β1,4-galactosyltransferase recognizes terminal GlcNAc-6-*O*-sulfate on a number of acceptors represents a significant advance in our understanding of the synthesis of 6-sulfo-sialyl Lewis X structures.

*Corresponding author. Fax: (81) (3) 3294-2656.
E-mail: yamashit@sasaki.or.jp

Abbreviations: GalT, galactosyltransferase; GL, GlcNAcβ1→3Galβ1→4Glc; GP, GlcNAcβ1→2Manα1→3(Glc NAcβ1→2-Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc; NEM, *N*-ethylmaleimide; RCA, *Ricinus communis* agglutinin; 6S-GN, GlcNAc-6-*O*-sulfate; 6S-GP, SO_3^-→6GlcNAcβ1→2Manα1→3(Manα1→6)Manβ1→4GlcNAcβ1→4 (Fucα1→6)GlcNAc; SulT, sulfotransferase; TAPS, *N*-tris(hydroxymethyl)-methyl-3-aminopropanesulfonic acid; UDP-hexanolamine, uridine 5′-diphosphohexanolamine

2. Materials and methods

2.1. Materials and enzymes

Uridine diphosphate [^{3}H]galactose {UDP-Gal, galactose-4,5-[^{3}H](N)} (1.79 TBq/mmol) was purchased from DuPont/NEN (Boston, MA). UDP-Gal, UDP, human α-lactalbumin, bovine milk β1,4GalT were purchased from Sigma Chemical Co. (St. Louis, MO). *Ricinus communis* agglutinin $(RCA)_{120}$-agarose (4.1 mg protein per ml gel) was purchased from Seikagaku Co. (Tokyo, Japan).

GlcNAcβ1→3Galβ1→4Glc(GL) and Neu5Acα2→6GlcNAcβ1→3Galβ1→4Glc (agalacto-LST-b) were prepared from lacto-*N*-tetraose and LST-b [6], respectively, by *Streptococcus* 6646K β-galactosidase digestion. GlcNAcβ1→2Manα1→3(GlcNAcβ1→2Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc (GP) was prepared from egg yolk glycopeptide SGP [7] by sequential steps of hydrazinolysis-re-*N*-acetylation, mild acid hydrolysis, and *Streptococcus* 6646K β-galactosidase digestion. *N*-Acetyl-D-glucosamine-6-*O*-sulfate (6S-GN) was prepared from D-glucosamine-6-*O*-sulfate (Sigma) by *N*-acetylation [8]. SO_3^- → 6GlcNAcβ1 → 2Manα1 → 3(Manα1 → 6)Manβ1 →4GlcNAcβ1→4(Fucα1→6)GlcNAc (6S-GP) was prepared from porcine thyroglobulin (Sigma) [9] by sequential steps of pronase E digestion, hydrazinolysis-re-*N*-acetylation, and digestion with *Arthrobacter ureafaciens* sialidase, *Streptococcus* 6646K β-galactosidase, and jack bean β-*N*-acetylhexosaminidase.

2.2. Preparation of affinity resins

UDP-hexanolamine (Sigma) was conjugated to CNBr-activated Sepharose 4B (Pharmacia Biotech, Sweden). The amount conjugated was 7 μmol of UDP-hexanolamine per ml of gel.

Ovomucin was prepared from egg white according to the method of Brooks and Hale [10], and digested sequentially with pronase E, *Salmonella typhimurium* LT2 α2,3-sialidase and *Streptococcus* 6646K β-galactosidase. Asialo-agalacto-ovomucin glycopeptides obtained were conjugated with CNBr-activated Sepharose 4B. The amount conjugated was 6.7 mg per ml of gel.

2.3. Assay of GL:β3GalT and GL:β4GalTs

The assay was performed by the method described previously [11]. Briefly, a 4 μl reaction mixture consisting of 50 mM HEPES-NaOH buffer (pH 7.2), 10 mM $MnCl_2$, 0.5% (v/v) Triton X-100, 0.5 μM UDP-[^{3}H]Gal (2.2×10^5 dpm), 130 μM UDP-Gal, acceptor oligosaccharides, 1 mM dithiothreitol (DTT) and enzymes, was incubated at 37°C for 20 min. ^{3}H-Labeled products were purified by paper electrophoresis and paper chromatography. The [^{3}H]Gal residue linkage was determined by RCA_{120}-agarose affinity chromatography [12].

2.4. Separation of colorectal βGalTs

The normal mucosal tissue was obtained from an area at least a 10 cm distance from the margin of the carcinoma of patients under-

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going surgical resection, and was separated completely from the muscle layer and stored frozen at −80°C before use. The procedure of microsome preparation was described previously [11]. Crude microsomes derived from 10 individuals (17.3 g) were suspended in 8 ml of 20 mM HEPES-NaOH (pH 7.3), 10 mM $MnCl_2$, 20% (v/v) glycerol, and 1 mM DTT (buffer A) containing 0.15 M NaCl and 1% (v/v) Triton X-100, and the sample was gently stirred for 1 h. After ultracentrifugation, the precipitate was further extracted twice and three detergent-extracted fractions were collected. The fraction was dialyzed against buffer A and applied to an UDP-hexanolamine-Sepharose column (1.6×3 cm; equilibrated with buffer A containing 0.1% Triton X-100 (buffer B); flow rate: 10 ml/h). After washing with buffer B, βGalT activities were eluted with buffer B containing 1 M NaCl (fraction I) and thereafter buffer B containing 1 M NaCl and 1 mM UDP (fraction II). Each of the fractions was dialyzed against buffer A and rechromatographed. Fraction I was further applied to an asialo-agalacto-ovomucin-Sepharose column (0.7×2.6 cm; equilibrated with buffer B; flow rate: 10 ml/h). After washing with buffer B, βGalT activity was eluted by applying a linear gradient of NaCl (0–0.2 M) in buffer B and thereafter buffer B containing 0.5 M NaCl. The protein contents were estimated using the Bio-Rad Protein Assay dye reagent with bovine serum albumin as a standard.

3. Results and discussion

3.1. Partial purification of human colorectal 6S-GN:β4GalT

βGalTs in crude microsomes of human colorectal mucosa were extracted three times with 1% Triton X-100. When the supernatant fractions were applied to UDP-hexanolamine-Sepharose affinity chromatography, the GL:βGalT activity was separated into two fractions, I and II, as shown in Fig. 1A. Fraction I was eluted with 1 M NaCl and contained mainly GL:β3GalT activity. Fraction II was eluted with 1 mM UDP/1 M NaCl and contained only GL:β4GalT. The linkages of [^{3}H]Gal in the products were determined by RCA_{120}-agarose lectin column chromatography [12]; [^{3}H]Galβ1→3GL was retarded, and [^{3}H]Galβ1 → 4GL bound to the column and was eluted with 10 mM lactose. When 6S-GN was used as acceptor, a single type of βGalT activity was found in fraction I (Fig. 1A). It was suggested that not only β3GalT but also 6S-GN-specific βGalT exists in fraction I. This fraction was rechromatographed and then applied to an asialo-agalacto-ovomucin-Sepharose column, because ovomucin contains a Galβ1 → 4(SO_3^- → 6)GlcNAc moiety in the region peripheral to the *O*-linked chains [13]. As shown in Fig. 1B, two types of βGalTs, 6S-GN:βGalT (△ in Fig. 1B) and GL:β3GalT (● in Fig. 1B), were separated from each other. The yield of 6S-GN:βGalT from crude extract is summarized in Table 1.

The linkage of [^{3}H]Gal in the enzyme product, [^{3}H]Gal → (SO_3^- → 6)GlcNAc, was determined by two methods. First, upon digestion with β1→4 specific diplococcal β-galactosidase, [^{3}H]Gal was released from [^{3}H]Gal→ (SO_3^- →6)GlcNAc. Second, after mild methanolysis (0.05 N HCl/MeOH, 25°C, 4 h) [14], the desulfated product [^{3}H]Gal-GlcNAc was bound to an RCA_{120}-agarose column and eluted with 10 mM lactose-PBS, indicating that [^{3}H]Gal is linked to the C-4 position of the GlcNAc residue. These results indicate that this enzyme is 6S-GN:β4GalT. Next, the biochemical properties of the 6S-GN:β4GalT in human colorectal mucosa were investigated.

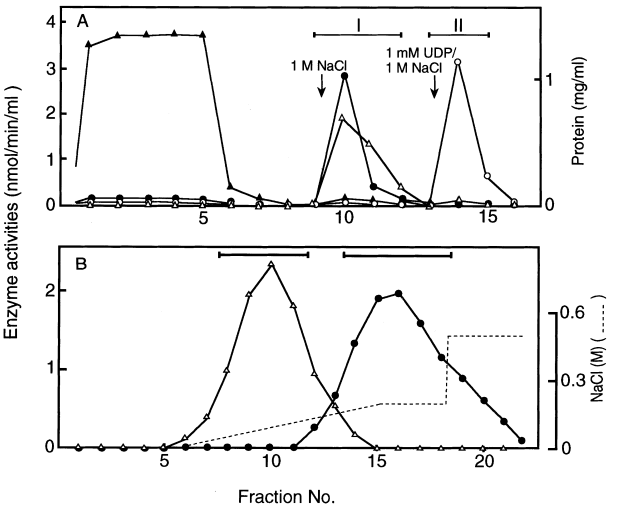


Fig. 1. A: Chromatography of a Triton X-100-extracted fraction containing βGalT activities on UDP-hexanolamine-Sepharose. The column (1.6×3 cm) was washed (each 18-ml fraction) with buffer B followed by buffer B containing 1 M NaCl and then buffer B containing 1 M NaCl-1 mM UDP. Protein (▲), GL:β3GalT (●; 1 = 10 nmol/min/ml), GL:β4GalT (○; 1 = 1 nmol/min/ml) and 6S-GN:β4GalT (△; 1 = 1 nmol/min/ml) were assayed as described in Section 2. B: Chromatography of fraction I containing β3GalT and 6S-GN:β4GalT on asialo-agalacto-ovomucin-Sepharose. The column (0.7×2.6 cm) was eluted (each 2-ml fraction) with buffer B followed by a linear gradient of NaCl (dotted line) and thereafter 0.5 M NaCl in buffer B.

3.2. The biochemical properties of 6S-GN:β4GalT

The reaction rate was proportional to protein concentration (at least 1 mg/ml of protein) under the standard conditions employed and was linear for at least 2 h (data not shown). The activity of the enzyme as a function of pH is shown in Fig. 2A. The optimum pH was within the range of pH 6.5–7.5. From a double reciprocal plot of the substrate concentration (6S-GN) versus reaction rate, the K_m and V_{max} values for 6S-GN were calculated to be 0.43 mM and 450 nmol/min/mg protein, respectively (data not shown). Furthermore, K_m and V_{max} values for UDP-Gal were calculated to be 16 μM and 450 nmol/min/mg protein, respectively (data not shown).

The relative activity in the presence of various compounds is shown in Fig. 2B. The enzyme was inhibited by 5 mM EDTA (△), suggesting that 6S-GN:β4GalT is dependent on divalent cations. $MnCl_2$, $MgCl_2$, and $CaCl_2$ were good activators of 6S-GN:β4GalT (▼, ▽, and □, respectively in Fig. 2B). 6S-GN:β4GalT activity was inhibited by 10 mM NEM

Table 1
Partial purification of 6S-GN:β4GalT from human colorectal mucosa

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Yield (%)	Purification (fold)
Crude extract	568	68.8	0.121	100	–
Microsome	142	61.9	0.436	90	3.6
First UDP-hexanolamine-Sepharose	1.93	31.0	16.1	45	133
Second UDP-hexanolamine-Sepharose	0.176	30.8	175	45	1450
Asialo-agalacto-ovomucin-Sepharose	0.038	15.1	397	22	3280

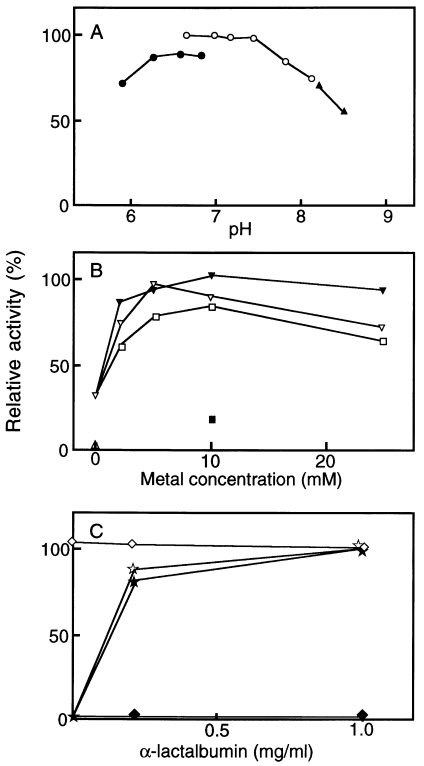


Fig. 2. Effects of pH (A), divalent cations (B) and α-lactalbumin (C) concentration on 6S-GN:β4GalT activity. A: The pH effect was analyzed using 50 mM sodium cacodylate buffer (pH 5.90–6.83, ●), 50 mM HEPES-NaOH buffer (pH 6.66–8.12, ○) or 50 mM TAPS-NaOH buffer (pH 8.19–8.49, ▲). B: The enzymatic activity was assayed in the presence of indicated concentrations of $MnCl_2$ (▼), $MgCl_2$ (▽), or $CaCl_2$ (□). The enzymatic activity displayed in the presence of 5 mM EDTA (△) or upon preincubation at 4°C for 10 min in the presence of 5 mM NEM and 10 mM $MnCl_2$ (■) is also indicated. C: β4GalTs (fraction II in Fig. 1A) (☆), bovine milk β4GalT (★), and 6S-GN:β4GalT (♦) activities using 20 mM Glc as acceptor were assayed in the presence of indicated concentrations of human α-lactalbumin and are indicated relative to the activity of bovine milk β4GalT in the presence of 1.0 mg/ml α-lactalbumin as 100%. 6S-GN:β4GalT activity (◇) using 3 mM 6S-GN as acceptor was also assayed and is indicated relative to the activity in the presence of 1.0 mg/ml α-lactalbumin as 100%.

(■), showing only 18% of the control level (10 mM $MnCl_2$), suggesting that the enzyme has thiol residues which are important for activity.

The substrate specificity of 6S-GN:β4GalT was investigated in comparison with β3GalT (● in Fig. 1B) and β4GalTs (fraction II in Fig. 1A) using various acceptors. As summarized in Table 2, when GL, GP, or GlcNAc was used as the acceptor, 6S-GN:β4GalT showed only weak activity. In contrast, when 6S-GN or 6S-GP was used as the acceptor, each of these substrates was recognized as a good acceptor by 6S-GN:β4GalT. However, 6S-GN:β4GalT did not recognize Neu5Acα2 → 6GlcNAcβ1 → 3Galβ1 → 4Glc as a good acceptor. These results show that the enzyme displays strict substrate specificity limited to SO_3^- → 6GlcNAc-R and it was categorized as 6S-GN:β4GalT. To the best of our knowledge, this is the first demonstration of the occurrence of a 6S-GN-specific β4GalT. Its character is very different from that of other βGalTs including GL:β3GalT (● in Fig. 1B) and β4GalTs (fraction II in Fig. 1A). β3GalT preferentially recognizes GL, while β4GalTs broadly recognize GL, GP, and GlcNAc, however, they do not recognize 6S-GN or 6S-GP as good substrates. The substrate specificities of the β4GalTs in human colorectal mucosa (fraction II in Fig. 1A) are similar to those of β4GalT in bovine milk, which is categorized as β4GalTI. In the presence of α-lactalbumin, 6S-GN:β4GalT activity with 6S-GN as the substrate did not change (Fig. 2C, ◇) and 6S-GN:β4GalT did not show any activity with Glc as the substrate, (Fig. 2C, ♦), whereas β4GalTs (fraction II in Fig. 1A) and bovine milk βGalT I activities with glucose as the substrate markedly increased (Fig. 2C, ☆ and ★, respectively).

Recently cDNAs for β4GalTs other than β4GalTI have been cloned and sequenced [15–19]. Almeida et al. [17] isolated cDNAs for β4GalTII and β4GalTIII, and showed that β4GalTII is similar to β4GalTI in terms of the effect of α-lactalbumin and that β4GalTIII activity is not influenced substantially by α-lactalbumin. Sato et al. [18] isolated a cDNA for β4GalT, which shows a rather strict substrate specificity limited to *N*-linked glycans. However, it is unclear whether or not the β4GalTs can preferentially galactosylate 6S-GN residues. It should be resolved in a subsequent study whether 6S-GN:β4GalT is distinct from the six β4GalTs so far reported.

The Galβ1 → 4(SO_3^- → 6)GlcNAc structure is broadly distributed in *O*-linked and *N*-linked glycans of various glycoproteins. The extensive distribution of this structure suggests that 6S-GN:β4GalT could occur in various tissues. In biosynthesis of the Galβ1 → 4(SO_3^- → 6)GlcNAc structure, it seems to be important which residue is first transferred to the non-reducing GlcNAc, β1 → 4-linked Gal or → 6-linked SO_3^-. We recently found that GlcNAcβ1 → 2Man is a good substrate for human colorectal mucosa GlcNAc: → 6SulT (A. Seko, S. Yonezawa, T. Utsunomiya and K. Yamashita, submitted). It has been shown that non-reducing GlcNAcβ1 → is a much better substrate for GlcNAc: → 6SulTs from rat organs and human respiratory mucus than Galβ1 → 4GlcNAcβ1 → [3–5]. These results indicate that the β1 → 4-galactosylation follows sulfation at the C-6 of GlcNAc residues and β1 → 4-galactosylation

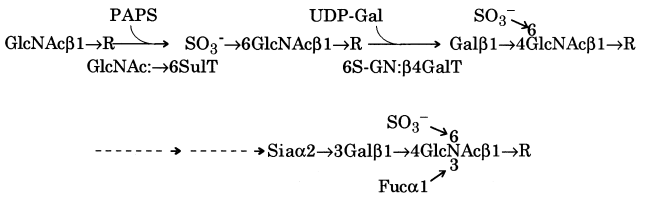
Table 2
Substrate specificities of human colorectal β-galactosyltransferases

Substrate[a]	6S-GN:β4GalT (△ in Fig. 1B)	β3GalT (● in Fig. 1B)	β4GalTs (fraction II in Fig. 1A)	Bovine milk β4GalT
GlcNAcβ1 → 3Galβ1 → 4Glc (GL)	3.9	100	100	100
$GlcNAc_2$·Man_3·$GlcNAc_2$ (GP)	2.9	0.5	90	95
GlcNAc	1.1	1.5	43	60
SO_3^- → 6GlcNAc (6S-GN)	100	0	2.0	1.1
SO_3^- → 6GlcNAc·Man_3·GlcNAc·Fuc·GlcNAc (6S-GP)	129	0	8.3	13.1
Neu5Acα2 → 6GlcNAcβ1 → 3Galβ1 → 4Glc	9.1	2.5	2.6	4.7

[a]The substrate concentration was 1 mM in each instance.

of the Galβ1 → 4(SO_3^- → 6)GlcNAc structure may be specifically catalyzed by 6S-GN:β4GalT.

It has been reported that L-selectin and their glycan ligands are involved in the attachment of leukocytes to high endothelial venules in lymph nodes. It was reported that sulfated sialyl Lewis X containing a Gal-6-*O*-sulfate or GlcNAc-6-*O*-sulfate moiety occurs in endothelial glycoproteins and serves as a good ligand for L-selectin [1,2,20]. It still remains unclear which glycosyltransferases are rate-limiting in the biosynthesis of sulfated glycans. It might be speculated that GlcNAc: → 6SulT or 6S-GN:β4GalT, which is likely to be involved in the early steps of biosynthesis, might play an important role in expression of the 6-sulfo-sialyl Lewis X structure. The possible biosynthetic pathway is considered to be as follows:



Cloning of the genes encoding GlcNAc: → 6SulT and 6S-GN:β4GalT will open the way to resolving the regulatory mechanism of the biosynthesis of the sulfated glycans.

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