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Specificities of *N*-Acetylglucosamine-6-*O*-sulfotransferases in Relation to L-selectin Ligand Synthesis and Tumor-associated Enzyme Expression*

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***N*-Acetylglucosamine-6-*O*-sulfotransferase (GlcNAc-6ST) catalyzes the transfer of sulfate from adenosine 3′-phosphate,5′-phosphosulfate to the C-6 position of the non-reducing GlcNAc. Three human GlcNAc6STs, namely GlcNAc6ST-1, GlcNAc6ST-2 (HEC-GlcNAc6ST), and GlcNAc6ST-3 (I-GlcNAc6ST), were produced as fusion proteins to protein A, and their substrate specificities as well as their enzymological properties were determined. Both GlcNAc6ST-1 and GlcNAc6ST-2 efficiently utilized the following oligosaccharide structures as acceptors: GlcNAcβ1–6[Galβ1–3]GalNAc-pNP (core 2), GlcNAcβ1–6ManOMe, and GlcNAcβ1–2Man. The ratios of activities to these substrates were not significantly different between the two enzymes. However, GlcNAc-6ST-2 but not GlcNAc6ST-1 acted on core 3 of GlcNAc-β1–3GalNAc-pNP. GlcNAc6ST-3 used only the core 2 structure among the above mentioned oligosaccharide structures. The ability of GlcNAc6ST-1 to sulfate core 2 structure as efficiently as GlcNAc6ST-2 is consistent with the view that GlcNAc6ST-1 is also involved in the synthesis of L-selectin ligand. Indeed, cells doubly transfected with GlcNAc6ST-1 and fucosyltransferase VII cDNAs supported the rolling of L-selectin-expressing cells. The activity of GlcNAc6ST-2 on core 3 and its expression in mucinous adenocarcinoma suggested that this enzyme corresponds to the sulfotransferase, which is specifically expressed in mucinous adenocarcinoma (Seko, A., Sumiya, J., Yonezawa, S., Nagata, K., and Yamashita, K. (2000) *Glycobiology* 10, 919–929).**

Sulfated sugar residues of glycoconjugates have been shown to elicit diverse biological functions (1–4). The addition of a sulfate group on the carbohydrate chains is catalyzed by various sulfotransferases with rigorous acceptor specificities (5). Recent studies (5, 6) have achieved purification and molecular cloning of a series of glycoconjugate sulfotransferases, which are classified into several groups with regard to substrate specificities and primary structures. 6-*O*-Sulfation of *N*-acetylglucosamine (GlcNAc)[1] residues, which occurs during the synthesis of keratan sulfate (1) and is also observed in *N*-linked (7) and *O*-linked (8, 9) glycans, is catalyzed by GlcNAc-6-*O*-sulfotransferase (GlcNAc6ST). The enzymatic activities of GlcNAc6ST in the rat liver (10), human respiratory mucosa (11), and porcine lymph nodes (12) were characterized, and it was suggested that GlcNAc6ST recognizes non-reducing GlcNAc but not internal GlcNAc residues in *N*-acetyllactosamine-based oligosaccharides.

We have previously cloned mouse (13) and human (14) GlcNAc6STs based on their sequence similarity to chondroitin 6-sulfotransferase (15, 16). In our previous study (13), *N*-acetyllactosamine oligomer, GlcNAcβ1–3Galβ1–4GlcNAc, was used as an acceptor for the assay of the expressed enzyme. Subsequently, another member of the GlcNAc6ST family was isolated and designated as HEC-GlcNAc6ST/LSST (17, 18). HEC-GlcNAc6ST/LSST is preferentially expressed in high endothelial venules. Both GlcNAc6STs are involved in the synthesis of sialyl-6-sulfo-Lewis-X, a key structure in L-selectin ligands (17–19). The third member of the GlcNAc6ST family, I-GlcNAc6ST, is preferentially expressed in the intestinal tissues (20), whereas the fourth member, C6ST-2/GST-5/GlcNAc6ST-4, is expressed in many organs (21–23) as GlcNAc6ST (13, 14). Recently, a fifth member, C-GlcNAc6ST, expressed in the human cornea was found (24). The mutation in the enzyme gene has been identified as a cause of macular cornial dystrophy. Despite the highly significant roles of GlcNAc6STs, their specificities and enzymological properties have not been elucidated. In the investigation reported here, we produced protein A fusion proteins of human GlcNAc6STs and comparatively analyzed their substrate specificities as well as enzymological properties. The human GlcNAc6STs studied were GlcNAc6ST (14, 25), HEC-GlcNAc6ST (17), and

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[1] The abbreviations used are: GlcNAc, *N*-acetylglucosamine; GlcNAc6ST, *N*-acetylglucosamine-6-*O*-sulfotransferase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PAPS, adenosine 3′-phosphate,5′-phosphosulfate; GlcNAcβ1–6ManOMe, methyl 6-*O*-(2-acetamido-2-deoxy-β-*O*-glucopyranosyl)-α-D-mannopyranoside; core 2, GlcNAcβ1–6[Galβ1–3]GalNAc-pNP; core 3, GlcNAcβ1–3GalNAc-pNP; TLC, thin layer chromatography; RT, reverse transcription; Fuc-T VII, fucosyltransferase VII; GST, galactose/*N*-acetylgalactosamine/*N*-acetylglucosamine-6-*O*-sulfotransferase; HEC, high endothelial cell.

I-GlcNAc6ST (20) (for the sake of simplicity, here we call these enzymes according to the order of cloning, GlcNAc6ST-1, GlcNAc6ST-2, and GlcNAc6ST-3, respectively).

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained commercially from the sources indicated. [^{35}S]PAPS (1.9 Ci/mmol) was from PerkinElmer Life Sciences; GlcNAcβ1–6ManOMe, 6-*O*-sulfated GlcNAc, and 3-*O*-sulfated GlcNAc were from Sigma; GlcNAcβ1–2Man was from Dextra Laboratories (Reading, UK); GlcNAcβ1–6[Galβ1–3]GalNAc-pNP and GlcNAcβ1–3GalNAc-pNP were from Toronto Research Chemicals (North York, Ontario, Canada); and *Streptococcus* β-galactosidase and jack bean β-*N*-acetylhexosaminidase were from Seikagaku Corporation (Tokyo, Japan). GlcNAcβ1–3Galβ1–4Glc and GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc were prepared from Galβ1–4GlcNAcβ1–3Galβ1–4Glc and Galβ1–4GlcNAcβ1–3Galβ1–4-GlcNAcβ1–3Galβ1–4GlcNAc, respectively, by β-galactosidase digestion as described previously (13).

Construction and Purification of GlcNAc6STs Fused with Protein A—The human HEC-GlcNAc6ST cDNA (17) containing the open reading frame and a FLAG-tag sequence at its 5′ region was introduced into the *Hin*dIII site of the pcDNA3.1 vector (Invitrogen). The constructed vector, pcDNA3.1(+)/FLAG-GST-3, and recombinant plasmids, pcDNA3-GlcNAc6ST (13) and pcDNA3.1-human GST-4 (20), were used as templates for PCR to amplify truncated forms of GlcNAc6STs. To amplify the cDNAs encoding the truncated forms of GlcNAc6ST-1 lacking the first 35 amino acids, GlcNAc6ST-2 lacking the first 29 amino acids, or GlcNAc6ST-3 lacking the first 32 amino acids, 5′ and 3′ primer sets were used, respectively, as follows: a 5′ primer containing an in-frame *Eco*RI site (5′-TGGAATTCCTGCAGCAGTGCAACCCCGAT-3′) and a 3′ primer containing an *Eco*RI site (5′-GAGAATTCTTA-GAGACGGGGCTTCCGA-3′); a 5′ primer containing an in-frame *Eco*RV site (5′-ATGTACGATATCCAACATCAGCTCCCTGTCT-3′) and a 3′ primer containing an *Eco*RV site (5′-CGAAGCGATATCCTTAGT-GGATTTGCTCAGGGAC-3′); and a 5′ primer containing an in-frame *Eco*RI site (5′-CTGAATTCTCATCCCCAGCCGGCGGCGAG-3′) and a 3′ primer containing an *Eco*RI site (5′-CTGAATTCTCAGTCAGGC-GATGCCCAGCT-3′). The amplification was carried out at 94 °C for 3 min with 35 cycles of 94 °C for 0.5 min, 56 °C for 0.5 min, and 72 °C for 1 min. The amplified fragments of GlcNAc6ST-1 and GlcNAc6ST-3 were subcloned into the *Eco*RI site of the fusion vector pcDSA (26) to yield pcDSA-GlcNAc6ST1 and pcDSA-GlcNAc6ST3, respectively. The amplified fragment of GlcNAc6ST-2 was digested with *Eco*RV and then subcloned into the blunted *Eco*RI site of the pcDSA. The obtained plasmid was named pcDSA-GlcNAc6ST2. In all cases, the plasmids encoded fusion proteins of GlcNAc6STs to the IgM signal sequence and protein A in the N-terminal region. COS-7 cells on 10-cm dishes were transfected with 4 μg of relevant plasmids using LipofectAMINE PLUS (Invitrogen) according to manufacturer instructions. After 24 h of culture in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, the medium was replaced with Dulbecco's modified Eagle's medium containing 2% IgG-free fetal calf serum. The cells were cultured for an additional 48 h. Subsequently, the culture medium was collected, and the protein A fusion GlcNAc6STs expressed in the medium were adsorbed to IgG-Sepharose (10 μl of resin/10 ml of culture medium) at 4 °C for 3 h. The resin was collected by centrifugation and washed three times with Dulbecco's phosphate-buffered saline. Finally, the resin was suspended in 30 μl of 50 mM Tris-HCl, pH 7.5, and used as the enzyme.

Assay of GlcNAc6ST Activities toward Various Oligosaccharides—The standard reaction mixture contained 1 μmol of Tris-HCl, pH 7.5, 0.2 μmol of $MnCl_2$, 0.04 μmol of AMP, 2 μmol of NaF, 20 nmol of oligosaccharides, 150 pmol of [^{35}S]PAPS (1.5×10^6 cpm), 0.05% of Triton X-100, and 1 μl of the fusion protein suspension in a final volume of 20 μl. After incubation at 30 °C for 1 h, aliquots of 2 μl of the reaction mixture were applied to TLC plates, which were then developed with ethanol-pyridine-*n*-butyl alcohol-water-acetate (100:10:10:30:3 (v/v)), and the radioactivity was visualized with a BAS2000 bioimaging analyzer (Fuji Film, Tokyo, Japan). For analysis of substrate specificity, half-aliquots of the reaction mixture were subjected to Superdex 30 gel chromatography to confirm the radioactivity of the ^{35}S-labeled products as described previously (13). Digestion with jack bean *N*-acetylhexosaminidase and TLC of the products were performed as described previously (23). Sulfotransferase reactions of GlcNAc6ST-1, GlcNAc6ST-2, and GlcNAc6ST-3 toward various oligosaccharides analyzed in this study proceeded linearly up to 4 h under the standard assay conditions.

Clinical Sample, Cell Culture, and RNA Extraction—Surgical specimens were obtained from two colon cancer patients during surgery and processed as described previously (27). These cases were histologically diagnosed as mucinous adenocarcinoma. Malignant and non-malignant portions of the specimen were used for RNA extraction. Sample was frozen rapidly and stored at −80 °C until extraction of total RNA. The specimen was powdered in liquid nitrogen, and total cellular RNA was extracted with guanidine isothiocyanate and purified by cesium chloride gradient centrifugation. Cultured human colon cancer cell lines LS180 and LS174T, which have characteristics of mucinous adenocarcinoma (28, 29) were maintained in RPMI 1640 medium (Invitrogen) containing 10% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2 in air. Total RNA was isolated from 1×10^6 cells according to the acid guanidinium thiocyanate-chloroform extraction method using an Isogen kit (Nippon Gene, Tokyo, Japan), and the amount of RNA was quantitated by spectrophotometry at 260 nm.

Reverse Transcription (RT)-PCR Analysis—The first strand cDNA was synthesized by reverse transcription of the extracted total RNA (5 μg) using the first strand cDNA synthesis kit (Invitrogen) according to manufacturer protocol. One microliter of the retrotranscription reaction was subjected to PCR amplification using sulfotransferases primers. For detection of GlcNAc6ST-1 mRNA, the upstream primer 5′-CTTA-AGGTCATCCACTTGGTGCG-3′ and the downstream primer 5′-GGG-TCTTGCTGAGGTCTTTGACC-3′ were used. For GlcNAc6ST-2 detection, the upstream primer 5′-GCAGCATGAGCAGAAACTCAAG-3′ and the downstream primer 5′-TCCAGGTAGACAGAAGATCCAG-3′ were used. For GlcNAc6ST-3, the upstream primer 5′-CAAGACAGTGACA-GTGCTCC-3′ and the downstream primer 5′-TACGTCCTGCTTGCTG-ATGG-3′ were used. The cycle numbers most suitable for semiquantitative RT-PCR were determined in preliminary experiments, *i.e.* 30 cycles for GlcNAc6ST-1, 35 cycles for GlcNAc6ST-2, and 30 cycles for GlcNAc6ST-3. As an internal control for the RT-PCR analysis, the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) transcripts were amplified from the same cDNA samples. The upstream and downstream for G3PDH genes were 5′-TGAAGGTCGGAGTCAACGGATTT-GGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′, respectively. Aliquots of each reaction product were fractionated by electrophoresis through a 2% agarose gel containing ethidium bromide. The intensities of the bands were quantified by the Densitograph apparatus and software (AE-6920WLSA, ATTO, Tokyo, Japan).

Rolling Assays—The production of 300.19 cells stably expressing L-selectin or E-selectin has been described previously (30). The interaction of 300.19/L-selectin cells was assayed in a parallel plate flow chamber (Glycotech, Rockville, MD). Monolayers of LS12 cells (19) were grown in 35-mm tissue culture plates and served as the rolling substrate. LS12 cells are cloned ECV304 cells stably transfected with both GlcNAc6ST-1 and fucosyltransferase VII (Fuc-T VII) cDNAs and have previously been shown to significantly adhere to L-selectin-expressing cells in non-static monolayer adhesion assays (19). Cells were introduced into the flow chamber at a concentration of 10^6 cells/ml in a buffer of RPMI 1640 medium supplemented with 0.1% serum. The shear stress in the flow chamber was maintained constant at 1.0 dyne/cm^2 using a syringe pump (Harvard Apparatus, Holliston, MA), and images were obtained using a Nikon Eclipse TE300-inverted microscope (Nikon, Tokyo, Japan). Data analysis was performed using Celltrak software developed by Compix (Cranberry Township, PA) as described previously (31, 32). A rolling event is defined as a rolling cell that can be tracked between sequential images separated by a defined time delay. The time defined here is 0.5 sec. Velocities of individual rolling cells were also simultaneously obtained during analysis, and the mean rolling velocity of the rolling cell population was determined. The total number of rolling events and velocities were collected for 50–100 sequential images. Each experiment was repeated at least four times. COS-7 cells stably transfected with Fuc-T VII and/or GlcNAc6ST-1 were prepared using pRC/CMV-FT VII and/or pIRES1hyg-6ST-1 by employing methods similar to those described for ECV304 transfectant cells (19). Rolling assays for COS-7 transfectant cells were performed using the flow chamber as described by Lawrence *et al.* (33), a syringe pump (Model 944, Harvard Apparatus), and an Olympus IX70–22FL/PH inverted microscope equipped with air-curtain incubator (Model IX-IBM, Olympus, Tokyo, Japan) at a constant shear stress of 1.0 dyne/cm^2. The digital images were recorded with a digital video camera system (Model CS-220, Olympus). Expression of L-selectin ligands on the transfected cells was ascertained by flow cytometric analyses using the G152 antibody (murine IgM) specific to sialyl-6-sulfo-Lewis-X according to the methods described previously (19).

TABLE I
Comparison of K_M values for sulfate acceptor and donor among GlcNAc6ST-1, GlcNAc6ST-2, and GlcNAc6ST-3

Substrate	K_M values[a]		
	GlcNAc6ST-1	GlcNAc6ST-2	GlcNAc6ST-3
	mM	*mM*	*mM*
GlcNAcβ1–6ManOMe	0.2	1.14	n.d.[b]
GlcNAcβ1–6[Galβ1–3]GalNAc-pNP (core 2)	0.41	0.41	0.2
PAPS	0.0012[c]	0.0059[c]	0.014[d]

[a] Data are representative of one of two series of independent experiments, which exhibited essentially identical results.
[b] n.d., not determined.
[c] The acceptor substrate was GlcNAcβ1–6ManOMe.
[d] The acceptor substrate was GlcNAcβ1–6[Galβ1–3]GalNAc-pNP (core 2).

RESULTS

Enzymological Properties of GlcNAc6ST-1, -2, and -3—We compared enzymological properties of GlcNAc6ST-1, -2, and -3 to determine whether there are significant differences among the three enzymes. The activities of GlcNAc6ST-1 and GlcNAc6ST-2 were measured by using GlcNAcβ1–6ManOMe as a substrate, whereas core 2 was used as a substrate of GlcNAc6ST-3. GlcNAc6ST-1, -2, and -3 showed an optimum pH of approximately 7.5–8.0. GlcNAc6ST-3 activity was higher in sodium HEPES than Tris-HCl buffer, whereas GlcNAc6ST-1 and GlcNAc6ST-2 showed better activity in Tris-HCl buffer. With respect to the effects of divalent cations at a concentration of 10 mM, GlcNAc6ST-1 activity was stimulated 1.8-fold by the addition of Mn^{2+}, whereas Mn^{2+} showed no effects on GlcNAc6ST-2 and inhibited GlcNAc6ST-3 to one-third of the activity without it. Ca^{2+} and Cu^{2+} strongly inhibited all three enzymes. Co^{2+} showed strong inhibitory effects on GlcNAc6ST-2 and GlcNAc6ST-3 but not on GlcNAc6ST-1.

The K_m value of GlcNAc6ST-1 for GlcNAcβ1–6ManOMe as an acceptor was lower than that of GlcNAc6ST-2 (Table I), indicating that GlcNAc6ST-1 has a higher affinity to mannosyl-linked GlcNAc residues than GlcNAc6ST-2. K_m values for the core 2 oligosaccharide were the same between GlcNAc6ST-1 and GlcNAc6ST-2, whereas the value for GlcNAc6ST-3 was the lowest. GlcNAc6ST-1 had the lowest K_m value for PAPS as the sulfate donor among the three enzymes (Table I). Both protein A-fused soluble GlcNAc6ST-1 and full-length GlcNAc6ST-1 as microsomal fractions of the cells transfected with pcDNA3-GlcNAc6ST showed the same enzymological properties, namely kinetical parameters and effects of pH and divalent cations (data not shown). Because microsomal fraction gave only weak activities of GlcNAc6ST-2 and GlcNAc6ST-3, production of the fusion protein became necessary to determine the activities of these enzymes precisely. Thus, fusion proteins were used to compare the properties of the three GlcNAc6STs.

Substrate Specificities of GlcNAc6ST-1, -2, and -3—The series of oligosaccharides shown in Table II was tested as acceptors to evaluate the substrate specificities of GlcNAc6ST-1, -2, and -3. The TLC of ^{35}S-labeled products revealed that both GlcNAc6ST-1 and GlcNAc6ST-2 utilized efficiently GlcNAcβ1–6Man, GlcNAcβ1–2Man, and core 2 oligosaccharide (GlcNAcβ1–6[Galβ1–3]GalNAc-pNP) as acceptors (Fig. 1, Table II). As microsomal fractions of the cells transfected with the expression vector, GlcNAc6ST-1 showed the same specificity as that of the protein A-fused enzyme (data not shown). As compared with GlcNAc6ST-1, GlcNAc6ST-2 showed higher activity against GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc. The most notable difference was that GlcNAc6ST-2 used core 3 oligosaccharide (GlcNAcβ1–3GalNAc-pNP) as an acceptor, whereas GlcNAc6ST-1 did not (Fig. 1 and Table II). In contrast, GlcNAc6ST-3 acted only on core 2 and did not act on GlcNAcβ1–6Man, GlcNAcβ1–2Man, core 3 oligosaccharide, or GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Gal β1–4GlcNAc. Quantitative analysis revealed that both GlcNAc6ST-1 and GlcNAc6ST-2 utilized core 2 oligosaccharide, GlcNAcβ1–6Man, and GlcNAcβ1–2Man structures with similar velocities (Table II).

Expression of GlcNAc6ST-2 in Mucinous Adenocarcinoma—Among three GlcNAc6STs examined in this study, only GlcNAc6ST-2 acted on core 3. Because Yamashita and co-workers (34) recently reported that a GlcNAc6ST acting on core 3 is specifically expressed in mucinous adenocarcinoma of the colon, we examined whether GlcNAc6ST-2 was expressed in the tumor. Indeed, semiquantitative RT-PCR analysis revealed that GlcNAc6ST-2 was expressed in two specimens of mucinous adenocarcinoma of the colon (Fig. 2). However, its expression was either very weak (Case 1) or not detectable (Case 2) in the adjacent normal mucosal tissue (Fig. 2). Furthermore, two cell lines with the characteristics of mucinous adenocarcinoma, LS180 and LS174T, strongly expressed GlcNAc6ST-2 (Fig. 2). Densitometric analysis yielded relative values for the intensity of GlcNAc6ST-2 bands as follows: Case 1, normal colon, 27; Case 1, colon cancer, 123; Case 2, normal colon, $<$10; Case 2, colon cancer, 362. The values for the bands of G3PDH were similar in these samples and were in the range of 1396–1683. On the other hand, the expression of GlcNAc6ST-1 or GlcNAc6ST-3 did not increase in tumors as compared with the normal tissue (Fig. 2).

The Rolling of L-selectin-expressing Cells on Cells Doubly Transfected with GlcNAc6ST-1 and Fuc-T VII cDNAs—GlcNAc6ST-1 showed substrate specificities similar to GlcNAc6ST-2 in agreement with the view that GlcNAc6ST-1, which is expressed in high endothelial venules (13), is involved in the synthesis of L-selectin ligand together with other GlcNAc6STs. Indeed, a co-transfection of GlcNAc6ST-1 and Fuc-T VII cDNAs resulted in the expression of sialyl-6-sulfo-Lewis-X (13, 19) and adhesion of the cells to L-selectin-expressing cells, 300.19/L-selectin cells (19, 30).

We examined whether double transfection with GlcNAc6ST-1 and Fuc-T VII cDNAs resulted in the rolling of 300.19/L-selectin cells, because this is considered to be the most reliable method to evaluate the physiological activity of L-selectin ligands. The ECV304 cells doubly transfected with GlcNAc6ST-1 and Fuc-T VII cDNAs, (LS12 clone, for review, see Ref. 19) significantly expressed sialyl-6-sulfo-Lewis-X (Fig. 3*a*) and supported the rolling of a large number of 300.19/L-selectin cells (Fig. 3*b*). Transfection with Fuc-T VII cDNA only caused rolling of a relatively small number of cells. In this case, the rolling velocity was not reduced as compared with parental cells (Fig. 3*b*). In the case of COS-7 cells, transfection with only Fuc-T VII cDNA resulted in no increase in the rolling of 300.19/L-selectin cells, whereas efficient rolling was observed for E-selectin-expressing cells, 300.19/E-selectin cells (30) (Fig. 4*b*). Double transfection with GlcNAc6ST-1 and Fuc-T VII cDNAs induced strong expression of sialyl-6-sulfo-Lewis-X (Fig. 4*a*) and supported the rolling of the L-selectin-expressing cells (Fig. 4*b*). From these results, we concluded that GlcNAc6ST-1 can collaborate with Fuc-T VII in the formation of physiologically significant L-selectin ligands.

TABLE II
Comparison of the substrate specificities of GlcNAc6ST-1, GlcNAc6ST-2, and GlcNAc6ST-3 secreted into the culture medium by transfected COS-7 cells

Acceptor	Enzyme activity[a] pmol/h/ml of medium (%)[b]		
	GlcNAc6ST-1	GlcNAc6ST-2	GlcNAc6ST-3
GlcNAcβ1–6ManOMe	20.6 (100)	6.8 (100)	N.D.[c]
GlcNAcβ1–2Man	26.3 (128)	10.3 (151)	N.D.[c]
GlcNAcβ1–6[Galβ1–3]GalNAc-pNP (core 2)	39.3 (191)	9.9 (145)	5.7[d]
GlcNAcβ1–3GalNAc-pNP (core 3)	N.D.[c]	12.5 (184)	N.D.[c]
GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc	4.3[d] (21)	5.0[d] (73)	N.D.[c]

[a] The values represent the averages of two independent experiments.
[b] The percentage of the activity compared to that of GlcNAcβ1–6ManOMe is also shown.
[c] N.D. indicates <0.1 pmol/h/ml of medium.
[d] The actual observed radioactivities were approximately 30,000 cpm, whereas the assay without the enzyme or with IgG-Sepharose exposed to the culture supernatant of mock-transfected cells gave values <500 cpm.

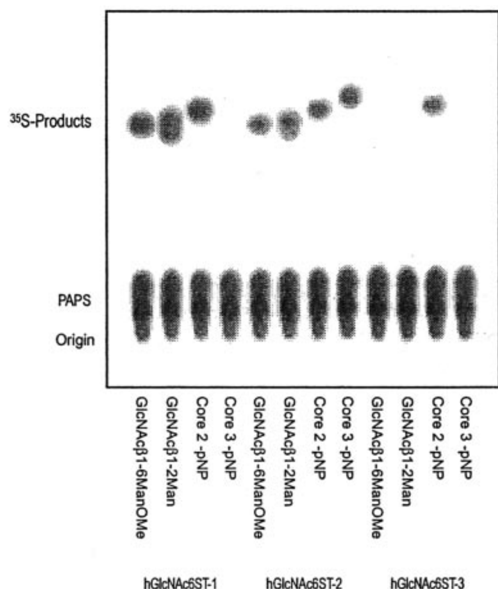

FIG. 1. **Sulfation of various oligosaccharides by human GlcNAc6ST-1, -2 , and -3.** Various oligosaccharides were incubated with the protein A-fused GlcNAc6ST-1, -2, and -3 under standard assay conditions described under "Experimental Procedures." Aliquots of 2 μl of each reaction were applied to a TLC plate and then developed with ethanol-pyridine-*n*-butyl alcohol-water-acetate (100:10:10:30:3 (v/v), respectively). ^{35}S-Labeled products were visualized, and the radioactivity was measured with a BAS2000 bioimaging analyzer (Fuji Film).

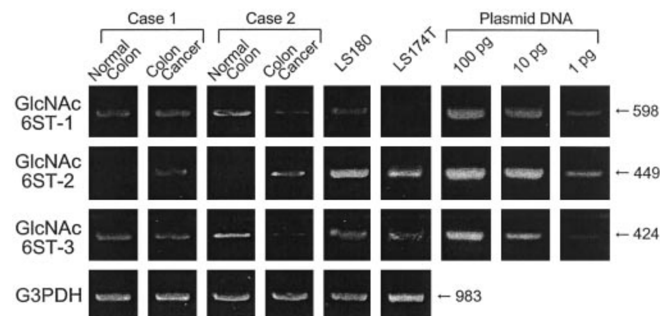

FIG. 2. **Expression of GlcNAc6STs in mucinous adenocarcinoma.** RT-PCR was performed to detect mRNA of GlcNAc6STs as described under "Experimental Procedures." *Markers* to the *right* indicate the size of the products (base pairs). *Lanes* labeled as *Colon Cancer* and *Normal Colon* indicate specimens of mucinous adenocarcinoma and that of adjacent normal mucosa, respectively. LS180 and LS174T are cultured cells with the character of mucinous adenocarcinoma of the colon. Results of PCR performed using known amounts of plasmid harboring full-length cDNA of each 6-sulfotransferase are also indicated.

DISCUSSION

Using five oligosaccharides or their derivatives as substrates, we compared substrate specificities of GlcNAc6STs; all oligosaccharides were naturally occurring ones, namely two mannosyl oligosaccharides, two mucin oligosaccharides, and an *N*-acetyllactosamine oligomer. Consequently, we found that substrate specificities of GlcNAc6ST-1, -2, and -3 were different from each other. The specificity of GlcNAc6ST-4 reported previously is somewhat similar to that of GlcNAc6ST-1, but the former acted on mannosyl oligosaccharides more efficiently (23). The existence of multiple GlcNAc6STs with different substrate specificities and tissue distributions is consistent with the view that the GlcNAc-6-sulfated structures play important roles in many intercellular recognition systems.

Bowman *et al.* used substrates based on GlcNAcβ1–6Gal and compared substrate specificities of GlcNAc6ST-1, -2 and -3 (35). GlcNAcβ1–6Gal is an artificial substrate to examine substrate specificities to a mucin-type substrate; GalNAc in GlcNAcβ1–6GalNAc structure is replaced to Gal. GlcNAcβ1–6Gal also lacks Galβ1–3 structure, which is the biosynthetic precursor of the core 2 oligosaccharide (36). Nevertheless, Bowman *et al.* (35) obtained highly interesting results (35). GlcNAc6ST-1, -2, -3, and -4 (C6ST-2/GST-5) utilize GlcNAcβ1–6Gal structure as a substrate but not Galβ1–4GlcNAcβ1–6Gal structure. GlcNAc6ST-2, -3, and -4 but not GlcNAc6ST-1 use the Siaα2–3Galβ1–4GlcNAcβ1–6Gal structure (35). Therefore, the sialylated structure can be a precursor of the 6-sulfated structure. GlcNAc6ST-3 distinguishes the C-4 hydroxyl group of the penultimate sugar residue in the substrate, because it acts on GlcNAcβ1–6Gal structure (35) but not on GlcNAcβ1–6Man structure as reported here. However, the enzyme is insensitive to the presence or absence of an *N*-acetyl group at the C-2 position of the penultimate sugar residue. As substrates, it uses both GlcNAcβ1–6Gal (35) and GlcNAcβ1–6GalNAc structures as shown in this study. Among five different substrates examined in this study, GlcNAc6ST-3 acted only on the core 2 structure. The inability to act on GlcNAc-Man structures and on *N*-acetyllactosamine oligomer is consistent with the view that GlcNAc6ST-3, which is present in the intestine, is involved in sulfation of mucin oligosaccharides in the organ. Human C-GlcNAc6ST, the fifth member of the GlcNAc6ST family, and mouse GlcNAc6ST-3 have the ability to produce keratan sulfate (37). The latter enzyme has been proposed to be an orthologue of human C-GlcNAc6ST (37). However, as reported here, human GlcNAc6ST-3 did not act on *N*-acetyllactosamine oligomer. The orthologous relationships between human C-GlcNAc6ST and human and mouse GlcNAc6ST-3 remain to be established.

Recently, Yamashita and co-workers (34) reported the presence of a GlcNAc6ST, which acts on the core 3 structure in mucinous adenocarcinoma and in adenocarcinoma with a mucinous component . Here, we showed that only GlcNAc6ST-2 acted on core 3. It is likely that GlcNAc6ST-2 is the enzyme with cancer-associated expression and is involved in the syn-

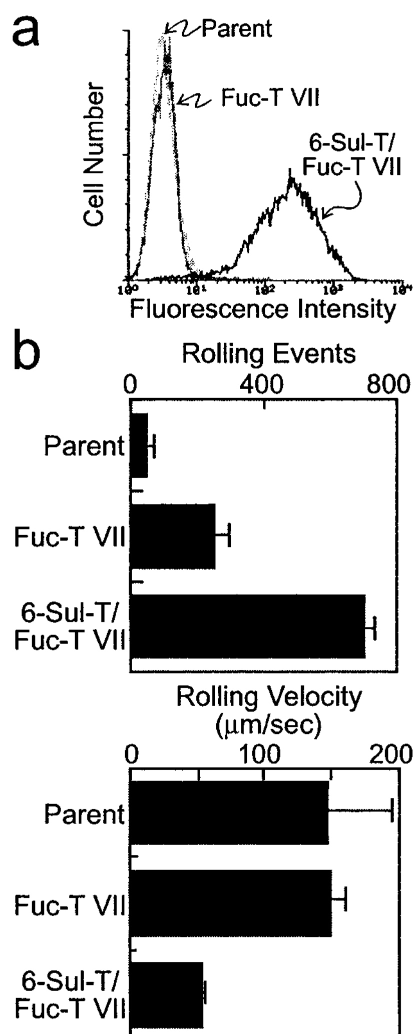

FIG. 3. **Rolling of L-selectin-expressing cells in shear flow on ECV304 cells transfected with GlcNAc6ST-1 and/or Fuc-T VII cDNAs.** *a*, flow cytometric analysis of ECV304 cells transfected with or without Fuc-T VII cDNA or double-transfected with GlcNAc6ST-1 and Fuc-T VII cDNAs using the G152 antibody specific to sialyl-6-sulfo-Lewis X. *b*, rolling events and rolling velocity of L-selectin-expressing cells (300.19/L-selectin, for review see Ref. 27) on the ECV304 cells at 1.0 dyne/cm^2 were analyzed as described under "Experimental Procedures." Data are presented as mean ± S.D. of total rolling events.

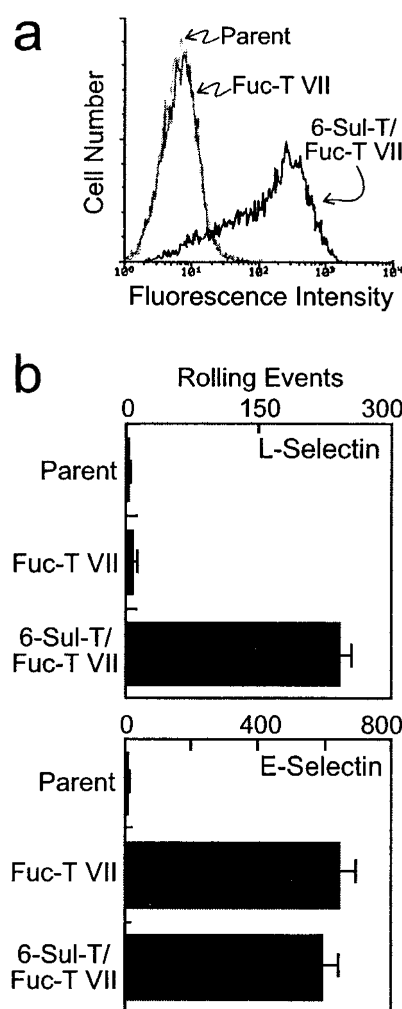

FIG. 4. **Rolling of L- or E-selectin-expressing cells in shear flow on COS-7 cells transfected with GlcNAc6ST-1 and/or Fuc-T VII cDNAs.** *a*, flow cytometric analysis of COS-7 cells transfected with or without Fuc-T VII cDNAs or double transfected with GlcNAc6ST-1 and Fuc-T VII cDNAs using G152 antibody specific to sialyl-6-sulfo-Lewis-X. *b*, rolling events of L-selectin-expressing cells (300.19/L-selectin, for review, see Ref. 27) or E-selectin-expressing cells (300.19/E-selectin, for review, see Ref. 27) at 1.0 dyne/cm^2 were analyzed as described under "Experimental Procedures." Data are presented as the mean ± S.D. of total rolling events.

thesis of a cancer-associated carbohydrate epitope. Indeed, the expression of GlcNAc6ST-2 mRNA was clearly detected in mucinous adenocarcinoma but only very weakly or not detectable in adjacent normal mucosa. Two cell lines with the characteristics of mucinous adenocarcinoma also strongly expressed GlcNAc6ST-2.

These results are important in terms of the participation of GlcNAc6STs in the synthesis of L-selectin ligands. The interaction of L-selectin ligands in high endothelial venules with L-selectin is the initial step of lymphocyte homing to lymph nodes (38). A sulfate group is essential for ligand function (39), and recent studies (19, 40) have revealed the importance of sialyl-6-sulfo-Lewis-X as the ligand structure. Because the L-selectin ligand is carried by sialomucins such as CD34 (41) and GlyCAM-1 (42), the sialyl-Lewis-X epitope is expected to be present on *O*-linked glycans such as those with core 2 oligosaccharides. Indeed, knockout mice deficient in core 2 oligosaccharide structure have reduced L-selectin ligand activity (43). Thus, the ability to act on branched *O*-linked glycans might be necessary to participate in the synthesis of L-selectin ligand. The present results demonstrated that GlcNAc6ST-1 as well as GlcNAc6ST-2 fulfilled these requirements. The capability to form sialyl-6-sulfo-Lewis-X in cooperation with Fuc-T VII has been verified in the case of GlcNAc6ST-1 (19) and GlcNAc6ST-2 (17). *In situ* hybridization analyses revealed GlcNAc6ST-1 and GlcNAc6ST-2 transcripts in high endothelial venules of lymph nodes (13, 17, 18). The adhesion of L-selectin-expressing cells to ECV304 cells doubly transfected with GlcNAc6ST-1 and Fuc-T VII cDNAs has been demonstrated (19). However, in terms of the rolling of L-selectin-expressing cells in shear flow, L-selectin ligand activity was not detected in cells doubly transfected with GlcNAc6ST-1 and Fuc-T VII cDNAs (18), whereas double transfection with GlcNAc6ST-2 and Fuc-T VII cDNAs successfully reconstituted L-selectin ligand activity (18, 44). Because the results of Hiraoka *et al.* (18) contrasted with our observations regarding the substrate specificities of the two enzymes, we re-examined the ability of GlcNAc6ST-1 in reconstitution of L-selectin ligand involved in rolling. As reported here, GlcNAc6ST-1 in cooperation with Fuc-T VII successfully reconstituted the rolling-inducing activity in two different cell lines. We consider that the different results were obtained by the usage of different cell lines. GlcNAc6ST-1 has a lower K_m value

than GlcNAcβ1–6ManOMe as compared with GlcNAc6ST-2 (Table I), although K_m value for core 2 (Table I) and relative activities of GlcNAcβ1–6ManOMe and core 2 (Table II) were not different between GlcNAc6ST-1 and GlcNAc6ST-2. Thus, in cells that abundantly express Asn-linked oligosaccharides, sulfation of mucin-type oligosaccharides by GlcNAc6ST-1 might be hindered.

Our tentative conclusion is that both GlcNAc6ST-1 and GlcNAc6ST-2 are involved in formation of the L-selectin ligand, even though GlcNAc6ST-1 has a broad distribution, and GlcNAc6ST-2 is preferentially located in high endothelial venules. In terms of the involvement of fucosyltransferases in the formation of selectin ligands, Fuc-T VII, which shows a restricted expression pattern, is primarily involved in the formation of sialyl-Lewis-X structures in high endothelial venules but with lesser contribution of Fuc-T IV (45). Furthermore, both Fuc-T IV and Fuc-T VII participate in the formation of selectin ligands in skin venules (46) and neutrophils (47). An analogous situation may be present also for GlcNAc6ST-1 and GlcNAc6ST-2. Very recently, GlcNAc6ST-2 gene has been deleted (48). Lymphocyte homing in the deficient mice was significantly reduced to both peripheral lymph nodes and mesenteric lymph nodes to 50 and 69% of wild-type levels, respectively (48). These data clearly establish the importance of GlcNAc6ST-2 in lymphocyte homing but suggest the additional L-selectin ligands that are formed by an enzyme different from GlcNAc6ST-2. GlcNAc6ST-1 is implicated as a candidate enzyme (48). Definitive conclusions concerning the role of GlcNAc6ST-1 must await the deletion of the gene.

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