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Redefined Substrate Specificity of ST6GalNAc II: A Second Candidate Sialyl-Tn Synthase

Mari Kono,[1] Tetsuro Tsuda,* Shunichiro Ogata,† Shou Takashima,[2] Hong Liu,[3] Toshiro Hamamoto,[4] Steven H. Itzkowitz,† Shinichiro Nishimura,* and Shuichi Tsuji[5]

*Molecular Glycobiology, Frontier Research Program, Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-0198, Japan; *Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, 060-0810 Japan; and †Division of Gastroenterology, Mount Sinai Medical Center, One Gustave L. Levy Place, New York, New York 10029-6574*

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The acceptor substrate specificities of ST6GalNAc I and II, which act on the synthesis of O-linked oligosaccharides, were reexamined using ovine submaxillary mucin, [Ala-Thr(GalNAc)-Ala]$_n$ polymer (n = 7–11). It has been suggested that only ST6GalNAc I can synthesize carbohydrate structures of sialyl-Tn-antigen; i.e., NeuAcα2-6GalNAc-*O*-Thr/Ser [Kurosawa *et al., J. Biol. Chem.* 269, 19048–19053 (1994)] based on the result that ST6GalNAc I, not ST6GalNAc II, exhibited activity toward asialoagalacto-fetuin. In this study, we present evidence that both ST6GalNAc I and II exhibit activity toward asialo-OSM (ovine submaxillary mucin) and [Ala-Thr(GalNAc)-Ala]$_n$ polymer (n = 7–11) which have only the GalNAc-*O*-Thr/Ser-structures. These results strongly indicate that not only ST6GalNAc I but also II are candidates for sialyl-Tn synthases.

Key Words: **sialyl-Tn; sialyltransferase; ST6GalNAc I; ST6GalNAc II.**

Abbreviations used: OSM, ovine submaxillary mucin; BSM, bovine submaxillary mucin; kb, kilobase(s); PCR, polymerase chain reaction; STn, sialyl-Tn antigen. The abbreviated nomenclature for cloned sialyltransferases follows the system of Tsuji *et al.* (1).

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[1] Present address: Genetics of Development and Disease Branch, Building 10, Room 9D-11, National Institutes of Health, 10 Center DR MSC 1810, Bethesda, MD 20892-1810.

[2] Present address: Laboratory for Neural Regeneration, Brain Science Institute, RIKEN, Wako, Saitama 351-0198, Japan.

[3] Present address: Department of Biochemistry and Molecular Biology, Georgetown University, Medical Center, 3900 Reservoir Road, NW, Washington, DC 20007.

[4] Present address: Department of Biochemistry, Jichi Medical School, Minamikawachi, Tochigi 329-0498, Japan.

[5] To whom correspondence should be addressed. Fax: +81-463-85-5301. E-mail: stsuji02@yhb.att.ne.jp.

The sialyl-Tn antigen (STn; NeuAcα2-6GalNAc-*O*-Thr/Ser) is a mucin-associated carbohydrate antigen, an epitope that can be expressed in a tumor-associated fashion in different organs (2). The addition of a sialic acid through an α2-6-linkage to GalNAc-*O*-Thr/Ser prevents the elongation of *O*-linked oligosaccharides (3). Thus, the enzyme which catalyzes this reaction is a key enzyme for *O*-linked oligosaccharide biosynthesis. The transfer of sialic acid with an α2-6-linkage to N-acetylgalactosamine (GalNAc) from CMP-sialic acid is catalyzed by a family of sialyltransferases, GalNAc α2-6-sialyltransferases (ST6GalNAc-family).

The cDNAs of six members of ST6GalNAc-family (ST6GalNAc I–VI) have been currently cloned from chicken (cST6GalNAc I and II) (4, 5), mouse (mST6GalNAc II–VI) (6–10, 13), rat (rST6GalNAc III) (11) and human (hST6GalNAc I, II, and VI) (12). Among them, ST6GalNAc III–VI exhibit restricted substrate specificity, utilizing only the NeuAcα2-3Galβ1-3GalNAc-sequence as an acceptor. However, there are some differences in their substrate preferences. ST6GalNAc III can transfer sialic acid to both NeuAcα2-3Galβ1-3GalNAc-*O*-Ser/Thr and ganglioside GM1b. ST6GalNAc IV exhibits strong activity toward NeuAcα2-3Galβ1-3GalNAc and *O*-glycans. ST6GalNAc V and VI may be the candidate for GD1α synthase. On the other hand, ST6GalNAc I exhibits the broadest substrate specificity toward GalNAc-*O*-Ser/Thr, Galβ1-3GalNAc-*O*-Ser/Thr, and NeuAcα2-3Galβ1-3GalNAc-*O*-Ser/Thr (4, 6, 12). ST6GalNAc II was reported to exhibit activity toward Galβ1-3GalNAc-*O*-Ser/Thr but not toward GalNAc-*O*-Ser/Thr (5). Thus, only ST6GalNAc I was considered to be a candidate for STn synthase. In this study, however, we found that ST6GalNAc II also exhibits activity toward GalNAc-*O*-Ser/Thr, suggesting that ST6GalNAc II is a second candidate for STn synthase.

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TABLE I

Comparison of Acceptor Substrate Specificity of Mouse ST6GalNAc I and II

Acceptor	Representative structures of carbohydrates	Relative activity (%)	
		ST6GalNAc I	ST6GalNAc II
Fetuin	NeuAcα2-3Galβ1-3GalNAc-*O*-Ser/Thr NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAc-*O*-Ser/Thr NeuAcα2-6(3)Galβ1-4GlcNAc-R	88.0	92.0
Asialofetuin	Galβ1-3GalNAc-*O*-Ser/Thr Galβ1-3GalNAc-*O*-Ser/Thr Galβ1-4GlcNAc-R	100.0	100.0
BSM	NeuAcα2-3Galβ1-3GalNAc-*O*-Ser/Thr NeuAcα2-6GalNAc-*O*-Ser/Thr	4.5	4.0
Asialo-BSM	Galβ1-3GalNAc-*O*-Ser/Thr GalNAc-*O*-Ser/Thr	160.0	160.0
OSM	NeuAcα2-6GalNAc-*O*-Ser/Thr GalNAc-*O*-Ser/Thr	4.3	3.5
Asialo-OSM	GalNAc-*O*-Ser/Thr	46.0	41.0
α1 acid glycoprotein	NeuAcα2-6(3)Galβ1-4GlcNAc-R	0.0	0.0
Asialo α1 acid glycoprotein	Galβ1-4GlcNAc-R	0.0	0.0
AsialoGM1	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer	0.0	0.0
GM1b	NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer	0.0	0.0
[Ala-Thr(GalNAc)-Ala]$_n$	GalNAc-*O*-Thr	0.7	0.8

Note. R represents the remainder of the *N*-linked oligosaccharide chain. The table shows the enzyme activities relative to the incorporation of sialic acid into asialofetuin. Each glycoprotein was used at the concentration of 5 mg/ml. [Ala-Thr(GalNAc)-Ala]n, Aialo GM1 and GM1b were used at the concentration of 0.1 mM. A value of 0.0 indicates less than 0.1%.

MATERIALS AND METHODS

Materials. The materials used in this study were essentially the same as in previous studies (6, 7). LipofectAMINE and macrophage SFM were purchased from GIBCO BRL. IgG-sepharose and CMP-[^{14}C]NeuAc were from Amarsham Pharmacia. Glycoproteins (fetuin, asialofetuin, α1 acid glycoprotein, bovine submaxillary mucin (BSM) and asialo BSM) were purchased from Sigma. The synthesis of [Ala-Thr(GalNAc)-Ala]$_n$ polymer (n = 7–11) was reported elsewhere (14). Ovine submaxillary mucin (OSM) was purified according to Tettamanti and Pigman (15). Asialo α1 acid glycoprotein and Asialo OSM were prepared as follows: These glycoproteins (10 mg/ml) were incubated at 50°C in 0.1 N H_2SO_4. After incubation, the solutions were neutralized by 1 N $Ba(OH)_2$ and desalted by ethanol precipitation. The precipitates were dissolved in water and used as substrates for enzyme reactions.

Sialyltransferase assay. Truncated forms of mouse ST6GalNAc I and II, lacking N-terminal putative cytosolic and transmembrane region, were prepared by PCR amplification as described before (7). The amplified fragments were inserted into a pcDSA vector (16). The resulting plasmid was designated as pcDSA-ST6GalNAc I or II, which encodes a fusion protein of the IgM signal peptide sequence, a protein A IgG binding domain and a truncated form of mST6GalNAc I or II.

COS-7 cells on a 100-mm plate (5×10^6) were transiently transfected with pcDSA-ST6GalNAc I or II DNAs (10 μg) using LipofectAMINE (GIBCO) reagent. After 5 h and 24 h of transfection, medium was changed to 2% DMEM containing FCS and macrophage SFM (GIBCO), respectively. From 48 h to 72 h after transfection, medium was collected and centrifuged at 800*g* for 10 min at 4°C to remove cell contamination. The protein A fusion enzyme, which was expressed in culture medium of COS-7 cells, was absorbed to IgG–Sepharose gel at 4°C for 16 h, and the IgG–Sepharose gel bound protein A fusion enzyme was used as enzyme source. Each reaction mixture comprised 50 mM MES buffer (pH 6.0), 10 mM $MgCl_2$, 2 mM $CaCl_2$, 200 μM CMP-[^{14}C]NeuAc (7.1 kBq), acceptor substrate (5 mg/ml glycoprotein, 0.1 mM glycolipid and 0.1 mM [Ala-Thr(GalNAc)-Ala]$_n$ polymer), and enzyme preparation, in total volume of 20 μl. After 4 h of incubation at 37°C, the reaction was terminated by the addition of SDS-PAGE loading buffer, and then the mixtures were directly subjected to SDS-PAGE (for glycoprotein acceptors). For glycolipid acceptors, the incubation mixtures were applied on a C-18 column (Sep-Pak Vac, 100 mg; Waters, Milford, MA), as described previously (16). When [Ala-Thr(GalNAc)-Ala]$_n$ polymer was used as acceptor substrate, the reaction mixture was directly subjected to HPTLC with a solvent system of 1-propanol:1-buthanol:water = 3:1:2. The radioactive materials were visualized with a BAS2000 radio image analyzer (Fuji Film, Japan).

RESULTS AND DISCUSSION

The substrate specificity of ST6GalNAc II was previously described using fetuin, asoalo-fetuin, agalactoasialo-fetuin, and other substrates (7). These results demonstrated that ST6GalNAc II requires Gal-residues in Galβ1-3GalNAc-Ser/Thr structure, since the enzyme does not exhibit activity toward agalactoasialo-fetuin which is considered to have GalNAc-Ser/Thr structure. Using the recently obtained substrates ovine submaxillary mucin (OSM) and [Ala-Thr(GalNAc)-Ala]$_n$ polymer (n = 7–11), which have the GalNAc-Ser/Thr structure, we re-examined the substrate specificity of mouse ST6GalNAc I and II. To achieve this, we constructed expression vectors, pcDSA-ST6GalNAc I and II, respectively, which express the fusion gene encoding the secretable form of ST6GalNAc I and II fused to the IgG-binding domain of *Staphylococcus aureus* protein A. Each vector was

TABLE II

Comparison of Substrate Specificities of the Mouse ST6GalNAc Family

Substrate	ST6GalNAc					
	I	II	III(8)	IV(8)	V(9, 10)	VI(13)
GalNAc-Ser/Thr	C	C	–[b]	–	–	–
Galβ1-3GalNAc-Ser/Thr	A	A	–	–	–	–
NeuAcα2-3Galβ1-3GalNAc-Ser/Thr	B	B	A	B	B	–
Galβ1-3GalNAc	–	–	–	–	–	–
NeuAcα2-3Galβ1-3GalNAc	–	–	C	A	–	–
GM1b[a]	–	–	B	C	A	A

Note. A, B, C: Significant enzyme activity could be detected. The activity strength order is A > B > C. Note that this is only the priority order within each enzyme, not across enzymes.

[a] NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′-Cer.

[b] Enzyme activity could not be detected or was negligible.

transfected into COS-7 cells, and the protein A-fused ST6GalNAc I and II was successfully secreted into the media. The secreted enzyme was adsorbed to IgG-Sepharose gel and was used as an enzyme source.

Table I shows the substrate acceptor specificity of ST6GalNAc I and II. As previously reported (6, 7), both ST6GalNAc I and II efficiently transferred sialic acids to asialo fetuin and asialo BSM. No significant activity was observed toward α1 acid glycoprotein, which has *N*-linked oligosaccharides. In addition, oligosaccharides and glycosphingolipids did not serve as acceptors for ST6GalNAc I and II.

Surprisingly, we found that both enzymes have almost the same activity toward asialo-OSM and $[Ala-Thr(GalNAc)-Ala]_n$ polymer. The carbohydrate structures of OSM are reported to be (NeuAcα2-6)GalNAc-*O*-Ser/Thr (17), thus almost all the carbohydrate structures of asialo-OSM are GalNAc-*O*-Ser/Thr-structure. These results clearly show that not only ST6GalNAc I but also II exhibit activity toward GalNAc-*O*-Ser/Thr structure. According to these results, we revised the table of substrate specificity of ST6GalNAc-family as shown in Table II. We believe that ST6GalNAc II is also the candidate for the sialyl-Tn synthase.

Although ST6GalNAc I and II exhibit similar substrate specificity, the expression patterns of these genes were quite different. The expression of the ST6GalNAc II gene was ubiquitously observed in mouse tissues (7), whereas the expression of the mouse ST6GalNAc I gene was limited in submaxillary gland, mammary gland, colon, and spleen. In addition, the expression level of the ST6GalNAc II gene was much higher than that of the ST6GalNAc I gene (6). Siaα2-6GalNAc-*O*-Ser/Thr structures occur as a cancer-associated carbohydrate antigen, STn. It is known that elevation of STn antigen correlates with a poor prognosis of gastric cancer (18) and colon cancer (19) patients. In order to better elucidate the relationship between STn antigen expression and the biology of cancers, it would be important to examine the expression of STn synthases in carcinogenesis. Since the mRNA expression of ST6GalNAc I was not well correlated with the expression of sialyl-Tn antigen during the maturation of goblet cells in intestinal metaplastic glands (13), we are now investigating the expression patterns and biological functions of STn synthases.

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