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Incorporation of *N*-acetylgalactosamine into consecutive threonine residues in MUC2 tandem repeat by recombinant human *N*-acetyl-D-galactosamine transferase-T1, T2 and T3

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Abstract An oligopeptide containing three consecutive Thr residues mimicking the tandem repeat portion of MUC2 (PTTTPLK) was investigated for the acceptor specificity to UDP-*N*-acetyl-D-galactosamine:peptide *N*-acetylgalactosaminyltransferase isozymes, UDP-*N*-acetyl-D-galactosamine:peptide *N*-acetylgalactosaminyltransferase-T1, T2 and T3. The enzymatic reaction products were fractionated by the reversed-phase high performance liquid chromatography, then characterized by matrix-assisted laser desorption ionization time of flight mass spectrometry and by a peptide sequencing analysis. A maximum of two, one or three *N*-acetyl-D-galactosamine residues was transferred by UDP-*N*-acetyl-D-galactosamine: peptide *N*-acetylgalactosaminyltransferase-T1, T2 or T3, respectively. The preferential orders of *N*-acetyl-D-galactosamine incorporation were Thr-2, then Thr-4 for UDP-*N*-acetyl-D-galactosamine:peptide *N*-acetylgalactosaminyltransferase-T1, Thr-2 for UDP-*N*-acetyl-D-galactosamine:peptide *N*-acetylgalactosaminyltransferase-T2 and Thr-4, Thr-3, then Thr-2 for UDP-*N*-acetyl-D-galactosamine:peptide *N*-acetylgalactosaminyltransferase-T3.

Key words: Mucin; *N*-acetylgalactosaminyltransferase; *O*-glycosylation; MUC2; Tn antigen

1. Introduction

UDP-*N*-acetyl-D-galactosamine (GalNAc):polypeptide GalNAc-transferases (GalNAc-Ts, EC 2.4.1.41) transfer GalNAc to Ser and Thr residues in a polypeptide. Four isozymes of human GalNAc-T have been identified and characterized to date: T1, T2 [1], T3 [2] and T4 [3]. Additional isozymes have been identified in rat and *Caenorhabditis elegans* [4–6]. Gene cloning studies suggested that there are many GalNAc-T isoforms having different acceptor substrate specificities [7]. For example, Wandall and co-workers reported, using recombinant human GalNAc-T1, T2 and T3, that these three enzymes had different kinetic properties where T1 and T3 showed a strong preference for TNTS and T2 showed a preference for TNST. However, they did not have a positional preference towards Thr residues in the tandem repeat portion of MUC1 mucin [8].

Previously, we reported that consecutive Thr residues from the MUC2 tandem repeat domain were fully glycosylated using detergent-soluble microsome fractions from the human colon carcinoma cell line LS174T as a source of GalNAc-Ts [9]. In the present study, we focused to define substrate specificities towards three consecutive Thr residues mimicking MUC2 tandem repeats, PTTTPLK. Different specificities and different kinetic properties were observed with GalNAc-T1, T2 and T3.

2. Materials and methods

2.1. Synthesis of acceptor substrates

Synthetic oligopeptides used as acceptor substrates were synthesized with a Model 9020 peptide synthesizer (Milligen, Burlington, MA, USA). Peptides corresponding to the tandem repeat domain of the MUC2 mucin core polypeptide (PTTTPITTTTTVTPTPTPTGTQT) [10] were synthesized with a lysine residue attached at the C-terminus. These are PTTTPITTTTK (FM2-1), TVTPTPTPTGK (FM2-3) and PTTTPLK (FM2-12). All peptides were labelled with fluorescein isothiocyanate at its N-terminal amino acid at pH 7.5 adjusted with 100 mM HEPES buffer.

2.2. Preparation of recombinant GalNAc-Ts

Soluble recombinant GalNAc-T1, T2 and T3 were prepared as described previously [8]. Briefly, plasmid pAcGP67-GalNAc-T1-sol, pAcGP67-GalNAc-T2-sol and pAcGP67-GalNAc-T3-sol were co-transfected with Baculo-GOLD DNA (Pharmingen) to Sf9 cells. Recombinant GalNAc-T1, T2 and T3 were purified from the spent media. One unit of enzyme is defined as the amount of enzyme that will transfer 1 μmol of GalNAc in 1 min using different acceptor substrates [8].

2.3. Assays of GalNAc transferase activity

The standard enzyme reaction mixture consisted of 20 mM HEPES buffer (pH 7.5), 5 mM $MnCl_2$, 5 mM 2-mercaptoethanol, 0.1% Triton X-100, 1 mM UDP-GalNAc (Sigma, St. Louis, MO, USA), 20 μM peptide and 0.25 mU recombinant enzyme or 100 μg microsome fraction from the LS174T human colon carcinoma cell line [9] in a final volume of 50 μl. Reactions were performed at 37°C for up to 24 h and were terminated by adding 300 μl of 5 mM EDTA. An equal amount of additional enzyme and UDP-GalNAc was added after 6 h. For kinetic studies, 0.1 mU of recombinant enzyme and UDP-[^{3}H]GalNAc (0.1 mCi, Dupont-NEN, Boston, MA, USA) were used. Reaction mixtures were applied on columns packed with C18-silica, washed with solvent A (0.05% trifluoroacetic acid (TFA) in water) and eluted with solvent B (0.05% TFA, 70% propanol in acetonitrile). Radioactivity incorporated into peptides was measured with a liquid scintillation counter using RedyCap (Beckman, Fullerton, CA, USA).

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Abbreviations: GalNAc, *N*-acetyl-D-galactosamine; GalNAc-T, UDP-GalNAc:polypeptide α-GalNAc-transferase (EC 2.4.1.41); HPLC, high performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid

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2.4. Monitoring of in vitro O-glycosylation by reversed-phase HPLC

The glycosylated peptides were separated by reversed-phase high performance liquid chromatography (HPLC) (JASCO, Tokyo, Japan). A PURESIL (C18, 6×150 mm, Nihon Waters, Tokyo, Japan) was used. The column was eluted with a linear gradient ranging from 10 to 30% solvent B in solvent A at a flow rate of 2 ml/min for 30 min. Eluates were monitored by the fluorescence intensity at 520 nm (ex: 492 nm).

2.5. Matrix-assisted laser desorption ionization mass spectrometry analysis of glycosylated peptides (MALDI-TOF MS)

Glycosylated peptides were applied on a tip and mixed with a 10 mg/ml solution of α-cyano-4-hydroxycinnamic acid dissolved in 0.1% TFA-50% ethanol in water. All mass spectra were obtained on a Voyager Elite (Nippon PerSeptive Biosystems, Tokyo, Japan) in the linear mode with the delayed extraction setting.

2.6. Amino Acid Sequencing

Pulsed liquid Edman degradation amino acid sequencing of glycopeptides was performed on the Applied Biosystems 490 Procise protein sequencing system (Perkin Elmer, Norwalk, CT, USA). With this system, a phenylthiohydantoin (PTH) derivative of GalNAc-attached Thr was identified as a pair of peaks eluted near the positions of PTH-Ser and PTH-Thr [11]. Amino acid sequencing of fully glycosylated peptide ($PT_{GalNAc}T_{GalNAc}T_{GalNAc}PLK$) confirmed these eluting positions.

3. Results

3.1. Incorporation of GalNAc into three consecutive threonine residues using a microsomal fraction of LS174T cells

We reported previously that the FM2-1 peptide (PTTTPITTTTLK) having three and four consecutive Thr residues was fully glycosylated by a microsome fraction of the colon carcinoma cell line LS174T (Iida, S. et al., submitted for publication). An oligopeptide mimicking the N-terminal portion of FM2-1 (FM2-12, PTTTPLK) was prepared and examined for whether the crude enzyme also achieved complete glycosylation. FM2-12 peptide was incubated with the microsome fraction from colon carcinoma cell line LS174T up to 18 h and subjected to reversed-phase HPLC. Four peaks including the original oligopeptide were observed. Materials corresponding to these peaks were isolated and analyzed by MALDI-TOF MS. The results showed that three GalNAc residues can be transferred in the presence of the microsome fraction to the FM2-12 peptide (Fig. 1). The major peak from the reversed-phase HPLC separation gave a mass of 1758.5 (Fig. 1d), indicating that three GalNAc residues were incorporated. Peptides with one or two GalNAc residues were also

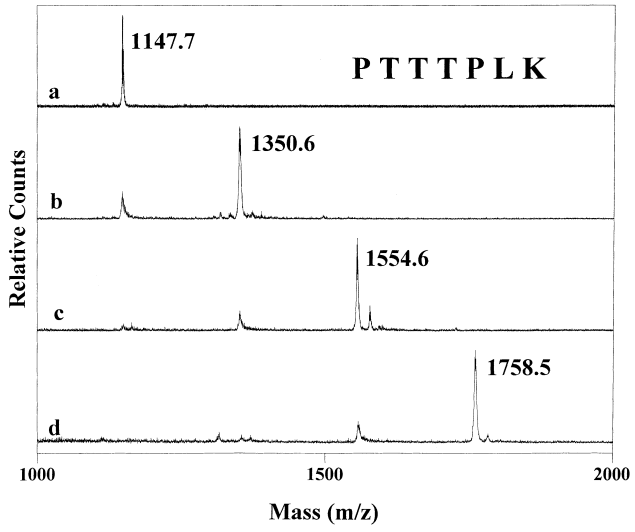

Fig. 1. Representative profiles of MALDI-TOF MS of glycosylated FM2-12 peptide separated by reversed-phase HPLC. FM2-12 peptides were incubated with detergent-solubilized microsome fractions from human colon carcinoma LS174T cells for 18 h. The products were separated by reversed-phase HPLC on a C18 column. Eluates corresponding to four major peaks were pooled and evaporated to dryness. Analysis with MALDI-TOF MS was performed as previously described [9]. Mass indicates (M+H)+form. a: The profile of unglycosylated FM2-12 peptide (predicted mass: 1146.3). b: FM2-12 peptide with a single GalNAc residue attached (predicted mass: 1349.5). c: FM2-12 peptide with two GalNAc residues attached (predicted mass: 1552.7). d: FM2-12 peptide with three GalNAc residues attached (predicted mass: 1755.9).

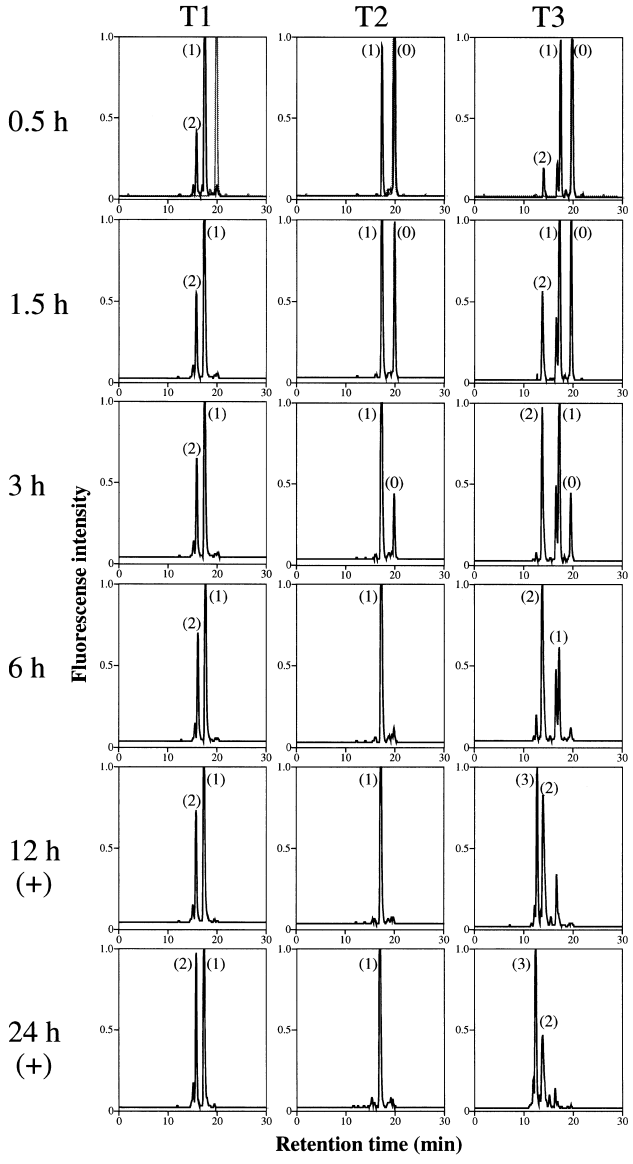

Fig. 2. Elution profiles of FM2-12 peptides incubated with recombinant GalNAc-T1, T2 or T3 on the reversed-phase HPLC with a C18 column. FM2-12 peptide was incubated with each recombinant GalNAc-T for the indicated periods and separated by reversed-phase HPLC. After incubation for 6 h, additional enzyme and UDP-GalNAc were added as indicated by (+). (1), (2) and (3) indicated eluting positions of the peptide with one, two and three GalNAcs attached as revealed by MALDI-TOF MS.

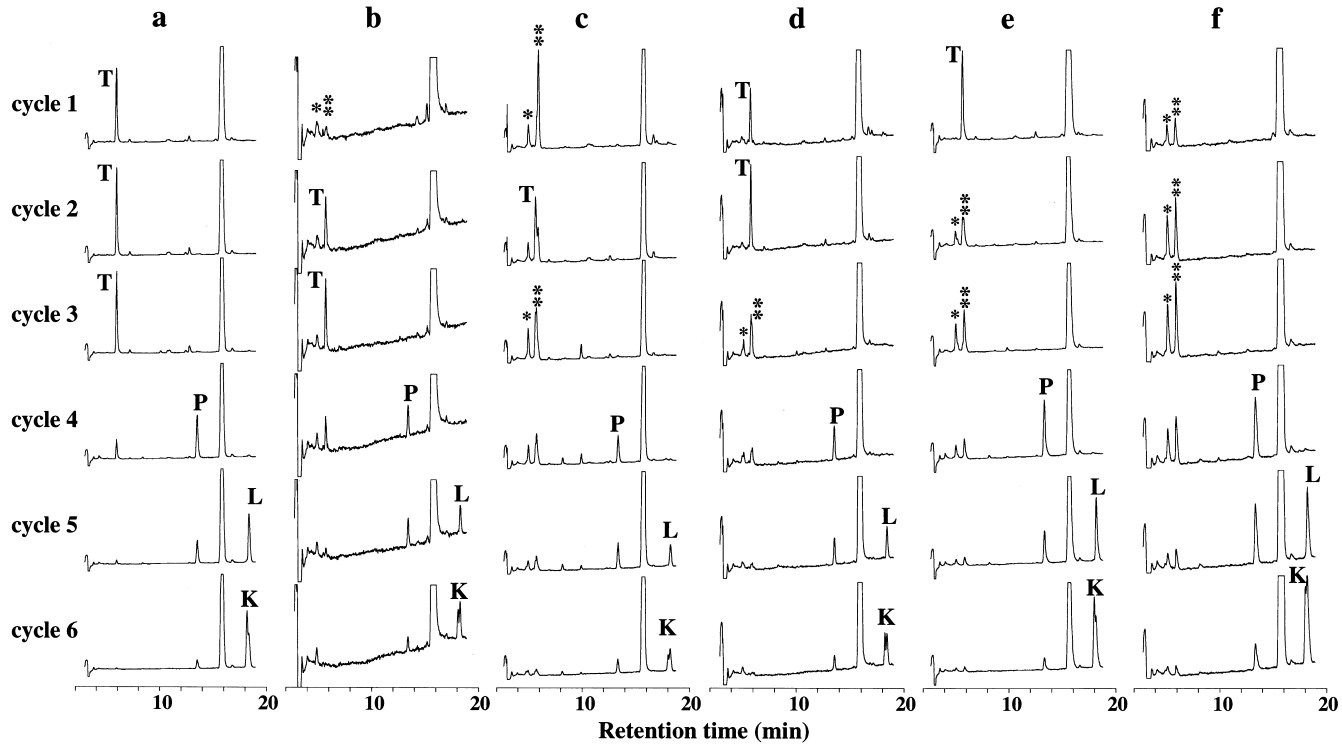

Fig. 3. Profiles of amino acid sequencing chromatograms of the oligopeptide FM2-12 and its derivatives containing incorporated GalNAc residues after incubation with GalNAc-T1, T2 and T3. Peaks after separation by the reversed-phase HPLC were analyzed on the Applied Biosystems 490 Procise protein sequencing system. a: Untreated FM2-12. b and d: FM2-12 with one GalNAc residue. c and e: FM2-12 with two GalNAc residues. f: FM2-12 with three GalNAc residues. Samples shown in b and c were obtained from an incubation mixture with recombinant GalNAc-T1. d, e and f were obtained from an incubation mixture with recombinant GalNAc-T3. Asterisks indicate putative peaks of PTH derivatives of GalNAc-Thr (T_{GalNAc}).

observed having masses of 1350.6 and 1554.6, respectively (Fig. 1b, c), which were prominent when the incubation time was less than 6 h. Thus, the FM2-12 peptide was shown to be useful as a good acceptor of GalNAc-Ts.

3.2. *Acceptor substrate specificity of three isozymes, GalNAc-T1, T2 and T3*

To define acceptor specificities of GalNAc-T1, T2 and T3 to the three consecutive Thr residues in FM2-12, this peptide was used as an acceptor for recombinant GalNAc-Ts. The peptide was incubated with recombinant GalNAc-T1, T2 or T3 for various time periods. The degree of glycosylation was monitored with reversed-phase HPLC (Fig. 2). Each peak was analyzed by MALDI-TOF MS, which revealed the number of attached GalNAc residues (data not shown). Under the condition used, GalNAc-T1 transferred a maximum of 2 mol of GalNAc residues within a short period. However, even after a prolonged incubation and with supplemented recombinant GalNAc-T1, an additional peak corresponding to the peptide with three GalNAc residues was not observed. With the same amount of GalNAc-T2, one GalNAc was transferred to the FM2-12 peptide after 24 h incubation. A small peak, apparently corresponding to the same glycopeptide with two GalNAc residue as seen with GalNAc-T1, was observed after prolonged incubation with an addition of GalNAc-T2 (not shown). Therefore, GalNAc-T1 and GalNAc-T2 are clearly different in their acceptor specificity. The preferential occupancy of GalNAc incorporation into three consecutive Thr residues by GalNAc-T1 was apparently two residues whereas that by GalNAc-T2 was one residue. On the contrary, GalNAc-T3 transferred three GalNAc to FM2-12 peptide within a 12 h time period. A peptide with two GalNAc residues formed with GalNAc-T3 eluted at a retention time of 13.5 min, which was different from the retention time of similar products obtained by the incubation of FM2-12 with GalNAc-T1 (15.1 min). Thus, apart from the different occupancy, GalNAc-T3 seemed to perform glycosylation in a reverse order compared to T1 and T2.

3.3. *Identification of N-acetylgalactosaminylated threonine residues*

Glycopeptides corresponding to three peaks were subjected to the amino acid sequencing analysis (Fig. 3). Analysis of the FM2-12 peptide with three attached GalNAc residues generated by the action of GalNAc-T3 (Fig. 3f) revealed that the PTH derivative of GalNAc-Thr was identified as a pair of peaks near the positions of PTH-Ser and PTH-Thr [10]. The peptide with a single GalNAc residue generated by the action of GalNAc-T1 was shown to have GalNAc at Thr-2 (Fig. 3b). The second GalNAc residue was shown to be attached to Thr-

Table 1
Preferential orders of the incorporation of GalNAc residues into a synthetic peptide FM2-12 mimicking a portion of the tandem repeat sequence of MUC2 incubated with UDP-GalNAc and GalNAc-T1, T2 or T3

	Pro	Thr-2	Thr-3	Thr-4	Pro	Leu	Lys
T1		1	–	2			
T2		1	–	–			
T3		3	2	1			

Table 2
Kinetics of GalNAc-T1, T2 or T3 when FM2-1, FM2-12 and FM2-3 are used as acceptors. The K_m and V_{max} values are calculated by the initial incorporation of [^{3}H]GalNAc into these peptides as described in Section 2

	T1		T2		T3	
	K_m (μM)	V_{max} (pmol/min)	K_m (μM)	V_{max} (pmol/min)	K_m (μM)	V_{max} (pmol/min)
FM2-1						
PTTTPITTTTK	23.9	51.5	25.2	4.5	30.7	8.3
FM2-12						
PTTTPLK	37.4	38.5	27.9	2.4	80.2	9.5
FM2-3						
TVTPTPTPTGK	16.0	50.2	8.7	28.2	50.4	61.8

4 (Fig. 3c). GalNAc-T2 seemed to be different from GalNAc-T1 because Thr-4 was only marginally glycosylated by this enzyme. In contrast, the peptide with a single GalNAc residue generated by the action of GalNAc-T3 was shown to have a glycosylated Thr-4 (Fig. 3d). The second GalNAc was apparently transferred to Thr-3 (Fig. 3e). Therefore, the preferential acceptor site for GalNAc-T3 should be different from that of GalNAc-T1 and T2. The estimated order of the GalNAc incorporation is summarized in Table 1. In the reversed-phase HPLC profile of the product with GalNAc-T3, an additional peak eluted slightly earlier than PTTT$_{GalNAc}$PLK. The sequence of this peptide was not analyzed due to the amount of available sample but is likely to be PTT$_{GalNAc}$TPLK.

3.4. Kinetic properties of three isozymes

Two oligopeptides corresponding to two different domains of the tandem repeat portion of MUC2 and FM2-12 peptide were compared for their acceptors specificity for GalNAc-T1, T2 and T3 (Table 2) using kinetic analysis as a parameter. Under the condition used in this analysis, the first GalNAc was incorporated. The FM2-1 and FM2-3 peptide contain consecutive and alternative Thr residues, respectively. The V_{max} of GalNAc-T1 was high with FM2-1 and FM2-3 (51.5 and 50.2 pmol/min). The V_{max} of T2 and T3 was higher with FM2-3 than with FM2-1 or FM2-12. Also, the K_m of GalNAc-T2 with FM2-3 was much lower than that with FM2-1 or FM2-12. These results indicated that GalNAc-T1, T2 and T3 possessed different specificities towards consecutive and alternating Thr residues. Also, the results suggested that FM2-12 was a suitable model of an acceptor with consecutive Thr residues.

4. Discussion

It is widely accepted that the glycosylation sites and the pattern of elongation of *O*-glycan on mucins depends both on the type of glycosyltransferases involved and on the structures of core polypeptides [12–14]. The profile of *O*-glycan attachment on MUC1 has been a focus of attention and it is believed that MUC1 of mammary fat globule contained more *O*-glycans than carcinoma-associated MUC1, although little is known about the distribution of *O*-glycan in MUC2, which contains consecutive Thr residues [10]. In the present study, substrate specificities of GalNAc-T1, T2 and T3 towards three consecutive Thr residues in a peptide mimicking the tandem repeat sequence of MUC2, PTTTPLK, was investigated. In this peptide, Leu was used in place of Ile in the MUC2 sequence. GalNAc-T3 was shown to have a unique acceptor specificity different from that of T1 or T2. (i) The preferential Thr residue of the initial GalNAc attachment was different from that of T1 or T2. (ii) All three Thr residues were derivatized with GalNAc by the action of T3 but not T1 or T2. These three enzymes were previously shown to share a similar acceptor site specificity towards the tandem repeat sequence of MUC1, which has three Thr residues separated within its tandem repeat unit [8]. Thus, GalNAc-T3 might play an important role in the glycosylation of consecutive Thr residues, at least when relatively short peptides used in this study were the substrate. In the present study, we used acceptor substrates with very short flanking sequences and attached fluorescein that might influence their acceptor specificity as indicated by Nishimori et al [12]. It was suggested by Nehrke and co-workers that charged amino acid residues at the amino-terminal side of Thr suppressed the GalNAc incorporation [15].

Three consecutive Thr residues of the tandem repeat portion of MUC2 were also shown to be completely glycosylated by a microsome fraction of LS174T colon carcinoma cells. Furthermore, the attachment positions of GalNAc residues of partially glycosylated FM2-1 in the presence of the microsome fraction was similar to those after incubation with GalNAc-T3 (not shown). These results suggest that the pattern of GalNAc incorporation into MUC2 in the presence of microsome fractions of LS174T cells may be attributed to GalNAc-T3. Because GalNAc-T3 was not previously detected in normal colonic epithelia [2], the expression might be associated with the growth of malignant epithelial cells in colons. Recently, Bennett and co-workers reported that two sites in the MUC1 tandem repeat portion, left unglycosylated after other GalNAc-Ts glycosylated the first three sites, were glycosylated by recombinant GalNAc-T4 [3]. Therefore, GalNAc-T4 might also contribute to dense glycosylation of MUC2 peptides generated in the presence of the microsome fraction of LS174T cells if this enzyme is expressed.

Using recombinant GalNAc-Ts and MUC2 peptide (PTTTPISTTTMVTPTPTPTC), kinetic parameters of GalNAc-T1, T2 and T3 were previously compared by Wandall and co-workers [8]. In the present study, the detailed specificity towards two different portions of the tandem repeat sequence of MUC2 with consecutive or alternating Thr residues was examined. Therefore, their and our results should not be directly compared. None the less, GalNAc-T2 showed a low K_m value with FM2-3 which corresponds to a portion having alternating Thr residues. This result was consistent with Wandall and co-workers' finding showing that GalNAc-T2 had a high affinity with the MUC2 peptide. Although alternating Thr residues were observed to serve as preferential substrates of bovine GalNAc-T1 [16], the significance of such a sequence in the regulation of *O*-glycosylation remains to be elucidated

In summary, the difference in the acceptor specificity of

GalNAc-Ts strongly suggests that *O*-glycosylation of mucins is a highly regulated process. Thus, the pattern of *O*-glycosylation should have a biological niche yet to be explored.

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