Glycoconj J (2010) 27:267–276
DOI 10.1007/s10719-009-9275-4

Loss of UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase 3 and reduced *O*-glycosylation in colon carcinoma cells selected for hepatic metastasis

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Received: 18 September 2009 / Revised: 4 December 2009 / Accepted: 9 December 2009 / Published online: 14 January 2010

Abstract *O*-glycosylation of mucin is initiated by the attachment of *N*-acetyl-D-galactosamine (GalNAc) to serine or threonine residues in mucin core polypeptides by UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases (ppGalNAc-Ts). It is not well understood how GalNAc attachment is regulated by multiple ppGalNAc-Ts in each cell. In the present study, the expression levels of murine ppGalNAc-Ts (mGalNAc-Ts), T1, T2, T3, T4, T6, and T7 were compared between mouse colon carcinoma colon 38 cells and variant SL4 cells, selected for their metastatic potentials, by using the competitive RT-PCR method. The expression levels of mGalNAc-T1, T2, and T7 were slightly higher in the SL4 cells than in the colon 38 cells, whereas the expression level of mGalNAc-T3 in the SL4 cells was 1.5% of that in the colon 38 cells. Products of enzymatic incorporations of GalNAc residues into FITC-PTTTPITTTTK peptide by the use of microsome fractions of these cells as the enzyme source were separated and characterized for the number of attached GalNAc residues and their positions. The maximum number of attached GalNAc residues was 6 and 4 when the microsome fractions of the colon 38 cells and SL4 cells were used, respectively. When the microsome fractions of the colon 38 cells were treated with a polyclonal antibody raised against mGalNAc-T3, the maximum number of incorporated GalNAc residues was 4. These results strongly suggest that mGalNAc-T3 in colon 38 cells is involved in additional transfer of GalNAc residues to this peptide.

Keywords O-glycosylation · ppGalNAc-T · UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferases · Mucin

Abbreviations

ABA	*Agaricus bisporus* agglutinin
ACA	*Amaranthus caudutus* agglutinin
AP	alkaline phosphatase
BSA	bovine serum albumin
α-CHCA	α-cyano-4-hydroxycinnamic acid
GalNAc	*N*-acetyl-D-galactosamine
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
MAbs	monoclonal antibodies
MALDI-TOF	matrix-assisted laser desorption ionization
MS	time-of-flight mass spectrometry
NRF-1	nuclear respiratory factor-1
P/CAF	p300/camp-response-element binding protein (CREB)-binding protein (CBP) associated factor

Electronic supplementary material The online version of this article (doi:10.1007/s10719-009-9275-4) contains supplementary material, which is available to authorized users.

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PNA	*Arachis hypogaea* (peanut) agglutinin
ppGalNAc-T	UDP-*N*-acetyl-α-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferases
PTH	phenylthiohydantoin
SNA	*Sambucus nigra* agglutinin
TFA	trifluoroacetic acid
Tn	GalNAc-Ser/Thr
UDP	uridine diphosphate
VVA-B4	*Vicia villosa* agglutinin-B4

Introduction

O-glycosylation of mucin is initiated by the attachment of *N*-acetyl-D-galactosamine (GalNAc) residues to serine and threonine residues on mucin core polypeptides by UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases (ppGalNAc-Ts). Fifteen human (hGalNAc-Ts) [1–14] and 3 murine (mGalNAc-T) [15–17]. Enzymes in this large glycosyltransferase family have also been found in other species [18]. Studies from many laboratories have revealed that these enzymes have unique substrate specificities [2–8, 14, 19–28]. Moreover, we have shown that the sites and the orders of GalNAc attachment toward FITC-PTTTPITTTTK peptide were strictly regulated, even when microsome fractions of human colon carcinoma LS174T cells, which contain a mixture of ppGalNAc-Ts, were used as the source of enzymes [29]. However, it was not previously confirmed that the sites and the degree of O-glycosylation through differential expression of ppGalNAc-Ts influenced the biological behavior of the cells, such as cell surface bindings of lectins. In the present report, we found that mouse colon carcinoma colon 38 cells [30] and SL4 cells [31], a metastatic variant of colon 38 cells, expressed differential levels of mGalNAc-Ts (T1, T2, T3, T4, T6, and T7). It was also shown in our previous work that cell surface levels of Tn antigen (GalNAc-α-Ser/Thr) were higher on the surfaces of colon 38 cells than SL4 cells [32]. Therefore, we prepared microsome fractions of these cells, reacted with FITC-PTTTPITTTTK peptide and evaluated whether the site and the number of attached GalNAc residues were different between these closely related cells. Because mGalNAc-T3 expression was observed to be very low in SL4 cells, microsome fractions of colon 38 cells were treated with anti-mGalNAc-T3 antibody and tested for their ability to incorporate GalNAc residues into FITC-PTTTPITTTTK peptide. The results indicated that mGalNAc-T3 in colon 38 cells is involved in additional incorporations of GalNAc residues to this peptide.

Results

Comparisons of ppGalNAc-Ts expressed by colon 38 cells and SL4 cells Binding profiles of plant lectins specific for relatively short *O*-glycans, PNA, ABA, VVA-B4, SNA, and ACA, to electrophoretically separated lysates of colon 38 cells and SL4 cells were compared (Fig. 1). Significantly higher levels of binding of these lectins to components at approximate molecular weights of 200 kDa and 120 kDa were observed with the lysates of colon 38 cells than with the lysates of SL4 cells. These components were detected with lectins specific for Tn (GalNAc-α-Ser/Thr) antigen and related epitopes and higher bindings of these lectins to the surfaces of colon 38 cells than SL4 cells were previously shown by flow cytometry [32]. Membranes reacted with AP-streptavidin showed non-specific binding to 116 kDa and 66 kDa. A possibility that colon 38 cells express glycoproteins with relatively short *O*-glycans in a different density from SL4 cells was suggested but should further be explored as the basis for the increased bindings of lectins.

Differential expression patterns of mGalNAc-Ts between these cells have already been suggested by the microarray analysis in a previous report [32]. We compared the expression patterns of mGalNAc-Ts in these cells using the competitive RT-PCR method (Fig. 2). Among 6 mGalNAc-Ts (T1, T2, T3, T4, T6, and T7) we tested, the expression levels of mGalNAc-T1, T2, and T7 were slightly higher in SL4 cells than in colon 38 cells. The expression level of mGalNAc-T3 was 65 times higher in colon 38 cells than in SL4 cells (Fig. 2b). The expression level of mGalNAc-T4 was almost the same between these two cell types.

To confirm that the difference in mGalNAc-T3 was also found at the protein levels, microsome fractions of these cells (50 μg each) were electrophoretically separated on 12.5% polyacrlyamide gels and stained with anti-hGalNAc-T3 antibody (UH1, mouse IgG1), that was known to cross-react with mGalNAc-T3 (Fig. 3a). As a positive control, microsome fractions of human colon carcinoma LS174T cells were used. From the known electrophoretic profiles of hGalNAc-T3, which corresponds to the 67 kDa and 38 kDa bands, [28, 33], we concluded that a greater amount of mGalNAc-T3 was present at protein levels in colon 38 cells than in SL4 cells (Fig. 3a).

Differential GalNAc incorporations into mucin peptides by microsome fractions of colon 38 cells and SL4 cells Because differences in mGalNAc-Ts at mRNA and protein levels were confirmed between colon 38 and SL4 cells, we examined whether the numbers and the sites of GalNAc residues attached to mucin core peptides were different when microsome fractions of colon 38 cells and

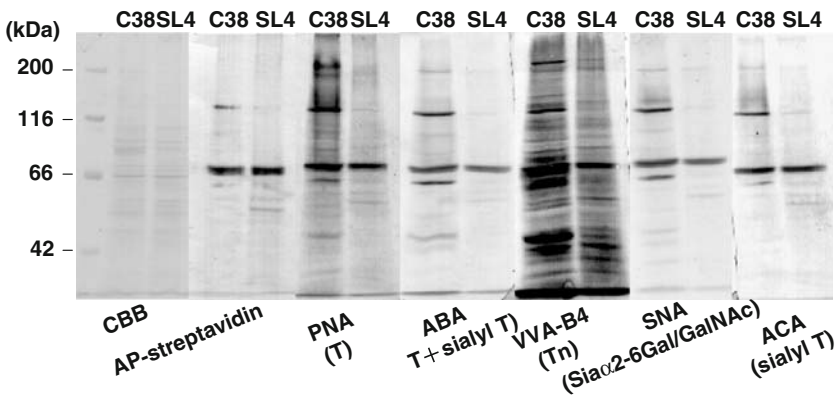


Fig. 1 Lectin staining profiles of electrophoretically separated lysates of colon 38 and SL4 cells. Glycoconjugates recognized by lectins are shown in parentheses. C38; colon 38, CBB; Coomassie brilliant blue staining, T; Galβ1-3GalNAc-Thr, Tn; GalNAc-Thr, sialyl T; NeuAcα2-3Galβ1-3GalNAc-Thr

SL4 cells were used as the enzyme sources. A portion of the human MUC2 tandem repeat (PTTTPITTTTK) was used as the substrate, and 700 μg/ml of microsome fractions were reacted for 1 h, 6 h, and 12 h and the products were separated using reversed-phase HPLC (Fig. 3b). The numbers in each panel indicate the number of GalNAc residues attached to the peptide confirmed by the MALDI-TOF MS analyses. In Fig. 3c, the profiles of MALDI-TOF MS analyses for the peaks are shown and the ratios of the glycopeptides obtained at each reaction period are shown in Table 1. G and number indicates the number of GalNAc residues attached on the peptides (eg. G2 indicates "peptide with 2 attached GalNAc residues"). After a 12-h reaction, the numbers of GalNAc residues attached to the peptide were 1, 3, 4, 5, and 6 when microsome fractions of colon 38 cells were used, whereas the numbers were 1, 2, and 4 when those of SL4 cells were used. The sites of attachment of GalNAc residues on the peptides were analyzed by Edman degradation and the results were summarized in Fig. 3d. When the glycopeptide products derived from incubations with microsome fractions of colon 38 and SL4 cells were compared, the attachment sites of GalNAc residues on the peptides were identical. However, when the microsome fractions of colon 38 cells were used, glycopeptides with five or six GalNAc residues were obtained. These glycopeptides had sequences PT*TT*PIT*T*T*TK and PT*T*T*PIT*T*T*TK, respectively (T* indicates GalNAc-Thr). From these results, it was strongly suggested that the efficiency of GalNAc attachment to mucins was different between colon 38 cells and SL4 cells although the site specificities in the two cell types appeared to be similar.

Effects of reduction of mGalNAc-T3 from the microsome fractions of colon 38 cells on the GalNAc incorporation to the substrate peptides As demonstrated above, microsome fractions of colon 38 cells were likely to contain significantly higher levels of mGalNAc-T3 than SL4 cells, which was potentially responsible for the differential GalNAc attachment to PTTTPITTTTK by the microsome fractions (Fig. 3). Thus, we tested whether microsome fractions of colon 38 cells after reduction of mGalNAc-T3 acted like those of SL4 cells. The results of western blotting analyses of microsome fractions after immunoprecipitation of mGalNAc-T3 are shown in Fig. 4a, Murine ppGalNAc-T3 was detected in the lysates of colon 38 cells near 67 kDa [33] and the relative intensity reduced (33% reduction analyzed using Image J program, data not shown) in the control after treatment with polyclonal antibody against mGalNAc-T3. The microsome fractions after treatment with the antibody were incubated with PTTTPITTTTK (Fig. 4b). The ratios of glycopeptides with differential numbers of GalNAc residues obtained after various reaction periods are shown in Table 2. After 12 h or 16 h of incubation there was a prominent difference in the elution profiles from HPLC of the products incubated with microsome fractions after reduction of mGalNAc-T3 immunoprecipitated by polyclonal antibody, as shown in Fig. 4b. The maximum number of GalNAc residues transferred to the peptides was 6 when microsome fractions treated with pre-immune serum were used as the enzyme source, while the maximum number of GalNAc residues attached to the peptides was 4 when the microsome fractions were treated with polyclonal anti-mGalNAc-T3 antibodies prior to the incubation with the substrate (Table 2). Differences in the GalNAc incorporations were not observed after similar treatments of microsome fractions of SL4 cells. These results strongly suggest that mGalNAc-T3, which is present in the microsome fractions of colon 38 cells but not SL4 cells, is responsible for the transfer of additional GalNAc residues to the peptides representing core polypeptides of mucins. These differences should strongly influence how lectins bind to the glycoproteins produced by these cells.

Discussion

We previously reported that the number and the positions of GalNAc residues incorporated into a peptide representing the tandem repeat portion of human MUC2 mucin PTTTPITTTTK were strictly regulated when this peptide

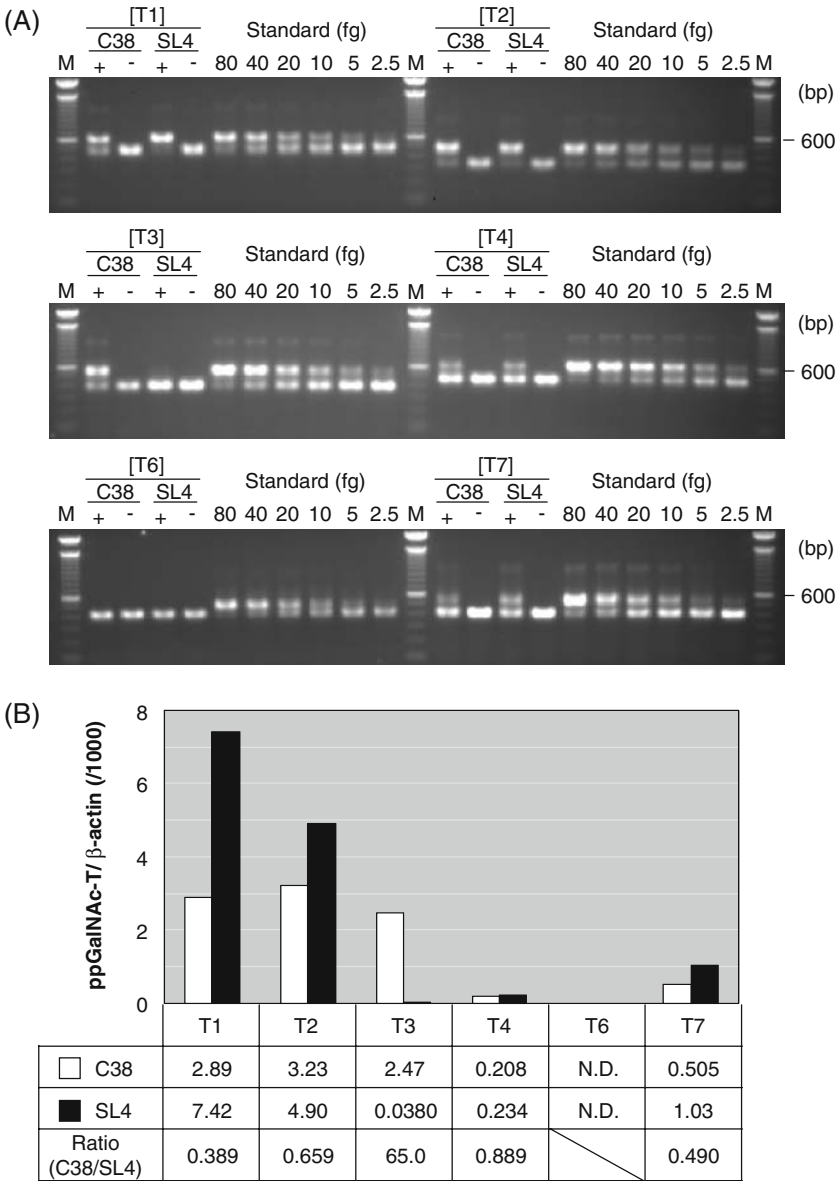
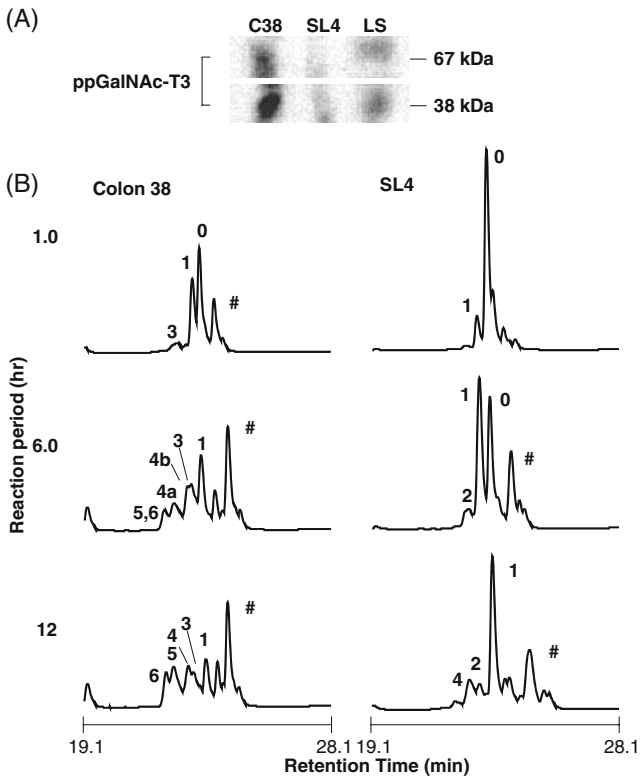


Fig. 2 The expression levels of mRNA of mGalNAc-Ts in colon 38 and SL4 cells. **a** The expression levels of mGalNAc-T1, T2, T3, T4, T6, and T7 were assessed by the competitive RT-PCR method. The relative expression levels of mGalNAc-T1, T2, and T7 were higher in SL4 cells than in colon 38 cells while those of mGalNAc-T3 were significantly higher in colon 38 cells than in SL4 cells. The expression of mGalNAc-T4 was almost the same between these cells, and mGalNAc-T6 was undetectable in both cells. The intensity of the bands was analyzed by an image analyzer and summarized in (**b**)

was incubated with UDP-GalNAc and microsome fractions of LS174T cells or with recombinant hGalNAc-Ts. Furthermore, the predicted orders of incorporations of each GalNAc residue were highly limited. There were only two initial attachment sites and usually a single "pathway" was assigned depending on the initial site [29]. Despite such high levels of fidelity, whether or not cells with different expression profiles of ppGalNAc-Ts produce differential arrangements of GalNAc residues attached to a glycopeptide has not previously been reported. In the present study, we elucidated that the expression pattern of mGalNAc-Ts was different between a mouse colon carcinoma cell line colon 38 and its metastatic variant line, SL4. This was done by competitive RT-PCR and by the western blotting analyses. We used microsome fractions of these cells and examined the incorporations of GalNAc residues into a peptide, PTTTPITTTTK, representing the tandem repeat portion of human MUC2. We found that the level of ppGalNAc-T3 was much higher in colon 38 cells than in SL4 cells and the figure for GalNAc attachment was higher when the microsome fraction of colon 38 cells rather than of SL4 cells was used. Because some ppGalNAc-Ts, such as ppGalNAc-T3 show tissue-specific expressions, these results strongly suggest that the pattern of GalNAc attachment is different among tissues, even on the same peptide. The expression levels of ppGalNAc-Ts in colon cancer cells were previously shown to be altered by cytokine stimuli [34]. These differences and alterations may lead to formations of mucins with unique properties to interact with endogenous and exogenous lectins [35].

Recently, the nuclear respiratory factor-1 (NRF-1) was shown to be responsible for the expression of hGalNAc-T3



(D)

Number of attached GalNAc residues	Colon 38	SL4
1	PTTTPITTTTK PTTTPITTTTK	PTTTPITTTTK PTTTPITTTTK
2	N.D.	PTTTPITTTTK
3	PTTTPITTTTK	N.D.
4	PTTTPITTTTK	PTTTPITTTTK
5	PTTTPITTTTK	N.D.
6	PTTTPITTTTK	N.D.

Fig. 3 Analysis of the enzymatic reaction products after incubations of UDP-GalNAc, FITC-PTTTPITTTK and microsome fractions of colon 38 or SL4 cells. **a** Western blotting analyses of the microsome fractions of colon 38 cells (C38), SL4 cells (SL4), and human colon adenocarcinoma LS174T cells (LS) by the use of anti-hGalNAc-T3 monoclonal antibody UH1. Murine GalNAc-T3 was known to be cross-reactive with this antibody. **b** Elution profiles of glycopeptides with attached GalNAc residues generated by incubation of PTTTPITTTTK with microsome fractions of colon 38 cells and SL4 cells on reverse phase HPLC. The # indicates a peak derived from a cleavage product. The numbers on each peak indicate the number of GalNAc residues attached to the peptide confirmed by MALDI-TOF MS analyses as shown in (**c**). The numbers of GalNAc residues on the peptides obtained by incubations with microsome fractions of colon 38 cells (*left*) or SL4 cells (*right*) are shown in Table 2. **d** Summary of the obtained glycopeptides by incubation of microsome fractions of colon 38 cells and SL4 cells for 12 h. *Filled circles* indicate GalNAc residues

[36], and the binding of NRF-1 to DNA was enhanced by its acetylation by p300/cAMP-response-element binding protein (CREB)-binding protein (CBP) associated factor (P/CAF) [37]. Therefore, we studied whether these proteins in colon 38 cells and SL4 cells influenced expression of mRNA of mGalNAc-T3. However, there was no difference in the expression of NRF-1 and P/CAF between colon 38 and SL4 cells by the DNA array or other methods (data not shown). Therefore, it seemed that there were other regulatory mechanisms related to the regulation of mGalNAc-T3.

Human ppGalNAc-T3 was previously shown to be expressed at higher levels in well-differentiated adenocarcinoma cells than in poorly differentiated counterparts [33]. Also, patients with high levels of ppGalNAc-T3 in colorectal carcinomas had longer survival than those with tumors with low levels of this enzyme. The expression was not associated with age, gender, tumor size, tumor location, or disease stage but was related to histologic differentiation and depth of invasion [38]. In gastric cancer patients, those having high hGalNAc-T3 tumors survived better [39]. However, these reports mainly discussed the expression level of ppGalNAc-T3 and none of them have shown the relationships between the expression of ppGalNAc-T3 and properties of mucins and glycans on mucins produced by carcinoma cells. SL4 cells were previously selected for increased metastatic potentials *in vivo* from the parental colon 38 cells and possessed poorly differentiated phenotypes [31]. Thus, low levels of mGalNAc-T3 of the cell line agree with the properties of metastatic and poorly differentiated colon carcinoma cells in the previous reports.

The present report is the first to show that the relationship between the expression levels of ppGalNAc-T3 and glycans of cancer cells, which express ppGalNAc-Ts to a different extent *in vitro*. Reduction of mGalNAc-T3 influenced the quantity of GalNAc attachment on the peptide and the maximum numbers of attached GalNAc residues decreased. So far only 3 mGalNAc-Ts were reported as active enzymes and other isoforms were potentially involved. Therefore, these observations could be resulted from other isoforms of mGalNAc-Ts with unique specificity different from mGalNAc-T3, which became the dominant enzymes in

Table 1 The ratio (%) of generated glycopeptides with attached GalNAc residues in various reaction periods after incubations of PTTTPITTTTK peptide with microsome fractions of colon 38 cells and SL4 cells. G0, G1, G2, G3, G4, G5, and G6 represent the peptide with 0–6 attached GalNAc residues, respectively

Reaction period		Colon 38	SL4
1 h	G0	57.6	85.0
	G1	34.3	14.9
	G3	8.15	N. D.
	G2, G4–G6	N. D.	N. D.
6 h	G0	N. D.	45.6
	G1	37.1	45.3
	G2	N. D.	9.09
	G3	17.7	N. D.
	G4	36.8[a]	N. D.
	G5 + G6	8.42	N. D.
12 h	G0	N. D.	N. D.
	G1	22.4	69.7
	G2	N. D.	11.5
	G3	19.2	N. D.
	G4	18.3	18.9
	G5	27.2	N. D.
	G6	12.8	N. D.

N. D. Not detected

[a] Sum of the ratio of 4a and 4b peptides in Fig. 3b

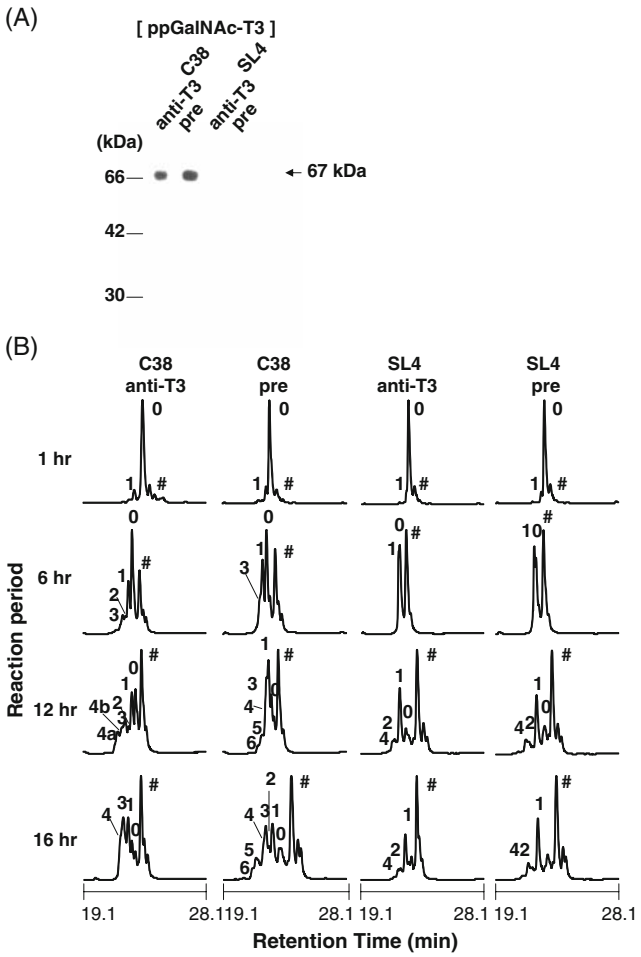


Fig. 4 Effects of anti-mGalNAc-T3 antibody on microsome fractions of colon 38 and SL4 cells. **a** Western blotting analyses of microsome fractions of colon 38 cells and SL4 cells by anti-hGalNAc-T3 (UH1) antibody after immunoprecipitation with polyclonal antibody raised against mGalNAc-T3 or with pre-immune serum. **b** Elution profiles from HPLC analyses of glycopeptides with attached GalNAc residues produced by incubations of PTTTPITTTTK peptide and UDP-GalNAc with microsome fractions of colon 38 cells and SL4 cells after immunoprecipitation with polyclonal antibody against mGalNAc-T3 or with pre-immune serum. Numbers attached above each peak indicate the number of GalNAc residues attached to PTTTPITTTTK peptide. These numbers were confirmed by MALDI-TOF MS analyses

microsome fractions after reduction of mGalNAc-T3. This assumption was supported at least in part by our previous data showing that the first GalNAc attachment sites determined the order of GalNAc attachment to the peptide [29].

Brooks *et al*. previously demonstrated that expression levels of hGalNAc-T3 and hGalNAc-T6 in human breast carcinoma cell lines correlated with the staining with *Helix pomatia* lectin (HPA), which recognizes terminal GalNAc residues [40]. The results were somewhat skewed because, BT 474 cells derived from primary, non-metastatic breast cancer, expressed a high level of hGalNAc-T3, even though HPA bindings to these cells were low. Other breast cancer cell lines they used in the study expressed high levels of hGalNAc-T3 and were strongly reactive with HPA, though the relationships with the metastatic phenotypes were unknown.

A non-redundant function of single isoform of hGalNAc-T was first reported by Topaz *et al*. [41]. It has been revealed that dysfunctions of hGalNAc-T3 are deeply involved in the cause of familial tumoral calcinosis [42] and hyperostosis-hyperphosphatemia syndrome [43]. This evidence suggests that even though there are at least 20 isoforms in human ppGalNAc-T family, each isoform has a distinct biological role. Therefore, it is possible that alterations of mucin structures due to differential degree of *O*-glycosylation might determine the metastatic potential of the cells in a direct or indirect manner. Colon 38 and the SL4 cell should provide useful tools to further elucidate the biological meanings of mucin *O*-glycosylation.

Materials and methods

Synthesis of acceptor substrates An oligopeptide used as the acceptor substrate, PTTTPITTTTK was synthesized with Pioneer™ peptide synthesizer (ABI) with a lysine residue attached at the C-terminus. This peptide was labeled with fluorescein isothiocyanate at its N-terminal

Table 2 The ratio (%) of glycopeptides attached with GalNAc residues obtained in various reaction periods after incubations of PTTTPITTTTK peptide with microsome fractions of colon 38 cells and SL4 cells untreated or treated with polyclonal antibody specific for mGalNAc-T3

Reaction period		Colon 38		SL4	
		αT3	pre	αT3	pre
1 h	G0	88.4	85.5	90.4	91.8
	G1	11.6	14.5	9.59	8.18
	G2-G6	N. D.	N. D.	N. D.	N. D.
6 h	G0	51.8	46.1	47.6	43.1
	G1	25.9	36.0	52.4	56.9
	G2	6.64	N. D.	N. D.	N. D.
	G3	15.6	18.0	N. D.	N. D.
	G4-G6	N. D.	N. D.	N. D.	N. D.
12 h	G0	27.2	21.6	30.9	28.5
	G1	25.4	36.3	52.3	48.3
	G2	8.08	N. D.	9.17	9.13
	G3	21.0	19.8	N. D.	N. D.
	G4	18.3[a]	8.06	7.64	14.1
	G5	N. D.	11.4	N. D.	N. D.
	G6	N. D.	2.85	N. D.	N. D.
16 h	G0	16.1	9.73	N. D.	N. D.
	G1	28.7	30.1	69.2	67.1
	G2	N. D.	9.79	15.4	11.4
	G3	37.7	19.1	N. D.	N. D.
	G4	17.5	12.7	15.4	21.5
	G5	N. D.	15.1	N. D.	N. D.
	G6	N. D.	3.45	N. D.	N. D.

N. D. Not detected

[a] Sum of the ratio of 4a and 4b peptides in Fig. 4b

amino acid at pH 7.5 adjusted with 100 mM HEPES buffer.

Preparation of microsome fractions of cells LS174T human colon carcinoma cells, colon 38 mouse colon carcinoma cells and SL4 cells [31] were maintained in an 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Nissui, Tokyo) containing 10% heat-inactivated FCS in a humidified atmosphere of 5% CO_2 at 37°C. Cells with 80% confluence were homogenized in 50 mM Tris buffer (pH 7.5) containing 250 mM of sucrose, 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, and 0.5 μg/ml of pepstatin A. After centrifugation of the lysates at 3,000×*g* for 10 min, the supernatant was further centrifuged at 100,000×*g* for 1 h. Pellets were suspended in 50 mM Tris buffer (pH 7.5) containing 250 mM of sucrose, 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, 0.5 μg/ml of pepstatin A, and 0.1% Triton X-100 and kept at −80°C until use. The concentration of protein in the microsome fraction was measured with a BCA protein assay kit (Pierce Chemical Co., Rockford,IL) using bovine serum albumin as a standard.

Enzymatic incorporations of GalNAc residues Enzymatic incorporations of GalNAc residues were conducted using PTTTPITTTTK peptide as a substrate. Reactions were performed in 50 μl of reaction mixtures (pH 7.5) containing 50 mM of HEPES, 5 mM of 2-mercaptoethanol, 20 mM of $MnCl_2$, 2 mM of PMSF, protease inhibitors (1 μg/ml of aprotinin, 1 μg/ml of leupeptin, 0.5 μg/ml of pepstatin A), 1 mM UDP-GalNAc (Sigma), 5 μM of synthetic peptide, and microsome fractions of colon 38 cells or SL4 cells (700 μg/ml). Reactions were conducted at 37°C for a maximum of 16 h and stopped by adding 0.5 M EDTA (pH 8.0) with 1/5 volume of reaction mixture.

To prepare microsome fractions depleted with mGalNAc-T3 from colon 38 cells or SL4 cells, Protein G-agarose (Amersham Biosciences) was incubated with rabbit anti-mouse mGalNAc-T3 serum or pre-immune serum (1 ml respectively) at 4°C overnight. The antibody-coated beads were pelletted by centrifugation at 14,000 rpm for 1 min and washed three times with 50 mM Tris buffer (pH 7.5) containing 250 mM of sucrose, 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, 0.5 μg/ml of pepstatin A, and 0.1% Triton X-100. Seven milligrams of microsome fractions of colon 38 cells or SL4 cells were incubated with the beads at 4°C overnight. Supernatants after centrifugation at 14,000 rpm for 1 min were used.

The percent ratios of glycopeptides with various numbers of GalNAc residues were calculated as "Ratio (%)=each pmol of each glycopeptide/ total pmol of glycopeptides x 100". The relative amount of each peptide was calculated according to the fluorescence intensity of the peptides in the HPLC charts as described below.

Characterization of glycopeptides The glycosylated peptides with GalNAc residues were separated by reverse-phase HPLC (JASCO, Tokyo, Japan). A Cosmosil ($5C_{18}$-AR, 10× 250 mm, Nacalai Tesque, Kyoto, Japan) was used. The column was eluted with a linear gradient of 0–50% of solvent B (0.05% TFA/2-propanol) in solvent A (0.05% TFA/H_2O) at a flow rate of 2 ml/min for 30 min. Eluates were monitored by fluorescence intensity at 520 nm under excitation at 492 nm.

The number and the sites of attached GalNAc residue were confirmed by MALDI-TOF MS analysis and Pulsed liquid Edman degradation amino acid sequencing respectively as described previously [25].

Lectins and polyclonal antibodies used in this study Biotinylated lectins, PNA (*Arachis hypogaea* (peanut) agglutinin, Seikagaku Corporation), ABA (*Agaricus bisporus*

agglutinin, Seikagaku Corporation), VVA-B4 (*Vicia villosa* agglutinin, Vector), SNA (*Sambucus nigra* agglutinin, EY Laboratories, Inc.), ACA (*Amaranthus caudutus* agglutinin, Vector) were used.

Rabbit anti-mGalNAc-T3 serum was obtained by immunizing a Japanese white female rabbit with KLH conjugated synthetic peptide GYYTAAELKPVFDRPPQDSC corresponding to 100–118th amino acids of mGalNAc-T3 with Freund complete adjuvant and with incomplete adjuvant for the boost. Measurement of antibody titer was conducted 10 days after boost. Antibody titer was assessed by the ELISA using the synthetic peptide.

Western blotting and lectin blotting analyses Thirty μg of cell lysates or 50 μg of microsome fractions of mouse colon carcinoma colon 38 cells and SL4 cells were separated by electrophoresis on 7.5% or 12.5% polyacrylamide gels under reducing conditions. Proteins were transferred onto PVDF membrane and the membranes were treated with 2% BSA/PBS or 5% skim milk/TBST at 4°C, overnight and incubated with biotinylated lectins or anti-hGalNAc-T3 (UH1, mouse IgG1) [33] for 1–2 h at room temperature. AP-streptavidin (Zymed) was used in lectin blotting and HRP-conjugated goat anti-mouse IgG(H + L) was used in the western blotting for the detection. For visualization, Alkaline phosphatase Substrate kit II (Vector) was used for AP and Aminoethyl Carbazole Substrate kit (Zymed) or ECL Western Blotting Detection Reagents (Amersham Biosciences) was used for HRP.

RT-PCR assay and competitive RT-PCR assay The expression levels of six mGalNAc-Ts were examined by the competitive RT-PCR method in mouse colon carcinoma colon 38 cells and SL4 cells following the methods reported previously [34]. The total RNA of each cell line was isolated using Ultraspec RNA kit (Biotecx Lab, Houston, TX). Five μg of total RNA were reverse transcribed by 200 U of Superscript™ II RNase H-free Reverse Transcriptase (Gibco-BRL, Rockville, MD). The reaction of reverse transcription was performed in 20 μl of 50 mM Tris-HCl (pH 8.3) containing 75 mM of KCl, 3 mM of $MgCl_2$, 10 mM of dithiothreitol, 0.5 mM of dNTP, 1 U/μl of RNase inhibitor (Ambion, Austin, TX), and 50 ng/μl of oligo$(dT)_{12–18}$ primer (Pharmacia Biotech, Buckinghamshire, UK) at 42°C for 50 min. After the reverse transcription, 2 U of Ribonuclease H (Gibco-BRL) was added and reacted at 37°C for 20 min. cDNA was amplified in 10 mM Tris-HCl buffer (pH 8.3) containing 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 5% DMSF, 0.25 μM of primers, and 0.025 U/μl of Ampli Taq Gold™ polymerase (ABI, Tokyo). Primer sets used in this study were: mGalNAc-T1 (599 bp), 5′-GTG CCG GTT CAT GTA ATT CG-3′ and 5′-TCC ATC CAT ACT TCT GCA AGC-3′; mGalNAc-T2 (466 bp), 5′-GTA TGT GGG CGC CTC TGC TGA CC-3′ and 5′-GAG CTC CAG CCT GCT CTG AAT ATT CC-3′; mGalNAc-T3 (561 bp), 5′-TAA CCA AGT TCG CCT TGC AG-3′ and 5′-GTT GCG TCA CAT GGC ACT AA-3′; mGalNAc-T4 (631 bp), 5′-CAT CTT GGT GGA TGA CTT GAG TG-3′ and 5′-CGC GGT ATT CTG TAG GAA GTT G-3′; mGalNAc-T6 (495 bp), 5′-TCG TCA CCA TCG ACC TTA ATA CC-3′ and 5′-AGA TGT CAC CGA AGT TGT TCT CC-3′; mGalNAc-T7 (538 bp), 5′-ACA TGA TCT CAC TGG ACC GCA G-3′ and 5′-ATA CTC CAG TCC CAT GCT CCT CG-3′. In the competitive RT-PCR of mGalNAc-Ts, a standard curve was obtained by PCR with coexistence of 2.5 fg, 5 fg, 10 fg, 20 fg, 40 fg and 80 fg of standard and 10 fg of competitors. The PCR condition was 94°C, 30 s for denaturation, 58°C, 30 s for annealing, and 72°C, 45 s for elongation for 45 cycles. For normalization, competitive RT-PCR of β-actin was also conducted using 5′-CTT CTA CAA TGA GCT GCG TGT GG-3′ and 5′-TGA TGA CCT GGC CGT CAG GCA-3′ primers (479 bp). Competitive RT-PCR of β-actin was conducted under the same conditions described above except Ampli Taq polymerases were used and the PCR cycle number was 24 cycles with denaturation at 94°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 45 s. After PCR, aliquots were separated by electrophoresis in 1.2% agarose gels, and the DNA was visualized by staining with ethidium bromide. The intensities of the amplified fragments were quantified by using a Fluor-S multi imager (BioRad, Richmond, CA) and NIH image software. The values for the transcripts were plotted on the respective standard curves to obtain the actual amount of each transcript. To normalize the data, the amount of transcript of each gene was divided by that of β-actin.

Acknowledgments We thank Ms. Kyoko Sakai and Ms. Miki Noji for their assistance in preparing this manuscript. This work was supported by grants-in-aid from the Ministry of Education, Science, Sports and Culture of Japan, the Research Association for Biotechnology, and the Danish Cancer Society. Kentaro Kato was a recipient of Fellowship from the Japanese Society for Promotion of Science.

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