High Density O-Glycosylation of the MUC2 Tandem Repeat Unit by N-Acetylgalactosaminyltransferase-3 in Colonic Adenocarcinoma Extracts

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ABSTRACT

A synthetic peptide corresponding to the human MUC2 tandem repeat unit was glycosylated in vitro using UDP-GalNAc and extracts of colonic adenocarcinoma and paired normal mucosa, followed by fractionation of the products by reverse phase high-performance liquid chromatography. Several peaks of glycopeptides with different numbers of GalNAc residues attached were detected. It is notable that the adenocarcinoma extract was capable of glycosylating peptides to a much greater extent than was normal mucosa. The levels of mRNA for N-acetylgalactosaminyltransferases-1, -2, and -3 were determined by reverse transcription-PCR. Only N-acetylgalactosaminyltransferase-3 mRNA was expressed at a higher level in the adenocarcinoma than in the normal tissue. When the MUC2 tandem repeat peptide was glycosylated with a mixture of the normal mucosa extract and recombinant N-acetylgalactosaminyltransferase-3, larger amounts of glycopeptides with higher contents of GalNAc residues were produced. The MUC2 tandem repeat peptide glycosylated extensively by recombinant N-acetylgalactosaminyltransferase-1, -2, or -3 were prepared and characterized. Substitution at each Thr residue, as revealed by Edman degradation sequencing, in conjunction with evidence obtained on mass spectrometry indicated a heterogeneous pattern of site-specific glycosylation within the MUC2 tandem repeat. It was found that maximum numbers of 6, 8, and 11 GalNAc residues were incorporated by N-acetylgalactosaminyltransferases-1, -2, and -3, respectively, and that only N-acetylgalactosaminyltransferase-3 could completely glycosylate both consecutive sequences composed of three and five Thr residues in the MUC2 tandem repeat unit. These results suggest that O-glycosylation of the clustered Thr residues is a selective process controlled by N-acetylgalactosaminyltransferase-3 in the synthesis of clustered carbohydrate antigens.

INTRODUCTION

Mucins are high molecular weight glycoproteins characterized by many O-linked oligosaccharides to the core polypeptide through Ser or Thr residues. Generally, tumor-associated carbohydrate antigens are produced through incomplete synthesis of carbohydrate chains, which causes the accumulation of precursor forms, or through neo-synthesis of carbohydrate chains through the activation of certain glycosyltransferases. The former involves Tn and T antigens and their tandem repeat domains, which include consecutive Thr/Ser residues incorporated by combining evidence from MALDI-TOF mass spectrometry with sequence data from Edman degradation. First, we found a remarkable difference between glycopeptides synthesized with extracts of colonic adenocarcinoma and with paired normal mucosa. The adenocarcinoma extract glycosylated the peptide to a greater extent than that of the normal mucosa, probably due to enhanced expression of GalNAc-T3. Next, the peptide glycosylated by rGalNAc-T1, -T2, and -T3 was prepared. Maximum numbers of 6, 8, and 11 GalNAc residues were transferred to the peptide by strongly reactive only with clustered O-glycans on peptide. For instance, the epitopic structures for anti-Tn and anti-sialylTn antibodies comprise three and four consecutive sequences of GalNAc-Ser/Thr and SAα2–6GalNAc-Ser/Thr, respectively (3–6). The clustering of a relatively common structure could lead to the formation of an uncommon structure exhibiting antigenicity. Although the roles of these truncated O-glycans in malignant behavior are not well understood, they are highly immunogenic and useful as a vaccine (7, 8). Therefore, it is important to elucidate a biosynthetic mechanism of clustered O-glycans aligned on a core peptide.

In a previous studies (3–5, 9), we reported that an extract of LS 180 cells glycosylated the MUC2 tandem repeat peptide in vitro, leading to the synthesis of Tn antigenic sites recognized by a monoclonal antibody (MLS 128), indicating the synthesis of clustered Thr-GalNAc.

Many mucins contain a large number of Thr and Ser residues within their tandem repeat domains, which include consecutive Thr/Ser residues except for MUC1 (10). The initial step in the regulation of O-glycosylation is the enzymatic transfer of GalNAc from UDP-GalNAc to Thr and Ser residues (11, 12). It is well known that there are multiple GalNAc-Ts expressed in various tissues (13). Thus, GalNAc transferases that regulate the initiation of O-glycosylation of mucins are important for understanding some aspects of tumor-associated aberrant O-glycosylation.

We used a synthetic peptide of MUC2 tandem repeat unit and extracts from a human colonic adenocarcinoma and paired normal mucosa and rGalNAc-Ts as a substrate and enzyme sources, respectively. Because MUC2 is a major secreted mucin of intestinal epithelia, and its tandem repeat unit contains two consecutive parts consisting of three and five Thr residues that potentially serve as a scaffold presenting clustered carbohydrate antigens (14).

Because the O-glycosylation of one site of a peptide may have some effects on other acceptor sites, we used a whole unit of the MUC2 tandem repeat as a substrate. The peptide was conjugated with DABITC, which has absorbance at 436 nm, to monitor the glycosylated peptides on reverse-phase HPLC. We determined the glycosylated sites of glycopeptides with the maximum number of GalNAc residues incorporated by combining evidence from MALDI-TOF mass spectrometry with sequence data from Edman degradation. First, we found a remarkable difference between glycopeptides synthesized with extracts of colonic adenocarcinoma and with paired normal mucosa. The adenocarcinoma extract glycosylated the peptide to a greater extent than that of the normal mucosa, probably due to enhanced expression of GalNAc-T3. Next, the peptide glycosylated by rGalNAc-T1, -T2, and -T3 was prepared. Maximum numbers of 6, 8, and 11 GalNAc residues were transferred to the peptide by

Received 4/24/00; accepted 11/29/00.
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1 Supported in part by Grant-in-Aid for Scientific Research on Priority Areas 10178102 from the Ministry of Education, Science and Culture of Japan; by the Foundation for Bio-venture Research Center from the Ministry of Education, Science and Culture of Japan; and by the Fogukku Trust for Medicinal Research.

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3 The abbreviations used are: GalNAc-T, UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase; rGalNAc-T, recombinant GalNAc-T; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight mass spectrometry; DABITC, 4-Dimethylaminobenzene-4'-isothiocyanate; RT-PCR, reverse transcription-PCR; PTH, phenylthiobutyramine.
rGalNAc-T1, -T2, and -T3, respectively, and both the consecutive Thr residues were fully glycosylated by rGalNAc-T3 but not by rGalNAc-T1 or -T2.

MATERIALS AND METHODS

Materials. A synthetic peptide, AAMAPTTPTPTTTTVTPTPTGQT, was obtained from Kurabo (Osaka, Japan). DABITC was purchased from Nacarai Tesque (Kyoto, Japan). Expression vector pSecTag was from Invitrogen (San Diego, CA). A reference glycopeptide (KGGGSGalNAc-TTGGGSGGG) was synthesized as described previously (15).

Cells. A human colorectal cancer cell line, LS 180, was obtained from the American Type Culture Collection and cultured in Eagle’s MEM supplemented with 10% FCS.

Preparation of Adenocarcinoma and Normal Mucosa Extracts. A fresh colonic adenocarcinoma and normal mucosa taken at a distance of more than 10 cm from the adenocarcinoma were frozen immediately after surgery with liquid nitrogen. Five adenocarcinomas and paired normal mucosae were always processed in parallel. Extracts of adenocarcinoma and normal mucosa were obtained as follows. The tissues were homogenized in 25 mM phosphate buffer (pH 7.5) and 0.15 M NaCl with a Bio-Mixer, and the resultant lysate was centrifuged at 10,000 g for 10 min. The supernatant was sonicated for 1 min and then centrifuged at 10,000 × g for 10 min. The supernatant was further centrifuged at 105,000 × g for 1 h. After solubilization with 2% octylglucoside, 2 mM phenylmethylsulfonyl fluoride, and 0.1 M Tris-HCl buffer (pH 7.4), the pellet was centrifuged at 105,000 × g for 1 h, and the resultant supernatant was used as the enzyme source.

RT-PCR of GalNAc-Ts. Preparation of total RNA from colonic adenocarcinoma and paired normal mucosa was performed using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer’s instruction. Total RNA (1 μg) was reverse transcribed using a reaction mixture comprising 5 mM MgCl2, 1 mM deoxynucleotide triphosphate mixture, 1 unit/μl RNase inhibitor, 1 μM oligo(dT)20 primer, and 0.25 unit/μl reverse transcriptase in a final volume of 20 μl. The mixture was incubated at 55°C for 40 min, at 99°C for 5 min, and then at 5°C for 5 min. The cDNA was then subjected to PCR. To obtain semiquantitative results, the cycle number and cDNA concentration were chosen so as to ensure that the amplification was not in the plateau phase. For GalNAc-T1 and -T2, 20, 26, and 30 cycles were performed, and for GalNAc-T3, 30, 35, and 40 cycles were performed. The cDNA amplification of GalNAc-Ts and β-actin was performed at the same time. The reaction mixture comprised 2.5 mM MgCl2, 20 pm forward and reverse primers, and 2.5 μl of TaKaRa Taq (TaKaRa, Tokyo, Japan) in a final volume of 100 μl. After polymerase activation step at 94°C for 5 min, samples were amplified for the indicated numbers of cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 74°C for 1 min. The amplified cDNAs were run on 1% agarose gels with 0.5 μg/ml ethidium bromide and visualized under UV light.

Preparation of rGalNAc-Ts. Total RNA was prepared from LS 180 cells using ISOGEN as described above. cDNAs encoding the putative ectodomains of rGalNAc-T1, -T2, and -T3 flanked by artificial sites for BamHI and EcoRV (rGalNAc-T1) and BamHI and XhoI (rGalNAc-T2 and -T3) were amplified by PCR. The PCR-generated DNA were digested with BamHI and EcoRV for rGalNAc-T1 or with BamHI and XhoI for rGalNAc-T2 and -T3 and then inserted into expression vector pSecTag containing an immunoglobulin κ chain leader sequence, a myc epitope, and a polyhistidine tag. The constructed plasmid (pSecTag-rGalNAc-T1, -T2, and -T3) was introduced into TOP10F cells and amplified according to the manufacturer’s instructions, and then COS-7 cells were transfected with pSecTag-rGalNAc-T1, -T2, and -T3 by the lipofection method. The culture medium was dialyzed against 25 mM phosphate buffer (pH 7.8) and 15 mM NaCl and then subjected to DEAE-cellulose column chromatography. This step was necessary to exclude endogenously synthesized GalNAc transferases. The excluded fraction was applied to a ProBond column, washed with 20 mM phosphate buffer (pH 7.8) and 0.5 mM NaCl, and then eluted with 0.3 mM imidazole, 0.5 mM NaCl, and 20 mM phosphate buffer (pH 6.0). Finally, rGalNAc-Ts were prepared on an immunoadfinity column using anti-myc/His antibodies according to the manufacturer’s instructions.

Transfer of GalNAc to a Synthetic Peptide. A synthetic peptide including 1 unit of the MUC2 tandem repeat was conjugated with DABITC as described by Chang (16). The incubation mixture comprised 5 mM MgCl2, 5 mM 2-mercaptoethanol, 5 mM CD-chole, 43 nmol of UDP-GalNAc, 4 μg of DABITC-MUC2 peptide, and an appropriate amount of a tissue extract or rGalNAc-Ts in a final volume of 100 μl. The mixture was incubated at 37°C for the times indicated in Figs. 1, 3, and 4.

Fractionation of the Glycopeptides. The glycosylated products were directly subjected to reverse phase HPLC, and elution was carried out with a linear gradient of 0–100% acetonitrile in 10 mM ammonium acetate buffer (pH 5.0) at a flow rate of 1 ml/min. Elution of the product was monitored by absorbance at 436 nm.

Mass Spectrometry of the Glycopeptides. Mass spectra of the glycopeptides were obtained by MALDI-TOF mass spectrometry (Kratos Analytical, Manchester). Samples (10 pmol) dissolved in 1 μl of distilled water were placed on a stainless steel probe tip along with 1 μl of the matrix (2,5-dihydroxybenzoin acid in 0.1% trifluoroacetic acid/acetonitrile, 7:3). Measurements were performed in the linear mode using an appropriate delay time and a potential to focus the ions of interest.

Identification of O-Glycosylation Sites of the Glycopeptides. The glycopeptides were degraded with cyanogen bromide (17) and then purified by the reversed phase HPLC.
Expression of GalNAc-T1, -T2, and -T3 mRNA in Colonic Adenocarcinoma and Normal Mucosa. The transfer of GalNAc to the peptide was investigated in vitro using the MUC2 tandem repeat unit containing 14 Thr residues and extracts of colonic adenocarcinoma and normal mucosa as described in “Materials and Methods.”

To detect the products directly, the synthetic peptide was conjugated with DABITC, which exhibits absorbance at 436 nm. Furthermore, as described in “Materials and Methods,” four amino acids including Met were inserted between DABITC and the NH₂-terminal of the MUC2 tandem repeat to minimize the influence of DABITC and to exclude DABITC released through cyanogen bromide degradation after fractionation of the glycopeptides. The effects of various assay parameters (cell extract, MUC2 peptide, and UDP-GalNAc) were evaluated, and appropriate conditions were selected. Five colonic adenocarcinomas and paired normal mucosae were examined. Fig. 1 shows a representative elution pattern on HPLC of the glycopeptides synthesized by the adenocarcinoma or normal mucosa extract. The nonglycosylated DABITC-MUC2 peptide was eluted at 30.5 min (fraction 6). Other fractions (fractions 1–5) contained glycopeptides with various numbers of GalNAc residues transferred. The peaks that eluted faster (fractions 1 and 2) contained glycopeptides with higher contents of GalNAc residues and showed the clustered Tn antigenicity, as reported previously (9). It is notable that the adenocarcinoma extract could transfer GalNAc to the MUC2 peptide to a much higher extent compared with that of the normal paired mucosa, as shown in Fig. 1, A and B.

Expression of GalNAc-T1, -T2, and -T3 mRNA was analyzed semiquantitatively by RT-PCR. Representative results for the same specimen seen in Fig. 1 are shown in Fig. 2. The mRNA levels for all GalNAc-T probes were quantified with a densitometer and normalized relative to the level of β-actin mRNA. It was found that the level of GalNAc-T3 mRNA in an adenocarcinoma was significantly higher than that in the paired normal mucosa.

Incorporation of GalNAc to the MUC2 tandem repeat unit and level of GalNAc-T1, T2, T3 mRNA were analyzed similarly by using other four adenocarcinomas and paired normal mucosae as summarized in Table 1. Because fractions 1 and 2 in Fig. 1 contained the clustered Tn antigenicity as reported previously (9), the relative activity producing the clustered GalNAc-Thr is shown by comparing the area of fractions 1 and 2.

The area for adenocarcinomas in fractions 1 and 2 increased about 4-fold compared with that of paired normal mucosa. The relative level of GalNAc-T3 mRNA also increased significantly (about 10-fold) in adenocarcinomas, whereas the relative levels of GalNAc-T1 and -T2 mRNA increased slightly.

Characterization of the MUC2 Peptide Glycosylated by rGalNAc-Ts. It is generally agreed that there are multiple polypeptide GalNAc-Ts expressed in various tissues (13). It was interesting to determine which GalNAc-T is responsible for the synthesis of clustered carbohydrate antigens. GalNAc-T1, -T2, and -T3 have been cloned and extensively characterized (19–22). rGalNAc-Ts were prepared as described in “Materials and Methods.” Each enzyme was subjected to SDS-PAGE followed by Western blotting. After staining through successive incubation with anti-MUC2 antibodies and protein G-peroxidase, the relative amounts of the enzymes were determined with a densitometer. Bands corresponding to molecular weights of 79,000, 73,000, and 85,000 were detected in the rGalNAc-T1, -T2, and -T3 lanes, respectively (data not shown). These molecular weights were larger than the sizes expected from the cDNAs inserted in the vector, and this difference was probably due to glycosylation during the synthesis in COS-7 cells.

The MUC2 tandem repeat unit conjugated with DABITC was incubated with each rGalNAc-T for up to 5 days to determine the maximal number of GalNAc residues transferred and identify the sites

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**RESULTS**

**GalNAc-Ts Activity and mRNA in Colonic Adenocarcinoma and Paired Normal Mucosa.** The transfer of GalNAc to the peptide was investigated in vitro using the MUC2 tandem repeat unit containing 14 Thr residues and extracts of colonic adenocarcinoma and normal mucosa as described in “Materials and Methods.”

To detect the products directly, the synthetic peptide was conjugated with DABITC, which exhibits absorbance at 436 nm. Furthermore, as described in “Materials and Methods,” four amino acids including Met were inserted between DABITC and the NH₂-terminal of the MUC2 tandem repeat to minimize the influence of DABITC and to exclude DABITC released through cyanogen bromide degradation after fractionation of the glycopeptides. The effects of various assay parameters (cell extract, MUC2 peptide, and UDP-GalNAc) were evaluated, and appropriate conditions were selected. Five colonic adenocarcinomas and paired normal mucosae were examined. Fig. 1 shows a representative elution pattern on HPLC of the glycopeptides synthesized by the adenocarcinoma or normal mucosa extract. The nonglycosylated DABITC-MUC2 peptide was eluted at 30.5 min (fraction 6). Other fractions (fractions 1–5) contained glycopeptides with various numbers of GalNAc residues transferred. The peaks that eluted faster (fractions 1 and 2) contained glycopeptides with higher contents of GalNAc residues and showed the clustered Tn antigenicity, as reported previously (9). It is notable that the adenocarcinoma extract could transfer GalNAc to the MUC2 peptide to a much higher extent compared with that of the normal paired mucosa, as shown in Fig. 1, A and B.

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The MUC2 tandem repeat unit conjugated with DABITC was incubated with each rGalNAc-T for up to 5 days to determine the maximal number of GalNAc residues transferred and identify the sites

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**Table 1. Relative amount of glycopeptides with the Tn antigenicity and level of GalNAc-T mRNA in colonic adenocarcinoma and paired normal mucosa**

<table>
<thead>
<tr>
<th>Area</th>
<th>GalNAc-T1</th>
<th>GalNAc-T2</th>
<th>GalNAc-T3</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.16 ± 2.20</td>
<td>1.40 ± 0.26</td>
<td>1.68 ± 0.73</td>
<td>10.85 ± 5.66</td>
</tr>
</tbody>
</table>

* Amount of glycopeptides with the Tn antigenicity is compared by the area of fractions 1 and 2 as shown in Fig. 1. These fractions contained clustered GalNAc-Thr residue with the Tn antigenicity.

* Relative mRNA level is compared as shown in Fig. 2.

* All values (mean ± SD) are normalized relative to those of paired normal mucosa.
of O-glycosylation. The glycopeptides synthesized with the recombinant enzymes were directly subjected to reverse phase HPLC, as shown in Fig. 3. Although the glycopeptides synthesized with rGalNAc-T1 and -T2 gave symmetrical peaks with apparent retention times of 28.07 and 27.32 min, respectively (Fig. 3, A and B), they contained glycopeptides with a few different numbers of incorporated GalNAc residues, as described later. The elution pattern of the peptides glycosylated by rGalNAc-T3 showed a broad peak even on incubation for 5 days (Fig. 3 C). The peak was tentatively divided into three fractions. The first fraction eluted at about 27.20 min was purified by reverse phase HPLC.

The DABITC-glycopeptides and -peptide were analyzed by MALDI-TOF mass spectrometry, as shown in Fig. 4. Peaks a–c and d showed DABITC-glycopeptides synthesized by GalNAc-Ts and DABITC-peptides, respectively. Peak a, which corresponds to the highest molecular weight synthesized by rGalNAc-T1, showed a $M_r$ of 4177.8, corresponding to the addition of six GalNAc residues to the peptide (Fig. 4A). Peptides with five or four GalNAc residues attached were also observed in the lower molecular weight region. The maximum numbers of GalNAc residues incorporated by rGalNAc-T2 and -T3 were calculated to be 8 and 11 from the molecular weights of 4582.3 (peak b) and 5187.0 (peak c), respectively, as shown in Fig. 4, B and C. To exclude DABITC, the glycopeptides were degraded by cyanogen bromide treatment and purified again by the same reverse phase HPLC. To analyze the glycopeptides with a maximum number of incorporated GalNAc residues, the first peak with a symmetrical elution profile was subjected to analysis by an amino acid sequencer. It has been reported that PTH-Thr/Ser-GalNAc derivatives are eluted as two diastereotopic peaks at unique positions on the chromatogram (18). To determine the retention time of PTH-Thr-GalNAc in our system, we used a reference glycopeptide containing Ser$_{GalNAc}$-Thr-Thr$_{GalNAc}$ (Fig. 5). Chromatography of PTH-Thr-GalNAc gave two peaks eluted at 5.24 min (peak $T^*$) and 5.94 min (peak $T^{**}$). The
second peak was slightly behind the peak of PTH-Thr (retention time, 5.91 min) on the chromatogram. Based on these data, the sites of O-glycosylation were determined. Fig. 6 shows the relative areas of the peaks (T*) derived from the PTH-Thr-GalNAc at each cycle. Based on the amount of the first peak (T*) and the retention time of the second peak (T**), we determined the O-glycosylation sites to be the Thr-2, -4, -10, -11, -13, and –15; the Thr-2, -4, -8, -10, -11, -15, -17, and –19; and the Thr-2, -3, -4, -7, -8, -9, -10, -11, -15, -17, and –19 residues of the MUC2 peptides glycosylated by GalNAc-T1, -T2, and -T3, respectively. In addition to these O-glycosylation sites, two Thr residues, i.e., Thr-13 (Fig. 6B) and Thr-21 (Fig. 6C), appeared to be poorly glycosylated in some cases.

DISCUSSION

Aberrant glycosylation of the core portion of O-glycans produces two kinds of immunogenic structures recognized by various monoclonal antibodies.

One is an unmasked core peptide produced by decreased glycosylation. MUC1 on breast cancer cells has been characterized by this unmasked core peptide and is assumed to permit preferential interaction with cytotoxic lymphocytes (23, 24). In contrast to these hypoglycosylation, Muller et al. (25) demonstrated that breast cancer cell line T47D glycosylates the MUC1 tandem repeat peptide at a higher density than lactating breast epithelia. This discrepancy may be explained by the finding (26) that a specific GalNAc-T named GalNAc-T4 is responsible for the glycosylation of the PDTR motif within the MUC1 tandem repeat peptide. Thus, the expression of these epitope structures seems to be dependent on the expression of particular GalNAc-Ts but not on the decrease or increase of generally expressed GalNAc-Ts.

Another is a truncated O-glycan, in which Tn and T antigens and their sialylated counterparts, sialylTn and sialylT antigens, are involved. Many mucins (except for MUC1) have unique tandem repeats containing consecutive Ser/Thr residues, MUC2 potentially serves as a scaffold, presenting a variety of carbohydrate epitopes on its abundant Thr residues. Byrd et al. (27) reported that 78% of the Thr residues are glycosylated in the LS 174T colon carcinoma cell line. However, whether or not the degree of glycosylation changes due to malignancy and how clustered O-glycosylation occurs have not been studied in detail. We have shown that the antigenic sites of the Tn and
sialylTn antigens are built up clusters of GalNAc-Ser/Thr and SAα2–6GalNAc-Ser/Thr, respectively (3–6). Although the function of these antigens is not understood, their increased expression has some correlation to advanced malignancy. It has been reported that about 73% and 96% of human colon cancers express the Tn and sialylTn antigens, respectively (28).

The present study was designed to compare the glycosylation of the MUC2 peptide by extracts of colonic adenocarcinoma and paired normal mucosa and to determine which GalNAc-Ts are relevant to the synthesis of consecutive Thr-GalNAcs. Our previous work showed that microsomal membranes of LS 180 cells glycosylated the MUC2 tandem repeat peptide, leading to the synthesis of clustered Tn epitopes, and the peptide glycosylated most extensively was determined to be a glycopeptide with 11 attached GalNAc residues. It is notable that the colonic adenocarcinoma extract exhibited a higher level of enzyme activity than did the normal mucosa (Fig. 1). In addition, as judged from the elution profile of the glycopeptides on reverse phase HPLC, the colonic adenocarcinoma extract could glycosylate the peptide at the same level as the microsomal membranes of LS 180 cells, as reported previously (9).

First, we estimated the expression of GalNAc-T mRNA semiquantitatively by RT-PCR. Only GalNAc-T3 mRNA was elevated remarkably in the colonic adenocarcinoma, whereas the level of GalNAc-T1 and -T2 mRNA increased slightly (Fig. 2 and Table 1), and these findings were consistent with previous reports that in all human organs examined, GalNAc-T1 and -T2 are expressed universally at low to moderate levels, but that the expression of GalNAc-T3 mRNA is highly tissue specific (19, 21, 22, 29). GalNAc-T4 was expressed at a very low level in both adenocarcinoma and normal mucosa (data not shown). These results prompted us to examine whether or not GalNAc-T3 could glycosylate the MUC2 peptide more extensively than the other enzymes could. Although O-glycosylation on the MUC1 tandem repeat unit has been studied extensively (25, 30, 31), little is known about the O-glycosylation sites on the MUC2 peptide. Iida et al. (32) demonstrated the acceptor specificity of rGalNAc-T1, -T2, and -T3 toward a short peptide, PTTTLK, mimicking a part of the MUC2 tandem repeat unit, in which rGalNAc-T3 was shown to have a unique acceptor specificity differing from those of GalNAc-T1 and -T2. Because substrate activity is significantly influenced by certain aspects of the primary amino acid sequence in the region adjoining the glycosylated position, it seems to be preferable to use a long peptide as a substrate within the range to be analyzed. In fact, Nishimori et al. (31) reported that the length for an enzyme-substrate interaction may influence its subsequent glycosylation, including both vicinal and distal glycosylation sites. Therefore, we used the whole MUC2 tandem repeat composed of 23 amino acid residues as a substrate.

The MUC2 peptide was glycosylated extensively on incubation with rGalNAc-T1, -T2, and -T3 for a long period with daily supplementation of the recombinant enzymes. Although the potential glycosylation sites were glycosylated completely or almost completely, the products comprised several glycopeptides with different numbers of incorporated GalNAc residues, as shown in Fig. 4. Muller et al. (25) reported that glycosylation of the tandem repeat peptides within individual MUC1 molecules is not uniform. However, the multiple Thr residues seem to be glycosylated in an ordered manner, at least under the experimental conditions used in which the peptides are glycosylated by a single enzyme in vitro. The fact that a preferential order for the incorporation of GalNAc to the Thr residues of the MUC2 peptide exists (32) supports this view. Although the order remains to be elucidated, it was clearly demonstrated that rGalNAc-T3 could glycosylate the MUC2 peptide most extensively, including both consecutive parts composed of three and five Thr residues (Fig. 6). This distinct substrate specificity of GalNAc-T3 is very significant in light of the fact that in contrast to the universal expression of GalNAc-T1 and -T2 mRNA in all human organs and malignant cells, GalNAc-T3 shows a quite unique expression pattern. These results are also consistent with previous reports (30, 32), which found that although there is overlapping of acceptor-substrate specificities due to some redundant functions of these enzymes, each enzyme gives a specific pattern of glycosylated Thr residues on the MUC2 peptide.

It has been reported that GalNAc-T3 is differentially expressed by adenocarcinoma cell lines, with a tendency for more differentiated cell lines to express it more extensively (29). More detailed analysis to determine the glycosylation pattern on the MUC2 peptide by using various adenocarcinomas differentiated to various degrees is necessary.

Recently, Bennett et al. (34) demonstrated that a new isozyme, designated GalNAc-T6, exhibits a similar substrate specificity to GalNAc-T3. GalNAc-T3 and probably GalNAc-T6 are essential for glycosylating consecutive Thr residues, which might play significant roles in the expression of not only clustered tumor-associated carbohydrate antigens but also of carbohydrate ligands for cellular lectins.

REFERENCES

EXPRESSION OF CLUSTERED CARBOHYDRATE ANTIGENS


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