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Glycosylation of the Tandem Repeat Unit of the MUC2 Polypeptide Leading to the Synthesis of the Tn Antigen

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A synthetic peptide corresponding to the human MUC2 tandem repeat domain containing 14 Thr residues was glycosylated *in vitro* using UDP-GalNAc and microsomal membranes of the colorectal cancer cell line, LS180. The products were fractionated by reverse phase HPLC, which gave seven glycopeptide fractions. Their molecular weights were estimated by matrix-assisted laser desorption/ionization mass spectrometry, the values obtained corresponding to glycopeptides containing from one to ten GalNAc residues. On solid phase radioimmunoassaying involving a monoclonal anti-Tn antibody (MLS128), it was found that the glycopeptides containing nine or ten GalNAc residues were strongly immunoreactive, whereas the glycopeptides containing less than six GalNAc residues were inactive, indicating that a cluster of GalNAc-Thr is essential for the Tn antigenicity.

Mucins are high molecular weight glycoproteins characterized by many O-linked oligosaccharides attached to the core polypeptide through Ser or Thr residues. It is well-known that the amounts of several human carbohydrate antigens on mucins increase with carcinogenesis. They are Tn and T , and their sialylated counterparts, sialylTn and sialylT, as well as the related structures representing blood group antigens (1).

Although most mucin core proteins contain numerous Ser and Thr residues, not all the residues are O-glycosylated. The distribution of O-glycans on the core polypeptide seems to be important for the expression of carbohydrate antigens. We have investigated the antigenic determinants of these carbohydrate chains and have shown that a cluster of GalNAc-Ser/Thr is essential for Tn antigenicity (2-4). The antibody used in this

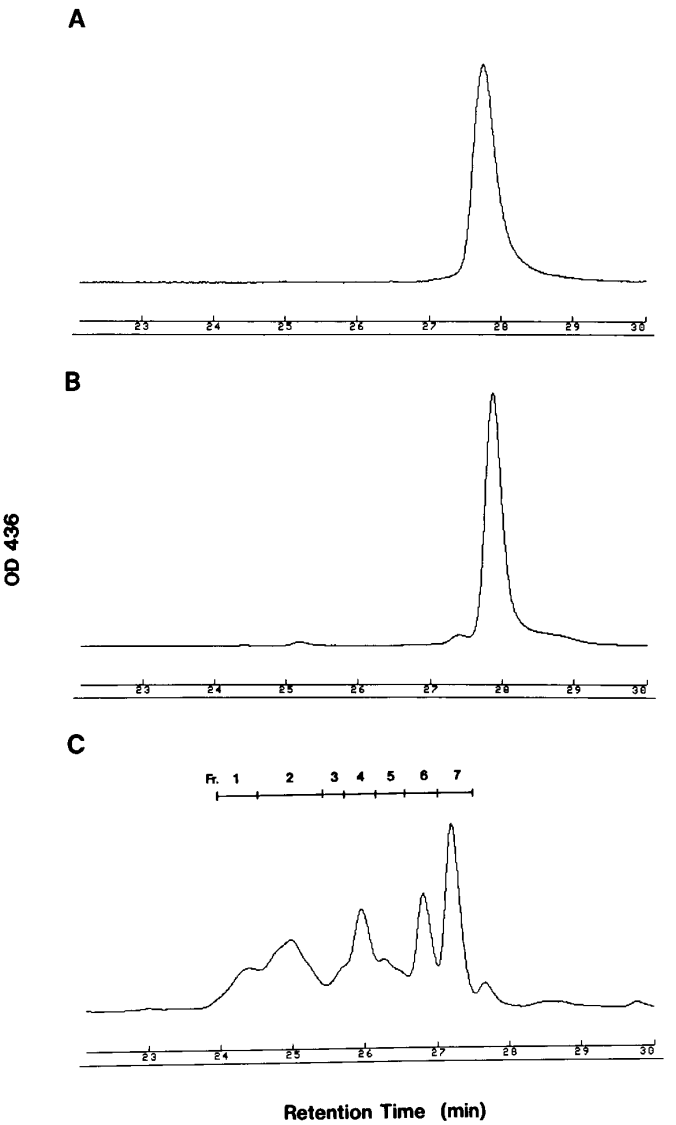

FIG. 1. Reverse phase chromatography of GalNAc transferred peptides. O-Glycosylation with UDP-GalNAc:polypeptide GalNAc transferase was carried out for 1 h in the presence (C) or absence (B) of UDP-GalNAc. The reaction products were directly subjected to reverse phase column chromatography as described under Materials and Methods. (A) shows the elution position of the DABITC-MUC2 peptide.

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Abbreviations: HPLC, high performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; DABITC, 4-N,N-dimethylaminoazo-benzene 4-isothiocyanate; MALDI mass spectrometry, matrix-assisted laser desorption/ionization; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

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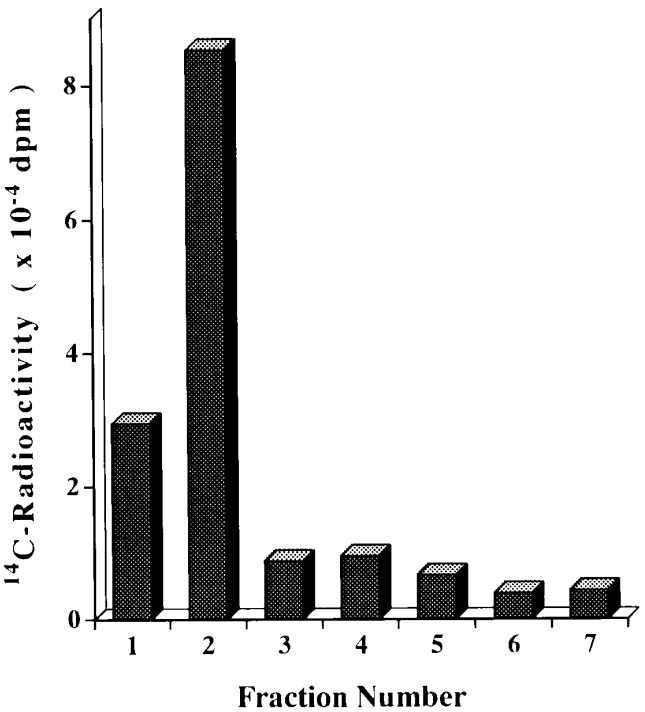

FIG. 2. Incorporation of ^{14}C-GalNAc into the MUC2 peptide. O-Glycosylation was performed for 1 h in the presence of UDP-^{14}C-GalNAc and then the radioactivity incorporated into each fraction was determined.

study was a monoclonal antibody, designated as MLS128, which was raised against a human colorectal cancer cell line, LS180 cells (5). The antigens, including the Tn antigen in LS180 cells have been shown to be the products of glycosylation of the polypeptide encoded by MUC2 gene (6,7).

We examined the expression of the Tn antigen *in vitro* using a synthetic peptide corresponding to the tandem repeat domain of the MUC2 gene and microsomal membranes of LS 180 cells.

MATERIALS AND METHODS

Materials. The synthetic peptide, GYPKAPTTTPITTTTTVTPTPTPTGTQT, was obtained from KURABO, Osaka. A monoclonal antibody, MLS128, recognizing the Tn antigen was prepared as described previously (5). UDP-^{14}C-GalNAc (2.07GBq/mmole) was purchased from Amersham. Phenylmethylsulfonyl fluoride (PMSF) and 4-N,N-dimethylamino azobenzene-4-isothiocyanate (DABITC) were from Nacarai Tesque, Kyoto.

Cells. LS180 cells were cultured in Eagles minimum essential medium (MEM) supplemented with 10 % fetal calf serum.

Transfer of GalNAc to a synthetic peptide. A synthetic peptide including one unit of the MUC2 tandem repeat was conjugated with DABITC according to Chang (8). LS180 cells were lysed in 25 mM Tris-HCl buffer, pH7.4, and 0.15 M NaCl by sonication, and then the lysate was centrifuged at 10,000 × g for 10 min. The supernatant was further centrifuged at 105,000 × g for 1 h. The pellet, after solubilization with 2 % octylglucoside, 0.1 M Tris-HCl buffer, pH 7.4, and 2 mM PMSF, was again centrifuged at 105,000 × g for 1 h. The resulting supernatant was used as the enzyme source. The incubation mixture comprised 5 mM $MnCl_2$, 5 mM 2-mercaptoethanol, 5 mM CDP-choline, 43 nmole UDP-GalNAc, 4 μg of DABITC-MUC2 peptide and 456 μg protein of the microsomal extract, in a total volume of 250 μl. The mixture was incubated at 37°C for the time indicated in the figure legends.

Characterization of the reaction products. The glycosylated products were directly subjected to reverse phase HPLC, elution being carried out with a linear gradient of 0-100 % acetonitrile in 0.1 % trifluoroacetic acid at the flow rate of 1 ml/min. Each peak was treated with trypsin and the digests were purified by re-chromatography. The molecular masses of the glycopeptides were determined by MALDI-mass spectrometry (Kratos Analytical, Manchester) according to Lapolla *et al.* (9). In some cases, labeled UDP-GalNAc was used and the radioactivity incorporated into each fraction was determined.

The glycopeptides labeled with ^{14}C-GalNAc were treated with 0.05 M NaOH and 1 M $NaBH_4$ at 45°C for 16 h according to Carlson (10), and then the released O-glycans were fractionated on Bio-Gel P-2.

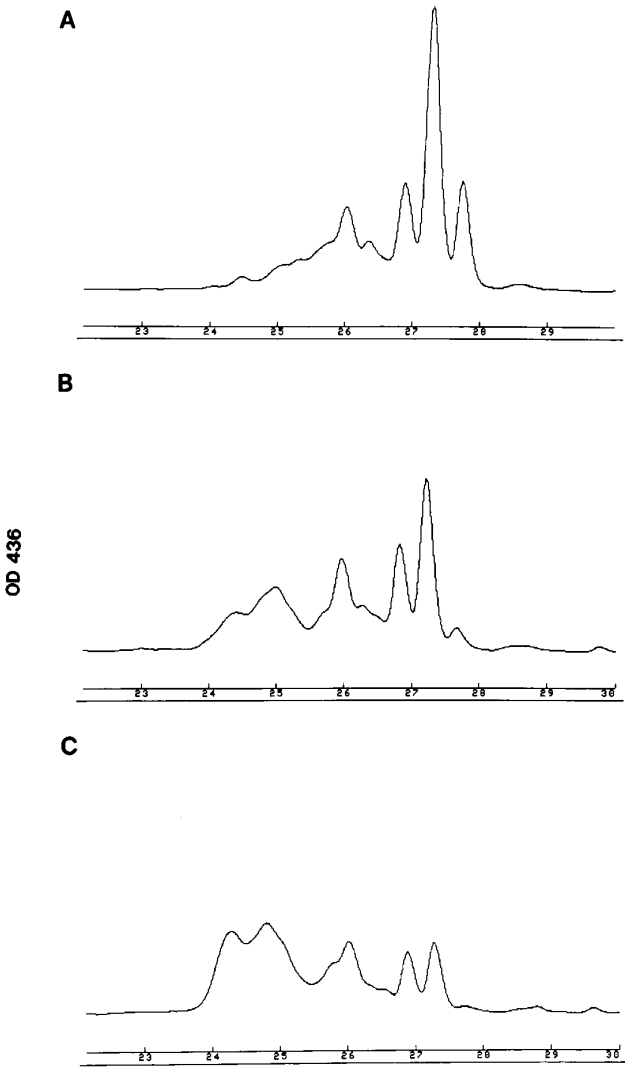

FIG. 3. GalNAc transferred peptides produced with different incubation times. MUC2 peptides were O-glycosylated for 30 min (A), 1 h (B), and 2 h (C). The products were then fractionated under described in Materials and Methods.

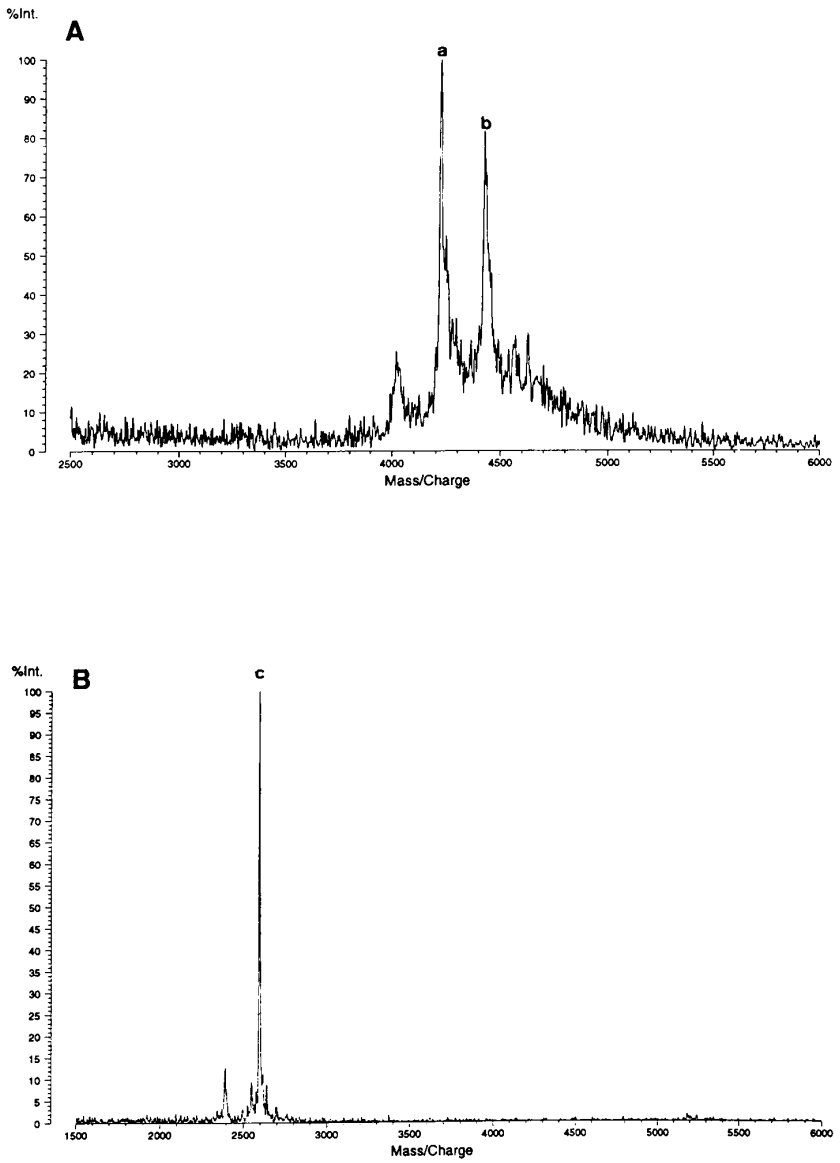

FIG. 4. MALDI-mass spectra of the glycopeptides. A: Fr.1, B: Fr.7, The molecular weights of Peaks a, b, and c were estimated to be 4220, 4424, and 2592, respectively.

Tn antigenicity of the glycopeptides. A polyvinyl chloride plate (96 wells; Coster) was coated with the glycopeptides according to Fukui *et al.* (11), and then the uncoated surfaces were coated with 1 % bovine serum albumin (BSA) in 20 mM phosphate-buffered saline (PBS), pH 7.2. An appropriate amount of MLS128 was added to each well and the plate was left standing overnight at 4°C, and then the wells were washed three times with 1 % BSA-PBS. For determination of the amount of MLS128 bound to the plate, ^{125}I-labeled protein A was added, followed by incubation for 2 h at room temperature. After washing the wells with 1% BSA-PBS, each well was cut out from the plate and the radioactivity was measured with a γ-counter (Aloka, Tokyo).

RESULTS AND DISCUSSION

Transfer of GalNAc to the MUC2 Peptide in Vitro

O-Glycosylation is initiated by the transfer of GalNAc from UDP-GalNAc to a polypeptide catalyzed by polypeptide : GalNAc transferase (12). The transfer of GalNAc to glycopeptides was investigated *in vitro* using synthetic peptides corresponding to the MUC2 tandem repeat with 14 Thr residues and microsomal membranes of human colorectal cancer cells, LS180, as described under Materials and Methods. The effects of various assay parameters (cell extract, MUC2 peptide and UDP-GalNAc) were evaluated and appropriate conditions were selected. To detect the products directly, the synthetic peptides were conjugated with DABITC, which exhibits an absorbance at 436 nm. Furthermore, five amino acids including Lys were inserted between DABITC and the amino-terminal of the MUC2 tandem repeat to minimize the influence of DABITC and to exclude DABITC released by trypsin digestion

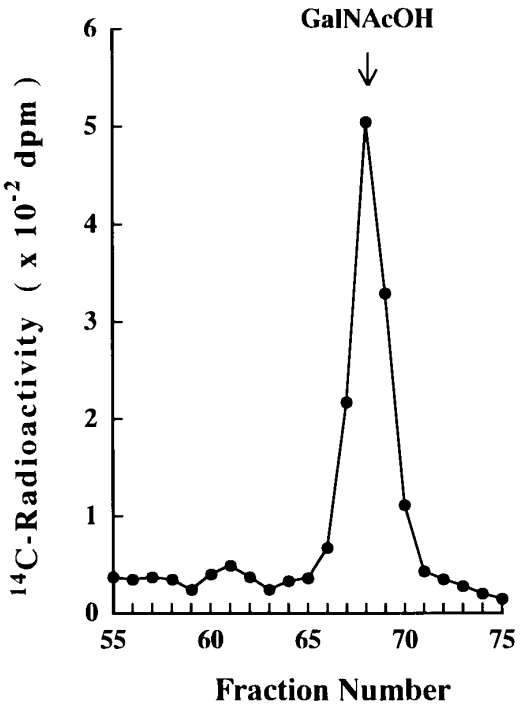

FIG. 5. Gel filtration of O-glycans released from GalNAc transferred peptides (Fr.1). O-Glycosylation was carried out for 1 h in the presence of UDP-^{14}C-GalNAc. The glycopeptides in Fr.1 were treated with alkaline-borohydride, followed by fractionation on Bio-Gel P-2 (1.0 × 109 cm). Fractions of 0.82 ml each were collected and then the radioactivity was determined.

after fractionation of the glycopeptides. As shown in Fig. 1, the products were fractionated by reverse phase HPLC.Non-glycosylated DABITC-MUC2 peptides were eluted at 27.8 min (Fig. 1A). Without UDP-GalNAc in the incubation mixture, a few small peaks were detected, probably due to degradation products (Fig. 1B). When incubated with UDP-GalNAc, several fractions (Fr.1-7) were obtained. With the use of labeled UDP-GalNAc, radioactivity was detected in these fractions (Fig. 2). The incorporated radioactivity per absorbance at 436 nm was maximum for Fraction 1 and minimum for Fraction 7 (data not shown).

As shown in Fig. 3, on longer incubation, the amounts of glycopeptides with shorter retention times increased, while later eluted glycopeptides decreased, suggesting that there seems to be a precursor-product relationship between these glycopeptides.

The glycopeptides in fractions 1-7 were digested with trypsin and then the digests were re-chromatographed on the same column. Each glycopeptide was applied to a gas phase protein sequencer to identify the glycosylation sites. However, we failed to assign the sites correctly because it was very difficult to analyze a peptide containing a large number of Thr and Pro residues.

Estimation of the Molecular Masses of Glycopeptides

The molecular masses of the glycopeptides were determined by MALDI-mass spectrometry as described by Lapolla *et al*. (9). Figs. 4 A and B show the mass spectra of fractions 1 and 7. The major glycopeptides in fraction 1 were estimated to have molecular weights of 4424 and 4220, which correspond to glycopeptides with 10 and 9 GalNAc residues attached, respectively. Fraction 7 contained a glycopeptide with a single GalNAc residue. Similarly, fractions 2-6 were mixtures of glycopeptides with intermediate numbers of GalNAc residues attached (data not shown). To confirm the transfer of GalNAc to the peptide, the glycopeptide (fraction 1) labeled with ^{14}C-GalNAc was treated with alkaline-borohydride, followed by gel filtration on Bio-Gel P-2, as shown in Fig. 5. The elution profile completely coincided with that of authentic N-acetylgalactosaminitol, indicating that GalNAc-Thr was synthesized exclusively, with no synthesis of other possible structures such as GalNAc-GalNAc-Thr.

Tn Antigenicity of the Glycopeptides

To detect the Tn antigenicity of the synthesized glycopeptides,the binding activity of MLS128 was determined as described under Materials and Methods (Fig. 6). Of the synthesized glycopeptides, fraction 1 with 9 or 10 GalNAc residues exhibited the strongest immunoreactivity. Fraction 2 also showed some reactivity, but other glycopeptides were practically inactive. Since fractions 2 and 3 were mixtures of glycopeptides with

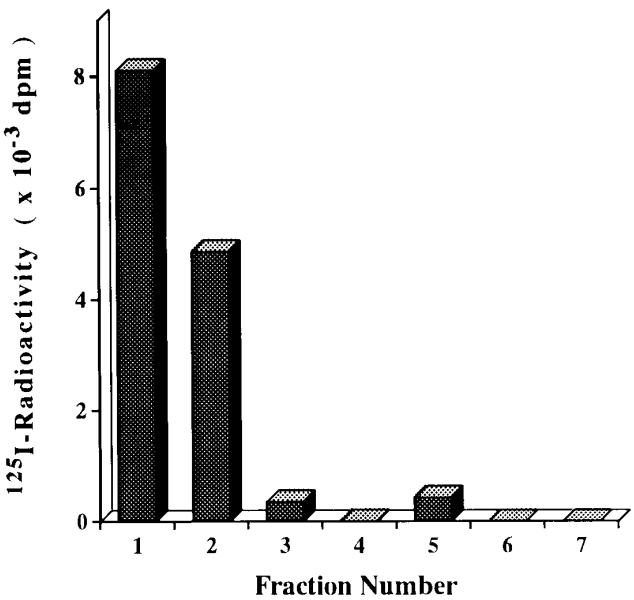

FIG. 6. Tn antigenicity of GalNAc transferred peptides. GalNAc transferred peptides were coated on a plate, and then the binding of the anti-Tn monoclonal antibody (MLS128) was estimated as described under Materials and Methods.

8 or 7, and 6 or 5 GalNAc residues, respectively, glycopeptides containing more than 7 or 8 GalNAc residues seem to be immunoreactive. Although the O-glycosylation sites have not been determined yet, these results seem to support the idea that consecutive sequences of three GalNAc-Thr are essential for the immunoreactivity, being consistant with our previous results (2-4).

It has been reported that multiple polypeptide GalNAc transferases exist (13). We also confirmed the occurrence of three GalNAc transferase genes, designated as GalNAc-T1,-T2 and -T3 by Clausen *et al.* (14,15), in LS180 cells using the polymerase chain reaction (data not shown). It would be interesting to determine which GalNAc transferases are responsible for this O-glycosylations.

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