Molecular Basis of Incomplete *O*-Glycan Synthesis in MCF-7 Breast Cancer Cells: Putative Role of MUC6 in Tn Antigen Expression

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Abstract

An incomplete elongation of *O*-glycan saccharide chains in mucins have been found in epithelial cancers, leading to the expression of shorter carbohydrate structures, such as the Tn antigen (GalNAc-*O*-Ser/Thr). This antigen is one of the most specific human cancer–associated structures and is capable of inducing effective immune responses against cancer cells. We aimed to investigate the causes of the expression of Tn antigen in the Tn-rich MCF-7 breast cancer cell line focusing on the first step of the *O*-glycosylation process. Interestingly, amino acid sequences derived from "nonmammary" apomucins (MUC5B and MUC6) were very good acceptor substrates for ppGalNAc-Ts, which are the enzymes catalyzing the Tn antigen synthesis. MUC6 peptide glycosylation with MCF-7 microsome extracts as source of ppGalNAc-T activity yielded 95% conversion of the peptide into MUC6-Tn. In addition, the MUC6-Tn glycopeptide was a poor acceptor substrate for core 1 β3Gal-T, the next enzyme involved in the saccharide chain biosynthesis, yielding only 5% conversion of MUC6-Tn into MUC6-TF. These results indicate that nonmammary apomucin expression could be responsible, at least in part, for Tn antigen expression in MCF-7 breast cancer cells due to a combined action on glycosyltransferases: an increase of ppGalNAc-T activity and a decrease of core 1 β3Gal-T activity. Our hypothesis is supported by experiments *in vivo* showing that (*a*) native MUC6 glycoproteins express the Tn antigen in MCF-7 cells and (*b*) Tn antigen expression is increased after transfection with a construct encoding for a MUC6 recombinant protein into the low Tn-expressing breast cancer cell T47D. These results open new horizons in breast cancer glycoimmunology, stressing the potential role of nonmammary apomucins. (Cancer Res 2005; 65(17): 7880-7)

Introduction

Mucins are high molecular weight proteins that are heavily *O*-glycosylated. In epithelial cancers, many of the markers for premalignant and malignant cells have been found on the carbohydrate and peptide moieties of mucins (1, 2), and these structures greatly contribute to the phenotype and biology of cancer cells. Abnormal *O*-glycans expressed by cancer cells have functional importance in cell adhesion, invasion, and metastasis (3). An incomplete elongation of *O*-glycan saccharide chains in mucins can lead to the expression of shorter carbohydrate structures, such as the TF, sialyl-Tn, or Tn antigens. The latter determinant (GalNAc-*O*-Ser/Thr: the innermost *O*-linked structure), which is usually masked by additional sugar residues in normal tissues, was characterized as one of the most specific human cancer–associated structures, and it was detected in ~90% of human carcinomas (4). It was reported that Tn could be an early biomarker of cancer, both in humans (5) and in animal models (6). A direct correlation has been shown between carcinoma aggressiveness and the density of expression of this antigen in the tumor (7). Moreover, Tn has been found to be capable of inducing an effective immune response against cancer cells (8). Although the chemical structure of the Tn determinant is GalNAc-*O*-Ser/Thr, its immunologic definition is more complex. Indeed, the characterization of the fine specificity of some antibodies showed that they require either the involvement of additional amino acids in the antigenic determinant or a high density of Tn residues. For example, the monoclonal antibody (mAb) PMH1 recognizes a mono-Tn determinant on the specific MUC2 apomucin peptide chain (9), whereas the anti-Tn mAbs 83D4 and MLS128 require the presence of at least two consecutive Tn residues for substrate recognition (10).

The first step of the mucin-type *O*-glycosylation pathway (i.e., the linking of a GalNAc residue to serine or threonine; synthesis of the Tn antigen) is catalyzed by a large family of isozymes called UDP-*N*-acetylgalactosamine: polypeptide *N*-acetylgalactosaminyltransferase (EC 2.4.1.41, ppGalNAc-Ts). To date, 15 ppGalNAc-Ts have been identified in mammals, and functional profiles of each member of the family have been characterized showing that these enzymes have not only different substrate specificities, but also specific tissue expression patterns (11–13). It is widely accepted that the glycosylation sites and the *O*-glycan pattern on mucins depend both on the type of glycosyltransferases involved and on the structures of core polypeptide acceptors (14). Thus, the expression of the Tn determinant could be the result of a deregulation of glycosyltransferases (e.g., changes in enzyme activity and/or in substrate specificity) or a shift of *O*-glycan biosynthesis in cancer cells, such as aberrant expression of apomucin genes.

In the present study, we investigated the causes of the expression of Tn antigen in breast cancer cells focusing on the first step of the *O*-glycosylation process. We studied the capacity of Tn-rich MCF-7 breast cancer cells to glycosylate synthetic peptides derived from different human apomucins. We found that the "nonmammary" apomucin MUC6, which is abnormally expressed in breast cancer, is a very good acceptor substrate for ppGalNAc-Ts present in breast cancer cells. Interestingly, the MUC6-Tn glycopeptide (recognized by two anti-Tn monoclonal antibodies) was a poor substrate for the core 1 β3Gal-T, which displayed 5-fold less activity than when using

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a MUC1-Tn glycopeptide as acceptor. Moreover, we show that transfection of the low Tn-expressing breast cancer cell T47D with a construct encoding for a MUC6 recombinant protein induces an increase of Tn expression. These results strongly suggest that the aberrant apomucin expression could explain, at least in part, Tn antigen expression by breast cancer cells.

Materials and Methods

Monoclonal Antibodies and Lectin

The mAb 83D4 (IgM), which binds specifically the Tn antigen (10), was produced from a mouse immunized with cell suspensions obtained from formalin-fixed paraffin-embedded sections of an invasive human breast cancer (15). The mAb was precipitated from ascitic fluids by dialysis against demineralized water at 4°C, dissolved in a small volume of 0.5 mol/L NaCl in PBS, and purified by gel filtration chromatography on Sephacryl S-200. The anti-Tn mAb MLS128 (IgG1), kindly given by H. Nakada (Department of Surgery, Saitama Medical School, Saitama, Japan) and collaborators, was established by immunizing mice with human colonic cancer cells (LS180; ref. 16) and purified by affinity chromatography on protein A-Sepharose. Both anti-Tn mAbs recognize Tn residues organized in clusters (10, 17). The biotinylated isolectin B4 from *Vicia villosa* seeds (VVLB4), which recognizes a single Tn determinant (10), was obtained from Sigma Chemical Co. (St. Louis, MO).

Synthetic (glyco)peptides

Peptides derived from the MUC1 sequence (Table 1) were synthesized by Dr L. Vernie (the Netherlands Cancer Institute, Amsterdam, the Netherlands) using a solid-phase procedure on a MilliGen 9050 synthesizer (B&L Systems, Maarssen, the Netherlands). Amino acids were coupled as fluoren-9-yl-methoxycarbonyl (Fmoc) derivatives. Peptides derived from MUC2, MUC5B, and MUC6 sequences (Table 1) were obtained from BioSynthesis Incorporated. The synthesis of the MUC6-Tn (GTT[α-GalNAc]PPPTTLK) and MUC1-Tn (ST[α-GalNAc]APPAHGV) glycopeptides was done as previously described using a protected glycosylated building block [Fmoc-Thr(α-GalNAc(OAc)3)-OH] at the appropriate place in the sequence (8). Peptides and glycopeptides were purified by reversed-phase high-pressure liquid chromatography (HPLC) and analyzed by mass spectrometry.

Breast Cancer Cell Line Extracts

Breast cancer cell line MCF-7 and T47D were grown to 90% confluence in DMEM (Life Technologies, Inc., Cergy Pontoise, France) with 10% fetal bovine serum, 1 mmol/L pyruvate, and 2 mmol/L glutamine and 5% CO_2 at 37°C. After trypsinization, cells were washed thrice with PBS and resuspended in 250 mmol/L sucrose and homogenized in a Potter-Helmer. Cells were centrifuged at 3,000 × *g* for 10 minutes at 4°C and at 100,000 × *g* for 1 hour at 4°C. The resulting pellet was resuspended in 0.1 mol/L imidazole (pH 7.2) and 0.1% Triton X-100. Cell extracts were aliquoted and stored at −80°C. Protein concentration was determined by the BCA method (Sigma).

Polypeptide GalNAc Transferase Activity Assay

The evaluation of the ppGalNAc-T activity was done as previously described (18). Briefly, the standard reaction mixture contained 150 μmol/L uridine-5′-diphospho-α-*N*-acetyl-D-[^{3}H]galactosamine (UDP-[^{3}H]GalNAc; ~80,000 dpm) and synthetic peptides (2 mmol/L) from the tandem repeat sequences of various human mucins (MUC1, MUC2, MUC5B, and MUC6; Table 1), in 50 mmol/L imidazole-HCl (pH 7.2), 10 mmol/L $MnCl_2$, and 0.5% Triton X-100 to a final volume of 50 μL. The mixture was incubated at 37°C for 2 hours. The reaction was terminated by adding 50 μL of 250 mmol/L EDTA. The glycosylated peptide was separated from unreacted UDP-[^{3}H]GalNAc on a 1 mL AG1X-8 (Cl^- form; Sigma) column with 2.6 mL of water as eluent. The rate of GalNAc transfer to peptide was measured by quantifying the radioactivity with an LS Analyzer Beckman scintillation counter. The enzyme activity was expressed as pmoles of [^{3}H]GalNAc transferred per milligram of protein per hour. All experiments were done in triplicate. Reactions lacking acceptor substrate yielded background values that were averaged for each protein extract and subtracted from each triplicate value. Error bars indicate SD.

Table 1. Synthetic peptide acceptors used for the ppGalNAc-T activity

Peptide	Amino acid sequence	Reference
Human MUC1-a	PDTRPAPGSTA	(43)
Human MUC1-b	HGVTSAPDTRP	(43)
Human MUC2-a	PTTTPITTTTTV	(44)
Human MUC2-b	VTPTPTPTGTQT	(44)
Human MUC5B	VLTTTATTPTA	(45)
Human MUC6	GTTPPPTTLK	(46)

Recognition of *In vitro* Glycosylated Peptides by Anti-Tn Molecules

Microtiter plates were coated with *in vitro* synthesized glycopeptides (100 μL/well) and dried overnight. Plates were washed thrice with 0.1% Tween 20 in PBS (PBS/T) and nonspecific binding sites were blocked with 1% gelatin in PBS (PBS/G) for 2 hours at 37°C. After washing, anti-Tn mAbs (83D4 and MLS128) or lectin VVLB4 were added and incubated for 2 hours at 37°C. After three washes with PBS/T, plates were incubated with peroxidase-conjugated anti-mouse (Sigma, 1/1,000) or avidin peroxidase (Sigma, 1/2,000) diluted in 0.5% gelatin, 0.1% Tween 20 in PBS (PBS/TG) for 1 hour at 37°C. The plates were revealed using 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) as described above. All experimental samples were analyzed in duplicate. Asialo OSM binding (30 ng/mL) was used as a positive control. Results are expressed in percentage of binding compared with the reactivity of the individual anti-Tn mAbs and the lectin with asialo–ovine submaxillary mucin (asialo-OSM; 100%).

Monitoring of *In vitro O*-glycosylation by Reversed-phase High-Pressure Liquid Chromatography and Semipreparative Synthesis of the MUC6-Tn Glycopeptide

In vitro glycosylation of MUC6 peptide in a semipreparative scale (0.5-1 mg) was carried out using microsome fractions of MCF-7 breast cancer cells and UDP-GalNAc (2 equivalents UDP-GalNAc/equivalent peptide) at 37°C. Aliquots (50-100 μL) of the reaction mixture were subjected to reversed-phase HPLC using a Perkin-Elmer pump system with an UV detector at 230 nm. The column was an AIT Kromasil C18 (5 μm, 100 Å, 4.6 × 250 mm). Elution was carried out with a linear gradient of 0% to 40% acetonitrile in 0.1% trifluoracetic acid in water at a flow rate of 1 mL/min (over 40 minutes). Each peak was collected and then lyophilized. The MUC6-Tn glycopeptide was characterized by amino acid analysis, mass spectrometry, and amino acid sequencing. Mass spectra were recorded by electrospray in the positive mode on a Quattro-LCZ or LCTOF mass spectrometer (Micromass, Manchester, United Kingdom). The sample was dissolved at 10 mmol/L concentration in water/acetonitrile (1:1) with 0.1% formic acid. The glycopeptide was sequenced by Edman degradation on the Applied Biosystems Procise Sequencer (ABI 494) system. With this system, the typical phenylthiohydantoin (PTH) derivative of GalNAc attached to Thr was eluted as a pair of peaks near the positions of PTH-Ser and PTH-Thr (19).

Enzymatic Transfer of GalNAc or Gal into MUC6 or MUC6-Tn, Respectively

The enzymatic mixture consisted of ~100 μg of MCF-7 microsome extract, 2 μmol of UDP-GalNAc, and 1 μmol of MUC6 peptide (GTTPPPTTLK; for GalNAc transfer) or 2 μmol UDP-D-galactose (UDP-Gal) and 1 μmol of MUC6-Tn glycopeptide (GTT[α-D-GalNAc]PPPTTLK; for Gal transfer) in 50 mmol/L Mes buffer (pH 7), 10 mmol/L $MnCl_2$, 0.1% Triton X-100, to a final volume of 50 μL. Reactions were done at 37°C. Samples were filtered in 10 kDa nanosep filters (Pall Life Sciences, Fontenay sous Bois, France), and glycosylation was monitored by reversed-phase HPLC as described above after 0, 6, and 40 hours of incubation. The transfer of Gal into the MUC1-Tn glycopeptide (ST[α-GalNAc]APPAHGV) was also tested under the same conditions.

Identification of Native MUC6-Tn Glycoproteins in Breast Cancer Cells

MUC6 glycoproteins expressing native Tn contained in MCF-7 cell extracts were analyzed by Western blotting using two different anti-MUC6 antibodies: (*a*) the anti-MUC6.1 polyclonal antibody (kindly given by C. Bolos and F.X. Real, Unitat de Biologia Cellular i Molecular, Institut Municipal d'Investigacio Medica, Barcelona, Spain; ref. 20) raised against the peptide SFQTTTTYPTPSHPATTLP and (*b*) an anti-MUC6 polyclonal serum (anti-MUC6.2) raised against a half tandem repeat of MUC6 (MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGSTSLVTPSTHTITP-THAQMTSASHHPTTIPPTTLHATGSTHTAPLITVTTSRTSQVHSSF-STAKTSTSLLSHASSTHHP.[4] Affinity-purified Tn glycoproteins, obtained according to the procedure described previously (21), and total MCF-7 extracts were separated in a 10% SDS-PAGE. Proteins were transferred to nitrocellulose sheets (Amersham, Saclay, France) at 60 V for 5 hours in 20 mmol/L Tris-HCl (pH 8.3), 192 mmol/L glycine, and 10% ethanol as already described (22). Residual protein-binding sites were blocked by incubation with 3% bovine serum albumin (BSA) in PBS overnight at 4°C. Nitrocellulose was then incubated either with the polyclonal anti-MUC6 sera or the anti-Tn mAb 83D4 for 2 hours at 37°C. After three washes with PBS containing 0.1% Tween 20 and 1% BSA, the membrane was incubated for 1 hour at room temperature with goat anti-rabbit and anti-mouse immunoglobulins conjugated to peroxidase (Sigma) diluted in PBS containing 0.3% Tween 20 and 3% BSA, and reactions were developed with enhanced chemiluminiscence (Amersham). The same procedure was done omitting the antibodies as a negative control.

Analysis of Tn Antigen Content in Cell Extracts

Tn quantification in MCF-7 and T47D breast cancer cell lines. The Tn antigen was quantified in breast cancer cell lines by a double-determinant immunolectin-enzymatic method (CA83.4 assay), using anti-Tn mAb 83D4 as the catcher, and Tn-reactive VVLB4 as the tracer. Microtiter wells (Nunc, Roskilde, Denmark) were coated with 100 μL of mAb 83D4 [10 μg/mL in 0.1 mol/L Na carbonate buffer (pH 9.6)] by overnight incubation at room temperature. The wells were washed with PBS/T and incubated with PBS/G at 37°C for 1 hour. After three washes, wells were incubated overnight at 4°C with 100 μL of test samples diluted in PBS, and then with biotinylated VVLB4 (5 μg/mL) in PBS/TG, at 37°C for 1 hour. Unbound material was then washed off and 100 μL of 1/2,000 avidin/peroxidase complex (Sigma) in PBS/TG was added for 1 hour at 37°C. Peroxidase activity was shown by incubation in ABTS (3 mg) and 30% hydrogen peroxide (7 μL) in phosphate-citrate buffer (pH 5.0; 10 mL). Reaction was allowed to proceed for 30 minutes at room temperature and absorbance was read at 405 nm with an ELISA reader. Sample concentrations of Tn glycoproteins were determined by interpolation against a standard curve done with asialo–OSM (a standard source of Tn). One antigen unit was defined as the amount of CA83.4 reactivity found in 10 ng of mucin. All experimental samples were analyzed in duplicate.

Tn content after MUC6 transfection of T47D cells. Microtiter plates were coated with 10 μg of wild-type or MUC6-transfected T47D extracts diluted in 0.1 mol/L Na carbonate buffer (pH 9.6) and dried overnight. Mock-transfected T47D extracts were used as control. Plates were washed thrice with PBS/T and nonspecific binding sites were blocked with PBS/G for 2 hours at 37°C. After washing, anti-Tn mAb 83D4 was added and incubated for 2 hours at 37°C. After three washes with PBS/T, plates were incubated with peroxidase-conjugated anti-IgM mouse (Sigma) diluted in PBS/TG for 1 hour at 37°C. The plates were revealed using *o*-phenylenediamine/H_2O_2 and read photometrically at 492 nm in an ELISA autoreader (Dynatech, Marnes la Coquette, France).

Transfection of MUC6 in T47D Cells

cDNA encoding one half of a tandem repeat of MUC6 isolated from total cDNA of MCF7 breast cancer cells was subcloned into pcDNA3 (Invitrogen, Cergy Pontoise, France). To this end, the following product coding one tandem repeat of human MUC6 (MUC6-1) was amplified by reverse transcription-PCR (RT-PCR) using *Pfu* DNA polymerase and the primers MUC6-F, 5′-cgggatccTCCACCTCCTTGGTGACT-3′ and MUC6-1R, 5′-ggaagcttTTAGAAAGGTGGAACGTG-3′ (lowercase letters indicate restriction sites for *BamH*I and *Hind*III in the forward and reverse primers, respectively). Following digestion with *BamH*I and *Hind*III, the product was cloned into the pET28a(+) vector (Novagen, Fontenay sous Bois, France), so as to encode for a protein carrying a six-histidine tail at the NH_2 terminus. A portion of the MUC6-1 sequence containing the His-tag was reamplified using the following primers: pcDNA-MUC6-F (5′-ccaagcttaccATGGGCAGCAGCC-3′) and pcDNA-MUC6-2R (5′-ccttaagTTA-GAAAGGTGGAACGTG-3′) to permit the expression of a MUC6 protein containing a half of the tandem repeat and an His-Tag at the NH_2 terminus (MUC6-2; lowercase letters indicate restriction sites for *Hind*III and *EcoR*I in the forward and reverse primers, respectively). The product was afterward digested with *Hind*III and *EcoR*I and cloned into the pcDNA3 vector (Invitrogen). The construct was used to transfect T47D breast cancer cells using Lipofectamine (Invitrogen). To generate stable clones, 0.25 mg/mL geneticin (Invitrogen) was added on day 2 after transfection. Positive clones expressing the MUC6 recombinant protein were selected by Western blotting (as described above) using an anti-His monoclonal antibody (Qiagen, Hilden, Germany), and then analyzed with the anti-MUC6 polyclonal serum (anti-MUC6.2) and with the anti-Tn mAb 83D4.

Results

Glycosylation of mucin peptides by MCF-7 cell extracts. Among different breast cancer cell lines evaluated, MCF-7 cell extract was the one that presented the highest level of both Tn antigen and ppGalNAc-T activity (data not shown). Therefore, we did *in vitro* glycosylation assays to evaluate the capacity of this cell extract to glycosylate peptides from various human mucins. As shown in Table 1, we selected peptides derived from the tandem repeat of MUC1 (a mucin highly expressed in breast tissue whose *O*-glycosylated chains are shortened, or even absent, in breast cancer) and from other nonmammary mucins previously identified in breast cancer cell lines and breast carcinoma extracts, such as MUC2 (23), MUC5B (24), and MUC6 (25). All peptides were glycosylated, although at different rates (Fig. 1). The glycosylation rate of the peptides derived from MUC1 (MUC1-a and MUC1-b) was different for each peptide, being higher for MUC1-b than for

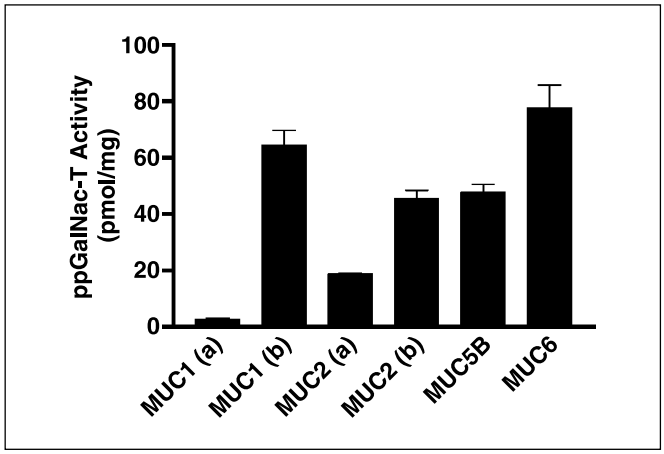

Figure 1. ppGalNAc-T activity using mucin synthetic peptide acceptors and MCF-7 microsome extracts. Six synthetic peptides (2 mmol/L; Table 1) were used to assess the ppGalNAc-T activity in the conditions described in Materials and Methods in a 1-hour incubation time. Results are expressed in picomoles of transferred [^{3}H]GalNAc per milligram of protein. *Error bars* indicate SD.

[4] T. Freire et al., in preparation.

MUC1-a. The same result was found for peptides MUC2-a and MUC2-b. The best substrates for ppGalNAc-Ts from MCF-7 cancer cells were MUC1-b and MUC6.

Tn antigenicity of the glycopeptides. To evaluate the Tn antigenicity of the *in vitro* synthesized glycopeptides, the binding properties of mAbs 83D4 and MLS128 and isolectin B4 from *V. villosa* (VVLB4) were determined using an ELISA assay, coating the wells with the glycosylated peptides as described under Materials and Methods. Interestingly, the three anti-Tn molecules poorly recognized most glycopeptides, whereas the MUC6 glycopeptide exhibited a strong immunoreactivity (Fig. 2).

Enzymatic synthesis and characterization of MUC6-Tn glycopeptide. Following the observation that the MUC6 peptide was a very good acceptor substrate for ppGalNAc-Ts in MCF-7 breast cancer cells and considering the antigenic properties of the resulting MUC6-Tn glycopeptide, we carried out preparative glycosylation assays of the MUC6 peptide to determine the number and the position of incorporated GalNAc residues. The reaction was monitored with reversed-phase HPLC. Nonglycosylated MUC6 peptide was eluted at 21.8 minutes (Fig. 3*A, 1*). When the peptide was incubated 6 hours with the enzymatic mixture containing UDP-GalNAc, one additional peak was obtained, which eluted at 20.7 minutes (Fig. 3*A, 2*). A 95% conversion of the peptide to glycopeptide was obtained after 40 hours incubation, as estimated by HPLC (Fig. 3*A, 3*). The peak was purified, lyophilized, and analyzed by analytic HPLC (Fig. 3*B*) and electrospray mass spectrometry (Fig. 3*C*). The fraction was shown to have a molecular weight of 1,214.6 Da, which corresponds to a glycopeptide with a single GalNAc (calculated 1,214.64 Da; Fig. 3*C*). Therefore, under the conditions used, the MCF-7 microsome extract transferred 1 mol of GalNAc per mole of peptide. No extra peak was observed even after a prolonged incubation (>3 days) and with further addition of MCF-7 microsome extract as enzyme source (data not shown). The glycosylation site was determined by Edman degradation amino acid sequencing. A PTH-Thr conjugated with a GalNAc residue resulted in two diastereomer peaks that appeared at cycle 3, demonstrating that the peptide was glycosylated as followed: GTT[α-GalNAc]PPPTTLK (data not shown). The antigenicity of this purified glycopeptide was confirmed using the two anti-Tn mAbs 83D4 and MLS128 (Fig. 3*D*).

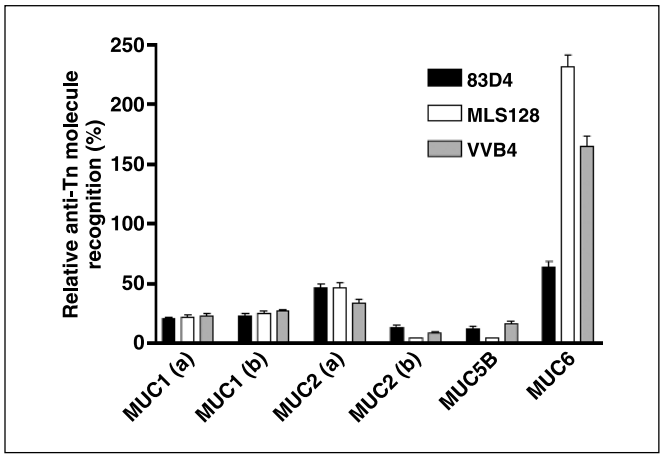

Figure 2. Antigenicity of the *in vitro* synthesized Tn peptides. Binding of two anti-Tn mAbs (83D4 and MLS128) or the anti-Tn lectin VVLB4 to the synthetic glycopeptides was evaluated using an ELISA assay. Results are expressed in percentage of binding compared with the reactivity of the individual anti-Tn mAbs and the lectin with asialo-OSM (100%).

Evaluation of the Gal transfer to the MUC6-Tn and MUC1-Tn glycopeptides. The peptide sequence can play an essential role in its *O*-glycosylation, as has already been reported for the ppGalNAc-Ts and core 1 β3Gal-T (26). To evaluate if the *in vitro* synthesized MUC6-Tn glycopeptide can be efficiently glycosylated by MCF-7 core 1 β3Gal-T, we carried out glycosylation assays using MUC6-Tn glycopeptide as substrate. The study was done in comparison with a MUC1-Tn glycopeptide (ST[α-GalNAc]APPAHGV) as acceptor substrate for core 1 β3Gal-T activity. When the MUC1-Tn glycopeptide was incubated with UDP-Gal and MCF-7 extract, the reaction yield for Gal transfer was 25% (Fig. 3*E, 2*). By contrast, in the same conditions, <5% of the initial glycopeptide was converted into the product MUC6-TF (GTT[α-GalNAc-β3Gal]PPPTTLK; Fig. 3*E, 4*). After purification, the identity of these enzymatically synthesized glycopeptides was confirmed by electrospray mass spectrometry: 1,376.8 Da for MUC6-TF (calculated 1,376.69 Da; Fig. 3*F*) and 1,200.6 Da for MUC1-TF (calculated 1,200.55 Da).

Identification of native MUC6 glycoproteins expressing the Tn antigen in MCF-7 cells. Previous works showed that MUC6 is expressed in breast cancer, including studies done on MCF-7 cells (25), pleural effusions associated to breast cancer (21), and mRNA expression of human breast tumors (24). To identify native MUC6 proteins bearing Tn antigen, we did Western blot analysis using the anti-Tn mAb 83D4 and two different anti-MUC6 antisera. Tn glycoproteins from total MCF-7 protein extracts were identified (using the mAb 83D4) as several components of high molecular weight (>100 kDa; Fig. 4, *lane 1*). Another group of apparent molecular weight of 45 kDa was also identified, which was also recognized by the anti-MUC6 antiserum, previously characterized on breast cancer cells by de Bolos et al. (Fig. 4, *lane 2*; ref. 25). When affinity-purified Tn glycoproteins were blotted, only the protein group of 45 kDa was identified (Fig. 4, *lane 3*). These results were confirmed using another anti-MUC6 polyclonal antiserum specific for a different amino acid sequence of MUC6 that corresponds to a 89 amino acid sequence of a MUC6 tandem repeat. This antiserum reacted with a component of the same apparent molecular weight (Fig. 4, *lane 4*).

Analysis of Tn expression in MUC6-transfected T47D breast cancer cells. The results obtained with MCF-7 cells suggest that the aberrant expression of MUC6 could be, in part, responsible for the expression of the Tn antigen. To have *in vivo* evidence, we analyzed the Tn content of a breast cancer cell line after transfection with a construct encoding for a MUC6 recombinant protein. To this end, we chose the T47D cell line that has a lower Tn antigen content than MCF-7 cells (Fig. 5*A*) and does not express MUC6 (as shown by Western blotting using a polyclonal antiserum; Fig. 5*B, 1, lane A*). After transfection with a half tandem repeat of MUC6 (see Materials and Methods), T47D clones expressed the MUC6 recombinant protein mainly as components of apparent molecular weights of 14 and 45 kDa (identified using anti-His mAb and anti-MUC6.2 polyclonal serum; Fig. 5*B, 1* and *2*). This could correspond to MUC6 recombinant proteins with different glycosylation levels (12.2 kDa being the expected molecular weight of the nonglycosylated MUC6 protein). Interestingly, the same cell extract analyzed by the anti-Tn mAb 83D4 presented two main components at 45 and 66 kDa (not observed in nontransfected T47D cells), suggesting that MUC6 on T47D clones expresses the Tn antigen (Fig. 5*B, 3*).

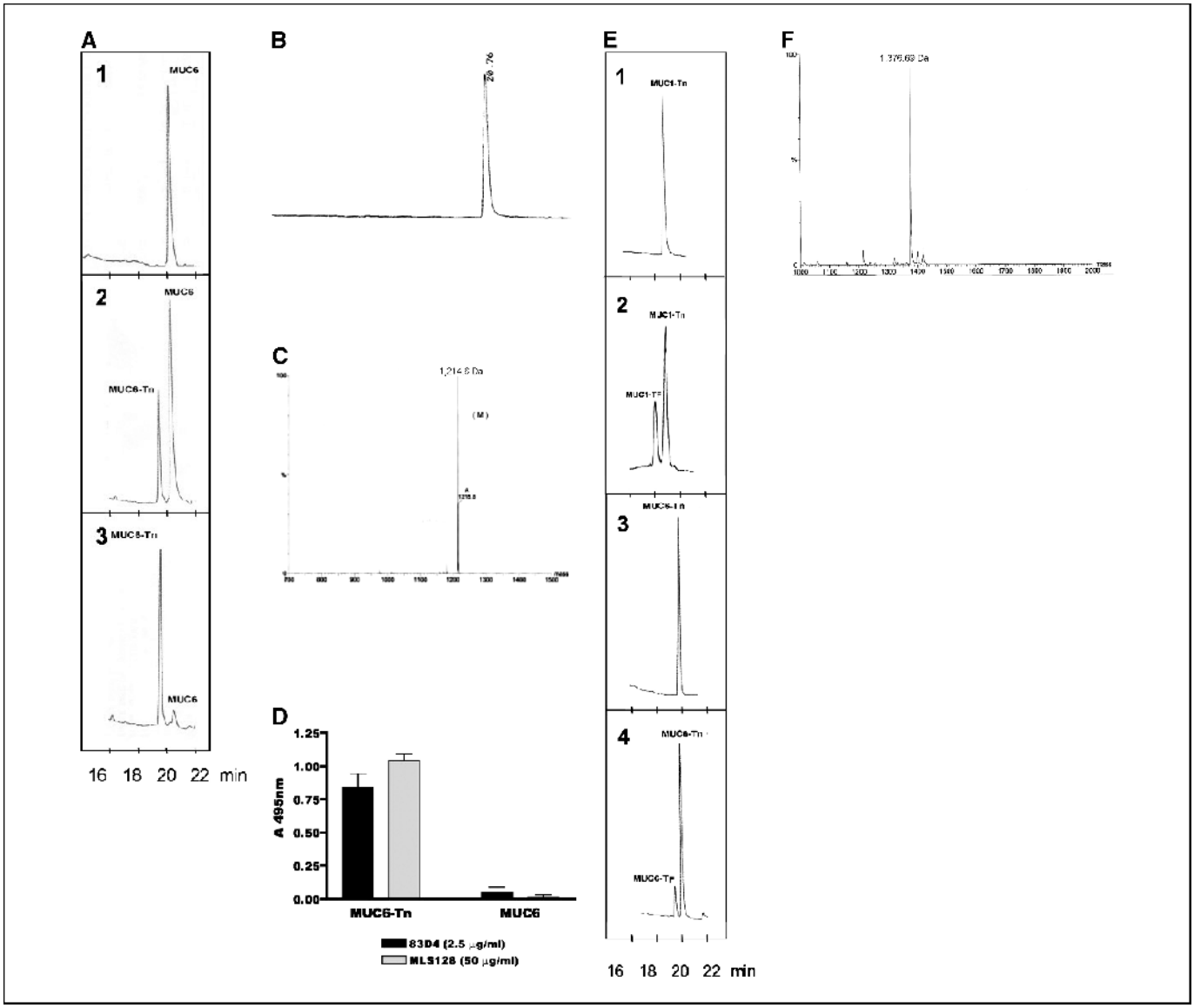

Figure 3. Semipreparative *in vitro O*-glycosylation reactions and characterization of the resulting MUC6 (glyco)peptides. *A,* for GalNAc transfer, UDP-GalNAc (2 μmol) was incubated with the MUC6 peptide (1 μmol) and MCF-7 microsome extracts at different incubations times: 0 hour (*1*), 6 hours (*2*), and 40 hours (*3*). The *O*-glycosylation rate was monitored by reversed-phase HPLC. The resulting glycopeptide was purified and analyzed by analytic HPLC (*B*) and electrospray mass spectrometry (*C*). *D,* antigenicity of the MUC6-Tn peptide was confirmed using the anti-Tn mAbs 83D4 (2.5 μg/mL) and MLS128 (50 μg/mL). The nonglycosylated MUC6 peptide was used as negative control. *E,* for β3Gal transfer, 1 μmol of MUC1-Tn or MUC6-Tn glycopeptides and MCF-7 microsome extract were incubated without (*1* for MUC1-Tn; *3* for MUC6-Tn) or with (*2* for MUC1-Tn; *4* for MUC6-Tn) 1.5 μmol of UDP-Gal. The resulting MUC6-TF glycopeptide was purified and analyzed by electrospray mass spectrometry (*F*).

To determine the influence of the MUC6 expression on the Tn content of T47D cells, we analyzed Tn expression levels in a MUC6-transfected T47D extract by ELISA using the mAb 83D4. As shown in Fig. 5*C*, the transfected clone expressed higher levels of Tn antigens than the wild-type T47D cells.

Discussion

Malignant transformation of epithelial cells is commonly associated with changes in the expression levels and glycosylation patterns of mucins, including exposure of simple mucin-type carbohydrates, like the Tn antigen. The expression of this antigen can be influenced by a number of factors, such as (*a*) the availability of functional ppGalNAc-Ts, which catalyze the first step of the *O*-glycosylation pathway and are expressed in an organ-specific manner; (*b*) the amino acid sequence environment around putative glycosylation sites that could influence catalytic action of glycosyltransferases; (*c*) posttranslational modifications of the peptide substrates, e.g., GalNAc substitutions proximal or adjacent to Ser/Thr residues that would inhibit or promote further glycosylation; and/or (*d*) the availability of glycosyltransferases that further elongate the saccharide chain on GalNAc-*O*-Ser/Thr.

Although important progress regarding the molecular basis of *O*-glycosylation have been obtained in the functional characterization of recombinant purified ppGalNAc-Ts on different peptide sequences, the native *O*-glycosylation profile of a cell is determined by the whole ppGalNAc-T mixture present in it. As it has already been reported for MUC1 and MUC2, several enzymes are involved in the glycosylation of a single substrate (27, 28). Correlations

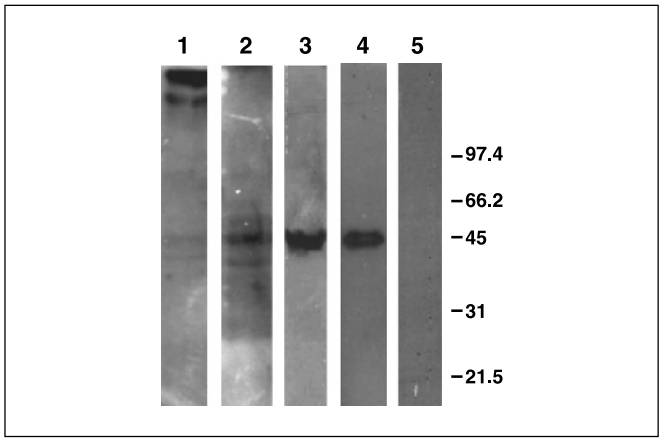

Figure 4. Identification of native MUC6 glycoproteins from MCF-7 cells carrying the Tn antigen. SDS-PAGE was done in a 10% gradient gel under reducing conditions. After transferring total MCF-7 proteins to nitrocellulose sheets, Tn proteins were detected using the mAb 83D4 (*lane 1*) and MUC6 was detected using MUC6.2 antiserum (*lane 2*). MUC6 from affinity-purified Tn glycoproteins was also detected with the antiserum MUC6.1 (*lane 3*) and anti-MUC6.2 (*lane 4*). The negative control (without antiserum) did not show any reactivity (*lane 5*).

between glycosyltransferase changes and Tn antigen expression have already been shown (29, 30).

Another factor that may contribute to the expression of Tn antigen in cancer could be a shift in the expression of acceptor polypeptides. An aberrant expression of MUC2 (23), MUC5B (24), and MUC6 (25) in breast cancer has been observed, suggesting that these apomucins are up-regulated during the course of malignant transformation of breast epithelium. It is possible that the aberrant expression of a specific type of apomucin could lead to the accumulation of simple *O*-glycan tumor-associated antigens, such as Tn. Recently, evaluating soluble apomucin in breast cancer pleural effusions, we observed that Tn is expressed not only on MUC1, the major breast mucin, but also on the three nonmammary apomucins evaluated (MUC2, MUC5AC, and MUC6; ref. 23). In the present study, the transfer of GalNAc to synthetic peptides corresponding to the MUC1, MUC2, MUC5B, and MUC6 apomucins was investigated *in vitro* using microsome membranes of human MCF-7 breast cancer cells. We showed that the ppGalNAc-T machinery present in breast cancer cells is capable of glycosylating "aberrantly" expressed mucins found in this type of cancer, such as MUC2, MUC5B, and MUC6. To our knowledge, this is the first report regarding the *in vitro* glycosylation of nonmammary apomucins by breast cancer cell extracts. Frequently studied models for *O*-glycosylation are MUC1 (for breast carcinomas) and MUC2 (for colon cancer; refs. 28, 31, 32), whereas little information is available on the glycosylation of MUC5AC (33), and no data concerning the glycosylation of MUC5B and MUC6 by cancer cells have been reported. MUC6 peptide was one of the best substrates for MCF-7 ppGalNAc-Ts, and the *in vitro* assay showed that these cells could achieve the synthesis of MUC6-Tn glycopeptide from MUC6 with a 95% yield. Semipreparative-scale glycosylation allowed us to purify this glycopeptide, which was shown to be monoglycosylated on the second Thr (GTT[α-GalNAc]PPPTTLK). Contrary to some previous results, where the *in vitro* glycosylation of MUC2 peptides using either cell extracts or recombinant purified ppGalNAc-Ts results in a heterogeneous mixture of different glycopeptides (9, 34–36), we obtained a single glycoform even after adding donor substrate and cell extract several times over 72-hour incubation. This result is very surprising because we have identified by RT-PCR various ppGalNAc-Ts in MCF-7 cells, including T1, T4, and T7 (data not shown), which are capable of adding GalNAc to peptides already glycosylated (37). Nevertheless, the MUC6 peptide, presenting four Thr residues, was monoglycosylated.

The combination of different apomucin peptides, multiple types of ppGalNAc-Ts, and the subsequent stepwise glycosylation events generates diverse *O*-linked glycan core mucin structures (14). The differential expression of mucins in carcinomas compared with normal tissues may lead to the appearance of structures that are not recognized by the glycosyltransferases responsible for subsequent glycosylation steps. Considering that the efficiency of ppGalNAc-Ts and of core 1 β3Gal-T for a given peptide substrate is significantly influenced by the primary amino acid sequence of the substrate (26, 33), we hypothesize that the Tn antigen in breast cancer is, at least partly, linked to and expressed on the aberrant MUC6 apomucin. To test this hypothesis, we evaluated core 1 β3Gal-T activity in MCF-7 extracts using MUC6-Tn and MUC1-Tn glycopeptides as acceptor substrates. We observed that the MUC6-Tn glycopeptide was five times less galactosylated than a MUC1-Tn glycopeptide (<5% of the MUC6-Tn glycopeptide was converted into MUC6-TF). These results indicate that MUC6 apomucin expression in MCF-7 cells determines an opposite effect on the

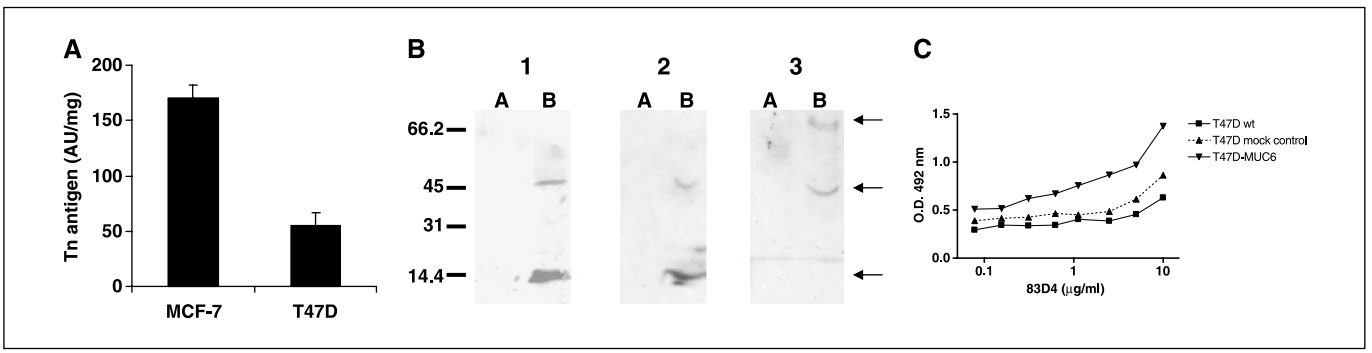

Figure 5. Tn expression in MUC6-transfected T47D breast cancer cells. *A,* Tn expression levels in wild-type MCF-7 and T47D breast cancer cell lines. Tn antigen levels [expressed as antigen units (AU) per protein (mg)] were determined by a sandwich ELISA using the mAb 83D4 and the VVB4 lectin (CA83.4 assay). *B,* identification of MUC6 in T47D cells by Western blotting. Expression of MUC6 and Tn antigen in wild-type T47D cells (*A*) and MUC6-transfected T47D cells (*B*) was analyzed by Western blotting using an anti-His mAb (*1*), the anti-MUC6.2 polyclonal serum (*2*), and the anti-Tn mAb 83D4 (*3*). *C,* anti-Tn mAb 83D4 reactivity of wild-type and MUC6-transfected T47D cell extracts. Anti-Tn mAb 83D4 reactivity was evaluated in wild-type, mock transfected controls and MUC6-transfected T47D cells by ELISA as described under Materials and Methods.

activity of two key enzymes of *O*-glycosylation, because the MUC6 peptide is a very good acceptor substrate for ppGalNAc-Ts and the MUC6-Tn glycopeptide is a poor substrate for the core 1 β3Gal-T. This process could explain the Tn accumulation in MCF-7 cells, at least for the MUC6 mucin, and could agree with the hypothesis we propose, that certain mucin sequences aberrantly expressed in cancer would not be recognized as efficiently as others, resulting in the accumulation of the Tn determinant. Higher levels of core 1 β3Gal-T activity than ppGalNAc-T activity have been reported in various breast cancer cell lines, including MCF-7 (38), and in colon cancer tissues (39), suggesting that it is not a lack of the enzyme but the sequence of the MUC6 peptide that is playing a role in the resulting glycosylation profile. The fact that we have identified MUC6 Tn-expressing glycoproteins from MCF-7 by Western blot supports this theory. MUC6 has been detected by Western blot in normal gastric lysates (20), gastric carcinoma patients (40), and gastric cancer cell lines (41) as components of >100 kDa, which corresponds to a high molecular weight glycosylated mucin. However, when tested in deglycosylated HT29 extracts, MUC6 was identified as a group of low molecular weight components (20). We identified MUC6 from MCF-7 cells and MCF-7–purified Tn glycoproteins as a component of an apparent molecular weight of 45 kDa, which could correspond to a low-glycosylated proteolytic product of MUC6 in these cancer cells.

To confirm *in vivo* the hypothesis that nonmammary apomucin expression contributes to the expression of the Tn antigen, we transfected T47D cells (which present lower Tn content than MCF-7 cells and which do not express MUC6) with a construct encoding for a recombinant MUC6 protein. We showed that the MUC6 transfectants express more Tn than the wild-type cells. This fact could be explained by the presence of a new MUC6-related acceptor substrate for ppGalNAc-Ts, which, as shown by the *in vitro* assays, is a poor substrate for the core 1 β3Gal-T. These results emphasize the importance of MUC6 expression in breast cancer cells and its relationship with tumor-associated antigens.

When the antigenicity of the synthesized Tn glycopeptides was evaluated, we found that both anti-Tn mAbs (83D4 and MLS128) bound strongly the MUC6-Tn glycopeptide in spite of the fact that a single GalNAc was incorporated. This is very interesting because previous studies showed that Tn residues organized in clusters are essential for the binding of these antibodies (10, 17, 42). The present results suggest that the vicinal amino acids play a role in the recognition of the Tn residues by the mAbs 83D4 and MLS128. Similarly, the mAb PMH1 can recognize single or multiple GalNAc-*O*-Ser/Thr on a specific MUC2 apomucin peptide chain (9). Attempts to determine the exact epitope of MLS128 and 83D4 in the MUC6-Tn glycopeptide should be carried out to understand the exact role of the primary sequence in this recognition. Considering that Tn is being tested for immunotherapy of breast cancer, it should be of interest to evaluate if nonmammary apomucins carrying Tn, such as MUC6-Tn, constitute better immunogens.

In summary, the results reported here bring new evidence on mucin-type *O*-glycan synthesis deregulation, which could account for Tn antigen accumulation in breast cancer cells. Several experiments are under way to determine the glycosylation profile of the *in vivo* synthesized MUC6 and to confirm its association with the Tn antigen. Further studies should address the glycosylation pattern of other peptide sequences derived from nonmammary apomucins and determine if this glycosylation pattern is exclusive of cancer cells.

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Molecular Basis of Incomplete *O*-Glycan Synthesis in MCF-7 Breast Cancer Cells: Putative Role of MUC6 in Tn Antigen Expression

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