β1,3-Galactosyltransferase β3Gal-T5 Acts on the GlcNAcβ1→3Galβ1→4GlcNAcβ1→R Sugar Chains of Carcinoembryonic Antigen and Other N-Linked Glycoproteins and Is Down-regulated in Colon Adenocarcinomas*  

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We attempted to determine whether β1,3-galactosyltransferase β3Gal-T5 is involved in the biosynthesis of a specific subset of type 1 chain carbohydrates and expressed in a cancer-associated manner. We transfected Chinese hamster ovary (CHO) cells expressing Fuc-TIII with β3Gal-T cDNAs and studied the relevant glycoconjugates formed. β3Gal-T5 directly synthesizes Lewis type 1 antigens in CHO cells more efficiently than β3Gal-T1, whereas β3Gal-T2, -T3, and -T4 are almost unable to directly synthesize. In the clone expressing Fuc-TIII and β3Gal-T5 (CHO-FT-T5), sialyl-Lewis α synthesis is strongly inhibited by swainsonine but not by benzyl-α-GalNAc, and sialyl-Lewis x is absent, although it is detected in the clones expressing Fuc-TIII and β3Gal-T1 (CHO-FT-T1) or Fuc-TIII and β3Gal-T2 (CHO-FT-T2). Endo-β-galactosidase treatment of N-glycans prepared from clone CHO-FT-T5 releases (±NeuAcα2→3Galβ1→3[Fucα1→4]GlcNAcβ1→3Gal but not GlcNAcβ1→3Gal or type 2 chain oligosaccharides, which are found in CHO-FT-T1 cells. This result indicates that β3Gal-T5 expression prevents poly-N-acetyllactosamine and sialyl-Lewis x synthesis on N-glycans. Kinetic studies confirm that β3Gal-T5 prefers acceptors having the GlcNAcβ1→3Gal end, including lactotrioseylceramide. Competitive reverse transcriptase mediated-polymerase chain reaction shows that the β3Gal-T5 transcript is expressed in normal colon mucosa but not or poorly in adenocarcinomas. Moreover, recombinant carcinoembryonic antigen purified from a CHO clone expressing Fuc-TIII and β3Gal-T5 reacts with anti-sialyl-Lewis a and carries type 1 chains on oligosaccharides released by endo-β-galactosidase. We conclude that β3Gal-T5 down-regulation plays a relevant role in determining the cancer-associated glycosylation pattern of N-glycans.

Type 1 chain oligosaccharides found in N- and O-glycans, as well as in glycolipids, contain the distinctive Galβ1→3GlcNAc disaccharide as their core structure. It is synthesized by β1,3-galactosyltransferases (β1,3Gal-Ts),1 a family of enzymes whose genes have been cloned very recently (1). The functional role of type 1 chains is not known, but several studies have indicated that some of them are differentially expressed in cancer. In particular, CEA expressed in colon cancer is characterized by the absence of such chains (2), which are abundantly present on the N-glycans of its normal counterparts synthesized by healthy colon mucosa, also referred to as the non-specific cross-reacting antigen-2 (3) and the normal fecal antigen-2 (4). At this regard, a β3Gal-T activity measured using GlcNAcβ1→3Galβ1→4Glc as acceptor was found lower in adenocarcinomas than in normal mucosa (5). However, type 1 chain Lewis antigens sialyl-Lewis a (sLeα) and Leα are considered tumor markers (6), and serum levels of sLeα (CA19-9 antigen) are used for clinical diagnosis and follow-up of epithelial cancers of the gastrointestinal tract (7). sLeα from cancer patient serum was found on mucins (8), and it is reported on both mucins (9) and glycolipids (10) in adenocarcinoma cell lines. Five β3Gal-T cDNAs are presently available, β3Gal-T1, cloned first from melanoma cells (11) and then from colon carcinoma cells (12), as well as β3Gal-T2 (13), were found to synthesize sLeα in CHO cells but to be very poorly expressed in cancer cell lines expressing sLeα. β3Gal-T3 and -T4 (14) were found expressed in various tissues, including colon and pancreas and in some cancer cell lines, but the expression levels do not correlate with those of type 1 chain Lewis antigens. Moreover, there is no evidence yet for their ability to synthesize the type 1 chain. In particular, β3Gal-T3 was very recently reported as a GalNAc transferase involved in globoside biosynthesis and renamed β3GalNAc-T1 (15). β3Gal-T5, cloned from colon carcinoma cells, was found to direct synthesis of sLeα in different cell lines, and its expression levels correlate with those of Lewis type 1 antigens in cancer cell lines from colon and pancreas (16). This enzyme was suggested to be the one previously characterized as the β1,3Gal-T active on glycolipids (17) and GlcNAc (18, 19), and involved in the synthesis of core acetylglicosaminyltransferase; FUT2, secretor type α1,2-fucosyltransferase; Fuc-TIII, α1,3(4)-fucosyltransferase; sLeα, sialyl-Lewis a [NeuAcα2→3Galβ1→3(Fucα1→4)GlcNAc]; Leα, Lewis a [Galβ1→3(Fucα1→4)GlcNAc]; sLeα, sialyl-Lewis x (NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAc); CEA, carcinoembryonic antigen; RT-PCR, reverse transcriptase mediated-polymerase chain reaction; lactotrioseylceramide, GlcNAcβ1→3Galβ1→4Glcpβ1→1′Cer; lacto-N-tetraosylceramide, Galβ1→4GlcNAcβ1→3Galβ1→4Glcpβ1→1′Cer; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; HPTLC, high performance thin layer chromatography; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; CHO-T-FT, CHO cells expressing Polyoma virus T antigen and Fuc-TIII; CHO-FT-T1, CHO clone permanently expressing Fuc-TIII and β3Gal-T1; CHO-FT-T2, CHO clone permanently expressing Fuc-TIII and β3Gal-T2; CHO-FT-T5, CHO clone permanently expressing human Fuc-TIII and β3Gal-T5.
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Table I

| Target gene | Primer pair* | PCR products | Restriction sites for competitor |
|-------------|--------------|--------------|---------------------------------|
| β3Gal-T2   | F: 5'-GCCAAAGGTCTGCCTGGCCGACCTC-3' | 817 bp | NdeI/PstI |
|            | R: 5'-GTTGGGTTGCCATATCTCTGCCATAGG-3' | 740 bp | XmnI/PstI |
| β3Gal-T3   | F: 5'-CGTGGAGCTCCACCTTACGATG-3' | 526 bp | SmaI/EcoRV |
|            | R: 5'-GCCAGAGAAAGGGCATTGCTGTCGAGG-3' | 709 bp | XmnI |
| β3Gal-T4   | F: 5'-AGGTTGGAAGGGGGAACCCAGGTT | 526 bp | SmaI/EcoRV |
|            | R: 5'-GCCAGATGACTGCAGAGACCTACCT-3' | 709 bp | XmnI |
| Mouse β-actin | F: 5'-GCCACCTGTCCTGCTCTCAGGACG-3' | 813 bp | Rca1 |
|            | R: 5'-GTGCACTAGGAGGGCGGGCACCT-3' | 534 bp | |

*F, forward primer; R, reverse primer.

3 O-glycans (20). The above data led to the working hypothesis that the biosynthesis of different type 1 chain oligosaccharides may depend on the expression of different and differentially regulated β3Gal-Ts. In particular, an enzyme able to act on N-glycans would be expected to be less active in cancer than in normal mucosa, whereas another active on O-glycans should be more expressed in cancer.

To evaluate whether β3Gal-T5 is actually involved in the biosynthesis of a specific subset of type 1 chain oligosaccharides and expressed in a cancer-associated manner, we have transiently transfected CHO cells expressing Fuc-TIII with β3Gal-T cDNAs and compared the relative ability of enzymes to direct synthesis of Lewis type 1 antigens. We then constructed CHO clones permanently expressing Fuc-TIII and β3Gal-T5, Fuc-TIII and β3Gal-T2, or Fuc-TIII and β3Gal-T1, determined the effect of drugs affecting glycosylation on antigen expression, and characterized the oligosaccharides that became radioactive in some clones upon metabolic labeling with tritiated Gal. Moreover, we studied the substrate specificity of β3Gal-T5 by calculating the kinetic constants toward different acceptors, and measured the expression levels of the transcript in colon adenocarcinomas and surrounding normal mucosa by competitive RT-PCR. We further modified the CHO clone expressing β3Gal-T5 to make it able to stably express human CEA and investigated the presence of type 1 chains in CEA purified from such clone.

**EXPERIMENTAL PROCEDURES**

**Materials**—GlcNAcβ1–3Galβ1–4Glc was prepared by digesting lacto-N-tetraose (IsoSep, Lund, Sweden), 5 mg/ml, in 0.1 M citrate phosphate buffer, pH 4.5, with 40 milliunits/ml bovine testis β-galactosidase (Sigma), for 20 h at 37°C. The obtained triascharide was purified from Gal and unrelated lacto-N-tetraose by repeated Bio-Gel P-2 columns monitored by HPTLC, as reported (21). Lactotriosylceramide was prepared by digesting lacto-N-neotetraosylceramide, 4 mg/ml in 50 mM cacodylate/HCl buffer, pH 6.5, with Diplococcus pneumoniae β-galactosidase (Sigma), 0.2 units/ml for 20 h at 37°C. The obtained compound was purified by a silica-Gel column (0.7 x 50 cm) using chloroform/methanol/water, 55:20:3 (v/v), as the eluting solvent system. Lacto-N-neotetraosylceramide was prepared by digesting sialyl-lacto-N-neotetraosylceramide, 5 mg/ml, in 0.1 M sodium cacodylate buffer, pH 6.0, with Clostridium perfringens sialidase, 1 unit/ml, for 20 h at 37°C. Ganglioside sialyl-lacto-N-neotetraosylceramide was purified from bovine erythrocytes using the procedure reported by Chien et al. (22).

**Cell Cultures, Transfections, and Treatments**—CHO cells expressing Polyoma virus T antigen and Fuc-TIII (CHO-T-FT), COLO-205, and WM266-4 cells were cultured as described previously (12, 19). Human gastric adenocarcinoma MKN-45 cells (a gift of C. Ponzetto, University of Turin), were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin, 1.0 mg/ml streptomycin, and 2 mM l-Glu. For transient transfection, 2.0 x 10⁶ CHO-T-FT cells were plated in 12-well plates 20 h before transfection, washed with serum free α-minimal essential medium, and incubated with 0.5 ml of transfection solution for 3 h under usual growing conditions. Transfection solutions, containing 1 μg/ml β3Gal-T DNA in pcDNA1 or pcDM8 vectors plus 0.065 μg/ml pcDNA1-Luc and 18 μg/ml DOTAP (Roche Molecular Biochemicals) were prepared and used as reported previously (12). Seventy-two hours after transfection, cells were harvested, washed, and resuspended with PBS. Ten-eighth of resuspended cells was processed for luciferase activity determination, using a commercial kit (Luciferase assay system, Promega) according to the manufacturer’s recommendations. The remaining material was stained and analyzed by flow cytometry as previously reported (12, 19). For treating cell lines and clones with drugs affecting glycosylation, 1 x 10⁶ cells were plated in 12-well plates and incubated overnight, and the medium was replaced with medium containing 0.1 μg/ml swainsonine (Sigma), 2 mM benzyll-α-GalNAc (Sigma), or 2 mM sodium butyrate. After 48 h, medium was replaced with fresh medium containing drugs. After additional 48 h, cells were collected, stained, and analyzed by flow cytometry (19).

**Construction of CHO Clones**—CHO clones expressing either Fuc-TIII and β3Gal-T5 (CHO-FT-T5), Fuc-TIII and β3Gal-T2 (CHO-FT-T2), or Fuc-TIII and β3Gal-T1 (CHO-FT-T1) were obtained by the calcium phosphate transfection method (23), using a modification of the procedure reported (24). Briefly, the DNA mixture (20 μg) contained 1 μg of EcoR1-linearized pSV2Neo, 10 μg of ScaI-linearized pcDNA1/Fuc-TIII, and 10 μg of ScaI-linearized pcDM8/β3Gal-T5, or 10 μg of KpnI-linearized pcDM8/β3Gal-T2, or 10 μg of ScaI-linearized pcDM8/β3Gal-T1, respectively. After selection with the corresponding G418, 30 (CHO-FT-T5 and CHO-FT-T1) or 60 colonies (CHO-FT-T2) were collected using cloning cylinders, grown in tissue culture slides, stained with anti-sLeα or anti-Lea antibody followed by secondary FITC-conjugated anti-mouse IgG, and analyzed by fluorescence microscopy. Two or three positive colonies were subcloned by limiting dilution in 96-well plates, and several subclones were analyzed as above. sLeα and Leα expression on positive colonies was quantitated by flow cytometry. Single colonies expressing a constant level of sLeα or Leα, named CHO-FT-T5, CHO-FT-T2, and CHO-FT-T1, respectively, were selected and used for further characterization and experiments. CHO cells expressing CEA (CHO-FT-T5-CEA) were obtained from clone CHO-FT-T5 by the transfection procedure described above but using 20 μg of KpnI-linearized pcDM8-CEA and 1 μg of pHAS8 plasmid in the DNA mixture, and 0.5 mg/ml hygromycin B (Roche Molecular Biochemicals) for selection. pHAS8 plasmid, a generous gift of P. Morandini (University of Milan), was derived from pSV2 vector and had the hygromycin B gene under the control of mouse phosphoglycerate kinase-1 promoter. Monoclonal anti-CEA antibody was used, at 1:100 dilution, for cell staining.

**DNA Preparation**—β3Gal-T1 cloning in pcDNA1 vector has been previously reported (12). For β3Gal-T2, -T3, -T4, -T5, and CEA cloning, the corresponding coding sequences were amplified using single-stranded cDNA as template, obtained by reverse transcription of total RNA extracted (12) from WM266-4 (β3Gal-T2), MKN-45 (β3Gal-T3 and CEA), or COLO-205 cells (β3Gal-T4 and -T5), in a reaction mixture containing, in 40-μl volume, 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM MgCl₂, 1000 units/ml human placental ribonuclease inhibitor, 1 μM of each deoxynucleotide triphosphate, 0.4 μM oligo(T)₁₆ primer, 1000
kinetic analysis, cotransfected with different protocol, using 2.5 m Taq template. Reactions were kept for 90 min at 42 °C. Amplifications were individual CHO-T-FT cells, expressing Polyoma virus T antigen and human Fuc-

b adaptors (all others), and cloned in pcDNAI (Amersham pcDNAI expression vectors. Seventy-two hours later, cells were har-

b3Gal-T5 was assayed upon transfection of pCDM8-

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b3Gal-T3 were analyzed using a Bio-Gel P-2 column (19). Reaction product with glycolipid acceptor, pooled from several reactions, was purified by Sep-Pak C-18 cartridge, desalted by partitioning in chloroform/methanol/water, 2:1:1 (v/v), and analyzed by HPTLC, using chloroform/methanol/water, 60:35:8 (v/v), as eluting solvent system, and visualized by fluorography (25).

Metabolic Labeling and Carbohydrate Analysis—CHO clones (2.0 × 10⁶ cells) were plated in 60-mm dishes containing 0.1 ml of ²H₂O (Amersham Pharmacia Biotech) in 2.5 ml of culture medium and incubated 40 h under regular conditions. Labeled cells were harvested as for immunostaining, resuspended in PBS at a density of 4 × 10⁶ cells/ml, and kept frozen until used. N-Glycans were released from cell suspension by N-glycanase (Glyko) digestion of the material denatured by heating in the presence of SDS, under the conditions recommended by the manufacturer. Released N-glycans were isolated by a Sep-Pak C-18 cartridge and further purified by Sephadex G-50 chromatography, as reported (24). Oligosaccharides released by endo-β-galactosidase were characterized following the procedure reported by Seuyoshi et al. (26) with some modifications. Purified N-glycans were passed through a Bio-Gel P-4 column (0.7 × 50 cm), and only the radioactivity eluted with water as a peak close to the exclusion volume was collected, lyophilized, and used for characterization. Endo-β-galactosidase digestion was performed on radioactive N-glycans, 10,000–20,000 cpm/µl, using the enzyme from Bacteriodes fragilis (Glyko), 0.5 milliunit/µl, in the buffer supplied by the manufacturer, for 20 h at 37 °C. The reaction mixture was then applied to the same Bio-Gel P-4 column as above, and the radioactivity was eluted with the exclusion volume collected and re-

b3Gal-T5 was digested with HinIII and XhoI (β3Gal-T5) or ligated to BstXI adaptors (all others), and cloned in pcDNAI (β3Gal-T3) or pcDNA8 vectors (all others) using a procedure reported previously (12). Direct DNA sequencing of the obtained constructs, performed by the dideoxy nucleotide chain-termination method using an automated procedure, indicated that the coding sequences are identical to those published. pcDNAI-Luc, expressing the luciferase gene, was constructed by removing the luciferase coding sequence from plasmid pGL3 (Promega) using HindIII and XhoI, and subcloning into the corresponding sites of pcDNA vector.

Enzyme Assays and Reaction Product Characterization—β3Gal-T5 and Fuc-TIII were assayed in cell clones as previously reported (19). For kinetic analysis, β3Gal-T5 was assayed upon transfection of pCDM8-β3Gal-T5 in COS-7 cells. Cells were transfected, harvested, washed, resuspended, and used as the enzyme source, as described previously (21). Enzyme activity was determined in a reaction mixture containing, in a final volume of 10 µl, 0.1 M Tris/HCl buffer, pH 7.0, 10 mM MnCl₂, 0.5 mg/ml Triton X-100, 1 mg/ml α-lactalbumin, 5 mM CDP-choline, 0.8 mM donor UDP-[¹⁴C]Gal, specific radioactivity (10 mCi/µmol), and 0.2-1.0 µg/ml cell protein, in the presence of different concentrations. In the case of glycolipid acceptor, it was dissolved in chloroform/methanol, 4:1 (v/v), mixed in the reaction tube with 15 µg of Triton CF-54 dissolved in the same solvent, and dried before adding the reaction mixture. Incubations were done at 37 °C for 60 min. At the end of incubation, reaction products were assayed by Dowex chromatography (oligosaccharide acceptors) or descending paper chromatography (glycolipid acceptor) according to previously reported protocols (19, 25). The oligosaccharide reaction products were identified by pooling several Dowex eluates, which were lyophilized and purified by Bio-Gel P-2 chromatography (21). The obtained saccharides were treated with D. pneumoniae or Xanthomonas manihotis β-galactosidases, specific for β1-4- and β1,3-linkages, respectively, and analyzed using a Bio-Gel P-2 column (19). Reaction product with glycolipid acceptor, pooled from several reactions, was purified by Sep-Pak C-18 cartridge, desalted by partitioning in chloroform/methanol/water, 2:1:1 (v/v), and analyzed by HPTLC, using chloroform/methanol/water, 60:35:8 (v/v), as eluting solvent system, and visualized by fluorography (25).

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was performed as previously reported (24) using monoclonal anti-CEA (1:200), anti-sLea (1:500), and anti-sLex (1:100) antibodies, followed by peroxidase-conjugated goat anti-mouse immunoglobulin (Amersham Pharmacia Biotech) (1:2000). ECL was developed with Renaissance chemiluminescence reagent (PerkinElmer Life Sciences). Homogeneous fractions were collected, lyophilized, and desalted on a Bio-Gel P-2 column. Purified [3H]CEA was treated with C. perfringens sialidase and almond meal α-fucosidase, and then with endo-β-galactosidase, under the conditions reported for N-glycan digestion. The reaction mixture was then applied to a Bio-Gel P-2 column (0.7 × 50 cm), eluted with water at a flow rate of 0.15 ml/min, 2.5 min/fraction. The obtained peaks, as well as the total purified CEA, were treated with β-galactosidases and analyzed by Bio-Gel P-2 chromatography as above described.

Competitive RT-PCR Analysis—For the analysis of human samples, biotic specimens were collected at surgery, immediately frozen in dry ice, and placed in liquid nitrogen until used. For RNA extraction, 1–2 mm² of material was homogenized with a rotary homogenizer in 0.5 ml of the lysis buffer from a Qiagen RNeasy minikit and processed in the presence of DNase according to the manufacturer’s recommendations. First strand cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase (Amersham Pharmacia Biotech) as previously reported (12). Control reactions were prepared by omitting the reverse transcriptase in the mixture. First strand cDNA was amplified in a reported (12). Control reactions were prepared by omitting the reverse transcriptase (Amersham Pharmacia Biotech) as previously described. First strand cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase (Amersham Pharmacia Biotech) as previously reported (12). Control reactions were prepared by omitting the reverse transcriptase in the mixture. First strand cDNA was amplified in a reported (12). Control reactions were prepared by omitting the reverse transcriptase (Amersham Pharmacia Biotech) as previously described. First strand cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase (Amersham Pharmacia Biotech) as previously reported (12). Control reactions were prepared by omitting the reverse transcriptase (Amersham Pharmacia Biotech) as previously described. First strand cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase (Amersham Pharmacia Biotech) as previously reported (12). Control reactions were prepared by omitting the reverse transcriptase (Amersham Pharmacia Biotech) as previously described.

RESULTS

Bioynthesis of sLeα and Leα in CHO Cells Transiently Transfected with β3Gal-T cDNAs—The ability of different cloned β3Gal-Ts to synthesize the type 1 chain was assessed to determine the amount of sLeα and Leα antigens expressed upon cDNA transfection on the surface of CHO-TFT cells, a clone permanently expressing Fuc-TIII and Polyoma virus T antigen. For this purpose, cells were transiently cotransfected with β3Gal-T and reporter luciferase cDNAs both placed in expression vectors having the Polyoma virus origin of replication. Luciferase activity from different transfections ranged between 0.6 and 1.4 units. Despite such differences in transfection efficiency, both sLeα and Leα antigens were easily detected in cells transfected with β3Gal-T5 (very bright) and β3Gal-T1, poorly detected with β3Gal-T2, and not at all detected with β3Gal-T3 and β3Gal-T4. In a typical experiment (Fig. 1), a peak representing up to 35% of cells transfected with β3Gal-T5 appears intensely bright mostly after anti-sLea antibody staining. Fluorescence intensity on positive cells was much lower upon β3Gal-T1 transfection, and faintly or not detectable in the other cases, although transfection efficiency was similar or sometimes higher, as assessed by luciferase activity assay. These results suggest that different substrate specificity exists among the different β3Gal-Ts, and only β3Gal-T5 finds large amount of suitable acceptors in CHO cells.

Characterization of CHO Clones Expressing β3Gal-T5 and Fuc-TIII, β3Gal-T2 and Fuc-TIII, or β3Gal-T1 and Fuc-TIII—To evaluate the hypothesis that the amount of sLeα and Leα antigens expressed in CHO cells transfected with β3Gal-Ts reflects the availability of the proper precursors, we constructed three CHO clones permanently expressing the antigens upon stable transfection with either β3Gal-T5 and Fuc-TIII, β3Gal-T2 and Fuc-TIII, or β3Gal-T1 and Fuc-TIII. All clones, named CHO-FT-T5, CHO-FT-T2, and CHO-FT-T1, respectively, express human Fuc-TIII transcript in a similar amount, as determined by competitive RT-PCR, whereas each clone expresses only its corresponding human β3Gal-T transcript (Fig. 2A). Flow cytometry analysis (Fig. 2B) of the obtained clones shows intense and homogeneous staining with anti-sLea and anti-Lea antibodies in CHO-FT-T5 cells, weak and heterogeneous staining in CHO-FT-T1, and very faint staining in CHO-FT-T2 despite the high expression level of the transcript, thus providing fluorescence patterns very similar to
Moreover, CHO-FT-T2 and CHO-FT-T1 cells are homogeneously bright with anti-sLex staining, as expected in CHO cells expressing Fuc-TIII, whereas CHO-FT-T5 cells are totally negative (Fig. 2B). This last result is surprising and suggests a competition between Gal-Ts in the synthesis of type 1 versus type 2 chain Lewis antigens.

Effect of Drugs Affecting Glycosylation on the Expression of sLea and sLex Antigens in Different Cells—CHO cells are known to express mostly complex type N-glycans and simple core 1 type O-glycans (28). Because the latter are not precursors of type 1 chains, our findings suggest that β3Gal-T5 has a distinctive ability to act on complex type N-glycans. To address this issue and to study the contribution of N- and O-linked glycosylation on the expression of sLea and sLex, cells were treated with swainsonine and benzyl-α-GalNAc, selectively affecting complex type N-glycan and O-glycan biosynthesis, respectively, before staining with anti-sLea or anti-sLex antibodies. sLea staining is 80% inhibited by swainsonine treatment in CHO-FT-T5, whereas benzyl-α-GalNAc treatment is ineffective and sLex staining is absent (Fig. 3). Conversely, in clone CHO-FT-T2 sLea staining is almost absent, whereas sLex staining is reduced by swainsonine treatment (about 65%) and slightly stimulated by sodium butyrate. In clone CHO-FT-T1 sLea staining is almost 70% inhibited by benzyl-α-GalNAc, whereas swainsonine treatment is ineffective; moreover, sodium butyrate treatment stimulates sLea synthesis 5-fold. sLex staining in this clone is reduced by swainsonine treatment and slightly stimulated by sodium butyrate, as in CHO-FT-T2. In control COLO-205 cells, sLea and sLex reactivity is totally abolished by benzyl-α-GalNAc, and only about 30% inhibited by swainsonine treatment (Fig. 3). These results indicate that sLea expressed in CHO-FT-T5 cells is carried by complex type N-glycans, presumably

| Table II |
| Radioactivity distribution in CHO clones metabolically radiolabeled with [3H]Gal |
| Values are expressed as cpm x 10^6/mg cell protein. |

| CHO-FT-T5 (%) | CHO-FT-T1 (%) | CHO-T-FT (%) |
|---------------|---------------|---------------|
| Total cell incorporation | 6.2 (100) | 8.4 (100) | 7.9 (100) |
| N-Glycans | 5.2 (83.8) | 6.4 (76.2) | 6.2 (78.4) |
| Oligosaccharides | | | |
| Endo-β-galactosidase-sensitive Neutral | 1.2 (19.3) | 1.6 (19.0) | 1.5 (18.9) |
| Neutralt | 0.84 (15.3) | 1.0 (11.9) | 0.92 (11.6) |
| Chargedb | 0.39 (6.3) | 0.52 (6.2) | 0.55 (6.9) |

a Unbound to QAE-Sephadex. 
b Bound to QAE-Sephadex.
the same carrying the sLe<sup>a</sup> epitope in CHO-FT-T1 and CHO-FT-T2 cells.

**Characterization of Complex N-Glycans Formed in Clone CHO-FT-T5**—To investigate the actual nature of the saccharides used as substrates by β3Gal-T5 in CHO cells, we studied the radioactive structures formed upon metabolic labeling with [3H]Gal in CHO-FT-T5 as well as in CHO-FT-T1 and CHO-T-FT cells, a clone not expressing β1,3Gal-T but Fuc-TIII only. The N-glycans were prepared from the total incorporated radioactivity and treated with endo-β-galactosidase, and the released oligosaccharides were separated in the neutral and charged fractions. As shown in Table II, the radioactivity recovered after each step is rather similar in the three clones. On the other hand, the composition of the neutral saccharides released by endo-β-galactosidase treatment of CHO-FT-T5 N-glycans is dramatically different from that of CHO-FT-T1 N-glycans. In fact, almost all radioactivity derived from clone CHO-FT-T5 is eluted as a single peak in the area of tetrasaccharides by Bio-Gel P-4 fractionation, whereas that from clone CHO-FT-T1 is distributed in several peaks, including some of higher molecular weight as well as one in the disaccharide area (Fig. 4, upper panel). The radioactive peak obtained from CHO-FT-T5 N-glycans is converted to a trisaccharide by almond meal α-fucosidase, to an equal mixture of disaccharide and monosaccharide by the action of both almond meal α-fucosidase and *X. manihotis* β-galactosidase, and to a monosaccharide by the combination of the above enzymes and *D. pneumoniae* α-acetylgalactosaminidase (Fig. 4, lower panel). Digestion of the tetrasaccharide peak with almond meal α-fucosidase and *D. pneumoniae* β-galactosidase determines the formation of a single peak in the area of trisaccharides but not detectable monosaccharides (not shown). The elution profile obtained after de-sialylation of the charged oligosaccharides is identical, indicating that Galβ1→3[Fucα1→4]GlcNAcβ1→3Gal and NeuAcα2→3Galβ1→3[Fucα1→4]GlcNAcβ1→3Gal account for the vast majority of oligosaccharides released by endo-β-galactosidase digestion of the N-glycans formed in clone CHO-FT-T5. However, the elution profile of the oligosaccharides released by endo-β-galactosidase treatment of CHO-FT-T1 and CHO-T-FT cells are very similar. Among such peaks, the one eluted as a disaccharide was identified as GlcNAcβ1→3Gal, because it is converted to a monosaccharide by *D. pneumoniae* β-N-acetylgalactosaminidase. Such a disaccharide is formed by the action of endo-β-galactosidase on multiple lactosamine repeats (29). The peak eluted at the size of tetrasaccharides was identified as Galβ1→4[Fucα1→3]GlcNAcβ1→3Gal, because it is converted to a monosaccharide only by the sequential action of almond meal α-fucosidase, *D. pneumoniae* β-galactosidase, and *D. pneumoniae* β-N-acetylgalactosaminidase. The larger peaks (fractions 51–55 and 46–50 in Fig. 4, upper panel) contain one and two other major oligosaccharides, respectively. They were identified by sequential exoglycosidase digestions and Bio-Gel P-4 filtration as the pentasaccharide, hexasaccharide, and heptasaccharide already reported (26). Such oligosaccharides contain double lactosamine repeats fucosylated on the inner GlcNAc, a substitution that makes the Gal β1→4GlcNAc linkage resistant to endo-β-galactosidase under usual reaction conditions (30). These results strongly suggest that the (±)-NeuAcα2→3Galβ1→3[Fucα1→4]GlcNAcβ1→3Gal oligosaccharide synthesized in CHO-FT-T5 replaces the poly-N-acetyllactosamine chains and sLe<sup>a</sup> structure present in CHO-FT-T1 as well as in CHO cells expressing Fuc-TIII only. To assess the presence of type 1 chain carbohydrates in endo-β-galactosidase-resistant N-glycans, we have determined the amount of radioactive Gal released by *X. manihotis* β-galactosidase. As shown in Table III, *X. manihotis* β-galactosidase is active only on the glycans derived from CHO-FT-T5, but not on those from CHO-FT-T1. The amount of Gal released by the enzyme is strongly dependent on the concurrent action of sialidase, but not on that of α-fucosidase, and is much lower than that released by *D. pneumoniae* β-galactosidase under the same reaction conditions. These results indicate that type 1 chains synthesized on endo-β-galactosidase-resistant N-glycans by β3Gal-T5 are present in limited amount and are not significantly substituted by fucose residues.

**In Vitro Properties of β3Gal-T5**—Using a cell homogenate prepared from COS cells transiently transfected with β3Gal-T5 cDNA as the enzyme source, we determined the optimal reaction conditions using GlcNAc as acceptor and found that the activity requires Mn<sup>2+</sup>, is maximal at neutral pH, is saturated by donor UDP-Gal concentrations above 0.5 mM, and is not affected by α-lactalbumin. Under the same reaction conditions, various oligosaccharides are also used. The obtained reaction products are over 95% affected by *X. manihotis* β-galactosidase and almost unaffected by *D. pneumoniae* β-galactosidase. In the presence of the proper detergent concentration, the same homogenate very efficiently transfers Gal to the glycolipid lactotriacylceramide, and the reaction product was identified as lactotetraacylceramide (Galβ1→3GlcNAcβ1→3Galβ1→4 Glcβ1→1Cer) by HPTLC mobility and differential sensitivity to β-galactosidases. The calculated *K<sub>m</sub>* and *V<sub>max</sub>* values (Table IV) indicate that the enzyme prefers the GlcNAcβ1→3Gal end but also distinguish among the different carrier molecules.
cDNA is well amplified using control primers such as those faintly detectable or undetectable in adenocarcinomas whose script is detected in all normal mucosa samples, although it is 35 times more abundant in eight normal mucosa and four adenocarcinoma cases, as determined by quantitative terms (Fig. 6), the expression levels of sLea and Leα antigens in colon adenocarcinomas—To evaluate whether the β3Gal-T5 transcript is differentially expressed during carcinogenesis, we analyzed its amount by competitive RT-PCR performed on total RNA extracted from human colon specimens collected at surgery, representing both adenocarcinomas and surrounding normal mucosa. Clinical features and tumor staging are outlined in Table V. The β3Gal-T5 transcript is detected in all normal mucosa samples, although it is faintly detectable or undetectable in adenocarcinomas whose cDNA is well amplified using control primers such as those for β-actin or other glycosyltransferases (Fig. 5). In quantitative terms (Fig. 6), the β3Gal-T5 transcript is on the average 30-fold less expressed in adenocarcinomas than in normal mucosa. In individual cases, the levels in adenocarcinomas range from 4-fold to over 100-fold less than in normal mucosa. For comparison we looked at the expression levels of the other β3Gal-T transcripts, as well as of Fuc-TIII and FUT2, two fucosyltransferases involved in type 1 chain sLea and Leα glycosyltransferases, respectively), and subjected to PCR (25 and 35 cycles for β-actin and glycosyltransferases, respectively). Primers and PCR product length are indicated in Table I. One-fifth aliquot of each amplification reaction was electrophoresed in 1% agarose gel and visualized by staining with ethidium bromide. N normal mucosa; A adenocarcinoma. Numbers denote patients according to Table V. Samples 1–6 and 7–10 were run on different gels.

| Case | Age | Sex | Localization | Grade | Stage (Dukes') | CEAa | CA19.9b |
|------|-----|-----|--------------|-------|---------------|------|--------|
| 1    | 61  | F   | Splenic flexure | G2    | C2            | 5.3  | 72.9   |
| 2    | 75  | M   | Hepatic flexure | G2    | C2            | 53.2 | 84.6   |
| 3    | 69  | M   | Rectum        | G2    | B2            | NAa | NA     |
| 4    | 76  | F   | Cecum         | G3    | C2            | 60.5 | 19.2   |
| 5    | 64  | F   | Ascending colon | G2    | B2            | 4.7  | 115    |
| 6    | 78  | M   | Ampulla recti | G2    | B2            | NAa | NA     |
| 7    | 77  | M   | Cecum         | G2    | D             | NAa | NA     |
| 8    | 77  | M   | Ascending colon | G2    | B2            | NAa | NA     |
| 9    | 66  | F   | Sigmoid colon | G2    | B2            | NAa | NA     |
| 10   | 65  | F   | Rectum        | G3    | C1            | NAa | NA     |

*a* M, male; *f* female.

with a preference for the glycolipid acceptor. GlcNAc linked to α-Man through β1,2- or β1,6-linkages is also a suitable acceptor, but with much lower affinity. These in vitro results are in good agreement with those obtained by the structural analysis of CHO-FT-T5 clone, where we found that endo-β-galactosidase-sensitive saccharides, having the GlcNAcβ1→3Gal acceptor sequence, are preferentially operated upon by β3Gal-T5, whereas endo-β-galactosidase-resistant N-glycans, mostly having GlcNAcβ1→2/6Man as acceptor sequences, are poorly utilized.

**Expression of β3Gal-T5 Transcript in Normal Colon Mucosa and in Adenocarcinomas—**To evaluate whether the β3Gal-T5 transcript is differentially expressed during carcinogenesis, we analyzed its amount by competitive RT-PCR performed on total RNA extracted from human colon specimens collected at surgery, representing both adenocarcinomas and surrounding normal mucosa. Clinical features and tumor staging are outlined in Table V. The β3Gal-T5 transcript is detected in all normal mucosa samples, although it is faintly detectable or undetectable in adenocarcinomas whose cDNA is well amplified using control primers such as those for β-actin or other glycosyltransferases (Fig. 5). In quantitative terms (Fig. 6), the β3Gal-T5 transcript is on the average 30-fold less expressed in adenocarcinomas than in normal mucosa. In individual cases, the levels in adenocarcinomas range from 4-fold to over 100-fold less than in normal mucosa. For comparison we looked at the expression levels of the other β3Gal-T transcripts, as well as of Fuc-TIII and FUT2, two fucosyltransferases involved in type 1 chain addition. β3Gal-T4 transcript is expressed at high and heterogeneous levels in normal mucosa and remains detectable in all adenocarcinomas. β3Gal-T1 transcript is detectable in eight normal mucosa and four adenocarcinoma cases, whereas β3Gal-T2 transcript is detectable in four normal mucosa and seven adenocarcinoma cases. β3Gal-T3, Fuc-TIII, and FUT2 transcripts are heterogeneously expressed in both normal mucosa and adenocarcinomas. Altogether, these data suggest that the β3Gal-T5 transcript, almost undetectable in colon adenocarcinomas, is strongly down-regulated during carcinogenesis.

**Characterization of CEA Purified from a CHO Clone Expressing β3Gal-T5—**To study the ability of β3Gal-T5 to act on CEA, a CHO clone permanently expressing this antigen, as well as β3Gal-T5 and Fuc-TIII, was obtained by transfecting CHO-FT-T5 cells with CEA (CHO-FT-T5-CEA). As assessed by flow cytometry, the expression levels of αLeα and Leα antigens in this clone are identical to those of the starting one, whereas those of CEA were comparable to that of human cancer cell lines. In particular, we measured a mean fluorescence intensity lower than in MKN-45 cells, but higher than in COLO-205 cells (not shown). The purified material from clone CHO-FT-T5-CEA metabolically radiolabeled with [3H]Gal runs on SDS-PAGE as a single radioactive band of an apparent molecular mass of 200 kDa (Fig. 7). Immunoblot analysis shows that this band strongly reacts with anti-CEA antibody. Sequential stripping and reprobing of the filter with anti-Lewis antigen antibodies reveals that the purified CEA reacts with anti-sLeα but not with anti-sLeα antibodies, indicating that type 1 chains are specifically present on CEA expressed in the clone. To directly assess the presence of type 1 chain carbohydrates on CEA and to determine whether they are preferentially bound to GlcNAcβ1→3Galβ1→4GlcNAcβ1→R outer sequences, the purified [3H]CEA, once de-fucosylated and de-sialylated, was treated with different β-galactosidases before and after endo-β-galactosidase treatment. To this end, the endo-β-galactosidase reaction mixture was submitted to Bio-Gel P-2 filtration and the endo-β-galactosidase-resistant and -sensitive radioactivity was recovered with the exclusion volume and at the size.
of trisaccharides, respectively (Fig. 8, upper panel). A small but detectable amount of Gal was removed by X. manihotis β-galactosidase from total de-sialylated and de-fucosylated CEA, and the amount released from the endo-β-galactosidase-resistant material (fractions 18–22 in Fig. 8) was minimal, whereas that released by D. pneumoniae β-galactosidase was more consistent in both cases. Conversely, almost 40% of the radioactivity from the endo-β-galactosidase-sensitive trisaccharide (fractions 32–39 in Fig. 8) was released by X. manihotis β-galactosidase, but poorly affected by the diplococcal enzyme (Fig. 8, lower panel). These data indicate that β3Gal-T5 preferentially acts on the GlcNAcβ1→3Galβ1→4GlcNAcβ1→R side chains present in CEA.

DISCUSSION

This report shows that β3Gal-T5, in transfected CHO cells, directs synthesis of type 1 chain oligosaccharides on CEA and other N-glycans having the GlcNAcβ1→3Galβ1→4GlcNAcβ1→R outer sequence, prevents poly-N-acetyllactosamine and sLea synthesis on N-glycans, and is down-regulated in colon adenocarcinomas.

We have found that β3Gal-T5 synthesizes sLea and Lea antigens very efficiently in CHO cells, much more than β3Gal-T1, which is still more efficient than β3Gal-T2, whereas β3Gal-T3 and -T4 are unable to synthesize these antigens at all. In the stable clone CHO-FT-T5, sLea expression is selectively affected by swainsonine, an inhibitor of complex type N-glycan processing (31), but not by benzyl-α-GalNAc, an inhibitor of O-glycan biosynthesis (32), although the opposite occurs in clone CHO-FT-T1. These data support the hypothesis that β3Gal-T5 acts on complex type N-glycans in CHO cells and suggest that β3Gal-T1 presumably affects unknown O-glycans available in these cells in a low amount. In this regard, it is interesting that sLea expression in CHO-FT-T1 is strongly stimulated by sodium butyrate, a commonly used activator reported to enhance core 2 GnT activity in CHO cells (33). However, results obtained with CHO-FT-T2 suggest that β3Gal-T2 requires dedicated substrates not available in these cells. Moreover, CHO-FT-T1 cells, as well as CHO-FT-T2, were found to express a large amount of sLea that is undetectable in CHO-FT-T5. We found that the oligosaccharides released from N-glycans by endo-β-galactosidase are completely different in the clones. In fact, ( NeuAcα2→3)Galβ1→3Galβ1→4GlcNAcβ1→3Gal is the only oligosaccharide identified in CHO-FT-T5, whereas in clone CHO-FT-T1 several oligosaccharides are released, which correspond to those reported in CHO expressing Fuc-TIII only (26). They include the sLea tetrasaccharide as well as poly-N-acetyllactosamine side chains, but not type 1 chain oligosaccharides. Among endo-β-galactosidase-resistant N-glycans, type 1 chain oligosaccharides are present in small amounts and not fucosylated in clone CHO-FT-T5 but not detectable in CHO-FT-T1 cells. On this basis we conclude that the expression of
are elongated by a single lactosamine unit. This is acted upon by iGnT, presumably on a β1,6-branched chain, which forms the GlcNAcβ1→3Glcα1→4GlcNAcβ1→R outer group. At this stage, β3Gal-T5 competes with β4Gal-T1 adding a β1,3-galactosyl residue that prevents further chain elongation by iGnT. Consequently, poly-N-acetyllactosamine chains could not be efficiently extended nor sLeα synthesized.

It is worth noting that the K_m values of β3Gal-T5 for GlcNAcβ1→3Glcα1→4Glc and GlcNAcβ1→2Man are virtually identical to those reported for a β1,3-GalT expressed in normal colon mucosa whose activity was found low in adenocarcinomas (5). These data prompted us to evaluate the expression level of β3Gal-T5 transcript in normal mucosa versus colon adenocarcinomas. Competitive RT-PCR analysis performed on 10 colon adenocarcinoma cases indicate that β3Gal-T5 transcript is constantly down-regulated in cancer, confirming that it is the enzyme previously detected by Seko et al. (5) and corroborating the hypothesis that in vivo it would prevent the expression of poly-N-acetyllactosaminas and sLeα on N-glycans. This behavior is unique among β3Gal-T and other glycosyltransferase transcripts studied to date (40–45).

Because CEA synthesized in normal mucosa is reported to express N-glycans having type 1 chains as Galβ1→3GlcNAcβ1→3Galβ1→4GlcNAcβ1→R outer sequences (3, 4), whereas CEA produced by colon adenocarcinomas lacks type 1 chains but have poly-N-acetyllactosamine and β1,6 branched type 2 chains on N-glycans (2, 44), our results suggest that β3Gal-T5 may act on CEA in vivo. To assess this hypothesis, we permanently expressed CEA in clone CHO-FT-T5 (CHO-FT-T5-CEA). We found that CEA purified from the clone reacts with anti-sLeα antibody by Western blot. Moreover, the type 1 chain carbohydrates present in the molecule are more abundant in endo-β-galactosidase-sensitive oligosaccharides than in those resistant. These results confirm that β3Gal-T5 acts on CEA and suggest that it is responsible for the differential glycosylation pattern of this protein in colon cancer.

The kinetic data calculated in this paper for lactotriosylceramide, as well as those reported (20) for GlcNAcβ1→3GlcNAc as acceptors, suggest that β3Gal-T5 is able to act on glycolipids as well as on core 3 O-linked glycoproteins. The possibility of a broad range of specificity of the enzyme is confirmed by the recent report that it also acts on the GaINαc end of globotriaosylceramide (45). On this basis, the down-regulation of β3Gal-T5 in cancer is expected to affect other glycoconjugates synthesized by the enzyme in normal mucosa. However, β3Gal-T5 expression is maintained in some colon adenocarcinoma cell lines (16), and we suggest that the results obtained with these cells must be cautiously interpreted, or reinterpreted (12, 19). In particular, further experimental data are needed to establish whether or not the CA19.9 antigen present in patient serum is synthesized by β3Gal-T5 and secreted from the tumor mass.

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β3Gal-T5 in CHO cells inhibits the synthesis of poly-N-acetyllactosamines and sLeα in N-glycans, replacing them with a short type 1 chain, whereas that of β3Gal-T1 does not affect N-glycans. Expression of N-linked poly-N-acetyllactosamines, which are often modified to express functional oligosaccharides such as sLeα, is considered to be associated with tumor progression and malignancy (34). Recent work on poly-N-acetyllactosamine extension in N-glycans (35) demonstrated that it is achieved mainly by β4Gal-T1 and β1,3-N-acetylglucosaminyltransferase IgNt (36) and is favored by β1,6GalNac branching, because the branched structure serves as a much better acceptor for such enzymes (37). Expression of GlcNAc-TV, responsible for branching, also correlates with metastatic potential of cell lines (38) as well as with metastasis and poor prognosis in colon cancer (39). Because β3Gal-T5 has a very high affinity for acceptors having the GlcNAcβ1→3Gal outer sequence, but much lower for those with the GlcNAcβ1→2Man or GlcNAcβ1→6Man sequence, the following scenario can be envisaged in CHO cells. In the presence of β3Gal-T5, β4Gal-T1 still acts on GlcNAc linked to Man, and complex type N-glycans

FIG. 8. Sensitivity to endo-β-galactosidase and different β-galactosidases of tritiated CEA purified from clone CHO-FT-T5. CEA labeled with [3H]Gal. Upper panel, radioactive CEA prepared as described in Fig. 7 was de-sialylated, de-fucosylated, and then subjected to Bio-Gel P-2 chromatography before (full circles) and after (empty circles) endo-β-galactosidase treatment. Column calibration is indicated at the top of the panel, standards are as in Fig. 4. Lower panel, the total de-sialylated and de-fucosylated material, as well as endo-β-galactosidase-resistant (fractions 19–22 in upper panel) and -sensitive (fractions 32–38 in upper panel) radioactivity were treated with D. pneumoniae or X. manihotis β-galactosidases, and the reaction mixtures were analyzed by Bio-Gel P-2 chromatography. Released Gal was quantitated by liquid scintillation counting.
β1,3-Galactosyltransferase β3Gal-T5 Acts on the GlcNAcβ1→3Galβ1→4GlcNAcβ1 →R Sugar Chains of Carcinoembryonic Antigen and Other N-Linked Glycoproteins and Is Down-regulated in Colon Adenocarcinomas

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