GalNAc-α-O-benzyl Inhibits NeuAcα2-3 Glycosylation and Blocks the Intracellular Transport of Apical Glycoproteins and Mucus in Differentiated HT-29 Cells

Guillemette Huet,* Sylviane Hennebicq-Reig,* Carmen de Bolos,‡ Fausto Ulloa,‡ Thécla Lesuffleur,§ Alain Barbat,§ Véronique Carrière,§ Isabelle Kim,‡ Francisco X. Real,‡ Philippe Delannoy,† and Alain Zweibaum§

*Unité de Recherches sur la Biologie et la Physiopathologie des Cellules Mucipares, Institut National de la Sante et de la Recherche Medicale (INSERM) U377, 59045 Lille Cedex, France; †Unité de Biologie Cellulaire i Molecular, Institut Municipal d’Investigació Médica, Universitat Autònoma de Barcelona, E-08003, Barcelona, Spain; §Unité de Recherches sur la Différenciation Cellulaire Intestinale, INSERM U178, 94807 Villejuif Cedex, France; and ‡Laboratoire de Chimie Biologique, Centre National de la Recherche Scientifique (CNRS) Unité Mixte de Recherche (UMR)-111, Université des Sciences et Technologies de Lille, 59655 Villeneuve d’Ascq Cedex, France

Abstract. Exposure for 24 h of mucus-secreting HT-29 cells to the sugar analogue GalNAc-α-O-benzyl results in inhibition of Galβ1-3GalNAcα2,3-sialyltransferase, reduced mucin sialylation, and inhibition of their secretion (Huet, G., I. Kim, C. de Bolos, J.M. Loguidice, O. Moreau, B. Hémon, C. Richet, P. Delannoy, F.X. Real, and P. Degand. 1995. J. Cell Sci. 108:1275–1285). To determine the effects of prolonged inhibition of sialylation, differentiated HT-29 populations were grown under permanent exposure to GalNAc-α-O-benzyl. This results in not only inhibition of mucus secretion, but also in a dramatic swelling of the cells and the accumulation in intracytoplasmic vesicles of brush border–associated glycoproteins like dipeptidylpeptidase-IV, the mucin-like glycoprotein MUC1, and carcinoembryonic antigen which are no longer expressed at the apical membrane. The block occurs beyond the cis-Golgi as substantiated by endoglycosidase treatment and biosynthesis analysis. In contrast, the polarized expression of the basolateral glycoprotein GP 120 is not modified. Underlying these effects we found that (a) like in mucus, NeuAcα2-3Gal-R is expressed in the terminal position of the oligosaccharide species associated with the apical, but not the basolateral glycoproteins of the cells, and (b) treatment with GalNAc-α-O-benzyl results in an impairment of their sialylation. These effects are reversible upon removal of the drug. It is suggested that α2-3 sialylation is involved in apical targeting of brush border membrane glycoproteins and mucus secretion in HT-29 cells.

Enteroctylic and mucus-secreting populations isolated from the human colon carcinoma cell line HT-29 have proven extremely useful for the study of intestinal cell differentiation (for review see Zweibaum et al., 1991). The differentiated populations used in this study were isolated from the mainly undifferentiated parental line (Fogh and Trempe, 1975) by selection with increasing concentrations of methotrexate (MTX)† (Lesuffleur et al., 1990, 1991). Cells selected with 10⁻⁶ and 10⁻⁵ M MTX form a homogeneous population of mucus-secreting cells (Lesuffleur et al., 1990, 1991), whereas selection with 10⁻³ M MTX results in a population of enterocytic phenotype (Lesuffleur et al., 1991). Whatever their phenotype, these populations share common differentiation characteristics, namely (a) a postmitotic onset of the differentiation process and (b) a polarized organization of the cell monolayer with the presence of an apical brush border endowed with several glycoproteins such as dipeptidylpeptidase-IV (DPP-IV), the carcinoembryonic antigen (CEA) and the mucin-like glycoprotein MUC1. Mucus-secreting cell populations selected by MTX have been extensively analyzed as to the characteristics of their mucins: at the mRNA level they mainly express MUC5AC (Lesuffleur et al., 1993, 1995); at the protein level they secrete mucus of gastric immunoreactivity (Lesuffleur et al., 1990) with a main oligosaccharide species similar to the clone HT29-

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Previous results have shown that short-term (24-h) exposure of postconfluent mucus-secreting HT-29 cells to benzyl-2-acetamido-2-deoxy-a-D-galactopyranoside (GalNAc-a-O-benzyl), an inhibitor of mucin O-glycosylation (Kuan et al., 1989; Huang et al., 1992; Byrd et al., 1995), results in a decrease of mucus secretion, a lower sialic acid content of newly synthesized mucins, and an increased content of T antigen (Galβ1-3GalNAc-R) (Huet et al., 1995). Similar effects of GalNAc-a-O-benzyl have been demonstrated in LS174-T colon cancer cells (Kuan et al., 1996) after several weekly passages of reversion to drug-free medium (10^{-4} to 10^{-7} M MTX; Sloan-Kettering Memorial Cancer Center, Rye, NY). The cells adapted to MTX 10^{-6} M (Sloan-Kettering Memorial Cancer Center, Rye, NY) was solubilized in DME. All experiments were performed in the presence of tissue culture-treated Transwell polycarbonate membranes with 24.5-mm diameter and a 0.4-μm pore size (Costar). For maintenance purposes, cells were passaged weekly, using 0.025% trypsin in 0.53 mM EDTA in PBS Ca^{2+}/Mg^{2+}-free (PBS−). The medium was changed daily in all culture conditions. For growth curves, cells grown in 24-well culture clusters were detached with trypsin and counted with a hemocytometer. Cell volume was determined in a hemocytometer. Control Caco-2 cells were cultured as previously reported (Pinto et al., 1983) and analyzed between passages 70 and 80.

Antibodies and Lectins

Mouse mAbs HBB 3/775/42 (Hauri et al., 1985) and G1/136 (Eilers et al., 1989) specific for human DPP-IV and a 120-kD basolateral glycoprotein respectively, were a gift of H.P. Hauri (Biocenter of the University of Basel, Basel, Switzerland). Rat mAb 4H3 against human DPP-IV (Gorvel et al., 1991) was obtained from S. Maroux (CNRS Unité de Recherche Associée 1820, Faculté des Sciences de Saint Jerôme, Marseille, France). Mouse mAb 517 (Le Bivic et al., 1988) against CEA was obtained from A. Le Bivic (Faculté des Sciences de Luminy, Marseille, France). Mouse mAb BC-2 (Xing et al., 1989), which recognizes a sequence in the tandem repeat of the MUC1 gene product was obtained from I. McKenzie (Austin Cancer Research Institute, Heidelberg, Victoria, Australia). Mouse mAb BT2.3 against sialyl-Tn (Nutl et al., 1982) was obtained from K.O. Lloyd (Memorial Sloan-Kettering Cancer Center, New York). Mouse mAb TS2/16 against the integrin β1 subunit (Arroyo et al., 1992) was obtained from F. Sanchez-Madrid (Universidad Autonoma de Madrid, Madrid, Spain). Rabbit polyclonal Abs against porcine villin (Robine et al., 1985) and the tight junction protein ZO-1 (Willot et al., 1992) were obtained from D. Louvard (Institut Curie, Paris, France) and J.M. Anderson (Yale University, New Haven, CT), respectively. For the detection of gastric mucus we used the same rabbit polyclonal Ab LS6/C as previously used for cloning the L31 mucus cDNA encoding the 3′ end of MUC5AC (Lesuffleur et al., 1995). Fluorescein-conjugated Maackia amurensis agglutinin (MAA) (Wang and Cummings, 1988), Sambucus nigra agglutinin (SNA) (Shibuya et al., 1987), and Peanut (Arachis hypogaea) agglutinin (PNA) (Latan et al., 1975), which recognize the oligosaccharide species NeuAcα2-3Gal-R, NeuAcα2-6Gal, and Galβ1-3GalNAc-R, respectively, were from Vector Labs Inc. (Burlington, CA).

Immunofluorescence and Histochemical Staining

Indirect immunofluorescence was performed on cryostat sections of cell layer rolls as reported (Lesuffleur et al., 1990). Briefly, late (day 21) cultures of cells grown in 25-cm² T flasks were rinsed with Ca^{2+}/Mg^{2+}-free PBS, the T flask was cut up with a soldering iron, and then the cell layer gently was scraped with a rubber policeman and poured in a bath of liquid nitrogen. The resulting frozen cell pellet was either stored in liquid nitrogen or homogenized in PBS for 16 h at 37°C. The resulting homogenate were centrifuged at 30,000 g for 10 min at room temperature using secondary antibodies fluorescein-coupled sheep anti-mouse or anti-rabbit Ig (Institut Pasteur Production, Marne-la-Coquette, France) or rhodamine-coupled sheep anti-galactosyl antibodies (Boehringer Mannheim Biochemicals). Desialylation was performed by incubation for 16 h at 37°C of paraformaldehyde-fixed cryostat sections with sialidase from Clostridium perfringens (Sigma Chemical Co.) (50 mU/ml in 50 mM citrate buffer, pH 6.0, 0.9% NaCl, 0.1% CaCl₂). For confocal microscopy analysis, cells grown on glass coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature under secondary antibodies fluorescein-coupled sheep anti-mouse or anti-rabbit Ig (Institut Pasteur Production, Marne-la-Coquette, France) or rhodamine-coupled sheep anti-galactosyl antibodies (Boehringer Mannheim Biochemicals). Desialylation was performed by incubation for 16 h at 37°C of paraformaldehyde-fixed cryostat sections with sialidase from Clostridium perfringens (Sigma Chemical Co.) (50 mU/ml in 50 mM citrate buffer, pH 6.0, 0.9% NaCl, 0.1% CaCl₂). For confocal microscopy analysis, cells grown on glass coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature.
Transmission EM and Ultrastructural Immunochemistry

Classical transmission EM was performed as previously reported (Lesuffleur et al., 1990, 1991) on cells grown in 25-cm² plastic flasks. Samples embedded in Epon (Polysciences, Inc., Warington, PA) were reembedded to make sections perpendicular to the bottom of the flask. Thin sections were stained with toluidine blue. Ultrastructural immunochemistry was performed as previously described (Hennebicq-Reig et al., 1996). After rinsing three times in PBS, cells cultured in 25-cm² flasks were fixed in phosphate buffer containing 4% paraformaldehyde and 0.05% glutaraldehyde. The cell layer was scraped with a rubber policeman, the cell pellet was infiltrated with phosphate buffer containing 2.3 M sucrose and 20% polyvinylpyrrolidone, and then frozen in liquid nitrogen. Ultrathin cryosections were then successively incubated with PBS containing 10% BSA, mouse mAb HBB 3/775/42 (DPP-IV), rabbit anti–mouse Ig antibody, and 8-nm gold-conjugated protein A. All antibodies and gold-conjugated protein A were diluted in PBS containing 10% BSA. The grids were finally counterstained with methylcellulose uranyl acetate and observed using an electron microscope (model 902, Carl Zeiss, Inc., Thornwood, NY). The same procedure was applied for mAb 517 (CEA) and mAb BC-2 (MUC1).

Northern Blot Analysis

For detection of DPP-IV and villin mRNAs, total RNA was isolated from the cells 16 h after medium change by lysis with guanidium isothiocyanate and centrifugation through a CsCl gradient (Chirgwin et al., 1979). Samples of total RNA, denatured in 1 M glyoxal (Thomas, 1980), were fractionated by electrophoresis through 1% agarose gels and then transferred to nitrocellulose (model Hybond N, Amersham Corp., Arlington, UK) in the presence of 20× SSC. Filters were incubated overnight at 42°C in prehybridization buffer containing 50% formamide, 5× SSC, 10× Denhardt’s solution, 50 mM sodium phosphate, pH 6.5, and 250 μg/ml sonicated and denatured salmon sperm DNA. Filters were then hybridized with the 32P-labeled probe for 20 h at 42°C in prehybridization buffer containing 10% dextran sulfate (Thomas, 1980). Blots were washed twice with 2× SSC, 0.1% SDS at room temperature, once with 0.1× SSC, 0.1% SDS at 50°C, and once, using the same solution, at 65°C for 15 min. Blots were then processed for autoradiography. To normalize for RNA, filters were dehybridized and stained with methylene blue. Methylene blue staining was preferred to hybridization with actin or glyceraldehyde 3-phosphate dehydrogenase since it was found that the levels of these transcripts differ in dividing and postconfluent cells. DPP-IV was detected with cDNA DPP-1 (Darmoul et al., 1990) and with cDNA V19 (Pringault et al., 1986), obtained from D. Louvard. The probe for human ST3Gal I (Kitagawa and Lesuffleur et al., 1990, 1991) was hybridized in prehybridization buffer containing 50× SSC in presence of 20× SSC, 0.1% SDS at 65°C for 1 h. The incorporation of [14C]-Neu5Ac was determined by subtration of the radioactivity measured in the absence of exogenous acceptors and results are expressed as average values in nmol of Neu5Ac transferred per milligram of protein and per hour.

Electrophoresis and Western Blotting

Cells were homogenized by sonication in Tris/Mannitol buffer. Immuno-precipitation of DPP-IV, CEA, and MUC1 was performed as in Hauri et al. (1985), using mAbs 3/775/42, 517, and BC-2 previously coated on protein A-Sepharose beads (Pharmacia Fine Chemicals, Uppsala, Sweden). SDS-PAGE was performed under reducing conditions on 4-20% gradient polyacrylamide gels (Laemmli, 1970) with 50 μg of total cellular protein per lane or with DPP-IV, CEA, or MUC1 immunoprecipitates. After electrophoresis, proteins were transferred to a nitrocellulose membrane (model BioTrace NT; Gelman Sciences Inc., Ann Arbor, MI) as described in Vaessen et al. (1981). The membranes were then treated for 2 h with polyvinylpyrrolidone K-30 (2% in TBS). Immunodetection of DPP-IV, CEA, and MUC-1 was performed with mAbs 4H3, 517, and BC-2, respectively, using secondary antibodies peroxidase-coupled anti-rat or anti-mouse Ig accordingly (Bioys, Compiègne, France). For glycan detection, membranes were incubated with digoxigenin-labeled lectins from Boehringer Mannheim Biochemicals at concentrations of 5 μg/ml in TBS for MAA and SNA, and 2 μg/ml in TBS for PNA-digoxigenin. Then, the nitrocellulose membranes were incubated for 1 h with alkaline phosphatase-labeled antidigoxigenin Fab fragments (1 μg/ml in TBS) (Boehringer Mannheim Biochemicals). After washing, labeled glycoproteins were revealed by 4-nitro blue tetrazolium chloride 5-bromo-4-chloro-3-indolyl phosphate staining. Desialylation was performed as described in Vassao et al. (1981). For glycan detection, membranes were incubated with digoxigenin-labeled lectins from Boehringer Mannheim Biochemicals at concentrations of 5 μg/ml in TBS for MAA and SNA, and 2 μg/ml in TBS for PNA-digoxigenin. Then, the nitrocellulose membranes were incubated for 1 h with alkaline phosphatase-labeled antidigoxigenin Fab fragments (1 μg/ml in TBS) (Boehringer Mannheim Biochemicals). After washing, labeled glycoproteins were revealed by 4-nitro blue tetrazolium chloride 5-bromo-4-chloro-3-indolyl phosphate staining. Desialylation was performed as described in Vassao et al. (1981).

Metabolic Labeling and Immunoprecipitation of DPP-IV

Cells were cultured in six-well plates with or without the presence of 2 mM GalNAc-α-O-benzyl until day 11. Subsequently, untreated cells were pulse labeled for 15 min with 200 μCi/well of [3H]-methionine (Amersham Corp.) in 1 ml of methionine-free medium, and then chased for the indicated periods of time with 1 ml 0.01 M methionine in regular medium. The same protocol was applied to treated cells, except for the presence of 2 mM GalNAc-α-O-benzyl throughout the experiment. Cells were rinsed in PBS and lysed in 1 ml of RIPA buffer (0.001 M Tris-HCl, pH 8.0, 0.01 M NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 1% phenylmethylsulfonylfluoride, 0.001 M sodium ethylene diamine tetraacetate). Aliquots of 50 μg of proteins from cell lysates were incubated with mAb 4H3 overnight at 4°C. Immunocomplexes were collected on protein G-Sepharose 4B (Sigma Chemical Co.), eluted in SDS sample buffer (0.2 M Tris-HCl buffer, pH 6.8, containing 2% SDS and 30% glycerol) at 60°C for 5 min, and then analyzed on 5–30% SDS–polyacrylamide gels. For autoradiography, x-ray film, washed in 40% ethanol, 10% glycerol, 10% acetic acid (by vol), soaked in Amplify (Amersham Corp.) for 20 min, dried on Whatman paper, and then exposed to Cronex 4 NIF film (Dupont, Les Ulis, France).

Results

GalNAc-α-O-benzyl Treatment Results in a Dose-dependent Decrease of Mucus Secretion and Welling of Mucus-secreting HT-29 Cells

To assay for a dose-dependent effect of GalNAc-α-O-benzyl, mucus-secreting HT-29 cells (RevMTX10-6) were treated, from the day of seeding on, with different concen-
trations of the drug in the 0.1–2 mM range. Regardless of the concentration used, no effect on cell viability was observed, as assessed by the absence of cells in suspension and trypan blue exclusion. As shown in Fig. 1, it has no effect on the doubling time of the cells in the first days in culture, but results in a dose-dependent lower cell density in the stationary phase. At the highest concentration (2 mM) the cells stop growing before reaching total confluence, with the cell layer occupying 75–80% of the surface of the flask (Fig. 1). This effect is associated with a dramatic swelling of the cells (Figs. 1 and 2), with their cytoplasm appearing filled with a honeycomb-like accumulation of vesicles of various sizes during transmission electron microscopy (see Fig. 4 e). Concomitant with these changes, the mucus gel is totally absent from the cells treated with the highest concentrations, even when the cells are maintained for a longer period (<30 d). The decrease in mucus content was further demonstrated by the analysis of sections of the cell layer (Fig. 2). At the highest concentration (2 mM), there was a total absence of alcian blue–stained material (Fig. 2 e). In addition, the dense immunofluorescent staining of apical mucus droplets observed in control cells with antigastric mucus antibodies was no longer observed in cells treated with 2 mM, having been replaced by a diffuse staining of the cytoplasm (Fig. 2 f).

**Treatment of Mucus-secreting HT-29 Cells with GalNAc-α-O-benzyl Leads to an Intracytoplasmic Accumulation of Brush Border Membrane–associated Glycoproteins**

Because the most clear-cut effect of GalNAc-α-O-benzyl was observed at 2 mM, all further experiments were done at this concentration. In control postconfluent HT29-RevMTX10^-5 cells, DPP-IV, MUC1, and CEA are exclusively associated with the apical brush border of the cells, as previously reported (Lesuffleur et al., 1993) and shown in Fig. 3, g, i, and k. In contrast, in GalNAc-α-O-benzyl–treated cells, DPP-IV, MUC1, and CEA are present in the totality of the cytoplasm (Fig. 3, h, j, and l); this occurs without modification of the morphological polarity of the cells, substantiated by the apical expression of villin andZO1 (Fig. 3, a–d). Interestingly, and in contrast to what observed for apical glycoproteins, the treatment had no effect on the basolateral expression of GP120 (Fig. 3, e and f). The cytoplasmic accumulation of apical glycoproteins in treated cells was further confirmed using immunoelectron microscopy: unlike in control cells where they are restricted to the apical brush border, DPP-IV, MUC1, and CEA are localized in the numerous vesicles that fill the cytoplasm (Fig. 4). Using DPP-IV as a marker of the effect, we further found that this altered distribution is associated with an increased level of expression of the enzyme at both mRNA (Fig. 5) and protein level as substantiated by higher enzyme activities and higher protein content, with a lower apparent molecular mass, however, as shown by Western blot (Fig. 5). The same results were obtained with mucus-secreting HT29-RevMTX 10^-5 cells and enterocytic HT29-RevMTX10^-3 cells (data not shown). The effect of GalNAc-α-O-benzyl on apical glycoproteins is not dependent on the support the cells are cultured on, as exemplified by the swelling of the cells and the intracytoplasmic accumulation of DPP-IV also observed in treated filter-grown cells (data not shown).

**The Effects of GalNAc-α-O-benzyl Are Reversible**

Switching back the cells to drug-free medium after 20 d of treatment results in a rapid reversal of the phenotype described above: within 24 h the volume of the cells decreases, and in the following days apical glycoproteins redistribute to the apical surface (Fig. 6 e). At the same time, mucus secretion resumes, judged by the occurrence of a visible gel on the surface of the cell layer demonstrated by alcian blue staining (data not shown) and immunofluorescence reactivity to antimucus antibodies of cell layer sections (Fig. 6 f).

**NeuAcα2-3Galβ1-3GalNAc Is a Major Oligosaccharide Species Associated with Mucins and Other Glycoproteins from Differentiated HT-29 Cells**

To characterize the oligosaccharide species associated with
mucins and other glycoproteins from HT-29–differentiated cells, we used the lectin MAA, which reacts with NeuAcα2-3Gal-R terminal sequence and PNA, which reacts with the O-linked T antigen (Galβ1-3GalNacα1-O-Ser/Thr). As shown in Fig. 7 via double immunofluorescence, mucus from control HT29-RevMTX10^6 cells is reactive with MAA (Fig. 7, top panel, a–c), whereas only a small proportion of the mucus reacts with PNA (Fig. 7, top panel, d–f). After treatment with sialidase from Clostridium perfringens, all the mucus is reactive with PNA (data not shown), confirming the fact that a large proportion of the mucus expresses the NeuAcα2-3Galβ1-3GalNacα1-O-Ser/Thr sequence. Because mucus droplets are concentrated in the apical cytoplasm and do not allow to distinguish the reactivity of brush border–associated glycoproteins to lectins in mucus-secreting cells, we used enterocytic HT29-RevMTX10^6 cells to further characterize the reactivity of these glycoproteins to lectins. As shown on cryostat sections from postconfluent cells (Fig. 7, middle panel), MAA shows a strong apical reactivity (Fig. 7, middle panel, a), whereas PNA is unreactive (Fig. 7, middle panel, e). After sialidase treatment, there is no longer any reactivity to MAA (Fig. 7, middle panel, c), whereas PNA shows a strong apical staining (Fig. 7, middle panel, g), thus testifying the presence of a large amount of sialyl-T antigen linked to brush border–associated glycoproteins. In contrast, no basolateral labeling was observed with MAA by confocal microscopy (Fig. 7, bottom panel), thus indicating that sialyl-T antigen is not associated with basolateral glycoproteins in differentiated HT-29 cells. The association of NeuAcα2-3Galβ1-3GalNacα1-O-Ser/Thr with glycoproteins in differentiated HT-29 cells was confirmed by Western blot analysis of cell homogenates from the different HT-29 cell populations (Fig. 8) which suggest, based on the reactivity to MAA and PNA before and after treatment with sialidase, that NeuAcα2-3Galβ1-3GalNacα1-O-Ser/Thr sequence is associated not only to mucins, but also to a number of glycoproteins with a molecular mass in the 80–400 kD range. Among the glycoproteins that react with MAA are DPP-IV, MUC1, and CEA as shown by immunoblot analysis with MAA of these immunoprecipitated proteins (see Fig. 10). NeuAcα2-3 glycosylation of T antigen is further supported by the observation that ST3Gal I is expressed at all stages of the culture in mucus-secreting as well as in enterocytic HT-29 cells, as substantiated by analysis of ST3Gal I mRNA level and enzyme activity (Fig. 8). Interestingly, no reactivity was observed by immunoblotting cell homogenates with mAb B72.3 against sialyl-Tn (data not shown) or SNA that recognizes the terminal oligosaccharide species NeuAcα2-6Gal (Fig. 9).

**Treatment with GalNAc-α-O-benzyl Results in a Decreased α2,3-Sialylation of Glycoproteins**

Western blot analysis of the reactivity to lectins of cell homogenates from postconfluent HT29-RevMTX10^6 cells treated with various concentrations of GalNAc-α-O-benzyl shows a dose-dependent decrease of MAA-reacting glycoproteins associated with a dose-dependent increase

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**Figure 2.** Effects of GalNAc-α-O-benzyl on cell morphology and mucus expression in HT29-RevMTX10^6 mucus-secreting cells. Cells were cultured in the absence or under permanent exposure to 2 mM GalNAc-α-O-benzyl and then analyzed after 21 d in culture. *Left column*, light microscopy of thin sections of the cell layer perpendicular to the surface of the flask. *Middle column*, alcian blue staining of cryostat sections of the same cell layers, counterstained with nuclear red. *Right column*, indirect immunofluorescence staining with pAb L56/C of cryostat sections of the same cell layers. (*a–c*) Untreated cells; (*d–f*), cells treated with GalNAc-α-O-benzyl. Bars: left column (17 μm); middle and right columns (40 μm).
in PNA-reacting glycoproteins, with a maximum effect observed at a drug concentration of 2 mM (data not shown). This was further confirmed by the observation, using immunofluorescence, that regardless of their phenotype, the cytoplasm of cells treated with 2 mM GalNAc-α-O-benzyl is heavily stained with PNA, in contrast to control cells (data not shown). Western blot analysis of immunoprecipitated DPP-IV, MUC1, and CEA with MAA and PNA shows that MAA reactivity of these glycoproteins is reduced in cells treated with GalNAc-α-O-benzyl. Concomitant to these changes, a reactivity to PNA was observed for MUC1 and DPP-IV, but not for CEA (Fig. 10). The changes in glycosylation are reversible upon removal of the drug; Western blot analysis of the reactivity to lectins of cell homogenates from cells reverted to drug-free medium shows a reappearance of MAA reactivity and the concomitant disappearance of PNA reactivity of a number of glycoproteins in the 80–400 kD range (data not shown). These include MUC1, DPP-IV, and CEA as shown by Western blot analysis with lectins of the immunoprecipitates of these glycoproteins (Fig. 10).

**The GalNAc-α-O-benzyl–dependent Secretory Block Occurs beyond the cis-Golgi Compartment of the Cells**

To further characterize at which level the block induced by GalNAc-α-O-benzyl occurs, immunoprecipitates of apical glycoproteins from control and treated cells were analyzed.
for their sensitivity to endoglycosidase H and endoglycosidase F treatment. As exemplified for DPP-IV and CEA for their sensitivity to endoglycosidase H and endoglycosidase F treatment. As exemplified for DPP-IV and CEA for their sensitivity to endoglycosidase H and endoglycosidase F treatment. As exemplified for DPP-IV and CEA for their sensitivity to endoglycosidase H and endoglycosidase F treatment.

Discussion

The present results suggest that in HT-29 cells, α2,3-sialylation plays a crucial role in intracellular transport of brush border membrane–associated glycoproteins and in mucus secretion. They rely on the exceptional conjunction of a number of factors: (a) the availability of polarized cells, isolated from the HT-29 cell line, expressing either an enterocyte-like or a mucus-secreting differentiated phenotype; (b) the neoplastic nature of these cells which, as such, show a modified pattern of protein glycosylation as compared with their normal counterpart with shorter oligosaccharide side chains (for review see Lesuffleur et al., 1994); (c) the fact that the main sialyltransferase activities expressed in HT-29 cells catalyze the transfer of sialic acid to the 3-position of Gal in the Galβ1-3GalNAc disaccharide sequence (Dall’Olio et al., 1993; Majuri et al., 1995; Delannoy et al., 1996) in contrast to most colon cancers (Sata et al., 1995) or cell lines, including the enterocytic cell line Caco-2, which mainly express the Galβ1-4GlcNAcα2,6-sialyltransferase, ST6Gal I as reported by others (Dall’Olio et al., 1992, 1996), and confirmed here by the reactivity to SNA of Caco-2 glycoproteins; (d) the availability of an O-glycosylation inhibitor, GalNAc-α-O-benzyl (Kuan et al., 1989; Huang et al., 1992; Byrd et al., 1995; Dilulio and Bhavanandan, 1995), which enters the cells and is metabolized into a compound that acts as a competitive inhibitor of Galβ1-3GalNAcα2,3-sialyltransferases (Huet et al., 1995; Delannoy et al., 1996); and (e) the fact that, in differentiated HT-29 cells, NeuAcα2-3Galβ1-3GalNAc-R is the main oligosaccharide species associated not only with mucus, as previously reported (Capon et al., 1992; Lesuffleur et al., 1993; Huet et al., 1995) but also, as shown here, with a number of glycoproteins of the brush border concomitantly expressed in these cells. The association of NeuAcα2-3Galβ1-3GalNAc-R to apical glycoproteins re-
lies exclusively on their reactivity to MAA, and not to a biochemical characterization that would require a huge amount of cells to be performed. However, the reliability of MAA characterization is validated by a recent structural characterization of carbohydrate chains of the mucus from HT29-RevMTX10²⁵ mucus-secreting cells (that can be easily performed since large quantities of mucus can be harvested daily) which has confirmed that NeuAcα²-3Galβ¹-3GalNAc-R is the main oligosaccharide species associated with the mucus of these cells (Hennebicq-Reig,

Figure 6. Reversibility of the effects of GalNAc-α-O-benzyl on cell morphology, distribution of DPP-IV, and mucus secretion. HT29-RevMTX10⁻⁶ cells were cultured until day 15 in the presence of 2 mM GalNAc-α-O-benzyl and then in drug-free medium. Cultures were analyzed on day 15 (a–c), and 5 d after removal of the drug: (d–f). Left column, thin sections of the cell layer; middle column, indirect immunofluorescence detection of DPP-IV with mAb 3/775/42; right column, indirect immunofluorescence detection of mucus with pAb L56/C in sections of the cell layer. Similar results for DPP-IV were observed as for MUC1 and CEA (data not shown). Bars: left column (16 μm); middle and right columns (80 μm).

Figure 7. Evidence that NeuAcα2-3Galβ1-3GalNαc1-O-Ser/Thr is the main oligosaccharide species associated with mucins and apical proteins from differentiated HT-29 cells. Top panel: cryostat sections of cell layers from HT-29 mucus-secreting cells (HT29-RevMTX10⁻⁶) were analyzed after 21 d in culture by double immunofluorescence labeling with antigastric mucus Ab L56/C followed by rhodamine-conjugated Ig (a and d) and fluorescein-coupled MAA (b) and PNA lectins (e). In c and f, a mixed filter for rhodamine and fluorescein was used. (a–c) Double labeling with Ab L56/C and MAA, showing that the totality of the mucus droplets reacts with MAA; (d–f), double labeling with Ab L56/C and PNA showing that only a small proportion of mucus droplets reacts with PNA. Middle panel (a–h): apical reactivity to MAA and PNA of enterocyte-like HT-29 cells. Frozen cryostat sections from postconfluent (day 21) HT29-RevMTX10⁻³ cells, treated or not treated with sialidase, were double labeled with fluorescein-conjugated MAA (a and c) or PNA (e and g) and antibodies against villin (b, d, f, and h), used as an apical marker, using rhodamine-coupled Ig as a second antibody. Note in sialidase-treated sections, the disappearance of MAA staining (c) contrasting with the appearance of an apical reactivity to PNA (g). Bottom panel: confocal microscopy analysis of postconfluent HT29-RevMTX10⁻⁶ cells (day 21). A section perpendicular to the cell layer was coimmunostained by MAA (red) and mAb TS2/16 against β1 integrin (green). Note the absence of basolateral reactivity to MAA. A similar basolateral pattern as observed with mAb TS2/16 was obtained with mAb G1/136 against GP120 (data not shown). Bars: top and middle panels (40 μm); bottom panel (12 μm).
Finally, it must be noted, as demonstrated by confocal microscopy, that no MAA reactivity could be detected on the basolateral membrane of the cells.

Based on both the sugar specificity of MAA and PNA lectins and the results of sialidase treatment, the present data show that in addition to mucins (i.e., in HT29-RevMTX10⁻⁶ and 10⁻⁵), apical O-glycosylproteins such as MUC1 express NeuAcα₂-3Galβ₁-3GalNAc-R terminal sequences. This being established, four main observations can be drawn from the experiments performed with GalNAc-α-O-benzyl. First, alteration of α₂,3-sialylation of mucins and of apical O-glycosylproteins is accompanied by their accumulation into intracytoplasmic vesicles. This accumulation, which is most likely responsible for the dramatic swelling of the cells, is not restricted to O-glycosylproteins, but is also observed for N-glycosylproteins such as DPP-IV and CEA. Regarding CEA, the absence of PNA binding in treated cells may be explained by a weak expression of the Galβ₁-3GalNAc sequence, not in contradiction with the fact that the binding of MAA is decreased. Second, the block induced by GalNAc-α-O-benzyl occurs after the cis-Golgi as substantiated by endoglycosidase H resistance of apical glycoproteins and normal processing of DPP-IV. Third, these effects are reversible upon removal of the drug, resulting in a concomitant restoration of α₂,3-sialylation and resumption of the secretion of mucins and MUC1, but not CEA, show a reactivity to PNA. The changes in treated cells are reversible upon removal of the drug.
other hand, the presence of a high concentration of the sialylation of ST3Gal IV can use both Galβ1,3GalNAcα-O-benzyl, an O-glycosylation inhibitor (Kuan et al., 1989; Huang et al., 1992; Byrd et al., 1995) which, in HT-29, is metabolized into a compound, acts as a competitive inhibitor of sialyltransferases expressed in HT-29 cells via the donor competitive substrate (i.e., Galβ1-3GalNAcα-O-benzyl) may also decrease the concentration of CMP-NeuAc in the Golgi lumen and therefore can compete with the other sialyltransferases expressed in HT-29 cells via the donor substrate.

Whatever the mechanism leading to the decrease of α2,3-sialylation, these results show that lack of terminal NeuAcα2-3Gal-R glycosylation is associated with a blockade of apical targeting of brush border membrane–associated glycoproteins and mucus secretion in HT-29 cells. They further suggest that α2,3-sialylation could play a role in regulating the intracellular traffic of these proteins, and in some way, support the view by Fiedler and Simons (1994) that “it may be that oligosaccharide side chains play a more important role in biosynthetic traffic than hitherto recognized”. Even if the role of glycans in the intracellular targeting of newly synthesized lysosomal enzymes via the mannose-6-phosphate receptor pathways was clearly demonstrated in human colon adenocarcinoma cell lines (Braulke et al., 1992), a role for glycans in the sorting machinery of membrane proteins in polarized cells has long been excluded on the basis of experiments using inhibitors of N-glycosylation such as tunicamycin, which blocks the transfer of Glc3Man9GlcNAc2 from dolichol to Asn, thus resulting in the accumulation of unprocessed proteins in the rough ER (Green et al., 1981), or castanospermine, or 1-deoxymannojirimycin which block processing before trimming of the oligomannose chains (Duronio et al., 1988). Very few recent observations suggest, however, a possible role for terminal glycans in this regulation. Growth hormone, which is nonglycosylated and secreted from both sides of MDCK cell layers, is secreted from the apical side when N-glycosylated (Scheiffele et al., 1995). The sialoglycoprotein gp114 that is expressed on the apical membrane of MDCK cells is misglycosylated and predominantly basolateral in the MDCK mutant MDCKII-RCA' (Le Bivic et al., 1993). The O-glycosylated stalk domain is required for apical sorting of neurotrophin receptors in polarized MDCK cells (Yeaman et al., 1997). In addition to these particularities that concern glycoproteins, it has been shown that among the increasing number of vesicular proteins presumably involved in protein sorting (Rothman, 1994), one of them, VIP36 (Fiedler et al., 1994), presents some homology with leguminous lectins (Fiedler and Simons, 1994) and binds GalNAc (Fiedler and Simons, 1996). Therefore, it is conceivable that terminal NeuAcα2-3Gal glycan sequences as signals, and lectins as receptors for these signals, could be involved in the sorting machinery of glycoproteins in polarized HT-29 cells. Because the glycosylation of both lectin-like resident Golgi proteins and in transit glycoproteins may be affected by GalNAcα-O-benzyl, the precise level at which the sorting machinery is affected needs to be elucidated.

Whether or not α2,3-sialylation is exclusively involved in the apical sorting of glycoproteins in HT-29 cells cannot be concluded from the present work since it was only focused on brush border membrane glycoproteins and mucins. From the results obtained with Western blot, it is clear that the effects of GalNAcα-O-benzyl are shared by a number of other proteins that remain to be characterized as to their nature and localization. Nevertheless, the present data show that a shift in the predominant glycos-
lation pattern of glycoproteins from NeuAcα2-3Galβ1-3GalNAc-R to Galβ1-3GalNAc-R results in what appears as a glycoprotein traffic jam that dramatically alters their normal delivery and also leads to cell hypertrophy.

Analysis of whether the present results uncover a more general involvement of terminal glycans in the intracellular protein transport machinery needs to consider two main evidences. First, there is the high polymorphism of terminal glycans which differ from one individual to another. Second, and with regard to a putative role of animal lectins as receptors for the glycan signal (Fiedler and Simons, 1994), there is the strict oligosaccharide specificity of lectins. This implies that if the results obtained with HT-29 cells rely on a general mechanism, each cellular system, whether from human or animal origin, is unique and only representative of the genetic background of the individual it originates from. With regard to normal or malignant intestinal cells it must be noted that there are only very few indications in which oligosaccharide species are associated with apical glycoproteins. The only available data concern the observation that, in the normal adult intestine, ABH blood group antigens are the main terminal glycans associated with the intestinal brush border hydrolases in humans (Triadou et al., 1983; Green et al., 1988) as well as in rabbits (Gorvel et al., 1982). With regard to differentiated colon cancer cells, the results reported here are the first observation showing that in HT-29 cells, the main terminal oligosaccharide species associated with apical glycoproteins is NeuAcα2-3Gal.

Preliminary results obtained in the laboratory indicate that, as in rat developing enterocytes (Roth, 1993), the apical membrane of intestinal enterocytes from 10–20 gestational wk fetuses is strongly reactive with MAA; in contrast, epithelial cells in the adult human small intestine lack MAA reactivity (our unpublished results). This absence of effect at concentrations (10 mM), on the morphology of the cells and the apical phenotype (Zweibaum et al., 1991) and on the assignment of the gene locus DPPIV to chromosome 2. Preliminary results obtained in the laboratory indicate that, as in rat developing enterocytes (Roth, 1993), the apical membrane of intestinal enterocytes from 10–20 gestational wk fetuses is strongly reactive with MAA; in contrast, epithelial cells in the adult human small intestine lack MAA reactivity (our unpublished results). This absence of effect at concentrations (10 mM), on the morphology of the cells and the apical phenotype (Zweibaum et al., 1991) and on the assignment of the gene locus DPPIV to chromosome 2.

With regard to the oligosaccharidic individual variability it is clear that the results obtained with HT-29 cells cannot be extrapolated as such to other cellular systems. For example, in Caco-2 cells, in which the main sialyltransferase is ST6Gal I (Dall’Olio et al., 1992, 1996) GalNAcα-O-benzyl has no effect, even at much higher concentrations (10 mM), on the morphology of the cells and the apical polarity of brush border–associated glycoproteins (our unpublished results). This absence of effect is obviously consistent with the fact that in Caco-2 cells, apical glycoproteins such as saccrose-isomaltase or DPP-IV most likely express NeuAcα2-6, as suggested from the SNA reactivity of Caco-2 glycoproteins and that GalNAcα-O-benzyl has no effect on ST6Gal I.

Finally the present results draw attention to the necessity of characterizing, in each experimental system, which oligosaccharide species is associated with the apical glycoproteins. This is a prerequisite for any further analysis of the role of terminal oligosaccharides in the intracellular traffic of apical glycoproteins. It also implies that new strategies should be developed to specifically block the terminal glycosylation of apical glycoproteins. One challenge is to analyze whether, for example, ABH blood group antigens are involved in the intracellular traffic of intestinal brush border hydrolases in humans.

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