In previous work we reported that long term treatment of polarized HT-29 cells by 1-benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (GalNAc-α-O-bn) induced undersialylation and intracellular distribution of apical glycoproteins such as dipeptidyl peptidase IV (DPP-IV), and we suggested therefore that sialylation could act as an apical targeting signal. In this work, the apical direct biosynthetic route was studied after transfection of polarized enterocyte-like HT-29 5M12 cloned cells with a murine cDNA coding for a soluble form of DPP-IV, which became blocked inside the cell. A similar short GalNAc-α-O-bn treatment also induced an intracellular distribution of both endogenous trans-Golgi network (TGN) and protein delivered in the regulation of the apical trafficking such as the apical t-SNARE syntaxin-3 and the raft-associated protein annexin XIIIb, whereas the basolateral t-SNARE syntaxin-4 kept its normal localization. These apical membrane proteins moved efficiently from trans-Golgi network to apical carrier vesicles but failed to be transported from carrier vesicles to the apical plasma membrane. Isolation of membrane microdomains showed that GalNAc-α-O-bn induced the formation of abnormal lipid-rich microdomains in comparison to normal rafts, as shown by their lower buoyant density and their depletion in annexin XIIIb. In conclusion, GalNAc-α-O-bn blocks the anterograde traffic to the apical surface of polarized HT-29 cells at the transport level or docking/fusion level of carrier vesicles.

In recent years, data of the literature have shown the role of glycosylation in the apical biosynthetic route in polarized epithelial cells. Association of proteins with glycosphingolipids has been proposed for the apical delivery (1, 2). Glycosylphosphatidylinositol (GPI)-anchored proteins and glycosphingolipids could be isolated from a Triton X-100-insoluble fraction (3). The dynamic clustering of glycosphingolipid-enriched microdomains in the TGN would constitute functional rafts for apical delivery of proteins (4). Thus, proteins of apical destination, such as GPI-anchored proteins, would be specifically recruited in these apical raft carriers. Besides, data obtained by mutation, deletion, or addition of glycosylation sites showed that O- or N-glycosylation could influence the targeting of glycoproteins toward the apical side of the cells (5–14).

The putative role of N- and O-glycans was also shown through the use of drugs affecting their processing, i.e. tunicamycin, deoxymannojirimycin, swainsonine for N-glycans, and 1-benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (GalNAc-α-O-bn) for O-glycans (11, 14–18). We previously observed that long term treatment of polarized goblet or enterocytic HT-29 cells by GalNAc-α-O-bn led (i) to a dramatic inhibition in the secretion of mucins, which are highly O-glycosylated; (ii) to a decrease in the apical membrane expression of brush border markers such as the transmembrane glycoprotein dipeptidylpeptidase-IV (DPP-IV), the GPI-anchored carcinoembryonic antigen, and the mucin-like glycoprotein MUC1; and (iii) to the abnormal presence of these glycoproteins inside the cells (19, 20). In contrast, basolateral glycoproteins such as gp120 and gp525 kept a normal localization under GalNAc-α-O-bn treatment (19, 21).

These data led us to analyze more deeply the interferences of exogenous GalNAc-α-O-bn with the intracellular processes of glycosylation in HT-29 cells. In a previous work, we showed that GalNAc-α-O-bn was highly converted into the benzyldisaccharide Galβ1–3GalNAc-α-O-bn, which acts as a potent competitive inhibitor of α2,3-sialyltransferase ST3Gal I and involved in the terminal elongation of O-linked glycans (22). Thereafter, we reported that GalNAc-α-O-bn was extensively metabolized beyond Galβ1–3GalNAc-α-O-bn, showing that the glycosylation of endogenous substrates by several glycosyltransferases could be inhibited, and in particular the sialylation of N-glycans by α2,3-sialyltransferase ST3Gal IV (21, 22). In this way, the sialylation of N- and/or O-glycans was found inhibited on the endogenous glycoproteins DPP-IV, MUC1, and GPI-anchored carcinoembryonic antigen, and we suggested that undersialylation of glycoproteins may induce a defect in the direct apical targeting of glycoproteins (19–21).

Later on, the effect of GalNAc-α-O-bn was studied by others using other cell lines, and the induction of a shift from the dase IV; sDPP-IV, secreted dipeptidyl peptidase IV; mAb, monoclonal antibody; MDCK, Madin-Darby canine kidney; MAA, M. amurensis lectin; MALDI, matrix-assisted laser desorption ionization; TOF, time-offlight; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

This paper is available on line at http://www.jbc.org
apical membrane to the basolateral membrane was reported in the targeting of the brush border glycoproteins in cell type-specific process that depends on the expression pattern of cell type specificity in the modification of cell type specificity in the cellular responses to GalNAc-O-bn was disrupted with intracellular trafficking (21, 29–27), suggesting that the cell type specificity in the cellular responses to GalNAc-O-bn was connected to the cell type specificity in the modification of the glycosylation pattern.

GalNAc-O-bn appeared to be an interesting tool to study polarized HT-29 cells, regarding the fact that, in this cell type specifically, (i) the terminal sialylation was identified as the target of inhibition, and (ii) the normal apical localization of brush border glycoproteins was disrupted with intracellular localization. Thus, we investigated the effect of this inhibitor of glycosylation on de novo apical trafficking in enterocyte-like HT-29 cells.

MATERIALS AND METHODS

Cell Culture—HT-29 clone 5M12 cells (cloned from a HT-29 cell subpopulation resistant to methylxanate (Ref. 28)) were cultured as previously described (19). Cells were cultured in 25-cm² T-flasks (Corning Glass Works, Corning, NY) for cell maintenance and on glass coverslips or 24.5-mm tissue culture-treated Transwell polyester membrane filters (0.4-μm pore size) (Costar, Cambridge, MA) for confocal microscopy and on 6-well culture dishes or 24.5-mm tissue culture-treated Transwell polyester membrane filters for metabolic labeling. GalNAc-O-bn was used at a concentration of 2 mM in Dulbecco's modified Eagle's medium with fetal bovine serum during a short-term treatment (from 0 to 44 h) starting from confluence. In all experiments, GalNAc-O-bn had no effect on cell viability, as assessed by the absence of cells in suspension and trypan blue exclusion. For the analysis of cell culture media by two-dimensional gel electrophoresis, cells were cultured for 24 h in serum-free medium before collection.

Transfection of Soluble Mouse DPP-IV—To generate a secreted mouse DPP-IV (sDPP-IV), the full-length mouse DPP-IV cDNA (29) was digested with PvuII restriction enzyme to remove the first 105 nucleotides and was subcloned downstream of a coding sequence for a cleavable peptide signal into the eukaryotic expression vector pTEJS that contains the G418 resistance gene (18). HT-29 5M12 cells were transfected using LipofectAMINE according to the instructions from the manufacturer (Invitrogen). Resistant colonies growing in the presence of 400 μg/ml G148 were isolated using cloning cylinders and screened for secreted DPP-IV activity in the medium. Clone 5M12 Cl2 was selected for further experiments. DPP-IV activity was measured by a kinetic method with the fluorogenic substrate H-Gly-Pro-AMC (Bachem) (21).

Antibodies—Mouse mAbs 525 against gp525, HBB 3/775/42 against human DPP-IV (30) were a gift from Dr. A. Le Bivic (IBDM, Marseille, France) and Dr. M. Kauppi (National Public Health Institute, Helsinki, Finland), respectively. Rat mAbs 1082 and 773 against mouse DPP-IV were obtained from Didier Marguet (Centre d’Immunologie INSERM-CNRS de Marseille Luminy, France). Rabbit antibodies against human syntaxin-3, human annexin XIIIb, and human Munc18-2 were obtained from Dr. A. Le Bivic, Dr. J. Gordon (Washington University School of Medicine, St. Louis, MO) and Dr. M. Kauppi (National Public Health Institute, Helsinki, Finland), respectively. Mouse mAbs against syntaxin-4 (S40220) and against flotillin-1 (clone 1) were purchased from BD Transduction Laboratories (Lexington, KY). Mouse mAb against human DPP-IV (M-A261) was purchased from BD Pharmingen (San Diego, CA).

Two-dimensional Gel Electrophoresis and MALDI Time-of-flight (TOF) Mass Fingerprinting of Tryptic Peptides—HT-29 5M12 Cl2 cells culture media were concentrated and precipitated with trichloroacetic acid. Amounts used for two-dimensional electrophoresis were normalized to the amount of secreted DPP-IV activity in the medium. The grids were counterstained with 0.3% uranyl acetate and 1.8% methylcellulose. Controls grids were processed without the second primary antibody (monoclonal anti-human DPP-IV antibody (1/20)) for 5 h at room temperature. The grids were counterstained with 0.3% uranyl acetate and 1.8% methylcellulose. Controls grids were processed without the secondary antibodies and showed the absence of detection of 12-nm gold particles. The perforated grids were collected from the filters.

Ultrastructural Immunocytochemistry—Immunolabeling was carried out on isolated carrier vesicles of HT-29 5M12 cells according to the procedure described by Wandering-Ness et al. (31). Briefly, HT-29 5M12 cells were seeded on 12-mm filters, which were cultured up to day 10. Six filters were treated by 2 mM GalNAc-O-bn for 18 h before the experiment. After the treatment of filters to 18 h, the filters were fixed with 2% paraformaldehyde for 5 min. Single or double labeling was performed. The grids were coated with a mixture containing 0.2% gelatin, then with anti-rabbit Ig-coupled 18-nm gold particles (monoclonal anti-human DPP-IV antibody (1/20)) for 5 h at room temperature and then with anti-mouse Ig-coupled 12-nm gold particles. The grids were counterstained with 0.3% uranyl acetate and 1.8% methylcellulose. Controls grids were processed without the secondary primary antibodies and showed the absence of detection of 12-nm gold particles.
control cells were pulse-labeled for 30 min with 200 µCi/well of \(^{35}\)S-methionine (ICN, Irvine, CA) in 1 ml of methionine-free medium, and then chased for the indicated periods of time with 1 ml of 0.01 M methionine in regular medium. A similar protocol was applied in the presence of 2 mM GalNAc-\(\beta\)-O-bn throughout the experiment. To follow the secretion of sDPP-IV, we collected the apical medium and we immunoprecipitated sDPP-IV with rat mAbs 1082 and 773. For analyzing the detergent extractability of cellular endogenous human DPP-IV, we used the procedure described by Alfalah \textit{et al.} (14). HT-29 5M12 cells were rinsed in PBS, and cells were solubilized in 25 mM Tris-HCl, pH 8.0, 50 mM NaCl, containing 1% Triton X-100 and protease inhibitors for 2 h at 4 °C. The detergent extracts were centrifuged at 100,000 x g for 1 h at

---

**FIG. 1.** Secretion of recombinant soluble murine sDPP-IV by control and GalNAc-\(\beta\)-O-bn-treated HT-29 5M12 Cl2 cells. A, cells were cultured on filters in standard conditions up to day 14. Subsequently, cells were cultured for 24 h in serum-free medium with or without GalNAc-\(\beta\)-O-bn. Apical and basolateral media were collected and analyzed by two-dimensional electrophoresis. In the apical medium of control cells, spots 1–5 were identified as sDPP-IV by MALDI-TOF mass spectrometry. The amount of sDPP-IV decreased under GalNAc-\(\beta\)-O-bn treatment, particularly the most acidic forms. sDPP-IV was not significantly secreted into the basolateral medium. B, pattern of apical secretion of sDPP-IV after pulse-chase metabolic labeling. HT-29 5M12 Cl2 cells were pulse-chase-labeled with \(^{35}\)S-methionine in the absence (control) or presence (treated) of GalNAc-\(\beta\)-O-bn. The apical medium was collected and immunoprecipitated with anti-murine DPP-IV antibody, and immunoprecipitates were subjected to SDS-PAGE. C, lectin blot with MAA lectin of immunoprecipitated sDPP-IV from the apical medium of control and GalNAc-\(\beta\)-O-bn-treated cells. Note that sDPP-IV secreted by control cells reacts with MAA but not sDPP-IV secreted by GalNAc-\(\beta\)-O-bn-treated cells.
4 °C, and the supernatant was immunoprecipitated with the anti-human DPP-IV mAb HBB 3/775/42. The pellets were then solubilized in the same buffer for 20 min at 37 °C and immunoprecipitated with the anti-human DPP-IV mAb HBB 3/775/42. Immunocomplexes were collected on protein G-Sepharose-4B (Sigma), eluted in SDS sample buffer (0.2 M Tris-HCl buffer, pH 6.8, containing 2% SDS, 5% β-mercaptoethanol and 30% glycerol) at 60 °C for 15 min, and then analyzed on a 7% SDS-polyacrylamide gels. For autoradiography, gels were fixed in 40% ethanol, 10% glycerol, 10% acetic acid, soaked in Amplify (Amersham Biosciences) for 20 min, dried on Whatman paper, and then exposed to Hyperfilm-max (Amersham Biosciences).

Isolation of Raft Microdomains—Rafts were isolated from series of 12 75-cm² culture flasks of HT-29 5M12 cells according to the procedure of Fiedler et al. (33) slightly modified. The cell pellet equilibrated in 10 mM HEPES buffer, pH 7.4, with 2 mM EGTA, 0.25 M sucrose, and protease inhibitors, was homogenized by 10 passages in a 1-ml tip, 15 passages in a 22-gauge needle, and 10 passages in a Dounce homogenizer. A total membrane fraction was obtained from a post-nuclear supernatant after ultracentrifugation (38,000 rpm, 20 h) in a SW41 swinging rotor at the interface (1.2 M; 0.8 M) of a discontinuous sucrose gradient, washed in TNE buffer (25 mM Tris-HCl, pH 7.5, with 150 mM NaCl and 5 mM EDTA), treated with a mixture of TNE/Triton X-100 at 1% for 30 min on ice, and separated by ultracentrifugation (39,000 rpm, 18 h) on a discontinuous sucrose gradient (0.15 M; 1.1 M; 1.2 M) in a SW41 rotor. Individual fractions of 1 ml were isolated all over the gradient and examined for their protein content. For Western blotting, half of the entire volume of each fraction was used. Proteins were precipitated with trichloroacetic acid, washed with acetone, and solubilized in SDS sample buffer.

Western Blotting—Samples were separated on a 5–30% SDS-PAGE (34), and the detection of proteins was carried out by luminescence using the ECL Western blotting system (Amersham Biosciences, Aylesbury, UK).

**RESULTS**

In this work, we studied the effect of GalNAc-β1,4GalNAc on the direct apical biosynthetic route using a cloned population of HT-29 cells of enterocytic phenotype (HT-29 5M12) and short exposure times to GalNAc-β1,4GalNAc. In addition to the study of endogenous transmembrane DPP-IV, which is endocytosed and recycled to the apical membrane, we studied a soluble form of murine DPP-IV, which was expected to be secreted in the medium under a polarized fashion after transfection in HT-29 5M12 cells. Indeed, after transfection of a soluble form of rat DPP-IV in MDCK cells, Weisz et al. (35) reported that this soluble form was predominantly secreted into the apical medium, indicating that the lumenal domain of DPP-IV was likely to contain the apical sorting information.

**Inhibition in the Secretion of a Soluble Murine DPP-IV Form**—HT-29 5M12 cells of enterocytic phenotype were stably transfected with an eukaryotic expression vector coding for a secreted mouse DPP-IV, and we selected the clone HT-29 5M12 Cl2 that secreted DPP-IV enzymatic activity in the medium.

We first investigated the secreted DPP-IV forms in the apical and basolateral medium using two-dimensional electrophoresis and protein identification by mass spectrometry of tryptic digests (Fig. 1A). Cells were cultured on filters in standard medium up to day 10 and then for 24 h in serum-free medium with...
Fig. 3. Analysis of the cellular distribution of syntaxin-3, Munc18b, annexin XIIIb, and syntaxin-4 in control and GalNAcα-O-bn-treated HT-29 5M12 cells using confocal microscopy. Control (upper row) and GalNAcα-O-bn-treated (lower rows) cells were successively analyzed with antibodies directed against syntaxin-3, Munc18b, annexin XIIIb, and syntaxin-4. In control cells, syntaxin-3 was faintly detected at the apical side, and Munc18b and annexin XIIIb were clearly visualized at the apical side. GalNAcα-O-bn treatment induced a shift in the distribution of these proteins. Syntaxin-4 was found at the basolateral side of HT-29 5M12 cells, and this expression was unchanged under GalNAcα-O-bn treatment. Bars, 44 μm (syntaxin-3, Munc18b, syntaxin-4) and 22 μm (annexin XIIIb).
Apical and basolateral cell culture media were collected, concentrated, and precipitated with trichloroacetic acid. The amounts of cell culture media used for two-dimensional electrophoresis were normalized in reference to the same number of cells. In the apical medium of control cells, a train of 5 spots of high intensity was visualized between 80 and 90 kDa with a range of pI (isoelectric point) from 5.5 to 6 approximately. These spots were excised, digested with trypsin and analyzed for their peptidic map by MALDI-TOF. Results showed that these spots corresponded to murine DPP-IV (data not shown). The two-dimensional pattern of the basolateral medium showed that sDPP-IV was not significantly secreted into this medium. This shows that the recombinant sDPP-IV is predominantly secreted in the apical medium of

Fig. 4. Analysis of carrier vesicles isolated from perforated control and GalNAcα-O-bn-treated HT-29 5M12 cells. A, immunogold single labeling of DPP-IV, annexin XIIIb, and syntaxin-3, and double labeling of DPP-IV/annexin XIIIb. In the double-labeling experiments, DPP-IV was labeled by 12-nm gold particles and annexin XIIIb by 18-nm gold particles. B, Western blotting of annexin XIIIb in the carrier vesicles and the membrane fraction from ghosts of perforated cells. Bars, 100 nm.

or without GalNAcα-O-bn. Apical and basolateral cell culture media were collected, concentrated, and precipitated with trichloroacetic acid. The amounts of cell culture media used for two-dimensional electrophoresis were normalized in reference to the same number of cells. In the apical medium of control cells, a train of 5 spots of high intensity was visualized between 80 and 90 kDa with a range of pI (isoelectric point) from 5.5 to 6 approximately. These spots were excised, digested with trypsin and analyzed for their peptidic map by MALDI-TOF. Results showed that these spots corresponded to murine DPP-IV (data not shown). The two-dimensional pattern of the basolateral medium showed that sDPP-IV was not significantly secreted into this medium. This shows that the recombinant sDPP-IV is predominantly secreted in the apical medium of
HT-29 5M12 C12 cells and confirmed the existence of apical targeting signal(s) in the ectodomain of the molecule, and also indicated the involvement of a specific machinery for its transport toward the apical side. In the apical medium collected after GalNAc-O-bn treatment, we visualized in the area of sDPP-IV only three spots (spots 1, 2, and 3) with lower intensity. Spots 4 and 5, corresponding to the most acidic forms, were not detected, and spot 3, which accounted for the major spot, showed a marked decrease in comparison to spots 1 and 2. sDPP-IV was not recovered in the basolateral medium of treated HT-29 5M12 cells, although a slight increase was detected at the area of sDPP-IV. To further assess the intracellular retention of sDPP-IV, we evaluated the DPP-IV enzymatic activity in the cytosol of control and treated cells. Results were consistent with an intracellular trapping of endogenous transmembrane DPP-IV in HT-29 5M12 cells after increasing exposures times to GalNAc-O-bn (from 6 to 44 h) using quantitative confocal microscopy. Results (Fig. 2) showed that DPP-IV progressively lost its localization at the apical membrane and displayed an overall cellular localization. The quantitative measure of the ratio apical fluorescence/inside fluorescence showed that, after a 18-h exposure time, most DPP-IV was localized inside the cells. To confirm that DPP-IV only redistributed to the inside of the cells and not to the basolateral membrane, we further analyzed the localization of DPP-IV by double-labeling experiment using E-cadherin as a marker of the basolateral membrane (data not shown). These results were consistent with an intracellular trapping of endogenous transmembrane DPP-IV.

Alteration in the Distribution of Endogenous Human DPP-IV—We then analyzed the cellular distribution of the endogenous transmembrane DPP-IV in HT-29 5M12 cells after increasing exposures times to GalNAc-O-bn. After the indicated periods of chase, cells were extracted with Triton X-100 at 4 °C and DPP-IV (detergent-soluble form) was immunoprecipitated from the supernatant. The pellet was then solubilized at 37 °C, and DPP-IV (detergent-insoluble form) was immunoprecipitated. Immunoprecipitates were subjected to SDS-PAGE. In both control and GalNAc-O-bn-treated cells, DPP-IV displayed detergent insolubility after a chase of 4 h. Subsequently, DPP-IV was found under a detergent-soluble form after both 6 and 24 h of chase in control cells, whereas in GalNAc-O-bn-treated cells, the detergent-soluble form was found at a low level after 6 h of chase, and was nearly undetectable after 24 h of chase.

Specific Alteration in the Distribution Proteins Involved in Apical Regulation—We then hypothesized that GalNAc-O-bn treatment could specifically affect the vesicular trafficking machinery to the apical surface. Both trafficking pathways toward the apical and basolateral membranes of polarized cells are regulated by the SNARE machinery, but different SNARE complexes are involved in each targeting. The t-SNAREs syntaxin-3 and -4 were, respectively, localized at the apical and basolateral membranes of polarized MDCK and Caco-2 cells (36, 37). Munc18-2/Munc18b is a Sec1 homologue, which interacts with the apical t-SNARE syntaxin-3 (38). Annexin XIIIb is an N-myristoylated protein, which was reported to participate in the apical transport via an association with the raft lipid microdomains (39, 40). We thus investigated the distribution of these SNARE or SNARE partners in control and GalNAc-O-bn-treated polarized HT-29 5M12 cells (Fig. 3). Syntaxin-3 was faintly detected at the apical side of control cells. After GalNAc-O-bn treatment, we observed a significant syntaxin-3 labeling inside the cells. Munc18-2/Munc18b and annexin XIIIb were clearly detected at the apical surface of control cells. After GalNAc-O-bn treatment, Munc18-2/Munc18b and annexin XIIIb became localized inside the cells. In contrast, syntaxin-4 was expressed at the basolateral membrane of HT-29 5M12 cells, and this localization was unchanged under Gal-
FIG. 6. Fractionation on a discontinuous sucrose gradient of the detergent extracts of the membrane fractions from control and GalNAcα-O-bn-treated HT-29 5M12 cells. Detergent-insoluble raft microdomains, present at the interface 0.15 M/1.1 M, corresponded to fraction 3, and detergent-soluble material was collected over fractions 4–12. A, protein content of each fraction, which shows that the detergent-insoluble...
NAcα-O-bn treatment. Altogether, these data showed that GalNAcα-O-bn also inhibited the trafficking of proteins involved in apical regulation.

**Correct Transport of Apical Proteins from TGN to Carrier Vesicles**—Apical delivery has been proposed to occur via the recruitment of apical proteins within rafts in TGN and the budding of raft carrier vesicles (4). Regarding published data on the role of N- and/or O-glycans on raft association, we searched to determine whether apical proteins were transferred into carrier vesicles under GalNAcα-O-bn treatment. Thus, we have isolated TGN-derived carrier vesicles from perforated HT-29 5M12 cells and analyzed them at the ultrastructural level by immunogold labeling (Fig. 4A) and at the biochemical level by Western blotting (Fig. 4B). We examined endogenous DPP-IV, annexin XIIIb, and syntaxin-3 by single labeling. As syntaxin-3 and annexin XIIIb were previously localized in apical carrier vesicles in MDCK cells (40, 41), two immunogold double labelings were performed: DPP-IV/syntaxin-3 (data not shown) and DPP-IV/annexin XIIIb. Results clearly showed the presence of these apical proteins in carrier vesicles in both control and GalNAcα-O-bn-treated cells. These data indicated that GalNAcα-O-bn treatment did not alter the pathway from TGN to the TGN-derived carrier vesicles.

We further tried to obtain quantitative data on the relative distribution of these apical proteins in the carrier vesicles and at the plasma membrane. The isolated carrier vesicles were analyzed by immunoblotting in comparison to the membrane fraction isolated from the corresponding ghosts of perforated cells. Samples were normalized to a similar amount of ghost fraction isolated from the corresponding ghosts of perforated cells. Samples were normalized to a similar amount of ghost fraction isolated from the corresponding ghosts of perforated cells. Samples were normalized to a similar amount of ghost fraction isolated from the corresponding ghosts of perforated cells. Samples were normalized to a similar amount of ghost fraction isolated from the corresponding ghosts of perforated cells.

**Raft Association of DPP-IV along Its Biosynthetic Pathway**—Raft association of endogenous DPP-IV was then followed along its biosynthetic pathway in control and GalNAcα-O-bn-treated HT-29 5M12 cells after pulse-chase metabolic labeling (Fig. 5). In control cells, results showed that DPP-IV displayed a detergent insolubility after 4 h of chase. At this time, the maturation of the protein precursor into the mature glycosylated form of higher molecular weight appeared nearly complete and only the mature form was detergent-insoluble. Subsequently, the relative proportion of detergent-insoluble DPP-IV form decreased, and the glycoprotein reappeared under a detergent-soluble form after 6 and 24 h of chase. After 24 h of chase, a second shift in the apparent $M_r$ of DPP-IV was visualized, suggesting a late maturation by post-translational processing. In GalNAcα-O-bn-treated cells, DPP-IV also showed the appearance of a detergent-insoluble form after 4 h of chase, which decreased thereafter. However, after 6 h of chase, the detergent-soluble form appeared only at a very low level, and became nearly undetectable after the chase of 24 h, showing that DPP-IV followed a degradative process after detergent insolubility in GalNAcα-O-bn-treated cells. We have checked this pattern of biosynthesis and trafficking of DPP-IV in two other immunoprecipitation experiments. These data indicated that DPP-IV was able to associate transiently with lipid microdomains in GalNAcα-O-bn-treated cells, but that the subsequent reversion of DPP-IV in a non-raft membrane fraction did not show a similar steady state to that in control cells.

**Association of Apical Proteins with Rafts**—To further analyze raft association, we used a slightly modified procedure of membrane fractionation using detergent insolubility and ultracentrifugation on discontinuous sucrose gradients as described by Fiedler et al. (33). The procedure was applied on control and GalNAcα-O-bn-treated HT-29 5M12 cells. Twelve fractions were collected all over the gradient (Fig. 6A). Detergent-insoluble raft microdomains described to be present at the interface 0.15 M/1.1 M corresponded to the collected fraction 3, whereas detergent-soluble membranes was collected along fractions 4–12. In control cells, a high amount of proteins were found in fractions 4–12, but a peak of proteins was clearly visualized in fraction 3. In GalNAcα-O-bn-treated cells, the peak of detergent-insoluble material shifted to fraction 2 of lower buoyant density.

The equilibrium distribution of DPP-IV and annexin XIIIb along this gradient was probed by Western blot after loading each lane with half of the entire volume of each fraction (Fig. 6B). Films were scanned, and the insoluble (fraction 2–3)/soluble (fractions 4–12) ratio was calculated. In control cells, DPP-IV was mainly present in detergent-soluble membrane fractions (ratio 0.09). We also observed an heterogeneity in the apparent $M_r$ of DPP-IV, as DPP-IV in fraction 4 showed a lower migration in comparison to other fractions, an observation connected to the pattern obtained by pulse-chase metabolic labeling. GalNAcα-O-bn induced an enrichment of DPP-IV in detergent-insoluble fraction 2 (ratio 1.15). For annexin XIIIb, the distribution in control cells also showed a predominance in detergent-soluble membrane fractions (ratio 0.17), but in contrast to DPP-IV, GalNAcα-O-bn markedly decreased the proportion of annexin XIIIb in the detergent-insoluble fractions (ratio 0.02).

Controls were then used to check the specificity of our data (Fig. 6C). The distribution of the raft marker protein flotillin-1/Reggie-2 was examined as a positive control. Flotillin-1 was found in raft fraction 3 of control cells but also in non-raft fractions 4–12. In GalNAcα-O-bn-treated cells, flotillin-1/Reggie-2 shifted to the fraction 2. The glycoprotein gp525, present at the basolateral membrane and not affected in its trafficking by GalNAcα-O-bn treatment (21), was used as a negative control. As shown in Fig. 6C, the distribution of this glycoprotein was exclusively localized in non-raft fractions and appeared unchanged by GalNAcα-O-bn treatment. Altogether, these data showed that GalNAcα-O-bn treatment modified the composition of lipid microdomains.

**DISCUSSION**

In our previous work, we reported that long term treatment of polarized HT-29 cells by the inhibitor of glycosylation GalNAcα-O-bn induced an abnormal intracellular localization of apical membrane glycoproteins. In this cell line, GalNAcα-O-bn was found to inhibit primarily the terminal sialylation by the sialyltransferases ST3Gal I and ST3Gal IV, and we suggested that sialylation of apical glycoproteins may act as a targeting signal for apical delivery (19, 22, 23). The goal of this work was to examine the anterograde traffic in cloned HT-29 5M12 cells
under GalNAc-O-bn exposure. Short exposure times were used to avoid the expression of a storage phenotype linked to the accumulation of GalNAc-O-bn metabolites (23, 27, 42).

It has been previously shown that rat and murine soluble recombinant DPP-IV were secreted predominantly into the apical medium of MDCK cells, indicating that the ectodomain of DPP-IV contained sorting signals for apical targeting (18, 35). To study the direct apical route in HT-29 5M12 cells, we transfected an expression vector coding for a secreted mouse DPP-IV. Murine sDPP-IV accounted for a major protein secreted into the apical medium of transfected HT-29 5M12 C12 cells, whereas it was barely detected in the basolateral medium. Interestingly, GalNAc-O-bn markedly decreased the apical secretion of this glycoprotein, and sDPP-IV did not shift to the basolateral medium but accumulated inside the cells.

Altogether, these data showed that GalNAc-O-bn blocked the apical anterograde traffic in HT-29 5M12 C12 cells. In addition, we observed that GalNAc-O-bn also induced undersialylation of sDPP-IV. Recently, Ulloa and Real (43) tried to rule out the hypothesis of a defect in the apical direct traffic caused by undersialylation, because the storage phenotype induced by prolonged GalNAc-O-bn treatment led to a defect in the recycling of endocytosed membrane glycoproteins which became accumulated in late endosomes. However, our analysis of various cell lines treated by GalNAc-O-bn clearly showed that the storage phenotype and the intracellular retention of apical glycoproteins are two independent events (27). In this work overall, the finding of the intracellular retention of a DPP-IV form, which does not follow the endocytosis/recycling process but is exocytosed into the apical medium allowed to assert clearly the occurrence of a block on the apical anterograde traffic in GalNAc-O-bn-treated HT-29 5M12 C12 cells.

We had shown that the block occurred beyond the cis-Golgi, as substantiated by endoglycosidase treatment and processing of endogenous DPP-IV (19). Thus, we investigated the distribution of different proteins known to be involved in the regulation of the vesicular transport. Proteins of SNARE complex are known to play a role in the regulation of the direct apical biosynthetic route: t-SNARE syntaxin-3 and v-SNARE Ti-VAMP (36, 37, 44, 45). In addition, the sec1-related protein Munc18-2 modulates the interaction of syntaxin-3 with other SNARE partners, and controls the vesicular apical transport (38). Annexin XIIb has been also previously described to play a role in apical delivery through an association with rafts (40). Syntaxin-3, Munc18-2, and annexin XIIb were localized at the apical side of control HT-29 cells, but shifted to an intracellular localization in GalNAc-O-bn-treated HT-29 5M12 cells. Therefore, GalNAc-O-bn also affected proteins regulating the apical trafficking such as proteins of the apical SNARE fusion machinery and raft-associated proteins, showing finally a general block in the vesicular transport toward the apex. The effect affected specifically the apical route, because the basolateral t-SNARE syntaxin-4 kept its normal basolateral localization.

Proteins of the apical membrane have been proposed to be transported via apical carrier vesicles (4). Furthermore, such apical vesicles are also known to carry proteins involved in the regulation of apical trafficking (41). Analysis of carrier vesicles isolated from perforated HT-29 5M12 cells showed the presence of DPP-IV, syntaxin-3, and annexin XIIb in carrier vesicles of both control and GalNAc-O-bn-treated cells, indicating that GalNAc-O-bn did not prevent the trafficking pathway from the TGN to the apical carrier vesicles, but rather inhibited the transport from the carrier vesicles up to the apical plasma membrane. Thus, undersialylated DPP-IV is still packaged into vesicles containing apical directed SNAREs. The correct transport from TGN to carrier vesicles was in accordance with the occurrence of an association of DPP-IV with detergent-insoluble microdomains in GalNAc-O-bn-treated cells as in control cells. This is in agreement with the fact that rafts have been described to constitute a dynamic platform for budding of raft carrier vesicles, which would transport the apical cargo up to the apical plasma membrane (4). It is not surprising that detergent insolubility of DPP-IV in control and GalNAc-O-bn-treated HT-29 5M12 cells occurred after processing by post-translational glycosylation, because N- or O-glycans were reported to favor raft association and apical delivery of membrane proteins in MDCK and Caco-2 cells (12, 14). In the literature, human DPP-IV has been described as preferentially non-raft or raft-associated in MDCK or Caco-2 cells (14, 15), and our results using HT-29 5M12 cells suggest that DPP-IV is raft-associated along a transient period of its biosynthetic pathway, i.e. in the Golgi apparatus and carrier vesicles, and subsequently stabilized in non-raft domains. Despite detergent insolubility and recruitment in budding carrier vesicles, DPP-IV failed to be transported to the apical membrane in GalNAc-O-bn-treated cells and appeared to follow in turn a degradation process, showing that carrier vesicles could not be normally processed for apical delivery, i.e. for transport along the microtubule and/or actin networks or for docking/fusion with the apical plasma membrane.

In this regard, the fractionation of a detergent extract of a membrane fraction on sucrose gradient showed an impact of GalNAc-O-bn on rafts. In control HT-29 5M12 cells, DPP-IV, annexin XIIb, and an ubiquitous marker of raft domains, i.e. flotillin-1/Reggie-2 (46, 47) were found in the typical raft fraction and also in non-raft membrane fractions. In GalNAc-O-bn-treated HT-29 5M12 cells, (i) these raft-associated proteins shifted to a detergent-insoluble fraction of lower buoyant density, characterized by a different lipid composition in comparison to rafts of control cells (the total amount of glycolipids is decreased by 50% by GalNAc-O-bn treatment, and their composition is changed, the major feature being the complete disappearance of GM1 ganglioside) and (ii) DPP-IV appeared 10-fold enriched in the lipid-rich microdomains, whereas annexin XIIb appeared 10-fold depleted. The enrichment of DPP-IV in a detergent-insoluble fraction was in accordance with the pattern observed by biosynthetic labeling, showing that DPP-IV failed to reverse in non-raft membrane fractions in turn degraded. In contrast, annexin XIIb showed an inability to associate with the lipid microdomains of GalNAc-O-bn-treated cells, and such finding might be connected to the abnormal apical delivery, as annexin XIIb has been proposed to play a function in apical delivery either as an adaptor for the binding of apical carrier vesicles to the microtubule motors or as a factor involved in the docking and fusion machinery. Until now, very few data have been available concerning the mechanisms involved in the transport of apical carrier vesicles and in the docking/fusion process. The association of annexin XIIb with a protein of the kinesin superfamily (KIF), the microtubule minus-end-directed motor KIFC3, in Triton-insoluble membranes in MDCK cells was recently reported (48). However, annexin XIIb is not directly associated with KIFC3 but involves other unknown intermediate partners (48). Our data can be connected to those obtained with the unmyristoylated recombinant form of annexin XIIb, which inhibited the apical delivery without affecting the release of apical carriers (40, 49, 50). However, unmyristoylated annexin XIIb was not bound to the membrane but exclusively present in the cytosolic fraction (50). Beyond the requirement of the myristoyl moiety for mem-

---

2 D. Delacour, A. Pons, V. Gouyer, C. Richet, H. Drobecq, E. Leteurtre, G. Grard, J. P. Zanetta, and G. Huet, manuscript in preparation.
brane binding (50), our data suggest that in HT-29 5M12 cells, the association of membrane-bound annexin XIIIb with lipid microdomains involves its interaction with sialylated epitopes, as we showed that GalNAc\(\alpha\)-O-bn inhibits sialylation of glycoproteins and likely glycolipids by the two sialyltransferases ST3Gal I and ST3Gal IV (22, 23).

In conclusion, our data bring a new insight on the effect of GalNAc\(\alpha\)-O-bn in the apical trafficking in polarized HT-29 cells. The fact that undersialylated DPP-IV is incorporated into apical transport vesicles indicates that the sialylation is not essential as an apical targeting signal. The defect is in transport or docking/fusion of apical carrier vesicles rather than impaired packaging of undersialylated DPP-IV. In this context, our data show the role of glycosylation in the organization of raft microdomains and their functions in the transport of carrier vesicles to the apical membrane and/or in the docking/fusion machinery.

Acknowledgments—We thank Dr. A. Le Bivic, Dr. M. Kauppi, Dr. H. P. Hauri, and Dr. J. Gordon for their gift of antibodies. Confocal microscopy was carried out in IFB 114 (Biologie et Pathologies des Régulations cellulaires). MALDI-TOF mass spectrometry was carried out in CNRS UMR 8525 (Prof. C. Sergheraert). We thank Dr. T. Galli and Dr. J. L. Desseyn for critical comments on the manuscript. We thank Dr. H. P. Hauri, and Dr. J. Gordon for their gift of antibodies. Confocal microscopy was carried out in IFB 114 (Biologie et Pathologies des Régulations cellulaires). MALDI-TOF mass spectrometry was carried out in CNRS UMR 8525 (Prof. C. Sergheraert). We thank Dr. T. Galli and Dr. J. L. Desseyn for critical comments on the manuscript. We thank Dr. J. P. Zanetta for glycolipid analysis. We thank B. Hénon, M. J. Dejonghe, and G. Grard for technical assistance.

REFERENCES
1. Simons, K., and Van Meer, G. (1988) Biochemistry 27, 6197–6202
2. Simons, K., and Wandinger-Ness, A. (1990) Cell 22, 207–210
3. Brown, D. A., and Rose, J. K. (1992) Nature 357, 569–572
4. Scheiffele, P., Peranen, J., and Simons, K. (1995) Nature 378, 96–98
5. Mirre, C., Monlaureu, L., Garcia, M., Delgrossi, M. H., and Le Bivic, A. (1996) Am. J. Physiol. 271, 887–894
6. Yeaman, C., Le Gall, A. H., Baldwin, A. N., Monlaureu, L., Le Bivic, A., and Rodriguez-Boulan, E. (1997) J. Cell Biol. 139, 929–940
7. Monlaureu, L., Breuza, L., and Le Bivic, A. (1998) J. Biol. Chem. 273, 30263–30270
8. Gut, A., Kappeler, F., Hyka, N., Balda, M. S., Hauri, H. P., and Matter, K. (1998) EMBO J. 17, 1919–1929
9. Alfalah, M., Jacob, R., Preuss, U., Zimmer, K. P., Naim, H., and Naim, H. Y. (1999) Curr. Biol. 9, 593–596
10. Benting, J. H., Rietvelo, A. G., and Simons, K. (1999) J. Cell Biol. 146, 313–320
11. Jacob, R., Alfalah, M., Grunberg, J., Obendorf, M., and Naim, H. Y. (2000) J. Biol. Chem. 275, 6656–6652
12. Alfalah, M., Jacob, R., and Naim, H. Y. (2001) J. Biol. Chem. 277, 10683–10690
13. Urban, J., Parczyk, K., Leutz, A., Kayne, M., and Kander-Koch, C. (1987) J. Cell Biol. 105, 2735–2743
14. Kitagawa, Y., Sano, Y., Ueda, M., Higashin, K., Narita, H., Okano, M., Matsumoto, S., and Sasaki, R. (1994) Exp. Cell Res. 213, 449–457
15. Naim, H. Y., Jobery, G., Alfalah, M., and Jacob, R. (1999) J. Biol. Chem. 274, 17961–17967
16. Aït-Slimane, T., Lenoir, C., Sapin, C., Maurice, M., and Trugnan, G. (2000) Exp. Cell Res. 258, 184–194
17. Alfalah, M., Jacob, R., Preuss, U., Zimmer, K. P., Naim, H., and Naim, H. Y. (1999) Curr. Biol. 9, 593–596
18. Benting, J. H., Rietvelo, A. G., and Simons, K. (1999) J. Cell Biol. 146, 313–320
19. Jacob, R., Alfalah, M., Grunberg, J., Obendorf, M., and Naim, H. Y. (2000) J. Biol. Chem. 275, 6656–6652
20. Alfalah, M., Jacob, R., and Naim, H. Y. (2001) J. Biol. Chem. 277, 10683–10690
21. Urban, J., Parczyk, K., Leutz, A., Kayne, M., and Kander-Koch, C. (1987) J. Cell Biol. 105, 2735–2743
22. Kitagawa, Y., Sano, Y., Ueda, M., Higashin, K., Narita, H., Okano, M., Matsumoto, S., and Sasaki, R. (1994) Exp. Cell Res. 213, 449–457
23. Naim, H. Y., Jobery, G., Alfalah, M., and Jacob, R. (1999) J. Biol. Chem. 274, 17961–17967
24. Aït-Slimane, T., Lenoir, C., Sapin, C., Maurice, M., and Trugnan, G. (2000) Exp. Cell Res. 258, 184–194