GalNAc-α-O-benzyl Inhibits Sialylation of de Novo Synthesized Apical but Not Basolateral Sialoglycoproteins and Blocks Lysosomal Enzyme Processing in a Post-trans-Golgi Network Compartment*

Received for publication, January 24, 2000, and in revised form, April 3, 2000
Published, JBC Papers in Press, April 5, 2000, DOI 10.1074/jbc.M000510200

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Glycosylation plays an important role in glycoprotein traffic. Our previous work has shown that long term treatment of mucus-secreting HT-29 cells with GalNAc-α-O-benzyl reversibly inhibits sialylation and causes the accumulation of apical glycoproteins in cytoplasmic vesicles. We have analyzed at the biochemical level the effects of GalNAc-α-O-benzyl on glycoprotein processing. Both apical and basolateral membrane glycoproteins were sialylated, but GalNAc-α-O-benzyl selectively inhibited the sialylation of apical glycoproteins. In addition, lysosomal α-glucosidase, which is partially targeted to the apical membrane, was abnormally processed leading to the accumulation of an immature molecular species. Several findings support the conclusion that accumulation of this protein occurs in a post-trans-Golgi network (TGN) compartment: 1) it is partially sialylated; 2) it does not occur when glycoprotein exit from the TGN is blocked at 20 °C; 3) upon Triton X-114 partition, it distributes to the aqueous phase, a characteristic that is acquired in a post-TGN compartment; and 4) its appearance is inhibited when cells are cultured in the presence of NH₄Cl. The processing of cathepsin D was also found to be affected by GalNAc-α-O-benzyl treatment. In conclusion, GalNAc-α-O-benzyl selectively inhibits sialylation of apical glycoproteins and perturbs lysosomal enzyme processing; these effects occur in a post-TGN acidic compartment and are reminiscent of the alterations found in sialic acid storage diseases.

In the secretory pathway, glycoproteins are sorted and subsequently targeted to specific cellular domains (for review, see Ref. 1). Targeting signals for basolateral proteins have been extensively studied, and they correspond to specific peptide sequences, such as dihydrophobic amino acid motifs and tyrosine-dependent motifs, in the cytoplasmic domain (1–3). Apical targeting signals have been less extensively studied and appear to be more diverse. The role of the glycosylphosphatidylinositol linkage (1, 4–6) and the transmembrane domain of viral glycoproteins has been well established (7–9); recent evidence supports a role for N- and/or O-glycans in the extracellular domain in the targeting of apical, but not basolateral, glycoproteins (10–15). Neither the sugars nor the putative lectins involved in the selection of glycoproteins for apical targeting have been identified until now (Ref. 15; see discussion in Ref. 16).

Epithelial cell lines that form a polarized monolayer provide useful in vitro systems to examine the signals involved in targeting. A series of subpopulations derived from HT-29 colon cancer cells have been obtained that exhibit various types of differentiated features (17, 18). Among them, the populations selected in 10⁻⁶ and 10⁻⁵ M methotrexate display a mucus-secreting phenotype (17); the mucins produced by these cells contain mainly O-glycans with core 1 structure, in particular NeuAc2,3-Galβ1–3GalNAc (19). To examine the relevance of glycosylation in mucin biosynthesis and secretion in mucus-secreting HT-29 cells, we have previously used the sugar analogue GalNAc-α-O-benzyl (BG) (19–22). BG was initially reported to selectively inhibit O-glycosylation through its ability to compete with GalNAc-O-Ser/Thr for the β1–3galactosyltransferases involved in the biosynthesis of O-glycans (23). However, short exposure (24 h) of mucus-secreting HT-29 cells to 5 mM BG resulted in a 13-fold decrease in the levels of mucin-associated sialic acid and an increase of T antigen, suggesting that the major step inhibited by treatment with this sugar analogue was sialylation rather than the transfer of Gal to GalNAc-α-O-Ser/Thr (20). These effects were associated with the metabolism of GalNAc-α-O-benzyl to Galβ1–3GalNAc-α-O-benzyl, which is a potent competitor of the o2,3-sialyltransferase activity present in HT-29 cell extracts (20, 21). To examine in more detail the effects of BG on mucin biosynthesis and secretion, mucus-secreting and undifferentiated HT-29 cells were cultured for 20 days in the presence of 2 mM BG; this was associated with a 6-fold increase in cell volume, an accumulation of small cytoplasmic vesicles containing electron-lucid material, a marked decrease in the sialylation of cellular glycoproteins, and a dramatic alteration in the subcellular distribution of apical glycoproteins (22). These effects were fully reversible upon withdrawal of the drug. BG appeared to selectively affect apical glycoproteins, such as MUC1, dipepti-

* This work was supported in part by Grant SAP97-0085 from Comisión Interministerial de Ciencia y Tecnología, Grant SGR 00433 from Comissió Interdepartamental de Recerca i Tecnologia (Generalitat de Catalunya), and a grant from the Mizutani Foundation for Glycience. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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† The abbreviations used are: BG, GalNAc-α-O-benzyl; AAG, α-glucosidase; APN, aminopeptidase N; DPP-IV, dipeptidylpeptidase IV; gp, glycoprotein; M6, HT-29 cells selected in 1 μM methotrexate; MAL, Maackia amurensis lectin; PNA, peanut agglutinin; SASD, sialic acid storage disease; SI, sucrase-isomaltase; TGN, trans-Golgi network; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; endo H, endoglycosidase H; ST, sialyltransferase.
dipeptidylpeptide IV (DPP-IV), and carcionoemobryonic antigen. The basolateral glycoprotein gp120 and non-glycosylated apical proteins, such as villin and ZO-1, did not accumulate in cytoplasmic vesicles as determined using immunofluorescence microscopy (22). In addition, immunoprecipitation and lectin-blotting experiments showed that, in control cells, apical glycoproteins express NeuAcO2,3-Gal-R reactive with the sialic acid-specific Maackia amurensis lectin (MAL); by contrast, apical glycoproteins immunoprecipitated from BG-treated cells display a decreased sialylation, a loss of reactivity with MAL, and an increased reactivity with peanut agglutinin (PNA), a lectin that binds Gal-R (22). When BG is removed from the cultures, sialylation is recovered. We and others have proposed that the effects of BG may be due to the accumulation of BG-derived metabolites that inhibit α2,3 sialytransferases (20–23).

These results led to the analysis of the reactivity of sialic acid-reactive lectins with mucus-secreting HT-29 cells, and sialic acid appeared to be restricted to the apical membrane, as determined by confocal immunofluorescence. This finding, together with the effect of BG on apical but not basolateral glycoproteins, led us to propose a possible role for sialic acid in processes related to apical targeting (22). In this work we have aimed at analyzing in closer detail at the biochemical level the presence of sialic acid in apical and basolateral glycoproteins, as well as the effects of BG on the processing of apical, basolateral, and lysosomal glycoproteins in polarized mucus-secreting HT-29 cells. Several glycoproteins from each of these groups were selected in order to assess if similar changes take place in proteins having a common destination and to rule out that the observed effects reflect the particular behavior of a given protein. DPP-IV and aminopeptidase N (APN) are brush-border-associated enzymes expressed in the apical membrane of intestinal cells; integrins are heterodimeric glycoproteins destined to the basolateral membrane, whereas the gp9525 glycoprotein also localizes; acid α-glucosidase (AAG) and cathepsin D are lysosomal enzymes that undergo proteolytic processing to generate a mature isofrom.

In this work, we present biochemical evidence that 1) BG perturbs the intracellular processing of apical glycoproteins as well as lysosomal enzymes, but not of basolateral glycoproteins; 2) despite its selective effects on apical glycoproteins, both apical and basolateral glycoproteins are sialylated; and 3) altered processing of lysosomal enzymes occurs in a post-trans Golgi network (TGN) acidic compartment. The alterations found in HT-29 cells treated with BG are reminiscent of those present in cells from patients with sialic acid storage diseases (SASD).

MATERIALS AND METHODS

Reagents—DMEM and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. (Glasgow, United Kingdom (UK)). All chemicals used were of the highest chemical grade and were purchased, unless otherwise indicated, from Sigma.

Cells and Antibodies—HT-29 cells selected with 10−7 m methotrexate (here designated as HT-29 M6 cells) and Caco-2 colon cancer cells were obtained from Drs. Alain Zweibaum and Thécla Lesuffleur (INSERM U505, Paris, France) and were maintained with DMEM supplemented with 10% FBS, respectively, were obtained from Dr. L. J. Old (Ludwig Institute for Cancer Research New York Branch, Sloan-Kettering Institute, New York) and Dr. H. P. Hauri (Biozentrum, Basel, Switzerland); mAb 525 detecting a basolateral glycoprotein of 38–40 kDa was obtained from Dr. A. Le Bey (IBDM, Marseille, France) (26); mAb A9 detecting β4 integrin was obtained from Dr. T. Carey (University of Michigan, MI) (27); rabbit anti-AAG polyclonal antibodies were obtained from Dr. A. Reuser (Erasmus University, Rotterdam, The Netherlands) (28); rabbit polyclonal anti-cathepsin D antibodies were purchased from Dako (Glostrup, Denmark).

Glycosidase Treatment—For digestion with endoglycosidase H (endo H), immunoprecipitates were boiled for 5 min in 0.1 M phosphate buffer, pH 6.1, containing 1% SDS and 50 mM EDTA, diluted 10-fold in 0.1 M phosphate buffer, pH 6.1 containing protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 100 μg/ml benzamidine HCl, 100 μg/ml soybean trypsin inhibitor), and 1% 2-mercaptoethanol, and divided in two aliquots, to one of which 2 milliunits of endo H (Genzyme, Boston, MA) was added. The reactions were incubated overnight at 37 °C, and the digestion was stopped with sample buffer. For neuraminidase treatment, immunoprecipitates were resuspended in 20 mM acetate buffer pH 5, containing 5 mM CaCl2 and 20 milliunits of neuraminidase from Arthrobacter ureafaciens (Roche Molecular Biochemicals), incubated at 37 °C for 3 h, washed with 20 m Tris/ HCl, and resuspended in sample buffer. Triton X-114 Fractionation—Triton X-114 phase separation was performed as described elsewhere (29). Briefly, cells were solubilized in 1% Triton X-114, 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 10,000 × g for 30 min at 4 °C, and supernatants were warmed at 30 °C; 5 min later, detergent and water phases were separated by centrifugation at 2000 rpm for 1 min at room temperature. This procedure was repeated three times.

RESULTS

Processing of Apical and Basolateral Glycoproteins in Benzyl GalNAc-treated HT-29 M6 Cells—We have previously shown...
that long term treatment of HT-29 M6 cells with 2 mM BG induces a dramatic accumulation of cytoplasmic vesicles containing apical glycoproteins. Among them are DPP-IV, carcinoembryonic antigen, and MUC1. By contrast, non-glycosylated apical proteins, such as villin or ZO-1, and the basolateral glycoprotein gp120 display a normal subcellular distribution (22). These changes are already detectable after culture of cells for 7 days in the presence of the drug (data not shown); this time point was selected for labeling because uptake of radiolabeled amino acids is higher under these conditions than at day 21 of culture. To examine the effects of BG at the biochemical level, HT-29 M6 cells were cultured with the drug starting on day 3 of culture. BG induces changes in the electrophoretic mobility of apical, but not basolateral, glycoproteins. DPP-IV from control and BG-treated cells (APNbg) showed a slightly higher mobility than APN from control untreated cells (APNc). Their estimated molecular masses were 123 and 128 kDa, respectively. Unlike these two apical glycoproteins, the electrophoretic mobility of three basolateral glycoproteins, gp525, β4 integrin, and α6 integrin was undistinguishable in control and BG-treated cells. BG did not affect the half-lives of these glycoproteins (Fig. 1). The same results were obtained when SDS-PAGE gels with variable composition (6–12%) were used in order to increase the resolution of the electrophoretic analysis. These results confirm and extend our previous findings (22).

BG has been shown to decrease the sialylation of glycoproteins in HT-29 cells, likely as a result of the ability of BG-derived metabolites to inhibit sialytransferases such as ST3Gal I, the main sialyltransferase expressed in these cells (21, 22). To determine if this effect might be responsible for the observed change in electrophoretic mobility of apical glycoproteins, we analyzed the sialylation of apical and basolateral membrane glycoproteins were performed. After 1 h of pulse with [35S]Met/Cys and in the absence of a chase period, immunoprecipitates obtained using anti-DPP-IV antibodies showed two components: the higher mobility one, designated DPP-IVc, and representing the immature protein, showed a similar apparent molecular mass (93 kDa) in control and BG-treated cells. By contrast, the component with a lower mobility, corresponding to mature DPP-IV, was detected and DPP-IVbg always showed a greater mobility than DPP-IVc from control cultures (Fig. 1). The half-life of DPP-IV was similar in control and BG-treated cells (Fig. 1). Using anti-APN antibodies, the immunoprecipitated molecules showed a similar migration 1 h after chase; by contrast, after 3 h of chase and at all later time points, APN from BG-treated cells (APNbg) showed a slightly higher mobility than APN from control cells (APNc). Their estimated molecular masses were 123 and 128 kDa, respectively. Unlike these two apical glycoproteins, the electrophoretic mobility of three basolateral glycoproteins, gp525, β4 integrin, and α6 integrin was undistinguishable in control and BG-treated cells. BG did not affect the half-lives of these glycoproteins (Fig. 1). The same results were obtained when SDS-PAGE gels with variable composition (6–12%) were used in order to increase the resolution of the electrophoretic analysis. These results confirm and extend our previous findings (22).
BG-treated cells increased after neuraminidase treatment and desialylated DPP-IV from both cultures showed the same mobility, indicating that BG reduces, but does not abolish, sialylation of DPP-IV and suggesting that it does not affect other glycosylation steps. The electrophoretic mobility of the basolateral glycoproteins gp525, β4 integrin, and αv integrin increased upon neuraminidase treatment, indicating that they are sialylated. The observed shift and the mobility of the neuraminidase-treated molecules were undistinguishable in control and BG-treated cells. These findings indicate that basolateral glycoproteins are also desialylated and that differential sialylation of apical and basolateral glycoproteins does not account for the selective effects of BG on apical glycoproteins.

**BG Affects the Processing of Lysosomal Enzymes in HT-29 M6 Cells**—To determine the extent of the effects of BG on glycoprotein traffic, we examined its ability to affect the endosomal-lysosomal pathway. AAG was chosen because: 1) a fraction of its precursor form has been shown to be targeted selectively to the apical membrane in Caco-2 colon cancer cells (31); 2) it allows the topological dissection of the effects of BG as, after glycosylation in the Golgi complex, it is processed by stepwise proteolytic cleavage in a post-trans Golgi network compartment (32–34); and 3) we have previously characterized its biosynthesis in detail in HT-29 M6 cells (34). First, we examined whether AAG also has a selective apical membrane distribution in HT-29 M6 cells using immunocytochemical techniques. Confocal microscopy did not yield conclusive results due to low levels of fluorescence. Using immunoelectron microscopy, AAG was detected in lysosomes in HT-29 M6 cells (data not shown). Fig. 3A illustrates the detection of AAG in the apical membrane, in close association with microvilli (Fig. 3A, arrows) and its absence from the basolateral membrane (Fig. 3A, arrowheads). Similarly, AAG was detected in the apical membrane of Caco-2 cells and normal colonic epithelial cells. The precursor form of AAG was mainly secreted through the apical route. HT-29 M6 cells were cultured in Transwells and 21 days after seeding; when the monolayer was impermeable, cells were labeled with [35S]Met/Cys for 1 h. After 36 h of chase, AAG was immunoprecipitated from apical and basolateral medium; as shown in Fig. 3 (B and C), >80% of AAG was secreted to the apical compartment.

AAG is synthesized as a polypeptide of 97 kDa that is glycosylated and phosphorylated, yielding a precursor form of 110 kDa that is subsequently proteolytically processed to an intermediate form of 95–100 kDa, and to mature forms of 70–76 kDa (33). In HT-29 M6 cells cultured in the absence of BG, only the precursor form is detected after a 1-h pulse and 3 h of chase. After 24 h of chase, the three forms of AAG are detected in control cells and the expected processing is observed thereafter (34) (Fig. 4). By contrast, in the presence of BG, all de novo synthesized protein is partially processed to a 100-kDa species with a mobility in between that of the precursor and intermediate forms, designated AAG_{bg}. N-Glycosidase F digestion of AAG immunoprecipitated from control and BG-treated cells showed a lower apparent molecular mass for AAG_{bg}, indicating that this form has undergone partial proteolytic processing (data not shown). In BG-treated cells, the normally processed forms of AAG are not detectable at any time of chase and labeled AAG is undetectable after 72 h of chase, suggesting that it is degraded (Fig. 4A). As with the precursor form, the electrophoretic mobility of the AAG_{bg} molecules immunoprecipitated after 24 h of chase increased slightly upon neuraminidase treatment (Fig. 4B), indicating that BG does not abolish the sialylation of AAG. Importantly, BG completely blocked the secretion of AAG in cells cultured on plastic (Fig. 4C).

To determine if the observed effects of BG on the processing of AAG are extensive to other lysosomal enzymes, cathepsin D was analyzed. This enzyme is synthesized as a 53-kDa precursor that is proteolytically processed to a single-chain form of 47 kDa and subsequently cleaved to two subunits, the large one having an apparent molecular mass of 28 kDa (35). In control cells, the mature 28-kDa component was already detectable after 1 h of chase and it was present until 48 h of chase (Fig. 5). By contrast, in BG-treated cells, the 28-kDa mature form was undetectable until the 3-h chase time point and a 32-kDa form was present instead. This molecular species was observed, together with the 28-kDa mature form, all throughout the chase period, indicating incomplete maturation of the enzyme (Fig. 5).

The Blockade of Processing of Acid α-Glucosidase Occurs in a Post-TGN Compartment—To determine where the blockade in glycoprotein processing takes place in HT-29 M6 cells treated with BG, we took advantage of the sequential maturation of AAG. We have previously shown DPP-IV acquires endo H
resistance in HT-29 cells treated with BG, indicating that at least it has undergone processing steps that typically occur in the cis-medial Golgi (22). The work shown here demonstrates that both DPP-IV and AAG are partially sialylated (Figs. 2 and 4B), suggesting that these glycoproteins have reached the medial/trans-Golgi cisternae where sialyltransferases are thought to be present (36–38). To determine more precisely the site at which the blockade of AAG processing occurs in BG-treated cells, we took advantage of the TGN accumulation of en route glycoproteins induced by culture at 20 °C (39–41). We have previously shown that, in untreated cells, culture at 20 °C results in the accumulation of the 110-kDa precursor form of AAG and, upon transfer to 37 °C, normal processing is resumed, indicating that proteolytic cleavage requires exit from the TGN (34). In BG-treated cells, the 20 °C block also results in the accumulation of the 110-kDa precursor form. In these cells, release from the 20 °C block leads to the proteolytic processing to the abnormally processed \( \text{AAG}_{bg} \) species described above (Fig. 6A). As in cells continually cultured at 37 °C, processing after release from the 20 °C is abnormal; these results indicate that the blockade observed in BG-treated cells takes place in a post-TGN compartment.

AAG is transported to the lysosomes as a transmembrane protein that is anchored to the membrane by its signal peptide (33). Removal of the signal peptide, rendering the enzyme water-soluble, also occurs in a post-TGN compartment (33, 34). Therefore, we examined the Triton X-114 partition properties of the partially processed form that accumulates in BG-treated cells; this molecular species is completely water-soluble (Fig. 6B), indicating that it has already undergone proteolysis of the transmembrane domain. Altogether, these findings support the contention that the main effects of BG on AAG processing take place in a post-TGN compartment.

To determine if AAG molecules reach acidic compartments in the presence of BG, HT-29 M6 cells were pulse-labeled and chased in presence of \( \text{NH}_4\text{Cl} \). In control cells treated with 10 \( \text{mM} \text{NH}_4\text{Cl} \), the precursor and intermediate forms were present 24 h of chase, but the mature form was undetectable, indicating that processing of the intermediate form takes place in acidic compartments. In cells exposed to BG for 7 days and treated with \( \text{NH}_4\text{Cl} \), the only detectable molecular species had a mobility that was in-between that of the precursor form and the \( \text{AAG}_{bg} \) form present in the absence of \( \text{NH}_4\text{Cl} \) (Fig. 7). These results indicate that, in BG-treated cells, AAG reaches an acidic compartment.

**DISCUSSION**

In this study, we show that prolonged exposure of HT-29 M6 cells to the sugar analogue BG leads to altered intracellular processing of apical and lysosomal glycoproteins, whereas no effects are observed on three basolateral glycoproteins. These findings confirm and extend prior immunocytochemical data showing that apical glycoproteins accumulate intracellularly upon prolonged treatment with this sugar analogue.

The altered processing of apical glycoproteins is associated with a marked decrease in sialylation causing changes in electrophoretic behavior of DPP-IV and APN. In the case of DPP-IV, several pieces of evidences support this contention. 1) The mobility of DPP-IV after neuraminidase digestion is undistinguishable in control and BG-treated cultures, suggesting that no other major changes in glycosylation take place; 2) DPP-IV from control cells reacts with MAL but not with PNA, whereas
DPP-IV from BG-treated cells reacts with PNA and does not react with MAL, supporting the reduced sialylation of the protein in treated cells (22); and 3) the activity of the enzyme is preserved in BG-treated cells, indicating the lack of major conformational changes (22). Nevertheless, these observations do not preclude minor changes in glycosylation in addition to the reduced sialylation. The above mentioned hypothesis is also supported by the known fate of BG in HT-29 cells; it is metabolized to Gal-GalNAc-a-O-benzyl as well as to NeuAc2,3Gal-GalNAc-a-O-benzyl (20, 21). The former is a competitive inhibitor of ST3Gal I, the main sialyltransferase expressed in HT-29 cells, and it may also act as an inhibitor of other sialyltransferases such as ST3Gal IV (see discussion in Ref. 22). It has recently been shown that DPP-IV, sucrase-isomaltase (SI), and APN are both N- and O-glycosylated, and it has been proposed that when O-glycosylation is perturbed in Caco-2 cells, the apical targeting of DPP-IV and SI (but not that of APN) is affected (42). Furthermore, treatment of Caco-2 cells with BG is associated with an inhibition of O-glycosylation of SI and a loss of its selective targeting to the apical membrane (43). Nevertheless, the overall effects of prolonged exposure to BG on HT-29 M6 and Caco-2 cells are very different, and the described biochemical effects should be considered in light of these differences (see below).

A remarkable finding in this work was that basolateral glycoproteins were not affected by BG despite the fact that they are sialylated. In our previous study we showed that a2,3NeuAc is mainly distributed in the apical membrane of polarized HT-29 M6 cells, as determined by confocal microscopy of the reactivity with MAL, and that there was no colabeling of MAL and antibodies recognizing β1 integrin (22). These findings do not appear unique to HT-29 M6 cells, nor artifactual; using confocal microscopy, a2,3NeuAc was detected predominantly in the apical membrane of HRT18 cells, the apical targeting of DPP-IV and SI (but not that of APN) is affected (42). Furthermore, treatment of Caco-2 cells with BG is associated with an inhibition of O-glycosylation of SI and a loss of its selective targeting to the apical membrane (43). Nevertheless, the overall effects of prolonged exposure to BG on HT-29 M6 and Caco-2 cells are very different, and the described biochemical effects should be considered in light of these differences (see below).

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These findings are also substantiated by a large amount of work showing that sialic acid is mainly found in the apical membrane of epithelia in tissues (reviewed in Ref. 44). Here, we show, using neuraminidase digestion of metabolically labeled immunoprecipitates, that three basolateral proteins whose processing is not affected by BG, gp525, β₁ integrin, and α₂ integrin, are indeed sialylated. The apparent contradiction between the immunocytochemical and biochemical findings may be accounted for in several ways. 1) De novo synthesized apical and basolateral glycoproteins may be sialylated, whereas, in the steady state, the latter would be predominantly desialylated, possibly through the action of sialidases; 2) apical glycoproteins may become hypersialylated through recycling after reaching the apical membrane; a precedent for this exists with MUC1, an apical mucin-type glycoprotein whose sialylation is inhibited by BG (22) and which acquires sialic acid as it is recycled from the plasma membrane (45); 3) basolateral glycoproteins may be sialylated by different enzymes, or in different compartments, than apical glycoproteins. This hypothesis might also explain the differential effects of BG on apical and basolateral glycoproteins, as the latter might not be exposed to the metabolites that are involved in the inhibition of sialylation of apical glycoproteins. In relationship with these possibilities, we have found that the apical membrane of MDCK strain II cells is strongly reactive with MAL and unreactive with S. nigra agglutinin, whereas the opposite is true for the basolateral membrane, suggesting that either apical and basolateral glycoproteins are selectively recognized by ST or that the enzymes reside in different compartments. These hypotheses are currently being examined in our laboratory.

Besides altering the intracellular processing of apical glycoproteins, BG affected the processing of lysosomal enzymes. A small fraction of AAG is normally targeted to the apical membrane but altered apical targeting cannot account for the observed effects as defective maturation affected all AAG synthesized. Furthermore, we have shown that in untreated HT-29 M6 cells, 30% of the synthesized molecules remain in the precursor form after 24 h of chase, whereas in the presence of BG all AAG molecules are processed to the AAGbg abnormal species described here, suggesting that proteolysis is more efficient. The precursor form has previously been shown to be soluble in Triton X-100 (33), suggesting that its absence from the immunoprecipitates in BG-treated cells is not due to its insolubility. AAGbg, the molecular species present in BG-treated cells, is sialylated and distributes to the aqueous phase upon Triton X-114 extraction, indicating that it has exited the TGN. The precise proteolytic steps leading to the intermediate and mature forms of AAG have been studied by Wisselaar et al. (33); based on this evidence and on the presence of multiple additional bands in various cell types (34), it is likely that multiple proteases (and perhaps several distinct compartments) are involved in this process. The lack of reagents to distinguish these molecular species hampers their precise biochemical characterization and the identification of the subcellular compartments in which they reside. The proteolytic processing of the intermediate form to the mature form depends on the action of thiol proteases (33, 34); Wisselaar et al. have shown that in their presence, a 100-kDa intermediate is apparent, the size of which is similar to that of AAGbg. It has been shown that thiol proteases, including cathepsins, are involved in the processing of other lysosomal enzymes (35, 46), and their altered maturation might, in turn, contribute to the accumulation of AAGbg described here. Although there is no evidence that AAG is O-glycosylated (33), it cannot be formally excluded that altered sialylation of both N- and O-glycans may be involved in its abnormal processing in HT-29 M6 cells.

The major questions raised by the findings described here are how this sugar analogue affects processing of apical and lysosomal glycoproteins and what is the nature of the intracellular vesicles that appear in BG-treated HT-29 M6 cells. Regarding the former, the BG-derived metabolites are likely to account for the effects on sialylation and targeting. N-Glycosylation has been shown to play a role in apical targeting of secreted, GPI-anchored, and transmembrane forms of rat growth hormone (10, 15). Recent work with MDCK cells transfected with the neurotrophin receptor has provided evidence that the O-glycosylation-rich stalk of this protein is involved in apical targeting (13, 14) and similar data have been published regarding SI in Caco-2 cells treated with BG (42) and in MDCK cells transfected with a CDNA encoding a pro-SI lacking the Ser/Thr-rich stalk domain (43). A role for intracellular lectins in these processes has been proposed, but there are no firm candidates yet (11, 15, 16, 47, 48). BG affects both apical and lysosomal glycoproteins. It seems unlikely that this might be due to the codistribution of both types of proteins in the same vesicles coming out of the TGN. Rather, we favor the hypothesis that the contents of independent vesicles targeted to the apical membrane and the lysosomes, respectively, meet at a later step, possibly in the cytoplasmic vesicles that accumulate in BG-treated HT-29 M6 cells (see below and Fig. 8).

It is remarkable that an inhibition of sialylation and apical targeting occurs both in HT-29 and in Caco-2 cells (19, 20, 42) because they display very distinct patterns of sialylation; Caco-2 cells contain mainly ST6 Gal I, and its glycoproteins are mainly α₂,6-sialylated (49, 50), whereas HT-29 cells contain mainly ST3 Gal I and its glycoproteins are mainly α₂,3-sialylated (22). Furthermore, prolonged exposure of Caco-2 cells to BG does not result in major changes of cell morphology at the light microscopy or ultrastructural levels nor in the accumulation of apical glycoproteins in cytoplasmic vesicles (Ref. 22 and data not shown). These observations indicate that inhibition of sialylation of glycoproteins cannot explain by itself the intracellular accumulation of abnormally glycosylated apical proteins. Differences in the structure and/or levels of BG metabolites in HT-29 and in Caco-2 cells may account for the accumulation of glycoproteins in vesicles.

Regarding the nature of such vesicles, the data reported here together with the endo H resistance and low sialylation of the accumulated apical proteins support the notion that they correspond to a post-TGN compartment. This proposal is compatible with the idea that such vesicles may contain BG-derived metabolites as a fraction thereof is sialylated and would, therefore, be synthesized in the late Golgi compartments. The accumulation of high concentrations of metabolites, including Gal-GalNAc-α-O-benzyl and NeuAco2,3Gal-GalNAc-α-O-benzyl (21), together with their osmotic activity due to their low liposolubility, would lead to a situation reminiscent of the accumulation of sialic acid in endocytic compartments in SASD. Cultured fibroblasts from patients with SASD contain small lysosomes, accumulate sialic acid, and display an abnormal processing of cathepsin B (51, 52). This phenotype can be mimicked by treatment of normal fibroblasts with the non-degradable disaccharide sucrrose, which accumulates within large vesicles (“sucrosomes”) resulting from the osmotic influx of water (53, 54). It is not clear whether such structures, which morphologically resemble the cytoplasmic vesicles of BG-treated HT-29 M6 cells, represent swollen lysosomes or vesicles from the late endosomal compartments. The accumulation of sucrose interferes with the formation of a dense lysosomal matrix (55). When normal fibroblasts are cultured in the presence of N-acetylmannosamine, the intracellular levels of sialic acid increase, but there is no effect on lysosomal enzyme processing.
Effects of BG on Glycoprotein Processing in HT-29 Cells

Intracellular pathways followed by apical and basolateral glycoproteins in untreated control cells are shown in panel A; the pathway followed by lysosomal AAG in control cells is shown in panel B. The asterisk in panel A indicates the recycling of glycoproteins from the apical membrane. Upon chronic treatment with BG, processing of apical and lysosomal glycoproteins is affected, whereas processing of basolateral glycoproteins is not. Panels C and D depict two hypothetical models that are not mutually exclusive and can account for the effects of BG on apical glycoproteins. In panel C, their cytoplasmic accumulation results from a blockage in targeting of Golgi-derived apical vesicles to the apical membrane and a re-routing to BG-induced cytoplasmic vesicles. In panel D, Golgi-to-apical membrane targeting is unaffected; apical glycoproteins undergo endocytosis but cannot recycle back to the apical membrane and accumulate in the cytoplasmic vesicles. In both models, the BG-induced cytoplasmic vesicles would correspond to an aberrant endosomal compartment (dotted lines). Panel E shows a model accounting for the effects of BG on lysosomal AAG; BG blocks apical membrane targeting and secretion as well as complete maturation to the 75-kDa form. The 100-kDa anomalous form of AAG (AAG bg) that accumulates in BG-treated cells is formed in an acidic, endosomal, compartment that may be aberrant in nature (dotted lines). It remains to be determined whether apical and lysosomal glycoproteins accumulate in the same vesicles or not.

Fig. 8. Model accounting for the effects of BG on glycoprotein processing in HT-29 M6 cells. Intracellular pathways followed by apical and basolateral glycoproteins in untreated control cells are shown in panel A; the pathway followed by lysosomal AAG in control cells is shown in panel B. The asterisk in panel A indicates the recycling of glycoproteins from the apical membrane. Upon chronic treatment with BG, processing of apical and lysosomal glycoproteins is affected, whereas processing of basolateral glycoproteins is not. Panels C and D depict two hypothetical models that are not mutually exclusive and can account for the effects of BG on apical glycoproteins. In panel C, their cytoplasmic accumulation results from a blockage in targeting of Golgi-derived apical vesicles to the apical membrane and a re-routing to BG-induced cytoplasmic vesicles. In panel D, Golgi-to-apical membrane targeting is unaffected; apical glycoproteins undergo endocytosis but cannot recycle back to the apical membrane and accumulate in the cytoplasmic vesicles. In both models, the BG-induced cytoplasmic vesicles would correspond to an aberrant endosomal compartment (dotted lines). Panel E shows a model accounting for the effects of BG on lysosomal AAG; BG blocks apical membrane targeting and secretion as well as complete maturation to the 75-kDa form. The 100-kDa anomalous form of AAG (AAG bg) that accumulates in BG-treated cells is formed in an acidic, endosomal, compartment that may be aberrant in nature (dotted lines). It remains to be determined whether apical and lysosomal glycoproteins accumulate in the same vesicles or not.

Acknowledgments—We thank the investigators mentioned in the text for providing reagents; P. Delannoy, G. Huet, A. Le Bivic, L. Mach, G. Trugnan, and A. Zweibaum for valuable discussions; G. Egea for performing the immunoelectron microscopy analysis and for a critical review of the manuscript; and A. Mallabiabarrena and X. Mayol for valuable discussions and critical review of the manuscript.

Note Added in Proof—A detailed description of BG-derived metabolites produced in HT-29 cells is reported by Zanetta, J. P., Gouver, V., Maes, E., Pons, A., Hemon, B., Zweibaum, A., Delannoy, A., and Huet, G. (2000) Glycobiology 10, in press.

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