Asynchronous Transport to the Cell Surface of Intestinal Brush Border Hydrolases Is Not Due to Differential Trimming of N-Linked Oligosaccharides*

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Karl Matter, William McDowell, Ralph T. Schwartz§, and Hans-Peter Hauri§

From the Department of Pharmacology, Biozentrum of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland, the Institute für Virologie der Justus-Liebig-Universität Giessen D-6500 Giessen, Federal Republic of Germany, and the Laboratoire de Chimie Biologique, Université des Sciences et Techniques de Lille, Flandres-Artois, F-59555 Villeneuve d’Ascq Cedex, France

Intestinal brush border enzyme glycoproteins are transported to the microvillar membrane at different rates in the differentiated intestinal cell line Caco-2. This asynchronism is due to at least two rate-limiting events, a pre- and an intra-Golgi step (Stieger B., Matter, K., Baur, B., Bucher, K., Höchli, M., and Hauri, H. P. (1988) J. Cell Biol. 106, 1853–1861). A possible cause for the asynchronous protein transport might be differential trimming of N-linked oligosaccharide side chains. The effects of two trimming inhibitors on the intracellular transport of sucrase-isomaltase, a slowly migrating hydrolase, and dipeptidylpeptidase IV, a rapidly migrating hydrolase, are described. 1-Deoxynojirimycin, an inhibitor of Golgi α-mannosidase I, had no influence on the rate of appearance of these hydrolases in the brush border membrane as assessed by subcellular fractionation. In the presence of N-methyl-1-deoxynojirimycin, an inhibitor of glucosidase I, 30–40% of the newly synthesized molecules appeared at the cell surface, and half-time for appearance of this pool was identical to that found in control cells. The reduced maximal transport to the cell surface observed with N-methyl-1-deoxynojirimycin may suggest that proper glycosylation is necessary for an efficient transport from the Golgi apparatus to the microvillar membrane. Inhibition of glucosidase I does not prevent the acquisition of endoglycosidase H resistance. Furthermore, evidence is presented that the processing in the presence of N-methyl-1-deoxynojirimycin leads to glucosylated endoglycosidase H-resistant glycoproteins.

The enterocyte of the small intestinal epithelium is a highly polarized cell in regard to function, morphology, and biochemical composition of its surface membrane domains. The major constituents of the apical, microvillar membrane are digestive hydrolases including peptidases and disaccharidases, which are large integral membrane glycoproteins (1, 2). Cell surface glycoproteins are synthesized on ribosomes attached to the endoplasmic reticulum membrane and are in most cases cotranslationally inserted into the lipid bilayer (3, 4). During translocation through the membrane of the endoplasmic reticulum oligosaccharide side chains of the Glc3Man9GlcNAc2 structure are transferred to specific asparagine residues and immediately trimmed by the sequential action of two glucosidases and possibly one α-mannosidase (5). The partially trimmed glycoproteins are subsequently transported to and through the Golgi apparatus (6) where further glycan processing occurs by sequential action of trimming enzymes and several glycosyltransferases (5). In some instances O-linked sugars are attached as well (5). After completion of the biosynthesis in the Golgi apparatus, the proteins are transported to their spatially correct plasma membrane domains by a mechanism which is at present only poorly understood. The intracellular transport of newly synthesized proteins was found to be asynchronous in many instances. Most of these studies have suggested that the exit from the endoplasmic reticulum is the rate-limiting step for efficient transport (7–11).

Previous work has shown that the human colonic adenocarcinoma cell line Caco-2 (12) is a valuable model for the study of biosynthesis of brush border hydrolases (13). Recently, we have reported that at least two events, a pre- and an intra-Golgi step, are the underlying cause for the different transport rates of the brush border enzymes sucrase-isomaltase (SI) and dipeptidylpeptidase IV (DPPIV) (14). This raises the interesting possibility that differential carbohydrate trimming may be responsible for the asynchronous transport of the two membrane glycoproteins.

In this paper, studies on the effect of N-methyl-1-deoxynojirimycin (MDNM) and 1-deoxynojirimycin (DMN) on the intracellular transport of SI and DPPIV in Caco-2 cells are presented. MDNM inhibits glucosidase I, the first enzyme that processes N-linked oligosaccharides after their attachment to proteins (15), and DMN is an inhibitor of Golgi α-mannosidase I the first trimming enzyme in the Golgi apparatus (16). It is shown that DMN has no influence on the rates of transport. In the presence of MDNM, 30–40% of the molecules appeared at the cell surface, and half-time for appearance of this pool was identical to that found in control

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¶ To whom correspondence should be addressed.

The abbreviations used are: SI, sucrase-isomaltase; DPPIV, dipeptidylpeptidase IV; DPPIVc, complex-glycosylated form of DPPIV; DPPIVb, high mannose form of DPPIV; SIC, complex-glycosylated form of SI; SIIh, high mannose form of SI; MDNM, N-methyl-1-deoxynojirimycin; DMN, 1-deoxynojirimycin; endo H, endo-β-N-acetylglucosaminidase II; endo F, endo-β-N-acetylgalactosaminidase F; O-glycanase, endo-α-N-acetylgalactosaminidase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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cells. It is concluded that the asynchronous intracellular transport of digestive hydrolases is not due to differential trimming of N-linked oligosaccharides, but proper glycosylation might be essential for efficient post-Golgi transport.

**EXPERIMENTAL PROCEDURES**

**Materials**

1.-[35S]Methionine (1125 Ci/mmol), EN'HANCE and Protosol tissue, and gel solubilizer were purchased from Du Pont-New England Nuclear. Endoglycosidase H (endo H), endoglycosidase F (endo F) and DMN were obtained from Boehringer Mannheim and O-glycanase from Genzyme Corp. (Boston, MA). α-Mannidase (from jack bean) and α-glucosidase (from rice, type V) were purchased from Sigma. Dulbecco's modified Eagle's medium, penicillin/streptomycin, and low glucose Iscove's medium were purchased from KCl Biologicals. Ponceau, minimal essential medium selectamine kit, and nonessential amino acids were obtained from GibCO. Protein A-Sepharose, CNBr-activated Sepharose, and concanavalin A-Sepharose were purchased from Pharmacia (Uppsala, Sweden). N-Methylhydroxyargininomycin was prepared by methylation of 1-deoxynojirimycin (kindly provided by Dr. E. Truscheit, Bayer AG, Wuppertal, Federal Republic of Germany (R.G.). All other chemicals were obtained from commercial sources and were of the highest purity available.

**Cell Culture and Labeling with [35S]Methionine and [2-3H]Mannose**

Caco-2 cells were kindly obtained from Dr. A. Zweibaum, Paris, and were grown as described (13, 14) labeling with [35S]methionine was carried out with cells grown on filters 5–15 days after confluence (13). Due to the low amounts of trimming inhibitors that were at our disposal the filter chambers were disassembled before labeling. This allowed cell culturing in a minimal volume of medium. In all pulse-chase experiments a labeling time of 15 min was used. For digestion experiments the cells were continuously labeled for the indicated time. In order to label cells with [2-3H]mannose, they were preincubated for 15 min in low glucose Iscove's medium and then labeled with 250 μCi [2-3H]mannose/filter for 5 h.

**Application of Trimming Inhibitors**

MDNM and DMN were used at concentrations of 2 mM. The inhibitors were diluted from 1 to 4 mM stock solutions which were prepared in 1% SDS. After cooling down on ice, the SDS was diluted with glucosidase-buffer (10 mM Na2HPO4, pH 6.8, containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml antipain, and 17.5 μg/ml benzanid). To two of the samples, 0.01 mM of MDNM and DMN were added and all samples were incubated at 37°C. After 1 h 4 milliunits of O-glycanase were added to one of the two neuraminidase-treated samples and to one of the other two samples. All four samples were further incubated at 37°C for 22 h. The reaction was stopped by adding 35 μl of three-times concentrated Laemmli sample buffer followed by boiling the samples for 3 min at 100°C.

**Endo-α- and Endo-β-N-Acetylgalactosaminidase**—Washed immunoprecipitates were divided into four samples. Each of these samples was resuspended in 50 μl of a 20 mM sodium-citrate, 20 mM Tris-malate buffer, pH 6.0, containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml antipain, and 17.5 μg/ml benzanid. To two of the samples, 0.01 mM of MDNM and DMN were added and all samples were incubated at 37°C. After 1 h 4 milliunits of O-glycanase were added to one of the two neuraminidase-treated samples and to one of the other two samples. All four samples were further incubated at 37°C for 22 h. The reaction was stopped by adding 35 μl of three-times concentrated Laemmli sample buffer followed by boiling the samples for 3 min at 100°C.

**endoglycosidase Treatments**

Endo-β-N-Acetylgalactosaminidase H—Washed immunoprecipitates were boiled in 50 μl of 0.1 M citric acid-NaOH, pH 5.5, for 3 min in the presence of a mixture of the following protease inhibitors: phenylmethylsulfonyl fluoride (1 mM final concentration), antipain (1 μg/ml), pepstatin (1 μg/ml), and benzamid (17.5 μg/ml). 2 μl (74 μg/ml) of endo H together with 25 μl of protease inhibitor mixture were added, and the samples were incubated for 22 h at 37°C. The reaction was stopped by adding 35 μl of three-times concentrated Laemmli sample buffer followed by boiling for 3 min at 100°C. The samples were then loaded onto a SDS-polyacrylamide gel. Controls were incubated at 37°C without endo H.

**endo-α- and Endo-β-N-Acetylgalactosaminidase**—Washed immunoprecipitates were divided into four samples. Each of these samples was resuspended in 50 μl of a 20 mM sodium-citrate, 20 mM Tris-malate buffer, pH 6.0, containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml antipain, and 17.5 μg/ml benzanid. To two of the samples, 0.01 mM of MDNM and DMN were added and all samples were incubated at 37°C. After 1 h 4 milliunits of O-glycanase were added to one of the two neuraminidase-treated samples and to one of the other two samples. All four samples were further incubated at 37°C for 22 h. The reaction was stopped by adding 35 μl of three-times concentrated Laemmli sample buffer followed by boiling the samples for 3 min at 100°C.

**Oligosaccharide Analysis**

Caco-2 cells were labeled with [2-3H]mannose and lyophilized. The samples were taken up in Pronase buffer and digested exhaustively with Pronase. The resulting glycopeptides were desalted by passage through a column of Bio-Gel P6. The complex and high mannose glycopeptides were digested with endo H and separated on a calibrated column of Bio-Gel P4. Columns of Bio-Gel P4 and P6 were calibrated and eluted as described previously (18, 19).
RESULTS

Effect of MDNM and DMN on the Posttranslational Maturation of SI and DPPIV—The maturation of SI and DPPIV in the presence of the glucosidase I inhibitor MDNM and the Golgi α-mannosidase I inhibitor DMN was investigated by the pulse-chase technique. Caco-2 cells were preincubated in the presence of 2 mM of the corresponding inhibitor. After 3 h the cells were labeled with [35S]methionine for 15 min followed by a chase with excess unlabeled methionine. After various intervals of chase, the cells were solubilized and SI and DPPIV were immunoprecipitated and analyzed by SDS-PAGE followed by fluorography.

Fig. 1A shows that in the presence of MDNM the newly synthesized DPPIV exhibits a higher apparent \( M_\text{s} \), than the normal high mannose form. With increasing times of chase this band becomes broader. However, its mean molecular weight never approaches that of the complex-type enzyme. The broadening is concomitant with the appearance of the complex-glycosylated form of DPPIV (DPPIVc) in the control. The effect of MDNM on the maturation of SI is very similar. The SI initially synthesized in the presence of the glucosidase I inhibitor exhibits an intermediate mobility between high mannose SI (SIh) and complex-glycosylated SI (SIC) from control cells. As with DPPIV, the broadening of the SI band correlates with the formation of the complex-glycosylated form in untreated cells. On a 6% gel this broad band could be resolved into two bands (Fig. 1B). It is worth mentioning that the upper band of this doublet still had a higher electrophoretic mobility than the normal SIC even after a 240-min chase.

The altered apparent \( M_\text{s} \) of the initially synthesized products in the presence of MDNM is indicative for the presence of glucosylated high mannose oligosaccharides on the glycoproteins. The described later events could be due to incomplete inhibition of \( N \)-linked carbohydrate trimming, to \( O \)-glycosylation, or to both.

The maturation of the two hydrolases in cells treated with the Golgi α-mannosidase I inhibitor DMN is different from that of control or MDNM-treated cells. No broadening of the DPPIV band was visible with time, and SI shifted only slightly to a higher apparent \( M_\text{s} \) (Fig. 1C). The initially synthesized molecules had the same \( M_\text{s} \), as those of control cells (not shown).

Evidence for \( O \)-Glycosylation of SI and DPPIV—Previous studies have provided evidence that the SI of rat (21) and hog (22) carries \( O \)-linked carbohydrates. In order to test if this is also applicable to human SI and DPPIV of Caco-2 cells, digestions with endoglycosidases were performed. Endoglycosidase H removes high mannose oligosaccharides by cleaving the \( \beta-1\-4 \) glycosidic linkage between the two core glucosamine hexoses. Endoglycosidase F removes high mannose and complex-type \( N \)-linked oligosaccharides by cleaving either the same linkage as endo H or the \( N \)-glycosidic linkage (23). The principle of this approach is as follows. Endo F digestion of a protein which possesses only \( N \)-linked oligosaccharides will lead to a single band with an identical apparent \( M_\text{s} \), as the endo H-digested high mannose form. If, however, both \( N \)- and \( O \)-linked glycans are present, the endo F digestion of the complex-glycosylated form will produce a molecule with a higher apparent \( M_\text{s} \), than the digested high mannose form. The resulting electrophoretic patterns of endo H- and endo F-digested SI (Fig. 2A) fits well with the presence of \( N \)- and \( O \)-linked glycans. It is worth mentioning that the endo H digestion also led to a small shift of the mature form indicating that it possesses some incompletely processed oligosaccharide side chains. DPPIV behaves somewhat different from SI (Fig. 2B). The endo F-digested form appears as a single but still broad band (lane 6). This is unlikely to be due to an incomplete action of endo F since up to four times higher concentrations of the endoglycosidase did not change the \( M_\text{s} \), further (not shown). Therefore the time-dependent apparent microheter-
ogeneity of DPPIV might not entirely be due to N-glycosylation.

The results described above could also be due to posttranslational modifications other than O-glycosylation. Therefore, O-glycosylation was directly assessed by the use of O-glycanase. This enzyme has a narrow substrate specificity. It removes oligosaccharides with the structure Gal-β-1-3-GalNAc α-ser/thr by cleaving the O-glycosidic linkage (24, 25). Since the presence of terminal sialic acid is known to interfere with the enzyme activity neuraminidase was included in some experiments. Fig. 3 shows that the O-glycanase digestion resulted in a small but reproducible mobility shift of the complex-type forms of SI and DPPIV toward the anode (lane 3). Since the high mannose forms were not affected, proteolysis can be excluded. Neuraminidase had no effect. These results confirm the notion that SI and DPPIV carry O-linked carbohydrates. Due to the narrow substrate specificity of O-glycanase, it is not possible to draw conclusions about the extent of O-glycosylation of the two hydrolases.

**MDNM Does Not Inhibit the Conversion to Endo H-resistant Glycoproteins in the Golgi**—Inhibition of glucosidase I or Golgi α-mannosidase I should result in endo H-sensitive forms of glycoproteins. To test this assumption for Caco-2 cells, SI and DPPIV were immunoprecipitated from metabolically labeled Caco-2 cells grown in the presence of MDNM, DMN, or both inhibitors, and their endo H sensitivity was assessed. Fig. 4 shows that SI and DPPIV derived from DMN-treated cells exhibit the expected sensitivity to endo H. In contrast, the late forms of brush border enzymes isolated from cells cultured in the presence of MDNM were completely resistant to endo H in the case of DPPIV (B, lane 6) or only sensitive to the same degree as the normal complex-glycosylated form in the case of SI (A, lane 6), whereas the initially synthesized forms were entirely sensitive (only shown for SI). Since endo H resistance may also originate from interactions between the oligosaccharides and the peptide backbone in native proteins (26) the immunoprecipitates were denatured with SDS prior to digestion. However, this procedure did not alter the above results. Interestingly, when MDNM and DMN were used in combination the immunoprecipitable SI and DPPIV exhibited identical electrophoretic behaviors as the originally synthesized forms from MDNM-treated cells and they remained endo H-sensitive even after a continuous labeling of 5 h (Fig. 4).

These results suggest that DMN but not MDNM is able to completely inhibit the further trimming of N-linked oligosaccharides in Caco-2 cells.

**Endo H-sensitive Oligosaccharides Are Glucosylated in Caco-2 Cells Treated with MDNM**—In order to confirm that MDNM indeed inhibits glucose trimming in Caco-2 cells an analysis of the endo H-sensitive oligosaccharides was carried out. Due to the limited sensitivity of this technique, it was not possible to analyze the glycans of individual brush border enzymes. Total endo H-sensitive oligosaccharides were therefore studied in control and MDNM-treated cells. The cell extracts were digested with Pronase, desalted by passage through a Bio-Gel P6 column, treated with endo H, and subjected to Bio-Gel P4 gel filtration (Fig. 5).

**FIG. 4.** Endo H digestion of SI and DPPIV derived from cells treated with DMN and MDNM. Caco-2 cells were preincubated in the presence of either MDNM, DMN, both, or none of the inhibitors. After 3 h the cells were continuously labeled with [35S]methionine for 4 h. The cells were solubilized, and SI (A) or DPPIV (B) were immunoprecipitated. After endo H digestion the labeled proteins were analyzed by SDS-PAGE. Shown are fluorograms of a 6% gel (A) or of a 7.5% gel (B), respectively.

**FIG. 3.** O-Glycanase treatment of SI and DPPIV. Caco-2 cells were continuously labeled with [35S]methionine for 3 h. Immunoprecipitates were digested either with neuraminidase, O-glycanase, or with both glycosidases. Shown is a fluorogram of a 6% gel that was run with the digested immunoprecipitates.
proteinase K, and the glycopeptides were separated by concanavalin A chromatography. As one would expect, the cells that the majority of the radioactive SI and DPPIV was converted to the endo H-resistant form. After immunoprecipitation the two major peaks are glucosylated was provided by the hapten sugar, the elution of complex sugars needs either no or only very low concentrations of hapten sugar (28). Confirmation of our analysis Caco-2 cells were continuously labeled with [35S]methionine for 5 h in the presence of glycosylation inhibitors. Figs. 7 and 8 show that in the presence of MDNM both the endo H-sensitive and endo H-resistant forms of SI had a higher electrophoretic mobility after treatment with α-glucosidase (Fig. 7, lanes 3 and 4). This is unlikely to be due to partial proteolysis since neither the protein synthesized by DMN-treated cells (lanes 6 and 7) nor the high mannose (lower band) and the complex-glycosylated form (upper band) of SI in untreated control cells were affected by this treatment a high efficiency and affinity (Fig. 6). In fact, 90% of the totally applied radioactivity was specifically elutable with high concentrations of α-methyl-mannoside indicating that a vast majority of oligosaccharides were of the high mannose type. In contrast, the treatment with MDNM alone led to glycopeptides with an intermediate affinity for concanavalin A. Under these conditions 62% of the radioactivity was recovered in the high affinity fraction, while in the control the corresponding value was 22%. Several interpretations can be offered to explain these results. 1) In view of the extended labeling time of 22 h, it appears possible that MDNM has led to glycoproteins which carry both high mannose and complex oligosaccharides. However, this would not be compatible with the results of the endo H experiments (Fig. 4), which showed that SI and DPPIV acquire the same resistance to this glycosidase irrespective of the presence or absence of MDNM. 2) A subpopulation of SI and DPPIV may be kept in a high mannose form while another fraction underwent complex glycosylation. This is unlikely since already after shorter labeling periods most of the radioactive protein is converted to the endo H-resistant form in the presence of MDNM (e.g. see Fig. 4B for DPPIV). 3) The enzymes may possess a novel type of oligosaccharides that bind to concanavalin A with high affinity (28). This structure may still carry the glucose residues on one branch and complex sugars, as for example galactose, on the other branches.

MDNM Leads to the Synthesis of Glucosylated Endoglycosidase H-resistant Glycoproteins—To test the possibility that MDNM leads to the synthesis of glucosylated endo H-resistant oligosaccharides, we used two exoglycosidases. Such glycoproteins would be sensitive to α-glucosidase but resistant to α-mannosidase. Since DPPIV displays less defined bands on gels than SI this analysis was performed for SI only. For these experiments Caco-2 cells were continuously labeled with [3H]mannose for 20 h with [2-3H]mannose, so that the majority of the radioactive SI and DPPIV was converted to the endo H-resistant form. After immunoprecipitation the two brush border hydrolases were digested with proteinase K, and the glycopeptides were separated by concanavalin A chromatography. The lectin concanavalin A can be used to differentiate between high mannose and complex-type oligosaccharides. Whereas high mannose oligosaccharides bind with a high affinity to this lectin and therefore can only be eluted with a high concentration of the hapten sugar, the elution of complex sugars needs either no or only very low concentrations of hapten sugar (28). Caco-2 cells were labeled for 20 h with [2-3H]mannose, so that the majority of the radioactive SI and DPPIV was converted to the endo H-resistant form. After immunoprecipitation the two brush border hydrolases were digested with proteinase K, and the glycopeptides were separated by concanavalin A chromatography. As one would expect, the cells treated with either DMN alone or with DMN and MDNM synthesized oligosaccharides which bound to the column with
were treated with either MDNM, DMN, or both and labeled with \[^{35}\text{S}\]methionine for 5 h. SI was immunoprecipitated and analyzed by SDS-PAGE (6% gel) and fluorography.

The proposed endo H-resistant oligosaccharides can be expected to lack terminal mannose residues, since on one branch the outer mannoses would be replaced by terminal sugars. Therefore, these glycans should be insensitive to exomannosidase. This was assessed as follows. Cells were treated with either MDNM, DMN, or both and labeled with \[^{35}\text{S}\]methionine for 5 h. SI was immunoprecipitated and digested with \(\alpha\)-mannosidase (\(\alpha\)-Man.). The digests were separated on 6% SDS-gels and visualized by fluorography.

The only other variant which exhibited sensitivity to \(\alpha\)-glucosidase was SI of cells treated with both MDNM and DMN (lanes 7 and 8) indicating that this variant was indeed a glycosylated high mannose form of SI.

The proposed endo H-resistant oligosaccharides can be expected to lack terminal mannose residues, since on one branch they are covered by terminal glucose residues and on the other branches the outer mannoses would be replaced by terminal sugars. Therefore, these glycans should be insensitive to exomannosidase. This was assessed as follows. Cells were treated with either MDNM, DMN, or both and labeled with \[^{35}\text{S}\]methionine for 5 h. SI was immunoprecipitated and digested with \(\alpha\)-mannosidase. The result is as expected (Fig. 8). In the sample derived from MDNM-treated cells, only the endo H-sensitive lower molecular weight band shifted after digestion with mannosidase whereas the endo H-resistant, higher molecular weight form did not change its mobility (compare lanes 3 and 4). Likewise the normal complex-glycosylated protein was not sensitive to the exomannosidase (lanes 1 and 2, upper band). All other forms of SI were sensitive to \(\alpha\)-mannosidase as for example the high mannose form in control cells (lanes 1 and 2, lower band). Interestingly, the protein synthesized in the presence of the mannosidase I-inhibitor DMN is more sensitive (lanes 5 and 6) than that derived from cells which were additionally treated with the glucosidase I-inhibitor MDNM (lanes 7 and 8). This is compatible with the results of the glucosidase experiments which suggested that SI in MDNM-treated cells is glycosylated (Fig. 7). Thus, terminal glucose protects one branch against the action of the exomannosidase.

Taken together these results strongly suggest that the inhibition of glucose removal at one branch by MDNM does not prevent trimming and complex-glycosylation of the other branches and thereby leads to glycosylated oligosaccharides that are resistant to endo H.

Effect of MDNM and DMN on Asynchronous Protein Transport to the Brush Border—In order to determine whether or not differential trimming of N-linked oligosaccharides is the underlying cause of the asynchronous intracellular transport of SI and DPPIV, the pulse-chase technique was combined with subcellular fractionation. Caco-2 cells were preincubated for 3 h with 2 mM MDNM and labeled with 250 \(\mu\text{Ci}\) of \[^{35}\text{S}\]methionine for 15 min followed by a chase for up to 5 h. At the end of the chase, the cells were subjected to subcellular fractionation (14) resulting in P1 (total membranes) and P4 (brush border membranes). SI and DPPIV were immunopurified and analyzed by SDS-PAGE. The fluorogram in Fig. 9 shows the result of an experiment with a chase of 3 h. As expected, in the P1 fraction derived from control cells SIh is clearly visible whereas DPPIV is already completely processed to the mature form. In P1 prepared from MDNM-treated cells, the broad DPPIV band and the two electrophoretically different forms of SI are visible as already described in Fig. 1. A careful comparison of the electrophoretic mobility of the molecular species of SI in the microvillar fraction (P4) revealed that it is always the upper band that appears in these membranes irrespective of whether the cells were treated with MDNM or not. The intensities of the bands in P4 indicate that MDNM in part interfered with the transport of SI and DPPIV to the cell surface. Quantification of these experiments (Fig. 10) revealed that MDNM affected the intracellular transport of the two enzymes in an identical way. Neither the rate of transport (that is the time of half-maximal appearance) nor the time of the first appearance changed significantly when compared with untreated control cells.

However, MDNM lead to a 60–70% decrease of the maximal radioactivity in the P4 fraction. This effect was due neither to an altered total incorporation of radioactivity nor to inf-
acid-precipitable radioactivity from homogenate samples was set radioactivity associated with the enzymes relative to trichloracetic radioactivity was measured in a p-counter. The maximal amount of DPPIV in the brush border membrane. Preincubation of the resistant glycans and the asynchronous transport of SI and section itself was not affected by MDNM as enrichment factors these parameters in the corresponding homogenates (data not shown). Furthermore, the procedure of subcellular fractiona-

FIG. 10. Effect of MDNM on the appearance of SI and DPPIV in the brush border membrane. Preincubation of the cells and the pulse-chase protocol was as in Fig. 1. Gel slices corresponding to SI or DPPIV were cut out of dried gels, solubilized, and radioactivity was measured in a β-counter. The maximal amount of radioactivity associated with the enzymes relative to trichloracetic acid-precipitable radioactivity from homogenate samples was set to 100%.

FIG. 11. Appearance of SI and DPPIV in the microvillar membrane of Caco-2 cells in the presence of DMN. After a preincubation in the presence (A and B) or in the absence (C) of 2 mM DMN, the cells were pulsed for 15 min with [35S]methionine. After a chase of 1.5 h (A) or 5 h (B and C), the cells were fractionated. The immunoprecipitated SI and DPPIV from P4 and a sample of the homogenate were loaded on a 7.5% SDS-polyacrylamide gel and visualized by fluorography.

DSPPV is in part due to an intra-Golgi rate-limiting step (14), we also tested the effect of DMN, which is known to inhibit the first trimming step in the Golgi apparatus. Fig. 11A (90 min chase) shows that the two hydrolases appear asynchronously in the brush border fraction when the trimming was inhibited by DMN. The maximal radioactivity of the enzymes in the P4 fraction was not altered by this treatment (Fig. 11, B and C).

DISCUSSION

In the present publication we have reported on the effects of MDNM, a glucosidase I inhibitor, and DMN, an inhibitor of Golgi α-mannosidase I, on the posttranslational maturation and intracellular transport of SI and DPPIV in Caco-2 cells.

Effects of Trimming Inhibitors on Glycosylation—Experiments with endo H showed that DMN completely inhibited the maturation of high mannose to complex oligosaccharides attached to SI and DPPIV. In contrast, MDNM was unable to prevent the acquisition of endo H resistance. The latter finding together with the time-dependent increase in apparent Mr of DPPIV and SI strongly suggests that MDNM does not completely inhibit the processing of N-linked carbohydrates to complex forms in this system. Furthermore, the use of exoglycosidases has led us to conclude that the endo H-resistant form of SI in MDNM-treated cells carries glucosylated oligosaccharides.

In untreated cells the increase in apparent Mr, with time of chase is not exclusively due to maturation of N-linked glycans. Indeed, the use of O-glycanase revealed that SI and DPPIV carry O-glycosidically linked carbohydrates. For DPPIV there is a discrepancy at late chase times between the molecular species synthesized in the presence of DMN and those obtained from untreated cells after digestion with endo F. Since DMN did not inhibit protein transport to the cell surface one would expect normal O-glycosylation in the presence of this inhibitor, and hence a time-dependent increase in the mean apparent Mr under the two conditions. This was clearly not obtained. It is possible therefore that DPPIV carries N-linked oligosaccharides which are not removable by the used mixture of endo F and peptide N-glycosidase F. Alternatively, DMN might not only inhibit Golgi α-mannosidase I but also interfere with other posttranslational modifications that lead to electrophoretic mobility shifts. Interestingly, we were not able to alter the electrophoretic behavior of DPPIV from DMN-treated cells by a digestion with O-glycanase (not shown). Other changes may concern sulfation a recently detected modification of brush border enzymes (29). It has been reported, for instance, that inhibition of N-linked glycosylation by tunicamycin induces tyrosine sulfation of hybridoma immunoglobulin G (30).

The initially synthesized forms of SI and DPPIV have higher apparent Mr in the presence of MDNM than their counterparts in control cells. This is due to untrimmed high mannose forms still carrying glucosylated oligosaccharides as assessed by an analysis of endo H-sensitive carbohydrates. However, this inhibition of glucosidase I did not prevent the conversion from endo H-sensitive to endo H-resistant glycoproteins. This conversion is not due to an incomplete inhibition of glucosidase I which would lead to a heterogenous population of glycoproteins carrying high mannose and complex oligosaccharides. Our experiments with endo H did not provide evidence for such a heterogeneity in MDNM-treated cells. Furthermore, digestion experiments with an α-exoglycosidase showed that the endo H-resistant SI in cells treated with MDNM is still glucosylated. What is the mechanism underlying the biogenesis of glucosylated endo H-resistant
glycoproteins? A possibility to be considered is that the glycoproteins synthesized in the presence of MDNM may be partially processed by Golgi endo-α-D-mannosidase which removes glucosylmannose disaccharides from glucosylated high mannose oligosaccharides (31, 32). This reaction would lead to glycoproteins carrying both glucosylated high mannose and complex carbohydrates. While this mechanism would fit with the increased affinity of glycopeptides from MDNM-treated cells to concanavalin A and the continued susceptibility of these glycoproteins to α-glucosidase it does not appear to explain other results including the apparent insensitivity of the glycoproteins toward α-mannosidase. This latter observation may, however, be due to insufficient resolution of the SDS-PAGE. More important, if generated by the Golgi endo-α-D-mannosidase the glycoproteins should exhibit partial endo H sensitivity since they would carry both glucosylated high mannose oligosaccharides and complex oligosaccharides, most likely in various proportions. Partial endo H sensitivity was clearly not observed. Based on all these results we favor a model in which the presence of MDNM leads to hybrid oligosaccharide structures similar to those postulated by Peyreras et al. (33) who found altered neuraminidase sensitivity of glycoproteins synthesized in 1-deoxynojirimycin-treated cells. However, these authors did not provide evidence for the continuous presence of glucose residues. In the present study we have also used an exomannosidase to probe for such hybrids. The endo H-resistant SI derived from MDNM-treated cells turned out to be insensitive to this enzyme what is to be expected if all the mannose residues are protected by other terminal sugars.

An interesting observation is that DMN inhibits the further trimming in MDNM-treated cells but that on gels SI and DPPIV have the same apparent M, as the initially synthesized products in cells incubated with MDNM alone. This suggests that the processing of the glucose-free branches of the high mannose oligosaccharides is catalyzed by the action of α-mannosidase I (or by a similar mannosidase which is inhibitable by DMN). If one assumes a cis-Golgi localization of the α-mannosidase I (5), it appears likely that the processing only starts in the Golgi apparatus in MDNM-treated cells. However, this trimming does not lead to the normal M, which is not surprising since in the presence of MDNM the N-linked oligosaccharides are definitely different from those synthesized in control cells. Furthermore, it is noteworthy that the activity of the enzymes involved in the processing of N-linked oligosaccharides critically depends on the structure of the substrate (24). In view of this knowledge, it is unlikely that the trimmed branches of the hybrid oligosaccharides are completely reglucosylated in an identical way as their counterparts in control cells.

Effects of Trimming Inhibitors on Intracellular Transport—Recent studies focusing on the effect of glucosidase inhibitors on the intracellular transport have shown that some secretory and lysosomal proteins are unable to leave the endoplasmic reticulum as long as they carry glucose residues (35–37). In the case of SI and DPPIV, the exit from the endoplasmic reticulum was not blocked by the presence of glucosylated oligosaccharides as the two proteins became endo H resistant without losing their glucose residues. This conversion was blocked by DMN which inhibits the cis-Golgi enzyme mannosidase I (5). As mentioned above the proteins synthesized in the presence of MDNM and DMN had the same apparent M, as their endo H-sensitive counterparts derived from Caco-2 cells treated with MDNM only, suggesting that under the latter conditions SI and DPPIV were first transported to the Golgi apparatus before their oligosaccharides were processed to the endo H-resistant forms.

The effect of N-linked carbohydrate trimming on the asynchronous intracellular transport of SI and DPPIV was studied by pulse-chase experiments in conjunction with subcellular fractionation. Neither DMN or MDNM affected the asynchronism. Recently, we have reported that the retardation of the intracellular transport of SI versus DPPIV is due to at least two rate-limiting steps, a pre-Golgi and an intra-Golgi step (14). The finding that DMN does not influence the rates of transport clearly indicates that the intra-Golgi rate-limiting step is not due to N-linked carbohydrate processing. A similar conclusion can be drawn for the pre-Golgi retardation of SI on the basis of the inhibitory effect of MDNM on the removal of glucose residues. Already a partial inhibition of a rate-determining step can be expected to alter the overall rate of transport to the brush border membrane. However, MDNM did not result in such an alteration.

An interesting finding of the present study is that MDNM treatment led to a 60–70% decrease of immunoprecipitable radioactivity in the microvillar membrane fraction. This result is at variance with the data of Danielewski and Cowell (38) who used the glucosidase inhibitor castanospermine and pig intestinal explants. These authors concluded that carbohydrate trimming is not required for the surface expression of intestinal hydrolases. However, in that study the results were not quantified and the degree of surface expression is difficult to estimate since castanospermine strongly inhibited protein synthesis. The reduced expression of SI and DPPIV in the microvillar membrane in the presence of MDNM indicates that aberrant glycosylation may reduce the efficiency of intracellular transport. MDNM seems to affect post-Golgi rather than pre- or intra-Golgi transport since the acquisition of endo H resistance of the two brush border hydrolases was concomitant in MDNM-treated and control cells.

Previous studies on the influence of trimming inhibitors on the intracellular transport and functional activity of glycoproteins have not led to a uniform concept. In many cases the trimming of glucose residues was shown to be important for an efficient transport of secretory (33, 35, 39) and lysosomal proteins (36, 37). However, other secretory proteins were not affected (33, 40). Membrane proteins seem to behave similarly. Whereas the intracellular transport of histocompatibility antigens (33, 41) and some viral glycoproteins (41, 42, 43) was not affected by glucosidase inhibitors, other viral proteins (27, 44) and the insulin and insulin-like growth factor-I receptors (45, 46) were transported inefficiently in the presence of these inhibitors. To our knowledge, whenever the inhibited step of intracellular transport was localized, it was the exit from the endoplasmic reticulum that was affected (35–37, 44). It is of interest therefore to note that the transport of SI and DPPIV in the presence of the glucosidase inhibitor was unaffected up to the level of the trans-Golgi. In fact, it was the efficiency of their further transport to the brush border membrane that was drastically reduced in the presence of this inhibitor. It is likely that this is due to conformational alterations of the protein backbone induced by the presence of artificial oligosaccharides. Such an interpretation is supported by the finding that the maturation of two viruses became temperature sensitive in the presence of glucosidase inhibitors (42, 43).

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