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Processing and transport of human small intestinal lactase-phlorizin hydrolase (LPH)

Role of N-linked oligosaccharide modification

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Abstract

The effect of glycosylation on the intracellular transport of human intestinal lactase-phlorizin hydrolase (LPH) was investigated by biosynthetic labeling of biopsy samples in the presence or absence of glycosidase inhibitors. In the presence of deoxynojirimycin (dNM) and deoxymannojirimycin (dMM), endo H sensitive LPH glycoforms of M_r = 135,000 in both cases were produced (LPH_{dNM} and LPH_{dMM}). The LPH glycoform generated in the presence of swainsonine had an apparent molecular mass of 141,000 (LPH_{Swa}) and was partially sensitive to endo H. By contrast to unmodified mature LPH (LPH_m, M_r = 160,000), these glycoforms are either not O-glycosylated (LPH_{dNM} and LPH_{dMM}) or partially O-glycosylated (LPH_{Swa}) indicating that processing of N-linked carbohydrates has direct effects on the O-glycosylation of pro-LPH. Analysis of transport kinetics of the various glycoforms strongly suggested that carbohydrate modification does not affect the transport of pro-LPH from the *cis*-Golgi to the cell surface, but could be rate limiting at the level of the ER.

Key words: Lactase-phlorizin hydrolase; Human, intestine; Carbohydrate processing; Intracellular transport

1. Introduction

Lactase-phlorizin hydrolase (LPH, EC 3.2.1.23-62) is an intestinal brush border glycoprotein that is abundant in almost all mammals. LPH converts lactose, the main carbohydrate in mammalian milk, to its two monosaccharide components. Studies in a variety of systems have established that LPH is synthesized as a large single-chain precursor (pro-LPH) that undergoes proteolytic cleavage to the final brush border form (LPH_m) [1–5]. In human intestinal cells [2,4,5] as well as in the pig small intestine [1] cleavage takes place in the Golgi after complex glycosylation of pro-LPH. The role of intracellular cleavage has been recently investigated by transfection of COS-1 cells with a full length LPH cDNA [6]. Here, it was shown that uncleaved forms of pro-LPH could be transported to the cell surface in COS-1 cells and that processing of the carbohydrate side chains is not required for the expression of enzymatically active LPH. Biosynthetic labeling studies of pro-LPH at reduced temperatures have demonstrated that at least a proportion of pro-LPH acquires complex glycosylated oligosaccharides before cleavage takes place [5]. However, it is not clear whether other forms of pro-LPH, for example differently glycosylated and/or improperly glycosylated molecules, could also be cleaved intracellularly. Moreover, the effects of carbohydrate modification on the intracellular transport and cleavage of pro-LPH have not been so far elucidated. This is particularly interesting in view of recent findings on the processing of LPH in several cases of adult hypolactasia [7,8] in which the intracellular transport and proteolytic cleavage of pro-LPH are substantially delayed.

In this paper the role of glycosylation on the intracellular transport and cleavage of pro-LPH has been investigated using a pulse-chase protocol of biopsy explants in the presence of inhibitors of glycosylation.

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Abbreviations: LPH, lactase-phlorizin hydrolase (EC 3.2.1.23-62); pro-LPH, pro-lactase-phlorizin hydrolase; LPH_m, mature brush border lactase-phlorizin hydrolase (160 kDa); dNM, deoxynojirimycin; dMM, deoxymannojirimycin; pro-LPH_{dNM} and LPH_{dNM}, pro-LPH and LPH obtained in the presence of dNM; pro-LPH_{dMM} and LPH_{dMM}, pro-LPH and LPH obtained in the presence of dMM; pro-LPH_{Swa} and LPH_{Swa}, pro-LPH and LPH obtained in the presence of swainsonine; endo F/GF, endo-β-*N*-acetylglucosaminidase F/glycopeptidase F; endo H, endo-β-*N*-acetylglucosaminidase H; IM, intracellular membranes; BBM, brush border membranes; SDS-PAGE, sodium-dodecyl sulfate-polyacrylamide gel electrophoresis.

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2. Experimental

2.1. Biosynthetic labeling of biopsy specimens and immunoprecipitations

Human small intestinal mucosa (approximately 5–10 mg wet weight) were obtained from patients biopsied for diagnostic purposes. They appeared normal when examined by light microscopy and expressed normal levels of brush-border disaccharidase activities. Biosynthetic labeling was performed according to Naim et al. [4]. In brief, the biopsy specimens were washed three times in methionine-free RPMI 1640 medium containing 10% dialyzed fetal calf serum (FCS) (Gibco), placed on stainless-steel grids in organ culture dishes (Falcon) [9] and incubated for 2 h at 37°C in a $CO_2 + O_2$ (5:95, v/v) incubator. Thereafter the tissue was labeled with 150 μCi of *trans*-[^{35}S]methionine (ICN) for 6 h. In some experiments labelings were performed in the presence of inhibitors of glycosidases, dNM, dMM and swainsonine (Boehringer-Mannheim). Here, 5 mM dNM, 5 mM dMM (5 mM) and 4 μg/ml swainsonine were used. The inhibitors were present in the preincubation and labeling media. After labeling, the specimens were washed three times in RPMI 1640 and homogenized at 4°C with a Teflon-glass homogenizer in 1 ml of 25 mM Tris-HCl (pH 8.1), 50 mM NaCl, and a cocktail of protease inhibitors containing 1 mM PMSF, 1 μg pepstatin, 5 μg leupeptin, 17.4 μg benzamidine and 1 μg aprotonin (all from Sigma). Processing of the biopsy homogenates for immunoprecipitation with monoclonal anti-human LPH (HBB 1/909/34/74) [2] and SDS-polyacrylamide gel electrophoresis were performed as described elsewhere [4].

2.2. Other procedures

Preparation of brush border membrane vesicles (BBM) and intracellular membranes (IM) from labeled mucosa, immunoprecipitations, endo-*N*-β-acetylglucosaminidase F/glycopeptidase F (endo F/GF) (EC 3.2.2.18 and EC 3.5.1.52, Boehringer-Mannheim), endo-*N*-β-acetylglucosaminidase H (EC 3.2.1.96, Boehringer-Mannheim) treatment were performed according to Naim et al. [4,10,11]. Treatment of immunoprecipitates with *O*-glycanase (EC 3.2.1.97, Boehringer-Mannheim) was essentially as described in [12] with the following modification. Immunoprecipitated proteins were eluted with 20 μl 1% SDS and 100 mM DTT in 0.1 M Tris-HCl, pH 8.0, by boiling for 5 min. To the eluates, 10 μg of bovine serum albumin as a carrier protein was added, and the mixture was precipitated in 3 vols. of ice-cold acetone for 20 h at −20°C. The proteins were recovered by centrifugation, dried at 37°C and treated or not treated with neuraminidase (EC 3.2.1.18, Boehringer-Mannheim) and *O*-glycanase as described previously [12]. One-dimensional SDS-polyacrylamide gel electrophoresis was conducted in 5% or 6% polyacrylamide slab gels containing 0.1% SDS according to Laemmli [13].

3. Results

3.1. Inhibition of oligosaccharide processing affects the ER to Golgi but not the Golgi to cell surface transport of pro-LPH

To investigate the effect of carbohydrate modification on the transport of pro-LPH to the cell surface inhibitors of carbohydrate processing were used in conjunction with biosynthetic labeling studies of biopsy specimens, subcellular fractionations, immunoprecipitations, endo F/GF, endo H treatments and SDS-PAGE. The effect of the glycosidase inhibitors was monitored by the appearance of modified and cleaved forms of LPH. Furthermore, cleavage of human pro-LPH in the Golgi provided a simple way by which the transport-competence of modified pro-LPH forms could be assessed. Here, the transport rates of pro-LPH from the ER to the Golgi in the presence or absence of glycosidase inhibitors were correlated with the proportion of pro-LPH that has undergone cleavage in the Golgi.

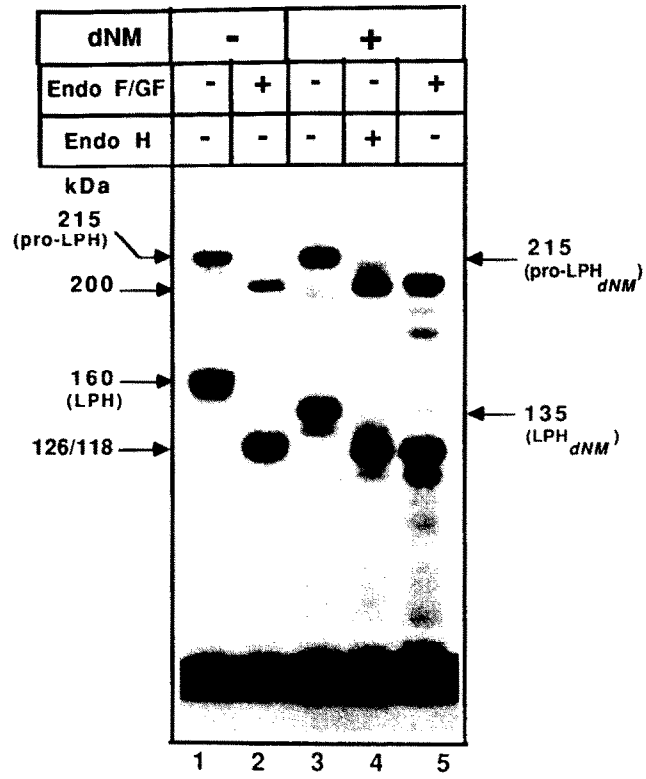

Fig. 1. Effect of deoxynojirimycin (dNM) on the processing of LPH. Biopsy samples were labeled for 6 h with [^{35}S]methionine in the presence (lanes 3–5) or absence (lanes 1 and 2) of 5 mM dNM and homogenized. Detergent extracts of the homogenates were immunoprecipitated with monoclonal anti-LPH antibodies. Part of the immunoprecipitates was treated with endo H (lane 4), endo F/GF (lanes 2 and 5) or untreated (lanes 1 and 3). The samples were analyzed by SDS-PAGE on 6% slab gels and fluorography.

3.1.1. Effect of deoxynojirimycin (dNM)

Deoxynojirimycin is a glucose analogue that inhibits the removal of the glucose residues from N-linked oligosaccharides chain, Glc_3 Man_9 $GlcNAc_2$, by α-glucosidases I and II (EC 3.2.1.20) of the endoplasmic reticulum [14]. Fig. 1 demonstrates that in the presence of dNM LPH is composed of two polypeptides of apparent molecular weights 215,000 (denoted pro-LPH_{dNM}) and 135,000 (denoted LPH_{dNM}) (lane 3). Therefore, the size of the cleaved LPH_{dNM} varied substantially from that of its counterpart in the dNM-non-treated sample, the M_r = 160,000 (denoted LPH_m) (Fig. 1, lane 1).

Pro-LPH_{dNM} and LPH_{dNM} showed similar sensitivities to endo H as well as to endo F/GF and were converted to M_r = 200,000 and M_r = 118,000, respectively, upon treatment with either enzyme (Fig. 1, lanes 4 and 5). Since endo H cleaves mannose-rich glycan chains, while endo F/GF cleaves mannose-rich as well as complex type N-linked oligosaccharides, we conclude that pro-LPH_{dNM} (M_r = 215,000) and LPH_{dNM} (M_r = 135,000)

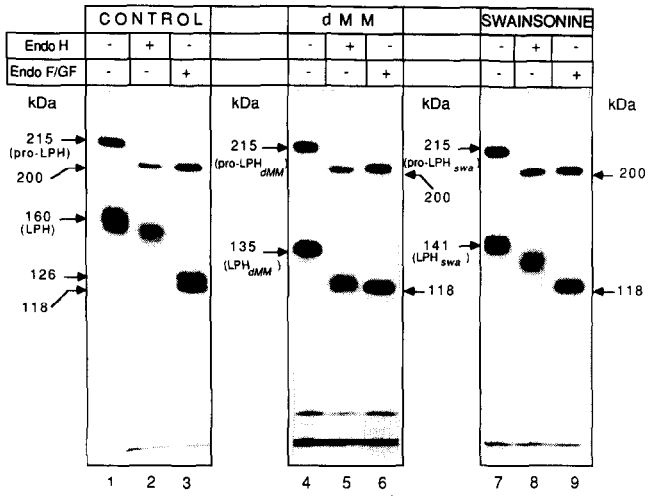

Fig. 2. Effects of deoxymannojirimycin (dMM) and swainsonine on the processing of LPH. Biopsy samples were labeled for 6 h with [^{35}S]methionine in the absence (lanes 1–3) or presence of 5 mM dMM (lanes 4–6) or swainsonine (lanes 7–9) and homogenized. The detergent extracts of the homogenates were immunoprecipitated with monoclonal anti-LPH antibodies and subjected to analysis by SDS-PAGE on 6% slab gels after treatment with endo H (lanes 2, 5, 8), endo F/GF (lanes 3, 6, 9) or without treatment (lanes 1, 4, 7). Lanes 4–6 were run on the same gel as lanes 1–3 and 7–9, but were taken from a longer exposed fluorogramme.

carry predominantly mannose-rich oligosaccharides. The results indicate that the mannose-rich pro-LPH$_{dNM}$ has been transported to and cleaved in the Golgi where the putative protease is located. It is interesting to note the variations in the proportions of the cleaved LPH forms in the total LPH protein in dNM-treated and non-treated samples. Densitometric scannings of the fluorograms revealed that almost 46% of the total LPH protein in the dNM-treated biopsy sample was cleaved to LPH$_{dNM}$ within 6 h of labeling and 53% persisted as uncleaved pro-LPH$_{dNM}$ (Table 1) resulting in a ratio of 0.87 of cleaved vs. uncleaved pro-LPH. In the control sample, on the other hand, 68% of total LPH protein was found in the cleaved form (LPH$_m$, M_r = 160,000) and 31.5% as uncleaved pro-LPH (M_r = 215,000). The resulting ratio of cleaved LPH$_m$ vs. uncleaved pro-LPH of 2.16 is therefore 2.5-fold higher than that obtained in the presence of the inhibitor (Table 1). This indicates that within 6 h of biosynthetic labeling significantly less pro-LPH$_{dNM}$ molecules have been cleaved in the Golgi to LPH$_{dNM}$ than in the absence of dNM (pro-LPH to LPH$_m$).

3.1.2. *Effects of deoxymannojirimycin (dMM) and swainsonine (Swa)*

Deoxymannojirimycin inhibits the first steps in the processing of mannose-rich oligosaccharides by blocking the removal of four α-1,2-linked mannose-residues by the *cis*-Golgi α-mannosidase I (EC 3.2.1.113) [15]. Swainsonine blocks the removal of α-1,3 and α-1,6 linked mannoses by Golgi α-mannosidase II (EC 3.2.1.114) [16]. In the presence of deoxymannojirimycin (dMM) the LPH band pattern revealed two polypeptides, a mannose-rich precursor (denoted pro-LPH$_{dMM}$, M_r = 215,000) and a modified cleaved product of M_r = 135,000 (denoted LPH$_{dMM}$) (Fig. 2, lane 4). Swainsonine effect is manifested by the appearance of a modified cleaved product of M_r = 141,000 (denoted LPH$_{Swa}$) (Fig. 2, lane 7). While LPH$_{dMM}$ was completely sensitive to endo H and converted to M_r = 118,000 (Fig. 2, lane 5), LPH$_{Swa}$ showed partial sensitivity to the endoglycosidase (Fig. 2, lane 8). Both LPH$_{dMM}$ and LPH$_{Swa}$ revealed similar products of digestion upon treatment with endo F/GF (Fig. 2, lanes 6 and 9). The results indicated that the glycosylation pattern of LPH$_{dMM}$ contains mainly mannose-rich oligosaccharide chains. The partial sensitivity of LPH$_{Swa}$ towards endo H is reminiscent of a hybrid type of glycosylation of this glycoform indicating that at least partial trimming of mannose-rich pro-LPH$_{Swa}$ has occurred.

Scanning densitometry revealed that the cleaved product LPH$_{dMM}$ constituted 65% of the total synthesized and processed LPH, which is almost 2-fold that of pro-LPH$_{dMM}$ (32%) in dMM-treated cells (Table 1). The ratio of LPH$_{dMM}$ to pro-LPH$_{dMM}$ (2.03) is therefore similar to that in non-treated cells (2.16). An almost similar ratio was also obtained for LPH$_{Swa}$ in total synthesized LPH protein. In this case, cleaved LPH$_{Swa}$ constituted almost 66% of the total LPH proteins which is similar to that found in the absence of the inhibitor (68%).

3.1.3. *Transport of LPH glycoforms from the Golgi to the brush border membrane*

Next the effect of glycosylation inhibitors on the transport of cleaved LPH to the cell surface was examined. Here, biopsy specimens were continuously labeled for

Table 1
Proportions of cleaved LPH in total synthesized LPH protein in the presence or absence of glycosidase inhibitors

Glycosidase inhibitor	LPH (M_r)	% in total LPH protein		LPH	LPH/pro-LPH (control)
		LPH	Pro-LPH	Pro-LPH	LPH/pro-LPH (inhibitor)
Not added	160,000	68	31.5	2.16	–
dNM	135,000	46	53	0.87	2.5
dMM	135,000	65	32.0	2.03	1.06
Swainsonine	141,000	66	35	1.9	1.14

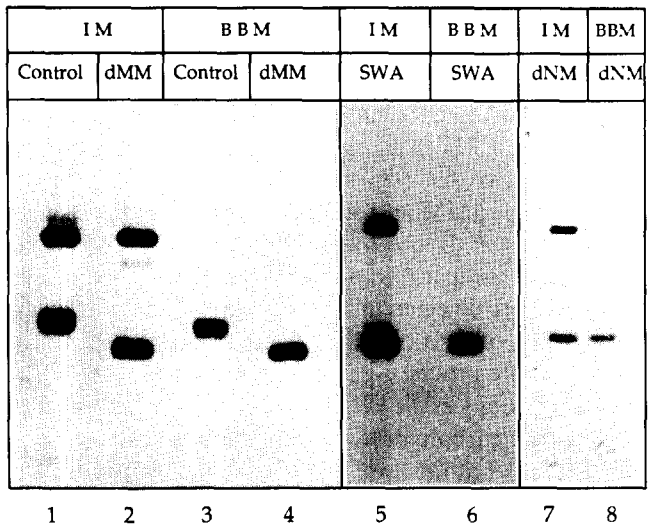

Fig. 3. Appearance of LPH glycoforms in the brush border membrane. Biopsy samples were labeled for 6 h with [^{35}S]methionine in the absence (lanes 1 and 3) or presence of 5 mM dMM (lanes 2 and 4), 6 μM swainsonine (Swa) (lanes 5 and 6) or 5 mM dNM (lanes 7 and 8) and homogenized. The homogenates were subcellularly fractionated into intracellular membranes (denoted IM) and brush border membranes (denoted BBM), solubilized, immunoprecipitated with anti-LPH antibodies and analyzed by SDS-PAGE on 6% slab gels. The figure is composed of three gels (lanes 1–4) (lanes 5 and 6) (lanes 7 and 8).

6 h in the presence or absence of dNM, dMM and swainsonine. The biopsy homogenates were subcellularly fractionated to intracellular membranes (IM) and brush border membranes (BBM) (see [4]). In the presence of dMM the modified cleaved LPH_{dMM} form was revealed in the brush border membrane (BBM) fraction (Fig. 3, lane 4). Its proportion in total cleaved LPH (the sum of cleaved LPH_{dMM} in the intracellular membranes (IM) (Fig. 3, lane 2) and brush border membrane (Fig. 3, lane 4)) was 46% (Table 2). The LPH modified cleaved forms generated in the presence of swainsonine (LPH_{Swa}, Fig. 3, lane 6) and dNM (LPH_{dNM}, Fig. 3, lane 8) were also transported to the brush border membrane. Their proportions in total cleaved LPH were 42% (swainsonine) and 47% (dNM). In the absence of glycosidase inhibitors the proportion of the cleaved LPH molecule (LPH_m, Fig. 3, lane 3) in the brush border membrane was 46.5%. The results indicate that the proportion of cleaved LPH molecule that reach the cell surface is similar in all cases regardless of the presence or absence of inhibitors of glycosylation suggesting that the modification of the carbohydrate residues seems not to have affected the transport of cleaved LPH to the brush border membrane.

3.2. *O-glycosylation of LPH is affected in the presence of glycosidase inhibitors*

Recently we have shown that the mature, brush border LPH molecule (LPH_m, 160 kDa) is composed of at least two families of differently glycosylated molecules, an N-glycosylated LPH molecules (LPH_N) and an N- and O-glycosylated ($LPH_{N/O}$) species [12]. Treatment of these two forms, i.e. the LPH_N and $LPH_{N/O}$ with endo F/GF generates 118 kDa and 126 kDa polypeptides, respectively. In fact, as shown in the control biopsy sample (Fig. 2, lanes 1–3) digestion of LPH_m (lane 1) with endo F/GF (lane 3) produced two products: the O-glycosylated 126 kDa species and the M_r = 118,000, which corresponds to the N-deglycosylated form of LPH_N (see [12]). Interestingly, the presence of the inhibitors dNM, dMM and swainsonine produced polypeptides that have only one product of deglycosylation with endo F/GF, which is represented by the M_r = 118,000 (Fig. 1, lane 5; Fig. 2, lanes 6 and 9) and is similar to the N-deglycosylation product of LPH_N. This strongly suggests that O-glycosylation of LPH did not occur/or is at least affected in the presence of dNM, dMM, and swainsonine. To examine this possibility, the different glycoforms of LPH, i.e. LPH_{dNM}, LPH_{dMM}, and LPH_{Swa} were treated with neuraminidase/*O*-glycanase to assess the presence or absence of O-glycosidically linked carbohydrates. The presence of neuraminidase in the reaction is required if the substrate of *O*-glycanase, Gal β (1–3) GalNAc, is substituted by sialic acid. Fig. 4 demonstrates that treatment of mature LPH_m with neuraminidase/*O*-glycanase resulted in a reduction in its apparent molecular weight (lanes 1 and 2). It is important to note that previous data [12] have shown that LPH does not carry sialic acid residues supporting the notion that the observed shift in the apparent molecular weight of LPH (Fig. 4, lane 2) is due to the presence of O-glycosidically-linked glycan units. By contrast, neuraminidase/*O*-glycanase did not affect the electrophoretic mobility of LPH_{dNM} (lanes 3 and 4), and LPH_{dMM} (lanes 5 and 6) indicating that these forms do not contain O-linked carbohydrates. On the other hand, a slight alteration in the electrophoretic appearance of LPH_{Swa} from a diffuse band to a sharper polypeptide could be observed upon treatment with neuraminidase/O-glycanase (Fig. 4, lanes 7 and 8). This suggests that LPH_{Swa} has undergone some O-glycosylation, but not as extensively as mature LPH_m. The mannose-rich precursors of pro-LPH, on the other hand, were devoid of O-linked sugars regardless of the presence or

Table 2
Proportions of brush border LPH[LPH(BBM)] in total cleaved LPH[LPH(IM + BBM)]* in the presence or absence of glycosidase inhibitors**

Glycosidase inhibitor	LPH (M_r)	% LPH(BBM)/ LPH(IM + BBM)
Not added	160,000	45.5
dNM	135,000	47
dMM	135,000	44.7
Swainsonine	141,000	43.4

*LPH(IM + BBM), total cleaved LPH that comprises LPH in the intracellular membranes (IM) and brush border membranes (BBM).
**The results are representative of two experiments.

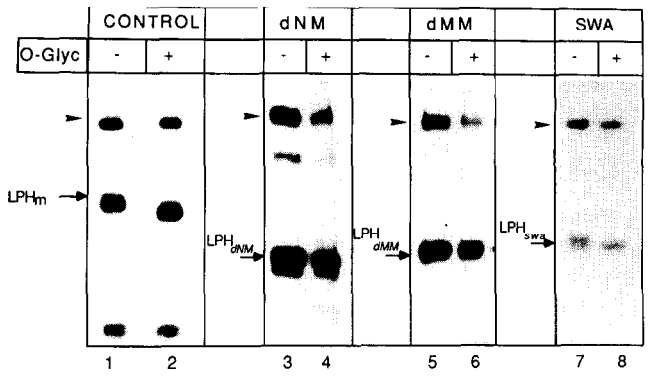

Fig. 4. Treatment of LPH glycoforms with neuraminidase/*O*-glycanase. Biopsy samples were labeled for 6 h with [^{35}S]methionine in the absence (lanes 1 and 2) or presence of 5 mM dNM (lanes 3 and 4), 5 mM dMM (lanes 5 and 6) or 6 μM swainsonine (Swa) (lanes 7 and 8), homogenized, solubilized and immunoprecipitated with anti-LPH antibodies. Each immunoprecipitate was divided into two equal parts, one of which was treated with neuraminidase/*O*-glycanase (lanes 2, 4, 6 and 8). The samples were analyzed by SDS-PAGE on 6% slab gels. The figure is composed of three gels (lanes 1 and 2) (lanes 3–6) (lanes 7 and 8). The shift in the apparent molecular weight of LPH (lane 2) is exclusively due to O-linked sugars and not due to neuraminidase treatment, since LPH does not carry sialic acids residues [12].

absence of glycosidase inhibitors, since no reactivity towards *O*-glycanase was observed (Fig. 4, lanes 1–8, upper bands indicated by the arrowheads). It should be noted that variations in the labeling intensities of the LPH glycoforms were observed after the incubations with or without neuraminidase/*O*-glycanase (Fig. 4, lanes 4, 6, 7 and 8). This could be due to varying susceptibilities of the different LPH glycoforms to contaminating proteases in the neuraminidase and *O*-glycanase preparations or due to spontaneous degradation during the long incubation procedure (24 h) at 37°C.

4. Discussion

The effect of glucosidase and mannosidase inhibitors on the transport and processing of pro-LPH could be clearly demonstrated by the appearance of cleaved LPH forms that varied in their glycosylation patterns. In the presence of dNM and dMM mannose-rich, endo H-sensitive cleaved LPH forms were detected, while in the presence of swainsonine a hybrid-type glycosylated, partially endo H sensitive LPH species was generated. These forms differ in their apparent molecular weights and in their endo H sensitivities from the cleaved LPH form (LPH_m) obtained in the absence of the inhibitors. Except for deoxynojirimycin (dNM), the presence of the glycosidase inhibitors (dMM/swainsonine) in the tissue did not generate significant variations in the proportions of pro-LPH that have undergone intracellular cleavage. The notable exception was the significant reduction in the proportion of cleaved LPH (LPH_{dNM}) observed upon inhibition of the early trimming events of the glucose residues in the ER. One possible explanation of these results is that carbohydrate processing of the pro-LPH precursor in the ER, but not in the *cis*-Golgi, is important for pro-LPH to acquire a correct, transport-competent conformation. Inhibition of carbohydrate processing by dNM, dMM and swainsonine did not have significant influence on the *trans*-Golgi to cell surface transport, since comparable proportions of the cleaved glycoforms were detected in the brush border membrane. In view of the observation that the only observed effect of carbohydrate processing is that in the presence of dNM, which functions in the ER, it is reasonable to assume that an effect of carbohydrate modification on the transport of LPH exists at the level of ER to Golgi transport. This in turn strongly supports the notion that the ER-Golgi transport constitutes the rate-limiting step along the secretory pathway of pro-LPH.

Perhaps the most important finding of this study is that inhibition of the processing of N-linked carbohydrates of pro-LPH has also inhibited or strongly interfered with O-glycosylation of LPH. In fact, the glycoforms generated in the presence of the inhibitors are either devoid of O-glycosidically-linked oligosaccharides (LPH_{dNM}, and LPH_{dMM}) or are not as extensively O-glycosylated (LPH_{Swa}) as the fully processed and complex glycosylated LPH molecule (LPH_m). It should be noted that dNM, dMM and swainsonine do not inhibit the addition of O-glycosidically-linked oligosaccharides per se. One possible explanation of this finding is that the inhibition of processing of N-linked carbohydrates of pro-LPH has induced alterations in the protein folding of the pro-LPH molecule, which in turn render the molecule inaccessible to glycosyltransferases involved in O-linked glycosylation. These alterations appear not to be directly involved in the transport of pro-LPH from the site of O-glycosylation in the *cis*-Golgi to the site of cleavage in the *trans*-Golgi, since in the presence of dMM and swainsonine, whose site of action is the *cis*-Golgi, normal transport to the *trans*-Golgi and cell surface was obtained. Moreover, the proteolytic cleavage of pro-LPH to LPH was not affected indicating that these conformational alterations do not involve regions in the molecule implicated in this event.

At present there is no uniform concept to understand the impact of carbohydrate processing of proteins on their intracellular transport. While in some cases efficient transport of secretory, membrane and lysosomal glycoproteins has been shown to be coupled to processing of their glycan units in the ER [17–23], several other investigations have demonstrated that these events are not rate-limiting of protein transport along the exocytotic pathway [17,24–27].

Our results have demonstrated that only inhibition of glucose trimming in the ER by dNM has significant effects on the intracellular transport of pro-LPH to the

cell surface. This suggests that a rate limiting step along the secretory pathway of pro-LPH and LPH does not exist in the Golgi. It is clear from all these examples that sugar modification in the ER and/or the Golgi induce alterations in the three dimensional structure or folding of proteins. Depending on the individual protein and the position of the N- and O-glycosylation sites within the polypeptide backbone these alterations could take place near or in structures that are implicated in the intracellular transport or perhaps in the function of the protein. Characterization of these regions may help unravel several aspects of the function and transport of these proteins.

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