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Analysis of lactase processing in rabbit

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The proteolytic processing of rabbit intestinal lactase-phlorizin-hydrolase (LPH) was studied by pulse-chase and continuous labeling experiments in organ culture from 15-day-old rabbits in the presence of glycosylation and processing inhibitors. Monensin and brefeldin A inhibited the two proteolytic cleavages of the precursor indicating that they are post-Golgi events as previously reported for the unique cleavage of LPH in man [1]. The inhibition was not related to a concomitant alteration glycosylation; in fact, if trimming was blocked by MDNM the abnormal glycosylated precursor was proteolytically processed normally. Finally the use of the anti-microtubular drug colchicine strongly inhibited both cleavages and caused accumulation of the complex-glycosylated precursor form in the brush border fraction indicating that proteolytic events depend on intact microtubule (transport).

Lactase; Biosynthesis; Proteolysis inhibition; Suckling; Rabbit

1. INTRODUCTION

Lactase-phlorizin-hydrolase (LPH; EC 3.2.1.22/62) is an integral glycoprotein of the small intestinal brush border membrane and of mammals. LPH carries two enzymatic activities: lactase and phlorizin hydrolase which are responsible for the hydrolysis of lactose and β-glycosylceramides [2–4]. Data on the primary structure of human and rabbit LPH from cDNA cloning [5] and biosynthetic labeling experiments [6–9] demonstrated that LPH is synthesized in a pre-pro form (215–245 kDa) which is split into the mature form (150–160 kDa). In particular, in rabbit, we [10] and others [11,12] found that a high-mannose glycosylated form of 200 kDa is first converted into a complex-glycosylated 215 kDa form. This is cleaved then into a 180 kDa and finally into the 150 kDa mature lactase. In a few cases of adult-type hypolactasia in humans delayed intracellular cleavage of pro-LPH to mature LPH has been demonstrated [13–15]. We have also observed quantitative differences in this processing along the whole small intestine and in suckling rabbit in comparison to the adult [10].

The cleavage of pro-LPH occurs intracellularly (in human and pig) [7,8,16], or perhaps close to or at the brush border membrane (in rat) [6]. In contrast to these results, Yeh et al., in experiments in vivo in rat, showed that the lactase precursor may indeed be cleaved by luminal proteases [17], possibly having escaped intracellular processing In organ culture at reduced temperature [1,18] and in the presence of brefeldin A [1] it was shown that the formation of mature LPH is preceded by complex glycosylation of the precursor form and that proteolytic processing occurs after passage of the pro-LPH through the Golgi complex [1]. Our findings in human [15] and rabbit [10] are in line with these conclusions. In a very recent work the full-length cDNA for human LPH was expressed in MDCK cells [19]; in these polarized cells proteolytic processing is not essential for sorting to the apical membrane domain, as both precursor and mature forms are routed to the brush border membrane.

In this study we have characterized the effect of some glycosylation and processing inhibitors on LPH synthesis to better define the localization and role of the proteolytic steps of the lactase precursor in rabbit. We show that both cleavages occur after the Golgi complex and that the proteolysis and not the transport of the precursor to the brush border membrane is blocked by colchicine.

2. MATERIALS AND METHODS

2.1. *Animals*

New Zealand white rabbits, 15 days old (sucklings), were used.

After sacrifice the middle jejunum was excised, extensively rinsed with cold 0.9% NaCl, and rapidly processed for organ cultures.

2.2. *Organ culture and in vitro biosynthesis*

Explants were cultivated as previously described [10] on stainless-steel grids in organ culture dishes. All explants were incubated for 1 h in methionine-free minimum essential medium supplemented with streptomycin (100 μg/ml) and penicillin (100 U/ml). Then 150 μCi/ml [^{35}S]-methionine was added for 30 min. After the labeling period the

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Abbreviations: LPH, lactase-phlorizyn hydrolase (EC 3.2.1.23/62); BFA, brefeldin A; MDNM, methildeoxynojirimicin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

grids were transferred to chase medium containing an excess of unlabeled methionine and explants incubated for different periods of time.

In the experiments with the inhibitors they were added to the preincubation medium and maintained during the whole incubation period. The inhibitors were used at the following concentrations: Brefeldin A (BFA), 10 μg/ml; monensin 50 μg/ml; methildeoxynojirimicin (MDNM), 350 μg/ml; colchicine, 50 μg/ml.

For subcellular fractionation and glycosydase treatment experiments cultures were continuously labeled for 1, 4 and 20 h. The collected samples were homogenized and LPH immunoadsorbed by the antilactase monoclonal antibody mlac 5 as previously described [10]. Following SDS-PAGE the gels were processed for fluorography [20]. Quantification of fluorographic bands was carried out by densitometric scanning with the laser densitometer Ultroscan XL (LKB, Bromma, Sweden).

2.3. *Glycosydase treatment*

LPH immunoadsorbed from explants labeled for 1, 4 and 20 h in the absence or presence of BFA or MDNM were digested with endoglycosidase H (endo H) or pN-glycopeptidase F (pNGase F) as previously reported [10].

2.4. *Subcellular fractionation*

The procedure was adapted from that of Danielsen et al. [7] and Shirazi-Beechey et al. [21]. Two labeled explants (about 40 mg) were resuspended and homogenized in 3.2 ml of 100 mM mannitol, 2 mM Tris-HCl, pH 7.1. $MgCl_2$ was added (10 mM final concentration) and the homogenate was slowly stirred at 4°C for 20 min. The sample was then centrifuged at $3000 \times g$ for 15 min to pellet the intracellular membrane fraction. The supernatant was removed and centrifuged 40 min at $20,000 \times g$ to obtain the brush border fraction pellet. The pellets were resuspended in homogenization buffer and solubilized by adding 0.5% Nonidet P-40 and 0.5% sodium deoxycholate. The effectiveness of the fractionation procedure was confirmed by measuring the enrichment of the lactase specific activity in the brush border fraction.

3. RESULTS

3.1. *Subcellular fractionation*

Fig. 1 shows the pattern of radiolabeled LPH proteins observed at various labeling times in the intracellular (lanes 1, 3 and 5) and brush border membranes (lanes 2, 4 and 6). The 200 kDa high mannose precursor was localized only in the intracellular membranes at all the labeling times. Practically no bands were observed in the brush border membranes at 1 h, while at 4 h the 215 kDa complex-glycosylated precursor, the 180 kDa and the 150 kDa mature forms [10] were clearly detected. At 20 h the complex precursor on the brush border fraction was largely converted into the other forms: in particular mature lactase increased and represented the predominant band.

3.2. *Monensin and BFA inhibit both proteolytic cleavages*

Monensin blocks or slows down protein transport through the Golgi complex [22–24]. Brefeldin A (BFA) causes redistribution of *cis*-, medial- and *trans*-Golgi, but not *trans*-Golgi network (TGN) enzymes back to the endoplasmic reticulum [25]. Fig. 2 shows pulse-chase experiments in the presence of these inhibitors. In the control experiment the 200 kDa high mannose precursor appeared after 30 min of pulse and predominated

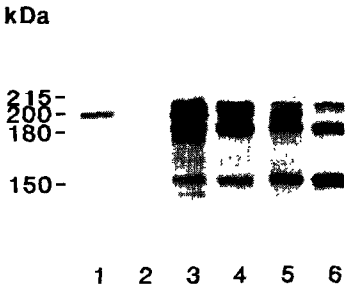

Fig. 1. Subcellular fractionation of radiolabeled LPH proteins. Explants were continuously labeled for 1 h (lanes 1 and 2), 4 h (lanes 3 and 4), or 20 h (lanes 5 and 6). After culture the intracellular membranes (lanes 1, 3 and 5) were separated from brush border (lanes 2, 4 and 6); LPH proteins were isolated from the two fractions and analyzed by SDS-PAGE and fluorography.

up until 2 h of chase, while the complex precursor and the 180 kDa bands were evident at 2 h and at 4 h, respectively. The 150 kDa mature lactase was visible at 4 h but only at 20 h of chase became the predominant band. Monensin inhibited the complex-glycosylation of the precursor (after 6 h of chase the 200 kDa high-mannose precursor was predominant) and proteolytic cleavages (after 4 h of chase the 180 and the mature 150 kDa forms were barely detectable; however, after 6 h both these products were present). After 20 h of chase there was a decrease of the label incorporation in all molecular forms, probably due to intracellular proteolysis.

Addition of BFA to the culture medium completely inhibited the proteolytic steps of lactase processing (Fig. 2). In fact only one form of LPH of approximatively 200 kDa was present at all chase times.

3.3. *Altered glycosylation does not inhibit proteolysis of LPH proteins*

Pulse-chase experiments were performed in the presence of (MDNM). MDNM inhibits glucosidase I, the first enzyme implicated in the trimming of the *N*-linked oligosaccharides [26] and causes alteration of the normal glycosylation pattern [27]. In fact, in the presence of this inhibitor the 200 kDa high mannose precursor of lactase was not transformed into the 215 kDa complex-precursor but only one band of approximately 220 kDa was evident till 6 h of chase (Fig. 2). Nevertheless transformation to the 180 and 150 forms occurred without apparent delay. In order to assess the glycosylation pattern of the LPH proteins after BFA and MDNM treatments, continuous labeling experiments were performed in the presence of these inhibitors and the immunoisolated proteins were digested with endoglycosydase-H (endo-H) or pN-glycosidase-F (pNGase-F). The results are shown in Fig. 3. Only the endo-H-sensitive high mannose precursor was detectable at 1 h in the control and in the presence of inhibitors (Fig. 3A, lanes 1–3). The presence of uncleaved glucose residues could

be responsible for the higher molecular weight (220 kDa) observed in the presence of MDNM, as all forms were transformed after glycosidase treatment into a 185 kDa band (lanes 4, 5 and 6). After 4 h of labeling the control showed three bands of 215, 180 and 150 kDa (Fig. 3B, lane 1) all endo-H-resistent (lane 4) and pnGase-F-sensitive (lane 7). Three bands were also detected in the presence of MDNM, but all of them showed higher molecular weights in comparison to control tissue (lane 2) and were endo-H-sensitive (lane 5). The molecular weight of the complex and intermediate forms after endo-H treatment were higher than after pNGase-F digestion. Similar results were obtained at 20 h (Fig. 3C).

As to the sole band (about 200 kDa) observed in the presence of BFA (Fig. 3B, lane 3) it was both endo-H-resistent (lane 6) and pNGase-F-sensitive (lane 9). At 20 h no bands were detected in the presence of BFA.

3.5. *Partial inhibition of the proteolytic steps by colchicine*

The pattern of biosynthesis of LPH proteins in the presence of BFA and monensin suggest that in rabbit both proteolytic steps occurred after the *trans*-Golgi. The possibility that proteolysis occurs between the Golgi complex and the brush border was tested by inhibiting the membrane transport traffic with colchicine, an anti-microtubular agent known to disturb the transport of microvillar proteins [28,29].

Table I shows data (means ± S.D., $n = 3$) of radiolabeling incorporation into LPH proteins from pulse-chase experiments in the absence or presence of colchic-

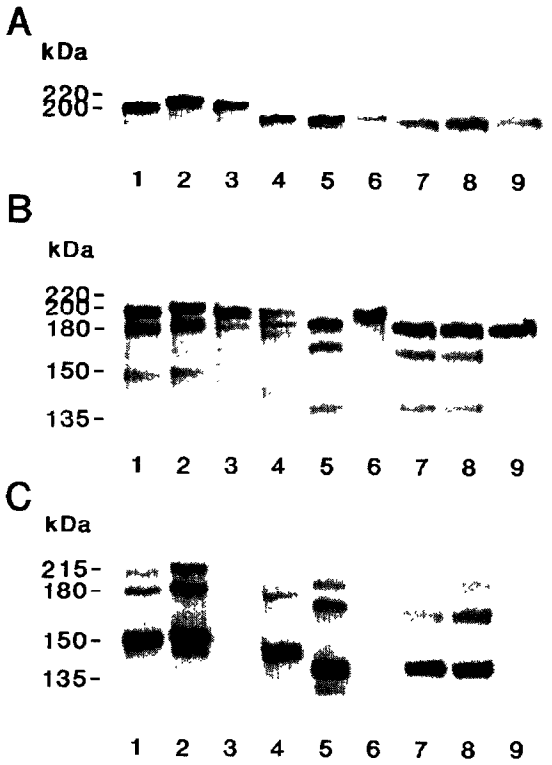

Fig. 3. Glycosylation pattern of LPH proteins isolated at 1 h (A), 4 h (B) and 20 h (C) of labeling in the presence of MDNM or BFA. Explants were continuously labeled for 1, 4 and 20 h in the absence (lanes 1, 4 and 7) or presence of MDNM (lanes 2, 5 and 8) or BFA (lanes 3, 6 and 9). LPH proteins were then isolated and incubated in absence of glycosidases (lanes 1–3) or subjected to endo-H (lanes 4–6) or pNGase-F (lanes 7–9) treatments before to be analyzed by SDS-PAGE and fluorography.

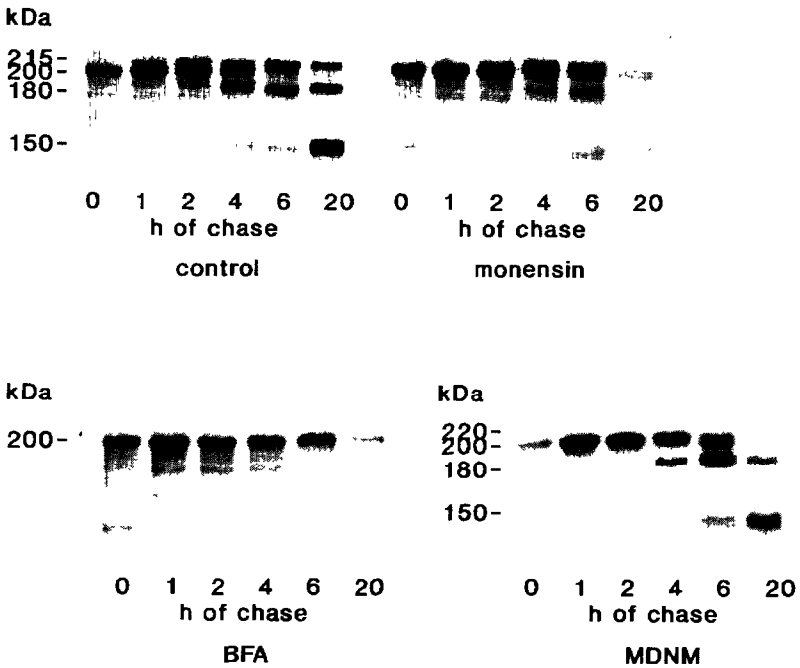

Fig. 2. Influence of monensin, BFA and MDNM on the lactase processing. Explants were pulsed for 30 min and chased for the indicated periods of times in the absence or presence of inhibitors of BFA, monensin or MDNM. After culture LPH proteins were immunoisolated and analyzed by SDS-PAGE and fluorography.

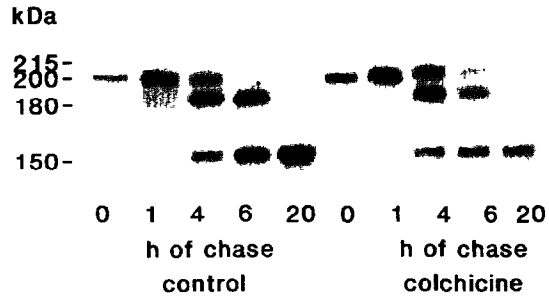

Fig. 4. Effect of colchicine on the LPH processing. Explants were pulsed for 30 min and chased for the indicated times in the absence or presence of colchicine added 1 h before the pulse. LPH proteins were then analyzed as described in Fig. 1.

ine. One of these experiments is shown in Fig. 4. After 4 h of chase the labeling of the 215 kDa complex-glycosylated precursor increased in the presence of colchicine (36%; 23.7% in the control), while the intensity of the 180 kDa band was apparently unchanged or little increased (31.7%; 24.3% in the control); the conversion into the 150 kDa mature form was instead clearly inhibited (32%; 51.7% in the control). At 6 h the effect of colchicine was also evident: the 180 and 150 kDa represented 24% and 44.7%, respectively (18% and 71% in the control) and the 215 kDa form increased to 31.7%; 10.7% in the control); however, after 20 h of chase no inhibitory effect was observed.

To study whether colchicine also influenced the cellular distribution of LPH proteins, explants were continuously labeled for 1 h and 4 h in the absence or presence of colchicine and intracellular and brush border membrane fractions were separated. The result shown in Fig. 5 is representative of two independent experiments.

Table I

Effect of colchicine on the processing of LPH proteins

Time of chase (h)	LPH proteins		
	150 kDa	180 kDa	200 + 215 kDa
Control			
0	0	0	100
1	0	0	100
4	51.6 ± 13.3	24.3 ± 11.9	23.6 ± 4.0
6	71.0 ± 19	18.0 ± 20.7	10.6 ± 1.2
20	96.7 ± 4.7	3.3 ± 4.7	0
Colchicine			
0	0	0	100
1	0	0	100
4	32 ± 3.5	31.7 ± 4.5	36 ± 1.4
6	44.7 ± 6.8	24.0 ± 11.5	31.7 ± 6.1
20	96.7 ± 4.7	3.3 ± 4.7	0

LPH proteins were immunoisolated from pulse-chase experiments performed in the absence or presence of colchicine. After SDS-PAGE and fluorography the different forms of lactase were quantified by densitometric scanning. Results from three independent experiments were averaged and expressed as mean ± S.D.

After 1 h of labeling the 200 kDa band was found in quite similar amounts in the control and colchicine-treated explants and only in the intracellular membrane (Fig. 5). In the presence of colchicine the 180 and 150 kDa forms were decreased and the 215 kDa complex-glycosylated precursor was increased both in the intracellular and brush border membrane fractions.

4. DISCUSSION

In the present paper we have studied in suckling rabbit the biosynthesis of LPH proteins in the presence of various inhibitors in order to localize where the proteolysis of the precursor into the 180 and 150 mature forms occurs and to study the role of these proteolytic steps for the correct expression of lactase on the brush border membrane.

The high-mannose 200 kDa precursor was found to be localized only in intracellular membranes, and not in brush border, while the other forms of LPH proteins were present in both fractions. This suggests that there is very low contamination (if any) of the brush border fraction by intracellular membranes. The amount of the complex-glycosylated precursor was found to decrease at increasing labeling times, in parallel to the increase of the 180 and 150 mature forms, suggesting that both proteolytic processes occurs in both membrane fractions. The complex glycosylated precursor has been found to be present in the brush border membranes in rat [6] and human (Rossi et al., unpublished), but not in pig [7], suggesting that the proteolytic cleavage into the mature form may occur also after insertion into the microvillar membrane.

The inhibition by monensin and BFA of the formation of the 180 and 150 kDa mature forms is strong evidence that both proteolytic cleavages occur after the Golgi complex, in agreement with previous studies in man [1]. This inhibition was not the consequence of the altered complex glycosylation as suggested by the experiment with MDNM. In fact this drug caused the appearance of a malglycosylated precursor form that was partially endo-H-sensitive and that was converted into 180 and 150 kDa mature forms. This result is in line with

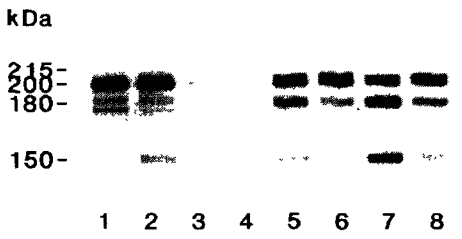

Fig. 5. Subcellular distribution of LPH proteins after colchicine treatment. Explants were continuously labeled for 1 (lanes 1–4) and 4 h (lanes 5–8) in the absence (lanes 1, 3, 5 and 7) or presence of colchicine (lanes 2, 4, 6 and 8). After culture the intracellular membranes (lanes 1, 2, 5 and 6) were separated from brush border (lanes 3, 4, 7 and 8); LPH proteins were isolated from the two fractions and analyzed by SDS-PAGE and fluorography.

results of previous studies [30] showing that after incubation with castanospermine LPH is still transported to the brush border membrane and proteolytically converted to the mature form. However, the labeled incorporation into LPH proteins was reduced after incubation with castanospermine whereas MDNM treatment did not reduce the lactase biosynthesis in our chase experiments (Fig. 2). Interestingly, the complex-precursor and the 180 kDa form were partially endo-H-resistent after MDNM treatment, whereas the mature form was completely endo-H-sensitive (Fig. 3). In Caco-2 cells MDNM led to incorporation of hybrid oligosaccharide structures, insensitive to endo-H, into sucrase molecules [27]. The discrepancies we observed could be explained with the hypothesis that hybrid carbohydrate chains are formed in the pro-peptide of LPH precursor but not in the mature form. These results need further elucidation.

The role of microtubules in the apical transport of microvillar enzymes is well known [28,29,31]. It has, in fact, been demonstrated that colchicine impairs the transport of enzymes to the brush border membrane. In Caco-2 cells this drug slows down the transport to the apical membrane of aminopeptidase-N and sucrase, suggesting that an intact microtubular system is crucial for the final polarity of these proteins [32]. In our study colchicine resulted in an inhibition of the splitting of the 215 kDa complex-glycosylated precursor, which then reached the brush border in larger amounts. Grunberg et al. [19] hypothesized that the protease(s) responsible for the cleavage of pro-LPH may be sorted along with the same pro-LPH in the vesicles destined for apical membranes and that splitting can occur also during transport. Our findings are compatible with the hypothesis that a subcellular compartment, preceding the microtubule-dependent vesicular transport, is present within the brush border fraction. In this organelle the 215 kDa form accumulates in the presence of colchicine when splitting is delayed. Alternatively we may suppose that the complex-precursor is transported to the brush border membrane in a microtubule independent way.

In conclusion proteolytic events apparently occur between the Golgi complex and the brush border membrane. Even if proteolysis of lactase seems not to be essential for proper sorting of the protein to the apical membrane [19], it could be important for proteins reaching the apical surface through the microtubule machinery.

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