Human lactase-phlorizin hydrolase expressed in COS-1 cells is proteolytically processed by the lysosomal pathway

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Abstract Lactase-phlorizin hydrolase (LPH) (EC 3.2.1.23/62), a major glycoprotein of the microvillus membrane of human small intestinal epithelial cells (enterocytes), is vital for the digestion of lactose during early infancy. The enzyme is synthesized in enterocytes as a single-chain precursor and subsequently proteolytically processed to the mature microvillus membrane-bound form. Because it has been reported that COS-1 cells were not able to proteolytically process LPH to the mature protein, these cells have been used as a model system to study potential roles of different proteases. COS-1 cells transfected with a full-length cDNA for human LPH synthesized enzymatically active enzyme. Immunoprecipitation of the expressed glycoproteins and their subsequent analysis by SDS-PAGE showed synthesis of two polypeptide species having apparent molecular masses of 210 and 220 kDa, respectively, corresponding to the high-mannose (pro-LPH$_h$) form and the complex glycosylated (pro-LPH$_c$) form of the LPH precursor. Surprisingly, an additional polypeptide species corresponding in size to the mature LPH found in human intestinal cells was also detected after longer chase periods. The source of this species was clearly pro-LPH, as its formation was inhibited by Brefeldin A. The cleaved form of LPH was not found on the cell surface; furthermore, its formation was prevented by an inhibitor of lysosomal function. We conclude from these data that in transfected COS-1 cells pro-LPH is transported to the cell surface, from which it is internalised and enters the lysosomal pathway, where proteolytic cleavage leads to a molecule not unlike mature LPH.

1. Introduction

Lactase-phlorizin hydrolase (LPH) (EC 3.2.1.23/62), an integral glycoprotein of the microvillus membrane of small intestinal epithelial cells, is responsible for the hydrolysis of lactose to its constituent monosaccharides. In enterocytes, LPH is synthesized as a single-chain precursor, pro-LPH (M_r = 215–245 kDa), which undergoes proteolytic processing to yield the mature microvillus membrane form, m-LPH (M_r = 160 kDa) [1–4]. The complete primary structure of rabbit and human pro-LPH has been deduced from cDNA cloning [5].

A number of studies have been published on the processing of lactase in organ cultured rat [3], pig [2] and human [4] mucosa. Büller et al. suggested that in rat intestine LPH processing is a two-step cleavage event, with the first cleavage taking place intracellularly and the second after insertion into the microvillus membrane. In pig [2] and in human intestine this proteolytic cleavage takes place intracellularly prior to insertion into the microvillus membrane [4]. In a human intestinal cell line, Caco-2, and in intestinal explants in organ culture, proteolytic processing of pro-LPH was shown to occur after passage of the precursor through the Golgi complex [6]. Surface immunoprecipitation of LPH from Madin Darby canine kidney (MDCK)-17 cells, which permanently expressed human LPH, demonstrated that LPH-processing occurs after sorting and is not necessary for surface expression [7].

Transfected COS-1 cells are a widely used model to study expression of eukaryotic proteins. Transfected with cDNA encoding human LPH, COS-1 cells have been reported to lack the processing enzymes responsible for maturation of LPH [8]. These cells thus promised to be a good model to investigate potential LPH-processing proteases.

We transiently transfected COS-1 cells with a full-length cDNA encoding human LPH precursor. Surprisingly, our data with LPH cDNA-transfected COS-1 cells show that human LPH is proteolytically processed in these cells to a molecule similar in size to mature LPH. This process was dependent on surface expression of pro-LPH and was arrested by an inhibitor of lysosomal function. The data further show that a partially hydrolysed LPH such as the mature enzyme is very resistant to further degradation, a property which helps the enzyme survive in the hostile environment of the duodenum.

2. Materials and methods

2.1. Reagents and materials

L-[^{35}S]methionine (1000 Ci/mmol) was from DuPont NEN. All SDS-PAGE chemicals were purchased from BioRad Laboratories. Endoglycosidase H (endo H) and endoglycosidase F/N-glycosidase F (endo F/GF) were purchased from Boehringer Mannheim. Protein A–Sepharose™ CL-4B was purchased from Pharmacia. Brefeldin A (BFA), bovine serum albumin (BSA), phenylmethane sulphonyl fluoride (PMSF), pepstatin, aprotinin, leupeptin, benzamidine and high molecular mass protein standards (cat. #SDS-6H) were from Sigma. Cell culture media, penicillin and streptomycin were obtained from Gibco BRL. Fetal calf serum (FCS) was from Biological Industries. All other chemicals were analytical grade from Merck. The pRB-1H vector containing the full-length cDNA coding for LPH was kindly provided by Dr. Ned Mantei and Rahel Bänzinger (Swiss Federal Institute of Technology, Zürich).

2.2. Determination of lactase activity in COS-1 cells

Two days post-transfection the COS-1 cells were harvested to determine lactase activity. First, cells grown in a 10 cm petri dish were washed twice in ice-cold PBS and then scraped with a rubber spatula in 1 ml of Tris–mannitol buffer. Cells were sonicated 20–30 s (homogenate). Rupture of the cells was checked by trypan blue staining.

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Abbreviations: LPH, lactase-phlorizin hydrolase; pro-LPH, high molecular mass precursor of lactase-phlorizin hydrolase; m-LPH, mature lactase-phlorizin hydrolase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; NP40, Nonidet P40; DOC, sodium deoxycholate; PBS, phosphate-buffered saline; DEAE-dextran, *o*-(dimethylaminoethyl)-dextran; endo H, endoglycosidase H; endo F/GF, endoglycosidase F/N-glycosidase F

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Nuclei were spun down at 3500 rpm for 10 min at 4°C (pellet=nucleus fraction). The supernatant was centrifuged at 100 000×*g* for 30 min to pellet the cell membranes (membrane fraction). Lactase activity was measured in cell homogenates, membrane pellets and in the final supernatant according to Dahlqvist [9] using lactose as substrate.

2.3. Cells and cell culture

COS-1 cells were a generous gift from Dr. J. Kruppa (Hamburg). COS-1 cells were grown as monolayers in Eagle's minimal essential medium with Earle's salts (EMEM) supplemented with 10% (v/v) FCS, 100 mU/ml of penicillin and 100 μg/ml of streptomycin.

MDCK-17 clone expressing human LPH permanently has been previously characterized [7]. To establish this cell clone, MDCK cells were cotransfected with pRB-1H containing the full-length cDNA for human LPH and pXT1 containing the neo^r resistance gene for selection in geneticin-containing medium [7,10]. The transfected MDCK cells were grown as monolayers in Earle's minimal essential medium with Earle's salts (EMEM) supplemented with 5% (v/v) FCS and 400 μg/ml G418. The medium was changed every 2 days. The expression of the transgene was enhanced by culturing the cells overnight in medium containing 8 mM sodium butyrate.

2.4. Transfection of COS-1 cells and biosynthetic labelling

Low passage number (<25) of COS-1 cells were used for DEAE–Dextran transfection. The method was as described by Cullen [11]. In brief, 1.5×10^6 COS-1 cells were seeded onto 100-mm dishes 24 h before transfection. The semi-confluent cells were transfected with 1 μg of recombinant DNA (pRB-1) using DEAE–Dextran. pRB-1H contains the full-length cDNA for human LPH. The transfection procedure used included a chloroquine shock [12]. After transfection, cells were maintained in fresh medium. Cells were used 48–72 h post-transfection. Prior to labelling, medium was removed and the cells were incubated for 1 h in cell culture medium, lacking methionine. Cells were either continuously labelled (different pulse times were used as indicated in the appropriate sections of the text) with 50 μCi of [^{35}S]methionine, or pulse labelled first, followed by a chase for different lengths of time in medium containing 10 mM non-radioactive L-methionine.

When used, BFA was added to cell cultures at a final concentration of 1–5 μg/ml. Inhibitor of lysosomal function was added 1 h before and during labelling at concentrations of 1–20 mM (NH_4Cl).

2.5. Immunoprecipitation of human LPH

Labelled COS-1 and MDCK cells were washed 3 times with 5 ml of ice-cold PBS and lysed by adding 1 ml of lysis buffer (25 mM Tris-HCl, pH 8.0, 50 mM NaCl supplemented with 1% NP-40, 1% DOC, 100 μg/ml PMSF, 10 μg/ml leupeptin, 2 μg/ml pepstatin, 34.8 μg/ml benzamidine, and 2 μg/ml aprotinin. The lysates were centrifuged at 100 000×*g* for 30 min prior to immunoprecipitation. Solubilised membrane proteins were precleared twice with protein A–Sepharose beads and transferred to tubes with 100 μl of antibody–protein A–Sepharose (containing 3 μl of the monoclonal antibody HBB 1/909/34/74 against lactase [13]). After an incubation period of at least 2 h at 4°C, beads were washed 3 times with 1 ml of PBS containing 0.5% NP-40, 0.05% DOC and 0.05% SDS and then twice with 125 mM Tris-HCl (pH 8.2), 500 mM NaCl, 1 mM EDTA, 0.5% NP-40.

2.6. Surface immunoprecipitation

COS-1 cells were grown in 60-mm dishes, transfected and labelled as described above. The cells were incubated for 30 min in serum-free EMEM before cell surface proteins were immunoprecipitated [14]. The cells were washed 4 times with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 6.47 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). After washing the cells, 3 μl of the monoclonal antibody against lactase [13] in 1 ml of PBS were added in the medium. After 30 min incubation with gentle agitation at 4°C, the cells were washed 4 times with PBS and an excess of unlabelled cell protein from pRB-1H-transfected COS-1 cells in 1 ml of PBS were added to the cells to saturate the remaining free antibody binding sites. After 15 min at 4°C, cells were washed again with PBS, scraped and lysed in 1 ml of 25 mM Tris-HCl (pH 8.0), 50 mM NaCl buffer supplemented with 1% NP-40, 1% DOC, 100 μg/ml PMSF, 10 μg/ml leupeptin, 2 μg/ml pepstatin, 34.8 μg/ml benzamidine, and 2 μg/ml aprotinin for 30 min on ice. After ultracentrifugation to remove cell debris the antigen–antibody complexes were adsorbed to protein A–Sepharose and analysed by SDS-PAGE. To isolate intracellular protein solubilized cell extracts were subsequently immunoprecipitated a second time with the lactase antibody bound to protein A–Sepharose.

2.7. Endoglycosidase H and endoglycosidase F-treatment (endo F/GF)

Digestion of immuno-isolated LPH with endo H and endo F/GF was carried out as previously described [4]. Treated proteins were recovered by precipitation with 30% (w/v) trichloroacetic acid, washed, and kept at −20°C until analysed by SDS-PAGE together with undigested proteins.

2.8. SDS-PAGE

Immunoprecipitates and proteins subjected to endo H treatment were solubilized with 30 μl of 2-fold concentrated electrophoresis sample buffer containing 4% SDS, 20% glycerol, 5% 2-mercaptoethanol or 5 μl of 2 M M-dithiothreitol, boiled for 4 min and submitted to electrophoresis on 6% acrylamide gels according to Laemmli [15]. The gels were stained with Coomassie blue R250, destained with 40% methanol/10% acetic acid, treated with sodium salicylate, and exposed to Kodak X-OMAT AR films at −80°C.

3. Results

3.1. Human LPH expressed in COS-1 cells is enzymatically active

The activity of human LPH expressed in COS-1 cells was determined in cell homogenates and an enriched plasma membrane-fraction. The specific activity of lactase was 5.9 and 19.8 IU/g protein in the homogenate and the membrane fraction respectively ($n=2$). In comparison, specific activity in mucosal homogenates was 35 IU/g protein ($n=3$), and in enriched microvillus membrane fractions it was 275 IU/g protein ($n=2$). The activity of lactase in the COS-1 cell membrane fraction was enriched by a factor of 3.4. 100% of the activity in the homogenised transfected COS-1 cells was recovered in the membrane-fraction. These results clearly showed that the lactase expressed in COS-1 cells was enzymatically active and membrane bound.

3.2. Lactase-phlorizin hydrolase expressed in COS-1 cells undergoes proteolytic processing

Fig. 1A shows a pulse-chase labelling experiment with pRB-1H transfected COS-1 cells. Expressed human LPH was synthesized as a 210 kDa high mannose precursor molecule (pro-LPH_h). After 2 h of chase a complex glycosylated 220 kDa precursor form (pro-LPH_c) appeared. These findings are consistent with data obtained from small intestinal epithelial cells and endo H analysis (Fig. 1B) [4]. A 145 kDa form corresponding in size closely to mature LPH (m-LPH) was detectable after 5 h of chase. Upon longer chases, the ratio pro-LPH/m-LPH decreased. This indicated that some proteolytic processing occurred in COS-1 cells resulting in the appearance of a molecular species similar in size to the mature LPH found in human intestine [4], Caco-2 [6] and transfected MDCK cells [7,16].

In Fig. 1B human LPH expressed in COS-1 cells is compared to that in human intestinal biopsies. As assessed by SDS-PAGE, pro-LPH synthesized in COS-1 cells (220 kDa) corresponded closely to the equivalent species synthesized in human intestinal biopsies (215 kDa). The processed form in COS-1 cells (145 kDa) showed somewhat different electrophoretic properties to the mature form in human intestine (160 kDa). Endo F treatment prior to SDS-PAGE in both cases yielded polypeptides with identical mobilities, indicating that mobility differences are due to differences in glycosylation.

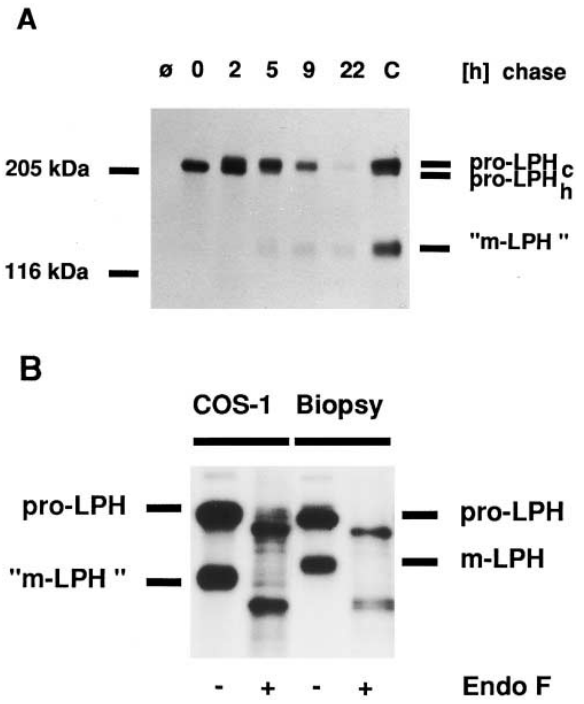


Fig. 1. A: Biosynthesis and post-translational processing of LPH expressed in COS-1 cells. The cells were pulse-labelled 48 h post-transfection for 1 h with 50 μCi/60 mm culture dish [^{35}S]methionine, then chased for the indicated time points. Analysis was by SDS-PAGE and fluorography. Exposure of the Kodak X-OMAT AR film was 4 h. Pro-LPH$_h$ = high-mannose form of the precursor molecule with an apparent molecular mass of 210 kDa. Pro-LPH$_c$ = complex glycosylated precursor molecule with an apparent molecular mass of 220 kDa. m-LPH = Putative mature 145 kDa form of LPH. ∅ = Mock transfected COS-1 cells pulse-labelled for 22 h and then treated as described above. C = LPH transfected COS-1 cells continuously labelled for 23 h. B: Comparison of LPH expressed in COS-1 cells and human intestinal explants. Transfected COS-1 cells and human intestinal explants were continuously labelled for 22 and 6 h, respectively with [^{35}S]methionine. Immunoisolated LPH was split into two aliquots, one control and one sample which was treated with endo F and analysed by SDS-PAGE and fluorography.

3.3. *Brefeldin A arrests the proteolytic processing of pro-LPH in COS-1 cells*

As shown in Fig. 2, different concentrations of BFA prevented the formation of 'mature' LPH after 7.5 h of chase. Furthermore, the effect of BFA was reversible, as m-LPH reappeared after a 21 h chase when the drug was removed from the culture medium during chase. These data clearly demonstrate a precursor/product relationship between the pro-LPH and the m-LPH in transfected COS-1 cells and correlate well with data obtained using Caco-2 cells and intestinal explants [6], which demonstrated that proteolytic processing of pro-LPH occurred after passage of precursor through the Golgi complex.

3.4. *The proteolytically processed form of LPH is not found on the cell surface*

Using cell surface immunoprecipitation assays the surface expression of human LPH in COS-1 cells was investigated. The pulse-chase experiment summarised in Fig. 3 shows surface expressed LPH on the left and intracellular LPH on the right. In addition, LPH species immuno-isolated after continuous labelling of cells for 24 h are shown (lanes C). Two hours after the initiation of chase the complex glycosylated precursor form (pro-LPH$_c$) appeared on the cell surface. The proteolytically processed form was not detectable on the cell surface. When lactase transfected COS-1 cells were labelled for 24 h, only traces of m-LPH and no pro-LPH$_h$ were detected on the cell surface (lane C, surface). These species were subsequently present in the total precipitate after surface immunoprecipitation (lane C, intracellular). The LPH immunoprecipitated from the intracellular compartment showed an electrophoretic pattern identical to that shown in Fig. 1. The lack of m-LPH on the cell surface of transfected COS-1 cells indicated a different proteolytic mechanism in these cells compared to intestinal epithelial cells.

3.5. *NH_4Cl inhibits the proteolytic cleavage of pro-LPH expressed in COS-1 cells but not in MDCK cells*

To investigate possible lysosomal involvement in the pro-

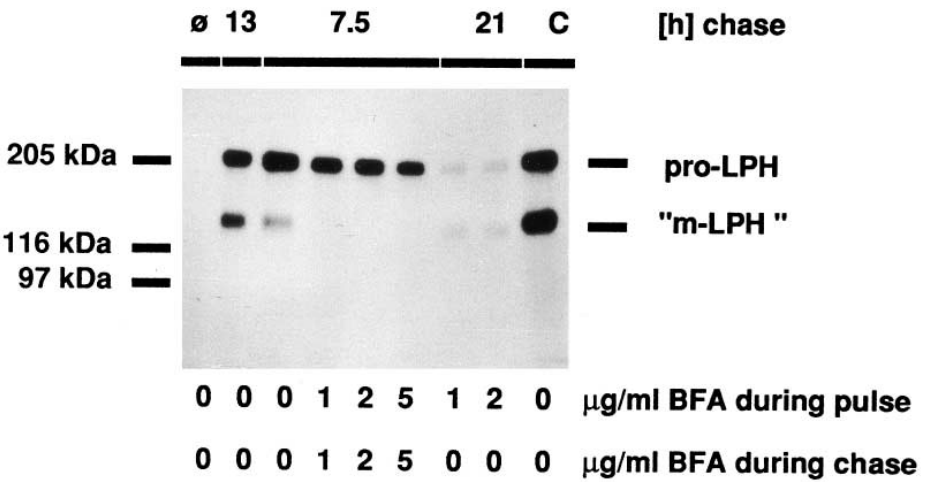


Fig. 2. Effect of BFA on processing of LPH expressed in COS-1 cells. Transfected cells were labelled with 50 μCi [^{35}S]methionine for 1 h in the absence or presence of different concentrations (μg inhibitor/ml medium) of BFA. ∅ = mock transfected COS-1 cells labelled for 1 h and chased for 21 h in the absence of BFA. C = LPH transfected COS-1 cells continuously labelled for 21 h in the absence of BFA. Pro-LPH = precursor form of LPH; m-LPH = putative mature form of LPH.

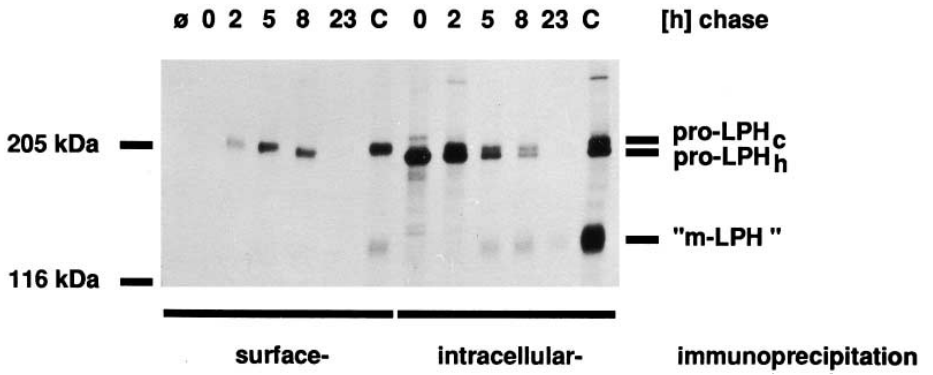
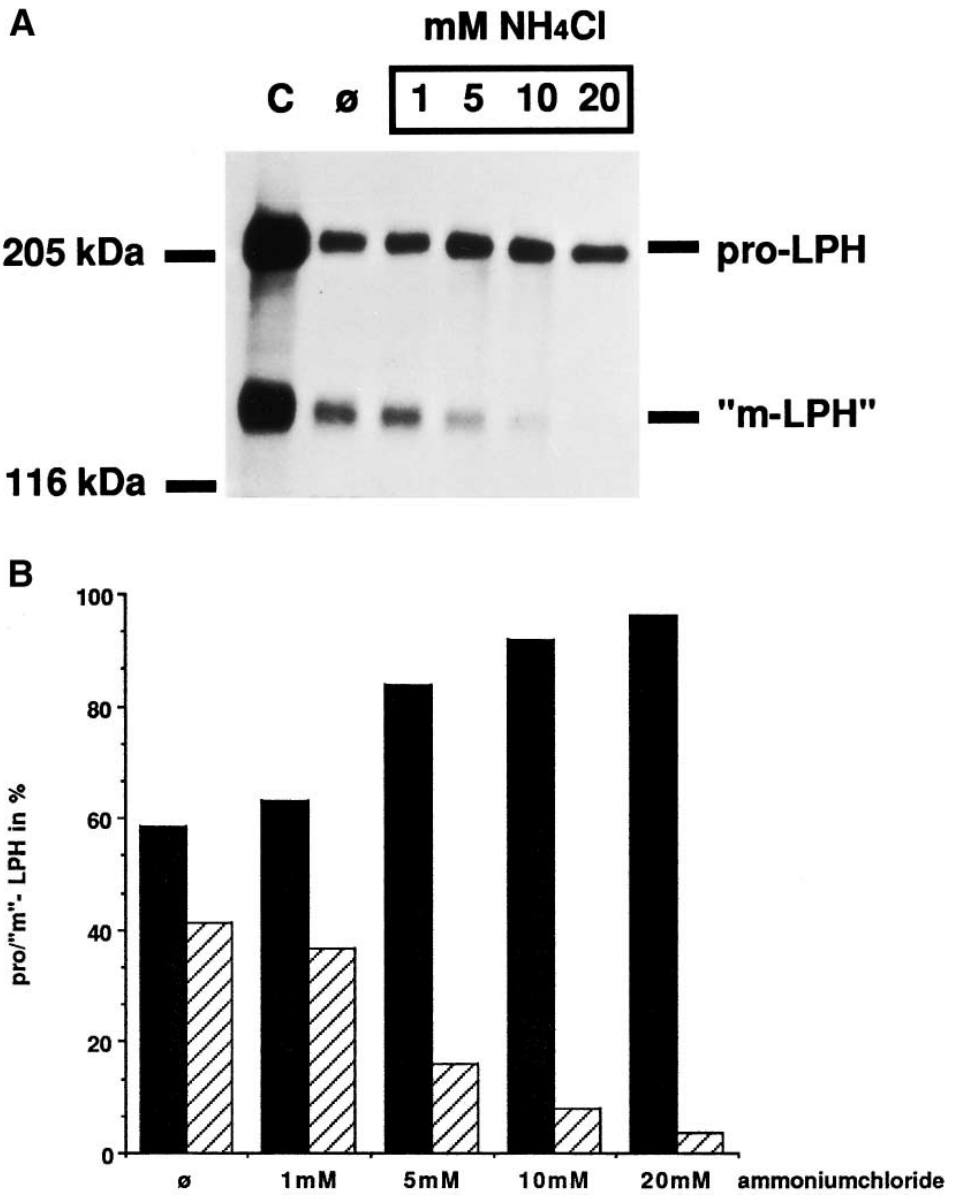


Fig. 3. Pulse-chase analysis and surface expression of LPH in COS-1 cells. After pulse-labelling for 1 h and subsequent chasing for 2, 5, 8, 23 h, surface expressed LPH was isolated by surface immunoprecipitation (see Section 2 for details). The remaining intracellular LPH was isolated by a further immunoprecipitation. ∅ = mock transfected COS-1 cells labelled for 23 h, lysed and total immunoprecipitated. C = LPH transfected cells continuously labelled for 24 h, surface expressed LPH immunoprecipitated first followed by intracellular LPH. Pro-LPH$_c$ = complex glycosylated form of precursor; pro-LPH$_h$ = high mannose glycosylated form of precursor; m-LPH = putative mature form of LPH.



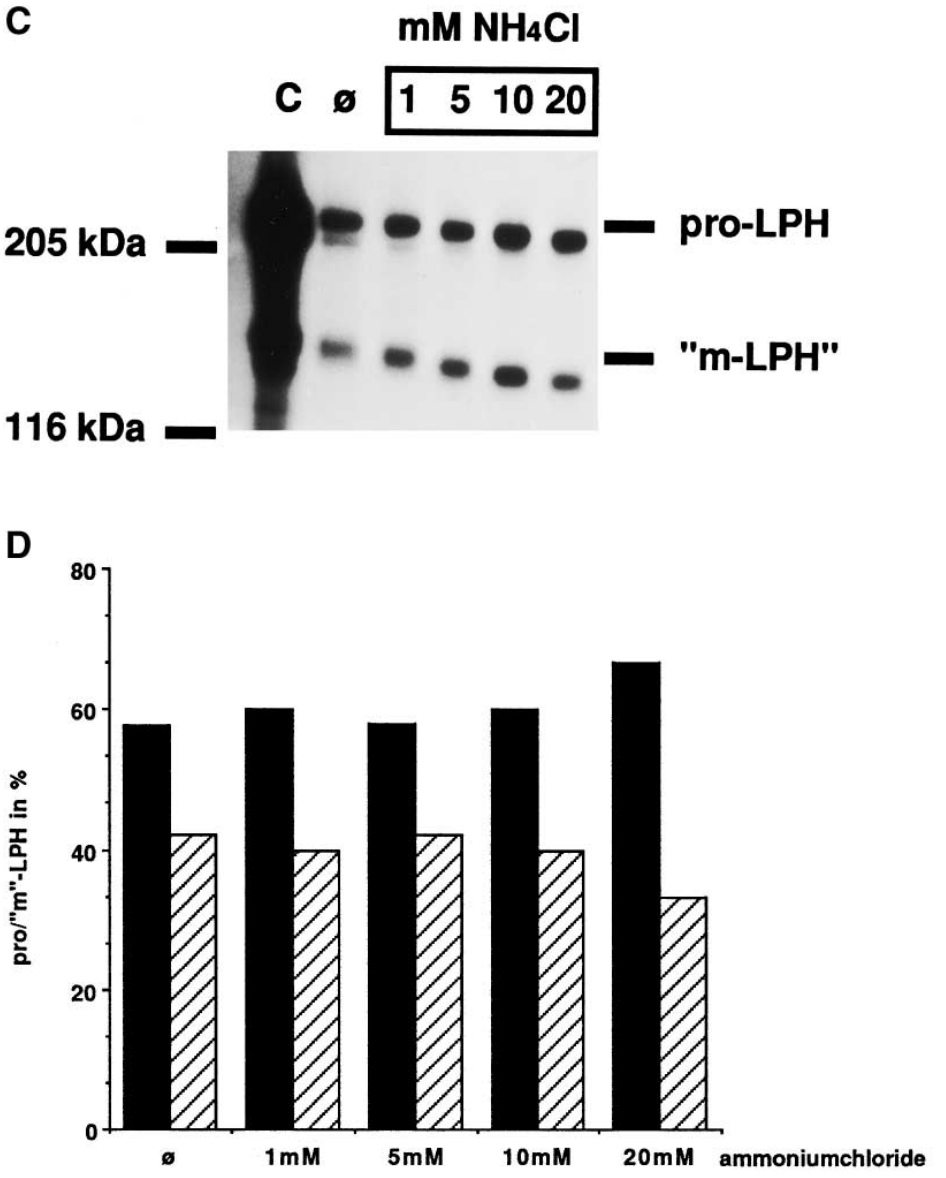


Fig. 4. Pulse-chase analysis in the presence of ammonium chloride. Panel A: LPH transfected COS-1 cells were pulsed for 1 h and chased for 16 h in the presence of the indicated inhibitor concentrations. After chasing cells were lysed, LPH was immunoprecipitated and subjected to SDS-PAGE and fluorography. Exposure of the film was 2 days. C = LPH transfected COS-1 cells continuously labelled for 16 h in the absence of inhibitor. Panel B: The relative intensities of the signals on the fluorographs were quantified using a laser densitometer (Applied Biosystems) and the ratios of pro-LPH to m-LPH of COS-1 cells were depicted as histograms. For each time point pro-LPH+mature LPH = 100%. pro-LPH (solid columns); mature LPH (hatched columns). Panel C: 16 h before labelling MDCK-17 cells were stimulated with sodium butyrate. MDCK-17 cells were then pulsed for 1 h and chased for 16 h in the presence of inhibitor identical to COS-1 cells. After chasing cells were lysed, LPH was immunoprecipitated and subjected to SDS-PAGE and fluorography. Exposure of the film was 2 days. C = LPH transfected MDCK-17 cells continuously labelled for 16 h in the absence of inhibitor. Panel D: The relative intensities of the signals on the fluorographs were quantified using a laser densitometer (Applied Biosystems) and the ratios of pro-LPH to m-LPH of MDCK cells were depicted as histograms. For each time point pro-LPH+mature LPH = 100%. pro-LPH (solid columns); mature LPH (hatched columns).

teolytic processing of LPH in COS-1 cells, we performed studies in which COS-1 cells were labelled for 1 h, followed by a chase of 16 h in the presence of ammonium chloride, a known inhibitor of lysosomal function [17]. The LPH species isolated from these cells are shown in Fig. 4, panel A. With increasing concentrations of inhibitor, the ratio of pro-LPH/m-LPH increased (Fig. 4, panel B). 20 mM ammonium chloride totally abolished proteolytic processing of pro-LPH. In parallel control experiments, MDCK-17 cells, a cell line that permanently expresses human LPH, were pulsed and chased identically to transfected COS-1 cells above in the presence of the same concentrations of inhibitor. In contrast to COS-1 cells, the ratio of pro-LPH/m-LPH did not change with increasing concentrations of inhibitor in MDCK-17 cells (Fig. 4, panels C and D). From this finding, we conclude that the observed 'mature' form of LPH in COS-1 cells was not the product of normal processing of LPH seen in intestinal epithelial cells and transfected MDCK cells but was the result of lysosomal degradation.

4. Discussion

Biosynthesis and post-translational processing of LPH has been the subject of intense investigations [2–4,6,8,16,18].

A crucial step in the maturation of this enzyme is the proteolytic conversion of the 215 kDa pro-LPH precursor to the 160 kDa mature form. For human LPH, using biosynthetic studies in Caco-2 cells and intestinal explants in organ culture at low temperatures and in the presence of BFA, we have shown that proteolytic cleavage of pro-LPH is a post-Golgi event [6]. In heterologous expression studies of human LPH in MDCK cells we have furthermore been able to show that proteolytic processing was not required for correct sorting of the enzyme to the apical (microvillus) membrane [7]. The latter findings have subsequently been confirmed by Jacob et al. [16]. Recently, two independent studies demonstrated that proteolytic processing of human LPH involves two steps [19,20]. A first cleavage at Arg_{734}–Leu_{735} occurring intracellularly, which generates an intermediate form of 160 kDa. After insertion of the intermediate into the microvillus membrane the second and final processing taking place at Arg_{868}–Ala_{869} by luminal trypsin.

Processing of LPH to the mature enzyme has been reported to be lacking in transfected COS-1 cells [8]. Hence these cells were thought to be an ideal cotransfection system for the study of potential processing enzymes in LPH-maturation.

The findings reported here confirm other studies in that they show LPH to be synthesized by COS-1 cells in an enzymatically active form. The specific activity in the homogenate of transfected COS-1 cells was in the range previously reported [8].

Furthermore, pulse-chase experiments led to the identification in transfected COS-1 cells of a mannose-rich 210 kDa pro-LPH_h species, which was converted into a complex-type 220 kDa pro-LPH_c species within 2 h of chase. This was in accordance to the molecular mass range reported by Naim et al. [8] for COS-1 cells, and within the molecular mass range of LPH expressed in small intestinal epithelial cells [4].

Contrary to previous findings [8] we also observed a proteolytically processed form of LPH in transfected COS-1 cells. This form appeared after 5 h of chase and was somewhat smaller (145 kDa) than the mature form found in intestinal epithelial cells (160 kDa). Deglycosylation by endo F/GF, however, reduced both species to an identical size of 122 kDa, indicating that the differences are due to differences in the glycosylation status. Continuous biosynthetic labelling of transfected COS-1 cells resulted in the detection of amounts of this 'mature' LPH comparative to those found in intestinal biopsies.

Previous failure to detect this processed LPH form may thus be explained by the shorter incubation times used in the biosynthetic labelling experiments.

It can therefore not be unequivocally determined if the enzymatic activity detected in transfected COS-1 cells is due to active pro-LPH or to the processed form or both.

The appearance of the processed m-LPH was inhibited by BFA. As BFA blocks intracellular protein transport to the cell surface, it is concluded that formation of this mature-like enzyme requires that pro-LPH is expressed on the plasma membrane of COS-1 cells. In contrast to other cell systems, the bulk of m-LPH was isolated from the intracellular compartment of transfected COS-1 cells. In addition, the complex glycosylated pro-LPH was predominantly detected as an ectoenzyme on the plasma membrane. These data suggest that in COS-1 cells pro-LPH is transported to the cell surface before being internalised again via the endocytic pathway where it is proteolytically cleaved by lysosomal hydrolases. This idea is clearly supported by experimental data obtained with an inhibitor of lysosomal function. Addition of the lysosomotropic agent NH_4Cl led to the abolition of proteolytic processing. In rat hepatocytes, it has been shown that late endosomes contain a trypsin-like protease [21], which is able to process internalised proteins after a basic amino acid residue like lysine. Pro-LPHc internalised into an endosomal–lysosomal compartment was only processed to m-LPH. In vivo, m-LPH anchored to the microvillus membrane of the enterocyte as an ectoenzyme is likewise not degraded further despite the potentially hostile proteolytic environment of the gut lumen. One explanation for this proteolytic resistance of mature LPH is that due to the compact folding only a few potential cleavage sites are exposed to the proteolytic enzymes of the gut. Support for this view comes from transfection studies with various LPH mutants. Only minor mutations in the 'pro' region of LPH resulted (a) in diminished complex glycosylation and thus a reduced transport competence and (b) in a complete degradation by trypsin (unpublished results). After the identification of the primary cleavage site in the two-step processing of human LPH [20] we suggest that the intermediate form of LPH plays an important role in the correct folding of this enzyme and that this leads to an relative resistance to proteolytic enzymes.

The appearance of the m-LPH in transfected COS-1 cells, on the other hand, demonstrates the potential limitations of these cells in studies where proteolytic processing of newly synthesised proteins is concerned.

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