

# Maturation of human lactase-phlorizin hydrolase

## Proteolytic cleavage of precursor occurs after passage through the Golgi complex

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Maturation of human intestinal lactase-phlorizin hydrolase (LPH) requires that a precursor (pro-LPH) be proteolytically processed to the mature microvillus membrane enzyme (m-LPH). The subcellular site of this processing is unknown. Using low-temperature experiments and brefeldin A (BFA), intracellular transport was blocked in intestinal epithelial cells. In Caco-2 cells incubated at 18°C, pro-LPH was complex-glycosylated but not cleaved, while at 20°C small amounts of proteolytically processed LPH were observed. These data exclude a pre-Golgi proteolytic event. BFA completely blocked proteolytic maturation of LPH and lead to an aberrant form of pro-LPH in both Caco-2 cells and intestinal explants. Therefore, proteolytic processing of LPH is a post-Golgi event, occurring either in the trans-Golgi network, transport vesicles, or after insertion of pro-LPH into the microvillus membrane.

Lactase-phlorizin hydrolase; Human; Enterocyte; Maturation; Proteolytic processing; Brefeldin A; Caco-2 cell

### 1. INTRODUCTION

Lactase-phlorizin hydrolase (LPH) (EC 3.2.1.23/62) is an integral glycoprotein of the microvillus membrane of small intestinal epithelial cells. With some notable exceptions, LPH is present in all mammals and is responsible for the hydrolysis of lactose, the major carbohydrate in milk, to its monosaccharide constituents prior to their absorption. The mature enzyme has two enzymic activities:  $\beta$ -D-galactoside galactohydrolase (EC 3.2.1.23), responsible for the hydrolysis of lactose, and the phlorizin hydrolase (glycosyl-N-acyl-sphingosine glucosylhydrolase) (EC 3.2.1.62) [1,2]. 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) [3–6]. The complete primary structure of rabbit and human pro-LPH has been deduced from cDNA cloning [7]. The cellular site of proteolytic processing has not been identified so far. According to the glycosylation status of the different LPH forms, proteolytic cleavage of the pro-LPH has been suggested to occur before the trans-Golgi [6], after the Golgi [8], or

after insertion into the microvillus membrane [5]. Two independent studies of the biosynthesis and processing of LPH in human subjects has indicated that proteolytic processing is delayed in adults having lactase restriction (lactase deficiency) [9,10]. It is conceivable, therefore, that this processing has a role in the regulation of surface expression of mature LPH on the enterocytes. The idea of a possible role of post-translational events in the regulation of lactase expression has been strengthened also by the finding of high LPH-specific mRNA in adult rabbits with low enzymatic LPH activity [11].

Further investigations reported in this paper using cultures of Caco-2 cells and intestinal explants incubated either at low temperature or with brefeldin A (BFA) to inhibit intracellular transport show that proteolytic processing of pro-LPH occurs after the precursor has passed the Golgi complex.

### 2. MATERIALS AND METHODS

#### 2.1. Reagents and materials

L-[<sup>35</sup>S]Methionine (> 1,000 Ci/mmol) was obtained from Amersham, England; BFA, phenylmethanesulphonyl fluoride (PMSF), pepstatin, aprotinin, leupeptin, benzamidin, and molecular weight markers for SDS-PAGE were from Sigma Chemical Co.; SDS-PAGE purity reagents were from Bio-Rad. Cell and organ culture dishes were from Falcon; penicillin, streptomycin, fetal calf serum (FCS) and cell culture medium (IMDM) were from Gibco, Basel; organ culture medium (RPMI) was from Amimed, Basel or Gibco, Basel. Endo- $\beta$ -N-acetylglucosaminidase H (endo H) was from New England Nuclear; protein A-Sepharose was from Pharmacia Fine Chemicals.

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Abbreviations: LPH, lactase-phlorizin hydrolase; SI, sucrase-isomaltase;  $\alpha$ -1-AT,  $\alpha$ -1-antitrypsin; BFA, brefeldin A; DOC, sodium dodecyl sulfate; TGN, trans-Golgi network.

## 2.2. Biological materials

Human small intestinal biopsies (5–10 mg) were obtained during routine diagnostic procedures with a Watson suction capsule. Explants from surgical specimens of small intestine were provided by Prof. M. Frey, Universitätsspital Zurich: a 3 cm segment of mid-jejunum was obtained by surgical resection from each of 4 patients during the course of surgical reconstruction of the larynx.

## 2.3. Immunological reagents

Monoclonal antibodies to LPH and sucrase-isomaltase (SI) were produced in our laboratory as described earlier [8]; monoclonal antibodies to  $\alpha$ -1-antitrypsin ( $\alpha$ -1-AT) were from Beckman, Zurich.

## 2.4. Biosynthetic labeling

Caco-2 cells (passage #37–70) were cultured in Iscove's modified Dulbecco's medium (IMDM) containing 25 mM glucose, 44 mM  $\text{NaHCO}_3$ , 0.2 mM L-methionine, supplemented with 10% FCS, 1% non-essential amino acids (Gibco), 50 U/ml penicillin, 50  $\mu\text{g/ml}$  streptomycin, and 2.5  $\mu\text{g/ml}$  amphotericin, pH 7.2, in an atmosphere of 5%  $\text{CO}_2$  in air at a constant humidity. 9–15 days post-confluent cells (in 60 mm culture dishes) were used for the labeling experiments. Cells were first cultured in 1 ml methionine-free medium for 1 h before being labelled with 150  $\mu\text{Ci}$  of L-[ $^{35}\text{S}$ ]methionine. Cells were either continuously labelled (different pulse times were used as indicated in the appropriate sections of the text), or pulse-labelled first, followed by a chase for different lengths of time in medium containing 10 mM non-radioactive L-methionine. Mucosal explants were cultured and labelled as previously described [6,9]. Continuous labeling was performed for 1 h or 5 h. After that time the explants were immediately processed as indicated in section 2.5, or were washed once with RPMI 1640 medium containing 2.5 mM unlabelled methionine and incubated for 5 h with the same medium. When used, BFA was added to cultures at a final concentration of 1–25  $\mu\text{g/ml}$ .

## 2.5. Immunoprecipitation

Labelled Caco-2 cells were washed twice with 2 ml 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% NP40, 1% DOC, 100  $\mu\text{g/ml}$  PMSF, 10  $\mu\text{g/ml}$  leupeptin, 2  $\mu\text{g/ml}$  pepstatin, 34.8  $\mu\text{g/ml}$  benzamidin, and 2  $\mu\text{g/ml}$  aprotinin). The lysates were filtered through 0.45  $\mu\text{m}$  filters prior to immunoprecipitation. Labelled mucosa explants were processed for immunoprecipitation as described earlier [6,9]. Solubilized membrane proteins were precleared once with protein A-Sepharose beads and transferred to tubes with 80  $\mu\text{l}$  of antibody-protein A-Sepharose (containing 4  $\mu\text{l}$  of ascites fluid). After an incubation period of 1.5 h at room temperature, the beads were washed three times with 1 ml PBS containing 0.5% NP-40, 0.05% DOC and 0.05% SDS and then three times with 125 mM Tris-HCl (pH 8.2), 500 mM NaCl, 1 mM EDTA, 0.5% NP-40.

## 2.6. Endo H-treatment

Digestion of immunoprecipitates with endo H was performed essentially by the method of Owen [13]. In brief, immunoprecipitated proteins were eluted from Sepharose beads by boiling in 0.1 M Tris-HCl (pH 7.5), 1% SDS, 1% 2-mercaptoethanol for 4 min. This solution was diluted with 9 vols. of 0.15 M sodium citrate buffer (pH 5.5) containing 4 mM PMSF and 4 mU of endo H and incubated for 16 h at 37°C. Proteins were recovered by precipitation with an equal volume of 30% (w/v) trichloroacetic acid and the pellet was washed twice with acetone and kept at –20°C.

## 2.7. SDS-PAGE

Immunoprecipitates and proteins subjected to endo H treatment were solubilized with 50  $\mu\text{l}$  of 2-fold concentrated electrophoresis sample buffer containing 4% SDS, 10% glycerol, 5% 2-mercaptoethanol or 10  $\mu\text{l}$  of 0.1 M D-thiothreitol, boiled for 4 min and submitted

to electrophoresis on 6% acrylamide gels according to Laemmli [14]. 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

As  $\alpha$ -1-AT is strongly expressed and secreted in Caco-2 cells, we were able to use this protein as a reporter protein for the secretory pathway. By lowering incubation temperatures or adding BFA, the inhibitory effects on the secretory transport could thus be monitored independently from the processing of LPH.

### 3.1. Processing of LPH and $\alpha$ -1-AT in Caco-2 cells at reduced incubation temperatures and in the presence of BFA

Reduction of the incubation temperature in pulse-chase experiments has the effect of slowing down intracellular transport. The use of different temperatures was employed to 'dissect' the transport of LPH. To assess proteolytic maturation of LPH, cells were labelled with [ $^{35}\text{S}$ ]methionine for 2 h at 37, 15, 18, or 20°C, and subsequently chased for up to 21 h at the appropriate temperature. In some experiments, the initially low temperatures were raised to 37°C to show the reversibility of the temperature effects. After culture the cells were homogenized, and LPH and  $\alpha$ -1-AT were immunoprecipitated and analyzed by SDS-PAGE.

#### 3.1.1. At 15°C pro-LPH is not proteolytically cleaved, and secretion of $\alpha$ -1-AT into the culture medium is strongly retarded

Processing of LPH at various temperatures is shown in Fig. 1A. At 37°C the first molecular species observed was of  $M_r = 210$  kDa, corresponding to the high-mannose form of pro-LPH [6,9] (as demonstrated by endo H sensitivity; data not shown). At 4 h of chase the complex-glycosylated form of pro-LPH was clearly visible ( $M_r = 220$  kDa) and, in addition, the mature form of the protein appeared ( $M_r = 150$  kDa). At 21 h virtually all of the LPH protein was in the mature form. Incubation at 15°C prevented the formation of complex-glycosylated pro-LPH and of the proteolytically processed mature form, evidence that cleavage of pro-LPH is not a pre-Golgi event. Changing the incubation temperature back to 37°C lead to a delayed but essentially normal processing of LPH.  $\alpha$ -1-AT was complex-glycosylated within 30 min and secreted into the medium after 1 h at 37°C. In comparison, at 15°C glycosylation and secretion of  $\alpha$ -1-AT into the culture medium was essentially blocked (Fig. 1B). However, after prolonged chase periods (21 h) complex-glycosylated  $\alpha$ -1-AT could be immunoprecipitated from the medium, showing that the temperature effect was not absolute.

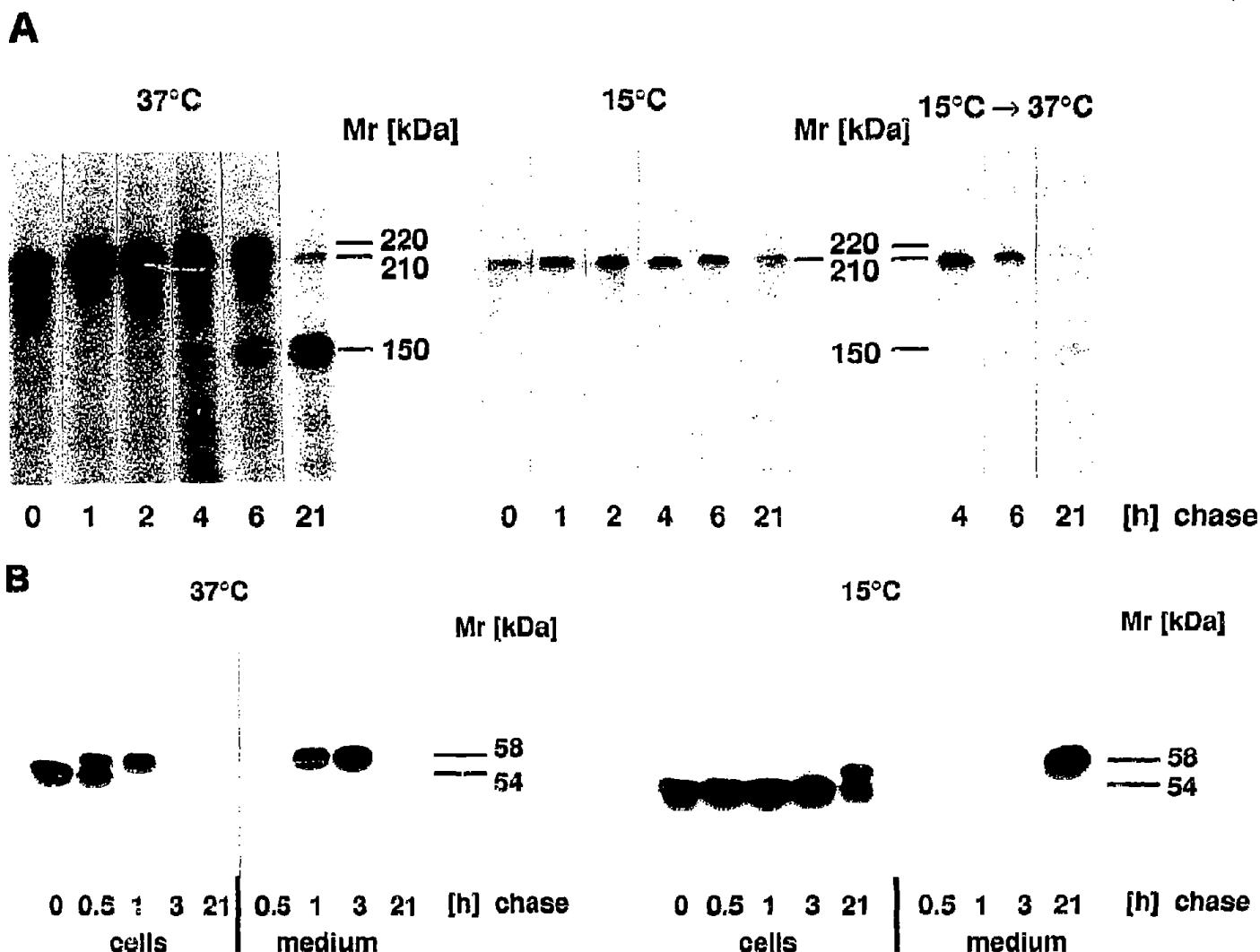


Fig. 1. Biosynthesis and post-translational processing of LPH and  $\alpha$ -1-AT in Caco-2 cells at 37°C and 15°C. The cells were pulse-labelled for 2 h at 37°C or 15°C with 150  $\mu$ Ci/ml [ $^{35}$ S]methionine and subsequently chased for the indicated time points at the appropriate temperature with 2.5 mM unlabelled L-methionine. Following incubation, cells were processed, LPH and  $\alpha$ -1-AT were isolated by immunoprecipitation and analyzed by SDS-PAGE and fluorography. (A) LPH. The molecular species of LPH obtained at 37°C (left) and 15°C (middle) are shown. The three lanes to the right show the LPH signals obtained from cells chased initially at 15°C for 2 h and then switched to the higher temperature of 37°C for further chasing.  $M_r$  210 kDa = pro-LPH<sub>H</sub> (high-mannose form);  $M_r$  220 = pro-LPH<sub>C</sub> (complex-glycosylated form);  $M_r$  150 = mature LPH (complex-glycosylated). Exposure time for fluorography was 10 days. (B)  $\alpha$ -1-AT. The molecular species of  $\alpha$ -1-AT obtained in cell extracts and in culture medium at 37°C (left) and 15°C (right) are shown.  $M_r$  54 kDa = high-mannose form of  $\alpha$ -1-AT;  $M_r$  58 kDa = complex-glycosylated form of  $\alpha$ -1-AT. Exposure time of the film was 7 h (for the 37°C samples) and 3 days (for the 15°C samples).

3.1.2. At 18°C pro-LPH is complex-glycosylated but not proteolytically processed; at 20°C some proteolytic processing occurs

Fig. 2 shows glycosylation and proteolytic processing at 18 and 20°C. At both these temperatures complex glycosylation of pro-LPH occurred, although at a much slower rate than at 37°C (see Fig. 1A). Traces of processed mature form were detectable at 20°C after 21 h of chase. When pulse-labeling at 18°C was followed by chase at 37°C accumulated endo H-sensitive pro-LPH was first detected, which was subsequently complex-

glycosylated and then proteolytically processed (data not shown). These data clearly showed that proteolytic processing was preceded by complex-glycosylation of pro-LPH in Caco-2 cells. Secretion of  $\alpha$ -1-AT into the culture medium was delayed about 5 h at 18°C and 1 h at 20°C (results not shown).

3.1.3. BFA leads to aberrant glycosylation and an arrest of proteolytic processing of pro-LPH

As shown in Fig. 3A, in the presence of BFA, chase periods up to 12 h did not lead to the formation of

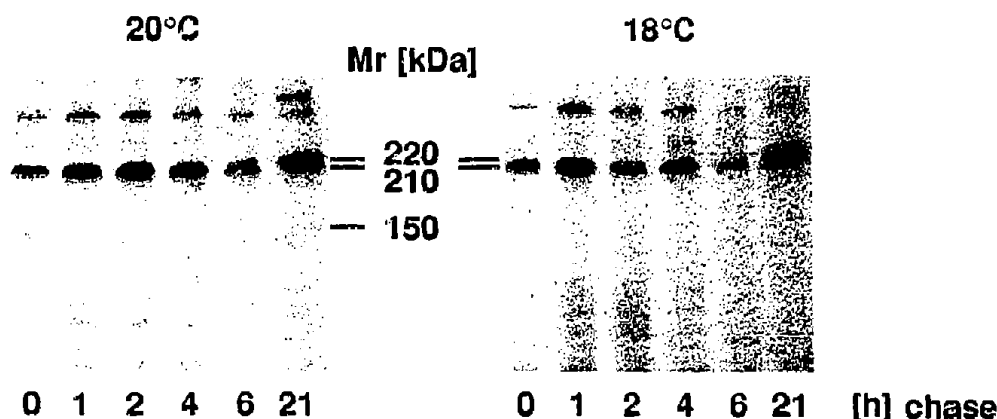


Fig. 2. Biosynthesis and post-translational processing of LPH in Caco-2 cells at 20°C and 18°C. The cells were pulse-labelled for 1 h at 20°C or 18°C followed by a chase at the appropriate temperature for the times indicated. Immuno-isolated LPH was further analyzed by SDS-PAGE and fluorography.  $M_r$  210 kDa = pro-LPH<sub>H</sub> (high-mannose form);  $M_r$  220 = pro-LPH<sub>C</sub> (complex-glycosylated form);  $M_r$  150 = mature LPH (complex-glycosylated). Exposure time of the film for fluorography was 21 days (20°C samples) and 29 days (18°C samples).

mature LPH. When BFA was removed from the culture medium, this effect was reversed, i.e. m-LPH appeared 5 h later. The pro-LPH was incompletely glycosylated, as assessed by its electrophoretic mobility and its partial resistance to endo H treatment (data not shown).  $\alpha$ -1-AT was prevented from being secreted into the medium by BFA (Fig. 3B). These results indicated that proteolytic processing of pro-LPH occurred after the precursor of LPH had passed the trans-Golgi complex.

### 3.2. Processing of LPH in cultured human small intestinal explants at reduced temperature and in the presence of BFA

In order to assess the relevance of the above data in Caco-2 cells to normal human intestinal epithelial cells, we investigated the effect of low incubation temperatures and BFA on the processing of LPH in organ-cultured human small intestinal explants. Parts of small intestinal explants taken from diagnostic biopsies or from surgical tissue were incubated in organ culture dishes as previously described [6,9]. The tissue proteins were labelled with [<sup>35</sup>S]methionine, either continuously for 5 h or pulsed for 1 h and chased for 5 h at normal (37°C) or at reduced (18°C) temperature or in the presence of BFA. Fig. 4 shows the result of a pulse-chase experiment at 37°C in the absence and presence of BFA, and at the reduced incubation temperature of 18°C. In contrast to Caco-2 cells, only one form of pro-LPH was detected in the control explants after 1 h of pulse and 5 h of chase (Fig. 4, lanes 1 and 2), with most of the labelled LPH being in the form of mature protein after the 5 h chase period. In the presence of BFA, by contrast, no mature LPH was detectable (Fig. 4, lanes 3 and 4). Culture at 18°C also blocked the appearance of mature LPH (Fig. 4, lane 5). Fig. 5a shows the molecular species of LPH and their glycosylation status in the absence and presence of BFA. In the control explants, m-LPH was partially sensitive to endo-H, and pro-LPH

was totally sensitive, verifying our earlier finding [6] of the absence of a complex-glycosylated pro-LPH in organ-cultured intestinal explants as compared to Caco-2 cells. Incubation in the presence of BFA, in addition to blocking maturation, lead to a partially endo H-resistant form of pro-LPH. This effect of BFA on glycosylation was also observed for SI, which was used as a control protein (Fig. 5b).

## 4. DISCUSSION

Transport, processing, and cell surface expression of enzymes of the microvillus membrane in small intestinal epithelial cells differ. While aminopeptidase N and dipeptidylpeptidase IV are synthesized as monomers [8], the disaccharidases, SI and LPH, are synthesized as precursors which are subsequently proteolytically processed [2-4,6,15,16]. In contrast, maltase-glucoamylase does not appear to be processed in analogy to the other disaccharidases in man [17]. SI has been shown to be proteolytically cleaved after insertion into the microvillus membrane by pancreatic proteases [12,16,18]. The site of processing of LPH, however, has not been identified yet. Recent investigations on the processing of LPH precursor to the mature form of LPH in biopsy explants from adults with lactase restriction (lactase deficiency) have attested to a possible link between the decline in enzyme activity to a slowed intracellular transport and a delayed proteolytic processing leading to an accumulation in some cases of LPH in the Golgi complex [9,10]. In contrast to earlier studies using intestinal biopsies from individuals with lactase persistence [6], complex-glycosylation of pro-LPH prior to proteolytic processing was observed in some of the cases with lactase restriction. These findings are analogous to those from Caco-2 cells where proteolytic processing of pro-LPH is significantly slower and occurs after complex-glycosylation of pro-LPH [8].

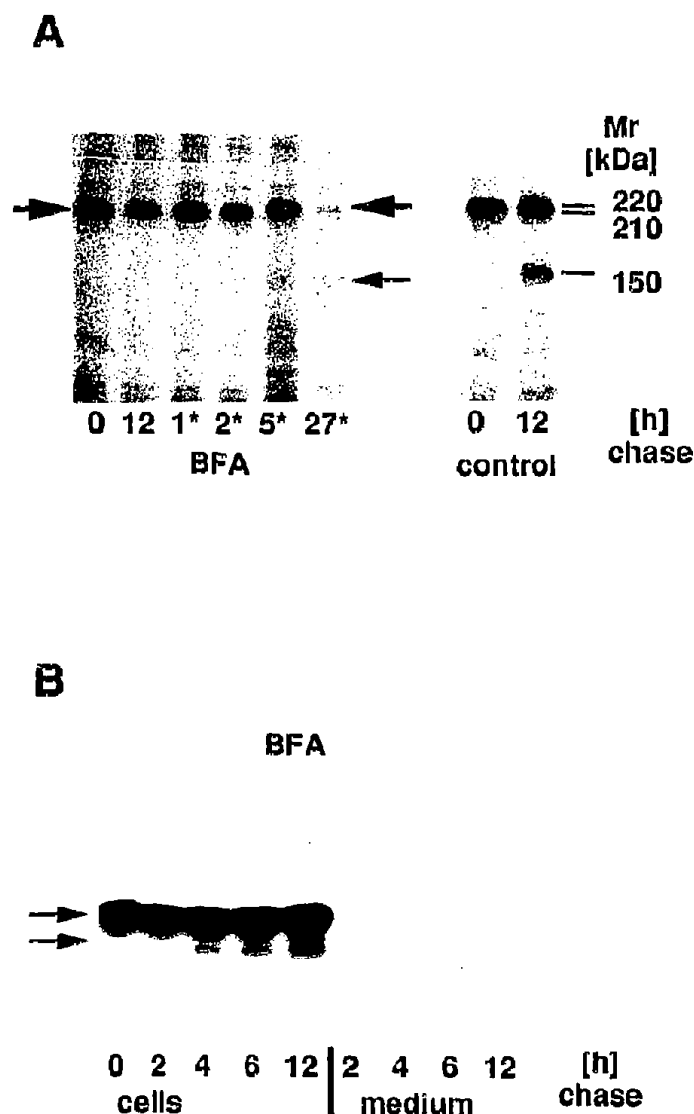


Fig. 3. Post-translational processing of LPH and  $\alpha$ -1-AT in Caco-2 cells in the presence of BFA. Pulse-labeling of the cells with 150  $\mu$ Ci/ml [ $^{35}$ S]methionine (1 h) and a 'first' chase up to 12 h was carried out in the presence of 2  $\mu$ g/ml of BFA. Following this chase, cells were washed 3 times with normal medium and chased for a second period for the indicated times without BFA (lanes labelled 1\* to 27\*). LPH and  $\alpha$ -1-AT were immuno-isolated from lysed cell homogenates and culture medium and analyzed by SDS-PAGE and fluorography. (A) LPH. Molecular species obtained in the absence of any inhibitors are shown in the 2 lanes on the right (control). The thick arrows indicate the position of pro-LPH, and the thin arrows the position of mature LPH.  $M_r$  210 kDa = pro-LPH<sub>H</sub> (high-mannose form);  $M_r$  220 = pro-LPH<sub>C</sub> (complex-glycosylated form);  $M_r$  150 = mature LPH (complex-glycosylated). Exposure time of the film was 15 days. (B)  $\alpha$ -1-AT. The species of  $\alpha$ -1-AT obtained in cell extracts and culture medium in the presence of BFA. The arrows indicate the major bands found (top arrow) and putative degradation products formed during incubation with inhibitors. Exposure time was 1 day.

In this study we altered the intracellular transport in Caco-2 cells both by reducing the incubation temperature and by treatment with BFA. Lowering incubation temperatures has been shown to significantly alter intra-

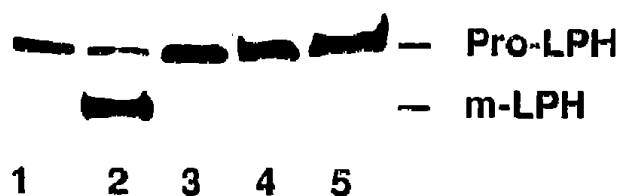


Fig. 4. Post-translational processing of LPH in organ-cultured human small intestinal explants in the presence of BFA and at an incubation temperature of 18°C. The explants were either pulsed for 1 h with 150  $\mu$ Ci/ml [ $^{35}$ S]methionine at 37°C (lane 1) or pulsed for 1 h and chased for 5 h at 37°C (lanes 2–4). For the low-temperature experiment (lane 5) explants were pulse-labelled at 37°C and chased at 18°C. LPH was immuno-isolated from the lysates and analyzed by SDS-PAGE and fluorography. Lane 1 (control), 1 h pulse at 37°C; lane 2 (control), 1 h pulse and 5 h chase at 37°C; lane 3, 1 h pulse, then 5 h chase at 37°C in the presence of 5  $\mu$ g/ml BFA; lane 4, as lane 3, but 25  $\mu$ g/ml BFA; lane 5, 1 h pulse at 37°C and 5 h chase at 18°C.

cellular transport of glycoproteins. At the appropriate temperatures an arrest of glycosylation at different stages has been observed [19–22]. In Caco-2 cells pulse-chased at 15°C, complex-glycosylation of pro-LPH was totally blocked and no mature LPH was detected. Glycosylation of  $\alpha$ -1-AT was also blocked, as was its secretion into the culture medium. Only after prolonged chase times of 21 h was complex-glycosylated  $\alpha$ -1-AT observed in the medium. From this we concluded that, at this temperature, LPH accumulates in the endoplasmic reticulum (ER) or in an intermediate, pre-Golgi compartment. At 18°C, complex-glycosylation of pro-LPH occurred but no mature LPH was observed, indicating that pro-LPH was not transported beyond the trans-Golgi. Finally, increasing the incubation temperature to 20°C led, in addition to complex-glycosylation of pro-LPH, to the appearance of trace amounts of mature LPH. Thus, from the low-temperature experiments described, it can be concluded that proteolytic processing of pro-LPH in Caco-2 cells is preceded by complex-glycosylation, i.e. it is not likely to occur in the Golgi complex. The same conclusions may be drawn from the experiments with organ-cultured intestinal explants at 18°C, and are in agreement with recently published data in cultured intestinal explants [23].

Pulse-chase labeling of Caco-2 cells and organ-cultured intestinal explants in the presence of BFA led to a complete arrest of proteolytic processing of pro-LPH to mature LPH, this effect being reversible. The pro-LPH which accumulated was partially endo H resistant. Interestingly, this was also the case in BFA-treated intestinal explants, where complex-glycosylation of pro-LPH is not normally observed in control experiments. These findings are in agreement with the effects of BFA on intracellular transport of glycoproteins [24–26] and may be interpreted as follows: BFA leads to a redistribution of Golgi-associated membranes and proteins, including trans-Golgi, back to the ER [24]. Newly synthesized pro-LPH accumulates in a compartment which



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