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Proteolytic processing of human intestinal lactase-phlorizin hydrolase precursor is not a prerequisite for correct sorting in Madin Darby canine kidney (MDCK) cells

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Maturation of lactase-phlorizin hydrolase (LPH) (EC 3.2.1.23–62) requires proteolytic processing of precursor (pro-LPH) to mature microvillus membrane enzyme (m-LPH). Subcellular site and function of this processing are unknown. We studied the processing and sorting of human LPH expressed permanently in MDCK cells. LPH was inserted into the apical membrane and small amounts were found basolateral. Of the LPH immunoprecipitated from the apical membrane, 42% was in the mature, i.e. proteoytically processed form; on the basolateral membrane it was 20%. Thus, LPH-processing occurs after sorting and is not necessary for surface expression.

Lactase-phlorizin hydrolase; Human; Enterocyte; Proteolytic processing; Sorting; Surface expression

1. INTRODUCTION

The β-glycoside complex (EC 3.2.1.23–3.2.1.62), widely known as lactase-phlorizin hydrolase (LPH), contains two enzymic activities: lactase (β-D-galactoside galactohydrolase (EC 3.2.1.23), which is responsible for the hydrolysis of lactose, and phlorizin hydrolase (phlorizin glucohydrolase) (EC 3.2.1.62) [1]. The primary structure of human and rabbit LPH has been deduced from cDNA cloning [2]. The human LPH complex is composed of a single polypeptide chain which contains a cleavable signal peptide sequence comprising 19 amino acids, a large pro-peptide of 849 amino acids, and the mature enzyme of 1059 amino acids which contains a hydrophobic membrane-spanning domain of 19 amino acids near the C terminus and a hydrophillic cytosolic domain of 26 amino acids at the C terminus. Thus, in contrast to sucrase-isomaltase [3], the catalytic sites for both lactase and phlorizin hydrolase are located on the same polypeptide chain. These data and data from biosynthetic labelling experiments [4–7] have led to the currently favoured concept that LPH is synthesized in a pre-pro form (M_r = 215–245 kDa) which is proteolytically modified to the mature enzyme (M_r = 160 kDa) which is found in the microvillus membrane only. The fate of the large pro-piece is not yet known. In contrast to sucrase-isomaltase though, this processing is catalyzed by an enterocyte-specific mechanism and not by pancreatic proteases [4–7]. The cellular site of processing is still subject to debate. The failure to detect complex glycosylated precursor forms in organ cultured human intestinal mucosa suggested that proteolytic maturation was an early Golgi event [6]. Later, studies with tissue from subjects with adult lactase restriction have shown proteolytic processing to be delayed, resulting in the detection of complex glycosylated pro-LPH [8]. The authors concluded from these data that the localization of proteolytic cleavage and the final glycosylation steps must be in close proximity along the secretory pathway of enterocytes. In organ cultured pig mucosa and in Caco-2 cells proteoytic processing seemed to occur later [5,6]. A recent study in our laboratory using Caco-2 cells and organ-cultured intestinal biopsies in an experimental design using low-temperature incubations and brefeldin A has revealed that proteolytic processing of LPH occur after the precursor has passed through the Golgi complex [9]. From in vivo labelling experiments in the rat it has been suggested that proteolytic processing occurs in two steps, the first in the Golgi and the second possibly in the microvillus membrane itself [10]. The question of a possible role of proteolytic maturation in sorting of LPH to the correct surface domain also remains unanswered. Recent data from heterologous expression studies of human LPH in COS-1 cells have shown proteolytic processing to be necessary neither for surface expression nor for enzymatic activation [11].

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Abbreviations. LPH, lactase-phlorizin hydrolase; TGN, trans-Golgi network; SDS-PAGE, sodium dodecylsulfate-polyacrylamid-gel electrophoresis; PBS, phosphate buffered saline.

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In this paper we report the expression of a full-length cDNA for human LPH in MDCK cells, and show that in these polarized cells proteolytic maturation of the enzyme does not appear to be essential for proper sorting to the apical membrane domain.

2. EXPERIMENTAL

2.1. *Reagents and materials*

Cell-culture media, penicillin and streptomycin were obtained from Gibco Laboratories. Transwell polycarbonate filter units (tissue-culture treated units of 24.5 mm diameter and 0.4 μm pore size) were from Costar Corp. Geneticin (G418) and sodium butyrate were purchased from Sigma Chemical Co. The pXT1 vector was from Stratagene Cloning System. The pRB-1H vector was kindly provided by Dr. Ned Mantei and Rahel Bänzinger (Swiss Federal Institute of Technology, Zürich).

2.2. *Cells and cell culture*

MDCK cells (strain II) were a generous gift from Dr. Kai Simons (European Molecular Biology Laboratory). MDCK cells were grown as monolayers in Eagle' s minimal essential medium with Earle' s salts (EMEM) supplemented with 5% (v/v) fetal calf serum (FCS), 100 milliunits/ml of penicillin and 100 μg/ml of streptomycin. Subconfluent cells were trypsinized and diluted 1:5 in new cell-culture dishes. The medium was changed every 2 days.

2.3. *Transfection of MDCK cells*

Low passage number (under 25) of MDCK cells were used for $CaPO_4$ transfection. The method was as described by Rodriguez-Boulan et al. [12] 1×10^6 cells per transfection were incubated overnight. 20 μg of pRB-1H and 2 μg of pXT1 (selectable DNA with the neomycin resistance gene) were dissolved in 440 μl of 10 mM Tris-HCl, pH 7.5 and 500 μl of 2X HBS (HEPES-buffered saline: 50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.10) were added. 60 μl of 2 M $CaCl_2$ was dropped into the DNA mix and the $CaPO_4$DNA precipitate allowed to stand for 20 min at room temperature. The MDCK cells were trypsinized and resuspended in 2 ml Eagle's MEM-10% FCS. The cells and the DNA precipitate were added into a 10 cm cell-culture dish. After 20 min at room temperature, 7 ml EMEM-10% FCS were added and the cells incubated for 16 h at 37°C. The medium was removed and 4 ml of 15% glycerol in HBS were added for 1 min at 37°C. The cells were washed twice with PBS and grown for 2–3 days in 10 ml EMEM-10% FCS. The cells were trypsinized and replated onto 10 new 100 mm cell-culture dishes in medium supplemented with 5% FCS and 400 μg/ml of G418, which was changed every 3 days. Resistant colonies were isolated with cloning rings and propagated.

2.4. *Domain-specific surface immunoprecipitation*

MDCK cells were plated on Costar polycarbonate filters (1.5×10^5/cm^2) and the medium was changed every day. After 4–5 days the tightness of the monolayer was checked by measuring the transepithelial electrical resistance using the millicell ERS voltohmmeter (Millipore Corp.) [13].

The expression of the human lactase was enhanced by adding 8 mM sodium butyrate to the cell culture medium 16 h before metabolic labelling of the cells [14]. The cells were labelled from the basolateral side with 150 μCi [^{35}S]methionine (1 Ci = 37 GBq) in methionine-free media supplemented with 5% dialyzed FCS for 30 min or 16 h, and subsequently chased for the times indicated in the figure legends. The cells were incubated for 30 min in serum-free EMEM before cell surface proteins were immunoprecipitated [15]. The cells were washed 4 times with ice-cold PBS$^+$ (PBS with 0.1 mM Ca^{2+} and 0.5 mM Mg^{2+}). After washing the cells, 3 μl of the monoclonal antibody against lactase [16] in 1 ml PBS$^+$ were added to the apical or basolateral side of the cells. After 30 min incubation with gentle agitation at 4°C, the cells were washed 4 times with PBS$^+$ and 400 μg of unlabelled cell proteins from mock-transfected MDCK cells in 1 ml PBS$^+$ were added to the corresponding side of the cells to saturate the remaining free binding sites of the antibodies. After 15 min at 4°C, the cells were washed again with PBS$^+$ and lysed in 25 mM Tris-HCl (pH 8.0), 50 mM NaCl buffer supplemented with 1% NP40, 1% DOC, 100 μg/ml PMSF, 10 μg/ml leupeptin, 2 μg/ml pepstatin, 38.4 μg/ml benzamidine, and 2 μg/ml aprotonin for 30 min at 4°C under gentle agitation. After centrifugation to remove cell debris the antigen–antibody complexes were adsorbed to protein A Sepharose and analysed by SDS-PAGE. The cell extracts were once again immunoprecipitated with the lactase antibody bound to protein A Sepharose.

2.5. *Endo H-treatment and endo F-treatment*

Digestion of immuno-isolated LPH with endo H and endo F was carried out as previously described [6]. Treated proteins were recovered by precipitation with 30% (w/v) trichloroacetic acid, washed, and kept at −20°C until analyzed by SDS-PAGE together with undigested proteins.

2.6. *Sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE)*

Immunoprecipitates and endo H- or endo F-digested proteins were solubilized with 50 μl of 2-fold concentrated electrophoresis sample buffer and analyzed by SDS-PAGE and fluorography as described previously [6].

3. RESULTS

Fig. 1 shows the MDCK-expression systems applied. A two-vector system was chosen, one vector containing the neomycin-resistence gene, the other containing a full-length cDNA for human LPH. Several clones were

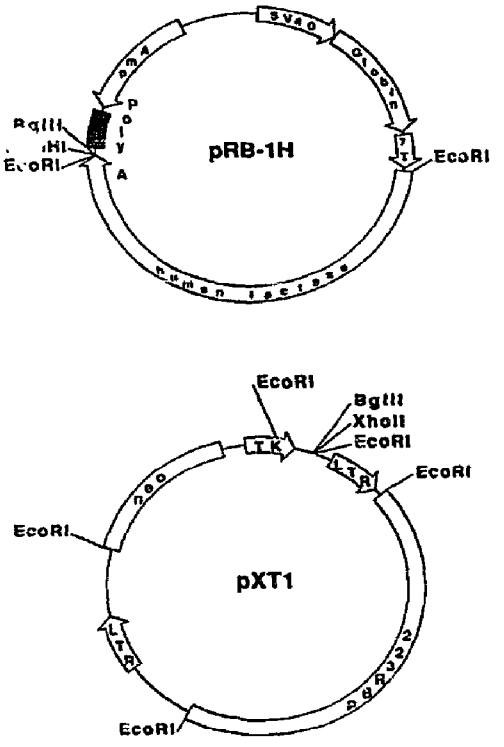

Fig. 1. Vectors used for the transfection of MDCK cells. A two-vector transfection system was employed, using pXT1 containing the neo^r resistance gene for selection in geneticin containing medium, and pRB-1H containing the full-length cDNA for human LPH.

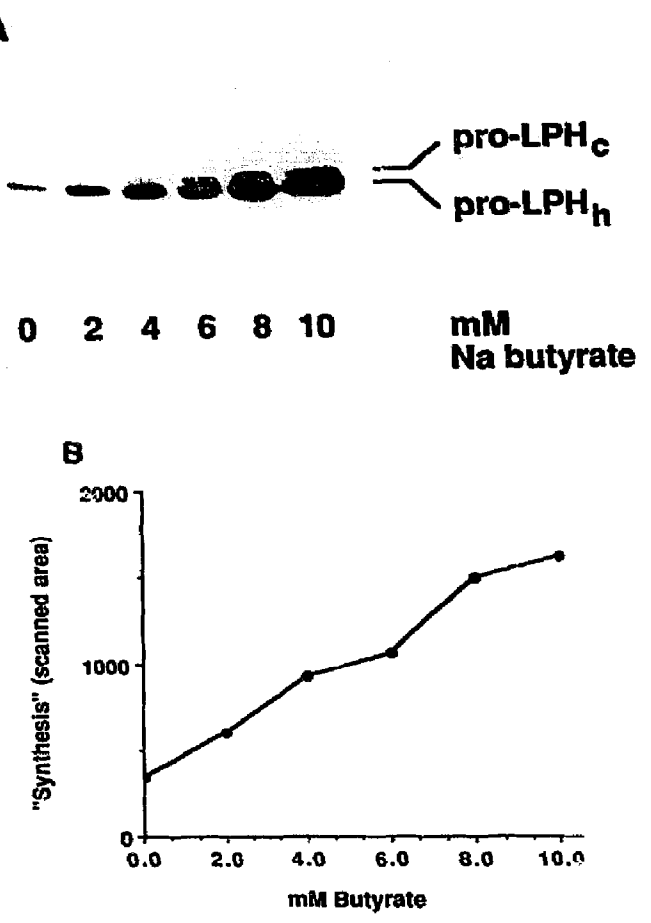

Fig. 2. Expression of human LPH in MDCK cells and its stimulation by sodium butyrate. Transfected and cloned MDCK cells (MDCK-17) grown in 60 mm culture dishes were treated with sodium butyrate 16 h prior to metabolic labelling. Labelling with [^{35}S]methionine was continuous for 5 h, after which cells were lysed and LPH was immunoprecipitated. Analysis was by SDS-PAGE and fluorography. Exposure of film was 1 day. A. Molecular forms of LPH expressed in MDCK cells after 5 h of pulse labelling. Pro-LPH$_h$: high-mannose form of the precursor molecule with an apparent M_r = 215 kDa. Pro-LPH$_c$: complex glycosylated precursor molecule with an apparent M_r = 220 kDa. B. Quantitation of stimulated expression of LPH by sodium butyrate. The fluorogram shown in Fig. 2A was scanned with a flat-bed scanner and the scans were analyzed by 'Image 1.33' on a Macintosh IIci computer.

selected using Geneticin (G418) and tested for LPH expression. The results presented herein have been obtained with clone MDCK-17. However, other clones were tested and gave identical results with respect to LPH processing.

3.1. *Expression of human LPH is stimulated by sodium butyrate*

Fig. 2 depicts the expression of human LPH in MDCK cells after transfection and stimulation by sodium butyrate. The molecular species of human LPH isolated from MDCK-17 cells after continuous labelling for 5 h are shown in Fig. 2A. In these cells two forms of pro-LPH were isolated, corresponding to a high-mannose form (pro-LPH$_h$, M_r = 215 kDa) and a complex-glycosylated form (pro-LPH$_c$, M_r = 220 kDa). Quantitation of LPH-expression by gel-scanning is shown in Fig. 2B. Expression of LPH was in direct correlation with the sodium butyrate concentration used for stimulation of cells prior to biosynthetic labelling. For all subsequent experiments 8 mM of sodium butyrate was used for stimulation of MDCK-17 cells.

3.2. *Synthesis and processing of LPH is the same as in intestinal epithelial cells*

In Fig. 3 a pulse-chase labelling experiment with MDCK-17 cells is shown. The expressed human LPH is synthesized as a high-mannose precursor (pro-LPH$_h$) as indicated by its total susceptibility to endo H digestion (Fig. 3, lane 2). Two h after the initiation of the chase period a complex glycosylated form (pro-LPH$_c$), resistent to endo H was evident (Fig. 3, lane 8). Furthermore, in MDCK-17 cells pro-LPH$_c$ was proteoytically processed to the mature enzyme (m-LPH, M_r = 160 kDa) which was detectable after 4 h of chase. The mature enzyme showed a partial resistence to endo H (Fig. 3, lane 14) and full sensitivity to endo F (Fig. 3, lane 15). These results indicate that human LPH is synthesized and processed normally in MDCK cells.

3.3. *Surface expression of LPH is not dependent on proteolytic processing*

The sorting of human LPH in MDCK-17 was investigated using surface immunoprecipitation assays. The results are summarized in Fig. 4 and show that 72% of LPH is found on the apical (microvillus membrane) surface and 28% on the basolateral surface. Of the apically expressed LPH 42% has been processed to the mature enzyme (Fig. 4A, lane 1), while 80% of the basolaterally expressed LPH was still in precursor form (Fig. 4A, lane 2). Only traces of mature enzyme were found in the total immunoprecipitate of the sample following apical surface immunoprecipitation (Fig. 4A, lane 5), while significant amounts were recovered after basolateral surface immunoprecipitation (Fig. 4A, lane 6), presumably mature enzyme from the apical membrane. Only complex glycosylated pro-LPH was found on the surface of MDCK-17 cells (lanes A and B, left), all the high-mannose pro-LPH being isolated from the total precipitate after surface immunoprecipitation.

Comparison of the results from surface immunoprecipitation in MDCK-17 and Caco-2 cells indicated that LPH was essentially processed the same in both cell types (not shown).

4. DISCUSSION

Intestinal lactase activity in mammals shows a distinct developmental pattern characterized by high levels

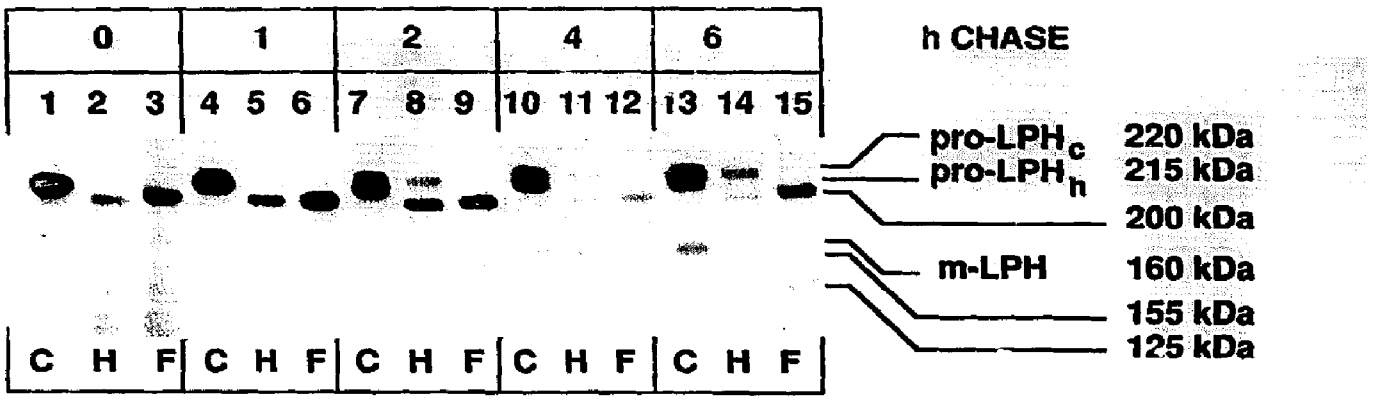

Fig. 3. Pulse-chase labelling of transfected MDCK cells. MDCK-17 cells were treated with 8 mM sodium butyrate 16 h prior to the experiment. Pulse labelling was for 30 min, followed by incubation in medium with unlabelled methionine (2.5 mM) for the times indicated. Cells were lysed and LPH immunoprecipitated. Immunoprecipitates were split into 3 equal aliquots, one control (C), one sample treated with endo H (H), one sample treated with endo F (F), and analyzed by SDS-PAGE and fluorography. Exposure of film was 13 days.

of enzymatic activity during the suckling period followed by a decline after weaning to a low residual activity in adult animals [17]. In man, two distinct adult phenotypes exist, i.e. that of lactase restriction (late onset lactase deficiency) and of lactase persistence [18]. It has been shown that LPH-specific mRNA levels in intestinal mucosa of adult rats and rabbits, as well as in human adults with lactase restriction, are comparatively high compared to the low levels of lactase activity [19]. Two recent independent studies on the biogenesis of LPH in organ-cultured mucosa of adults with lactase restriction have shown a delay in the proteolytic processing of LPH [8,20]. The postsynthetic proteolytic processing of pro-LPH is thus a potential regulatory target for the correct surface expression of mature enzyme. The cellular site as well as the enzymes involved in this proteolytic processing are not known. Very recent data from our laboratory on the processing of LPH in Caco-2 cells and in organ-cultured human small intestinal mucosa at reduced temperature and in the presence of brefeldin A have shown that proteolytic processing occurs after passage of the pro-LPH through the Golgi complex [9]. These findings are in agreement with another study using organ-cultured human small intestinal mucosa [21].

For the present work in order to study biosynthesis, intracellular transport, proteolytic maturation and surface expression in a controlled system, we have trans-

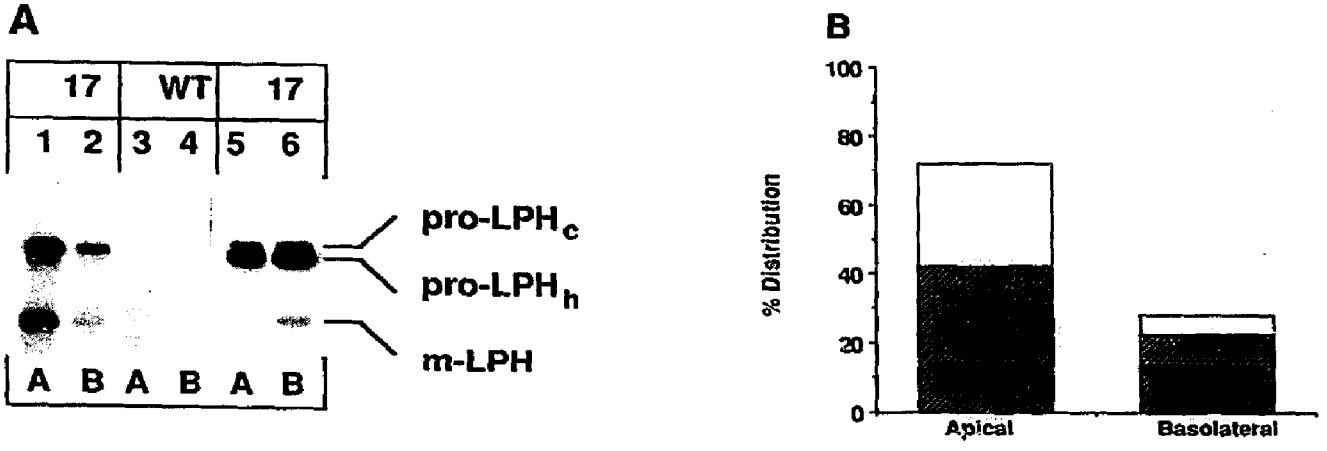

Fig. 4 Surface expression of LPH in MDCK cells. MDCK-17 cells were grown on Transwell filters as described in section 2. After stimulation with 8 mM sodium butyrate for 8 h, cells were labelled with 150 μCi [^{35}S]methionine from the basolateral side for 16 h. A. After labelling, surface-expressed LPH was removed by direct surface immunoprecipitation (see section 2 for details) (lanes 1 and 2). The remaining LPH was isolated by a further immunoprecipitation with LPH-specific antibodies (lanes 5 and 6). Lanes 3 and 4 show total immunoprecipitation from the untransformed wildtype MDCK cells. A = apical, B = basolateral; pro-LPH$_h$ = high mannose form of precursor, pro-LPH$_c$ = complex glycosylated form of precursor, m-LPH = mature form of LPH. B. The gel in Fig. 4A was quantitatively scanned and the distribution in percent of total LPH on the apical and basolateral surface calculated. In addition, the distribution of pro- LPH and m-LPH on the two polar surfaces was calculated. The column on the left shows the amount (in %) of total LPH expressed on the apical surface, the column on the right the amount on the basolateral surface. Within each column the ratio pro-LPH (striped) to m-LPH (white) is shown.

fected MDCK cells with a full-length clone of human LPH and isolated several clones which permanently expressed this enzyme. We have observed an essentially identical pattern of processing and surface expression in MDCK cells as has been reported for Caco-2 cells [9,16]. In contrast to LPH expressed in non-polarized COS-1 cells [11], proteolytic processing of pro-LPH does occur in the polarized MDCK cells. However, this processing was not found to be essential for expression of LPH on the cell surface, as pro-LPH could be immunoprecipitated from the surface membrane. 28% of the LPH protein was found in the basolateral domain, and of this 80% was in the form of pro-LPH. In contrast, 42% of the LPH found on the apical membrane surface had been processed to the mature form. The fact that 60% of LPH on the apical surface domain was in the uncleaved precursor form is strong evidence that the sorting of LPH occurs independently of and prior to proteolytic processing. It is generally agreed that sorting in MDCK cells occurs in the trans Golgi network [22], where distinct transport vesicles mediating the delivery of plasma membrane proteins to the apical or basolateral domain are formed [23]. The protease(s) responsible for cleavage of pro-LPH may be sorted along with pro-LPH to the same vesicle population destined for the apical membrane, and carry out processing on route to that surface. If this were the case, processing could be expected to continue after insertion of pro-LPH into the apical membrane, explaining the finding of uncleaved precursor on the apical surface. Erroneous inclusion of small amounts of the processing protease into basolateral vesicles would then result in the limited maturation of basolaterally expressed LPH observed in our study.

The question whether apical and basolateral glycoproteins themselves require inherent sorting signals has not been resolved. As proteins specific to epithelial cells are localized to the apical membrane, it has been suggested that specific signals are required for apical sorting [24,25]. Recently however, it has been reported that basolateral sorting in MDCK cells requires specific cytoplasmic domain determinants [26]. It is not known whether LPH has any specific sorting signals. One hypothesis was that intracellular removal of the pro-sequence results in conformational changes leading to the creation of a structure-dependent sorting signal guiding mature LPH to the microvillus membrane. Our data presented here are not compatible with such a hypothesis.

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