Routing and Processing of Lactase-Phlorizin Hydrolase in Transfected Caco-2 Cells*

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Human lactase-phlorizin hydrolase (LPH) is a digestive enzyme that is expressed in the small intestinal brush-border membrane. After terminal glycosylation in the Golgi apparatus, the 230-kDa pro-LPH is cleaved into the 160-kDa brush-border LPHβ and the 100-kDa profragment (LPHα). Since LPHβ is not transport-competent when it is expressed separately from LPHα in COS-1 cells, it was suggested that LPHα functions as an intramolecular chaperone. What happens to LPHα after cleavage is still unclear.

To analyze and localize LPHα in polarized epithelial cells, wild type and tagged LPH were stably expressed in Caco-2 cells. In tagged LPH, a vesicular stomatitis virus epitope tag was inserted into the LPHα region. Wild type and tagged proteins were processed at similar rates, and both cleaved LPHβ forms were expressed at the apical cell surface. Pro-LPH was recognized by antibodies against LPH, a profragment epitope and the vesicular stomatitis virus tag. LPHα alone, however, could not be recovered by these antibodies. Our data suggest that LPHα is degraded immediately after cleavage.

Human lactase-phlorizin hydrolase (LPH) is a disaccharidase that is localized at the microvillar membrane of epithelial cells in the small intestine. It is responsible for the hydrolysis of lactose, the main carbohydrate in mammalian milk.

LPH is synthesized as a 1927 amino acid precursor, propro-LPH. The first 19 amino acids of this precursor form the signal sequence that is cleaved off in the endoplasmic reticulum. The remaining 1908 amino acids form the pro-LPH, which is complex glycosylated on its way through the Golgi apparatus. In Fig. 1, the pro-LPH is schematically drawn. The first two regions, I and II, are localized in LPHα and LPHβ forms are expressed at the apical cell surface. Pro-LPH was recognized by antibodies against LPH, a profragment epitope and the vesicular stomatitis virus tag. LPHα alone, however, could not be recovered by these antibodies. Our data suggest that LPHα is degraded immediately after cleavage.

In Fig. 2, prepro-LPH is schematically drawn. The first two amino acids following the signal sequence of prepro-LPH are Ser-20-Thr-31 (2). The molecular mass of the immunoprecipitated profragment in these experiments was ~100 kDa (2). Because none of the used glucosaminidases (endo-N-acetylglucosaminidase H (endo H) and endo-N-acetylglucosaminidase F/glycopeptidase F (endo F/GF)) influenced the apparent molecular weight, it was concluded that the 5 consensus N-glycosylation sites were not glycosylated (2), although some conflicting data exist (1). In addition it was shown that LPHα does not form a stable complex with LPHβ after cleavage in intestinal biopsy specimens (2). These observations and further expression studies of the intestinal form of LPHβ (from Ala-869 to the C terminus) in COS-1 cells lead to the hypothesis that LPHα functions as an intramolecular chaperone. Where exactly in the cell the cleavage takes place as well as what happens to LPHα after cleavage is still uncertain despite extensive published data (2, 5).

To track down LPHα after cleavage, we decided to express and analyze LPH in Caco-2 cells. These cells are able to express LPH endogenously, and to perform the cleavage between the α and β domain of pro-LPH (6). Furthermore, we introduced a VSV tag into LPHα to generate additional recognition possibilities. Immunoprecipitation and localization studies were performed in Caco-2 cells that were stably transfected with wild type and VSV-tagged LPH cDNA. The results strongly suggest that the LPHα profragment is immediately degraded after cleavage in Caco-2 cells and therefore argue in favor of the intramolecular chaperone function.

EXPERIMENTAL PROCEDURES

Antibodies—HBB1/909 is an epitope-specific monoclonal antibody directed against LPH (6). This product of the hybridoma HBB1/909/34/74 was provided by Hans-Peter Hauri, Biozentrum der Universität Basel, Switzerland. V496 is a polyclonal antiserum directed against the first 12 amino acids following the signal sequence of propro-LPH (2). PSD4 is a monoclonal antibody against a specific epitope in VSV-G-protein (7), the hybridoma was provided by Thomas Kreis, Dept. of Cell Biology, University of Geneva, Switzerland.

DNA Constructs—To insert a VSV epitope tag into the LPH cDNA,
the LPH cDNA (8) was cloned into a pBluescript KS + vector in which the SstI site was removed. This construct was digested with SstI, blunt-ended with T4 DNA polymerase (New England Biolabs, Beverly, MA) and treated with calf intestinal phosphatase (Boehringer Mannheim BV, Almere, The Netherlands). For the construction of a 43-base pair VSV-tag insert, coding for 11 amino acids of the epitope, two oligonucleotides (5'-GGAGATCTTTATACGACATAGAGATGAA-3' and 5'-GGGGATCCCTTTCCAGTTGCTCCTCATCGTGTA-3') were annealed and treated with Klenow (Boehringer Mannheim BV) in the presence of dNTPs to fill in the 5' overhangs. The double-stranded product was digested with BamHI and BglII, and the 5' overhangs were filled in by Klenow treatment. This blunt-ended BamHI/BglII insert was ligated into the blunt-ended SstI-digested LPH cDNA so that the reading frame remained intact. The orientation of the insert was determined by sequencing. Fig. 3 depicts the sequence of the insert and the flanking LPH sequences, as well as the amino acid sequence of the wild type LPH and the tag insert in this region. This construct, denoted LPHST, was cloned into a modified pSG5 (9) expression vector containing a puromycin resistance cassette (pSpGuro), which resulted in the pLPHST plasmid. Wild type LPH cDNA was cloned into pSpGuro as well (pLPHwt).

To generate the pSpGuro vector the SstI fragment of the pKSpuro vector, which was kindly provided by Peter Laird (University of Southern California School of Medicine, Norris Comprehensive Cancer Center, Los Angeles, CA) (10) was inserted into the partially SstI digested pSG5 (a pSG vector (9)) with a multiple cloning site.

Cell Lines—Monkey kidney COS cells (ATCC CRL-1650) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Caco-2 TC7 and PD10 cells (a gift from Dr. Monique Rousseau, Ref. 11) were cultured in Dulbecco's modified Eagle's medium supplemented with 20% heat-inactivated fetal calf serum, 1% nonessential amino acids and antibiotics (all from Life Technologies, Inc.). All cell lines were cultured at 37 °C in a humidified 5% CO2 incubator.

Transfection and Selection—COS-1 cells were transfected via electroporation as described before (12). Caco-2 cells were transfected by LipofectAMINE treatment. One or two days prior to transfection, 0.2 × 10⁶ Caco-2 TC7 cells were seeded per 35-mm culture dish, so that on the day of transfection the culture was 60–80% confluent but not polarized. During the whole transfection procedure, no antibiotics were used. On the day of transfection, cells were washed twice with OptiMEM (Life Technologies, Inc.), Solution A, consisting of 2 μg of linearized DNA in 200 μl of OptiMEM, was gently mixed with Solution B, consisting of 6 μl of LipofectAMINE (Life Technologies, Inc.) and 200 μl of OptiMEM. The mixture was incubated at room temperature for 30 min, and 1600 μl of OptiMEM was added. The resulting 2ml was added to the cells and incubated at 37 °C and 5% CO2 for 6 h. Cells were washed with normal Caco-2 medium and incubated in this medium for 24 h, refreshed, and incubated for another 24 h. Cells were trypsinized and seeded into 96-well plates at a density of 5 × 10⁴ cells per well. After 24 h, selection medium was added, which consisted of normal Caco-2 medium with an (empirically determined) puromycin concentration of 13 μg/ml. After 1 week, wells with only one colony were selected. After another week, these colonies were trypsinized and cultured further in selection medium.

Metabolic Labeling and Immunoprecipitation—Caco-2 cells or transiently transfected COS-1 cells were metabolically labeled with [35S]Tran35S-label (ICN Biomedicals) as described by Naim et al. (8). After the labeling period, the cells were scraped in lysis buffer (1% Triton X-100, 0.2% bovine serum albumin in 100 mM phosphate buffer, pH 8.0, containing 1 tablet of complete protease inhibitor mixture (Boehringer Mannheim BV) per 25 ml) and lysed at 4 °C for 1 h. For COS cells usually 1 ml of ice-cold lysis buffer was used for each 100-mm culture dish (about 2–4 × 10⁶ cells). For Caco-2 cells, 0.5 ml was used per filter. Lysates were stored at −135 °C until use. Detergent extracts of cells were centrifuged for 1 h at 100,000 × g at 4 °C and the supernatants were immunoprecipitated as described by Schweizer et al. (13).

SDS-PAGE—SDS-PAGE was performed according to Laemmli (14), and the apparent molecular weights were assessed by comparison with high molecular weight markers (Bio-Rad) run on the same gel. In some experiments, deglycosylation of the immunoprecipitates with endo H and endo F/GF (also known as PNGase F) (both from New England Biolabs) was performed prior to SDS-PAGE analysis as described before (15).

Trypsin Sensitivity Assay—The sensitivity for proteatic digestion of LPH and LPHST was compared using a trypsin sensitivity assay. Transfected COS-1 cells were metabolically labeled and lysed. LPH and

**Fig. 1. Cartoon of the processing of LPH.** LPH is synthesized as prepro-LPH. Removal of the signal sequence results in pro-LPH. After complex glycosylation in the Golgi apparatus, pro-LPH is cleaved into prepro-LPH. Removal of the signal sequence results in pro-LPH. After

**Fig. 2. Schematic overview of several structural features in LPH.** The encircled Glu residues depict the essential residues in the active sites. The homologous regions are denoted by roman numerals. The introduction site of the VSV tag is depicted as well. The αβ cleavage site is located at Arg-734/Leu-735, intestinal LPH has Ala-869 at its N-terminus.

**Fig. 3. Alignment of the amino acid and DNA sequences of wtLPH and LPHST around the introduction site of the VSV tag.** Lines connect the corresponding amino acids. The box is drawn around the VSV tag sequence, which is shown in boldface type. Base pairing numbering is given above the sequence, and amino acid numbering is given below.
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LPHST were immunoprecipitated (see above) using the HBB1/909 antibody. Precipitates were divided into several fractions and treated with 50 μg/ml trypsin at 37 °C for the indicated incubation times. Samples were subjected to SDS-PAGE on a 8% gel that was analyzed by fluorography.

Enzyme Activities—Disaccharidase activities of immunoprecipitated LPHs were measured according to Dahlqvist (16) using lactose as a substrate. The method was essentially the same as described by Naim et al. (8). LPH was immunoprecipitated using the HBB1/909 antibody from lysates of 165-cm² culture flasks of transfected Caco-2 cells. The precipitates were dissolved in 2 ml of phosphate-buffered saline containing 0.3% Triton X-100 of which 25-μl aliquots were used for the enzyme activity assay. The amount of precipitated LPH was estimated in 500-, 300-, 200-, and 100-μl aliquots on an SDS-PAGE gel with a bovine serum albumin concentration standard series run on the same gel; the gel was stained with Coomassie Brilliant Blue.

Immunofluorescence and Confocal Microscopy—Cellular localization of expressed proteins in COS-1 cells and Caco-2 cells was studied with cells grown on coverslips. Cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. Immunolabeling was carried out using (as primary antibodies) monoclonal antibodies HBB1/909 against human LPH, P5D4 against the VSV epitope tag, and the polyclonal antibody V496 against the 12 amino acids directly after the signal sequence of pro-LPH. The secondary antibodies employed fluorescein isothiocyanate-conjugated goat anti-mouse or swine anti-rabbit IgG, Texas Red-conjugated goat anti-mouse or anti-rabbit IgG (all were from Boehringer Mannheim BV). Surface localization of proteins was assessed in transfected cells that were not fixed nor permeabilized. Labeling was carried out at 4 °C. Label was visualized using a Bio-Rad MRC1000 confocal scanning laser microscope using a double channel for fluorescein isothiocyanate and Texas Red or on a routine fluorescence microscope.

Immunoelectron Microscopy—Ultrastructural localization studies were performed on transfected Caco-2 TC7 clones grown on a filter that had been confluent for 5 days. They were fixed with 1% paraformaldehyde and stored until use in 1% paraformaldehyde. Filters were stacked in 10% bovine serum albumin concentration standard series run on the same gel; the gel was stained with Coomassie Brilliant Blue.

RESULTS AND DISCUSSION

Processing of wtLPH and LPHST in COS-1—To examine the influence of the introduction of the VSV tag into LPH, the processing, localization, and protease sensitivity of both wtLPH and LPHST were compared in COS-1 cells. Immunoprecipitations using the HBB1/909 antibody directed against pro-LPH and mature LPH resulted in precipitation of high mannose (~215 kDa) and complex glycosylated (~230 kDa) LPH (Fig. 4, first four lanes). No mature form (~160 kDa) could be observed, due to the absence in these cells of the protease that is responsible for the α/β cleavage (1, 2, 8, 18). However, both LPHST and wtLPH were present at the cell surface as shown by immunofluorescence labeling (see below). The results clearly show that introduction of the VSV tag did not affect the transport competence of the mutant.

P5D4, the antibody against the VSV epitope tag, did not recognize wild type pro-LPH but did precipitate the tagged LPH construct LPHST (Fig. 4, middle lanes). The V496 antibody directed against an epitope of LPHα precipitated both constructs (Fig. 4, last four lanes). The pro-LPH species precipitated by the V496 antibody corresponds to the mannos-rich precursor as was determined by endo H treatment of the precipitates (not shown).

Trypsin Sensitivity Assay—To compare sensitivities toward protease treatment of both LPH and LPHST proteins, they were precipitated from transfected COS-1 cells by the HBB1/909 antibody against LPH. The precipitates were treated for several time intervals with trypsin. In Fig. 5 is shown that insertion of the tag did not alter the sensitivity toward trypsin since both proteins show exactly the same pattern. Trypsin treatment results already after 75 s in a complete cleavage of the high mannose and complex forms of wtLPH and LPHST into two bands of around 140 kDa that remain trypsin insensitive for at least 1 h.

After 75 s, a band of about 100 kDa can be observed that gradually disappears after 15 min. In the same time, a band of about 69 kDa appears. Most likely these bands comprise LPHα as proposed earlier (1, 2).

Since differently folded molecules are expected to behave differently toward the same protease, in this case trypsin, the results lend strong support to the notion that the folding of LPH and LPHST in COS-1 cells is similar.

Processing and Enzymatic Activity of wtLPH and LPHST in Caco-2—A Caco-2 clone with an undetectable endogenous LPH expression (TC7) (11) was transfected with the pLPHST and pLPHwt constructs. Stable transfectants were selected with puromycin and screened using immunofluorescence labeling with antibodies directed against LPHβ. From each transfec-
tion, the clone with the highest expression was chosen for further studies. For the LPHST construct, this clone is called ST54, the wtLPH clone is WT2. As a control, a clone with an endogenous LPH expression (PD10) (11) was used.

Immunoprecipitation of pro-LPH and mature LPH from pulse-chased WT2 cells shows that LPH is synthesized as a 215-kDa single-chain polypeptide (Fig. 6, lanes 1 and 2), which becomes complex glycosylated to 230 kDa and therefore endo H insensitive after 4 h of chase (lanes 3 and 4). At this point, LPHβ appears as a faint band of 160 kDa. This band is more intense after 24 h of chase when the high mannose form has disappeared (lanes 5 and 6). This processing pattern matches the pattern observed in PD10 cells (lanes 13–18). In intestinal explants, the processing appears to be somewhat faster (2) but is essentially the same.

Introduction of a VSV tag in LPH did not influence the processing since LPHST was processed in ST54 cells (Fig. 6, lanes 7–12) in a comparable fashion to wild type LPH in both WT2 and PD10 cells. Sometimes a band of about 180 kDa is observed in Fig. 6 (lanes 1, 3, 7, 9, 11, and 13). This is a nonspecific background band that can be observed in other immunoprecipitations as well, like with anti-sucrase-isomaltase antibodies from not only ST54 but from other Caco-2 cells as well (data not shown).

The enzymatic activity of immunoprecipitated lactase from a 165-cm² culture flask wtLPH or LPHST transfected Caco-2 cells was determined. The lactase precipitated from both cell lines was capable of cleaving 59 and 61 nmol of lactose per minute, respectively. The protein quantities as determined by Coomassie staining of an SDS-PAGE gel with the immunoprecipitates were essentially the same. Therefore we conclude that both constructs display comparable lactase activities and that the introduction of the tag has no influence on this activity.

Immunoprecipitation of LPHα—V496 is an antibody directed against the 12 amino acids following the signal sequence of pro-LPH (2). In previous studies, a 100-kDa band could be observed after immunoprecipitations from intestinal explants using this antibody, which was suspected to be LPHα (2). A band of this size was never observed in immunoprecipitation studies from Caco-2 clones in this study. Only the high man-

![Fig. 6. Trypsin sensitivity assay of LPH and LPHST expressed in COS-1 cells. Transfected COS-1 cells were labeled with Tran35S-label for 4 h. LPH was precipitated using the HBB1/909 antibody. Precipitates were treated with 50 μg/ml trypsin for the indicated time intervals and analyzed by SDS-PAGE followed by fluorography.](image)

![Fig. 7. Immunofluorescence microscopy of permeabilized COS-1 (panels A-F) and Caco-2 cells (panel G-L), transfected with wtLPH (panels A, B, C, G, H, and I) or LPHST (panels D, E, F, J, K, and L) using antibodies against pro-LPH and mature LPHβ (HBB1/909), LPHα (V496), or the VSV epitope tag (P5D4). Bar, 25 μm.](image)
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Localization of wtLPH and LPHST in COS-1 Cells—In immunofluorescence studies using HBB1/909 on permeabilized 5 day confluent Caco-2 WT2 and ST54 cells, a patchy labeling pattern was visible on some clusters of cells, whereas others did not show label (Fig. 7, panel F). From the apical membrane, P5D4 against the VSV tag was able to show a faint label (Fig. 7, panel A). After subcloning using a limiting dilution protocol, the same distribution was observed (not shown). A comparable mosaic expression has been described for in vivo expression of LPH and for other transfections in Caco-2 cells before (11, 19). Labeling of nonpermeabilized cells also showed a patchy labeling pattern, indicating that mainly the cell surface was labeled (Fig. 8, panel A and D). This surface was shown to be the apical membrane by confocal microscopy (Fig. 9).

Immunoelectron microscopy studies on WT2 and ST54 cells confirmed the immunofluorescence data. Some cells did not have any label, whereas others were clearly labeled the brush border (Fig. 10). At the basolateral membrane (Fig. 10, arrow), no labeling was observed. Some gold particles could be found in the endoplasmic reticulum. The labeling pattern of WT2 (Fig. 10, panel A) and ST54 (panel B) was essentially the same.

Despite the fact that V496 labeled some intracellular structures in transfected COS-1 cells in immunolocalization studies, no labeling could be found under the same conditions in transfected Caco-2 cells (Fig. 7, panels H and K). This is a consequence of the differences in the steady state in COS-1 and Caco-2 cells. In transiently transfected COS-1 cells, the protein synthesis level is much higher then in stably transfected Caco-2. Therefore relatively more LPH in COS-1 cells is present in its high mannosyl glycosylated form, and recognized by V496. P5D4 against the VSV tag was able to show a faint label at the surface of a few (less then 0.1%) Caco-2 cells expressing tagged LPH (Fig. 7, panel L; Fig. 8, panel D). This surface staining is most likely labeling of complex glycosylated pro-LPH, which could be precipitated in surface immunoprecipitation experiments on Caco-2 WT2 and WT54 cells using the HBB1/909 antibody (Fig. 11). From the apical membrane, LPHβ and complex glycosylated pro-LPHST could be precipitated (lane 1). From the basolateral membrane, some pro-LPHST but not LPHβ could be precipitated (lane 2). Expression of pro-LPH at the cell surface has been found before in biopsy samples (20). In transfected Madin-Darby canine kidney cells, it was found at both membrane domains as well (21, 22). The ultimate fate of pro-LPH on the basolateral membrane, degradation, or transport to the brush border needs further analysis.

Concluding Remarks—Many proteins undergo proteolytic processing after translation. Among these proteins are lysosomal (23), secretory (24, 25), and some plasma membrane proteins. Most disaccharidases are plasma membrane proteins that are cleaved into their subunits (26). LPH is an example of a disaccharidase that undergoes proteolytic cleavage. Unlike other examples LPH has a C-terminal instead of a N-terminal transmembrane region, and both its active sites are located on one cleavage product. Furthermore, it consists of four homolo-
gous regions instead of two, which are probably derived from a double gene duplication (20). Two of these regions, III and IV, contain the active sites and are both localized on the same cleavage product, LPHβ, which is expressed on the brush-border membrane. The second cleavage product, LPHα, possesses the other two homologous regions, I and II, but does not show any activity toward disaccharides (2, 4). Two separate studies have reported a role for LPHα as an intramolecular chaperone (2, 5). An additional function could be hypothesized because of the internal homologies and because LPHα is relatively large (714 residues). Therefore we developed a model in which we could study LPHα in more detail. This model consists of Caco-2 cells expressing wild type or modified LPH containing a VSV epitope tag in its LPHα domain for additional recognition possibilities. Immunoprecipitation studies from these cells using both an antibody against LPHα and an antibody against the LPHα-inserted tag did not result in a specific profragment band. Furthermore, localization studies in Caco-2 cells did not result in specific labeling in any LPH-positive cell. We suggest that in Caco-2 cells LPHα is degraded soon after cleavage since no intra- or extracellular accumulation could be observed. Our results do not ascertain whether degradation of LPHα occurs on its way to the brush border or at the brush border itself.

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