Protein Domains Implicated in Intracellular Transport and Sorting of Lactase-Phlorizin Hydrolase*

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The roles of various domains of intestinal lactase-phlorizin hydrolase (pro-LPH) on its folding, dimerization, and polarized sorting are investigated in deletion mutants of the ectodomain fused or not fused with the membrane-anchoring and cytoplasmic domains (M Akt). Deletion of 236 amino acids immediately upstream of M Akt has no effect on the folding, dimerization, transport competence, or polarized sorting of the mutant LPH1646M Akt. By contrast, LPH1646, an anchorless counterpart of LPH1646M Akt, is not transported beyond the ER and persists as a mannose-rich monomer during its entire life cycle.

The further deletion of 87 amino acids generates a correctly folded but transport-incompetent monomeric LPH1559M Akt mutant. The results strongly suggest that dimerization and transport of pro-LPH implicate a stretch of 87 amino acids in the ectodomain between LPH1646M Akt and LPH1559M Akt. In addition, dimerization of pro-LPH requires at least two further criteria: (i) a correctly folded ectodomain of pro-LPH and (ii) the presence of the transmembrane region. Neither of these requirements alone is sufficient for dimerization. Finally, the sorting of pro-LPH appears to be mediated by signals located between the cleavage site of pro-LPH and the LPH1646M Akt mutant.

Human lactase-phlorizin hydrolase (LPH)* (EC 3.2.1.23/62) is an integral glycoprotein of the intestinal brush border membrane. It reveals two hydrolytic activities on the same polypeptide chain (1); lactase hydrolyzes the milk sugar lactose, the main carbohydrate in mammalian milk, and phlorizin hydrolase digests β-glycosyleramides, which are part of the diet of most vertebrates. Whereas reduced lactase activity leads to newborns and adults (2–4), a physiological role of phlorizin hydrolase activity is still unknown (5–7).

In human small intestinal epithelial cells, LPH is synthesized as a single chain precursor (Mr = 215,000) that undergoes core N-glycosylation while translocating into the ER. Possibly in the ER or in an intermediate compartment, LPH monomers form homodimers prior to Golgi processing (8). Along the pathway through the Golgi apparatus en route to the cell surface, the N-linked sugar chains are truncated, and carbohydrates are added, resulting in a complex-glycosylated protein (Mr = 230,000), which is intracellularly cleaved to the mature brush border form of 160 kDa (9–11). This intracellular processing was also observed in a transfected MDCK cell line, localizing the cleavage of the profragment to intracellular compartments after the trans-Golgi network (12). In contrast, the expression of a full-length cDNA encoding LPH in COS-1 cells reveals an uncleaved pro-LPH at the cell surface with an enzymatic activity similar to that of the intestinal mature brush border 160-kDa species (13). Consequently, the intracellular proteolytic processing of pro-LPH in intestinal epithelial cells is not essential for acquisition of transport competence and biological function (13). Furthermore, neither the cleavage process nor the large profragment LPHα that is generated upon proteolytic cleavage of pro-LPH are implicated in the sorting event of LPH to the apical membrane (13). Recent observations have strongly suggested that putative sorting signals of pro-LPH are exclusively located in the domain corresponding to the brush border-associated LPHβ.

The gene coding for human LPH is located on chromosome 2 and contains 17 exons (14, 15). The cDNA consists of 6274 base pairs, coding for 1927 amino acids. LPH is a type I protein with a NH2-terminal extracellular domain followed by a transmembrane domain comprising 19 hydrophobic amino acids and a COOH-terminal cytoplasmic domain of 26 amino acids (1, 17). The extracellular domain itself consists of a NH2-terminal cleavable signal peptide (19 amino acids) necessary for translocation into the ER (18) followed by four homologous regions with 38–55% identity to one another (1). The catalytic activity of lactase is localized to glutamine 1273 in the homologous region III, and the activity of phlorizin hydrolase is localized to glutamine 1749 in the homologous region IV (17). Because of the four homologous regions, LPH may have arisen from two gene duplications in evolution (1).

In the work presented here, we analyzed the intracellular transport and polarized sorting of deletion mutants in the extracellular domain IV of LPH contiguous to the transmembrane domain as well as the role of the membrane anchor in a transport-competent mutant, emphasizing the quaternary structure of the proteins. In a variety of membrane proteins such as low density lipoprotein receptor, sucrase-isomaltase and aminopeptidase N (19–21) approximately 17–37 amino acids from the membrane anchor are highly O-glycosylated,
revealing an unfolded tertiary protein structure. This region, referred to as the “stalk region,” serves as a link between the globular protein and the plasma membrane. Putative factors implicated in a postulated active intracellular protein transport might interact with this region because of the membrane proximity. We demonstrate that a region that reveals features of a stalk region is neither obligatory for intracellular transport of membrane-bound LPH nor for its polarized sorting to the apical membrane. Furthermore, the deletion of 236 amino acids containing more than one-third of phlorizin-hydrolase (homologous region IV) including the catalytic domain has almost no influence on LPH dimerization and transport to the plasma membrane. In contrast, the further deletion of 87 amino acids in the phlorizin-hydrolase domain had significant effects on LPH dimerization and thus on intracellular transport of the protein without significant alterations in the tertiary protein structure. We propose an important role for parts of the phlorizin hydrolase domain in protein-protein interactions and thus in intracellular transport in addition to the physiological role, which is still unknown (5–7).

EXPERIMENTAL PROCEDURES

Materials—Trans-[35S]Methionine was obtained from ICN. Tissue culture dishes were acquired from Falcon Division (Becton Dickinson Labware). Dulbecco’s modified Eagle’s medium, minimum essential medium, streptomycin, penicillin, and fetal calf serum were purchased from Life Technologies Inc. DEAE-dextran, pepstatin, leupeptin, antipain, aprotinin, molecular weight standards for SDS-PAGE, n-dodecyl b-β-maltose, trypsin, β-amylose, apoferritin, and thyroglobulin were obtained from Sigma. Acrylamide, N,N,N′-methylenebisacrylamide, SDS, TEMED, ammonium persulfate, 2-mercaptoethanol, diithiothreitol, and Triton X-100 were acquired from Bio-Rad. Phenylmethanesulfonyl fluoride was purchased from Boehringer Mannheim and protein A-Sepharose from Amersham Pharmacia Biotech. All other reagents were of superior analytical grade.

Reagents for Recombinant DNA Techniques—Restriction enzymes, T4 polymerase, and T4 ligase were obtained from New England Biolabs. Taq DNA polymerase was obtained from Perkin-Elmer. For PCR synthesis, the following oligonucleotides were used: LPH5658 (5′-GGATTCAAGGTACGCTCTTCTTCTTCTTCA-3′) and CLPH6270 (13). Plasmid pGEM 4Z was digested with EcoRI and BamHI to release the COOH-terminal Cla/EcoRI fragment of LPH (black arrow) as described previously for the cloning of LPH1646M, were inserted into the vector pCMV2 (Fig. 2). The resulting plasmid pLPH was used for further cloning. The COOH-terminal Cla/EcoRI fragment of LPH (black arrow) was subcloned for the construction of the deletion mutants. amp, ampicillin resistance gene.

human growth hormone polyadenylation site and transcription terminator, the CMV promoter, and the SV40 early region promoter/enhancer and origin of replication (13) (Fig. 2). Correct gene orientation was examined by HindIII mapping. To construct mutant LPH1646, depleted of the membrane anchor and the cytoplasmic domain, the two extracellular LPH fragments, as described previously for the cloning of LPH1646M, were inserted into the vector pCMV2 (Fig. 2). This construction resulted in HPH1646 expression with the additional amino acid leucine located at the COOH terminus.

Transfection, Generation of Stable Cell Lines, and Biosynthetic Labeling of Cells—COS-1 and MDCK cells were grown in Dulbecco’s modified Eagle’s medium containing 292 mg/ml glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum. They were transfected without DNA (mock) or 5 μg of the appropriate recombinant DNA using DEAE-dextran (for COS-1 cells) essentially as described before (13) or polybrene (for MDCK cells) (12). Expression of LPH1646M in stable MDCK cells was assessed by immunoprecipitation of total cell extracts with anti-LPH mAb. Transiently transfected COS-1 cells were labeled 48–60 h posttransfection with 80 μCi of [35S]methionine in methionine-free minimum essential medium containing 2% fetal calf serum. The labeling was either for 1 h, followed by a chase with an excess of nonlabeled methionine (2.5 mm) for different periods of time, or continuously.

MDCK cells stably expressing LPH1646MACT were grown on filters as described before (12) and labeled with [35S]methionine for 18 h. Surface antigens were immunoprecipitated by the addition of mAB anti-LPH to either the apical or basolateral compartments.

Immunoprecipitation of Cell Extracts and SDS-PAGE—The labeled cells were rinsed twice with phosphate-buffered saline. Cells were solubilized with 1 ml/dish of cold lysis buffer containing 25 mm Tris-HCl, pH 8.0, 50 mm NaCl, 0.5% sodium deoxycholate, 0.5% Triton X-100, and the following mixture of protease inhibitors: 1 mm phenylmethanesulfonyl fluoride, 1 μg/ml pepstatin, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml aprotinin, and 100 μg/ml soybean-trypsin inhibitor. The cell extracts were centrifuged for 20 min at 17,000 × g and 4 °C to remove nuclei and debris. The supernatants were incubated with anti-LPH antibodies and precipitated with protein A-Sepharose. The immunoprecipitates were washed according to Ref. 11. One-dimensional electrophoresis was performed on 6% acrylamide gels according to Ref. 24. Immunoprecipitates were dissolved in 80 mm Tris/HCl, pH 6.8, 0.1 mm diithiothreitol, 7% SDS, 10% glycerol, 0.01% bromphenol blue at 100 °C for 5 min. Gels were fixed and soaked in 16% salicylic acid for signal amplification, dried, and exposed to Kodak XAR 5 films at −70 °C. The following molecular mass standards were used: myosin (202 kDa),

FIG. 1. Schematic drawing of the recombinant plasmid pLPH. The full-length cDNA encoding human LPH (dotted and black arrow) was inserted into the unique EcoRI site of vector pGEM 4Z (13). The resulting plasmid pLPH was used for further cloning. The COOH-terminal Cla/EcoRI fragment of LPH (black arrow) was subcloned for the construction of the deletion mutants. amp, ampicillin resistance gene.
FIG. 2. Schematic drawing of wild type and mutant LPH molecules. The amino (N) and carboxyl terminus (C) determine the borders of wild type and mutant proteins, respectively. Homologous domains I, II, and III of LPH are denoted in white bars. Homologous region IV, which is partly deleted in the mutant proteins, is marked with a dotted bar. The transmembrane and cytoplasmic domains of LPH (MACT) are shown in striped bars. The number of amino acids (aa) of wild type LPH from the amino terminus to the beginning of the transmembrane domain are indicated in each protein. CMV, CMV promoter; hGH, human growth hormone polyadenylation site and transcription terminator; SV40-ori, SV40 early region promoter/enhancer and origin of replication; Amp, ampicillin resistance gene.

β-galactosidase (116 kDa), phosphorylase b (97.5 kDa), bovine serum albumin (68 kDa), and ovalbumin (46 kDa).

Processing of Immunoprecipitated Glycoproteins and Trypsin Treatment of Cell Extracts—Endo-N-β-acetylglucosaminidase H (endo H) was used for the removal of N-linked mannose-rich carbohydrates and endo-N-β-acetylglucosaminidase F/N-glycopeptidase F (PNGase F) was applied to deplete mannose-rich as well as complex N-linked sugar chains from the glycoproteins. Both endoglycosidases were purchased from Biolabs Corp. Cell extracts were immunoprecipitated with mAb anti-LPH as described above. The immunoprecipitated proteins bound to protein-A-Sepharose beads were treated as described by the manufacturer, using 0.5 unit of endo H and 1 unit of PNGase F. For trypsin treatment, cells were solubilized in cold lysis buffer on ice without protease inhibitors. After centrifugation for 20 min at 17,000 × g and 4 °C, the supernatants were incubated with 50 μl of 3% Triton X-100 after fixation. ER localization was confirmed by double-immunofluorescence staining with polyclonal anti-protein-disulfide isomerase and rhodamine-conjugated goat anti-rabbit IgG at 1:50 and 1:100 dilutions, respectively.

Cell Lysate Fractionation on Sucrose Density Gradients—The molecular mass of proteins in cell lysates was determined according to Ref. 25. COS-1 cells were transfected and biosynthetically labeled with [35S]methionine. Cell extracts were analyzed with anti-LPH M1/45 mAb. Three equal aliquots were treated with endo H or PNGase F or without any glycosidases. The dot in the second lane of LPH1559MACT represents complex-glycosylated protein of mutant LPH1559MACT. The molecular masses were estimated from standard molecular weight markers run in parallel.

FIG. 3. Glycosylation patterns of mutant and wild type LPH proteins. Transfected COS-1 cells were biosynthetically labeled for 18 h with [35S]methionine. Cell extracts were analyzed with anti-LPH M1/45 mAb. Three equal aliquots were treated with endo H or PNGase F or without any glycosidases. The dot in the second lane of LPH1559MACT represents complex-glycosylated protein of mutant LPH1559MACT. The molecular masses were estimated from standard molecular weight markers run in parallel.

RESULTS

Expression of LPH Wild Type and Deletion Mutants in COS-1 Cells—To investigate the intracellular transport of LPH mutants as compared with wild type LPH, COS-1 cells were transfected with the plasmid pCMV2 containing the recombinant DNA of wild type and mutant LPH (Fig. 2). All cell extracts were immunoprecipitated with mAb anti-LPH M1/45 (23). Samples were treated in parallel with endo H to identify mannose-rich LPH molecules predominantly in the ER or with PNGase F to detect complex-glycosylated proteins in the Golgi apparatus and at the plasma membrane. Untreated immunoprecipitations were used as a control. Biosynthetic labeling for 18 h revealed that LPH1646MACT showed at steady state level almost the same amounts of mannose-rich (193 kDa) and mannose-rich complex-glycosylated (200-kDa) molecules as wild type LPH (215 and 230 kDa) (Fig. 3). The ratio of Golgi-localized LPH to ER-concentrated molecules is best shown in endo H-treated samples. The slight decrease in molecular weight of the complex-glycosylated proteins in these samples is due to the depletion of mannose-rich sugar chains in predominantly complex-
glycosylated proteins. Either protein could be deglycosylated with PNGase F to molecular masses of 200 kDa for wild type LPH and 176 kDa for LPH1646MACT. In contrast, mutant LPH1559MACT exhibited only a slight amount of complex-glycosylated molecules (195 kDa), whereas the majority of the synthesized intracellular protein pool remained mannose-rich (190 kDa) (Fig. 3). The low quantity of Golgi-localized molecules relative to mannose-rich species was calculated for each time point.

Depletion of N-linked sugar chains could be achieved with PNGase F, resulting in a polypeptide with increased mobility to 166 kDa. These results indicate an inefficient intracellular transport of mutant protein LPH1559MACT from the ER to the Golgi and the plasma membrane. The deletion mutant LPH1646 (192 kDa), lacking the membrane anchor and the cytoplasmic domain was endo H-sensitive, resulting in deglycosylated molecules with apparent Mr = 175,000 (Fig. 3). The findings suggest that this mutant remains in the ER as mannose-rich precursors at steady state.

To determine the kinetics of intracellular transport of LPH mutants in COS-1 cells, pulse-chase experiments with [35S]methionine were performed. An interval of 7 h proved to be adequate for the transport of wild type LPH to the plasma membrane (13). Fig. 4A depicts the results of the electrophoretic analyses of the immunoprecipitates, and Fig. 4B represents the quantification of these fluorograms. 1 h into the chase, 49% of wild type LPH and 31% of mutant LPH1646MACT were converted to the corresponding complex-glycosylated forms. The amount of complex-glycosylated molecules toward the high mannose forms increased during a prolonged chase. 96% of wild type LPH achieved complex-glycosylation after 5 h of chase, whereas LPH1646MACT attained the mature form of the protein after 7 h of chase. Both proteins seem to have different transport kinetics from the ER to the Golgi with a delay of LPH1646MACT of approximately 2 h. Nevertheless, mutant LPH1646MACT with a deletion of 236 amino acids in the ectodomain could be slowly but efficiently transported to the Golgi. By contrast, no more than 10% of mannose-rich LPH1559MACT was converted to the final glycosylated form (Fig. 4). Taken together, these and the data obtained after 18 h of continuous labeling of LPH1559MACT (Fig. 3) demonstrate that this mutant protein is inefficiently transported from the ER to the Golgi. We propose that a deletion of an additional extracellular 87 amino acids in the transport-competent mutant LPH1646MACT results in ER localization of the LPH1559MACT product.

Cell Surface Expression of LPH Mutants—The mature LPH molecule is expressed at the cell surface of small intestinal brush border membranes (9, 11) as well as of transfected COS-1 cells (13). To investigate the cellular distribution of LPH mutants, transfected COS-1 cells were grown on coverslips and subsequently prepared for indirect immunofluorescence microscopy of intact and permeabilized cells. The results are shown in Fig. 5. Nonpermeabilized cells showed bright
immunofluorescence staining with the antibody MLac1 at the cell surface. This clearly demonstrated that LPH1646MACT is correctly transported to the plasma membrane in COS-1 cells as predicted by its final complex-glycosylated molecules and consistent with the biochemical data shown in Figs. 3 and 4. The permeabilization of the transfected cells with Triton X-100 revealed the intracellular distribution of the transfected protein (Fig. 5b). The mAb MLac1 strongly labeled the Golgi and the ER of the transfected cells, but further MLac1 staining was observed at the periphery of the permeabilized cells. In contrast, the construct LPH1559MACT displayed relative faint immunofluorescence signals at the cell surface after staining with the mAb MLac1 (Fig. 5c), while the prominent staining was confined to intracellular compartments (Golgi and ER) after permeabilization with Triton X-100 (Fig. 5d). The low staining of LPH1559MACT at the cell surface shows that a minor proportion of this construct has reached the cell surface. This is consistent with the biochemical data (Figs. 3 and 4) in which a low proportion of LPH1559MACT has been shown to acquire complex type of glycans. In contrast to LPH1646MACT, COS-1 cells transfected with the mutant LPH1646, lacking the membrane anchor and cytoplasmic domain, could not be localized at the cell surface (data not shown). However, the protein showed clear ER localization after permeabilization with Triton X-100 (Fig. 5f) as assessed in double labeling experiments using protein-disulfide isomerase as a protein marker of the ER (Fig. 5, compare e and f). Further, the subcellular localization of LPH1646 is consistent with the biochemical data of this mutant (Fig. 3), in which we demonstrated that LPH1646 persists as a mannose-rich species.

**Structural Requirements for Exit of LPH Mutants from the ER**—Previous investigations demonstrated that wild type LPH assembles as a homodimer in the ER and is subsequently transported to the Golgi apparatus and the plasma membrane (8). To investigate the quaternary structure of the mutant LPH proteins, cell lysates containing these proteins were separated on sucrose density gradients, and fractions were immunoprecipitated and analyzed by SDS-PAGE (Fig. 6). The molecular mass of proteins in each fraction was estimated by comparisons with standard proteins and with brush border proteins (8, 25) that were separated on similar sucrose gradients. In particular, β-amylase and apoferritin were previously found to be suitable markers for estimation of the size of intestinal glycoproteins such as dipeptidyl peptidase IV, aminopeptidase N, and monomeric and dimeric pro-LPH (8, 25).

COS-1 cells transfected with LPH1646MACT revealed after 1 h of pulse labeling with [35S]methionine mannose-rich glycosylated protein peaks in fractions 7 and 9, respectively (Fig. 6A). The protein in fraction 9 colocalizes with β-amylase (200 kDa) and apoferritin (450 kDa) and represents therefore a monomeric form of LPH1646MACT with a molecular mass of about 193 kDa as estimated from SDS-PAGE (Fig. 6A). The LPH1646MACT species recovered in fraction 7 could represent only a homodimer of LPH1646MACT with approximate Mr = 386,000, since it appeared in fraction 7, which lies between monomeric LPH1646MACT (Mr = 193,000) and the marker protein apoferritin of Mr = 450,000 found in fraction 5 of the denser phase. Further, a dimeric form of LPH1646MACT appeared before complex-glycosylation, and a significant proportion of the synthesized protein dimerized in the ER within the first hour of chase. At 2 h of chase, the complex-glycosylated molecules appeared exclusively in the denser fraction 7, suggesting that LPH1646MACT has egressed the ER as a dimer, which was subsequently transported through the Golgi apparatus. After 6 h of chase, most of the proteins were found as mannose-rich or complex-glycosylated dimers in fractions 6 and 7 (Fig. 6A).

Additionally, a third protein peak comprising fractions 2–4 and containing exclusively complex-glycosylated LPH1646MACT molecules could represent trimers (Mr = 772,000) or higher oligomers, since thyroglobulin of Mr = 660,000 was predominantly found in fraction 4. These oligomers should have been assembled in the Golgi apparatus or later in the secretory pathway. This oligomer formation was not expected, since wild type pro-LPH dimerizes only (8). We do not yet know the impact of this event on the intracellular transport of LPH1646MACT. Nevertheless, we can be certain that this process is not essential for the transport of LPH1646MACT from the ER.

LPH1559MACT revealed after 1 h of pulse labeling only one single peak comprising fractions 9 and 10, which contained the mannose-rich LPH1559MACT polyepitope (Fig. 6B). In comparison with the LPH1646MACT forms, which were run on similar gradients, the single peak contained monomers of LPH1559MACT of approximate Mr = 190,000. At 2 h of chase, a small proportion of mannose-rich and complex-glycosylated LPH1559MACT were revealed in the denser phase in fractions 5–7. These fractions contained a homodimer of LPH1559MACT of Mr = 380,000 in fraction 7 and presumably a higher order oligomer in fraction 5, since this fraction overlapped perfectly with apoferritin of Mr = 450,000.
Interaction of LPH1559MACT with cellular proteins cannot be excluded. However, such proteins could not be detected by SDS-PAGE.

Mutant LPH1646 depleted of the membrane anchor and the cytoplasmic domain revealed exclusively mannose-rich species after prolonged metabolic labeling (Fig. 3). This mutant appeared predominantly in fraction 9 of the sucrose density gradients after 7 h of biosynthetic labeling (Fig. 6B, lowest panel) and represented therefore a monomeric form of LPH1646.

Altogether, our results demonstrate that the membrane anchor of LPH as well as a stretch in the ectodomain of 87 amino acids missing in the LPH1559MACT mutant appear to be directly implicated in the dimerization event required for ER exit and transport to the Golgi and the plasma membrane. In fact, LPH1646MACT with efficient dimerization in the ER (Fig. 6A) has acquired complex-glycosylated forms in the Golgi with kinetics comparable with those of wild type LPH (Fig. 4). LPH1559MACT with slight oligomerization in the ER (Fig. 6B) was inefficiently complex-glycosylated in the Golgi (Fig. 4), whereas LPH1646 did not dimerize (Fig. 6B) and was retained in the ER as a mannose-rich species (Figs. 3 and 5).

**FIG. 7. Epitope mapping of mutant and wild type LPH.** COS-1 cells were transfected with the specific recombinant DNA and pulse-labeled for 2 h with [35S]methionine and chased for 5 h with an excess of cold methionine. Cell lysates were divided into equal aliquots and immunoprecipitated with different anti-LPH mAb. The immunoprecipitated proteins were analyzed by SDS-PAGE.

The deletion mutants showed different profiles of quaternary structure with subsequent effects on intracellular transport kinetics. The persistence as a monomer, as in the case of LPH1646, or the inefficient dimerization, as observed with LPH1559MACT, could be the consequence of altered tertiary structure of the ectodomains of the mutant enzymes, since oligomerization of proteins requires complementary amino acid sequences of the subunits (26, 27). We therefore attempted to analyze the globular structure of the mutant proteins by employing two sensitive approaches. In the first approach, several anti-LPH mAb that recognize diverse epitopes of native LPH were used. The rationale is that if an antibody fails to bind a LPH mutant, then this would indicate either an altered globular protein structure or a deletion of the specific epitope. In the second approach, the stability of the mutant proteins was probed with trypsin. The idea is that misfolded proteins exhibit protease-sensitive domains, which are normally shielded in the core of the native wild type protein.

Transfected COS-1 cells were labeled with [35S]methionine, and cell lysates were immunoprecipitated with different anti-LPH mAb (MLac series). All of these antibodies reacted with wild type LPH and the mutants LPH1646MACT, LPH1559MACT, and LPH1646 (Fig. 7). The antibodies tested were all raised against mature LPH, and it is likely therefore that conformation-specific antibodies are among those used (23). The results, therefore, strongly suggest that the LPH epitopes tested were present or were not substantially modified in the mutants as compared with wild type LPH. Together with the observation that the antibodies used react only with solubilized native LPH species and do not react with denatured LPH on Western blots (23, 28) the data suggest that the tertiary structures of the deletion mutants are comparable with their counterparts in wild type LPH.

These data were corroborated by a second approach. Here, the stability of the mutants was compared with that of wild type pro-LPH in a protease sensitivity assay using trypsin. Pro-LPH is cleaved at potential trypsin sites to the 160-kDa mature LPH (LPHβ) (13, 29). Trypsin is therefore a useful protease that could be employed to examine the conformation of pro-LPH and its mutants.

Transfected COS-1 cells were labeled biosynthetically with [35S]methionine and solubilized on ice without protease inhibitors. Lysates were treated with trypsin for different times, and the reaction was arrested with soybean trypsin inhibitor followed by immunoprecipitation with mAb anti-LPH. Fig. 8 shows that at 0 min of trypsin treatment, wild-type pro-LPH consisted of three bands, the mannose-rich 215-kDa form, the complex-glycosylated 230-kDa form, and the 160-kDa mature form. 2 min of trypsin treatment were sufficient to convert the entire complex-glycosylated 230-kDa pro-LPH species to the 160-kDa polypeptide. The intensity of the 160-kDa polypeptide did not change after 4 min of trypsin, confirming previous findings (8, 13, 29) that the 160-kDa LPH form displays relative resistance toward trypsin. The mutant LPH1646MACT consisted at 0 min of trypsin treatment of the mannose-rich 193-kDa and complex-glycosylated 200-kDa polypeptides. Trypsin cleaved these polypeptides within 2 min to the respective mannose-rich and complex-glycosylated forms with apparent molecular masses around 70 kDa. These two forms were resistant to further trypsin digestion after 4 min. Similarly, the LPH1559MACT and LPH1646 were converted by trypsin to species with approximate apparent molecular masses of 70 kDa that were stable toward further trypsin treatment. The control employed a mutant of LPH, LPH1365MACT, from which 518 amino acids were deleted immediately upstream of the membrane-anchoring domain. This mutant appears as a mannose-rich species that could be immunoprecipitated only with a monoclonal antibody (MLac6) that recognizes malfolded forms of LPH (8, 23, 28) (Fig. 8). Trypsin treatment of this mutant form of LPH resulted in its complete degradation within 2 min.

The stability of the final trypsin products of the mutants LPH1646MACT, LPH1646, and LPH1559MACT strongly suggests that no additional trypsin sites were exposed similar to the situation in wild type pro-LPH. As a consequence, it is
reasonable to propose that no gross alterations in the tertiary structure have occurred in these mutants as a result of the deletions. By contrast, the control LPH1365MACT was misfolded and hence completely degraded by trypsin.

Together, the epitope mapping analyses and trypsin sensitivity assay strongly suggest that the tertiary structures of LPH1646MACT, LPH1646, and LPH1559MACT are comparable with their corresponding domains in wild type pro-LPH.

Sorting of LPH1646MACT—LPH is sorted to the apical membrane in intestinal cells and in polarized MDCK cells with high fidelity. It is proposed that putative apical sorting signals are located in the ectodomain of the LPH mature form. As shown above, the deletion mutant LPH1646MACT is transported to the cell surface with almost wild type kinetics. This observation prompted us to analyze its sorting in a polarized cell line and to determine whether or not the deleted region of LPH contains putative signals for apical sorting of LPH. The LPH1646MACT was expressed in a stable cell line, and its sorting was analyzed in a membrane filter system as described previously (12). Fig. 9 demonstrates that LPH1646MACT is expressed predominantly at the apical surface of MDCK cells. In fact, 90% of the mutant was found at the apical side, indicating that the sorting of LPH1646MACT is similar to wild type LPH. These results demonstrate that the deleted region is most likely devoid of putative apical sorting signals, since its deletion did not influence the sorting behavior of LPH.

DISCUSSION

Oligomerization of a variety of proteins (for a review, see Ref. 30) occurs in the ER and constitutes an essential step for protein exit from this organelle. These events in the ER are crucial for the efficient expression of enzymes, receptors, or other biological molecules at the plasma membrane, since in most of the cases the ER egress is rate-limiting along the exocytic pathway of membrane and secretory proteins.

The data presented in this paper provide insights into the dimerization, transport, and sorting of a brush border membrane protein and into the role of particular subdomains in these events. Our model protein, intestinal LPH, does not exit the ER prior to homodimerization (8). The requirement of the membrane-anchoring domain for homodimerization has been suggested by a mutant, pro-LPH mact, from which the entire transmembrane and cytoplasmic tail has been deleted. This mutant does not dimerize, resides in the ER during its entire life cycle, and is ultimately degraded.

Here, we use several deletion mutants of pro-LPH to provide a conclusive evidence for the importance of the membrane-anchoring domain and also the ectodomain in the dimerization process. One of the mutants, LPH1646, is most likely a correctly folded molecule, as corroborated by epitope mapping with six different mAbs and by protease sensitivity assays with trypsin. The intracellular transport behavior of this mutant changes dramatically depending on the presence or absence of the transmembrane domain of pro-LPH. LPH1646MACT containing the membrane anchor is a dimeric and transport-competent molecule, while its anchorless counterpart, LPH1646, is retained in the ER as a monomeric, mannose-rich species. The region encompassing the deleted 236 amino acids lies immediately upstream of the membrane-anchoring domain. It is most likely not essential in the dimerization event of wild type pro-LPH, since its absence from LPH1646MACT is not accompanied by substantial effects on dimer formation of the mutant. It is interesting that deletion of a 236-amino acid large domain of pro-LPH in the homologous region IV does not generate misfolded monomers. One possible explanation is that the four homologous regions of pro-LPH (1) are autonomous and that each folds independently. Along this line of reasoning, it is likely that intramolecular protein-protein interactions in pro-LPH ensue after protein folding of the individual subdomains has reached completion.

The LPH1646MACT protein does not only dimerize; it also
forms higher order oligomers after complex glycosylation in the Golgi. This is surprising, since wild type pro-LPH dimerizes only. We do not have any experimental evidence yet to explain this behavior. However, it is reasonable to speculate that deletion of a rigid, unfolded negatively charged O-glycosylated domain may reduce the repulsion of the individual subunits. A post-ER oligomerization, although not very common, is known to occur in some secreted proteins (31, 32).

The membrane anchor and the cytosolic domain apparently do not suffice for efficient homodimerization of another mutant, LPH1559MACT, with a further deletion of 87 amino acids upstream of LPH1646MACT. This mutant is not transport-competent, maintains a predominant mannose-rich type of glycosylation, and accumulates most likely in the ER. The failure to dimerize is in all likelihood not the consequence of misfolded tertiary structure, since this mutant reacts with epitope-specific antibodies as wild type pro-LPH and LPH1646MACT and moreover shows also comparable sensitivity toward trypsin. It is likely that a protein structure in the deleted stretch of amino acids is important in the dimerization event. This in turn sheds light on the importance of the ectodomains in the dimerization event and on the role of amino acid complementarity in protein-protein interaction. In fact, it has been shown that the ectodomains of different subtypes of the hemagglutinin virus glycoprotein cannot trimerize due to sterical hindrance (26, 27), and a single amino acid mutation in an immunoglobulin chain prevents assembly as well (33).

Alternatively, it is possible that the 87-amino acid stretch contains binding sites for ER components that halt the LPH1559MACT until it acquires a competent quaternary structure. In the absence of this stretch, binding does not occur, and a monomeric LPH1559MACT molecule diffuses into the plain of the ER.

Our results are consistent with the view that topology of protein-protein interactions is not entirely restricted to a single domain and is different in distinct membrane proteins. In line with this are the findings that trimerization of the hemagglutinin glycoprotein implicates most likely the ectodomain (34) as well as the transmembrane domain (35). On the other hand, the transmembrane domain of glycophorin (36) and the cytoplasmic tail of low density lipoprotein (37) play striking role in the assembly, although the possibility of interaction of the globular domains of these proteins is not excluded. We could here show that a correctly folded ectodomain without the membrane anchor is not sufficient for dimerization. Nevertheless, fulfillment of these two criteria per se is also not sufficient for ensuing protein dimerization as shown in the mutant from which an 87-amino acid stretch has been deleted. It seems that the interaction of specific protein domains, in this case the 87-amino acid stretch, is more significant than others within a correctly folded ectodomain.

The highly polarized sorting of pro-LPH, with almost 90% of it being sorted to the apical membrane, renders pro-LPH particularly suitable in studies aimed at elucidating sorting mechanisms and identifying putative sorting signals. A major aim of the current study is to generate transport-competent deletion mutants that could be analyzed in polarized epithelial cells with respect to their sorting behavior. The large LPHα profragment (38) is not implicated in the sorting of pro-LPH to the apical membrane, but it is essential for acquisition of pro-LPH to a transport-competent configuration. In view of this, it could be concluded that any potential information for the sorting of LPH to the apical membrane must lie in the LPHβ domain (38).

By virtue of the transport competence of the LPH1646MACT mutant, we examined the possibility that structures within the 236-amino acid deleted stretch may carry potential apical sorting signals. Our data clearly demonstrate that the deletion of this stretch has no effect at the sorting of LPH1646MACT, since this mutant is delivered with high fidelity to the apical membrane. We conclude that the domain containing the 236 amino acids is devoid of potential apical sorting signals. A part of the deleted region, perhaps the first 50 amino acids upstream of the membrane anchor, reveals structural features reminiscent of a stalked region that separates the globular domain of LPH from the membrane in a fashion similar to the stalked regions of other membrane glycoproteins (19–21). According to data derived from a survey of large number of O-glycosylation sites (40), the first 50 amino acids of the deleted region contain several potential O-glycosylation sites, which are presumably glycosylated in the mature form of LPH (amino acids 869–1927) (41). Furthermore, structural predictions of the first 20 amino acids of the deleted region (42) reveal an α-helical structure similar to the stalk of aminopeptidase N (43).

One potentially possible role of stalked regions is their implication in the polarized sorting of glycoproteins destined for the apical membrane as has been recently shown for the neurotrophic receptor (39). Our results with the LPH1646MACT mutant, however, suggest that this role may be restricted to some proteins and cannot be considered as a general sorting mechanism.

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