# Impaired Trafficking and Subcellular Localization of a Mutant Lactase Associated With Congenital Lactase Deficiency

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***Background & Aims:*** **Congenital lactase deficiency (CLD) is a cause of disaccharide intolerance and malabsorption characterized by watery diarrhea in infants fed breast milk or lactose-containing formulas. The molecular basis of CLD is unknown. Mutations in the coding region of the brush border enzyme lactase phlorizin hydrolase (LPH) were found to cause CLD in a study of 19 Finnish families. We analyzed the effects of one of these mutations, G1363S, on LPH folding, trafficking, and function.** ***Methods:*** **We introduced a mutation into the *LPH* complementary DNA that resulted in the amino acid substitution G1363S. The mutant gene was transiently expressed in COS-1 cells, and the effects were assessed at the protein, structural, and subcellular levels.** ***Results:*** **The mutant protein LPH-G1363S was misfolded and could not exit the endoplasmic reticulum. Interestingly, the mutation creates an additional N-glycosylation site that is characteristic of a temperature-sensitive protein. The intracellular transport and enzymatic activity, but not correct folding, of LPH-G1363S were partially restored by expression at 20°C. However, a form of LPH that contains the mutations G1363S and N1361A, which eliminates the N-glycosylation site, did not restore the features of wild-type LPH. Thus, the additional glycosyl group is not required for the LPH-G1363S defects.** ***Conclusions:*** **This is the first characterization, at the molecular and subcellular levels, of a mutant form of LPH that is involved in the pathogenesis of CLD. Mutant LPH accumulates predominantly in the endoplasmic reticulum but can partially mature at a permissive temperature; these features are unique for a protein involved in a carbohydrate malabsorption defect implicating LPH.**

Human small intestinal lactase-phlorizin hydrolase (LPH; EC 3.2.1.23/62) is a physiologically important digestive enzyme of the brush border membrane. It hydrolyzes lactose, the most essential carbohydrate in milk and is therefore the primary diet source of newborns.[1] The generated monsaccharides, galactose and glucose, are transported across the brush border membrane to the cell interior. Phlorizin-hydrolase (PH) is a glycosylceramidase with a broad specificity comprising various substrates like glycosyl-N-acylsphingosines, phlorizin,[2] and dietary flavonoid glycosides.[3,4]

LPH is synthesized as a single chain precursor molecule (prepro-LPH) of 1927 amino acids comprising 4 homologous domains, I to IV, a membrane anchoring region, and a short C-terminal cytoplasmic tail.[5] The biosynthesis and processing of pro-LPH has been investigated in great detail (for a review see Naim[1] and Figure 1). An important event along the secretory pathway of LPH is the assembly of 2 subunits into 1 homodimer[6] that is absolutely required for efficient transport of pro-LPH from the endoplasmic reticulum (ER) to the Golgi apparatus, where terminal N- and O-complex glycosylation occurs.[7,8] O-glycosylation results in up to 4-fold increase in the enzymatic activity of LPH.[9] The propeptide (LPH$\alpha$) is cleaved in the Golgi apparatus proteolytically at $Arg^{734}/Leu^{735}$ to generate LPH$\beta_{initial}$ (160 kilodaltons),[10,11] which is efficiently sorted to the brush border membrane where the mature form, LPH$\beta_{final}$ (145 kilodaltons), is generated by cleavage by luminal pancreatic trypsin at $Arg^{868}/Ala^{869}$.[10] Brush border LPH$\beta$final consists of the homologous domains III and IV, the transmembrane region, and the cytoplasmic tail and exerts its enzymatic functions as phlorizin-hydrolase, assigned to $Glu^{1273}$ in domain III, and as lactase, assigned to $Glu^{1749}$ in domain IV.[2] The N-terminally located LPH$\alpha$ profragment ($Ser^{20}/Arg^{734}$) acts in the overall maturation pattern of pro-LPH as an intramolecular chaperone required for a correct folding of pro-LPH.[12]

Pathologic reduction of the lactase activity leads to osmotic diarrhea and accompanying symptoms after intake of lactose containing foods.[13–15] This limits the range of dietary sources as in the case of adult-type hypolactasia (lactose intolerance) affecting most of the adults worldwide[16,17] and patients suffering from congenital lactase deficiency (CLD).[1,18]

CLD is a rare autosomal recessive disorder. Only a few cases of this disease have been described since the first

*Abbreviations used in this paper:* CLD, congenital lactase deficiency; endo F, endoglycosidase F/glycopeptidase F; endo H, endoglycosidase H; ER, endoplasmic reticulum; LPH, lactase-phlorizin hydrolase (all forms); pro-LPH, uncleaved precursor of LPH.


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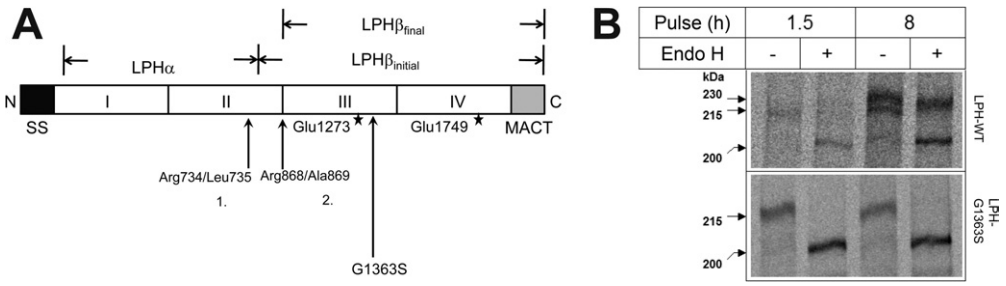


**Figure 1.** Location of the G1363S mutation in LPH and expression of wild-type and mutant LPH in COS-1 cells. (*A*) Main features of intestinal LPH structure. Prepro-LPH consists of a cleaveable signal sequence (SS; $Met^{1}$-$Gly^{19}$) and an extracellular region comprising homologous domains I–IV ($Ser^{20}$-$Thr^{1882}$). The initial cleavage step takes place between $Arg^{734}$ and $Leu^{735}$ generating LPH$\beta_{initial}$; removal of the polypeptide stretch $Leu^{735}$/$Arg^{868}$ occurs by a luminal trypsin creating LPH$\beta_{final}$. Cleavage and mutation sites are indicated by *arrows*; location of the phlorizin-hydrolase ($Glu^{1273}$) and lactase ($Glu^{1749}$) activities, respectively, are indicated by *asterisks*. MACT refers to the membrane anchor (MA) and cytoplasmic tail (CT). (*B*) COS-1 cells were transiently transfected with cDNAs encoding wild-type LPH and LPH-G1363S, biosynthetically labeled for 1.5 hours and 8 hours followed by cell lysis and immunoprecipitation. The immunoprecipitates were divided into equal aliquots and treated with endo H or not treated. The proteins were subjected to SDS-PAGE on 5% slab gels and autoradiography.

report in 1959.[19–22] Whereas the molecular basis of this deficiency is still obscure, posttranslational mechanisms are likely to be implicated in the lactase activity in these cases in a fashion similar to several pathomechanisms that are associated with protein trafficking defects, such as congenital sucrase-isomaltase deficiency[23] and cystic fibrosis.[24]

The typical symptoms of CLD are diet-induced liquid and acid diarrhea, meteorism, and severe malnutrition and commence a few days after birth with the onset of breast (or lactose-containing formula) feeding. Some patients present with hypercalcemia and nephrocalcinosis, which may be provoked by the metabolic acidosis and/or the calcium absorption-increasing effect of lactose.[25] Other disaccharidases as well as the morphology of the intestinal mucosa are usually in a normal range.

Recently, 5 point mutations have been identified within the coding region of the lactase gene in CLD patients, from which 3 result in early truncation.[22] Another mutation leads to a substitution of a glycine by a serine at amino acid residue 1363 (G1363S) in homologous domain III of LPH. In this study, we investigate at the protein and cellular levels the pathogenesis of CLD elicited by this mutation by analyzing the biosynthetic, structural, and functional features of mutant LPH. Interestingly, this mutation generates an additional glycosylation site in LPH, which reveals the characteristics of a temperature-sensitive mutant.

## Materials and Methods

### *Materials and Reagents*

Tissue culture dishes were obtained from Greiner (Hamburg, Germany). Dulbecco's modified Eagle medium (DMEM), minimum essential medium (MEM), streptomycin, penicillin, glutamine, fetal calf serum (FCS), and trypsin-EDTA were purchased from BioWest (Essen, Germany). Diethylaminoethyl-dextran, pepstatin, leupeptin, antipain, aprotinin, trypsin inhibitor, phenylmethanesulfonyl fluoride, trypsin, Triton X-100, SDS, molecular weight standards for SDS-PAGE, and phlorizin were acquired from Sigma Chemical Co. (Deisenhofen, Germany). L-[$^{35}$S]-methionine (>1000 Ci/mmol) and protein A-Sepharose were obtained from Amersham Biosciences Inc (Freiburg, Germany). Acrylamide, *N,N′*-methylenebisacrylamide, tetramethylethylenediamine, ammonium persulfate, dithiothreitol, and G418 were purchased from Carl Roth GmbH (Karlsruhe, Germany). Restriction enzymes were obtained from MBI Fermentas (St. Leon-Rot, Germany); Isis DNA polymerase was purchased from Qbiogene (Heidelberg, Germany). All other reagents were of superior analytical grade.

### *Immunochemical Reagents*

Monoclonal antibodies (mAbs) against human intestinal LPH were HBB 1/909[8] and the MLac 1 to 10.[26] The polyclonal antibody V496 is directed against the N-terminal part of the LPH prodomain.[12] Secondary anti-mouse antibody conjugated to Alexa Fluor 488 was purchased from Invitrogen (Karlsruhe, Germany).

### *Construction of Complementary DNA Clones*

The mutation G1363S was introduced by site-directed mutagenesis into the wild-type LPH complementary DNA (cDNA) cloned in the vector pSG5 (Stratagene, Amsterdam, The Netherlands) with the *Eco*RI sites of pLPH[7] using the following primers: 5′-GAGGTCATTACCAACAACAGTATGCCACTGGCCAGGGAGGATGAG-3′ and 5′-CTCATCCTCCCTGGCCAGTGGCATACTGTTGTTGGTAATGACCTC-3′. Oligonucleotides were obtained by Sigma Chemical Co., and DNA sequencing was performed by MWG (Ebersberg, Germany).

### *Transient Transfection of COS-1 Cells, Metabolic Labeling of Cells, Immunoprecipitation of Cell Extracts, and SDS-PAGE*

COS-1 cells were transiently transfected with DNA using diethylaminoethyl-dextran essentially as described