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Structure, biosynthesis and regulation of lactase-phlorizin hydrolase

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ABSTRACT

Lactase-phlorizin hydrolase (EC 3.2.1.108; 3.2.1.62) (LPH) is an enterocyte specific enzyme localised to the intestinal brush border. It has a key role in the digestion of lactose. LPH is an ectoenzyme anchored to the microvillar membrane via a C-terminally located hydrophobic stretch and it has an internal homology. LPH is sythesized as a large precursor which during the intracellular transport becomes N- and O- glycosylated. During the transport a large propeptide, which serves as a chaperone, is cleaved off before LPH reaches the brush border.

LPH-expression is regulated at the level of LPH-mRNA during both differentiation/tissue specific expression and post-weaning down regulation. A series of regulatory elements together with their transcription factors have been identified. It is suggested that lactase-persistence is caused by a mutation that either destructs a repressive cis-element (or a cis-element binding a destabilising protein) or creates an enhancing cis-element interacting with activating factors which increase after weaning.

Keywords: Hypolactasi, lactase, LPH-gene, LPH-promoter, phlorizin hydrolase

Structure and biosynthesis

Structure

Lactase-phlorizin hydrolase (EC 3.2.1.108; 3.2.1.62) (LPH) is an enterocyte specific enzyme carrying out its function in the luminal membrane – the brush border (for a detailed review see (1)). LPH contains two active sites. One is the main player in the intestinal hydrolysis of the β–galactoside lactose. It hydrolyses also other natural β–glycosides like cellobiose, cellotriose, cellotetrose and cellulose but with much less efficiency. The other active site hydrolyses β–glycosides with large hydrophobic alkyl chains like those in galactosyl- and glycosyl-β–ceramides. The lactase-activity was initially described and characterized by Dahlqvist (2) and Doell and Kretchmer (3) and its presence in mammals has been thoroughly documented (4).

LPH exerts its function in the membrane by an extracellularly located dimer built up of two identical 160 kDa polypeptide chains (Figure 1) (5). The deduced amino acid sequence of LPH infers that the enzyme is anchored to the membrane directly by a hydrophobic C-terminally located stretch rather than via a GPI anchor as the enzyme is not released by phospholipase C and is not present in the detergent insoluble fraction of the microvillus membrane (6,7). The enzyme that has a short (26 amino acid long) cytoplasmic part can be phosphorylated (8) but the biological importance of this finding is unknown.

The primary translation product of human LPH (pre-pro-LPH) is 1927 amino acid long and shows a fourfold internal homology between the four domains (I-IV) (6). It has a cleavable signal sequence (aa 1-19) and a large pro-peptide (domain I and II) comprising about half of the molecule. Domain III carries the active site responsible for the phlorizin hydrolysis and domain IV harbours the lactase active site (9,10).

Biosynthesis

Posttranslational modifications: By the use of pulse-chase experiments it was demonstrated that LPH is synthesized as a high mannose N-glycosylated single chain form (pro-LPH) (11-13). LPH dimerizes during ist biosynthesis (14,15). In the case of the human enzyme this occurs during the passage out of ER. In the Golgi apparatus the N-linked high mannose glycosylation is converted into an endo H resistant complex type glycosylation and here LPH also acquires O-linked glycosylation. This increases the enzymatic activity fourfold (16).

The propeptide of pro-LPH is cleaved off during its transport across the Golgi apparatus. For the human enzyme this occurs after ^{734}Arg leaving a 1192 amino acid long processed form of LPH (mature-like LPH) which is further transported to the brush border membrane. The cleavage is carried out by furin or by a furin-like convertase (17,18). On its way to or in the brush border the mature-like LPH is further processed by a combination of unknown peptidases (e.g. other furins, some aminopeptidases, cathepsins or might be granzyme A) in combination with a final trimming by trypsin (19).

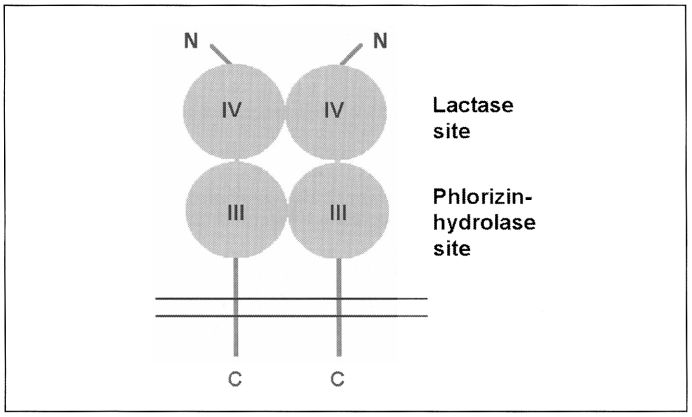

Figure 1. Schematic representation of the mature lactase-phlorizin hydrolase (LPH) complex on the outer side of the apical membrane of the enterocytes. The complex is composed of two complex N-glycosylated and O-glycosylated 160 kDa polypeptides. III and IV refer to homologous domains of LPH which harbour the phlorizin hydrolysing and lactose hydrolysing sites respectively.

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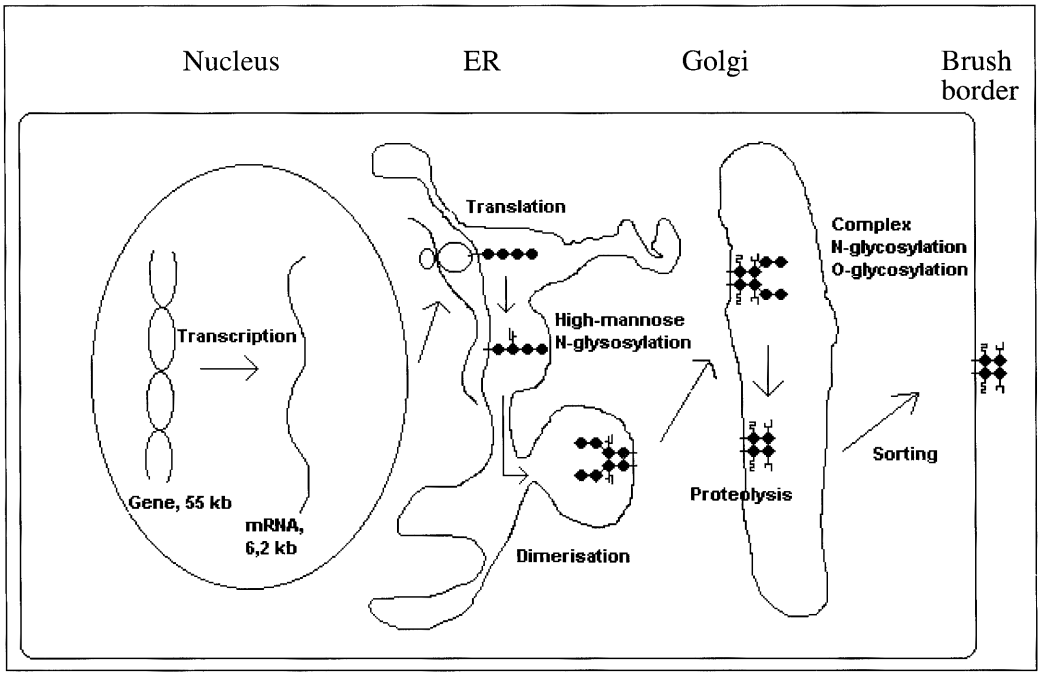

Figure 2. Overview of the biosynthesis of human lactase-phlorizin hydrolase.

The propeptide, which contains domain I and II does not contain structures similar to those carrying out the enzymatic activities on domain III and IV (20) and a search for possible enzymatic activities was without result (21). The cleavage of the pro-LPH has no effect on the enzymatic activity, as the intact pro-LPH is fully active (22). Separately expressed mature-like LPH devoid of the pro-peptide cannot be transported to the cell surface but is instead degraded intracellularly probably because of incorrect folding. It therefore seems as if the propeptide serves as an intramolecular chaperone securing correct folding of the mature-like LPH necessary for its proper transport to the cell surface (23), Figure 2.

Sorting: Both pro-LPH and the mature-like LPH are predominantly present on the apical membrane of MDCK-cells recombinantly expressing LPH (24,25) indicating that the cleavage process is without influence on the apical sorting of LPH. Studies on deletion mutants followed by expression in MDCK-cells point to the existence of an apical sorting signal in the ectodomain (26). This sorting signal is different from that of sucrase-isomaltase as this enzyme is found in the detergent insoluble fraction but the LPH is not. Furthermore it has elegantly been demonstrated that LPH and SI are transported by different vesicles from the Trans Golgi Network (TGN) to the apical membrane and that they segregate in the TGN (27).

Much focus has been on the sorting of the LPH-molecule. However there are also reports (28-30) reporting on an apical localization of LPH-mRNA. It thus seems as if the biosynthesis of LPH is carried out close to the site of function (the brush border) and that the polarized localisation of the LPH-mRNA is a part of the sorting process. The molecular mechanism for the LPH-mRNA sorting might be either an interaction between a sequence in the LPH-mRNA and an "address molecule" or a similar interaction with the growing LPH-protein.

Regulation of expression

Regulation of the level of LPH-mRNA

General: The *tissue specific expression* is mainly controlled by a series of transcription factors that regulate the transcription in a strictly controlled way during the *differentiation* of an adult stem cell to the mature, functioning cell. In the intestine adult stems cells are located in the crypts. Daughter cells divide and differentiate at the same time as they migrate towards the villi. Transcription factors bind to distinct segments of the DNA in the near upstream region (cis-elements) and thereby regulate transcription of the gene. Binding can also occur to cis-elements at farther distances and both upstream and downstream of the transcribed part of the gene. Once the transcription is activated, its efficiency, which is number of initiations per time, can be regulated by several mechanisms. Variations in the amount of critical transcription factors and their co-factors, other modulating transcription factors and degradation rate of mRNA are examples of such regulatory mechanisms. Degradation, although not described for the LPH-mRNA, is controlled by the binding of stabilising or destabilising proteins to another type of cis-elements rich in adenylate/uridylate (adenylate/uridylate-rich elements, AREs) and often located in the 3'-untranslated end (31). All these regulation mechanisms may be responsible for *variation of activity along the small intestine* and the *down-regulation after weaning.*

The LPH-gene: The human LPH-gene, which comprises 55 kb contains 17 exons with a length between 79 and 1551 bp (32). It is localised on human chromosome 2q21 and is together with some nearby genes transcribed towards the centromer. Thus 3.3 kb upstream from the start of transcription of the LPH gene, the next gene MCM6 (minichromosome maintenance deficient 6) is located, followed by the gene encoding aspartyl-tRNA synthetase. The region telomeric to these genes (upstream) has a relative low gene density. Thus in a segment of about 1,2 Mb only one gene (chemokine receptor 4) has been characterised. There is no evidence of more LPH-genes in the human genome, although three such genes have been suggested in the rabbit genome (33).

Cis-elements/Transcription factors of importance in differentiation/tissue specific expression: The characterisation of mutants/polymorphisms in relation to LPH expression levels, *in vitro* experiments for mapping important gene regions in combination

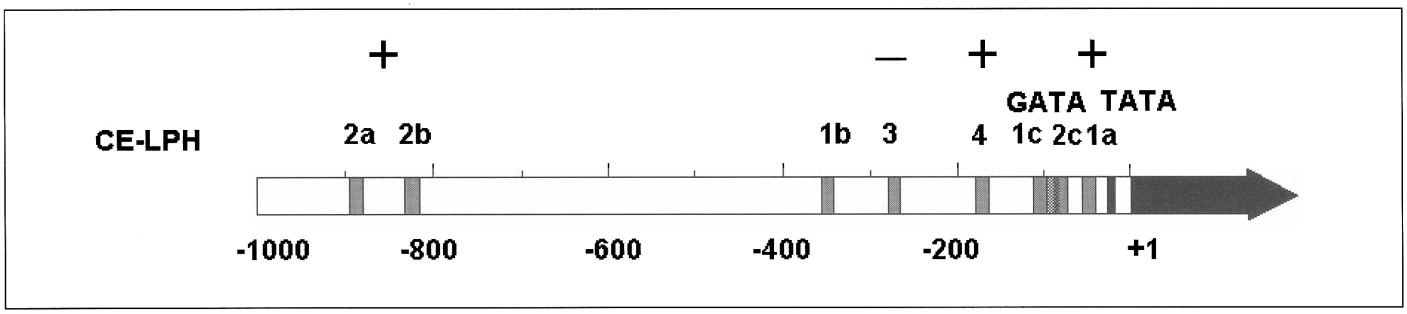

Figure 3. Schematic representation of proposed regulatory cis-elements in the 1 kb upstream segment of the pig lactase-phlorizin hydrolase (LPH) gene.

with cells in culture and finally studies on transgenic mice have provided information on functional cis-elements and their corresponding transfactors.

Computer searching for known cis-elements in the 1 kb upstream region of the human LPH-gene suggested that the transcription factors SP-1 (-210), SRF (-250), AP-2 (-410), CTF/NF1 (-525), CREB (-635) and Oct1/Oct2 (-980) might be of importance for the transcription. The region was furthermore shown to contain two Alu sequences (32). DNAse I footprint experiments showed that a pig intestinal nuclear extract contains transcription factors binding to the region between –40 to –54 of the pig promoter. These factors were not found in a liver nuclear extract (34). This finding was further characterised (35) and using a nuclear extract from Caco-2 cells, it was demonstrated that the major protein binding to this site, called CE-LPH1a, is a homeodomain protein related to *caudal* from *Drosophila* (36,37). This protein is in humans only expressed in intestinal tissue. Two more Cdx binding sites were later identified in the pig LPH-gene upstream region (CE-LPH1b and 1c) (38,39), and also another transcription factor (HOXC11) with a binding specificity similar to Cdx-2 (39). A detailed deletion analysis of the region between –17 to – 894 of the pig LPH promoter revealed both activating and repressing elements (38). Three cis-elements (CE-LPH2a, CE-LPH2b and CE-LPH2c) might be able to bind the transcription factors HNF1 α and β, which are homeodomain transcription factors being highly expressed in the intestine, liver and kidney. Functional experiments, including transfection experiments in Caco-2 cells, demonstrated that especially HNF1α, perhaps mostly by binding to CE-LPH2c, is of importance. The deletion experiments further showed that the region between –299 to –227 contains a repressive element. This region contains a binding site for transcription factors belonging to the FREAC family (CE-LPH3, -278 to – 264). Both FREAC 2 and FREAC 3 bind to CE-LPH3 in *in vitro* experiments and may thus be suggested to have a regulatory function *in vivo*. Recently, members of the GATA family transcription factors (GATA-4, -5 and –6) have been shown to activate LPH-gene expression in Caco-2 cells (40). There is no doubt an important interaction between some of the suggested factors. Thus interactions between HNF-1α and Cdx2 (41) and between HNF-1α and GATA-5 (42) have been demonstrated.

Experiments with transgenic mice carrying the 1 kb upstream region of the pig LPH-gene showed that this region contains the cis-elements necessary for correct transcription control in relation to differentiation and tissue-specific expression (43). Similar experiments performed with the rat lactase gene promoter suggested that the region from –2038 to +15 contains regulatory elements with the same functions (44).

Järvelä et al. (45) characterised Finnish families with congenital alactasia, a rare disease, which in opposition to adult-type hypolactasia, leads to complete absence of lactase activity. They suggested that mutation in a region located about 2 Mb upstream of the LPH gene was responsible for the disease. It seems most reasonable to suggest that such an element is responsible for LPH expression in connection with differentiation and tissue specific expression rather than for fine tuning/down-regulation, but further work has to be performed before it can be finally settled.

The current view on transcription factor/cis-elements of importance for the expression of LPH is depicted in Figure 3, which gives the current situation of the pig LPH-gene. Cdx-2 binds to CE-LPH1a(-54 to –40), and CELPH1c(-113 to –90), HNF1α to CE-LPH2a and 2c (-894 to –880 and –86 to -71), a FREAC factor or a FREAC related factor to CE-LPH3 (-278 to –264), GATA factors to an element between -97 and –73, and finally and an unknown activating factor to CE-LPH4 (in the region –178 to –164).

Cis-elements of importance for the tuning of LPH gene expression: It has been demonstrated in the rat that glucocorticoids and thyroid hormones may affact the lactase activity during the first weeks in life (46,47). These hormones work through receptors that bind to distinct DNA segments, thereby acting as transcription factors. However no glucocorticoid-responsive elements have been detected in the 5'-flanking region of the LPH gene. It may be that it is a more complex response as observed for two other intestinal glycosidases, trehalase and sucrase-isomaltase (48).

Down-regulation of LPH occurs in most mammals and in more than half of the world's human population. It seems very reasonable that the mechanism of this genetic switch is common to all studied species. After a period with conflicting results in the beginning of the 90s (see *Regulation at the posttranscriptional level*, below), it is now generally agreed that the down-regulation is due to decreased amounts of LPH-mRNA. It may, however, be difficult to assess whether this is due to decreased synthesis or increased degradation of LPH-mRNA. Krasinski et al. (49) showed agreement between synthesis rate measured in nuclear run-on assays and steady-state levels of LPH-mRNA in the developing rat intestine, arguing for a transcriptional rate control.

Based on *in vitro* experiments on the pig promoter, it was suggested that CE-LPH1a might be of importance in the down regulation of lactase after weaning (34). This does however not seem to hold true for rat lactase promoter in a similar experimental approach (50).

More conclusive evidence can be obtained from studies with transgenic animals, containing different regions of promoter region fused to a reporter gene or by the search for mutans/polymorphisms in humans, that do not down regulate LPH expression after weaning (lactase persistence). By the transgenic mice approach Troelsen et al. (43), reported that the cis-element(s) for down regulation was located within a 1 kb upstream promoter segment of the pig LPH-gene, whereas Krasinksi et al.

(44) also by a transgenic approach did not find the corresponding elements in a 2 kb upstream segment of the rat LPH-gene. It might be that the responsible cis-element in the rat is located further upstream, as the homology between the rat and pig upstream segment is rather low.

Swallow and collaborators have performed an extensive characterisation on human polymorphisms both in the LPH gene exons and introns and also in the region between the LPH and the MCM6 gene (51). The lactase persistence allele was found to be dominant to the non-persistent using symptoms occurring after lactose ingestion for phenotyping. (Half a level of lactase activity is sufficient to digest 50 g of lactose). However individuals homozygotic for the persistence have higher intestinal LPH expression than the corresponding heterozygotes, allowing them to be differentiated. In an interesting study, characterising LPH-mRNA levels in individuals being heterozygous with respect to a distinct polymorphism, it was demonstrated (52) that only the gene with one of the two polymorphisms was expressed in high levels, whereas the other was responsible for 10% or less of the total LPH-mRNA level. The results distinctly demonstrate that down-regulation is due to a cis-element. However so far no correlation between the many characterised polymorphisms and the LPH persistence/non-persistence have been demonstrated. The different polymorphisms are associated to form some distinct haplotypes, one of which is associated (however not exclusively) to lactase persistence in Europe (53).

Regulation at the posttranscriptional level

Early studies on LPH regulation using laboratory animals and selected human cohorts made some investigators to suggest a post-translational mechanism for the post-weaning decline and the regulation along the small intestine (29,54,55). However these initial studies were not corroborated in other studies where a more precise experimental desing was used (49,56,57). In these it was reported that the level of LPH-mRNA co-varies with the rate of LPH-biosynthesis, the amount of LPH-activity and LPH-protein. These data suggest that the LPH-level is to a large extent regulated at the transcriptional level as direct studies were carried out on the rate of LPH-mRNA biosynthesis (49).

Is the regulation of the LPH-mRNA levels evenly regulated in all enterocytes or is the LPH-gene silenced in some enterocytes and kept active in others? Is the latter a process in the post-weaning downregulation and in other tuning events? The occurrence of a patchy-pattern of LPH seems to be a part of the post-weaning downregulation as this has been reported in the post-weaning period of rabbit, rats and humans (58-60). No studies aiming at unravelling the molecular mechanisms behind the patchy pattern have been published but it can be hypothesised that either an inherent genetic programme of the enterocytes determines the LPH-gene activity or alternatively external signals from the submucosa are progressively and unevenly changed and is regulating the LPH-gene expression in the neighbouring enterocytes.

Even if it is largely correct to say that the expression of LPH at different levels along the small intestine and at different stages of development is proportional to the LPH-mRNA levels, it is evident that also other factors are modulating the levels of LPH-protein. This is clear as the LPH-mRNA is low or absent on the tips of the enterocyte villi even if the LPH protein is present in significant amounts in the villi (29, 61). This indicates that the LPH-protein has a longer half life than LPH-mRNA. Furthermore, a clear discrepancy (high LPH-mRNA *vs.* low LPH protein/activity) has been observed in the proximal duodenum (43,60) indicating that, e.g. pancreatic proteases reduce the brush border levels of LPH.

Perspectives in relation to adult-type hypolactasia

A recent publication (62) mentions unpublished data indicating that a cis-element controlling down regulation is located within a 300 kb region including the LPH gene (Poulter M, Hollox E, Swallow DM, *unpublished data*). Based on this information it seems very reasonable to suggest that lactose-persistence is caused by a mutation resulting in either destruction of a repressive cis-element (alternatively a cis-element binding a destabilising protein) or the creation of an enhancing cis-element, responding to a transcription factor that increases after weaning.

ABBREVIATIONS

Cis-elements/Transcription factors
see ref (63) for a detailed description

AREs	Adenylate/uridylate-rich elements
Alu sequences	Repetitive sequences recognised by the restriction enzyme AluI
Caco-2 cells	Colorectal adenocarcinoma cell line
ER	Endoplasmic reticulum
GPI	Glycosylphosphatidylinositol
LPH	Lactase-phlorizin hydrolase
LPH-mRNA	Lactase-phlorizin hydrolase messengerRNA
MCM6	Minichromosome maintenance deficient 6
MDCK-cells	Madin-Darby canine kidney cells
TGN	Trans Golgi Network

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