Characterization of natural and artificial mutants of human intestinal lactase phlorizin hydrolase

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Wenn die Nacht am tiefsten ist,
ist der Tag am nächsten.

Ton Steine Scherben

Für Kathy

Zusammenfassung

Kürzlich wurden neun unterschiedliche Mutationen in der codierenden Region des Gens der humanen intestinalen Laktase-Phlorizin-Hydrolase (LPH) identifiziert. Die Mutante LPH-G1363S weist eine zusätzliche N-Glykosylierungsstelle auf und stellt sich bei Expression in COS-1-Zellen als missgefaltetes, enzymatisch nicht aktives Protein dar, welches das Endoplasmatische Retikulum (ER) nicht in Richtung Golgi-Apparat (GA) verlassen kann. Es ist eine partielle Temperatursensitivität zu beobachten, wobei sowohl der intrazelluläre Transport als auch die enzymatische Aktivität von LPH-G1363S bei 20°C zum Teil wiederhergestellt werden können, nicht jedoch die korrekte Faltung. Des Weiteren zeigt sich, dass eine LPH-Form, welche die Mutationen G1363S und N1361A beinhaltet, und somit eine Re-Eliminierung der zusätzlichen N-Glykosylierungsstelle aufweist, nicht die Eigenschaften des Wildtyp-Proteins besitzt, sondern die von LPH-G1363S. Daraus lässt sich ableiten, dass die zusätzliche Glykosyl-Gruppe nicht die Defekte von LPH-G1363S verursacht. Diese Analyse ist die erste Charakterisierung einer LPH-Mutante auf molekularer und subzellulärer Ebene, die in die Pathogenese der Congenitalen Laktase-Defizienz involviert ist und offenbart einen bislang unbekannten Mechanismus der Ursache von Laktose-Malabsorption.

Der transportkompetente Protein-Vorläufer der LPH (pro-LPH) umfasst vier homologe Domänen. Der Einfluss jeder dieser Bereiche auf die strukturellen und funktionalen Eigenschaften von pro-LPH wurde durch gerichtete Umstrukturierung der Domänen-Zusammensetzung analysiert. Dabei zeigte sich, dass das Entfernen von Domäne IV, welche die Laktase-Aktivität trägt, eine beschleunigte Transportkinetik nach sich zieht. Außerdem weist die Mutante (LPHΔ4) eine verzögerte Dimerisierung im GA und eine reduzierte Verbundenheit mit Tween 20-resistenten Membranen im ER auf. Darüber hinaus kolokalisiert LPHΔ4 teilweise mit dem Wildtyp-Protein in Laktase tragendenden apikalen Vesikeln (LAVs) aber nicht mit der Saccharase-Isomaltase (SI) in SI tragenden apikalen Vesikeln (SAVs). Das Fehlen von Domäne II führt zu einer verminderten Phlorizin-Hydrolase- und nicht mehr nachweisbaren Laktase-Aktivität, verzögerter Dimerisierung, jedoch kaum veränderter Transportkinetik. Die Mutanten, welchen die homologe Domäne III oder I fehlt sind nur teilweise gefaltet und verlassen das ER nicht. Die Domäne III alleine hingegen ist *per se* transportkompetent, enzymatisch aktiv und wird effizient zur apikalen Seite polarer Zellen sortiert, lässt sich jedoch nur als Monomer nachweisen. Daraus ergibt sich ein hierarchisches Modell der frühen Faltungsvorgänge der naszierenden pro-LPH, wobei der membranassoziierten Domäne IV bei der Protein-Zielsteuerung der pro-LPH eine regulatorische Rolle zukommt. Domäne III hingegen stellt die autonome Kern-Domäne des Gesamtproteins dar, fungiert als zweites intramolekulares Chaperon und trägt die Informationen für die apikale Sortierung.

Schlagworte: Laktase-Phlorizin-Hydrolase, Congenitale Laktase-Defizienz, Faltung, intramolekulare Organisation, Zielsteuerung, Detergenz-resistente Membranen.

Abstract

Recently, nine distinct mutations within the coding region of the human intestinal lactase phlorizin hydrolase (LPH) gene have been identified. The mutant LPH-G1363S carries an additional N-glycosylation site and constitutes a misfolded, enzymatically inactive protein, which is not able to leave the endoplasmic reticulum (ER) towards the Golgi apparatus (GA) when expressed in COS-1 cells. It displays partial temperature-sensitivity, whereby its intracellular transport as well as its enzymatic activity can be restored in part during expression at 20°C, but correct folding can not. However, a form of LPH that contains the mutations G1363S and N1361A, which lead to re-elimination of the additional N-glycosylation site, does not display the features of wild type LPH but of LPH-G1363S. Thus, the additional glycosyl group is not required for the LPH-G1363S defects. This is the first characterization, at the molecular and subcellular levels, of a mutant form of LPH that is involved in the pathogenesis of congenital lactase deficiency. Mutant LPH accumulates predominantly in the ER but can partially mature at a permissive temperature; these features are unique for a protein involved in a carbohydrate malabsorption defect implicating LPH.

The transport-competent protein precursor of LPH (pro-LPH) contains four homologous domains. The influence of each of the homologous domains on the structural and functional characteristics of the pro-LPH polypeptide has been analyzed by directed restructuring of the domain composition. Removal of domain IV, which carries the lactase activity, results in accelerated transport kinetics and altered patterns of quaternary structure and membrane association. These novel biosynthetic features of the mutant (LPHΔ4) are directly associated with a retarded dimerisation of this mutant in the GA and a reduced association with Tween 20-resistant membranes in the ER. Furthermore, LPHΔ4 co-localises partly with the wild type protein in lactase-carrying apical vesicles (LAVs) but not with sucrase-isomaltase in SI-carrying apical vesicles (SAVs). Moreover, deletion of domain II leads to a reduced phlorizin hydrolase and not detectable lactase activity, retarded dimerisation, but almost normal transport kinetics. The mutants, which lack domain I or III, respectively, are badly folded and not capable of leaving the ER. However, domain III alone is *per se* transport-competent, enzymatically active and efficiently sorted to the apical membrane in polarized cells, but can only be detected as monomeric forms. Altogether, the data strongly suggest a hierarchical model of the early folding events of nascent pro-LPH. Here, membrane associated domain IV has a regulatory role of in the trafficking of pro-LPH, while domain III constitutes the core domain of the whole protein, functions as a second intramolecular chaperone and comprises the information for apical sorting.

Key words: Lactase phlorizin hydrolase, congenital lactase deficiency, folding, intramolecular organisation, trafficking, detergent resistant membranes.

Parts of this work have been published

Full papers:

Behrendt, M., Keiser, M., Hoch, M., and Naim, H. Y. *Impaired trafficking and subcellular localization of a mutant lactase associated with congenital lactase deficiency*. Gastroenterology. 2009 Jun; 136(7):2295-303. Epub 2009 Jan 24.

Behrendt, M., Polaina, J., and Naim, H.Y. *Structural hierarchy of regulatory elements in the folding and transport of an intestinal multi-domain protein.* J Biol Chem. 2010 Feb 5; 285(6):4143-52. Epub 2009 Dec 2.

Abstracts and posters:

Behrendt, M., and Naim, H.Y. *Structural hierarchy and implication of the homologous domains of intestinal lactase phlorizin hydrolase in its folding and intracellular transport.* 28th Annual Meeting of the German Society for Cell Biology (DGZ), Heidelberg 2005.

Behrendt, M., and Naim, H.Y. *Structural hierarchy and implication of the homologous domains of intestinal lactase phlorizin hydrolase in its folding, quaternary structure, intracellular transport and apical sorting.* 16th Symposium of the German Veterinary Society (DVG), Section Physiology und Biochemistry, Gießen 2006.

Behrendt, M., and Naim, H.Y. *Structural hierarchy and implication of the homologous domains of intestinal lactase phlorizin hydrolase in its folding, quaternary structure, intracellular transport and apical sorting.* 30th Annual Meeting of the German Society for Cell Biology (DGZ), Frankfurt 2007.

Behrendt, M., and Naim, H.Y. *Elimination of a homologous domain of an intestinal hydrolase enhances trafficking and affects polarized sorting via altered membrane association.* 18th Symposium of the German Veterinary Society (DVG), Section Physiology und Biochemistry, Leipzig 2008.

Behrendt, M., and Naim, H.Y. *Elimination of a homologous domain of an intestinal hydrolase enhances trafficking and affects polarized sorting via altered membrane association.* Annual Meeting of the American Society for Biochemistry and Molecular Biology (ASBMB) at the Experimental Biology Meeting, San Diego 2008.

Behrendt, M., and Naim, H.Y. *Interrelationship between the homologous domains of intestinal lactase phlorizin hydrolase.* 19th Symposium of the German Veterinary Society (DVG), Section Physiology und Biochemistry, Hannover 2010.

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1. Introduction

The biological cell is the central unit within the self-organisation of an organism, the maintenance of its functions therefore is essential. One crucial presupposition is the supply of distinct subcellular compartments with specific proteins. The basis for correct protein trafficking and consequently the fulfillment of its biological role is the acquisition of a native conformation via correct folding.

In virtue of the fact that misfolded polypeptides constitute the molecular basis of many inherited diseases, investigations on the mechanisms which contribute to and guarantee correct folding constitute a major and important part of current research. These mechanisms contain interactions between cellular protein components and structural elements within a polypeptide [Hammond and Helenius, 1994; Hutt et al., 2009]. Therefore, insights into the intramolecular organisation of proteins are of great significance for the understanding of fundamental molecular and cell biological processes. That is why an extension of knowledge in this field is of use as a basis for diagnostic and therapeutic progress concerning disorders associated with this subject.

1.1 Protein biosynthesis, folding, processing, and transport

The achievement of the native conformation is the condition to be fulfilled that a protein can be transported to its target site and be functional. Therefore, correct folding is a prerequisite for its full functionality.

Theoretically, a peptide with n amino acids could form 8^n conformations, because in the peptide backbone stereochemically eight bond angles are possible; however, under cellular conditions only one is realized. In this most stable structure about 95% of all intracellular proteins are found [Lodish et al., 2004].

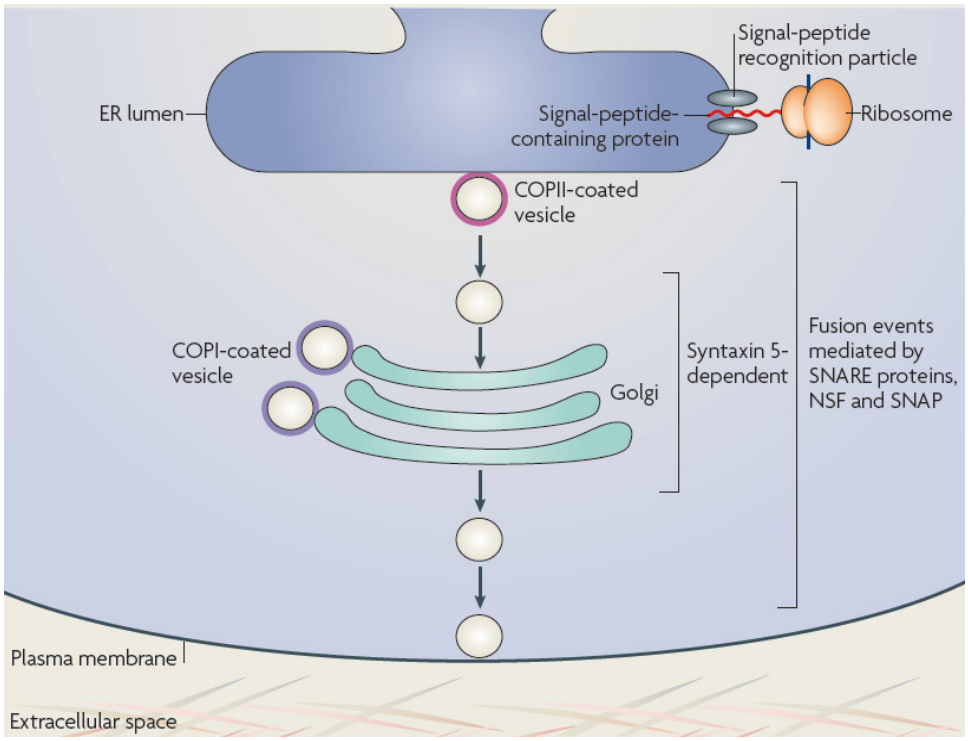
1.1.1 The secretory pathway

Most of the proteins of a eucaryotic cell are encoded within the nucleus, but their biosynthesis occurs in two different ways.

The mRNA of cytoplasmic, nuclear, mitochondrial, plastidal, and peroxisomal proteins is translated by free cytoplasmic ribosomes. After correct folding, the

polypeptides either remain in the cytoplasm or are transported to final destinations by means of their compartment-specific target and entry sequences. The proteins of the secretory pathway – like proteins of the ER, the Golgi apparatus, the lysosomes, as well as secreted and integral membrane proteins [Griffiths and Simons, 1986; Palade, 1975] – are synthesized by cytoplasmic ribosomes bound to the membrane of the rough ER during translation. Fig. 1.1 gives an overview over the secretory pathway.

A



B

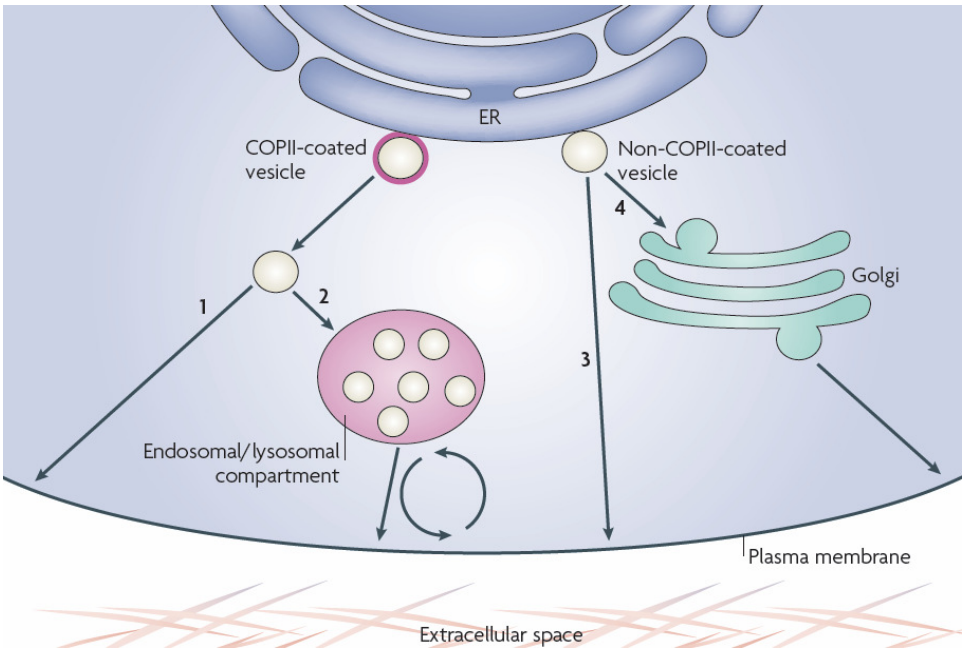


Fig. 1.1: Conventional secretory pathway (A) and unconventional transport of signal-peptide-containing proteins (B). 1-4 indicate different routes and mechanisms of unconventional protein transport. (From [Nickel and Rabouille, 2009])

1.1.2 Translocation in the ER

Signal sequences within a pre-pro-peptide precursor play a crucial role for the interaction between ribosomes and the ER. These sequences are predominantly localized N-terminally and are revealed and exposed co-translationally. The hydrophobic core within a signal sequence of a nascent polypeptide binds to a signal-recognition particle (SRP). This SRP directs the ribosome to an SRP-receptor in the ER membrane and interacts with it. Subsequently, the ribosome binds a translocase associated with the SRP-receptor [Gilmore, 1993; Johnson and van Waes, 1999]. In coordination with other ER-specific proteins, the nascent, unfolded polypeptide [Rapoport et al., 1996; Shaw et al., 1988] is translocated through the translocase channel into the ER lumen or it is inserted into the ER membrane, respectively.

Integral membrane proteins can be inserted in the lipid bilayer in different ways. Either a covalent bound glycosyl-phosphatidylinositol (GPI)-anchor is attached [Brown et al., 1989; Englund, 1993; Lisanti et al., 1989; Lisanti and Rodriguez-Boulan, 1990; Lisanti et al., 1988], or one or more transmembrane domains exist as topogenic amino acid sequences.

Topogenic sequences are responsible for the co-translationally determined orientation of the protein in the membrane [Hartmann et al., 1989]. Type-II-membrane proteins have a cytoplasmic N-terminus and a luminal or extra-cellular C-terminus, respectively, which is translocated in the ER after completed biosynthesis [Shaw et al., 1988]. Type-I-membrane proteins have an N-terminal, cleavable signal sequence and a C-terminally following stop-transfer signal-anchor sequence. Therefore, the N-terminus is located in the lumen or extra-cellularly, respectively, and the C-terminus is in the cytoplasm.

1.1.3 Folding in the ER

The ER is the secretory pathway site-of-entry for newly synthesized proteins. Its lumen constitutes a specialized folding environment with several molecular chaperones and folding factors and because of this enables rapid folding into a native structure [Gething and Sambrook, 1992]. Therefore, in this compartment the pre-conditions for the achievement of transport competence and oftentimes of biological functions of many secretory and integral membrane proteins are created [Gething and Sambrook, 1992; Hurtley and Helenius, 1989; Hutt et al., 2009; Kornfeld and Kornfeld, 1985; Rose and Doms, 1988; Wiseman et al., 2007].

Co- and post-translational modifications influence biological activity, lifespan as well as intra- and extra-cellular localization of proteins and are therefore of decisive importance. These processes can be differentiated in partly reversible chemical modifications and generally irreversible proteolytic alterations.
Reversible reactions are e.g. acetylation of terminal regions, phosphorylation and de-phosphorylation of serine, threonine and tyrosine residues as well as glycosylation of asparagines, serine and threonine residues.
Protein processing contains irreversible removal of C- or N-terminal amino acid residues by proteases or by auto-proteolysis [Lodish et al., 2004]. Existing cleavable signal sequences are removed proteolytically during translation by a signal peptidase and subsequentially degraded [Dalbey and Von Heijne, 1992].
The formation of intramolecular – and possibly later intermolecular – disulfide bonds occurs co-translationally, too, in the context of the folding process [Gething, 1997]. Disulfide bonds oftentimes are necessary for stability, further maturation, intracellular transport and protein function. This kind of chemical bonds can only form within the non-reducing milieu of the ER lumen [Wilson et al., 1998] and can therefore only be found for secreted proteins and exo-plasmatic domains of membrane proteins. Additionally, the spontaneous formation of disulfide bonds during ongoing protein biosynthesis can afterwards be re-organised by protein-disulfide isomerase (PDI) to guarantee the correct pairing of the cysteine residues [Bulleid and Freedman, 1988; Wilson et al., 1998].
Such isomerisation reactions, and others like the rotation of peptidyle-prolyle bonds by peptide-prolyle isomerase (PPI), are in many cases the rate-limiting events during

protein domain folding. Generally, small domains are stabilized at first and distant regions later [Lodish et al., 2004].

The information for the achievement of the native conformation is determined within the amino acid sequence of a protein, therefore folding can be considered as an auto-regulated process. However, a high folding efficiency at given intra-cellular conditions is only facilitated by chaperones [Ellis and Hemmingsen, 1989; Gething and Sambrook, 1992]. Chaperones inhibit any impermissible intermolecular interaction [Ellis and van der Vies, 1991] and thus avoid aggregation of unfolded proteins, and their early folding and assembly, respectively. They support folding processes and are capable of binding misfolded proteins and to adjust the structure of the latter [Wilson et al., 1998].
Two families of chaperones can be distinguished: molecular chaperones and chaperonines. Molecular chaperones protect unfolded or only partly folded proteins from degradation by intracellular peptidases. Chaperonines display an ATPase activity and are directly involved in protein folding. Some of the eucaryotic proteins like actin or tubuline depend on the presence of chaperonines like TciPs during their folding process, whereas bacterial chaperonines like GroEL and GroES play a more important role [Lodish et al., 2004].
Molecular chaperones are part of the Hsp70 protein family. Characteristic representatives are Hsp70 (in the cytoplasm and the mitochondrial matrix), BiP, Hsc70 (in the ER), and DnaK (in prokaryotes). They are fundamentally important for the formation of a transport-competent conformation [Munro and Pelham, 1986; Ou et al., 1993].

1.1.4 Glycosylation

Protein glycosylation is initiated co-translationally by covalent attachment of sugar chains. Most of the secreted and plasma membrane proteins carry at least one carbohydrate chain. Glycosylation is the most important and most frequent modification of these polypeptides [Reuter and Gabius, 1999].
The attachment of sugar chains begins in the ER. Subsequently, glycans are modified stepwise in this compartment and the Golgi apparatus; hence, glycan status and structure indicates the intracellular region of glycosylation. Cytosolic and nuclear proteins usually are not glycosylated [Lodish et al., 2004].

Co- and post-translational attachment and modification of carbohydrate side-chains can be differentiated in two types of glycosylation: O- and N-glycosylation, which both can be found together with different frequency for one polypeptide.

O-glycosylation

As to O-glycosylation [Roth, 1984], oligosaccharides are short and comprise at most four monosaccharides. Its transfer occurs stepwise and is catalyzed by glycosyl-transferases, which are highly specific, integral membrane proteins.

Monosaccharides contained in the glycans of glyco-proteins and -lipids originate from sugar residues of cytoplasmic nucleotide precursors. These precursors are build up in the cytoplasm from nucleoside-tri-phosphate and monosaccharide-phosphates and transported in the lumen of the appropriate cellular compartment via antiport [Lodish et al., 2004]. O-glycosylation is initiated by the binding of N-acetyl-α-D-galactosamine (GalNAc) to the hydroxyl group of a serine or threonine in the *cis*-Golgi. After the adding of one galactose (Gal) molecule within the *trans*-Golgi, more GalNAc and Gal molecules can bind to this core structure. Finally, sialic acid and L-fucose are added [Reuter and Gabius, 1999].

N-glycosylation

As to N-glycosylation, a sugar residue is bound to an amino group of an asparagine residue over an N-acetyl-glucosamine (GlcNAc) molecule. Thereby, the asparagine has to be part of the consensus sequence Asn-X-Ser/Thr, with the X being any amino acid except proline [Kornfeld and Kornfeld, 1985; Reuter and Gabius, 1999]. The actual addition of carbohydrate residues can not happen at all potential glycosylation sites, because not all sites are sterically accessible. Generally, potential N-glycosylation sites are more accessible for unfolded proteins [Allen et al., 1995].

Oligosaccharides contain mannose (Man) and GlcNAc and branches with a terminal, negatively charged sialic acid residue. Additionally, glucose (Glc), Gal and fucose can be bound. These carbohydrates are added to the protein co-translationally as a complex of fourteen sugar molecules *en block* by an oligosaccharide protein transferase (OSPT). This OSPT is anchored in the ER membrane and transiently associated with the glyco-protein synthesizing ribosome generating a spatial vicinity to the nascent polypeptide [Lodish 2004]. Before its transfer to the protein, the sugar

complex is bound to dolichol – an unsaturated isoprenoide, incorporated in the ER membrane – via a pyro-phosphate residue as oligosaccharide precursor [Kornfeld and Kornfeld, 1985]. The composition of three Glc, nine Man and two GlcNAc molecules [Trombetta and Helenius, 1998] is identical for plants, animals, and eucaryotic protozoa; a core of two GlcNAc and three Man molecules constitutes the structural element of all N-glycosidically bound oligosaccharides [Lodish et al., 2004].
After the oligosaccharide transfer its processing begins with the removal of one Man and three Glc molecules [Kornfeld and Kornfeld, 1985]. If the glyco-protein has not yet achieved its native conformation at this moment or is it misfolded, a Glc residue is re-added by the UDP-glucose:glycoprotein-glycosyl-transferase [Ponnambalam and Banting, 1996]. The latter identifies specific protein regions, which have an incorrect structure and therefore functions as a folding sensor [Ritter and Helenius, 2000]. Absence of N-glycosylation results in ER retention for many proteins. Secreted proteins, however, rarely accumulate, but in general are structurally more instable outside of the cell [Lodish et al., 2004].

Most of the glyco-proteins are transported to the Golgi apparatus as Man-rich protein forms by COP-II vesicles [Klumperman, 2000]. There, glycans are variously modified by different enzymes of the *cis*-, medial and *trans*-Golgi cisterns [Elbein, 1991; Kornfeld and Kornfeld, 1985]. By that, the diversity of complex glycosylated protein forms – typical for mammalian cells – is generated step by step. If one of these steps does not occur, the following ones are omitted, too [Elbein, 1991].
Each O- and N-glycosylation site typically possesses several glycan structures. Therefore, a glyco-protein constitutes a mixture of different glyco-forms with a varying number, localization, and sequence of glycans [Elbein, 1991]. Glycosylation influences folding, oligomerisation, transport, proteolytic degradation, and enzymatic activity [West, 1986]. Furthermore, glycans play an important role for protein-protein and protein-lectin interactions *inter alia* in the context of cell adhesion and cell migration [Zhao et al., 2008].

1.1.5 ER-to-Golgi-transport

The ER – and probably the Golgi apparatus, too – comprises a quality control system that retains most un- or misfolded proteins as well as monomers of proteins with an oligomeric quaternary structure [Anelli and Sitia, 2008; Ellis and Hemmingsen, 1989;

Fagioli and Sitia, 2001; Fink, 1999; Hurtley and Helenius, 1989; Propsting et al., 2005]. Proteins, which do not achieve their energetically most advantageous conformation, do not have to be restricted in their function. However, they are structurally more instable and therefore more susceptible for degradation in general.

Protein folding is a multi-step process, which is regulated on different levels. Firstly, there is a general folding control mechanism, which is characterized by the fulfillment of a particular folding order with few intermediate forms. Secondly, a cellular system exists, in which e.g. an increasing number of unfolded polypeptides in the ER induces an increase of (compartment-specific) chaperone and folding catalyst expression like Hsc70, PPI, and PDI [Lodish et al., 2004]. Additionally, gene expression of proteosomal proteins seems to be transcriptionally regulated because its increase is induced by the accumulation of misfolded polypeptides [Kaufman, 1999; Mori et al., 1992; Ng et al., 2000].

The majority of incorrectly folded proteins does not reach the Golgi apparatus and accumulates [Hurtley and Helenius, 1989], their detection possibly occurs because of membrane spanning regions [Lodish et al., 2004]. Moreover, there seems to be a quality control system at the Golgi apparatus level. It identifies misfolding indicating structures and retains the corresponding proteins [Doms et al., 1987; Hauri et al., 1985a; Naim et al., 1988; Propsting et al., 2005]. Most of them are translocated in the cytoplasm [Brodsky and McCracken, 1999; Wiertz et al., 1996], deglycosylated, ubiquitinated, and degraded by the proteasome [Hershko and Ciechanover, 1998; Vembar and Brodsky, 2008]. Furthermore, a rapid, directed protein degradation can also be induced by proteolytic sequences localized within the polypeptide itself, like the PEST sequence (Pro-Glu-Ser-Thr) [Rechsteiner and Rogers, 1996]. Generally, protein degradation limits lifespan of all proteins. Fig. 1.2 summarizes glycosylation and degradation events.

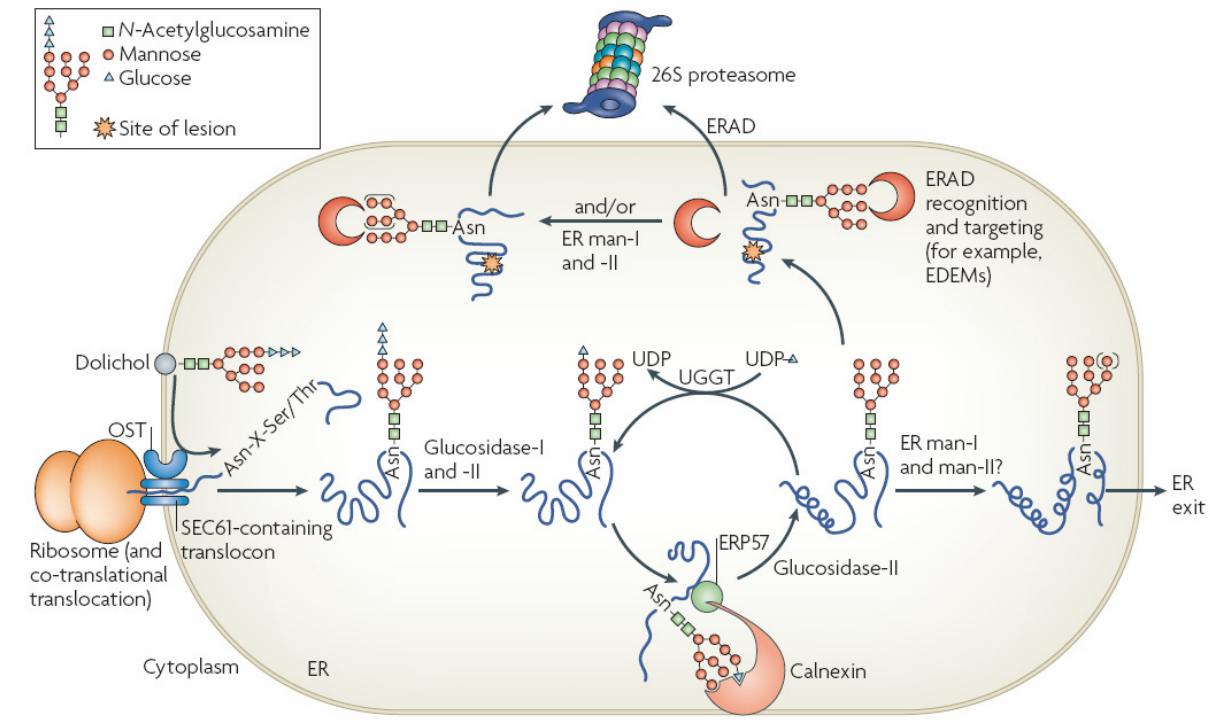


Fig. 1.2: N-linked glycosylation and the degradation of glycosylated proteins. (Taken from [Vembar and Brodsky, 2008])

Beside the selective retention and re-location of incorrectly folded proteins, these processes can be observed for several compartment-specific polypeptides, too. One of the underlying retention signals [Rothman and Wieland, 1996] is the C-terminally localized KDEL-peptide (Lys-Asp-Glu-Leu) [Munro and Pelham, 1987], which results in the retention of luminal ER proteins [Lodish et al., 2004]. Another signal is the also C-terminally localized di-lysine motif of membrane-anchored ER proteins, e.g. the KDEL-receptor. This motif together with COP-I vesicles directs the corresponding polypeptides from the Golgi apparatus back to the ER [Pelham, 1995].

1.1.6 Transport from the Golgi apparatus to the final destination

Within the cisterns of the Golgi apparatus, more glycans can be added, present ones be modified, and further proteolytic events occur. The anterograde transport through this compartment occurs by the progression of cisterns. By that, *cis*-Golgi cisterns (including their contents) migrate to the *trans*-Golgi network (TGN) [Lodish et al., 2004]. However, the cisternal maturation/progression model is not applicable for all cell types, e.g. for cells with a high secreting activity [Kartberg et al., 2005]. From the Golgi, the proteins which are not Golgi-resident are transported in vesicles to lysosomes, endosomes, or the plasma membrane, respectively, after sorting has

occurred [Griffiths and Simons, 1986]. Sorting itself depends on the interaction of distinct protein structures with specific receptors, i.e. cellular sorting machineries [Ikonen and Simons, 1998]. Particular secretory proteins are constitutively secreted, if no sorting signals exist, which could induce transport to certain intracellular regions. Other secretory proteins are concentrated and stored in secretory vesicles for regulated secretion in the form of exocytosis after a neuronal or hormonal signal.

Moreover, concerning protein and lipid targeting in polarized epithelial cells, one has to differentiate between an apical and a basolateral membrane domain, which differ structurally as well as functionally [Rodriguez-Boulan and Powell, 1992; Simons and Wandinger-Ness, 1990]. Their spatial separation originates and is maintained by tight junctions. These cell-cell-connections prevent the flow of extracellular liquid through the epithelium as well as protein and lipid diffusion between both plasma membrane domains. These domains possess different protein and lipid compositions because of directed supply [Egan et al., 2004; Massey-Harroche, 2000; Mostov et al., 2000; Simons and Wandinger-Ness, 1990; van Meer and Simons, 1986]. The generation and the maintenance of this asymmetry are fundamentally important for the physiological processes in polarized cells, e.g. signal transduction, absorption, and secretion.
During transport, basolateral and apical proteins are at first localized in the same TGN vesicles. However, they are found to be in different transport vesicles afterwards [Keller et al., 2001]. Thereby, signals within the transported proteins induce their segregation into different carrierss. Particular proteins on the surface of these vesicles – e.g. Rab [Chavrier et al., 1990] and v-SNARE proteins [Lodish et al., 2004] – are crucial for correct transport to one or the other membrane domain and induce membrane fusion.

A variety of intramolecular sorting signals could be found, which are identified by cellular components and carry the information for a directed transport [Casanova et al., 1991a; Casanova et al., 1991b; Delacour and Jacob, 2006; Hunziker et al., 1991]. Basolateral sorting signals appear in cytoplasmic domains of basolateral proteins [Brewer and Roth, 1991; Casanova et al., 1991a; Hunziker et al., 1991], e.g. as short amino acid sequences like the di-leucine [Hunziker and Fumey, 1994] and tyrosine motif [Brewer and Roth, 1991]. Basolateral proteins which lack the cytoplasmic

domain normally are sorted apically, wherefore apical sorting signals are likely localized in the transmembrane or ectodomain. Here, – and generally in protein sorting – N- and O-glycans could play a crucial role [Alfalah et al., 1999; Fiedler and Simons, 1995; Krahn et al., 2010; Naim et al., 1999; Scheiffele et al., 1995; Yeaman et al., 1997]. However, basolateral sorting signals are not dominant on apical signals on principle [Jacob et al., 1999].

The ER-to-Golgi transport kinetics varies between proteins. The reason for this could be the different development of folding and oligomerisation processes [Gething and Sambrook, 1992; Hurtley and Helenius, 1989]. The transport from the Golgi apparatus to the cell surface develops asynchronously in comparison to the ER-to-Golgi transport because of the modification within the Golgi apparatus [Jascur et al., 1991].

1.1.7 Lipid rafts and detergent resistant membranes

The association of several apically transported proteins with membrane microdomains, detergent resistant membranes (DRMs) or rafts [Simons et al., 1997] influences the transport mechanism [Jacob and Naim, 2001; Lindner and Naim, 2009; Pralle et al., 2000; Simons and Fuller, 1985; Simons and van Meer, 1988] for several transmembrane proteins [Krahn et al., 2010; Lin et al., 1998] and GPI-anchored proteins [Harder and Simons, 1997] as well. For other apically transported membrane proteins no relationship between sorting and the association with membrane microdomains could be demonstrated. Therefore, a raft-dependent and a raft-independent transport mechanism exist [Danielsen, 1995; Roper et al., 2000; Weisz and Rodriguez-Boulan, 2009; Yeaman et al., 1997; Zheng et al., 1999] indicating the existence of sorting signals within proteins.

Lipid rafts are central accumulations of cholesterol and glyco-sphingolipids in plasma membranes, characterized by a reduced flow capability in comparison to the surrounding lipids. They constitute microdomains of the liquid-ordered (lo) phase in biological membranes [Simons and Ikonen, 2000]. They have been investigated by the isolation of DRMs of the liquid-ordered phase. New insights into the formation of lipid rafts show that they are not the same as DRMs [Lindner and Naim, 2009].

The liquid-ordered phase is a sterol-dependent state, in which the lipid-acyl-chains are tightly packed and extended and lateral diffusion of single lipid molecules occurs [Veatch and Keller, 2005]. It is supposed that membrane domains of the ordered phase form also in sterol-rich membranes like plasma membranes resulting in a co-existence of the ordered and the dis-ordered phase (ld) [Lagerholm et al., 2005]. However, the rafts hypothesis results from the discovery that glyco-sphingolipids accumulate in the Golgi apparatus already before they are transported to the apical membrane of polarized epithelial cells [Simons and van Meer, 1988]. Fig. 1.3 illustrates the spatial lipid-lipid and lipid-protein associations found in rafts.

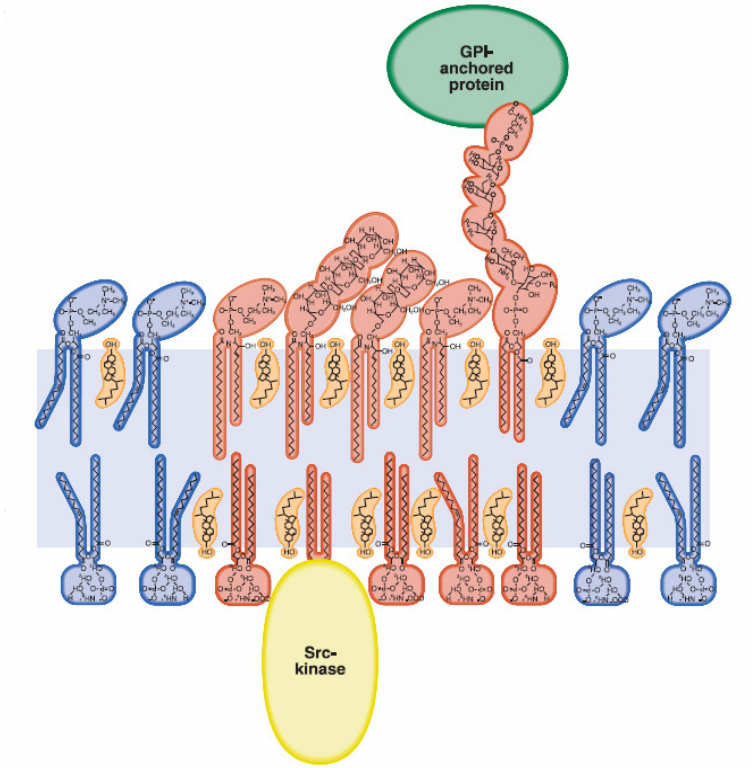


Fig. 1.3: Model of a raft with two intercalated proteins. The liquid-ordered phase is shown in red and the liquid-disordered phase in blue. (From [Simons and Ikonen, 2000]).

An early sign of the cellular existence of rafts was the observation that cellular membranes are not completely soluble in non-ionic detergents like Triton X-100 or CHAPS. Therefore, the unsoluble DRMs of the liquid-ordered phase remained, which were rich in cholesterol and GPI-anchored proteins and could be isolated by saccharose gradients [Brown and Rose, 1992]. The DRMs achieved a high density because of the interaction of cholesterol with the carbohydrate chains of the

sphingolipids, facilitated by stabilizing hydrogen bonds [Simons and Ikonen, 2000]. This high lipid density in the liquid-ordered phase inhibits the penetration of detergents in the lipid bilayer [Xu et al., 2001]. DRM isolation results in the concentration of mainly plasma membrane proteins. Enrichment of DRMs shows that these proteins were concentrated in lipid rafts and that raft targeting has great importance. In spite of all do the isolated DRMs not correspond to lipid rafts in living cells, *inter alia* because DRM isolation with Triton X-100 occurs at low temperatures [Melkonian et al., 1995].

1.2 Intramolecular protein organisation

1.2.1 Protein domains

Most of the secreted and some plasma membrane proteins are synthesized as pre-pro-forms. These are modified proteolytically on their way from the TGN to the cell surface leading to mature or active protein forms, respectively [Barr, 1991; Robakis et al., 2008; Steiner et al., 1984]. Here, irreversible removal of N-terminally localized pro-peptides plays an important role [Barr, 1991; Steiner et al., 1984].

These pro-peptides consist – like the rest of the protein – of one or more structural domains of the tertiary structure. These domains can be compact globule modules or linking domains [Brunger, 2001]. Moreover, they can appear as clearly outlined regions with distinct boundaries, which border on flanking areas like membrane regions or long domain-separating coiled-coiled helices [Abrahams et al., 1994].
Structural domains contain one- to three-hundred amino acid residues [Gething, 1997] and comprise various combinations of α-helices, β-sheets, hairpin structures and random balls. The frequency of the appearance of a particular amino acid in a particular protein region oftentimes is a special structural characteristic [Lodish et al., 2004]. Frequently, protein domains contain specific conserved sequences and secondary structure motifs like zinkfingers or loops. They constitute semi-independent three-dimensional units within proteins, which are capable to fold independently [Gething, 1997; Jaenicke, 1987] – and in eukaryotes also sequentially during translation [Bergman and Kuehl, 1979; Braakman et al., 1992; Gething, 1997; Kolb et al., 1994; Peters and Davidson, 1982].

Protein domains oftentimes are associated with specific functions, whereby a distinct area – e.g. a catalytic or binding domain – is related to the biological function of a protein. Such a functional domain can comprise several structural domains. This functional definition of a domain is less strict as the structural definition, but is of particular relevance, when the three-dimensional structure is unknown [Lodish et al., 2004].

The subdivision of the whole tertiary structure in structural domains corresponds to the principle, that complex molecules are comprised of simple elements. In different proteins recombination [Teichmann et al., 2001] of similar domains can occur by gene fusion or exon shuffling [Gething, 1997]. Homologous proteins display an amino acid sequence similarity of the whole length and usually a similar function. These sequence similarities mostly result from evolutionary relationship and indicate one ancestor, wherefore homologous proteins can be classified in families together. In the context of rapid evolution of eucaryotic multi-domain proteins facilitated by the development of complex genes [Gething, 1997], domains could constitute units of evolution [Holm and Sander, 1994].

The knowledge of the exact boundaries of structural domains is especially important as well as for the determination of the three-dimensional structure [Gething, 1997] as for the functional characterization of protein regions. The prediction of a three-dimensional structure (biomodeling) occurs on the basis of the amino acid sequence under the consideration of the frequency probability of single amino acids in distinct secondary structures [Lodish et al., 2004].

1.2.2 Intramolecular chaperones

For a particular region of some proteins a function as intramolecular chaperone is postulated [Chen and Inouye, 2008], whereby specific domains support or regulate the folding process of other domains [Naim et al., 1994; Oberholzer et al., 1993; Sagherian et al., 1994]. The region, which functions as intramolecular chaperone can be part of the mature protein like for sucrase-isomaltase [Jacob et al., 2002b]. However, for the majority of the proteins with identified intramolecular chaperone a cleavable pro-peptide carries this function, e.g. for subtilisin [Barr, 1991; Zhu et al., 1989], the bovine pancreatic trypsin inhibitor [Weissman and Kim, 1992], activin A

and TGF-β-1 [Gray and Mason, 1990], cathepsin C [Cigic et al., 2000], type-1-matrix-metalloproteinase [Cao et al., 2000], and lactase phlorizin hydrolase [Jacob et al., 2002a]. The pro-regions of these proteins are localized N-terminally, probably to facilitate an early participation in the folding process of the remaining protein part. However, C-terminally located intramolecular chaperones could be identified in mammalian [Jacob et al., 2002b] and non-mammalian cells [Conesa et al., 2001; Feller et al., 1998; Muhlenhoff et al., 2003].

1.3 Lactase phlorizin hydrolase

Intestinal lactase phlorizin hydrolase (LPH; EC 3.2.1.62/108) is a physiologically important digestive enzyme in the mammalian small intestine. It belongs to the group of brush border membrane hydrolases, which include disaccharidases and peptidases [Buller et al., 1987; Danielsen et al., 1984; Mantei et al., 1988; Naim, 1987].

1.3.1 Physiology and pathophysiology

Based upon its site-of-function and its disaccharidase activity, LPH together with sucrase-isomaltase, maltase-glucoamylase [Naim, 1987], and trehalase builds the more narrow group of intestinal disaccharidases. LPH is a membrane-bound β-glycosidase with two physically similar family-1-glycosyl hydrolase-domains [Colombo et al., 1973; Naim, 2001; Schlegel-Haueter et al., 1972; Skovbjerg et al., 1981] localized on one polypeptide chain [Mantei et al., 1988] and comprises two enzymatic activities, lactase and phlorizin hydrolase activity.

Phlorizin hydrolase (PH; glycosyl-N-acylsphingosine gluco-hydrolase) [Colombo et al., 1973; Kraml et al., 1972; Schlegel-Haueter et al., 1972; Skovbjerg et al., 1981] is a β-glycosyl-ceramidase with a broad specificity. The substrate range contains amongst others vertebrate alimentary β-glycosyl-ceramides [Leese and Semenza, 1973], some aryl-β-glycosides like phlorizin [Leese and Semenza, 1973; Zecca et al., 1998] as well as flavonoid-glycosides [Day et al., 2000]. The exact physiological role of PH still needs to be determined [Naim, 2001].

Most of the disaccharides found in the food of mammals consist of α-glycosidic linked monosaccharides, but one example of β-linked monosaccharides is lactose, which is

the most important carbohydrate in milk. The portion of lactose in human breast milk is about 7% and therefore higher than in other mammals. All mammals produce milk, which is therefore the main nutrient for newborns. The most important physiological task of LPH is therefore the cleavage of lactose with its lactase activity, which has lactose as its specific substrate [Zecca et al., 1998]. This disaccharide is hydrolysed in the lumen of the small intestine to galactose and glucose [Colombo et al., 1973]. Absorption of monosaccharides occurs via the microvilli of the brush border membrane by means of a galactose-specific, membrane-bound, and energy-dependent glucose/galactose transporter.

Lactose can not be uptaken by the enterocytes as an uncleaved molecule. However, the limiting factor of lactose digestion is not monosaccharide uptake, but hydrolysis of lactose by lactase. In contrast to other disaccharidases, lactase has a distinctly lower enzymatic activity [Henning, 1981]. A further, pathological decrease or an complete absence of lactase activity leads to osmotic diarrhoea with accompanying symptoms after ingestion of lactose containing food [Buller and Grand, 1990; Phillips et al., 1978]. This is the case for adult type hypolactasia concerning many mammals [Doell and Kretchmer, 1962; Henning, 1981]. However, the majority of human grown-ups are confronted with this widespread intestinal disorder also. Here, lactase activity declines rapidly and drastically after suckling period during early childhood or youth, respectively [Lebenthal et al., 1975]. This results in a substantially limited range of foods [Harvey et al., 1995; Wang et al., 1995]. Not afflicted are Northern Europeans, their descendants, and isolated groups in Africa and Asia, because lactase activity does not decrease and stays at high levels all one's lifetime [Harvey et al., 1995; Wang et al., 1995].

This phenomenon is determined genetically, with an autosomal-dominant allele being the cause for lactase persistence and an autosomal-recessive variant of the same gene locus causing adult-type hypolactasia [Sahi et al., 1973]. However, ethnic and geographic variations of lactose malabsorption exist, e.g. the different rate of lactase persistence between North- and South-India is connected to the -13910C/T polymorphism [Babu et al., 2010], which constitutes the first discovered variant correlated to lactase intolerance [Enattah et al., 2002]. Moreover, other single-nucleotide polymorphisms of the lactase gene are also linked to lactase persistence, whereby the alleles can be found in different pastoral populations. The -13910C/T polymorphism, for example, is not significantly correlated to lactase persistence in a

Chinese population, whereas the -22018A allele is [Xu et al., 2010]. Meanwhile, a database has been build up, which includes the worldwide distribution of lactase persistence and its correlation to the -13910T allele [Ingram et al., 2009a]. Interestingly, the European -13910T variant and the East-African -13907G allele, both associated with lactase persistence, were found to share the identical ancestral background – and probably history – because of the same cattle domestication event [Enattah et al., 2008]. A recently identified lactase persistence allele found in Arab populations has a different and divergent ancestral haplotype, suggesting that this variant developed independently, probably because of beginning camel milk consumption [Enattah et al., 2008].

The convergent lactase persistence evolution in different populations most likely reflects the historical diversity of milk drinking culture adaption. Furthermore, several lactase persistent individuals could be identified, who do not have the allele supposed to be associated with lactase persistence, but other (rare) single-nucleotide polymorphisms in the same genome region. For example, within a group of Somali camel-herds in Ethiopia eight new polymorphic sites were found including two significantly connected to lactase persistence [Ingram et al., 2009b]. However, a significant correlation between the hypolactasia genotype -13910C/C of a cohort of young Russians in North-West-Russia and the consumption of milk and milk-containing formula could not be detected [Khabarova et al., 2009]. Additionally, the T allele of the -13910C/T polymorphism seems to be associated with the body mass index and increased prevalence of obesity [Kettunen et al., 2010], whereas -13910C/C individuals are supposed to drink less milk and have a reduced calcium uptake [Laaksonen et al., 2009]. Besides, a correlation of milk consumption and lumbar bone mineral content as well as bone mineral density could also be detected [Esterle et al., 2009]. Very recently, another mechanism of lactase regulation was proposed. Here, the RNA-binding protein ‘quaking’ regulates differentiation of colon epithelia, acts as tumor suppressor and increases lactase expression after forced expression of ‘quaking’ [Yang et al., 2010].

Further analyses of the molecular mechanisms and the forces driving evolution are necessary to understand lactase regulation completely. The latter constitutes a valuable model to elucidate gene-culture co-evolution as well as susceptibility towards diseases. Moreover does it show the limits of single-nucleotide tagging and

clarifies the potential and the importance of distant regulator elements [Ingram et al., 2009a].

1.3.2 Biosynthesis

Biosynthesis of LPH in humans is representative for other investigated species [Buller et al., 1987; Danielsen et al., 1984; Naim, 1987]. LPH is synthesized as a single-chain precursor molecule. This pre-pro-LPH comprises an N-terminal cleavable signal peptide (Met^{1}-Gly^{19}), four homologous domains (Ser^{20}-Thr^{1882}), and a membrane-anchoring region (Ala^{1883}-Leu^{1901}), which crosses the lipid bilayer of the cellular membranes in a helical conformation and fixes the protein at the membrane. Moreover, a cytoplasmatic domain can be found at the C-terminal end (Ser^{1902}-Phe^{1927}) [Mantei et al., 1988].

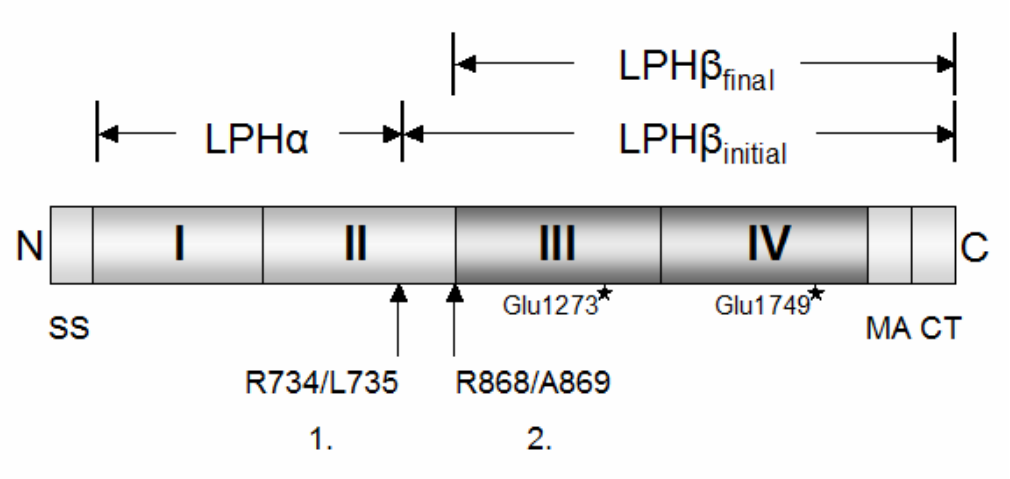


Fig. 1.4: Structural features of human pre-pro-LPH.

The existence of four homologous, highly conserved domains and in particular the similarity of the profragment with the region containing domains III and IV suggest that LPH has evolved from two partially gene duplications of one ancestral gene [Mantei et al., 1988]. Additionally, each of the homologous domains shares similarities in size with prokaryotic β-glycosidases as well as sequence similarities with β-glycosidases of archaea, eubacteria and fungi. Therefore, LPH likely is a member of a super family of β-glucosidases and β-galactosidases [Mantei et al., 1988; Naim, 2001].

The gene locus of human LPH is found on chromosome 2 [Kruse et al., 1988] and contains 17 exons within approximately 55kb [Boll et al., 1991]. The cDNA comprises

6274 bp [Boll et al., 1991; Kruse et al., 1988; Mantei et al., 1988] and pre-pro-LPH as primary product of translation is composed of 1927 amino acids [Mantei et al., 1988].

The signal peptide of LPH is essential for translocation into the ER [von Heijne, 1986] and is removed cotranslationally at the amino acid residues Gly^{19}/Ser^{20} within the ER lumen [Mantei et al., 1988; von Heijne, 1986]. The resulting pro-LPH is fixed in the ER membrane via its C-terminal membrane-anchoring domain. Because of its C_{in}-N_{out}-orientation, LPH is a type-I membrane protein [Blobel, 1980]. During the achievement of a correctly folded transport-competent conformation, highly mannose-rich glycosylation occurs (-> pro-LPH_h; 215 kDa), whereas 15 potential N-glycosylation sites exist within the molecule [Naim, 1992; Naim and Naim, 1996; Zecca et al., 1998]. Furthermore, a correctly folded transport-competent conformation is a precondition of the assembly of two subunits into one homodimer [Naim and Naim, 1996; Sterchi et al., 1990].
The molecular chaperone BiP has a stabilizing effect on unfolded or partially folded proteins [Gething and Sambrook, 1992] and is involved in the pro-LPH folding process in the ER [Jacob et al., 1995]. The C-terminally located membrane-anchor plays an important role for pro-LPH dimerisation, and dimerisation itself is essential for the protein in order to leave the ER and to acquire enzymatic activity [Naim and Naim, 1996].

Within the Golgi apparatus, complex N-glycosylation occurs in consequence of the final processing of the N-glycans [Hauri et al., 1985b; Naim et al., 1991]. Also, O-glycosylation of the complex is taking place [Hauri et al., 1985b; Naim, 1992; Naim et al., 1991; Naim, 1987], resulting in a four-fold increase of LPH enzymatic activity (-> pro-LPH_c, 230kDa) [Naim and Lentze, 1992].
After that, the propeptide Ser^{20}-Arg^{734} (LPHα) including complete homologous domain I and more than two thirds of domain II is removed in the *trans*-Golgi network [Jacob et al., 1994; Naim, 1992]. This occurs during a second proteolytic cleavage step [Danielsen et al., 1984] with a trypsin-like protease [Hauri et al., 1985b; Naim, 1987; Skovbjerg et al., 1984] at Arg^{734} /Leu^{735} (-> LPH$\beta_{initial}$, 160kDa) [Jacob et al., 1996; Wuthrich et al., 1996; Yeh et al., 1991]. Probably, LPHα is degraded subsequently [Naim et al., 1994; Ouwendijk et al., 1998].

Finally, the protein is transported in vesicles from the *trans*-Golgi network to the apical surface of the enterocytes where the mature form of the polypeptide (-> LPHβ_{final}, 145kDa) can display its physiological function. LPHβ_{final} is generated by cleavage of luminal pancreatic trypsin within the small intestine at Arg^{868}/Ala^{869} [Jacob et al., 1996; Wuthrich et al., 1996]. The cleavage sites are indicated in Fig. 1.4.
The sorting signals are independent of glycosylation [Buller et al., 1989; Naim, 1994; Panzer et al., 1998] and the association with Triton X-100 resistant membrane microdomains [Alfalah et al., 1999; Danielsen, 1995; Jacob et al., 1999; Naim, 1994] but are presumably located in particular subdomains in the ectodomain [Jacob et al., 1994; Jacob et al., 1999; Jacob et al., 1997; Panzer et al., 1998] or the transmembrane region [Jacob et al., 1999].

The brush-border LPH consists of 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 [Arribas et al., 2000; Zecca et al., 1998]. Nevertheless, the cleavage steps occurring in the Golgi apparatus of intestinal cells and in the small intestinal lumen are not required to generate a transport-competent [Naim et al., 1991], a correctly sorted [Grunberg et al., 1992; Jacob et al., 1994; Jacob et al., 1996] and an enzymatically active molecule [Naim et al., 1991].
The expression pattern of LPH on the mRNA and on the protein level is similar during development. The protein itself and the glycosylation pattern do not differ between LPH persistence and hypolactasia. Therefore, glycosylation seems not to be weightily important for the regulation of LPH expression [Naim, 1987]. However, post-translational mechanisms modify the final gene product, although the main mechanism of LPH regulation is transcriptional [Troelsen, 2005; Troelsen et al., 1994a; Troelsen et al., 1994b]. LPH is hardly expressed in the crypts, the maximal expression occurs between the low and middle villus and decreases towards the villus top [Buller et al., 1989; Hauri et al., 1985b].

The cytoplasmic region contains an endocytic signal in close vicinity of the membrane, which is suppressed by the strong apical sorting signal localized in the

ectodomain or the transmembrane domain. Therefore, this signal is hardly accessible for interactions with chlathrin coated pits [Naim, 1992].

1.3.3 Intramolecular organisation

The proportion of the complete pro-fragment Ser^{20}-Arg^{868} from pre-pro-LPH is about 45% and therefore is a sign of an important role of LPHα and the polypeptide stretch Leu^{735}-Arg^{868}. The significant homology with $LPH\beta_{final}$ led to the suggestion that the pro-fragment might have a glucosidase function in another cellular compartment [Mantei et al., 1988]. However, this was refuted by showing that pro-LPH and $LPH\beta_{final}$ have identical enzymatic activities [Naim et al., 1991], and by identifying the enzymatic centers [Zecca et al., 1998].

LPHα is devoid of sorting signals [Grunberg et al., 1992; Jacob et al., 1994; Mantei et al., 1988] and catalytic activity [Zecca et al., 1998], neither complex N- nor O-glycosylated [Naim et al., 1994] but rich in cysteine and hydrophobic amino acid residues suggesting a rapid folding to a compact globular domain [Jacob et al., 2002a].

Individual expression of LPHα [Jacob et al., 2002a] and $LPH\beta_{final}$ [Naim et al., 1994; Oberholzer et al., 1993] in COS-1 cells leads to localization of both polypeptides in the ER. Here, LPHα shows compact structural features stabilized by disulfide bonds [Jacob et al., 2002a], whereas $LPH\beta_{final}$ is identified predominantly as mannose-rich glycosylated protein form [Naim et al., 1994] with no measurable enzymatic activity [Jacob et al., 2002a].

Co-expression of LPHα and $LPH\beta_{initial}$ results in the mature, trypsin-resistant, correctly folded, enzymatically active, and transport-competent $LPH\beta_{initial}$ [Jacob et al., 2002a]. Individual expression of $LPH\beta_{initial}$, including the polypeptide stretch Leu^{735}/Arg^{868} of homologous domain II, results in a temperature-sensitive and correctly sorted protein. It folds properly at 20°C, interacts with BiP [Jacob et al., 2002a] in the ER, and is stable, trypsin-resistant, and enzymatically active. However, at 37°C it resides predominantly in the ER [Jacob et al., 2002a; Naim et al., 1994] as trypsin-sensitive and enzymatically inactive protein. During its biosynthesis at 37°C, $LPH\beta_{initial}$ interacts sequentially with BiP and calnexin [Jacob et al., 2002a]. A small proportion reaches the Golgi apparatus and gets complex glycosylated. However, it is enzymatically inactive and trypsin-sensitive, because folding and N-glycosylation

pattern – especially considering the sialic acid residues – are different [Jacob et al., 2002a].

These data show that LPHα indeed has the proposed intramolecular chaperone function [Mantei et al., 1988; Naim et al., 1994; Oberholzer et al., 1993] in enabling the correct folding of LPH$\beta_{initial}$ during pro-LPH biosynthesis [Jacob et al., 2002a]. Furthermore, LPHα is expected to facilitate the formation or disulfide bonds like other profragments [Jacob et al., 1995] and to bind LPH$\beta_{initial}$ directly. Its tendency to rapidly form a trypsin-resistant, enzymatically inactive core structure as folding template for the homologous domain LPH$\beta_{initial}$ is LPH-specific and this intramolecular chaperone function can not be compensated by calnexin [Jacob et al., 2002a].
The data suggest that the stretch (Leu^{735}/Arg^{868}) together with LPHα plays an important role in the correct folding of the pro-LPH.

1.4 Aim of the study

Especially β-galactosidase, or lactase, has important applications in medicine, food technology and the environmental sciences. But preparation, for instance from the yeast *Kluyveromyces lactis*, is ineffective and therefore expensive due to the low stability and intracellular nature of the enzyme [Adam et al., 2004; Becerra et al., 2004; Kim et al., 2004; Tahoun et al., 2002]. Consequently, inquiries on the structure-function relationship of this and other homologous enzymes have economic relevance.

The catalytic sites of the LPH are localized in homologous domains III and IV [Arribas et al., 2000; Zecca et al., 1998]. This has raised the question if these domains behave as autonomous regions and attain their enzymatically active conformation independently of each other in the context of the folding of the pro-LPH. And, supposing that these domains are indeed autonomous ones, one would ask if the possibility does exist to generate LPH mutants comprising more than two enzymatically active regions facilitating an increase of enzymatic activity.

Furthermore, the question which of the homologous domains, I or II, is more important, concerning the function of the removed polypeptide Ser^{20}/Arg^{868} as an intramolecular chaperone, remained until present unanswered.

LPH three-dimensional structure has not been worked out so far. A further characterisation of this protein with biochemical and cell biological procedures is necessary to achieve this goal and is a step in this direction. One aim of this study therefore was to investigate the influence of each of the four homologous domains on the structural and functional features of pro-LPH by directed change of domain composition. Moreover, the pathobiochemical and pathophysiological contexts of CLD have to be further investigated. In general, monogenetic diseases represent appropriate model systems to elucidate molecular, biochemical, cellular and physiological mechanisms and their analyses help to accelerate the development of individual diagnostic and therapeutic strategies.

2. Materials and methods

2.1 Materials

2.1.1 Chemical reagents

Acetic acid	Carl Roth GmbH (Karlsruhe, GE)
Acrylamide Rotiphorese Gel30	Carl Roth GmbH (Karlsruhe, GE)
Agarose, electrophoresis grade	Sigma Chem. Co. (Deisenhofen, GE)
Ammonium peroxydisulfate (APS)	Carl Roth GmbH (Karlsruhe, GE)
Ampicillin (D[-]-α-Aminobenzyle penicillin)	Sigma Chem. Co. (Deisenhofen, GE)
Bacto Agar	Carl Roth GmbH (Karlsruhe, GE)
Bacto-Trypton	Carl Roth GmbH (Karlsruhe, GE)
Bacto yeast extract	Carl Roth GmbH (Karlsruhe, GE)
Bromphenole blue	Merck (Darmstadt, GE)
Coomassie Brilliant Blue	Merck (Darmstadt, GE)
Dimethyle sulfoxide (DMSO)	Carl Roth GmbH (Karlsruhe, GE)
Dodecyl-β-m-maltoside	Sigma Chem. Co. (Deisenhofen, GE)
Ethanol	Carl Roth GmbH (Karlsruhe, GE)
Ethylenediaminetetraacetate (EDTA)	Serva Electrophoresis GmbH (Heidelberg, GE)
Fetal calf serum (FCS)	BioWest (Essen, GE)
Glycerol	Carl Roth GmbH (Karlsruhe, GE)
Isopropanol	Carl Roth GmbH (Karlsruhe, GE)
Kanamycin	Sigma Chem. Co. (Deisenhofen, GE)
Lactose	Sigma Chem. Co. (Deisenhofen, GE)
Lubrol	MP Biomedicals GmbH (Eschwege, GE)
L-[^{35}S]methionine (>1000 Ci/mmol)	Amersham Biosci. Inc. (Freiburg, GE)
N,N,N',N'-tetramethylethylendiamine (TEMED)	Carl Roth GmbH (Karlsruhe, GE)
Phenol	Carl Roth GmbH (Karlsruhe, GE)
Phlorizin	Sigma Chem. Co. (Deisenhofen, GE)
Protein A-sepharose (PAS)	Amersham Biosci. Inc. (Freiburg, GE)
Saponine	Sigma Chem. Co. (Deisenhofen, GE)
Sodium deoxycholate	Carl Roth GmbH (Karlsruhe, GE)

Sodium dodecyl sulfate (SDS)	Carl Roth GmbH (Karlsruhe, GE)
Sucrose	Carl Roth GmbH (Karlsruhe, GE)
Trypsin inhibitor (from soya beans)	Sigma Chem. Co. (Deisenhofen, GE)
Tris-hydroxymethyl-aminomethane (Tris)	Carl Roth GmbH (Karlsruhe, GE)
Triton X-100	Sigma Chem. Co. (Deisenhofen, GE)
Tween 20	Carl Roth GmbH (Karlsruhe, GE)

All other reagents were of superior analytical grade.

2.1.2 Enzymes

Endo-β-N-acetylglucosaminidase H (Endo H)	Roche Diagnostics (Mannheim, GE)
Endo-β-N-acetylglucosaminidase F (Endo F)	Roche Diagnostics (Mannheim, GE)
Isis™ DNA-polymerase (and buffer)	Qbiogene (Heidelberg, GE)
Restriction enzymes (and buffers)	MBI Fermentas (St. Leon-Rot, GE)
RNAse A (from bovine pancreas) 10 mg/ml stock solution	Roche Diagnostics (Mannheim, GE)
T4 ligase	MBI Fermentas (St. Leon-Rot, GE)
Trypsin Typ II-S from porcine pancreas ca. 1800 BAEE units per mg solid	Sigma Chem. Co. (Deisenhofen, GE)

2.1.3 Antibodies

Monoclonal antibodies (mAbs) against human intestinal LPH were HBB 1/909 [Hauri et al., 1985b] and the MLac 1 to 10 [Maiuri et al., 1991]. The polyclonal antibody V496 is directed against the N-terminal part of the LPH prodomain [Naim et al., 1994]. Anti-GFP antibodies were purchased from Invitrogen (Karlsruhe, GE). Anti-FLAG andibodies were obtained from Sigma Chem. Co. (Deisenhofen, GE).

2.1.4 Media, solutions, and buffers

Media

LB-Medium:	1% w/v NaCl 1% w/v Bacto-Trypton 0.5% w/v Bacto yeast extract autoclaved

LB solid medium:	LB-Medium + 1.5% w/v Bacto-Agar
LB selection medium:	LB-Medium +30-50 mg/l Kanamycin or +50-100 mg/l Ampicillin

DMEM (Dulbecco's Modified Eagle Medium) Low Glucose (1 g/l (D-)glucose)
BioWest (Essen, GE)

Culture medium:	DMEM + 10% v/v FCS + 1% v/v PSG

MEM (Minimum Essential Medium) (w/o methionine) BioWest (Essen, GE)

Starving medium	MEM + 1% v/v PSG
Transfection medium:	DMEM+ 1% v/v PSG
Hepes culture medium:	DMEM + 10 mM Hepes

Solutions

ATP	Sigma Chem. Co. (Deisenhofen, GE)
Benzyl-GalNAc	Sigma Chem. Co. (Deisenhofen, GE)
Chloroquine:	Sigma Chem. Co. (Deisenhofen, GE) 50 mg/ml, sterile filtered storage at –20 °C away from light
Coloring solution: (for SDS-PAGE)	0.1% w/v Coomassie Brilliant Blue 25% v/v isopropanol 10% v/v acetic acid
DEAE-dextran: (Diethyle aminoethyle dextran)	Amersham Biosci. Inc. (Freiburg, GE) 50 mg/ml
Discoloring solution: (for SDS-PAGE)	25% v/v isopropanol 10% v/v acetic acid
(1,4-)Dithiothreitol (DTT)	Carl Roth GmbH (Karlsruhe, GE) 1 M (for SDS-PAGE) 0.2 M (for ligation)
dNTPs (Desoxynucleoside triphosphate)	MBI Fermentas (St. Leon-Rot, GE) 25 mM dATP,dCTP,dGTP,dTTP

Ethidium bromide	Merck (Darmstadt, GE)
	10 µg/µl
G418	Carl Roth GmbH (Karlsruhe, GE)
Hepes:	Carl Roth GmbH (Karlsruhe, GE)
(N-2-Hydroxyethyle)piperazine-N-2-ethan-sulphonic acid) 1 M	
Monensin	Sigma Chem. Co. (Deisenhofen, GE)
NaJ	Carl Roth GmbH (Karlsruhe, GE)
	6 M in TE-buffer
Penicillin, streptomycin, glutamine (PSG):	BioWest (Essen, GE)
	penicillin 50.000 U/l
	streptomycin 10 mg/l
	L-glutamine 292 mg/l
Proteinase inhibitors:	Sigma Chemical Co. (Deisenhofen, GE)
	per ml (final concentration)
	1 mM PMSF (phenylmethylsulfonyl fluoride)
	100 µg trypsin/chymotrypsin inhibitor
	1 µg pepstatin
	5 µg antipain
	5 µg leupeptin
	1 µg aprotinin
Solution 1 (for DNA preparation):	50 mM glucose
	10 mM EDTA
	25 mM Tris-HCl, pH 8.0
Solution 2 (for DNA preparation):	0.2 mM NaOH
	1% w/v SDS
Solution 3 (for DNA preparation):	3 M KAc, pH 4.8
Trypsin-EDTA:	BioWest (Essen, GE)
	0.05% w/v trypsin (100 U/µl)
	0.02% w/v EDTA in PBS

Buffers

Denaturing buffer	0.5% w/v SDS
	1% v/v β-mercaptoethanol
Electrophoresis buffer:	25 mM Tris

(for SDS-PAGE)	250 mM glycine
	0.01% w/v SDS
3x Laemmli buffer:	6% w/v SDS
	30% v/v glycerol
	150 mM Tris-HCl, pH 6.8
	0.02% w/v bromophenol blue
	150 mM DTT
10x Ligation buffer:	0.5 M Tris pH 7.4
	0.1 M $MgCl_2$
Matrix washing buffer:	50 mM NaCl
	10 mM Tris pH 7.5
	2.5 mM EDTA
	50% EtOH
PBS:	0.8% w/v NaCl
(phosphate buffered saline)	0.2% w/v KCl
	8 mM Na_2HPO_4
	1.5 mM KH_2PO_4
	pH 7.4; autoclaved
6x sample buffer:	0.25% (w/v) bromophenol blue
(for agarose gels)	0.25% w/v xylencyanol
	30% v/v glycerol (in H_2O)
Separating gel buffer:	1.5 Tris-HCl, pH 8
Stacking gel buffer:	1 M Tris-HCl, pH 6.8
Standard lysis buffer:	25 mM Tris-HCl, pH 8.0
(for immunoprecipitation)	50 mM NaCl
	0.5% w/v sodium desoxycholate
	0.5% w/v Triton X-100
TAE-buffer:	40 mM Tris-acetate
	1 mM EDTA, pH 8.0
TE-buffer:	10 mM Tris-HCl
	1 mM EDTA, pH 8.0
Washing buffer I:	0.5% v/v Triton-X-100 (in PBS)
(for immunoprecipitation)	0.005% w/v sodium desoxycholate in PBS
Washing buffer II:	500 mM NaCl

(for immunoprecipitation)	0.5% v/v Triton X-100
	10 mM EDTA
	125 mM Tris-HCl, pH 8.0

2.1.5 Standards, systems, and software

Molecular weight standards

DNA standard (for agarose gels):

As DNA marker a λ-phage-DNA - cleaved with the restricion enzymes *Eco*RI und *Hin*dIII - was used and purchased by MBI Fermentas (St. Leon-Rot, GE).

fragment sizes (bp):

21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564,125

Protein standard:

For the estimation of protein bands the standard SDS-6H (High Molecular Weight Standard Mixture for SDS Gel Electrophoresis; 29-205 kDa) from Sigma Chem. Co. (Deisenhofen, GE) was applied.

protein:	molecular weight (kDa):
myosin (rabbit muscle)	205
ß-galactosidase (*E.coli*)	116
phosphorylase B (rabbit muscle)	97.4
albumine (bovine)	66
albumine (chicken)	45
carboanhydrase (bovine erythrocytes)	29

Systems

Metafectene™ Pro	Biontex (Martinsried, GE)
Miniprep Express™ matrix	BIO 101 (La Jolla, USA)

Software

Image J software package	http://rsb.info.nih.gov/ij/
Quantity One®	BioRad (Munich, GE)

2.1.6 Equipment

General equipment

Vapor sterilizer (autoclave)	Integra Biosciences (Ruhberg, GE)
Freezer –20 °C	Premium, Liebherr (Ochsenhausen, GE)
Deep freezer –80 °C	C54285, New Brunswick Scientific (Wesseling-Berzdorf, GE)
Incubator	FunctionlineB12, Heraeus (Hanau, GE)
Cooling centrifuge	Biofuge-fresco, Heraeus (Hanau, GE)
Magnetic stirrer	RCT basic, IKA Labortechnik (Staufen, GE)
Photometer	Jenway, Genova (Staffordshire, UK)
pH-Meter	PH538, WTW (Weilheim, GE)
Precision balance	PT310, Sartorius (Goettingen, GE)
Ultra-pure water supply	Milli Q, Millipore (Schwalbach, GE)
Bench centrifuge	Biofuge-pico, Heraeus (Hanau, GE)
Dry sterilizer	Heraeus (Hanau, GE)
Vortexer	Minishaker MS2, IKA Labortechnik (Staufen, GE)
Water bath	E-BRU/PU19A, Jubalo (Seelbach, GE)

Agarose gel electrophoresis

Flat-bed apparatus, gel beds, combs	BioRad Laboratories (Munich, GE)
Fluor-S® -Multiimager	BioRad Laboratories (Munich, GE)
Chambers, Wide mini Sub® Cell GT	BioRad Laboratories (Munich, GE)
Power supply Power Pac 300	BioRad Laboratories (Munich, GE)
UV-table	UVT-20 S/W, Herolab (Wiesloch, GE)

SDS gel electrophoresis

Glas plates, gel cast, combs, spacer	BioRad Laboratories (Munich, GE)
Gel observer Molecular Imager® FX,	BioRad Laboratories (Munich, GE)
Gel chambers Protean® II XI Cell,	BioRad Laboratories (Munich, GE)
Gel drying device DC-3	Biometra (Goettingen, GE)
Power supply Power Pac 1000	BioRad Laboratories (Munich, GE)

Phosphorimaging screens BAS-IP MS2025	Fujifilm Co. LTD (Duesseldorf, GE)

Fluorescence microscopy

Confocal laser scanning microscope TCS SP	Leica Microsystems (Bensheim, GE)

Cell culture

Incubator Biosafe eco 70040	Integra Bioscience (Ruhberg, GE)
Microscope DMIRB	Leica Microsystems (Bensheim, GE)
Sterile bench Holten LaminAir S 2000 1.2	Thermo Fisher Scientific (GE)
Tissue culture dishes, Falcon tubes, Transwell filters, and single-use pipetts	Greiner (Hamburg, GE)

PCR

Programmable heater	Personal Cycler, Biometra (Goettingen, GE) Minicycler, MJ Research (Waltham, MA, USA)

2.1.7 Host bacteria

Escherichia coli XL Gold

Genotype:

Tet^r Δ (mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte (F`proAB $lacI^q$ZΔM15 Tn10 (Tet^r) Amy Cam^r)^a, purchased by Stratagene (Heidelberg, GE).

2.1.8 Plasmids and oligonucleotides

The mutation G1363S was introduced by site-directed mutagenesis into the wild type LPH cDNA cloned in the vector pSG5 (Stratagene, Amsterdam, The Netherlands) [Hoch, 2006] with the *Eco*RI sites of pLPH [Naim et al., 1991].[1]

pcDNA3-LPH was generated by cloning the wild type LPH complementary DNA (cDNA) in the vector pcDNA3 (Invitrogen, Karlsruhe, GE) with the *Eco*RI sites of pLPH [Naim et al., 1991]. LPH was fused to YFP by subcloning the *Eco*RI/*Sca*I fragment from pcDNA3-LPH containing full-length LPH in-frame into the *Eco*RI/*Sma*I-

[1] This was done by Melanie Hoch.

digested pEYFP-N1 vector (Clontech-Takara, Saint-Germain-en-Laye, France) to create pLPH-YFP. pLPH-GFP was generated as desribed previously [Jacob et al., 2000]. SI-cherry was constructed by replacing YFP in SI-YFP [Jacob and Naim, 2001] with cherry, an improved monomeric red fluorescent protein [Shaner et al., 2004].[2]

The applied oligonucleotides for Δ-mutant and domain constructs were purchased by Sigma Chem. Co. (Deisenhofen, GE) and are listed in Table 2.1 and Table 2.2.

Table 2.1. A list of the wild type and Δ-mutant constructs used in this study

Protein	Plasmid for biochemical/ confocal analysis	Oligonucleotides 5'-3'
WT	psG5-LPH	-
	pLPH-GFP/pLPH-YFP	-
LPHΔ4	pΔ4 /pΔ4-GFP/pΔ4-YFP	ccactggccagggaggatgaggctttgtacgttctcttttct*
LPHΔ3	pΔ3 /pΔ3-GFP/pΔ3-YFP	cccaagttcgaaagagatttgtttctgtacggacggtttc*
LPHΔ3_{7xGly}	pΔ3_{7xGly} /pΔ3_{7xGly}-GFP/ pΔ3_{7xGly}-YFP	cccaagttcgaaagagatttgggggggcggtggaggcgggggggtttctgtacggacggtttcctg*
LPHΔ2	pΔ2 /pΔ2-GFP/pΔ2-YFP	gtccagggcggaaagggatgccttctaccacgggacgtttcgg
LPHΔ1	pΔ1 /pΔ1-GFP/pΔ1-YFP	ctaagtttttcatgctgggggttcctgcaggatactttccctg

*Forward mutagenesis primer. The reverse primer is the complementary sequence of the forward primer.

Table 2.2. A list of the domain constructs used in this study

Protein	Plasmid for biochemical/ confocal analysis	Oligonucleotides 5'-3'
Domain-III	pDomain-III	gccactggccagggaggatgagtgagaattctttctgtacggacggtttcctg*
	pDomain-III-GFP	aaaagaattcatggagctgtcttggcatgtagtc (forward)
		aaaaaccggtaactcatcctccctggccagtggcatg (reverse)
Domain-I-II-III	pDomain-I-II-III	gccactggccagggaggatgagtgagaattctttctgtacggacggtttcctg*
Domain-I-II-III-IV	pDomain-I-II-III-IV	ggcaccacagaagcacagacatgaattcgctttgtacgttctcttttctc*
Domain-II-III	pDomain-II-III	gccactggccagggaggatgagtgagaattctttctgtacggacggtttcctg*
$LPH\beta_{final}$	pJB20-$LPH\beta_{final}$	-
$LPH\beta_{initial}$	pLPH$\beta_{initial}$	gctaagtttttcatgctgggggtcactgttgcagtttgtatccctgg*
$Domain\text{-}III_{stretch}$	pDomain-$III_{stretch}$	gctaagtttttcatgctgggggtcactgttgcagtttgtatccctgg*
LPHα	pcDNA3-LPHα	-
Domain-I-II	pDomain-I-II	aaaagaattcatggagctgtcttggcatgta (forward)
		aaaaaccggttcacaaatctctttcgaacttggg (reverse)
Domain-II	pDomain-II_{3xFLAG}	aaaagaattcatggagctgtcttggcatgta (forward)
		aaaactcgagcaaatctctttcgaacttggg (reverse)
Domain-I	pDomain-I	aaaagaattcatggagctgtcttggcatgta (forward)
		aaaaaccggttcaggcatccctttccgccctgg (reverse)

* Forward mutagenesis primer. The reverse primer is the complementary sequence of the forward primer.

[2] SI-cherry was generated by Zeynep Hein.

2.1.9 Cell lines

COS-1

COS-1 cells are derived from CV-1 monkey kidney cells from the African green monkey *Cercopithecus aethiops.* These cells were transformed with an origin-defective SV40-mutant, which contains the wild type T-antigene [Gluzman, 1981]. The COS-1 cell line is an adherent, fibroblast-like, unpolar one, suitable for transient transfection with SV40-origin-of-replication-containing recombinant DNA.

Madin Darby Canine Kidney II-cells (MDCK-II)

The MDCK-II cell line is one strain of cells derived from cocker spaniel kidney cells isolated by S. H. Madin and N. B. Darby in 1958. These cells form a monolayer *in vitro,* and therefore are appropriate e. g. for the analysis of transport behavior of intestinal proteins [Leighton et al., 1969; Richardson et al., 1981].

All cell lines were obtained from the American Type Culture Collection (Rockeville, USA).

2.2 Methods

2.2.1 Manipulation of DNA

2.2.1.1 Amplification of cDNA fragments (PCR)

The polymerase chain reaction (PCR) [Saiki et al., 1988] is a method, with which copies of a particular DNA sequence can be generated specifically and in great numbers. For the amplification of particular LPH cDNA fragments a 50µl PCR reaction mixture comprised 50ng template DNA, 5µl 10x PCR buffer, 1µl dNTP mix, 50pmol forward primer, 50pmol reverse primer, 1U Isis DNA polymerase and aqua bidest. The PCR parameter program included a pre-denaturation step (95°C for 4min), 30 cycles of denaturation (95°C for 1min), annealing (55°C for 45sec), and elongation (2min/1kb at 72°C), as well as one final elongation step (72°C for 10min). The reaction was carried out in a heated-lid (98°C) thermocycler and performed as *hot-start* PCR, whereby the polymerase was added after the pre-denaturating step to avoid unspecific products.

2.2.1.2 Generation of sticky ends by restriction digestion

Before ligation, PCR product ends were digested with restriction enzymes (5U) within the reaction mixture to generate sticky ends. Moreover, the vector was prepared for ligation by mixing plasmid DNA (5µg), aqua bidest., enzyme buffer, and restriction enzymes (5U). The mix was incubated at optimal enzyme working temperature for 2h.

2.2.1.3 Agarose gel electrophoresis

After the reactions were finished the complete mixtures were added to 1/5 volume of 6x sample buffer and loaded on a 0.8% ethidium bromide stained agarose/TAE gel. The PCR product, the vector, and the λ-DNA standard were separated with 120V, the gel was analyzed by visualizing the DNA on a UV-table, and the bands corresponding to the LPH fragment and the vector, respectively, were cut out from the gel. Subsequently, the DNAs were isolated from the gel pieces and purified with Miniprep Express™ matrix.

2.2.1.4 Purification of DNA from agarose gels

The gel pieces were put into a 1.5ml tube, overlayed with 6M NaJ, and incubated at 52°C until the gel has melted. Then, the matrix was resuspended by vortexing, 100µl were added to the DNA, and the mixture was incubated at 52°C for 5min. After resuspension of the pellet, incubation was repeated, and the DNA-matrix pellet was washed three times with matrix washing buffer. The DNA was eluted by adding 10µl of aqua bidest. to the pellet and incubation at 52°C for 5min. The elution step was repeated, the eluates were combined, and the DNA concentration was determined.

2.2.1.5 Determination of DNA concentration via band intensity comparison

From the eluate, 2µl were mixed with 8µl aqua bidest. and 2µl 6x sample buffer. The mix and different amounts of λ-DNA standard were loaded on a 0.8% ethidium bromide stained agarose/TAE gel. After the gel run the sample band intensity was compared to the marker bands intensities which correspond to known DNA amounts and by that the sample DNA amount could be determined.

2.2.1.6 Ligation

For ligation of the LPH cDNA fragment into a vector, the total volume of the ligation mixture was 20µl. Generally, the number of insert molecules was 3 to 4 times of the acceptor molecules and the total DNA amount did not exceed 80ng. Before ligation, vector and insert DNA were incubated at 37°C for 5min in the required volume of aqua bidest. Then, the DNA was put on ice and 2µl of 10x ligation buffer, 1µl of 20x DTT (0.2M), 2µl of 10x ATP (10mM), as well as 1µl T4 ligase (5U) were added. The mix was pipetted up and down, spinned down, and incubated for 4h at room temperature or over night at 16°C. Subsequently, T4 ligase was inactivated by incubation at 65°C for 10min, the ligation mix was put on ice, and 5µl was used for transformation of 100µl competent *E.coli*.

2.2.1.7 Generation, transformation, and storage of competent *E.coli*

Generation and transformation of competent *E.coli* was performed according to Inoue [Inoue et al., 1990]. Competent *E.coli* were stored at –80°C.

2.2.1.8 Isolation of plasmid DNA from *E.coli*

Bacterial colonies grown on LB agar plates with selective medium were used for the inoculation of 5ml over night fluid cultures and bacteria grew at 37°C in a shaker. Plasmid DNA was isolated from *E.coli* by the rapid alkaline extraction procedure of Birnboim and Doly [Birnboim and Doly, 1979], purified and concentrated via ethanol precipitation. The DNA pellets were resuspended in TE-buffer and the over night culture remainders were stored in the refrigerator until the identification of positve clones.

2.2.1.9 Identification of positive clones by fragment restriction analysis

Putative positive clones were identified by fragment restriction analysis. For that, 2µl of the DNA from the over night clultures were mixed with aqua bidest., restriction enzyme buffer, and 2U of one or more restriction enzymes according to the manufacturers recommendation and dependent of the supposed recombinant DNA molecule. As a control, empty vector DNA was digested also. After incubation at the enzyme-specific temperature for at least 2h, the reaction was stopped by adding 1/5 volume of 6x sample buffer. Sample, control and marker DNA was separated on an ethidium bromide stained agarose gel, and the restriction patterns were analyzed by

visualizing the DNA on a UV-table. Overnight culture remainders of positive clones were mixed with glycerol to 50% v/v and stored at –80°C.

2.2.1.10 Large-scale DNA preparation

In order to receive large amounts of plasmid DNA, 10µl of a small over night culture or a piece from a glycerol culture were used to inoculate 200ml of LB selection medium. Bacteria were incubated at 37°C over night in a shaker and were afterwards centrifuged at 5000x*g* for 5min. The rapid alkaline extraction procedure of Birnboim and Doly [Birnboim and Doly, 1979] was performed as follows.

The pellet was resuspended in 8ml solution 1 with RNAse A and incubated for 10min at room temperature. Then, the cells were lysed by adding 16ml of solution 2 and gently mixing, as well as incubation for another 10min at room temperature. Subsequently, 12ml of solution 3 were added, mixed gently, and incubated on ice for 10min. The precipitate of chromosomal DNA and proteins was removed by filtering the mixture through two folded filters. The flow-through was added to 0.8x volumes of isopropanol, mixed intensively, and centrifuged for 20min at 15000x*g* and 4°C to precipitate plasmid DNA. The Pellet was washed with 70% ethanol and centrifuged again for 5min at 15000x*g* and 4°C. The pellet was dried and resuspended with 600µl TE-buffer.

For further purification, the DNA was mixed with 1 volume of phenol, centrifuged for 15min at 15000x*g* and 4°C. The resulting upper watery phase was transferred to another tube, mixed with 1 volume of chloroform, and centrifuged for 2min at 15000x*g*. This washing step was repeated once. To precipitate plasmid DNA, the upper watery phase was again transferred to another tube, mixed intensively with 0.8x volumes of isopropanol and 150mM sodium acetate (pH 7.4; final concentration), and centrifuged for 20min at 12000x*g* and 4°C. The pellet was washed with 70% ethanol, dried, and resuspended in 200-500µl of TE-buffer (depending on the pellet size).

2.2.1.11 Determination of DNA concentration via UV-light

The DNA concentrations of solutions from large-scale DNA preparation were determined by measuring the UV-absorption at 260nm and application of a quartz cuvette with a thickness of 1cm. The purity of nucleic acids was estimated by the ratio of extinctions at 260nm and 280nm, whereby the optimum is between 1.8 and 2.

2.2.1.12 Site-directed mutagenesis and loop-out PCR

In order to change only small parts of a cDNA, e. g. single nucleotides, site-directed mutagenesis was performed. Here, two complementary oligonucleotides carrying both a mutation were used as primers. They bind double-stranded plasmids sequence-specifically during a PCR and are elongated by the DNA-polymerase; the PCR products are complete plasmids carrying the mutation. The template plasmids were eliminated afterwards by adding *Dpn*I (5U per PCR) and incubation for 2h at 37°C. The restriction enzyme *Dpn*I digests the methylated template DNA, but not the non-methylated PCR products. Subsequently, *Dpn*I was inactivated at 65°C for 10min, the mixture was cooled down on ice and 5µl were used for transformation followed by subsequent experiments (cp. 2.2.1.7 - 2.2.1.11). In order to facilitate a rapid detection of positive clones by restriction fragment analysis, a second (silent) mutation had been introduced in the oligonucleotides, resulting in either an elimination or addition of a restriction site but not in changes of amino acids.

For the deletion of a larger cDNA area a special form of site-directed mutagenesis was applied, namely loop-out PCR. Here, the primers are designed in a way, that the first half of one oligonucleotide is complementary to the sequence directly in front of the area to be deleted, and the second half is complementary to the area localized directly behind the cDNA area to be deleted. During the annealing and elongation step of the PCR, the template cDNA area to be deleted forms a loop while the primers bind in front and behind it. This loop is not amplificated and the result is a smaller plasmid, with a cDNA deleted in part.

The carrying out of the mutagenesis PCR differed from the PCR described above (cp. 2.2.1.1) in some points. Firstly, only 5pmol per primer were used. Secondly, because of the amplification of whole plasmids, the elongation times were longer and only 18 cycles of denaturation, annealing, and elongation were performed. Thirdly, the PCR mixture was used directly for transformation after incubation with *Dpn*I.

2.2.1.13 DNA Sequencing

The correctness of every generated construct was verified by sequencing. This was done by MWG (Ebersberg, GE) according to the companies protocols.

2.2.2 Cell culture procedures and transfection

2.2.2.1 Transient transfection of COS-1 cells

COS-1 cells were transiently transfected with DNA using DEAE-dextran essentially as described previously [Naim et al., 1991].

2.2.2.2 Establishment of stable MDCK-II cell lines

MDCK cells were transfected using Metafectene™ Pro following manufacturer's instructions. Stable transfected MDCK cells were selected in the presence of 0.5mg/ml active G418 and after 14-21 days colonies were isolated, subcultured and stable transformants were screened by immunoprecipitation.

2.2.3 Isolation, detection, and analysis of proteins

2.2.3.1 Biosynthetic labeling

The cells were biosynthetically labeled with 80 µCi of [^{35}S]methionine in MEM after 2h of incubation with methionine-free MEM. Labeling was either performed continuously or following a pulse-chase protocol where the labeled cells were chased with non-radioactive methionine for different periods of time.

2.2.3.2 Standard cell lysis and immunoprecipitation

Standard cell lysis and immunoprecipitation of LPH from detergent extracts of the labeled cells was performed according to Naim et al. [Naim, 1987] using a mixture of mAb anti-LPH (HBB 1/909 and MLac1, MLac2, MLac4, MLac6 and MLac10) and V496. This mixture recognizes different conformations of LPH. GFP- and FLAG-tagged proteins were isolated with anti-GFP and anti-FLAG antibodies, respectively.

2.2.3.3 Manipulation of glycosylation

Deglycosylation

A further characterization of glycoproteins can occur by the use of endoglycosidases [Maley et al., 1989]. Immunoprecipitates were treated with endo H or endo F where indicated according to Naim et al. [Naim, 1987] followed by analysis using SDS-PAGE.

Inhibition of glycosylation

To test the role of inhibitors of the maturation process of D3, inhibitors of glycosylation were separately added to the medium during the preincubation of MDCK-D3 cells in methionine-free medium as well as during the labeling with [^{35}S]methionine. Benzyl-GalNAc was used as an inhibitor of O-glycosylation at 4mM final concentration, whereas monensin as inhibitor of N-glycosylation was given to cells at 1µM final concentration.

2.2.3.4 Trypsin treatment of immunoprecipitates

To assess the sensitivity of the mutants to trypsin, immunoprecipitated proteins were washed for additional two times with PBS containing 0.2% Triton X-100, were supplemented with 10 µg bovine serum albumin (BSA) as a carrier and incubated with 0.33 mg/ml trypsin for the indicated times at 37°C. The reaction was stopped by boiling for 5 min in SDS-PAGE sample buffer prior to gel electrophoresis.

2.2.3.5 Trypsin treatment of COS-1 cell surface proteins

Detection of deletion mutants at the cell surface was performed by adding 50µg/ml trypsin to the cell culture medium for 15min at 37°C during biosynthetic labeling or chase period to remove LPHα. The reaction was stopped by incubating the cells with cold FCS containing 500µg/ml trypsin inhibitor for 15min at room temperature.

2.2.3.6 Enzymatic activity assay

Lactase and phlorizin-hydrolase activities were assessed as follows. The ^{35}S-labeled immunoprecipitates were washed with PBS containing 0.2% Triton X-100 and incubated with 100 µl of this buffer containing lactose or phlorizin at 28 mM final concentrations. The samples were incubated at 37°C for 2h and the amount of released glucose was assessed by high-performance liquid-chromatography (HPLC).[3] The quantification of the specific activity was related to the radioactive protein band detected by autoradiography and the number of methionines for each construct.

[3] HPLC was performed by Uwe Glockenthör.

2.2.3.7 SDS-polyacrylamide gel electrophoresis

One-dimensional SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [Laemmli, 1970].

stacking gel:		separating gel:	5%	6%	7%	9%	10%
aqua bidest. (ml)	15.6	aqua bidest. (ml)	17.5	16.5	15.5	13.4	12.3
Acrylamide (ml)	3.9	Acrylamide (ml)	5.2	6.2	7.2	9.2	10.3
1M Tris, pH 6.8 (ml)	2.9	1.5M Tris, pH 8.8 (ml)	7.7	7.7	7.7	7.7	7.7
10% SDS (µl)	230	10% SDS (µl)	310	310	310	310	310
TEMED (µl)	23	TEMED (µl)	23	23	23	23	23
10% APS (µl)	230	10% APS (µl)	310	310	310	310	310

Table 2.3: Ingrediens for polyacrylamide gels.

The radioactively labeled protein bands were visualized by phosphorimaging. Protein bands were quantified with Quantity One® software.

2.2.3.8 Immunofluorescence

Immunofluorescence was performed essentially as described previously [Castelletti et al., 2006].

2.2.3.9 Confocal fluorescence microscopy

COS-1 cells grown on cover slips were transfected and confocal images of living cells were acquired 2 days after transfection on a Leica TCS SP2 microscope with a x63 water planapochromat lens (Leica Microsystems, Bensheim, GE) [Jacob and Naim, 2001] and processed with the public domain Image J software package. For colocalization studies the cells were co-transfected with GFP- or YFP-tagged LPH cDNA or the mutant LPH cDNA and the protein marker for the ER dsRed2-ER and for the Golgi apparatus Golgi-CFP (Clontech-Takara, Saint-Germain-en-Laye, France).

2.2.3.10 Cell lysate fractionation on sucrose density gradients

Quaternary structures of LPH wild type and mutants were analyzed according to Naim [Naim and Naim, 1996]. In brief, COS-1 cells were biosynthetically labeled and solubilized in 6mM dodecyl-β-m-maltoside, 50mM Tris-HCl, 100mM NaCl, pH 7.5 and protease inhibitors. The cell extracts were centrifuged at 100000x*g* for 1h at 4°C and

the supernatant was loaded onto an 11.5ml sucrose gradient that consisted of 10 to 30% or 15 to 25% sucrose (w/v), 50mM Tris-HCl, 100mM NaCl, pH 7.5, 6mM dodecy-β-m-maltoside and the protease inhibitors mentioned above except PMSF. The gradient was centrifuged at 100000x*g* for 18h at 4°C and divided into 0.5ml fractions followed by immunoprecipitation and SDS-PAGE.

2.2.4 Extraction of detergent resistant lipid microdomains

Extraction of detergent resistant lipid microdomains was performed essentially as described previously [Castelletti et al., 2008].

3. Results

3.1 Impaired trafficking and subcellular localization of a mutant lactase associated with congenital lactase deficiency

3.1.1 Location and description of the Gly1363 mutation in CLD

Diseases caused by one or more defects in one gene can result in individually different phenotypic appearances like those observed in cases of congenital sucrase-isomaltase deficiency [Fransen et al., 1991; Keiser et al., 2006; Naim et al., 1988]. Recently, nine distinct mutations in the coding region of the lactase gene have been identified in patients suffering from congenital lactase deficiency (CLD), one of them is G1363S [Kuokkanen et al., 2006; Torniainen et al., 2009]. Table 3.1.1 compiles the LPH primary sequence in different species and demonstrates that the residue Gly^{1363} is conserved. This together with the generation of a potential N-glycosylation site (cp. 1.1.4, *Glycosylation*) as result of G1363S exchange suggests that these amino acids play crucial roles in the context of folding determinants.

LPH G1363S	RYYTEVITNN**S**MPLAREDEFL
LPH human	RYYTEVITNN**G**MPLAREDEFL
LPH rabbit	SYYTELITNN**G**MPLPSEDEFV
LPH mouse	RYYTEVITNN**G**MPLAKEDEFL
LPH rat	RYYPDLIANN**G**MPLAREDEFL

Table 3.1.1: Comparison of the amino acid stretch encompassing the mutation G1363S. Sequence alignment between amino acids 1353 to 1373 of LPH from different species, indicating important functions of this region.

3.1.2 The G1363S mutation results in an intracellular localization and altered biosynthesis and processing of LPH

The influence of the residue Gly^{1363} on the folding and transport events of newly synthesized LPH was investigated by highlighting possible differences between wild type LPH and LPH bearing the G1363S mutation. Since this mutation appeared as a compound heterozygote together with an Y1390X mutation in the same patient

[Kuokkanen et al., 2006], it was also asked if this amino acid substitution *per se* can influence the enzyme function. For this, the mutation G1363S was introduced into wild type LPH (indicated LPH-G1363S).

Fig. 3.1.1 shows a schematic drawing of the structure and membrane association of LPH as well as the location of the mutation G1363S in the homologous domain III that comprises the phlorizin-hydrolase activity.

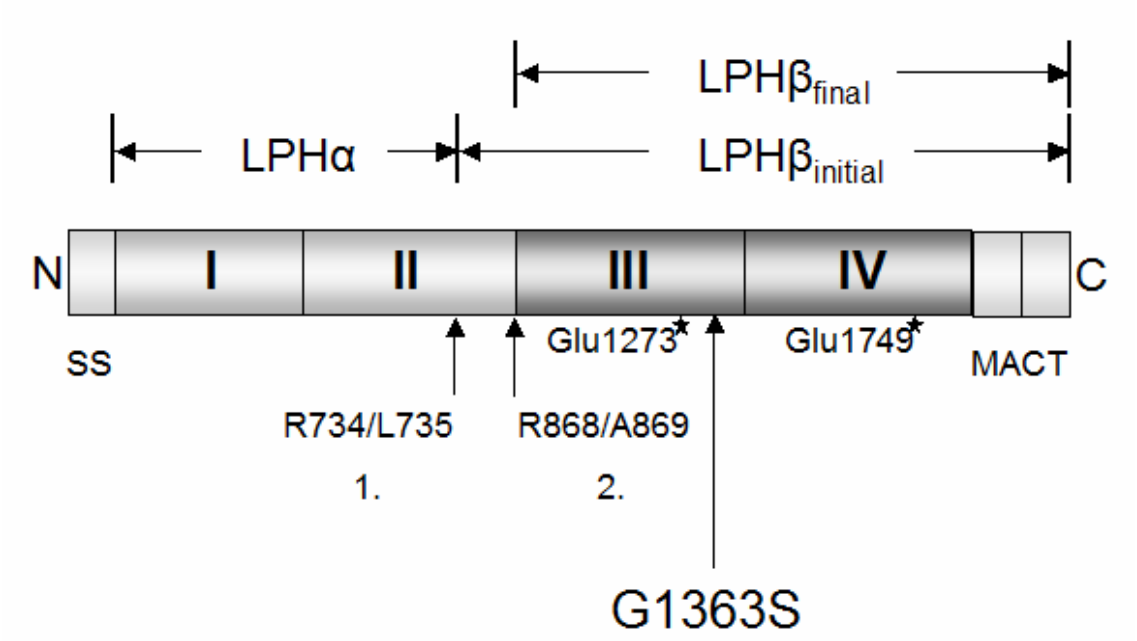


Fig. 3.1.1: Location of the G1363S mutation in LPH. Main features of intestinal LPH structure. Prepro-LPH consists of a cleaveable signal sequence (SS; Met^{1}-Gly^{19}) and an extracellular region comprising homologous domains I-IV (Ser^{20}-Thr^{1882}). The initial cleavage step takes place between 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).

The LPH-G1363S mutant and its wild type counterpart were expressed in COS-1 cells (Fig. 3.1.2), biosynthetically labeled and immunoprecipitated with anti-LPH antibodies. Fig. 3.1.2 (upper panel) shows that wild type LPH revealed an endo H-sensitive 215-kDa mannose-rich polypeptide within 1.5 h of labeling. Another glycosylated 230-kDa protein band appeared at 8 h of labeling that was endo H-resistant and represents therefore a complex glycosylated mature band that has been processed in the Golgi apparatus. By contrast, the LPH-G1363S mutant revealed exclusively an endo H-sensitive 215-kDa glycosylated protein that retained

its mannose-rich type of glycosylation even after 8 h of labeling and has not been therefore processed in the Golgi apparatus (Fig. 3.1.2, lower panel).

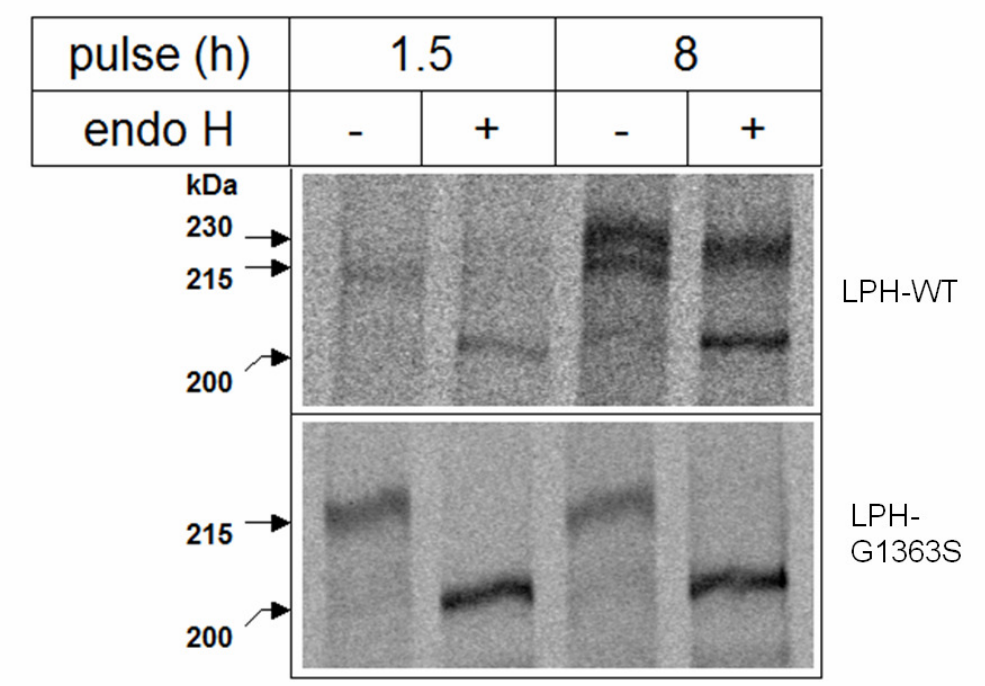


Fig. 3.1.2: Expression of wild type and mutant LPH in COS-1 cells. COS-1 cells were transiently transfected with cDNAs encoding wild type LPH and LPH-G1363S, biosynthetically labeled for 1.5h and 8h followed by cell lysis and immunoprecipitation. The immunoprecipitates were divided into equal aliquots and treated with endo H or not treated. The proteins were subjected to SDS-PAGE on 5% slab gels and autoradiography.

The biochemical data were corroborated by immunofluorescence analyses of the subcellular and cell surface localization of the wild type and mutant LPH-G1363S using confocal laser microscopy. As shown in Fig. 3.1.3 cell surface labeling revealed strong labeling of wild type LPH at the cell surface in contrast to LPH-G1363S which was barely detected (upper panel). In permeabilized cells, LPH-G1363S was predominantly located in intracellular compartments. In fact, LPH-G1363S was found to colocalize with an ER marker. Taken together, the biochemical and immunofluorescence data indicate that the LPH-G1363S mutant does not exit the ER.

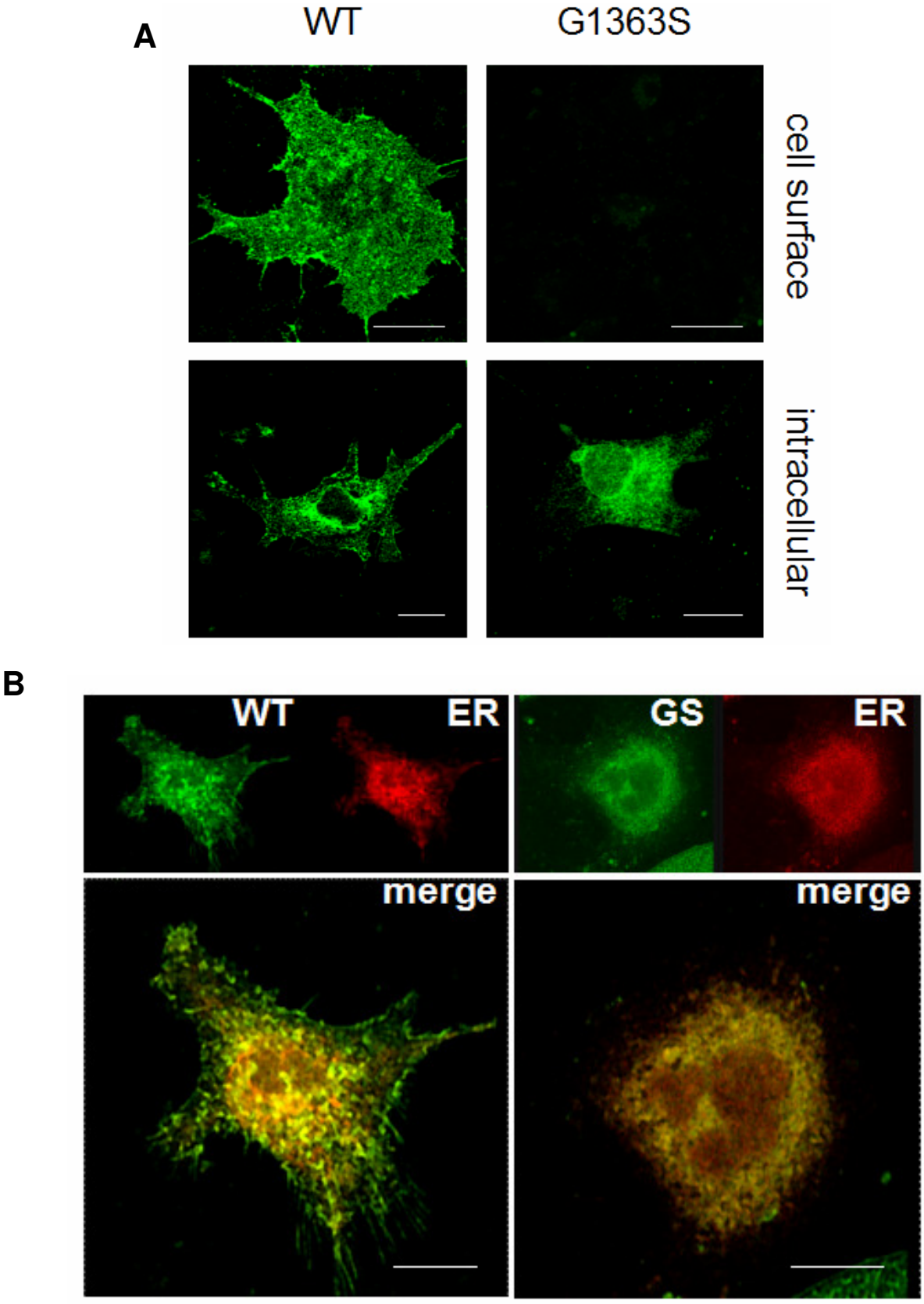


Fig. 3.1.3: Subcellular localization of LPH-G1363S in transiently transfected COS-1 cells. A) Wild type and mutant LPH forms were expressed in COS-1 cells grown on cover slips and their cell surface and subcellular localization was compared by immunofluorescence and confocal microscopy. B) Colocalization of wild type and mutant proteins with the ER marker DsRed2-ER (colocalizations are shown in yellow). Scale bars, 20 µm.

3.1.3 Mutant LPH-G1363S is a malfolded and enzymatically inactive protein

Given the predominant intracellular location of the mutant LPH-G1363S, one question was whether the trafficking arrest is the consequence of gross structural alterations elicited by the mutation G1363S. Firstly, the enzymatic activity of the mutant towards lactose and phlorizin were determined and were found to be below detection limit (Fig. 3.1.4). These measurements are indicative of an altered folding of at least around the activity centers of phlorizin-hydrolase, Glu^{1273}, and lactase, Glu^{1749} (cp. Fig. 3.1.1).

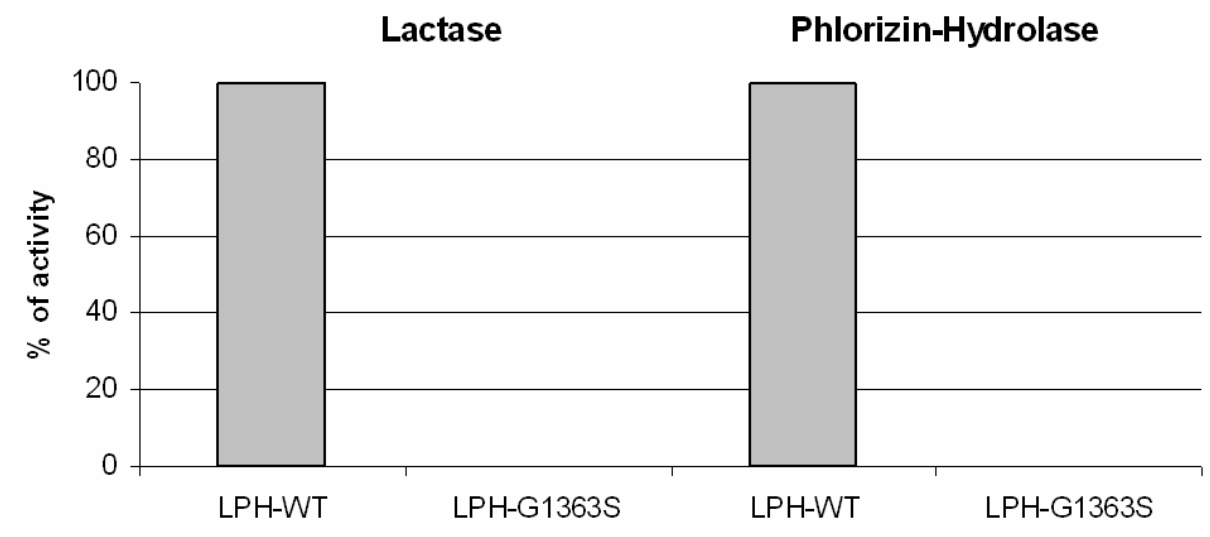


Fig. 3.1.4: Enzymatic activity of LPH and LPH-G1363S. COS-1 cells were transiently transfected with wild type and mutant LPH cDNA. 48h posttransfection the cells were lysed and immunoprecipitated with mAb anti-LPH, and the lactase and phlorizin activities were measured by determining the concentration of released glucose with HPLC.

Then, the enzymatic measurement data were corroborated by comparing the protease sensitivity of wild type and mutant LPH-G1363S towards trypsin. In the intestinal lumen a pancreatic trypsin cleaves wild type $LPH\beta_{initial}$ upon insertion into the brush border membrane to generate $LPH\beta_{final}$, which is trypsin resistant (cp. Fig. 3.1.1). As such trypsin is a convenient protease that could be used to probe possible folding alterations, whereby variations in the tryptic digestion pattern are indicative of a conformational change in LPH.

For this, wild type and mutant LPH were isolated from biosynthetically labeled COS-1 cells that are known not to cleave pro-LPH to $LPH\beta_{initial}$ intracellularly [Naim et al., 1991]. Subsequently, the immunoprecipitates were treated with trypsin at a

concentration capable of cleaving wild type LPH into the profragment and LPHβ_{final}. As shown in Fig. 3.1.5 at 0 min of trypsin treatment, wild type LPH consisted of two bands, the mannose-rich 215-kDa form, and the complex-glycosylated 230-kDa form. 2 min of trypsin treatment were sufficient to convert both forms to 160-kDa and 135-kDa polypeptides. The intensities of these polypeptides did not significantly change after 10 min of trypsin treatment strongly suggesting that no additional trypsin sites were exposed in wild type LPH[4]. LPH-G1363S appeared as a mannose-rich 215-kDa species after 0 min and was completely degraded within 2 min. The marked difference in the reactivity of this mutant and wild type LPH is indicative of altered folding patterns.

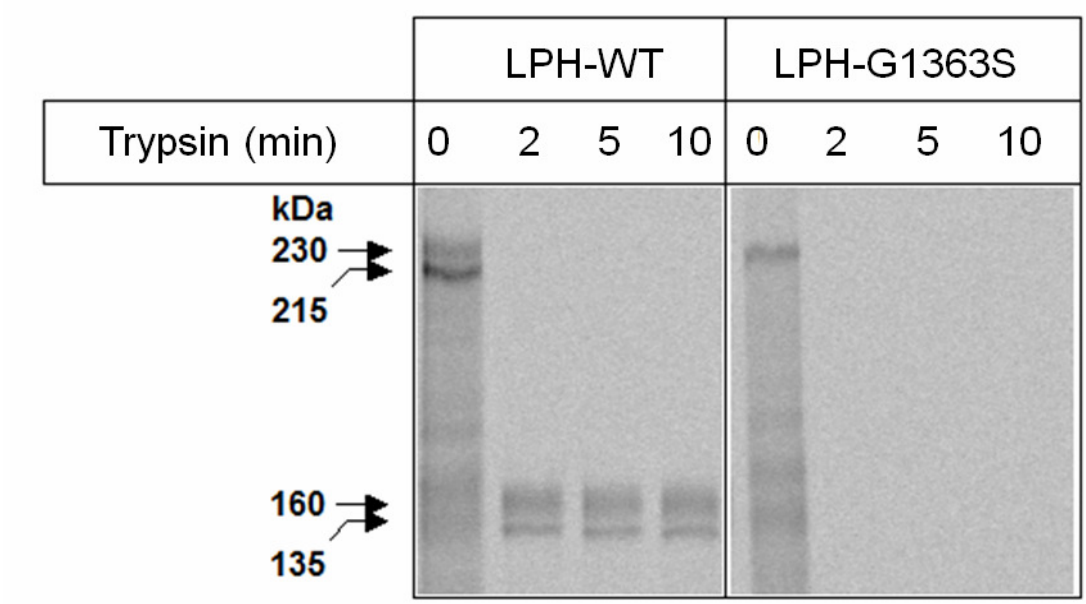


Fig. 3.1.5: Folding of wild type and mutant proteins. Trypsin sensitivity assay of LPH and LPH-G1363S. Transiently transfected COS-1 cells were biosynthetically labeled at 37°C followed by immunoprecipitation of LPH proteins from the cell lysates. The immunoprecipitates were treated with trypsin for the indicated times and analyzed by SDS-PAGE.

3.1.4 The mutation G1363S is responsible for an increased turnover rate of LPH-G1363S as compared with wild type LPH

The fate of the malfolded mutant was next addressed by delineating its trafficking kinetics as compared to the wild type protein utilizing pulse-chase experiments (Fig.

[4] The profragment LPHα is not shown, since it is further cleaved by trypsin to a lower molecular weight that runs at the front of the gel.

3.1.6). The conversion of the 215-kDa mannose-rich precursor to the complex glycosylated 230-kDa form occurs only for the wild type protein between 0.5 h and 1 h of chase (upper panel). By contrast, the LPH-G1363S mutant persisted as a mannose-rich glycosylated protein through out the chase and its intensity started to decrease at 4 h of chase with clear reduction observed at 8 h and 12 h of chase (lower panel) pointing to its degradation in the ER, perhaps by the ER-associated degradation pathway (ERAD) [Nakatsukasa et al., 2008]. The intensity of the complex glycosylated wild type LPH decreases also, but not to a similar extent as the mutant protein compatible with a longer turnover rate of the wild type as compared to the mutant.

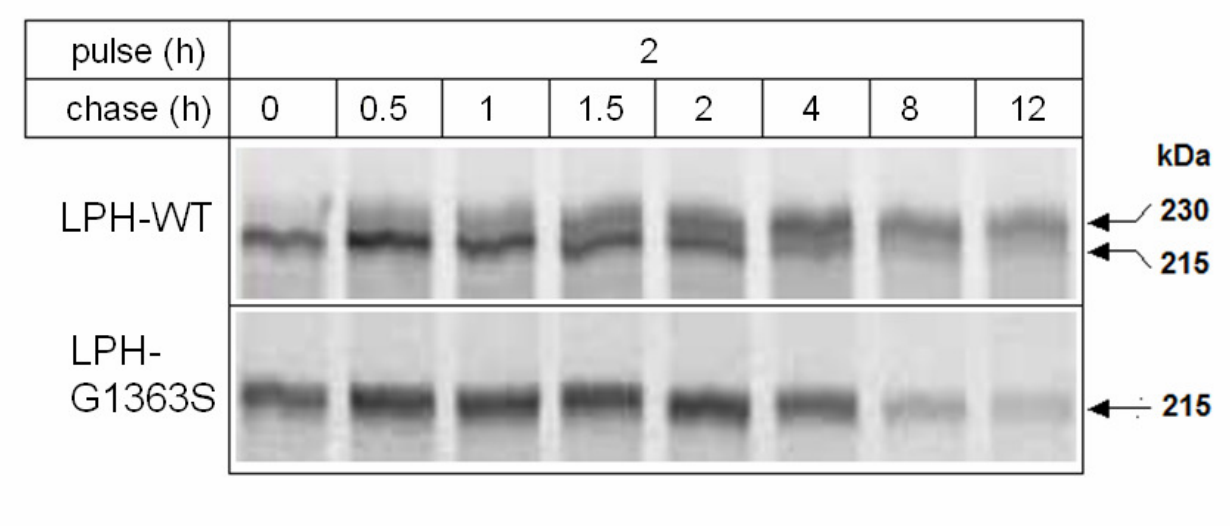


Fig. 3.1.6: Transport kinetics and turnover of wild type and mutant proteins. Transiently transfected COS-1 cells were pulsed with [^{35}S]methionine followed by chases for different times at 37°C. Cells were lysed, and the immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

3.1.5 LPH-G1363S is a temperature-sensitive mutant

The majority of naturally occurring mutants that are implicated in diseases are not capable of escaping the quality control machinery of the ER and are retained in that organelle. However, a few mutant phenotypes differ from this general scheme with respect to their *in vitro* sensitivity towards reduced non-physiological temperatures and the acquisition at these permissive temperatures to a partially correctly folded conformation that enables them to exit the ER. Known examples of this type of mutants are phenotype II in congenital sucrase-isomaltase deficiency [Propsting et al., 2005], the DeltaF508 mutant of the cystic fibrosis transmembrane conductance regulator [Cheng et al., 1990] and the temperature-sensitive ts045 mutant of the G

protein of the vesicular stomatitis virus [Doms et al., 1987]. Finally, individual expression of LPH$\beta_{initial}$ without its LPHα profragment at a permissive temperature of 20°C generates a stable, trypsin-resistant, and an enzymatically active polypeptide indicating that its individual expression results in a temperature-sensitive conformation [Jacob et al., 2002a].

Therefore the question arose whether LPH-G1363S belongs to this family of transport-defective mutants and meets the requirements of a temperature-sensitive mutant. For this, the influence of reduced temperatures on the trafficking behavior of LPH-G1363S was investigated in a pulse-chase experiment. Fig. 3.1.7 shows that biosynthetic labeling of cells expressing LPH-G1363S at a permissive temperature of 20°C revealed a complex glycosylated endo H-resistant protein band after 22 h of labeling. The proportion of the complex glycosylated form, on the other hand, was clearly below that detected for the wild type under similar labeling conditions. Nevertheless, the results unequivocally demonstrate that the mutant LPH-G1363S is temperature-sensitive and has been transported to and processed in the Golgi apparatus at the permissive temperature. The control sample in which the mutant was chased for 10 h at 37°C showed exclusively the mannose-rich polypeptide of mutant LPH-G1363S, while as expected the wild type revealed complex glycosylated forms under these conditions. Interestingly, complex glycosylated LPH-G1363S form is slightly larger than that of the wild type protein. This becomes more obvious when the patterns of the endo H-digested proteins are considered. Here, the difference in the electrophoretic mobilities of the endo H-products of the LPH-G1363S mutant is clearly higher than that of its wild type counterparts. This result demonstrates that LPH-G1363S is more glycosylated than the wild type protein and that the additional N-glycosylation site generated by the mutation (cp. Table 3.1.1) has been accessible to glycan transfer. The maturation of LPH-G1363S at 20°C is sufficient for its transport to the cell surface, when the temperature is raised to 37°C. In fact, immunofluorescence analysis of cells expressing LPH-G1363S that have been cultured at 20°C for 22 h followed by 2 h chase at 37°C reveal strong fluorescence signals corresponding to LPH-G1363S exposed at the cell surface (Fig. 3.1.7).

Finally, the question arose whether the observed transport-competence is due to acquired correct folding and probed therefore for its protease sensitivity with trypsin. The protein maintained its trypsin-sensitivity and was completely degraded in

contrast to the wild type protein, which revealed as shown above the trypsin-resistant polypeptides (160-kDa and 135-kDa) [Behrendt et al., 2009].

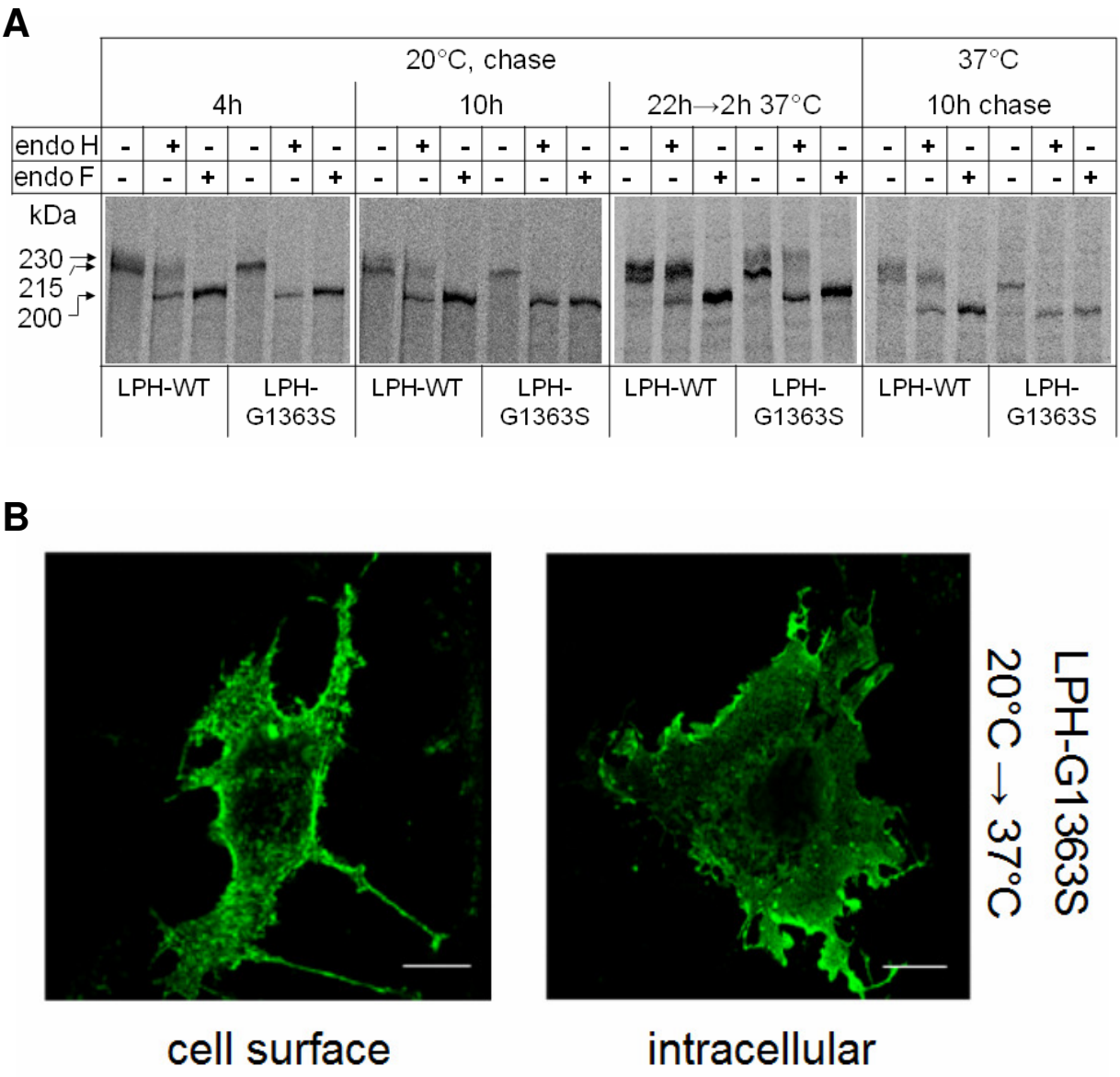


Fig. 3.1.7: Biosynthesis, transport kinetics, glycosylation pattern, and subcellular localization of wild type LPH and LPH-G1363S at 37°C and 20°C. A) Transiently transfected COS-1 cells were pulsed 48h after transfection with [^{35}S]methionine and chased for the indicated time points at 37°C, 20°C or both and treated with endo H or endo F or not treated. B) COS-1 cells grown on cover slips and expressing wild type LPH and LPH-G1363S were cultured at 20°C followed by incubation at 37°C. The subcellular localization was compared by immunofluorescence and confocal laser microscopy. Scale bars, 20 µm.

Further, the enzymatic activity pattern of the mutant at the permissive temperature was assessed and a slight increase in the activity towards lactose and phlorizin could be detected (Fig. 3.1.8). Together, these results demonstrate that LPH-G1363S did

not acquire a folding pattern similar to that of the wild type LPH at the permissive temperature. Nevertheless, this partial folding is apparently sufficient for proteins to leave the ER in line with novel concepts that have proposed the existence of quality control mechanisms beyond the ER and contradicting previous concepts of the absolute requirement for correct folding prior to exit from the ER [Ellgaard and Helenius, 2003].

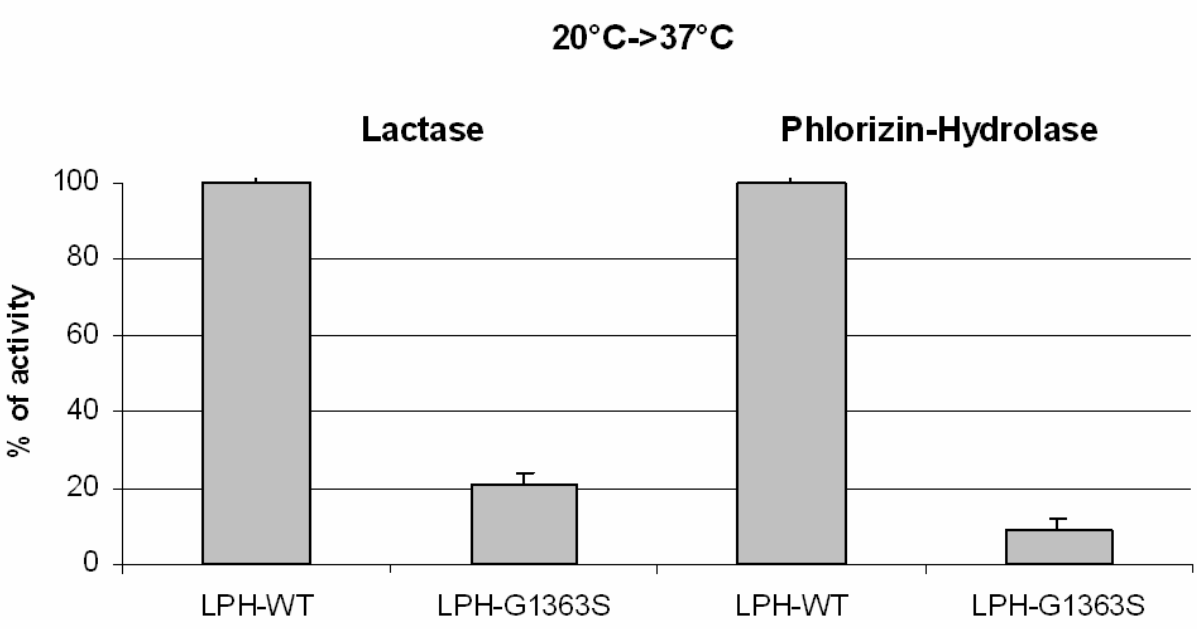


Fig. 3.1.8: Enzymatic activity of LPH and LPH-G1363S after incubation at 20°C. COS-1 cells were transiently transfected with wild type and mutant LPH cDNA. 48h posttransfection the cells were incubated at 20°C and 37°C, lysed and immunoprecipitated with anti-LPH antibodies, and the lactase and phlorizin activities were measured by determining the concentration of released glucose with HPLC. The error bars represent standard deviations.

3.1.6 The potential glycosylation site generated by the G1363S mutation is not the cause of defective trafficking of LPH-G1363S or its reduced enzymatic activity

Recent data show that addition of N-glycosylation sites can alter protein features as quaternary structure, transport [Jacob et al., 2000] and sorting [Vagin et al., 2005]. It was therefore necessary to elucidate, if the additional glycosylation of LPH-G1363S is the reason for impaired folding and trafficking of the protein. For this, the additional N-glycosylation site was re-eliminated by generating another mutant containing the amino acid substitutions N1361A and G1363S, denoted LPH-N1361A/G1363S. In a fashion similar to LPH-G1363S, this mutant was not capable of exiting the ER at

37°C, and showed a similar pattern to LPH-G1363S at 20°C (Fig. 3.1.9), suggesting that the additional glycosylation does not alone lead to altered LPH characteristics in LPH-G1363S.

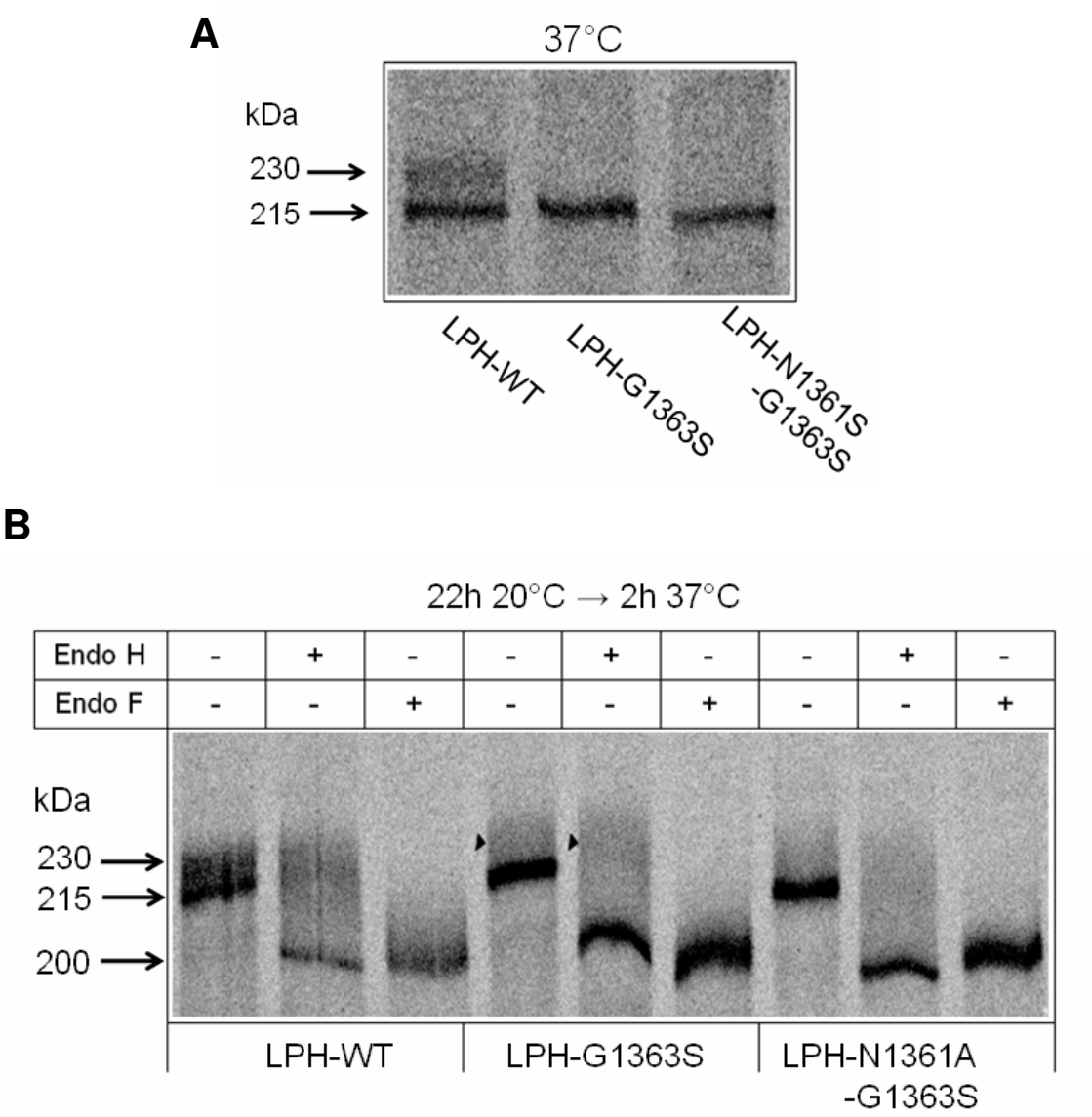
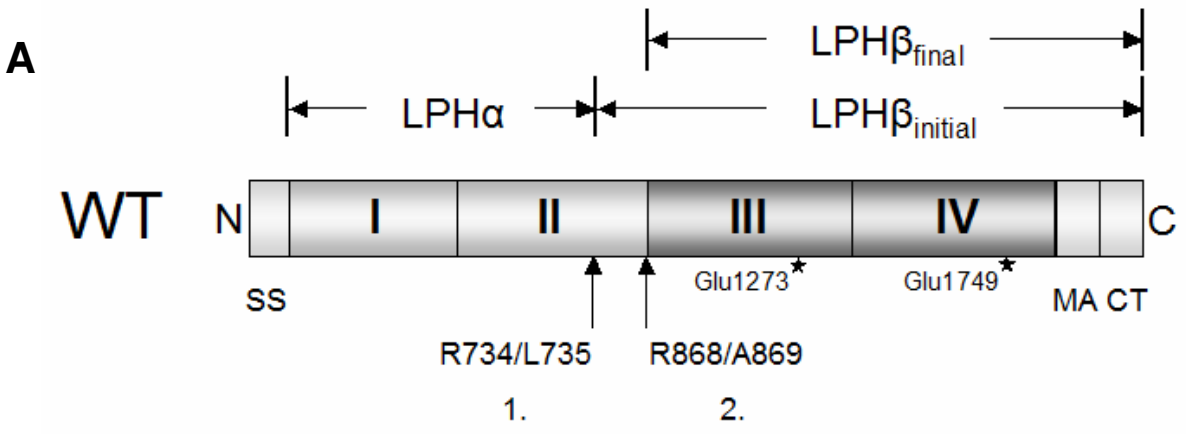


Fig. 3.1.9: Biosynthesis and glycosylation pattern of wild type LPH, LPH-G1363S and LPH-N1361A/G1363S at 37°C and 20°C. COS-1 cells were transiently transfected with the cDNAs encoding LPH or its mutants. 48h after transfection the cells were continuously labeled (A) or pulse labeled and chased at 20°C and 37°C (B). LPH proteins were immunoprecipitated and treated with endo H or endo F or not treated and analyzed by SDS-PAGE on 5% slab gels followed by autoradigraphy. Arrowheads indicate the complex glycosylated 235-kDa LPH-G1363S protein form.

3.2 Structural hierarchy of regulatory elements in the folding and transport of an intestinal multi-domain protein: Domain III constitutes the structural core while domain IV has a regulatory role

LPH comprises four extracellular regions which contain 38-55% identical residues [Mantei et al., 1988]. An interval of about 100 amino acids within each domain is even more homologous and this internal homology can also be found by comparison of LPH primary sequences of different species. While the function of domains I and II that constitute the profragment or proregion of LPH, LPHα, has been assessed before and shown to act as an intramolecular chaperone [Jacob et al., 2002a], the individual roles of the two other domains III and IV are poorly understood. Homology-based models of domains III and IV reveal typical TIM-barrel structures of family 1 glycoside hydolases and show distinct differences between these two domains [Behrendt et al., 2010]. The impact of these homologous domains on the generation of a transport-competent configuration of pro-LPH was addressed in conjunction with the question of whether either domain can fold independently. For this, several cDNA constructs each lacking the coding region of one homologous domain were generated (Fig. 3.2.1, B). Previous *in silico* analysis provided the potential domain boundaries as a basis for site-directed loop-out mutagenesis.[5] For better comparability, the main structural and functional features of human intestinal LPH are given in Figure 3.2.1, A.



[5] This was done by Julio Polaina, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, Apdo. de Correos 73, Burjassot, Valencia, E46100, Spain.

B

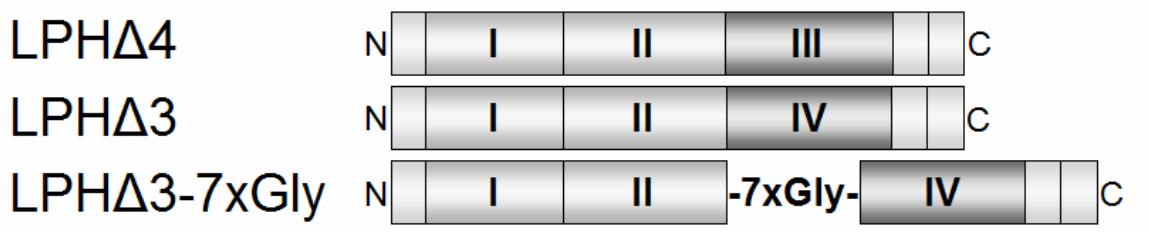


Fig. 3.2.1: Schematic presentation of wild type and LPH deletion mutants. A) Main features of intestinal LPH structure. B) Schematic drawing of domain deletion mutants generated by loop-out mutagenesis. Location of phlorizin hydrolase (Glu^{1273}) and lactase (Glu^{1749}) activities, respectively, are indicated by stars. MACT, membrane anchor and cytoplasmic tail.

3.2.1 Expression of wild type pro-LPH and domain deletion mutants in COS-1 cells

To examine the contribution of each of the two homologous domains to the structural, functional and trafficking features of LPH, the LPH deletion mutants were expressed in COS-1 cells and their characteristics compared to those of wild type LPH (Fig. 3.2.2). Cell lysates were immunoprecipitated and the precipitated proteins were treated with endo H to determine their glycosylated state as a measure of trafficking capacity. LPHΔ4 acquired endo H-resistance concomitant with complex glycosylation in and trafficking of these mutants to the Golgi apparatus. By contrast, the mutant lacking homologous domain III, LPHΔ3, was not transport-competent and the introduction of a spacer containing seven glycines to avoid possible sterical hindrances did not alter its trafficking characteristics. Assessment of the proportions of the mannose-rich and complex glycosylated forms after scanning of the gels revealed a substantial increase in the proportion of the complex glycosylated LPHΔ4 as compared to the wild type counterpart. This was surprising, since it indicated that the deletion of domain IV in LPHΔ4 leads to a more rapid processing of this deletion mutant than the wild type protein.

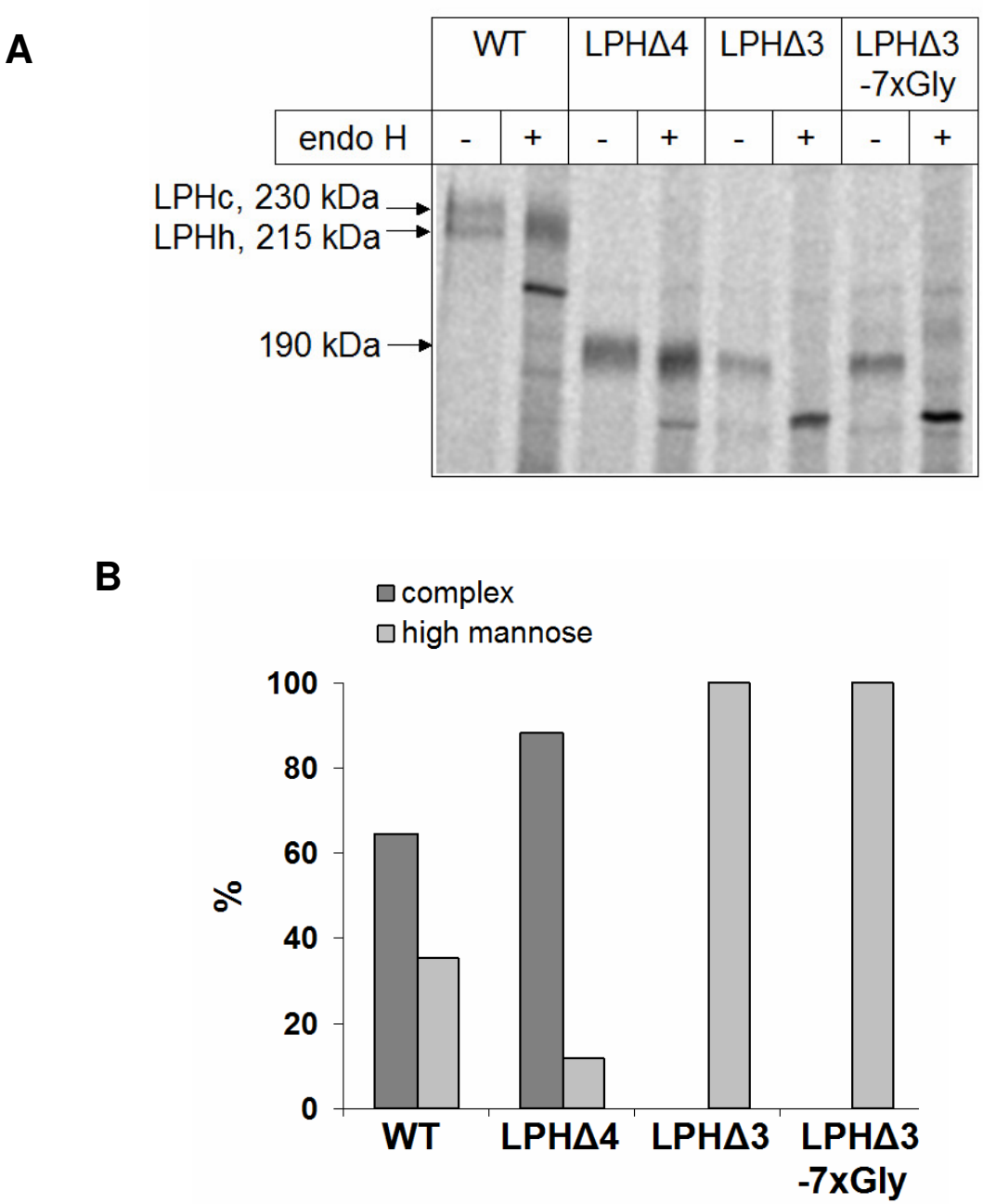


Fig. 3.2.2: Glycosylation pattern of LPH wild type and domain deletion mutants in COS-1 cells. A) Transiently transfected COS-1 cells were biosynthetically labeled for 8h with [^{35}S]methionine followed by immunoprecipitation. The immunoprecipitates were divided into two aliquots and treated with endo H, or not treated. The proteins were subjected to SDS-PAGE followed by autoradiography. B) densitometric scanning of the endo H treated biosynthetic forms of wild type and mutant LPH shown in (A).

The subcellular distribution of the mutant proteins was further investigated in more detail by confocal laser microscopy. As shown in Figure 3.2.3, LPHΔ3 was retained intracellularly and colocalized with the ER-DsRed marker. By contrast, and consistent with the biochemical data LPHΔ4 colocalized with markers of the ER, Golgi and was detected at the plasma membrane in a fashion similar to the wild type protein.

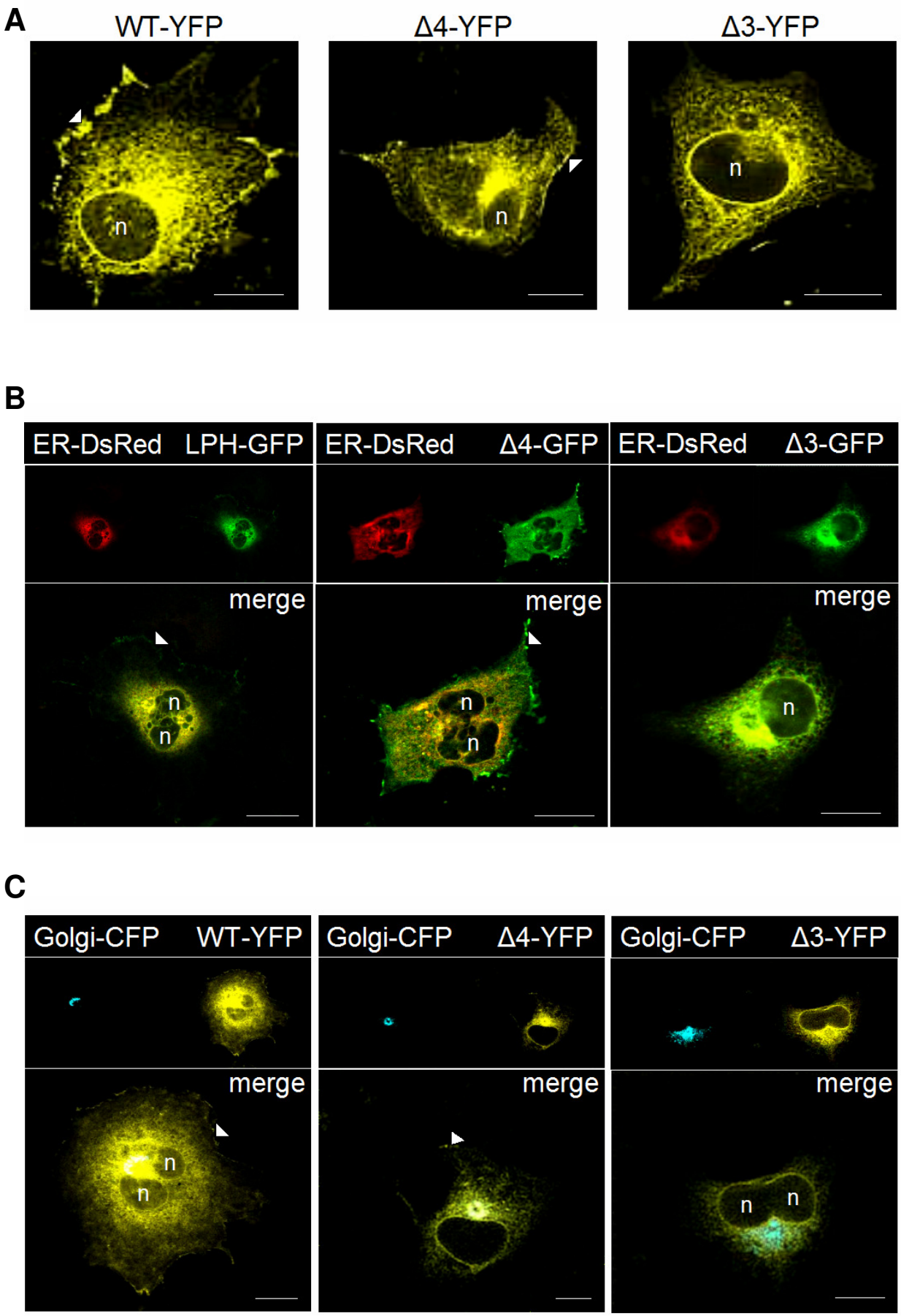

A
WT-YFP
Δ4-YFP
Δ3-YFP
n
B
ER-DsRed LPH-GFP
ER-DsRed Δ4-GFP
ER-DsRed Δ3-GFP
merge
C
Golgi-CFP WT-YFP
Golgi-CFP Δ4-YFP
Golgi-CFP Δ3-YFP

Fig. 3.2.3: Subcellular distribution of LPH wild type and domain deletion mutants in COS-1 cells. A) confocal analysis of transfected COS-1 cells. B-C), colocalization of LPH mutants with ER and Golgi markers, respectively, in transfected COS-1 cells. COS-1 cells were co-transfected with GFP-tagged LPH proteins and ER-DsRed or YFP-tagged LPH proteins and galactosyl transferase (GT)-CFP, respectively. Confocal analysis with living cells was performed 48h after transfection. n, nucleus; arrowheads, cell surface; bars, 20 µm.

3.2.2 Requirements for the LPH deletion mutants to exit the ER

Dimerisation of LPH in the ER is absolutely required for LPH to egress this organelle to the Golgi apparatus [Naim and Naim, 1996]. The differential intracellular distribution and maturation patterns of the deletion mutants as well as the variable proportions of the glycoforms have altogether lead to examine the quaternary structures of the mutants and assess their relevance to their transport out of the ER. Fig. 3.2.4 depicts the results obtained using sucrose density gradients. As has been previously shown, the mannose-rich LPH form was retained in the light as well as dense gradient fractions concomitant with its monomeric and dimeric states respectively and indicative of dimerisation occurring along the early secretory pathway. The complex glycosylated protein on the other hand is detected exclusively in the dense fractions indicating that the dimerisation of the mannose-rich form of LPH precedes its complex glycosylation and maturation in the Golgi [Naim and Naim, 1996]. Surprisingly, the transport-competent LPHΔ4 deletion mutant did not require dimerisation of its mannose-rich form in the ER prior to ER egress. As shown in Fig. 3.2.4 (the second top panel) the mannose-rich form of LPHΔ4 persisted as a monomeric protein and the complex glycosylated LPHΔ4 initially appeared in the monomeric fractions. The majority of the complex glycosylated molecules were mainly found in the denser gradient fractions. Interestingly, complex glycosylated LPHΔ4 was revealed in two peaks in the gradient compatible with two quaternary states, a dimeric and presumably a tetrameric state. A tetrameric LPHΔ4 form would be in line with the results obtained by [Panzer et al., 1998] for the LPH1646MACT mutant lacking 236 amino acids at the C terminus of homologous domain IV.

By contrast, LPHΔ3 was exclusively detected in the lighter fractions of the gradients in its mannose-rich glycoform compatible with retention in the ER as a monomeric protein.

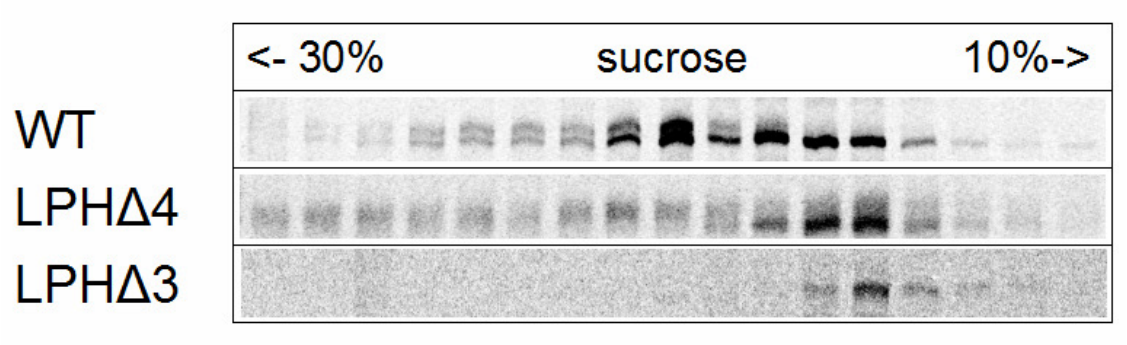


Fig. 3.2.4: Assessment of the quaternary structure. Transiently transfected COS-1 cells were biosynthetically labeled and solubilized in 6 mM dodecyl-β-m-maltoside. Cell lysates were layered on a sucrose density gradient. After centrifugation for 18h at 100,000 x *g*, fractions were collected, immunoprecipitated and analyzed on SDS-PAGE.

3.2.3 Transport kinetics of LPH wild type and deletion mutants

Next, the transport kinetics of the mutants in comparison to wild type LPH were analyzed in pulse-chase experiments. Complex glycosylated LPHΔ4 appeared within 1.5 h of chase and its proportion was higher than its counterpart in the wild type protein (Fig. 3.2.5, compare also Fig. 3.2.2) indicating that it is more efficiently transported to the Golgi apparatus than wild type LPH. By contrast, LPHΔ3 persisted as a mannose-rich polypeptide compatible with ER localization. Further, the labeling intensity of this mannose-rich form of LPHΔ3 decreased continuously within prolonged chase time points suggesting that this mutant undergoes degradation in the ER, presumably by ER-associated degradation (ERAD). The Gly-spacer containing LPHΔ3-7xGly mutant revealed also similar biosynthetic features as LPHΔ3 (not shown).

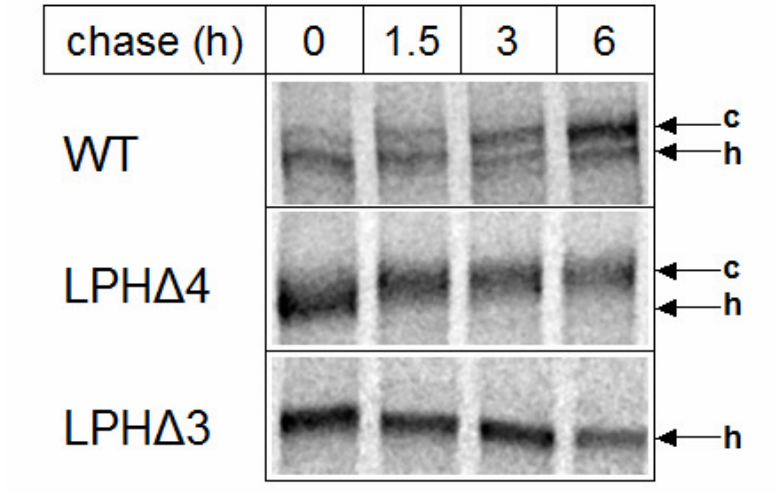


Fig. 3.2.5: Transport kinetics of wild type LPH and mutant proteins. Transfected COS-1 cells were pulse labeled for 1.5h with [^{35}S]methionine and chased for the indicated periods of time with cold methionine. The immunoprecipitates were analyzed by SDS-PAGE on 6% slab gels. c, complex glycosylated protein form; h, high mannose or mannose-rich protein form.

3.2.4 Folding of the LPH deletion mutants

The variations in the quaternary structure of the deletion mutants as well as in their transport kinetics raised the question of causal folding variations. Therefore, the folding of these mutants was examined by using three procedures. In the first the mutants were probed for their protease sensitivity using trypsin, in the second procedure the enzymatic activities of the mutants were measured and finally in the third reactivity of the mutants with epitope-specific antibodies were assessed.

Trypsin treatment

The tryptic digestion patterns of the wild type and mutant proteins are depicted in Fig. 3.2.6. Wild type LPH was digested to two main bands corresponding to cleaved mannose-rich and complex glycosylated LPH (cp. Fig. 3.1.5). This pattern did not change with prolonged digestion times. Similarly, LPHΔ4 pattern was also cleaved to two protein products that correspond to the mannose-rich and complex glycosylated forms. The smaller apparent molecular weights products fit well with a reduction corresponding to the size of the deleted domain IV. In a fashion similar to wild type LPH the cleaved products of LPHΔ4 were also resistant to trypsin. Importantly, the cleavage of LPHΔ4 to the final products was not preceded by major intermediate cleaved forms suggesting that one major trypsin site is exposed in the deletion mutant, which is in all likelihood the same as that in wild type LPH.

By contrast to wild type LPH and LPHΔ4, LPHΔ3 was completely degraded by trypsin already after 1 min of treatment concomitant with the exposure of several trypsin cleavage sites and thus altered folding in comparison to wild type LPH and LPHΔ4.

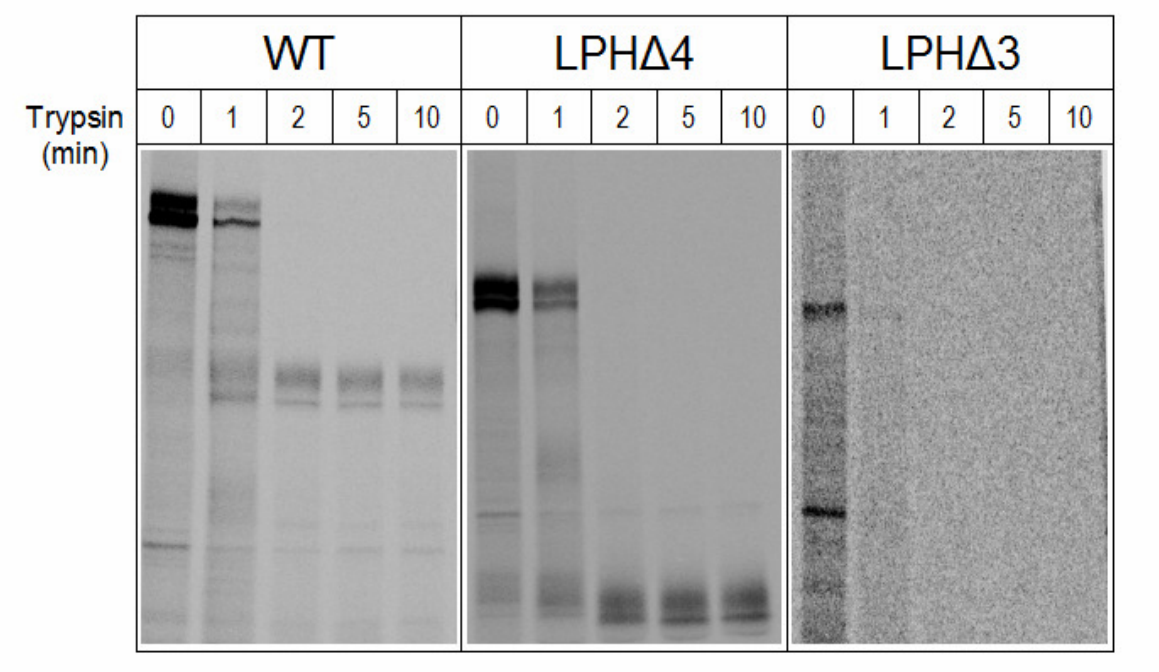


Fig. 3.2.6: Trypsin sensitivity assay of wild type and LPH mutants. Transiently transfected COS-1 cells were biosynthetically labeled followed by immunoprecipitation of LPH proteins from the cell lysates. The immunoprecipitates were treated with trypsin for different times and analyzed by SDS-PAGE on 7% slab gels.

Enzymatic activities of LPH deletion mutants

Another approach to examine the folding and maturation pattern of a protein is to assess its biological function. Therefore, the enzymatic activities of lactase and phlorizin-hydrolase in these mutants in comparison to their wild type counterparts were analyzed (Fig. 3.2.7). LPHΔ4 revealed slightly reduced activities of phlorizin hydrolase. The lactase activity was as expected absent, since the lactase active site is found in residue Glu^{1749} of domain IV. The lactase activity in LPHΔ3 was not detected. The data provides another support for malfolded LPHΔ3 and correct folding of LPHΔ4.

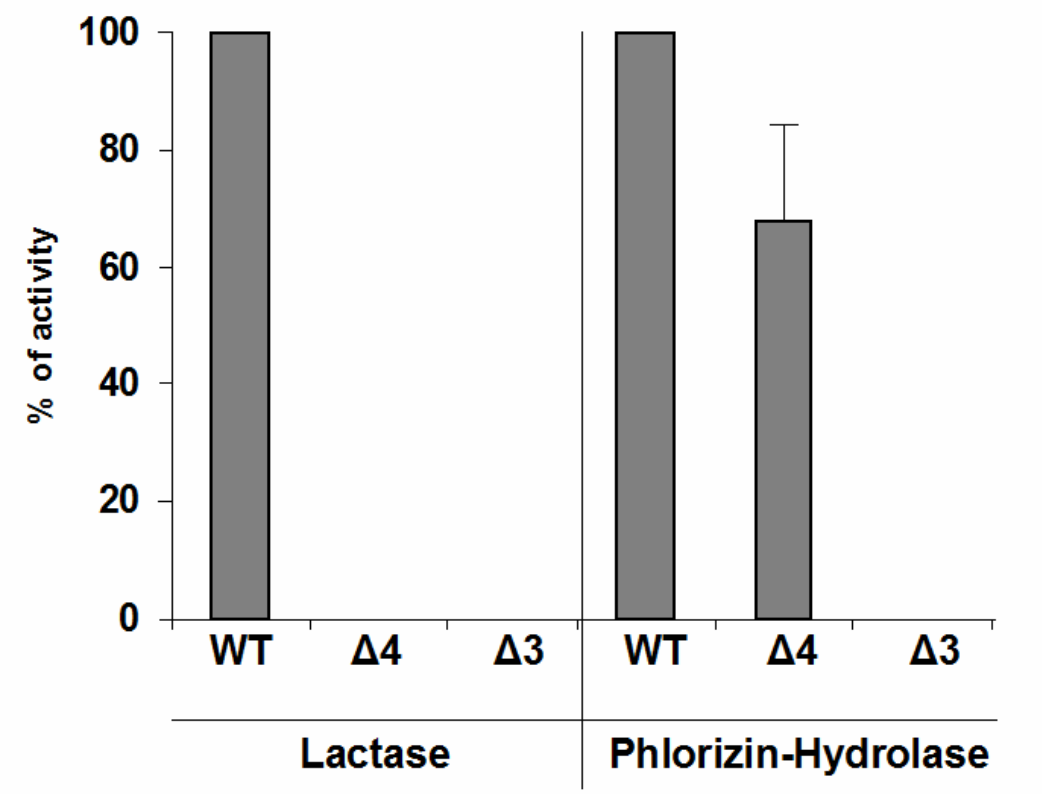


Fig. 3.2.7: Enzymatic activity of deletion mutants (Δ-mutants) of LPH. COS-1 cells were transiently transfected. 48h posttransfection, labeled cells were lysed and proteins immunoprecipitated. Immunoprecipitates were incubated with lactose and phlorizin, respectively, and the lactase and phlorizin hydrolase activities were measured by determining the concentration of released glucose by HPLC. The enzyme activities of the mutants were compared with those of wild type LPH. The error bar represents standard error.

Epitope mapping of domain deletion mutants

The deletion mutants were immunoprecipitated with a panel of mAbs, which are specific in recognizing native or unfolded conformations of LPH [Naim and Naim, 1996]. The control samples utilized immunoprecipitation of the GFP-tagged mutants with anti-GFP. Fig. 3.2.8 shows that LPHΔ4 and LPHΔ3 were isolated with anti-GFP antibody. Surprisingly none of the mAbs against LPH recognized LPHΔ3, even the two mAbs, MLac6 and MLac10, that recognize unfolded and denatured forms of LPH. LPHΔ4, on the other hand, reacted with all the antibodies utilized with the exception of MLac6 and MLac10. Given that the antibodies were raised against the mature form of LPH, i.e. LPHβ that comprises the two domains III and IV, it is obvious that all antibodies except MLac6 and MLac10 possess epitopes in domain III of LPH. Since MLac6 and MLac10 are directed against unfolded forms of LPH the results indicate that LPHΔ4 is properly folded lending a strong support to the protease sensitivity data. LPHΔ3, on the other hand, is malfolded and is therefore not recognized by the

antibodies. It is also likely that none of epitopes is found on LPHΔ3. This view is supported by the observation that in immunoprecipitation experiments LPHΔ3 does not react with MLac6 or MLAc10, which are directed against malfolded forms of LPH.

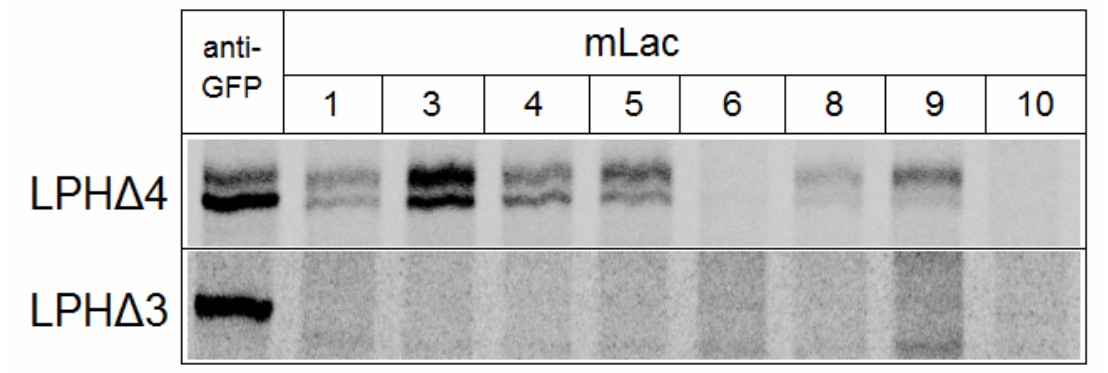
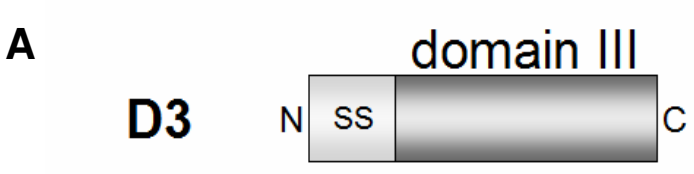


Fig. 3.2.8: Epitope mapping of LPH mutants. COS-1 cells were transfected with DNA coding for GFP-tagged deletion mutants and biosynthetically labeled 48 h after transfection. Cell lysates were divided into equal aliquots and immunoprecipitated with anti-GFP and different anti-LPH mAb. The immunoprecipitated proteins were analyzed by SDS-PAGE.

3.2.5 Domain III is a transport-competent and functional protein

The data gathered so far strongly suggest that domain III is a central autonomous component of LPH. The next step was therefore to express this domain independently and examine its trafficking and functional properties. As shown in Fig. 3.2.9 domain III expression in COS-1 cells revealed a predominant endo H- and endo F-sensitive protein band in the cell lysates indicating that it is a mannose-rich glycosylated form of domain III. The cell culture medium contained an endo H-resistant and endo F-sensitive protein compatible with a complex glycosylated domain III. These results clearly indicate that domain III is secreted into the cell exterior immediately and rapidly upon maturation in the Golgi apparatus. To substantiate the data with a further approach live cell imaging was performed. This shows that domain III was located in the ER compatible with the major mannose-rich form in the cell lysates. When the cells were subjected to a 20°C temperature block, domain III was found in the Golgi apparatus.



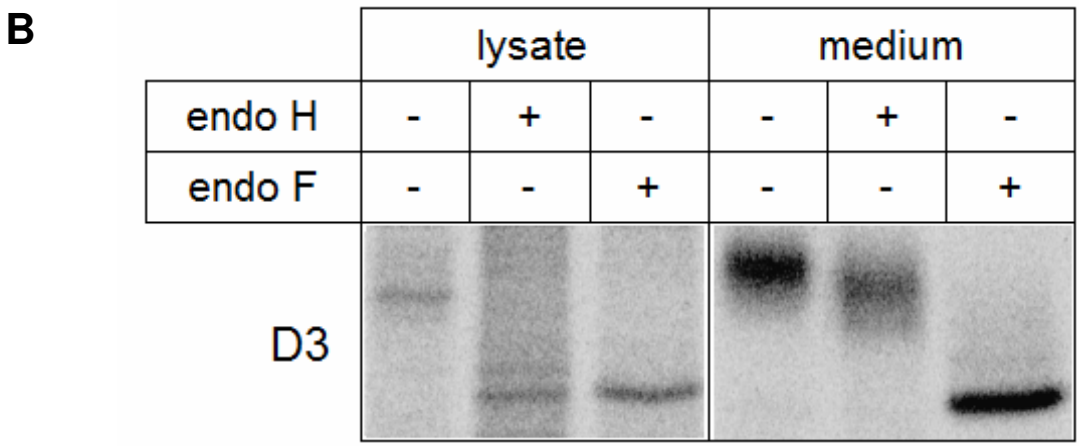


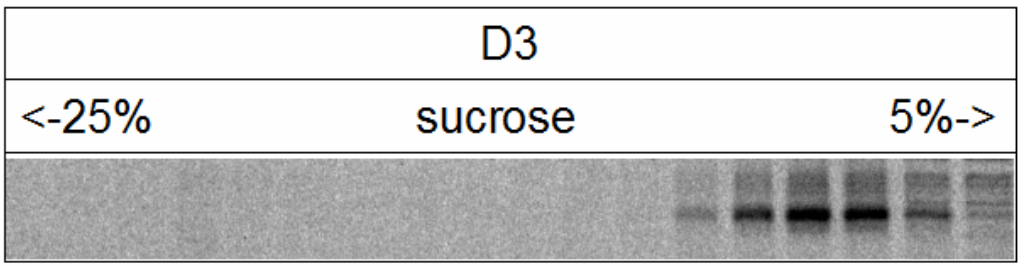
Fig. 3.2.9: Expression of domain III (D3) in COS-1 cells. A) schematic representation of the D3 construct. B) transfected COS-1 cells were biosynthetically labeled and proteins were immunoprecipitated from cell lysates and – where indicated – from cell culture media. Immunoprecipitates were treated with endo H or F, or not treated, analyzed by SDS-PAGE and visualized by autoradiography. C) confocal analysis of GFP-tagged D3 in transfected COS-1 cells. ER, endoplasmic reticulum; n, nucleus; G, Golgi apparatus. Scale bars, 20 µm.

Assessment of the quaternary structure of domain III – performed at 20°C in order to analyze mannose-rich and complex glycosylated proteins – revealed monomeric forms of the mannose-rich protein as well as the complex glycosylated form (Fig. 3.2.10). It should be noted that the overall labeling intensity of the complex glycosylated protein in all the lanes as compared to the mannose-rich polypeptide did not comprise more than 10% of total domain III in the cell lysates.

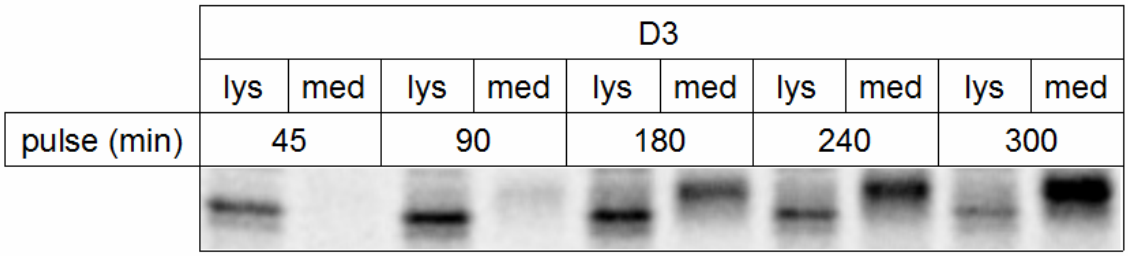
Continuous metabolic labeling was performed to determine the transport rate of domain III and domain III appeared in the medium after 90 min of labeling. Finally, the folding of domain III was probed utilizing trypsin sensitivity and measurement of

its enzymatic activity. Fig. 3.2.10 shows that domain III is predominantly resistant to trypsin. Its phlorizin hydrolase activity is, however, reduced by about 50%.

A



B



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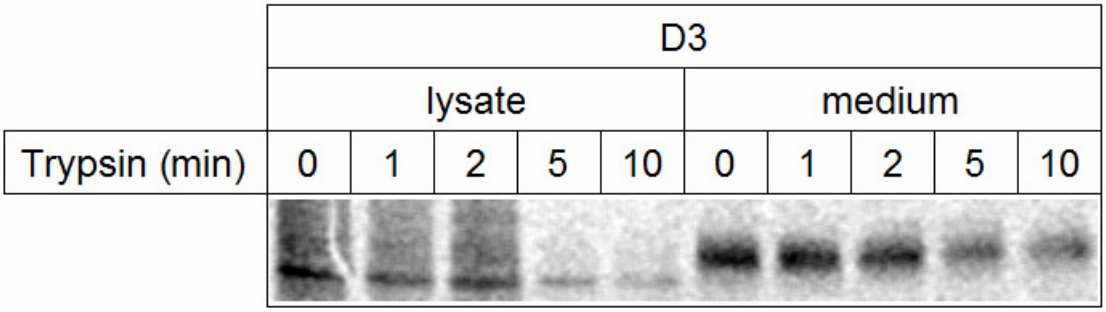


D

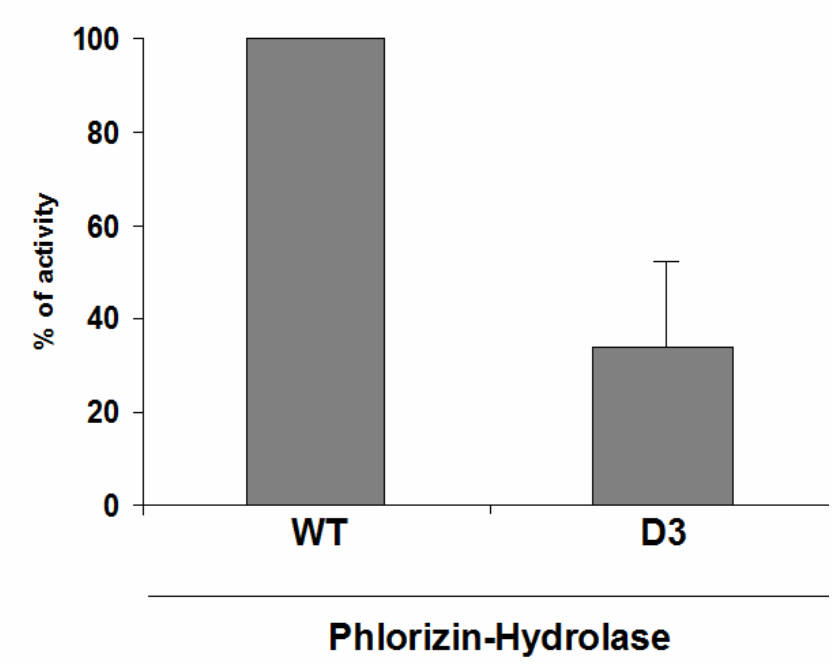


Fig. 3.2.10: Structural and functional features of domain III (D3). A) assessment of the quaternary structure of D3. Transiently transfected COS-1 cells were biosynthetically labeled at 20°C to avoid secretion and solubilized in 6 mM dodecyl-β-m-maltoside. Cell lysates were layered on a sucrose density gradient. After centrifugation for 18h at 100,000 x *g*, fractions were collected, immunoprecipitated and analyzed on SDS-PAGE. B) transport kinetics of D3. Biosynthetically labeled proteins were immunoprecipitated from cell lysates and cell culture media after the indicated labeling times and analyzed by SDS-PAGE. C) trypsin sensitivity assay with D3. Transiently transfected COS-1 cells were biosynthetically labeled followed by immunoprecipitation of LPH proteins from cell lysates and cell culture media. The immunoprecipitates were treated with trypsin for different times and analyzed by SDS-PAGE. lys, lysate; med, medium. D) enzymatic activity of domain III (D3). COS-1 cells were transiently transfected with wild type and D3 cDNA, respectively. 48h posttransfection cell lysates and cell culture media were immunoprecipitated with anti-LPH mAbs. The immunopreciptitates were incubated with phlorizin and the phlorizin-hydrolase activity was measured by determining the concentration of the released glucose by HPLC. The enzyme activity of the mutant was compared with wild type LPH. The error bar represents standard error. n=4.

3.2.6 Impact of N- and O-glycosylation on the transport of domain III in polarized MDCK cells

To investigate whether the extensive N- and O-glycosylation of D3 may modulate intracellular transport and delivery of D3 to the cell surface, glycosylation inhibitors were utilized in combination with immunoprecipitation of cell lysate and cell culture media proteins (Fig. 3.2.11). Here, it could be observed that inhibition of N-glycosylation has not more effect than inhibition of O-glycosylation, and that the ratio of intracellular and secreted domain III is similar for treated and untreated cells. These results are in line with the data obtained for wild type LPH [Naim and Lentze, 1992].

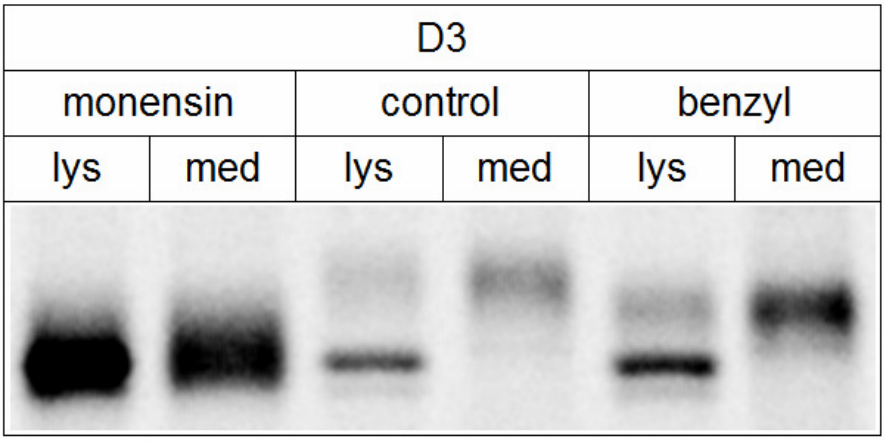


Fig. 3.2.11: Impact of glycosylation inhibitors on D3 transport. MDCK-II cells stably expressing D3 were biosynthetically labeled with [^{35}S]methionine in the presence or absence of modulators of N-glycosylation (monensin) as well as O-glycosylation (benzyl-GalNAc). D3 was immunopreciptated from cell lysates and cell culture media. The immunoprecipitates were finally analyzed by SDS-PAGE on 9% gels and subjected to a phosphorimaging device.

3.2.7 Sorting of domain III in polarized MDCK cells

LPH is sorted into the apical membrane in polarized MDCK cells and intestinal cells with high fidelity. Since it is proposed that putative apical sorting signals are located in the ectodomain of the LPH mature form and domain III builds one half of LPHβ, its sorting was analyzed in a polarized cell line to determine whether or not this region contains putative signals for apical sorting of LPH. Domain III was stably expressed in MDCK cells and its sorting was analyzed in a membrane filter system as described previously [Jacob et al., 1994]. Fig. 3.2.12 demonstrates that domain III is secreted predominantly at the apical surface of MDCK cells. In fact, more than 80% of this protein was found at the apical side, indicating that the sorting of domain III is not as efficient as for wild type LPH. These data suggest that domain IV is most likely devoid of putative apical sorting signals.

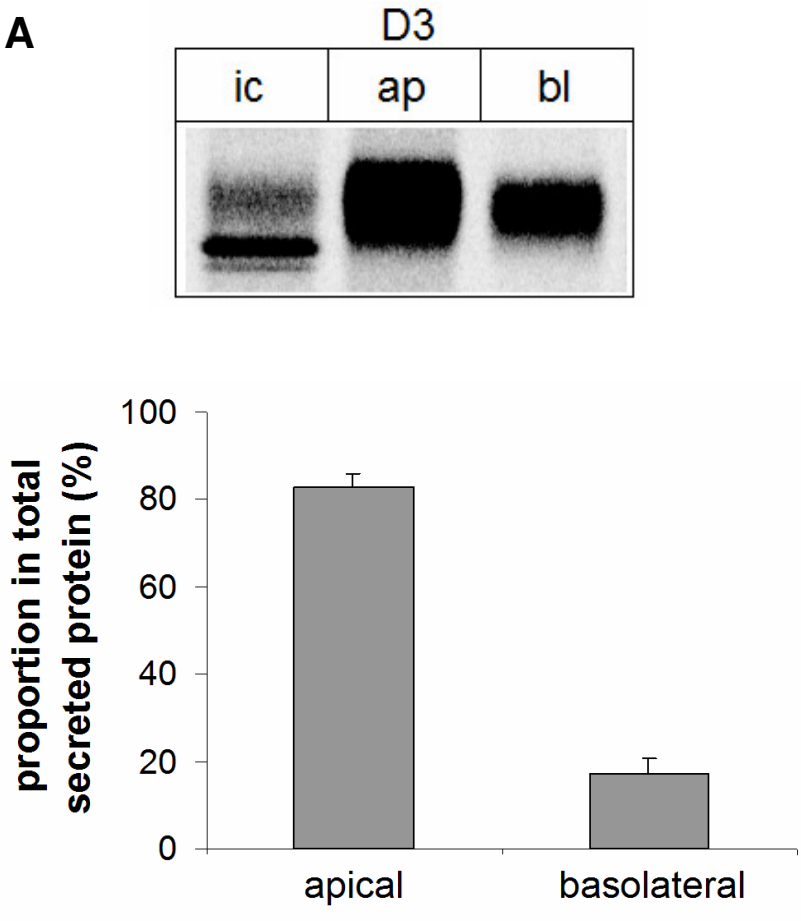


Fig. 3.2.12: Polarized sorting of domain III (D3). A) MDCK-II cells stably expressing D3 were cultured on transwell filters and biosynthetically labeled with [^{35}S]methionine. Proteins that have been secreted into the apical and the basolateral medium, respectively, were isolated by adding anti-LPH mAb and PAS to the collected media. Intracellular proteins were isolated by immunoprecipitation after cell lysis. The immunoprecipitates were analyzed by SDS-PAGE on 9% slab gels followed by phosphorimaging. B) The quantification of secreted D3 was performed with Quantity One® software. The error bars represent standard errors. ic, intracellular; ap, apical; bl, basolateral. n=5.

3.3 Structural hierarchy of regulatory elements in the folding and transport of an intestinal multi-domain protein: Domain I initiates folding of the whole protein while domain II regulates enzymatic activities

Having analyzed so far the roles of homologous domains comprised by mature LPH_{final} (III and IV), still open questions remain concerning the intramolecular organisation and function of the firstly synthesized part of LPH, its profragment encompassing domains I and II, or LPHα and the polypeptide stretch Leu^{735}/Arg^{868} (=$LPH_{stretch}$), respectively (cp. Fig. 3.1.1). The presence of $LPH_{stretch}$ within $LPH\beta_{initial}$ seems to be important during the transport from the ER to the apical plasma membrane, at least because it is not removed intracellularly (like the intramolecular chaperone LPHα) but at last by luminal trypsin, constituting the final step in LPH biosynthesis. Moreover, the fact that the theoretical boundary between homologous domains I and II revealed by intramolecular sequence alignment and *in silico* analysis does not correspond to the cleavage site between LPHα and $LPH\beta_{initial}$ (Arg^{734}/Leu^{735}) [Mantei et al., 1988] suggests that the folding of the LPH profragment is not a simple linear process. Therefore, the impact of these homologous domains on the generation of a transport-competent configuration of LPH was also addressed in conjunction with the question of whether either domain can fold independently. For this, several cDNA constructs each lacking the coding region of one homologous domain were generated (Fig. 3.3.1). *In silico* analysis again provided the potential domain boundaries as a basis for site-directed loop-out mutagenesis.[6]

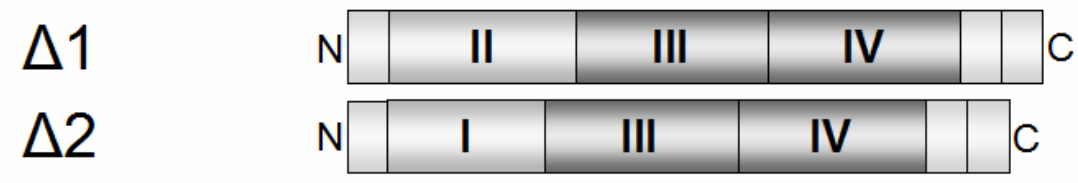


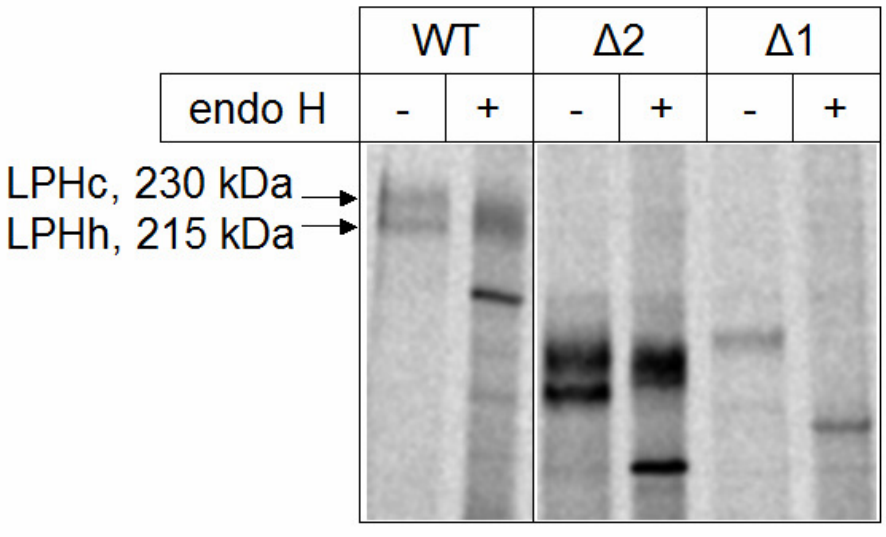
Fig. 3.3.1: Schematic presentation of the LPH deletion mutants Δ1 and Δ2.

[6] This was done by Julio Polaina, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, Apdo. de Correos 73, Burjassot, Valencia, E46100, Spain.

3.3.1 Expression of wild type pro-LPH and domain deletion mutants in COS-1 cells

To examine the contribution of each of the two homologous domains to the structural, functional and trafficking features of LPH, the LPH deletion mutants were expressed in COS-1 cells and their characteristics compared to those of wild type LPH (Fig. 3.3.2). For a better comparability, the wild type data already shown in chapter 3.2 were displayed again. Cell lysates were immunoprecipitated and the precipitated proteins were treated with endo H to determine their glycosylated state as a measure of trafficking capacity (Fig. 3.3.2, A and B). LPHΔ2 acquired endo H-resistance concomitant with complex glycosylation in and trafficking of these mutants to Golgi apparatus. By contrast, the mutant lacking homologous domain I, LPHΔ1, was not transport-competent. Assessment of the proportions of the mannose-rich and complex glycosylated forms after scanning of the gels revealed a similar proportion of the complex glycosylated LPHΔ2 as compared to the wild type counterpart (Fig. 3.3.2, B). This was surprising, since it indicated that the deletion of the complete homologous domain II in LPHΔ2 leads to a similar processing of this deletion mutant than the wild type protein.

A



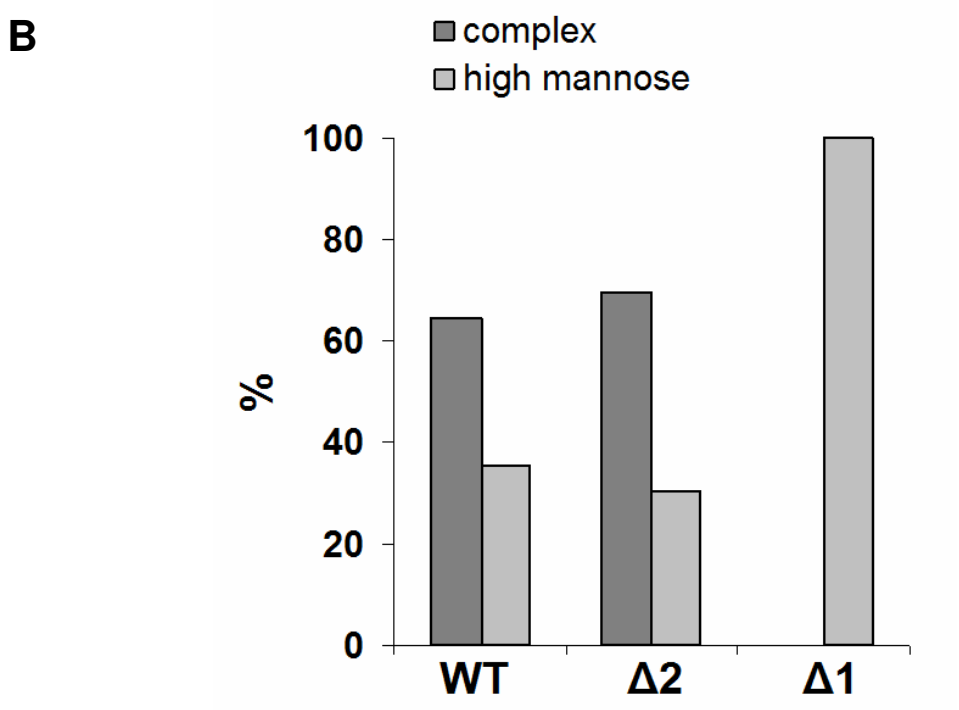
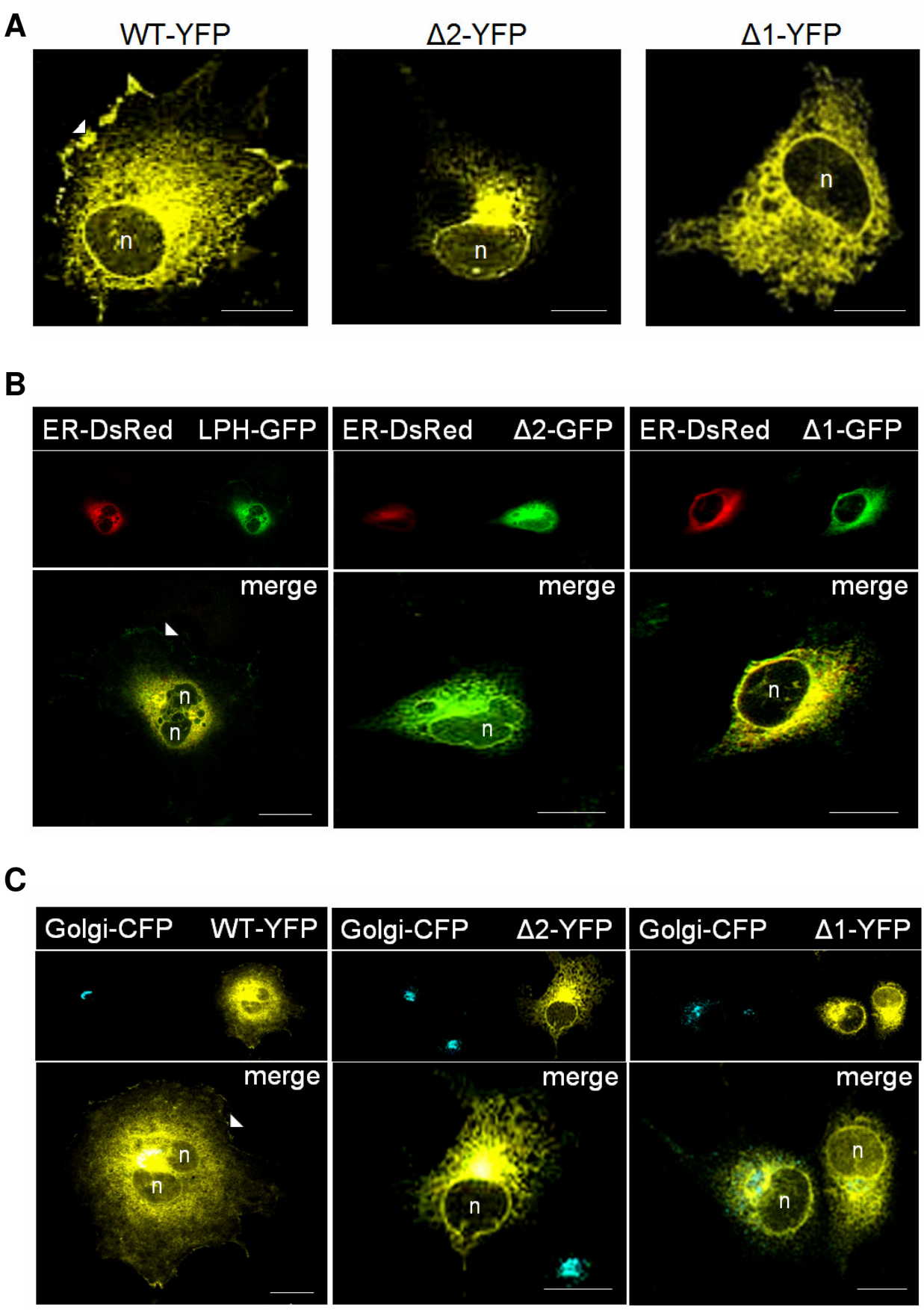


Fig. 3.3.2: Glycosylation pattern of LPH wild type and domain deletion mutants in COS-1 cells. A) Transiently transfected COS-1 cells were biosynthetically labeled for 8h with [^{35}S]methionine followed by immunoprecipitation. The immunoprecipitates were divided into two aliquots and treated with endo H, or not treated. The proteins were subjected to SDS-PAGE followed by autoradiography. B*)* densitometric scanning of the endo H treated biosynthetic forms of wild type and mutant LPH displayed in (A).

The subcellular distribution of the mutant proteins was further investigated in more detail by confocal laser microscopy. As shown in Figure 3.3.3, LPHΔ1 was retained intracellularly and colocalized with the ER-DsRed marker (Fig. 3.3.3, B) and not with the Golgi marker (Fig. 3.3.3, C). However, LPHΔ2 did colocalize with the ER marker, but only little with the Golgi marker and was not detected at the plasma membrane (Fig. 3.3.3, B-C), which is in contrast to the biochemical data (cp. Fig. 3.3.2). Since it is known that the transport kinetics of LPH-GFP/-YFP is slower than that of untagged LPH [Jacob and Naim, 2001], immunofluorescence was performed with COS-1 cells expressing untagged LPHΔ2. Here, the protein could be detected intracellularly and at the cell surface (Fig. 3.3.3, D), suggesting that the presence of a large reporter protein within LPHΔ2-GFP/-YFP reduces the kinetics beyond a certain threshold leading to degradation instead of ER exit.


A
WT-YFP
Δ2-YFP
Δ1-YFP
n
B
ER-DsRed LPH-GFP
ER-DsRed Δ2-GFP
ER-DsRed Δ1-GFP
merge
C
Golgi-CFP WT-YFP
Golgi-CFP Δ2-YFP
Golgi-CFP Δ1-YFP

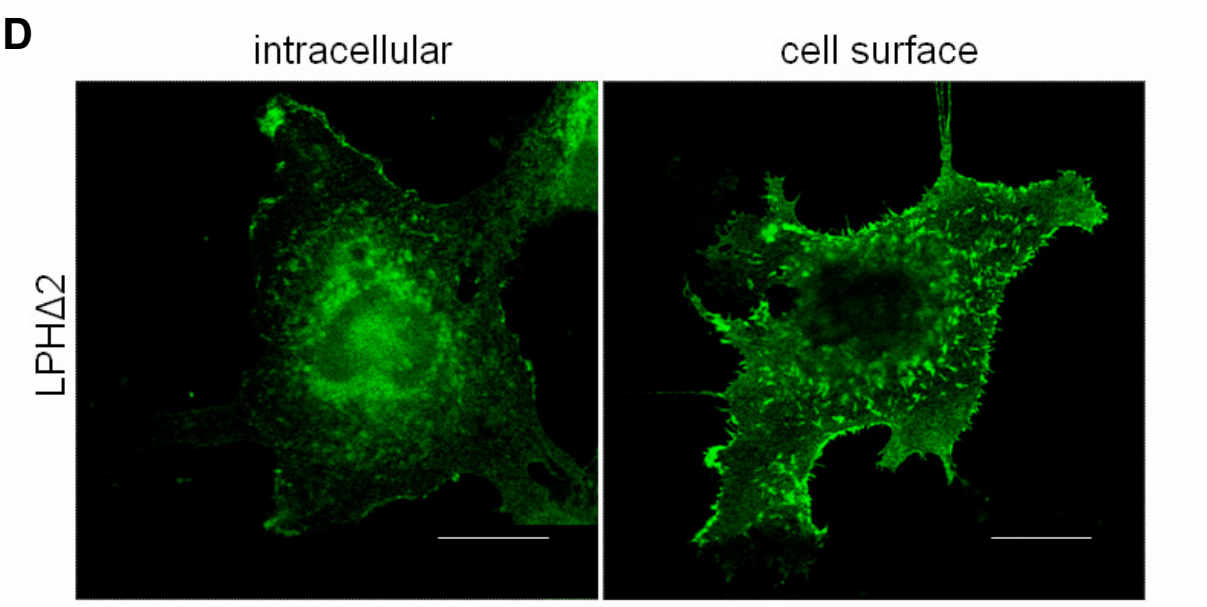


Fig. 3.3.3: Subcellular distribution of LPH wild type and domain deletion mutants in COS-1 cells. A) confocal analysis of transfected COS-1 cells grown on cover slips. B-C) colocalization of LPH mutants with ER and Golgi markers, respectively, in transfected COS-1 cells. COS-1 cells were co-transfected with GFP-tagged LPH proteins and ER-DsRed or YFP-tagged LPH proteins and galactosyl transferase (GT)-CFP, respectively. D) immunofluorescence was performed with COS-1 cells transfected with LPHΔ2 cDNA. Confocal analysis with living cells and immunofluorescence were performed 48h after transfection. n, nucleus; arrowheads, cell surface; bars, 20 µm.

3.3.2 Requirements for the LPH deletion mutants to exit the ER

Dimerisation of LPH in the ER is absolutely required for LPH to egress this organelle to the Golgi apparatus [Naim and Naim, 1996]. The differential intracellular distribution and maturation patterns of the deletion mutants as well as the variable proportions of the glycoforms have altogether lead to examine the quaternary structures of the mutants and assess their relevance to their transport out of the ER. Fig. 3.3.4 depicts the results obtained using sucrose density gradients. As has been shown previously (cp. 3.2.2), the dimerisation of the mannose-rich form of LPH precedes its complex glycosylation and maturation in the Golgi [Naim and Naim, 1996]. Surprisingly, the transport-competent LPHΔ2 deletion mutant did not require dimerisation of its mannose-rich form in the ER prior to ER egress. As shown in Fig. 3.3.4, A (the second top panel) the mannose-rich form of LPHΔ2 persisted as a monomeric protein and the complex glycosylated LPHΔ2 initially appeared in the monomeric fractions. The majority of the complex glycosylated molecules were mainly found in the denser gradient fractions. Interestingly, complex glycosylated LPHΔ2 was revealed in two peaks in the gradient compatible with a monomeric and a

dimeric state. By contrast, LPHΔ1 was exclusively detected in the lighter fractions of the gradients in its mannose-rich glycoform compatible with retention in the ER as a monomeric protein.

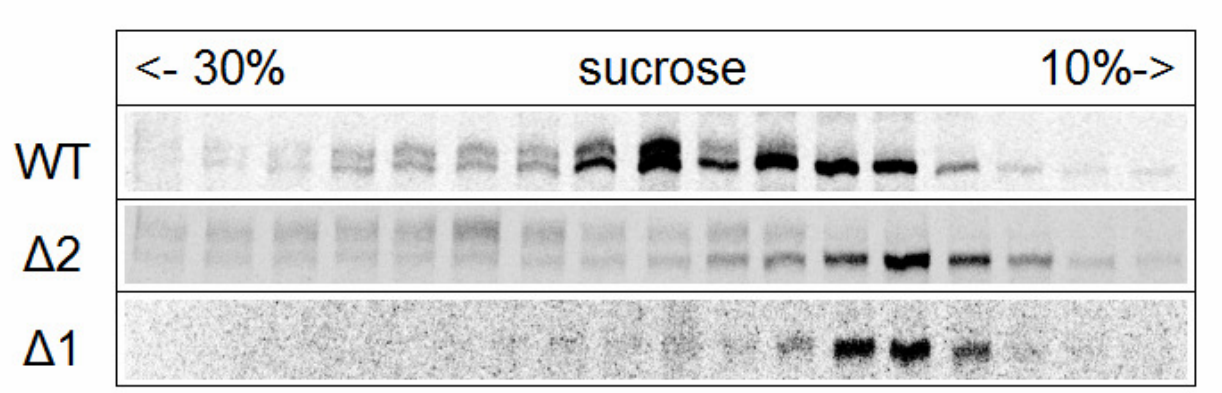


Fig. 3.3.4: Assessment of the quaternary structure. Transiently transfected COS-1 cells were biosynthetically labeled and solubilized in 6 mM dodecyl-β-m-maltoside. Cell lysates were layered on a sucrose density gradient. After centrifugation for 18h at 100,000 x *g*, fractions were collected, immunoprecipitated and analyzed on SDS-PAGE.

3.3.3 Transport kinetics of LPH and deletion mutants

Next, the transport kinetics of the mutants in comparison to wild type LPH in pulse-chase experiments was analyzed. Complex glycosylated LPHΔ2 appeared within 1.5 h of chase (Fig. 3.3.5, cp. also Fig. 3.3.2) indicating that it is as efficiently transported to the Golgi apparatus as wild type LPH. By contrast, LPHΔ1 persisted as a mannose-rich polypeptide compatible with ER localization. Further, the labeling intensity of this mannose-rich form of LPHΔ1 decreased continuously within prolonged chase time points suggesting that this mutant undergoes degradation in the ER, presumably by ER-associated degradation (ERAD).

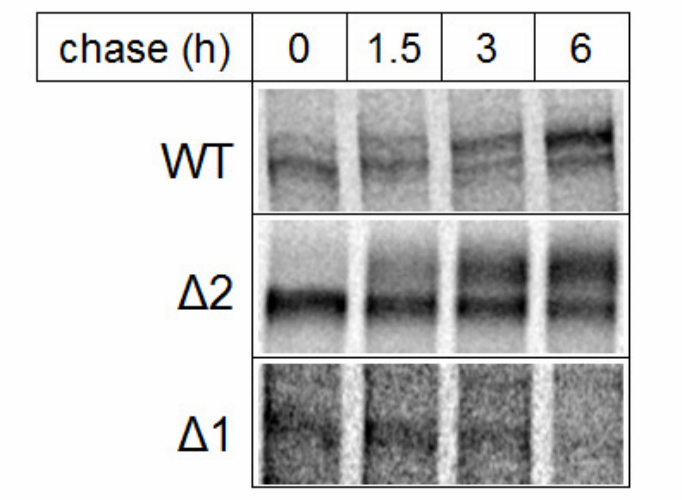


Fig. 3.3.5: Transport kinetics of wild type LPH and mutant proteins. Transfected COS-1 cells were pulse labeled for 1.5h with [^{35}S]methionine and chased for the indicated periods of time with cold methionine. The immunoprecipitates were analyzed by SDS-PAGE on 6% slab gels.

3.3.4 Folding of the deletion mutants

The variations in the quaternary structure of the deletion mutants as well as in their transport kinetics raised the question of causal folding variations. Therefore, the folding of these mutants was examined by using the same procedures described above (cp. 3.2.4).

Trypsin treatment

The tryptic digestion patterns of the wild type and mutant proteins are depicted in Fig. 3.3.6. Wild type LPH was digested to two main bands corresponding to cleaved mannose-rich and complex glycosylated LPH (cp. 3.2.4 and 3.1.5). However, LPHΔ2 pattern was also cleaved to two protein products that correspond to a mannose-rich and a complex glycosylated form although both cleavage sites are not present in LPHΔ2 because they are located within homologous domain II. In a fashion similar to wild type LPH the cleaved products of LPHΔ2 were also resistant to trypsin. Importantly, the cleavage of LPHΔ2 to the final products was preceded by major intermediate cleaved forms suggesting that several trypsin sites are exposed in the deletion mutant, indicating misfolded protein subdomains. Therefore, the cleavage products are supposed to contain domain III, which is resistant to trypsin (cp. 3.2.5) and which fit well to the smaller apparent molecular weights.

By contrast to wild type LPH and LPHΔ2, LPHΔ1 was completely degraded by trypsin already after 1 min of treatment concomitant with the exposure of several trypsin cleavage sites and thus altered folding in comparison to wild type LPH and LPHΔ2.

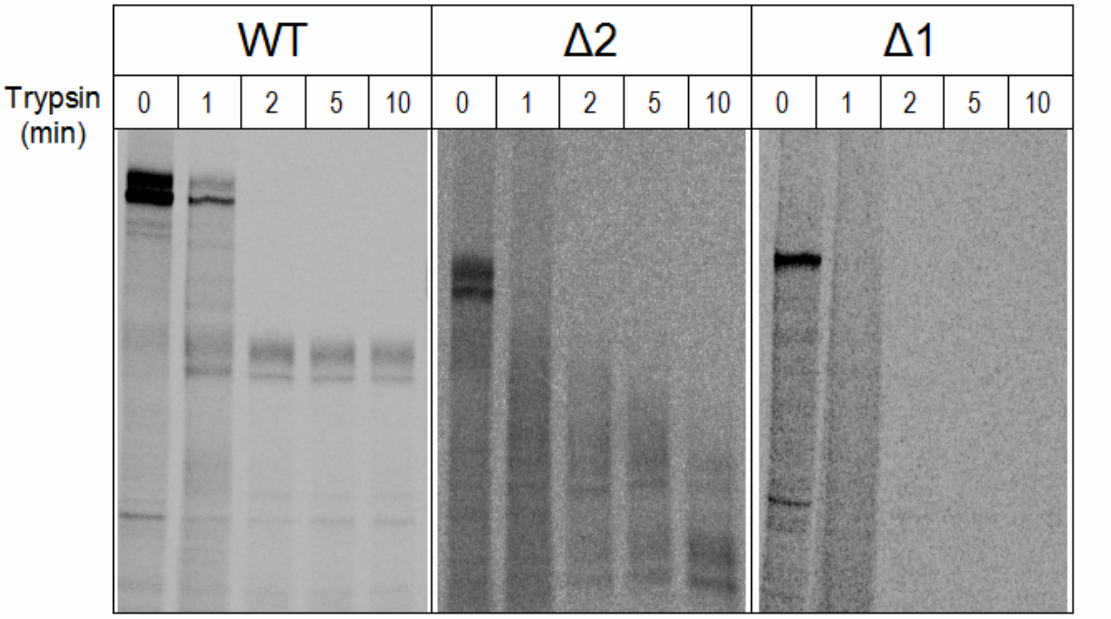


Fig. 3.3.6: Trypsin sensitivity assay of wild type and LPH mutants. Transiently transfected COS-1 cells were biosynthetically labeled followed by immunoprecipitation of LPH proteins from the cell lysates. The immunoprecipitates were treated with trypsin for different times and analyzed by SDS-PAGE on 7% slab gels.

Enzymatic activities of LPH deletion mutants

In order to assess its biological function, the enzymatic activities of lactase and phlorizin-hydrolase in these mutants were analyzed in comparison to their wild type counterparts (Fig. 3.3.7). LPHΔ2 revealed highly reduced activities of phlorizin hydrolase. The lactase activity was absent. Both enzymatic activities in LPHΔ1 were not detectable. The data provides another support for malfolded LPHΔ1 and not completely correct folding of LPHΔ2.

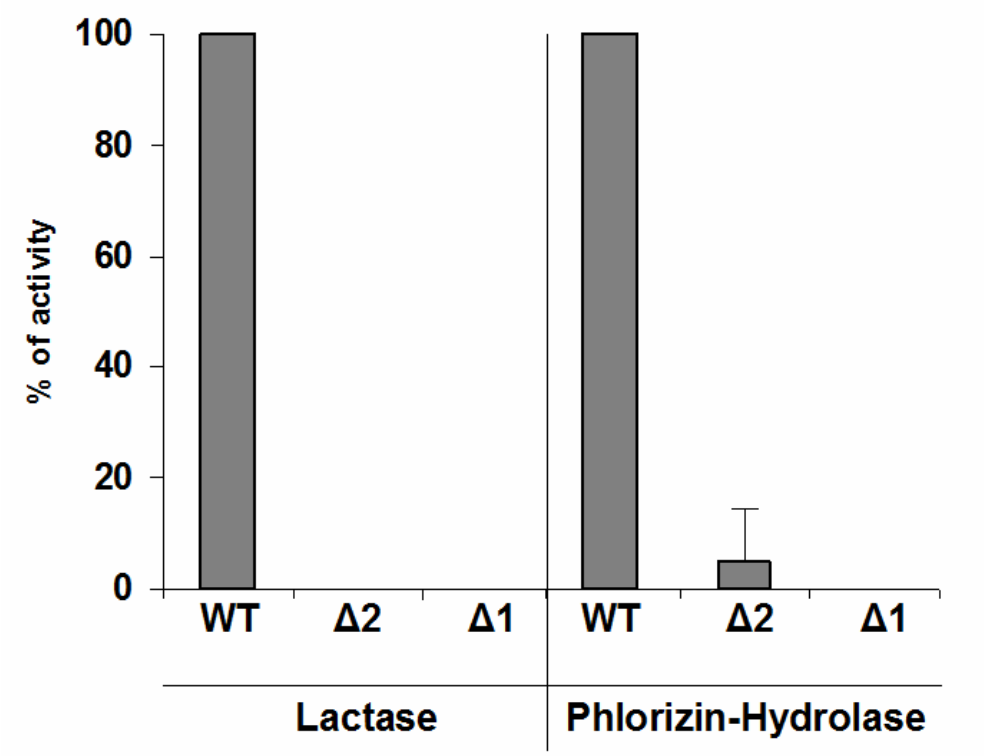


Fig. 3.3.7: Enzymatic activity of deletion mutants (Δ-mutants) of LPH. COS-1 cells were transiently transfected. 48h posttransfection labeled cells were lysed and proteins immunoprecipitated. Immunoprecipitates were incubated with lactose and phlorizin, respectively, and the lactase and phlorizin hydrolase activities were measured by determining the concentration of released glucose by HPLC. The enzyme activities of the mutants were compared with those of wild type LPH.

Epitope mapping of domain deletion mutants

The deletion mutants were immunoprecipitated with a panel of mAbs, which are specific in recognizing native or unfolded conformations of LPH [Naim and Naim, 1996]. The control samples utilized immunoprecipitation of the GFP-tagged mutants with anti-GFP. Fig. 3.3.8 shows that LPHΔ2 and LPHΔ1 were isolated with anti-GFP antibody. Interestingly, all of the mAbs against LPH recognized LPHΔ1, even the two mAbs, MLac6 and MLac10, that recognize unfolded and denatured forms of LPH. LPHΔ2, on the other hand, reacted well with all the antibodies utilized with the exception of MLac6 and MLac10.

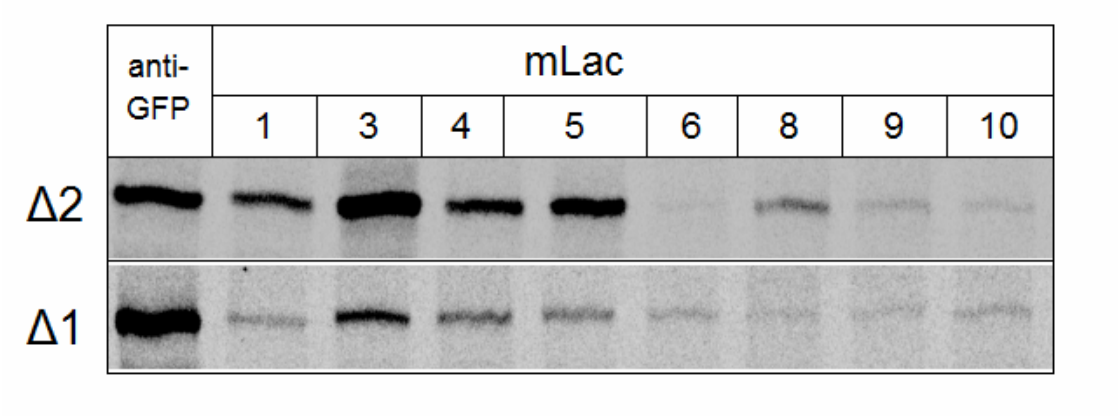


Fig. 3.3.8: Epitope mapping of LPH mutants. COS-1 cells were transfected with DNA coding for GFP-tagged deletion mutants and biosynthetically labeled 48h after transfection. Cell lysates were divided into equal aliquots and immunoprecipitated with anti-GFP and different anti-LPH mAb. The immunoprecipitated proteins were analyzed by SDS-PAGE.

3.3.5 Influence of $LPH_{stretch}$ on the transport competence of domain III and LPHβ

The results obtained so far strongly suggest that domain II is not a central autonomous component of LPH concerning the attainment of a fully transport-competent conformation, but crucially influences both enzymatic activities, lactase and phlorizin-hydrolase, respectively. The most interesting as well as uncharacterized part of homologous domain II constitutes $LPH_{stretch}$. The next step therefore was to express transport competent and enzymatically active proteins domain III and LPHβ with and without $LPH_{stretch}$ and examine the trafficking and functional properties of all constructs. As shown in Fig. 3.3.9, B, $LPH\beta_{initial}$ and $LPH\beta_{final}$ expression in COS-1 cells revealed that the presence of $LPH_{stretch}$ hampers the transport competence of LPHβ drastically, because no complex glycosylated protein form can be detected for $LPH\beta_{initial}$ in contrast to $LPH\beta_{final}$, which displays complex glycosylation (see also [Jacob et al., 2002a]). A similar effect of $LPH_{stretch}$ could be seen when it is fused to domain III alone. Here, the presence of $LPH_{stretch}$ also results in reduced protein transport. However, a small portion of $D3_{stretch}$ is secreted into the cell culture medium as N- and O-glycosylated protein forms, whereby the N-glycan/O-glycan-ratio seems to be shifted to the O-glycans indicating an effect of $LPH_{stretch}$ on glycosylation events.

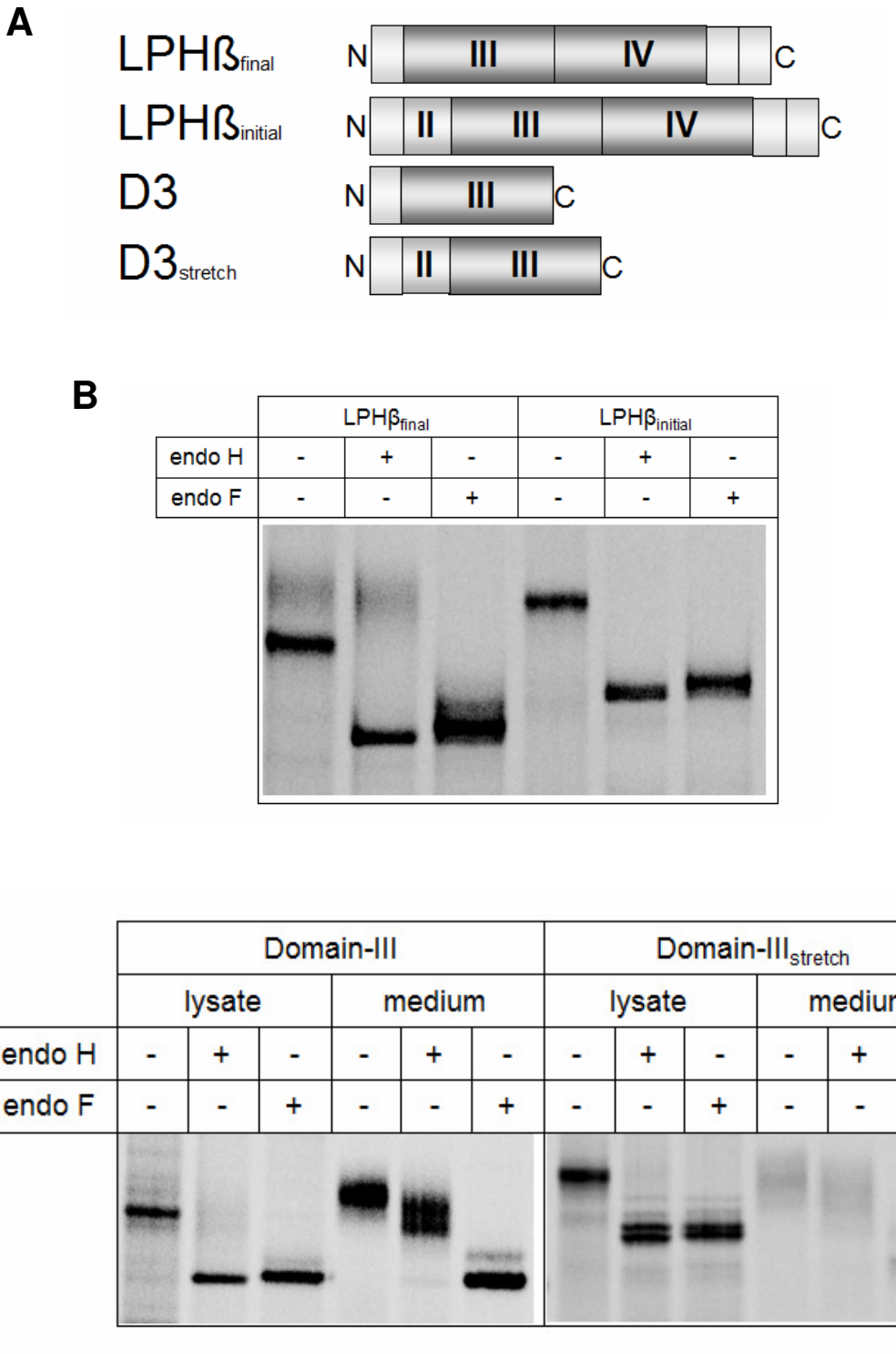


Fig. 3.3.9: Expression of LPHβ$_{final}$, LPHβ$_{initial}$, Domain III and Domain III$_{stretch}$ in COS-1 cells. A) schematic representation of the LPHβ$_{final}$, LPHβ$_{initial}$, Domain III and Domain III$_{stretch}$ constructs. B-C) COS-1 cells were biosynthetically labeled 48h posttransfection. Proteins were immunoprecipitated from cell lysates and - where indicated - from cell culture media, treated with endo H or F, or not treated, analyzed by SDS-PAGE and visualized by autoradiography.

3.3.6 Association of domain III with other extracellular LPH domains reduces its transport competence

In order to investigate the role of $LPH_{stretch}$ during LPH biosynthesis in more detail constructs were generated, which contain domain III – the autonomously folded core domain (cp. 3.2.5) – as well as $LPH_{stretch}$ and other LPH domains.

Interestingly, as shown by Fig. 3.3.10, B, the construct comprising LPHα, $LPH_{stretch}$ and domain III (denoted D123) is efficiently transported and secreted into the cell culture medium as endo H-resistant and endo F-sensitive protein similar as domain III (cp. 3.2.5 and 3.3.5). However, the protein composed of the complete LPH ectodomain (see also [Naim and Naim, 1996]) as well as the protein consisting of homologous domains II and III do not attain transport competent conformations and are retained in the ER.

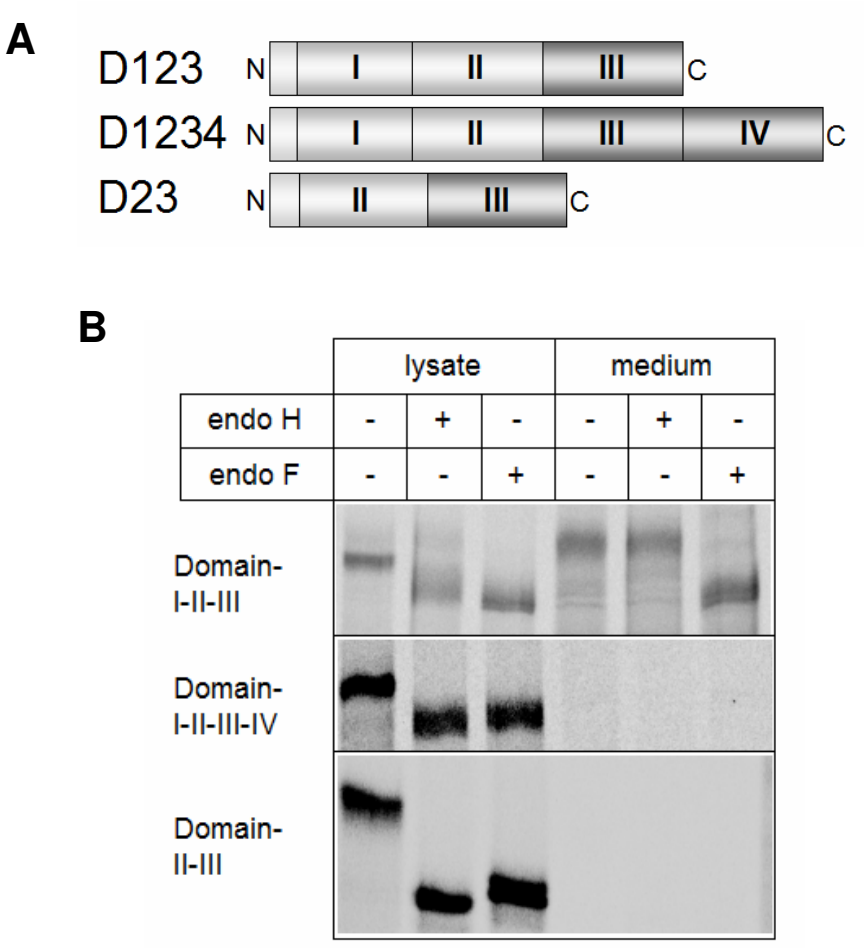


Fig. 3.3.10: Expression of homologous Domain III-containing LPH regions in COS-1 cells. A) schematic representation of the, Domain I-II-III, Domain I-II-III-IV, Domain II-III constructs. B) COS-1 cells were biosynthetically labeled 48h posttransfection. Proteins were immunoprecipitated from cell lysates and - where indicated - from cell culture media, treated with endo H or F, or not treated, analyzed by SDS-PAGE and visualized by autoradiography.

3.3.7 Expression of profragment subdomains reveals non-transport-competent proteins

The analysis of the role of $LPH_{stretch}$ on LPH biosynthesis and function so far revealed that $LPH_{stretch}$ containing proteins are only transport competent when i) domain III is present, ii) domain IV is absent, and iii) LPHα is completely present or absent and not in part, presumably because LPHα can positively act as intramolecular chaperone only as a whole.

In order to further elucidate the interactions and the structural hierarchy of LPHα and $LPH_{stretch}$, or homologous domains I and II, respectively, constructs containing parts of the LPH profragment were generated and expressed in COS cells (Fig. 3.3.11). pAb V496 binds the first part of homologous domain I and could therefore be used for immunoprecipitation of domain I, LPHα and the profragment (denoted D12). Domain II was fused to a FLAG-tag und isolated with mAb anti-FLAG. The results revealed that none of them attains transport competence and that all are retained in the ER.

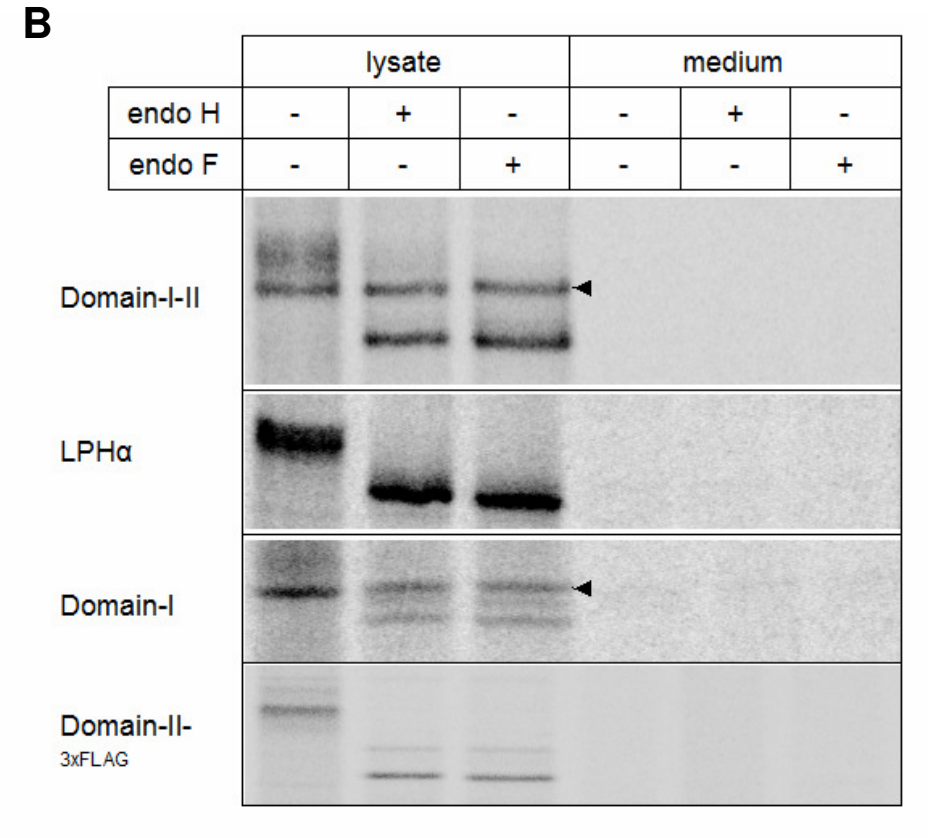


Fig. 3.3.11: Expression of LPH profragment domains in COS-1 cells. A) schematic representation of the LPHα, Domain I-II, Domain II_{3xFLAG} and Domain I constructs. B) transiently transfected COS-1 cells were biosynthetically labeled 48h posttransfection. Proteins were immunoprecipitated from cell lysates and cell culture media with pAb V496 or anti-FLAG antibodies, respectively, treated with endo H or F, or not treated, analyzed by SDS-PAGE and visualized by autoradiography. Arrowheads indicate unspecific protein bands.

3.4 Elimination of a homologous domain of an intestinal hydrolase results in changed intracellular trafficking via altered membrane association

The results gathered so far shed light on pathobiochemical mechanisms of CLD by characterizing a distinct mutation (cp. 3.1) as well as on the intramolecular organisation of the LPH protein by loop-out mutagenesis and construction of deletion or individual domain forms (cp. 3.2 and 3.3). Further experiments were planned and performed, in order to reveal LPH-related biochemical and physiological mechanisms. Here, LPH – besides other model proteins – is used as an experimental tool to elucidate general biochemical, physiological and cell biological mechanisms.

3.4.1 LPHΔ4's intracellular transport differs from that of wild type LPH and SI

Jacob and Naim could clearly show by confocal laser microscopy that LPH and another intestinal brush border membrane hydrolase, sucrase-isomaltase (SI), are transported from the Golgi apparatus to the cell surface in the same transport vesicles [Jacob and Naim, 2001]. In these vesicles areas could be detected, in which only SI is located in distinct clusters, whereas LPH is equally distributed over the vesicle surface. After both enzymes have passed the *trans*-Golgi network (TGN), they are distributed to separate vesicles [Jacob and Naim, 2001]. Subsequent experiments displayed that these different vesicles – denoted SI- and LPH-carrying apical vesicles (SAVs and LAVs), respectively – are transported with their divergent cargo by different cytoscelettal structures [Jacob et al., 2003]. In order to find out if the deletion of homologous domain IV has an effect on LPH trafficking in more detail, COS-1 cells grown on cover slips were co-transfected with cDNAs coding for fluorescence-tagged LPHΔ4 and wild type LPH or SI, respectively (Fig. 3.4.1). Interestingly, LPHΔ4 was predominantly found in vesicular structures and not in the Golgi apparatus, whereas the subcellular localization of LPH was *vice versa* when coexpressed in COS-1 cells and analysed after 4 h at 20°C followed by 20 min at 37°C (Fig. 3.4.1, A). Furthermore, LPH could be found in the same vesicles as LPHΔ4, but almost no colocalization was detectable within these vesicles (shown in white). When SI and LPHΔ4 were coexpressed, SI was also found in the Golgi and in vesicles like LPH (Fig. 3.4.1, B), but in contrast, LPHΔ4 and SI did colocalize moreoften within these vesicles (shown in yellow). The 20°C block and the 37°C

chase were performed in the presence of cycloheximide to eliminate background fluorescence by inhibition of protein synthesis.

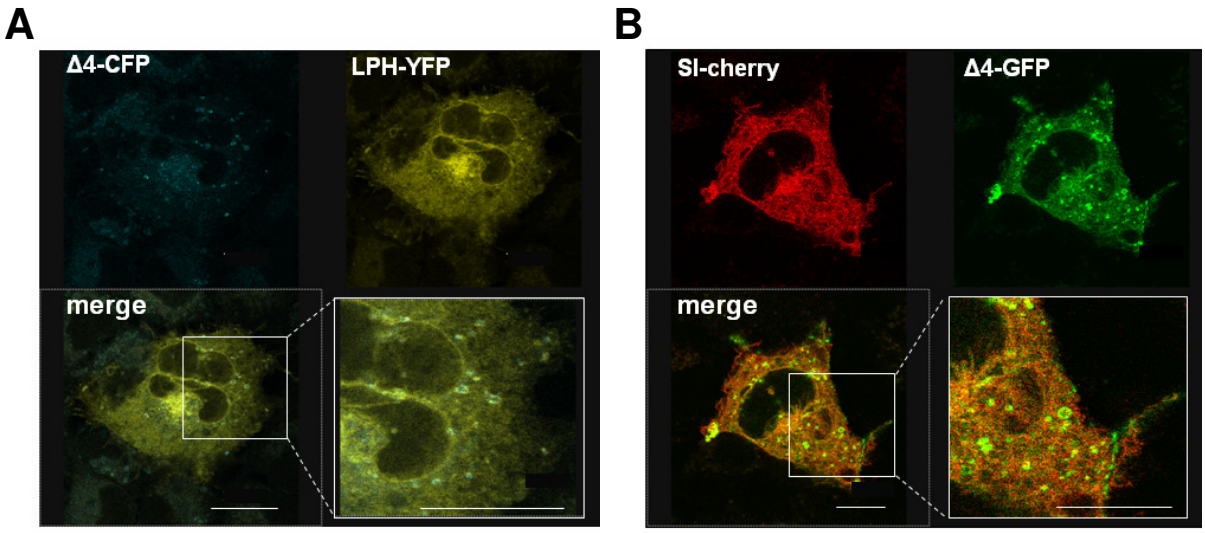


Fig. 3.4.1: Colocalization of LPHΔ4 with wild type LPH and sucrase-isomaltase. LPHΔ4 colocalizes partly with wild type LPH but not with sucrase-isomaltase (SI). COS-1 cells were cotransfected with LPH-YFP and LPHΔ4-CFP (A) or SI-cherry and Δ4-GFP (B). 36h posttransfection, the cells were incubated for 4h at 20°C to accumulate proteins in the Golgi apparatus. This incubation was performed in the presence of 1.6 mM cycloheximide to inhibit protein synthesis. After the block, the cells were incubated at 37°C for 20 min to chase the proteins out of the TGN, fixed and analyzed by confocal microscopy. Bars, 20 µm.

3.4.2 LPHΔ4 associates differently with detergent resistant membranes

Both apically sorted hydrolases, LPH and SI, are associated with the membrane via transmembrane protein domains. However, SI is located in Triton X-100-resistant, sphingolipid/cholesterol-enriched membrane microdomains or lipid rafts, while LPH is not [Jacob and Naim, 2001]. Lipid rafts are supposed to be sorting platforms [Alfalah et al., 2005; Lindner and Naim, 2009; Simons and Ikonen, 1997] and LPHΔ4 displayed different trafficking characteristics than wild type LPH and SI. Therefore, the next experimental step was to investigate, how the lack of homologous domain IV – which is in closest vicinity to the membrane in contrast to the rest of the LPH ectodomain (cp. Fig. 3.1.1) – effects the association of LPHΔ4 with different kinds of detergent resistant membranes in comparison to wild type LPH. In order to have comparable experimental conditions as in the colocalization study described above, cells were transfected with cDNAs coding for fluorescence-tagged SI, LPH and LPHΔ4 proteins.

3.4.2.1 Triton X-100-resistant membranes

The detergent used to isolate 'classical' rafts is Triton X-100 [Brown and Rose, 1992; Simons and Ikonen, 1997], therefore LPHΔ4 membrane association was analyzed by isolating Triton X-100-resistant membranes. SI showed the known pattern of membrane association in pelletation experiments, i.e. the complex glycosylated protein form could be isolated from the pellet, while the mannose-rich glycosylated portion was not [Alfalah et al., 1999]. However, although LPH was mainly found in the supernatant, a clear portion of complex as well as mannose-rich glycosylated protein was also found in the pellet, which is in contradiction to the published data [Jacob and Naim, 2001]. A contamination of the DRM fraction with non-DRM material can be excluded because of the control (SI) and the fact that a low-speed centrifugation step was performed before ultracentrifugation (cp. 2.2.4, *Extraction of detergent resistant lipid microdomains*). In order to further exclude artificial DRM-association, floating experiments should be performed. LPHΔ4 also showed a weak association with Triton X-100-resistant membranes.

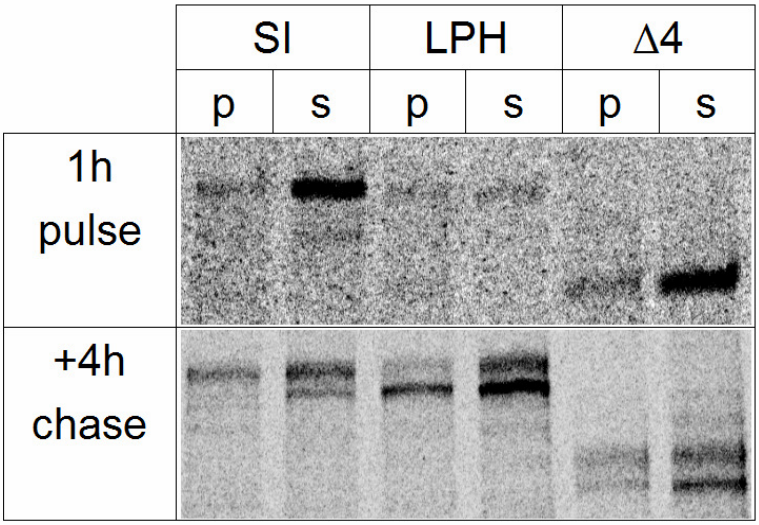


Fig. 3.4.2: Association of LPHΔ4 with Triton X-100-resistant membranes. Metabolic labeling of transfected COS-1 cells was performed for the indicated times, followed by solubilization in ice-cold Triton X-100. Proteins were immunoprecipitated from the detergent-soluble (s) and from the detergent-insoluble pellet (p) fractions.

3.4.2.2 Lubrol WX-resistant membranes

Lipids and proteins as main components of membranes are the basis for the heterogenous structures of cellular organelles [Sprong et al., 2001] and membrane subdomains [Roper et al., 2000], whereby the heterogeneity of the latter does not

only depend on selective lipid and protein enrichment, but also on their locations and functions. Based on this, it is likely that membrane-bound proteins associate with different kinds of membranes on their route to the final destination.

Many proteins, which are completely soluble in Triton X-100, show a certain insolubility in other mild detergents [Drevot et al., 2002; Holm et al., 2003; Roper et al., 2000]. Additionally, the membrane microdomains isolated with these detergents reveal differences in protein and lipid constitution [Schuck et al., 2003]. Castelletti *et al.* could show that the prostate-specific membrane antigen (PSMA) is detectable in two different kinds of detergent-resistant membranes [Castelletti et al., 2008]. Here, its biosynthetic forms associate with membrane microdomains of different constitution and suggest a role of compartment-specific detergent-resistant membranes in protein transport.

In order to investigate, if the results obtained for PSMA are reproducible for LPH and if the difference between LPH and LPHΔ4 in trafficking is due to an altered association with Lubrol-DRMs, pelletation experiments with Lubrol WX were performed (Fig. 3.4.3). Continous metabolic labeling revealed that both wild type LPH protein glycoforms – mannose-rich and complex glycosylated – are predominantly found in the DRMs (Fig. 3.4.3, A). However, complex glycosylated LPHΔ4 is mainly found in the DRMs, whereas the mannose-rich glycoform is mainly soluble. For the analysis of early trafficking protein-membrane interaction dynamics a pulse-chase protocol was followed (Fig. 3.4.3, B).

The data obtained so far suggest for both proteins – wild type and LPHΔ4 – that the mannose-rich protein form (which appears first) is predominantly soluble in Lubrol WX. Subsequently, this glycoform could also be detected in DRMs. Then, the complex glycosylated protein form appears first in the soluble fraction, before its portion in the DRM fraction increases.

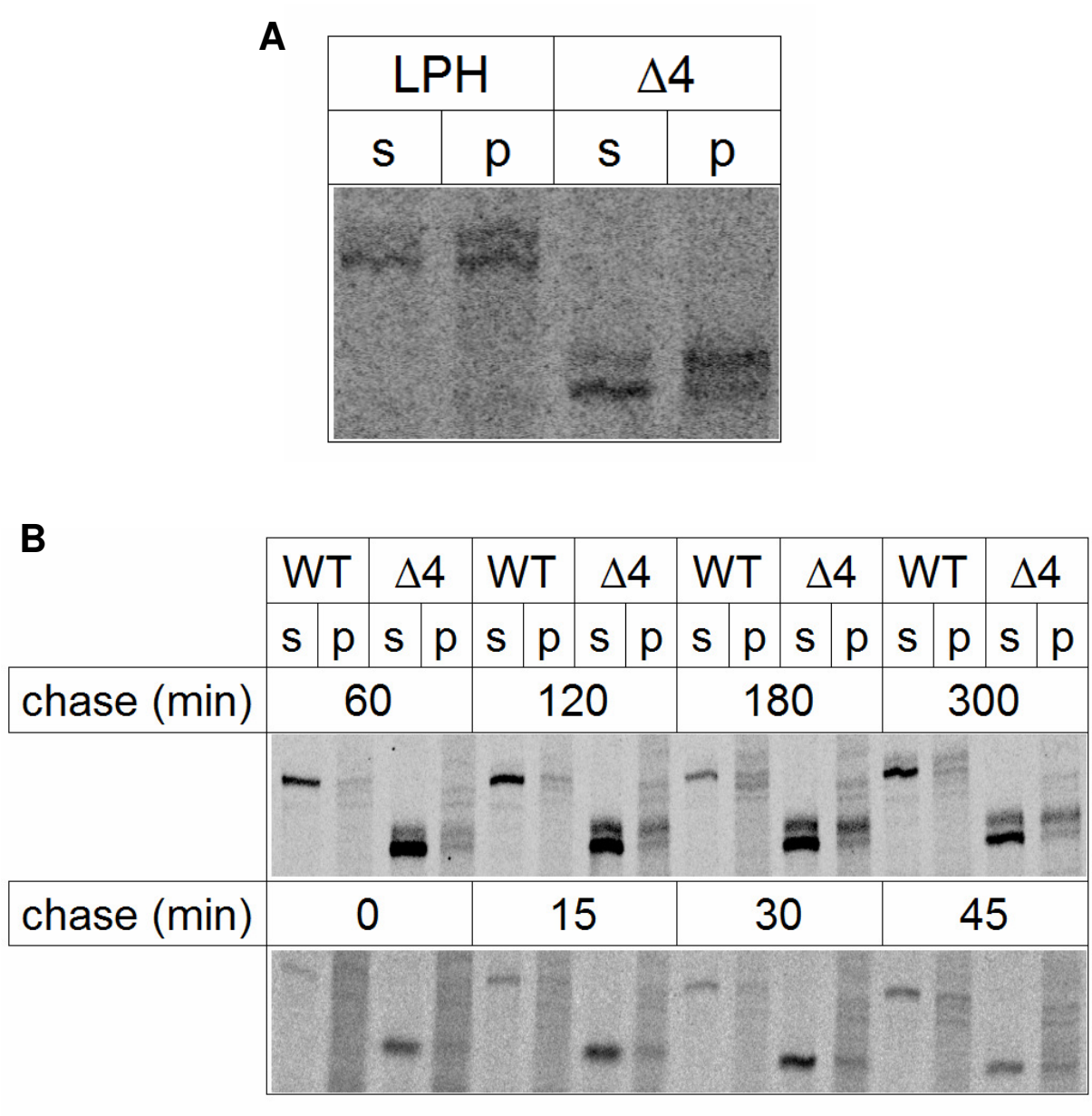


Fig. 3.4.3: Association of LPHΔ4 with Lubrol WX-resistant membranes. Metabolic labeling of transfected COS-1 cells was performed continuously (A) or following a pulse-chase protocol (B) with 1h pulse (upper panel) and 30 min pulse (lower panel), respectively, followed by the indicated chase times and solubilization in ice-cold Lubrol. Proteins were immunoprecipitated from the detergent-soluble (s) and detergent-insoluble pellet (p) fractions.

3.4.2.3 Tween 20-resistant membranes

Another mild non-ionic detergent utilzed in the investigations concerning protein transport, trafficking and sorting constitutes Tween 20. It was found to entirely solubilze basolateral but not apically sorted membrane proteins indicating protein sorting events at the stage of the ER [Alfalah et al., 2005]. Moreover, Tween 20-resistant membranes seem to be involved in early trafficking control mechanisms between the ER and the *cis*-Golgi apparatus [Hein et al., 2009].

Since LPH wild type and LPHΔ4 differences are mainly found in the early secretory pathway – e.g. ER-Golgi transport kinetics (cp. 3.2.3) and quaternary structure (cp. 3.2.2) – the association with Tween 20-resistant membranes was analyzed (Fig. 3.4.4). Biosynthetic labeling following a pulse-chase protocol revealed that mannose-rich protein forms of SI, LPH and LPHΔ4 are predominantely associated with DRMs after 1 h of labeling (Fig. 3.4.4, A). After 5 h of labeling, complex and mannose-rich glycosylated SI were mainly found in the pellet, whereas a large portion of mannose-rich LPH and LPHΔ4 could be detected in the soluble fraction and – in contrast to SI – the complex form was mainly found in the soluble fraction after 5 h chase. Surprisingly, almost no more mannose-rich LPHΔ4 was found to be associated with DRMs.

To further elucidate the mechanisms underlying early protein trafficking and its connection with intracellular membrane diversity and heterogeneity, the association with Tween 20-DRMs was investigated at 15°C, a temperature which blocks protein transport between the ER and *cis*-Golgi [Tomas et al., 2010]. Again, LPHΔ4 was less associated with DRMs than SI and LPH. Additionally, the soluble portion of LPHΔ4 was clearly smaller than its counterpart of the the DRM fraction, indicating a difference in glycosylation. This observed distribution of the investigated proteins could also be found, when the cells were treated with brefeldin A (BFA) during metabolic labeling, a drug known to inhibit retrograde protein transport from the Golgi leading to accumulation of proteins in the ER [Nebenfuhr et al., 2002], confirming the difference in membrane association between wild type LPH and LPHΔ4 in the early trafficking pathway.

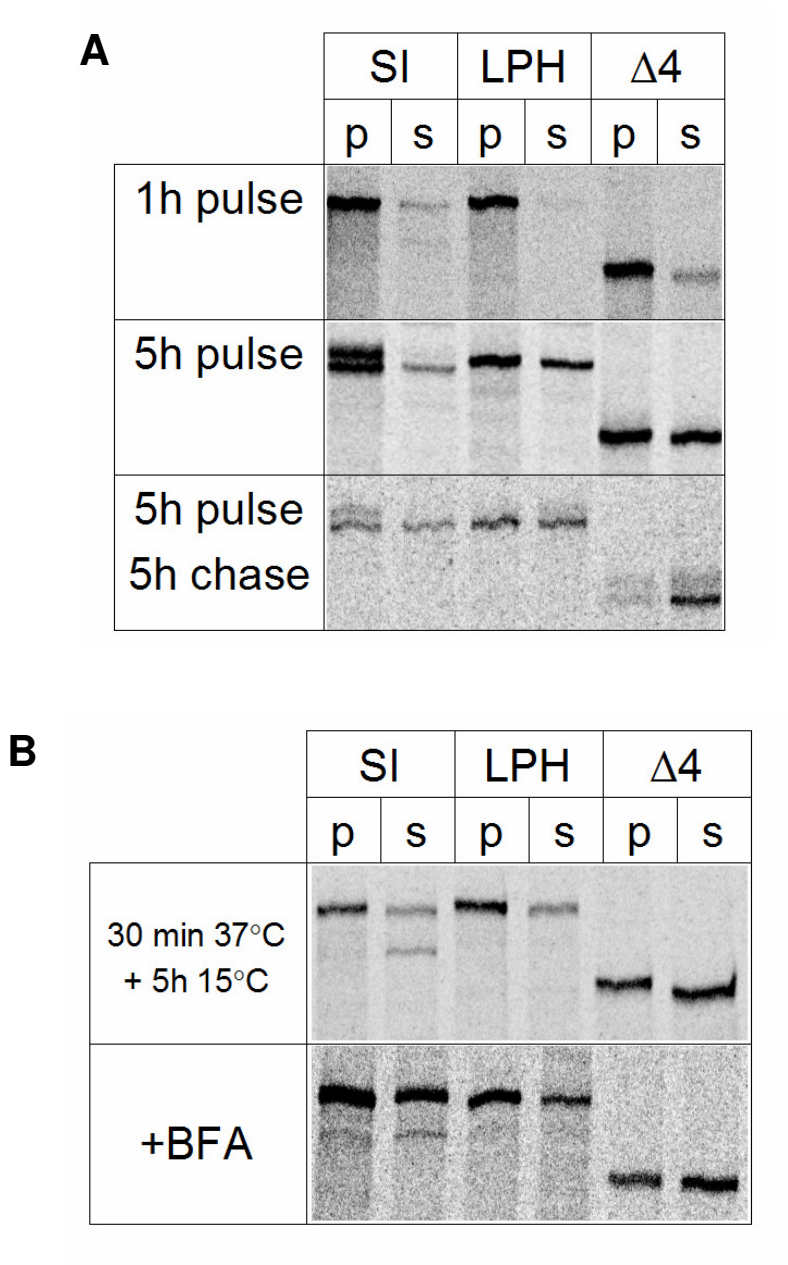


Fig. 3.4.4: Association of LPHΔ4 with Tween 20-resistant membranes. Metabolic labeling of transfected COS-1 cells was performed following a pulse-chase protocol at 37°C (A) or continuously at 37°C and 15°C (upper panel) or at 37°C in the presence of BFA (lower panel), respectively (B) followed by solubilization in ice-cold Tween 20. Proteins were immunoprecipitated from the detergent-soluble (s) and detergent-insoluble pellet (p) fractions.

4. Discussion

4.1 Impaired trafficking and subcellular localization of a mutant lactase associated with congenital lactase deficiency

Carbohydrate malabsorption associated with brush border LPH occurs in two forms, adult-type hypolactasia and congenital lactase deficiency or alactasia. Adult-type hypolactasia is mainly linked to a DNA variant, C/T-13910, about 14 kb upstream from the gene locus of LPH and also to another variant, G/A-22018, 8 kb telomeric to C/T-13910, both of which are associated with low lactase digesting capacity [Enattah et al., 2002]'. Congenital lactase deficiency, on the other hand, is a more severe and life-threatening form of lactose malabsorption in the newborn, where milk is the only food. While the symptoms of adult-type hypolactasia increase with age and many patients develop signs of lactose intolerance in adolescence and adulthood, the typical symptoms of CLD start a few days after birth with the onset of breast (or lactose-containing formula) feeding. They consist of liquid and acid diarrhea, meteorism, and severe malnutrition. Until very recently the genetic background of CLD has been completely unknown. Genetic analysis of several Finnish families has unraveled multiple mutations in the coding region of the lactase gene that are associated with CLD [Kuokkanen et al., 2006; Torniainen et al., 2009]. Strikingly, 84% of the patients were homozygous for a nonsense mutation, c.4170T-->A (Y1390X). This stop codon would generate a translated product of LPH from which the entire homologous domain IV that contains the lactase active site, Glu^{1749}, has been truncated. Obviously this deletion mutant is enzymatically inactive and therefore triggers the onset of lactose malabsorption.

The G1363S mutation, on the other hand, does not affect the length of the translation LPH product and has been therefore investigated further at the protein and cellular levels. As shown here substantial alterations in the posttranslational processing and maturation of LPH are elicited by this mutation. Interestingly the mutation occurs in a region that is highly conserved in different species (cp. Table 3.1.1) and additionally generates a potential N-glycosylation site suggesting that the Gly^{1363} residue plays an essential role in the context of folding of the homologous domain III in particular and the LPH in general. While N-glycosylation *per se* is required for correct folding and dimerisation of LPH, additional N-glycosylation in domain IV in the immediate

proximity of the membrane anchor sterically hinders the generation of stable dimeric LPH forms and reduces substantially the trafficking rates of LPH from the ER to the Golgi apparatus [Jacob et al., 2000]. While the data presented here reveal an increased N-glycosylation of mutant LPH-G1363S, this additional glycosylation site is not immediately implicated in the pathogenesis of the CLD phenotype. In fact, elimination of this site in the LPH-N1361A/G1363S mutant reveals similar biosynthetic, structural and functional features to LPH-G1363S. Remarkably, LPH-G1363S reveals the characteristics of a temperature-sensitive mutant that acquires partial transport-competence and is capable to exit the ER at a permissive temperature of 20°C. The partial transport-competence is paralleled by an increase in the enzymatic activity of lactase, but not by the acquisition of trypsin-resistance and hence wild type-like folding determinants. It appears that at this reduced temperature subdomains of the LPH protein implicated in the enzymatic activity acquire partial folding and hence partial enzymatic activity.

Temperature-sensitive and trafficking-impaired mutants of glycoproteins are associated with the pathogenesis of disease. Examples of these mutant proteins are sucrase-isomaltase phenotype II (SI-Q1098P) in congenital sucrase-isomaltase deficiency [Propsting et al., 2005], the DeltaF508 mutant of the cystic fibrosis transmembrane conductance regulator in cystic fibrosis [van Barneveld et al., 2006], the NPC1(I1061T) mutation in Niemann-Pick type C1 disease [Gelsthorpe et al., 2008] or the bile salt export pump in progressive familial intrahepatic cholestasis type 2 [Plass et al., 2004].

These mutants can exit the ER at the permissive temperature and are able to recycle between the ER and the *cis*-Golgi bypassing thus the ER quality control machinery [Propsting et al., 2005]. Another feature of these mutants is their sensitivity towards chemical chaperones that facilitate correct folding and subsequent acquisition of functional properties. Chemical chaperones are small cell- and ER-permeable molecules, which stabilize the native folding status of proteins within the ER [Ulloa-Aguirre et al., 2004] with the potential of altering the folding of mutant proteins, thus weakening distinct diseases that are caused by such misfolded protein mutants. One chemical chaperone is curcumin (1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), the yellow main substance of the spice curcuma, isolated from *Curcuma longa*. It shows anti-inflammatorial, anti-oxidative and anti-proliferative

features [Ammon and Wahl, 1991; Salvioli et al., 2007]. Another chemical chaperone is cyclosporin A, which constitutes an unpolar, cyclic oligopeptide and metabolite of the fungus *Tolypocladium inflatum*. It has strong immunosuppressive characteristics and influences T-lymphocytes [Borel et al., 1976] by inhibiting nuclear proteins related to T-cell activation [Emmel et al., 1989]. Chemical chaperoning is utilized in diseases like Chorea Huntington [Dikshit et al., 2006] and Cystic Fibrosis [Egan et al., 2004; Harada et al., 2007]. Exposure of the DeltaF508 of the cystic fibrosis transmembrane conductance regulator to curcumin [Egan et al., 2004] or to small chemical chaperones, such as osmolytes glycerol and trimethylamine N-oxide [Fischer et al., 2001; Zhang et al., 2003], rescue the folding defect of this mutant and restore partially its activity. It is tempting to propose that the temperature-sensitive LPH-G1363S mutant may behave in a similar fashion towards this type of chaperones.

4.2 Structural hierarchy of regulatory elements in the folding and transport of an intestinal multi-domain protein: Domain III constitutes the structural core while domain IV has a regulatory role

Characterization of the structure, biosynthesis and trafficking of the individual subdomains within pro-LPH, an essential brush border membrane enzyme, constitutes an important step towards understanding its function and impact to the intestinal epithelial cell physiology. Given that the three-dimensional structure of LPH has not been elucidated yet, alternative approaches have to be designed to determine the significance and relevance of the individual subunits to each other in the context of this multiple domain protein.

Lactases, or more properly, ß-galactosidases, are grouped within 4 of the near 100 families of glycosyl hydrolases (GHs) that have been characterized [Coutinho and Henrissat, 1999]. Mammalian intestinal lactase (LPH) is classified in the GH1 family, along with enzymes present in a variety of organisms acting against different types of ß-glycosides. While most GH1 enzymes (mostly bacterial) thus far characterized are conformed by a single domain, intestinal lactase is synthesized as a multidomain precursor protein that is encoded by a gene resulting from the fusion of 4 tandemly-arranged repetitions of an ancestor gene [Mantei et al., 1988; Naim et al., 1991]. Maturation of the enzyme generates the brush border form comprized by the last 2 domains, III and IV [Jacob et al., 2002a] each with differential activity. Domain III shows specificity towards glycosides, such as phlorizin, while domain IV is specifically active against lactose [Arribas et al., 2000]. Interestingly, individual expression of domain III of LPH reveals a correctly folded, transport-competent and rapidly secreted molecule underscoring thus its autonomous character that has been conserved from prokaryotes to eukaryotes.

Despite its strong homologies with domain III, domain IV is not a folding-competent, transport-competent or an enzymatically active species *per se*. This domain, however, plays a central regulatory role in the context of the function and trafficking of LPH. It contains the LAC236 stretch that is required for dimerisation of LPH [Jacob et al., 2000; Panzer et al., 1998]. The essential function of domain IV within the LPH complex becomes evident when considering its role in the dimerisation of LPH. In fact, domain III does not dimerise and the phlorizin-hydrolase activity of this domain

is elevated by a factor of 2.5 fold in the dimeric LPH molecule. Additionally, domain IV is rate-limiting along the secretory pathway of LPH from the ER to the Golgi. In fact, LPHΔ4, a deletion mutant that lacks the entire homologous domain IV, acquires more rapidly complex glycosylation than its wild type counterpart proposing a role of this domain in decelerating LPH processing. Importantly, LPHΔ4 does not dimerise in the ER lending a strong support to the view that dimerisation is initiated by homologous domain IV and supporting previous data that assigned a role of LAC236 an essential role in dimerisation. It is very likely therefore that the retarded trafficking of wild type LPH in comparison to LPHΔ4 is due to its dimerisation prior to the ER exit, while this additional step is not required for LPHΔ4 to acquire transport-competence. Interestingly, epitope mapping with a panel of mAbs against the mature brush border form of LPH (domains III and IV) demonstrated a similar pattern of recognition for LPHΔ4 and LPH strongly suggesting that the epitopes tested are located in domain III. Given that these antibodies (except MLac6 and MLac10) react only with native LPH species, but not with denatured LPH the data strongly suggest that the tertiary structure of LPHΔ4 is comparable with its counterparts in human wild type LPH and that domain III represents the structural core of the mature protein.

On the other hand, domain IV is not an autonomous region. It harbours the lactase catalytic site at Glu^{1749} [Arribas et al., 2000; Zecca et al., 1998] and acquires activity only when LPH dimerises [Naim and Naim, 1996]. Domain IV plays therefore a role as a regulatory switch that triggers the dimerisation of the LPH molecule thus activating itself and elevating the phlorizin hydrolase activities in domain III.

Concerning of the correctness of the chosen domain boundaries, the question can arise, how it can be ruled out that the reason for the negative results regarding deletion of homologous domain III is just that domain boundaries might extend beyond the deleted fragment. Here, the possibility exists that the evolutionary related domains have evolved into intertwined folds that can hardly be disentagled by simple deletions. However, in this case in which structural information about related proteins is abundant – as well as in other domain-shuffling strategies – the problem is not so much defining the limits of the domains as it is to sort out possible interactions between domains that have coevolved as parts of a single polypeptide. As an indication that the domain boundaries were defined correctly and that domain III, even in the absence of domain IV, it is able to fold properly into a viable structure serves in any case the fact that the LPH construction lacking domain IV retains

substantial phlorizin hydrolase activity. Moreover, it shows that the structural determinants of dimerisation are totally, or at least partially, outside domain III. For sure, one has to be more cautious in the interpretation of negative results, but the results of this work support the conclusion that domain IV requires of domain III to develop a stable conformation. It is likely therefore that the mutation G1363S within domain III has induced conformational changes in homologous domain IV that harbors the lactase activity.

The data present a hierarchical model of LPH in which the homologous domain III constitutes a fully autonomous core domain within the LPH molecule. In addition it represents another intramolecular chaperone of LPH besides the profragment LPHα (Fig. 4.2.1). This model assumes that the profragment [Jacob et al., 2002a] and homologous domain III (the data presented here) attain their native conformation autonomously. While domain III is transport-competent and enzymatically active *per se*, it requires homologous domain IV for elevation of its enzymatic activity and regulation of its trafficking kinetics. This occurs via dimerisation of the entire LPH molecule, an event that is triggered by domain IV. Nevertheless, domain IV is a non-autonmous domain that can not fold independently; it requires the profragment as well as domain III as templates for correct folding. Correctly folded domain IV is now capable of triggering the dimerisation of LPH in the ER [Naim and Naim, 1996], an event that is required for LPH to exit the ER, for regulation of its transport kinetics and elevation of its enzymatic activities. The profragment and domain III function therefore as switches for correct folding of domain IV, which in turn “pays back” by giving domain III an increased phlorizin-hydrolase activity and gaining more activity as the lactase active site.

To my knowledge, this is the first example of a mechanism, in which a protein has two intramolecular chaperones and is not activated by propeptide cleavage like described for zymogens, neuropeptides, and prohormones [Barr, 1991; Steiner et al., 1984], but by intramolecular organisation and oligomerisation.

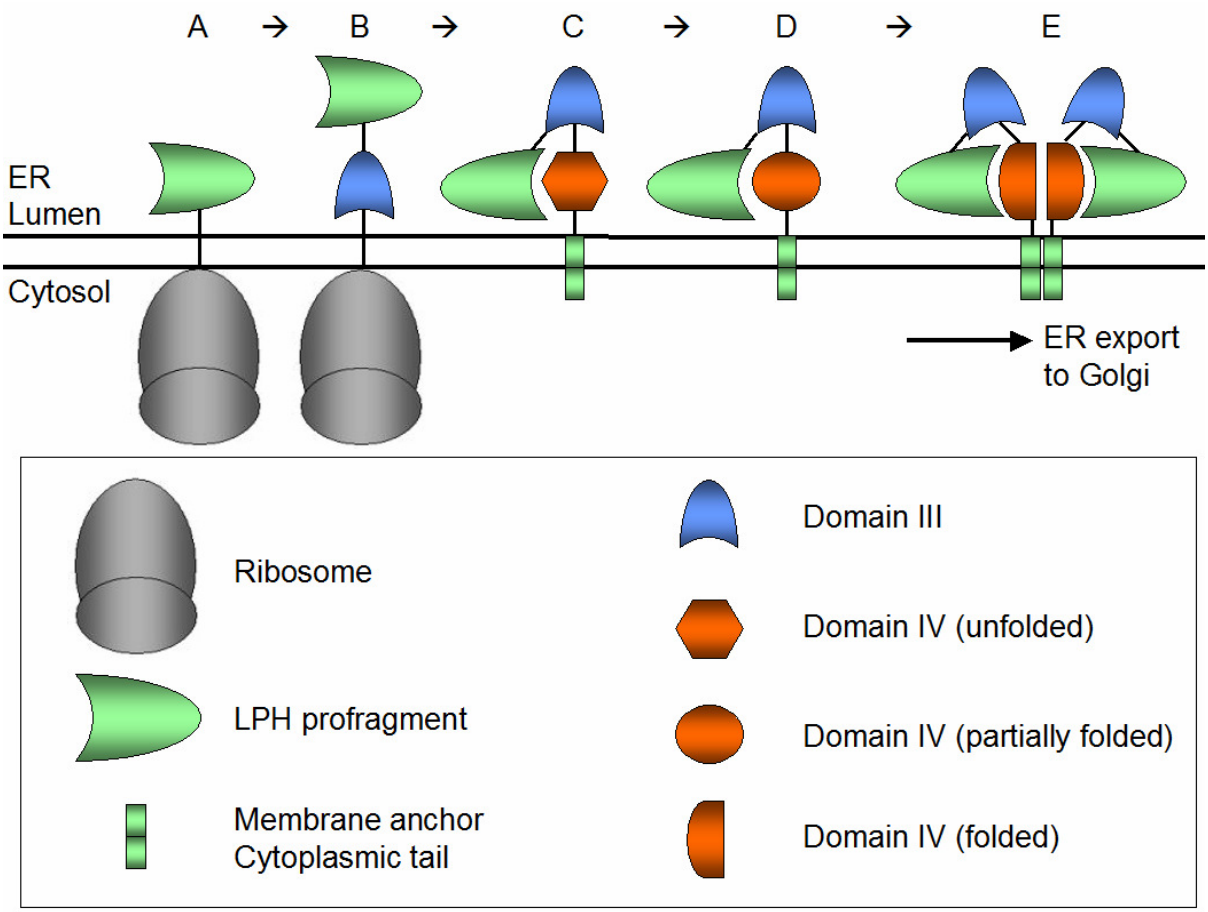


Fig. 4.2.1: Hierarchy model of LPH biosynthesis in the ER. The LPH precursor is synthesized at the ER and translocated in the ER lumen starting with the independently folding N-terminal profragment (*A*). Subsequently, autonomous domain III is synthesized and acquires correct folding independent of other domains (*B*), while homologous domain IV that associates LPH with the membrane is not capable of folding independently (*C*) and requires the profragment and domain III as folding templates (*D*). Finally, the correctly folded monomer LPH interacts with another monomer via the correctly folded domain IV to form a transport-competent LPH dimer that exits the ER and acquires full lactase and phlorizin hydrolase activities (*E*).

4.3 Structural hierarchy of regulatory elements in the folding and transport of an intestinal multi-domain protein: Domain I initiates folding of the whole protein while domain II regulates enzymatic activities

Many proteins comprise specific sequences, which are important for folding processes but not for protein function directly. They act as intramolecular chaperones and are eliminated in post-translational modification events [Chen and Inouye, 2008]. LPH contains an N-terminally located propeptide, which is almost as large as the mature protein [Naim et al., 1994]. For the major part of this profragment, LPHα, it could be shown that it acts as an intramolecular chaperone [Jacob et al., 2002a], but the role of the minor part, $LPH_{stretch}$, needs to be further elucidated. Moreover, the exact intramolecular organisation of LPH is a complex process, since i) homologous domain III constitutes the second intramolecular chaperone, ii) domain IV is supposed to have a regulatory function (cp. 3.2), and iii) the membrane anchor together with the cytoplasmic tail are also important for the attainment of a transport-competent conformation [Naim and Naim, 1996]. Additionally, recent findings of a first structural and dynamics study of a nascent protein support the hypothesis that a native tertiary structure of a polypeptide chain can be reached already on the ribosome [Hsu et al., 2007]. Therefore, the elucidation of early events in the biosynthesis of the nascent LPH polypeptide is of great interest for the understanding of the intramolecular organisation of multi-domain membrane-anchored proteins in general.

The absence of homologous domain II as part of the profragment including the polypeptide stretch Leu^{735}-Arg^{868} ($LPH_{stretch}$) results in a protein with a transport competence comparable to the wild type protein, therefore homologous domain II could be a slow folding domain like domain IV which decelerates LPH processing maybe by interacting with other proteins likely within the ER. Domain III and domain IV are the mainly glycosylated regions (each domain contains five potential N-glycosylation sites, whereas domains I and II contain two and three sites, respectively). That is why for LPHΔ2 - like for wild type LPH - the mannose-rich and the complex glycosylated protein forms are clearly distinguishable without endo H treatment, but not for LPHΔ4, because here the highly glycosylated domain IV is absent (cp. Fig. 3.2.2 and Fig. 3.3.2). The LPHΔ1 protein - lacking homologous

domain I, which is completely included within the intramolecular chaperone LPHα - is not transport competent and - like domain III - domain I seems to be also structurally important for the whole protein facilitating ER export and more important for the chaperone function than the first part of homologous domain II.

LPHΔ2 dimerises, but in the Golgi apparatus and not in the ER like wild type LPH. Like LPHΔ4 it seems to be involved in the oligomerisation process, but in contrast to LPHΔ4 it does not form higher oligomers and therefore domain II is not supposed to stabilize dimeric forms. Finally, domain I seems to be also important for LPH quaternary structure, since LPHΔ1 does not dimerise. This influence can be directly by facilitating dimerisation or indirectly by influencing other LPH regions by its chaperone effect.

The analysis of the LPHΔ-mutant transport kinetics revealed that indeed LPHΔ1 is not transport competent and what is more is degraded, because the band intensities of the mannose-rich forms decrease with time. Transport kinetics of LPHΔ2 is slower than for the wild type, but its mannose-rich protein form seems to be stable and converted to the complex glycosylated form and not degraded like for LPHΔ1. Therefore, domain II - as part of the intramolecular chaperone LPHα - is not a slow folding domain but supports folding of other LPH regions.

The protease sensitivity assay with trypsin shows that LPHΔ1 is degraded and therefore badly folded, because more potential trypsin cleavage sites are exposed. Moreover, LPHΔ2 is also reduced in size to two trypsin resistant protein forms like wild type LPH and LPHΔ4, but this reduction occurs over intermediate forms. One reason for this is most likely that the two cleavage sites exposed in wild type (cp. Fig. 3.2.1) are absent because domain II is deleted. Another reason is that LPHΔ2 seems not to be folded properly, because the resulting bands have a size comparable to the cleavage products of LPHΔ4 and not of wild type, although LPHΔ2 incorporates $LPH\beta_{final}$ like LPH wild type. Therefore, the presence of domain II, encompassing a part of LPHα and the polypetide stretch between the protease cleavage sites, seems to be crucial for the correct folding of the other domains and the whole protein.

The analyses of the enzymatic activity of the LPHΔ-mutants revealed that only LPHΔ4 and to a lesser extend LPHΔ2 show phlorizin-hydrolase activity, from which can be concluded that all LPH domains are crucial for the attainment of a correct lactase and phlorizin-hydrolase activity subdomain conformation except domain IV.

During the epitope mapping all of the antibodies reacted with all mutants except LPHΔ3 (cp. 3.2.4 and 3.3.4, *Epitope mapping of domain deletion mutants*). The antibodies tested were all raised against mature LPH, and it is likely therefore that conformation-specific antibodies are among those used. The results strongly suggest that the LPH epitopes tested were present or were not substantially modified in the mutants LPHΔ4, LPHΔ2 and LPHΔ1 as compared with wild type LPH, and that therefore all epitopes are localized in domain III.

Initial cleavage of mature pro-LPH in the Golgi occurs between Arg^{734} and Leu^{735} and generates $LPH\beta_{initial}$ that is transported with high fidelity to the apical membrane. In the intestinal lumen $LPH\beta_{initial}$ undergoes another cleavage at Arg^{868}/Ala^{869} by pancreatic trypsin to generate $LPH\beta_{final}$ [Jacob et al., 1996; Wuthrich et al., 1996]. The significance of the stretch between Leu^{735} and Arg^{868} in the context of trafficking and sorting of LPH has been until present obscure. The data presented here assign a role to this region (in association with domain III) in the sorting of LPH to the apical membrane. It is interesting to note that domain III *per se* is not as efficiently transported to the apical membrane in polarized MDCK cells as wild type LPH strongly proposing that the polypeptide stretch Leu^{735}-Arg^{868} in domain II could be important for the fine-tuning of polarized sorting.

Another reason, why $LPH_{stretch}$ is not already eliminated intracellularly like LPHα, but at the cell surface by luminal trypsin is its implication in the formation of an enzymatically active conformation. This hypothesis is supported by the observation that LPHΔ2 – which lacks $LPH_{stretch}$ – is transport competent but almost completely devoid of enzymatic activity. Moreover, LPHΔ2 is cleaved to two small protein forms by trypsin, although it lacks both trypsin cleavage sites exposed in wild type LPH, indicating that the lack of domain II does not result in a properly folded protein. Furthermore, $LPH_{stretch}$ is not capable to attain a transport competent conformation autonomously, because its presence reduces transport competence of domain III and prohibits ER exit of LPHβ. However, the presence of $LPH_{stretch}$ together with LPHα in D123 does not inhibit ER exit, indicating that the intramolecular chaperone LPHα counteracts the slow-folding effect of $LPH_{stretch}$. Interestingly, the presence of only a part of LPHα in D23 and LPHΔ1 is not sufficient to attain transport competence. Additionally, the presence of LPHα, $LPH_{stretch}$ as well as domain III and domain IV is also not enough to exit the ER, if the membrane anchor and the cytosolic tail are absent like in D1234 [Naim and Naim, 1996].

In order to further unravel the spatiotemporal hierarchy and the structural-functional correlation of LPH intramolecular organisation events, more comparative analyses have to be performed, e.g. on the sorting of efficiency LPHΔ2, domain $III_{stretch}$ and D123 as well as on enzymatic activities of all constructs containing phlorizin hydrolase, i.e. D123, D23, $LPH\beta_{initial}$, $LPH\beta_{final}$, domain $III_{stretch}$ and domain III. Finally, it will be inevitable to investigate the structure of LPH directly by cristallization studies, for which the transport competent proteins domain $III_{stretch}$, domain III and D123 constitute the appropriate constructs, because these are soluble and do not have hydrophobic regions.

The group of protein profragments can be classified into two subgroups, based upon their different ways of acting as an intramolecular chaperone [Chen and Inouye, 2008]. Type I intramolecular chaperones are mostly N-terminally located and support the formation of tertiary structures, like described for α-lytic protease [Anderson et al., 1999], nerve growth factor (NGF) [Kliemannel et al., 2004], and proinsulin [Munte et al., 2005]. Type II intramolecular chaperones are mainly found at the C-terminus and do not influence the formation of tertiary structures, but support the assembly of quaternary structures to functional protein complexes, e.g. the endosialidase of coliphage K1F [Schwarzer et al., 2009], fibril-forming collagen [Khoshnoodi et al., 2006], and the von Willebrand factor (VWF) [Rosenberg et al., 2002]. The question, if LPH belongs to one subgroup or the other remains to be answered. Finally, the possibility exists that this classification does not reflect the complexity of the folding processes of multi-domain membrane-anchored glycoproteins.

4.4 Elimination of a homologous domain of an intestinal hydrolase results in changed intracellular trafficking via altered membrane association

In polarized epithelial cells, like enterocytes, two distinct domains of the plasma membrane do exist, the basolateral and the apical domain. The latter is the site, to which LPH and other hydrolases of the brush border membrane, e.g. SI, dipeptidyl peptidase IV (DPPIV) or aminopeptidase N (APN), are delivered. The diverse and heterogeneous mechanisms of apical protein transport are reviewed by [Delacour and Jacob, 2006] as well as [Weisz and Rodriguez-Boulan, 2009].

Live cell imaging performed to elucidate LPH and SI apical sorting mechanisms revealed that SI – which is associated with Triton X-100 detergent resistant membranes (DRMs) – and LPH – which is not DRM-associated – can be found in different compartments in the same post-TGN vesicle. There, SI localizes at certain sites while LPH is equally distributed over the vesicle surface. Subsequently, both proteins are separated to smaller, different transport vesicles and for the first time a post-TGN separation event could be detected [Jacob and Naim, 2001]. Further experiments showed that transport of SAVs (SI-carrying apical vesicles), but not LAVs (LPH-carrying apical vesicles), is actin-dependent and requires the motor protein myosin Ia [Jacob et al., 2003] as well as the annexin II-S100A10 complex [Jacob et al., 2004] and alpha-kinase 1 (ALPK1) [Heine et al., 2005].
For LPH and LAVs, however, it could be shown that the beta-galactoside binding lectin galectin-3 (gal-3) is implicated in raft-independent, carbohydrate-dependent glycoprotein transport. Gal-3 acts as a sorting receptor by interacting directly with LPH in the LAV lumen and its depletion leads to basolateral sorting of LPH [Delacour et al., 2006]. Moreover, without gal-3 no high-molecluar-weight clusters are formed, in which non-raft-dependent glycoproteins are cross-linked prior to apical sorting [Delacour et al., 2007]. The direct interaction of gal-3 and LPH also occurs *in vivo* confirming the previous data from MDCK cells. Interestingly, for the raft-associated apical enzyme DPPIV this direct interaction could also be shown. Furthermore, gal-3 depletion results in cytoarchitectural defects of enterocytes, indicating an important role for this lectin not only in apical trafficking but also in epithelial morphogenesis [Delacour et al., 2008]. What is more, is that reduction of cellular gal-3 levels results

in changes in the microtubular network as well as in the membrane compartimentalization *in vitro* and *in vivo*. Moreover, gal-3 is transiently associated with the centrosome during cell differentiation and seems to be involved in centrosome formation and/ or stabilization [Koch et al., 2010]. It is tempting to speculate that – because of its connection to the centrosome – gal-3 is also implicated in early trafficking events, since the intermediate compartment (IC) is also connected to the centrosome as well as to the endocytic recycling compartment [Marie et al., 2009]. The question, if the differences between LPH and LPHΔ4 in ER-Golgi-transport, quaternary structure as well as membrane association are related to the lack of a gal-3-LPHΔ4 interaction still needs to be answered. The observation that LPHΔ4 can be found in the same vesicles as wild type LPH – and therefore is localized in LAVs – but does not colocalize within these carriers suggests that the absence of homologous domain IV rather than the difference in membrane association may cause a disturbed or inhibited gal-3-LPHΔ4 interaction together with reduced or even eliminated incorporation of LPHΔ4 into high-molecular.weight clusters. In general, galectins are known to have a variety of effects on the transport of proteins to their final destination [Delacour et al., 2009].
Very recently, the kinesin motor KIF5C was found to be involved in apical sorting of raft-dependent and raft-independent proteins directly after TGN-release [Astanina and Jacob, 2010]. Subsequently, SI and LPH are transported together in the same vesicle transendosomally and colocalize with Rab4-, Rab8-, and Rab11-positive endosomes before they are segregated to distinct vesicles [Cramm-Behrens et al., 2008]. If the absence of domain IV in LPHΔ4 influences the transendosomal transport still needs to be elucidated.

Protein-lipid and protein-protein interactions within biological membranes play key roles in arranging and regulating cellular mechanisms [Lindner and Naim, 2009; Lingwood and Simons, 2010]. For SI it could be shown that N- and O-glycosylation together with the localization of the enzyme in Triton X-100-DRMs enables the protein to become enzymatically active [Wetzel et al., 2009]. Another example is the PSMA protein. Here, three distinct glycoforms were identified with different detergents, displaying that the intracellular transport of each biosynthetic form happens through distinct membrane microdomains [Castelletti et al., 2008]. Furthermore, utilization of the mild non-ionic detergent Tween 20 made it possible to

discriminate apically and basolaterally sorted proteins early in the secretory pathway by isolation of ER-membrane enriched DRMs. By that, the existence of an early polarized sorting mechanism occurring before protein maturation could be proposed [Alfalah et al., 2005]. Although LPH and domain III are efficiently transported to the apical membrane of polarized cells, the possibility exists that LPHΔ4 is not, because it is weaker associated with Tween 20-DRMs.
Isolation and analysis of Tween 20-DRMs also suggested an early trafficking control for membrane proteins operating between the ER and Golgi apparatus, because an anchor-less mutant of the membrane dipeptidase (MDP) showed a retarded maturation together with complete solubility [Hein et al., 2009].

Similar to the prerequisition of gal-3-dependent high-molecular-weight clusters for apical sorting of raft-independent glycoproteins [Delacour et al., 2007], the oligomerisation of raft-associated GPI-anchored proteins prior to apical transport has also been observed [Paladino et al., 2004]. Additionally, it has been shown by the same group that some raft-independent apical transmembrane proteins do not oligomerise before they reach the apical plasma membrane [Paladino et al., 2007]. Moreover, coexpression of wild type and a mutant form of the prion protein resulted in an altered transport rate a well as increased DRM-association of both proteins most likely because the mutant influences the subcellular localization and membrane microenvironment of the wild type protein by direct interaction [Schiff et al., 2008]. It would be interesting to know if the LPH-G1363S mutant (or a deletion mutant) is able to form dimers with wild type LPH *in vitro* when coepressed – imitating a heterozygous *in vivo* situation – and if this pseudo-heterodimer is transport competent or not. By that, the intramolecular organisation of the LPH protein and the strengths of its intramolecular chaperones could be further elucidated.
Furthermore, cholesterol-dependent membrane domains exist at the apical and the basolateral sites of polarized cells, whereby the actin cytoskeleton is implicated in organizing apically sorted transmembrane proteins independently of rafts [Lebreton et al., 2008]. Finally, a connection between DRM-association, oligomerisation and apical sorting could be observed for GPI-anchored proteins. Interestingly, addition of cholesterol resulted in oligomerisation of a basolaterally sorted protein and subsequent missorting to the apical membrane [Paladino et al., 2008].

Taken together, the correlation of oligomerisation and membrane association in the context of polarized sorting is still obscure, but LPH and LPHΔ4 represent valuable tools for further analyses.

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6. Appendix

6.1 List of abbreviations

A	alanine; adenine
Ala	alanine
ALPK1	alpha-kinase 1
ap	apical
APN	aminopeptidase N
APS	Ammoniumperoxidsulfate
Aqua bidest.	*Aqua bidestillata*
Arg	arginine
Asn	asparagine
Asp	aspartate
ATP	adenosine triphosphate
bl	basolateral
bp	base pair(s)
BFA	brefeldin A
BiP	binding immunoglobulin protein
°C	degree Celsius
C	cytosine
cDNA	complementary DNA
CFP	cyan fluorescent protein
CLD	congenital lactase deficiency
cm	centimeter
conc.	concentration
COP	coat protein
cp.	compare
CT	cytoplasmic tail
D	asparagine
D3,DIII	domain III
Da	Dalton
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEAE	diethyle-amino-ethyle
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DPPIV	dipeptidyl peptidase IV
DRM(s)	detergent resistant membrane(s)
DTT	dithiotreitol (Cleland's reagent)
dTTP	deoxythymidine triphosphate
E	glutamate
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetate
e.g.	(exempli gratia) for example

Endo H	endo-β-*N*-acetylglucosaminidase H (from *Streptomyces plicatus*)
Endo F	endo-β-*N*-acetylglucosaminidase F (from *Flavobacterium meningosepticum)*
ER	endoplasmic reticulum; Endoplasmatisches Retikulum
ERAD	ER-associated degradation
et al.	et alii
evtl.	eventuell
F	phenylalanine
FCS	fetal calf serum
Fig.	figure
g	gram(s)
x *g*	accelaration of gravity
G	glycin; guanine
GA	Golgi apparatus; Golgi-Apparat
Gal	galactose
gal-3	galectin-3
GalNAc	N-acetyl-α-D-galactosamine
GE	Germany
GFP	green fluorescent protein
GH	glycosyl hydrolase
Glc	glucose
GlcNAc	N-acetyl-glucosamine
Glu	glutamate
Gly	glycine
GPI	glycosyl-phosphatidylinositol
GT	galactosyl transferase
h	hour(s)
HPLC	high-performance liquid chromatography
Hsp70	heat shock protein 70
ic	intracellular
IC	intermediate compartment
i.e.	id est
IgG	immunoglobulin G
k	kilo
K	lysine
kb	kilo base pair(s)
kDa	kilo Dalton
KOAc	potassium acetic acid
l	liter
L	leucine
LB	Luria Bertani
ld	liquid-disorderd
Leu	leucine
lo	liquid-ordered
LPH	lactase phlorizin hydrolase (all forms)
lys	lysate
Lys	lysine
M	molar
m	milli
µ	micro

mA	milliampere
mAb	monoclonal antibody
MA	membrane anchor; Massachusetts
Man	mannose
MDCK	Madin Darby Canine Kidney
MDP	membrane dipeptidase
med	medium
MEM	Minimum Essential Medium
Met	methionine
mg	milligram
min	minute(s)
ml	milliliter
µl	microliter
mM	millimolar
mRNA	messenger RNA
n	nano; nucleus
N	asparagine
NaCl	sodium cloride
NAD	nicotine amide dinucleotide
NaOAc	sodium acetic acid
NGF	nerve growth factor
NSF	N-ethylmaleimide-sensitive factor
OD_{xxx}	optical density at xxx nanometers
ORF	open reading frame
OS(P)T	oligosaccharide (protein) transferase
p	pico; pellet
P	proline
pAb	polyclonal antibody
PAS	protein A-sepharose
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDI	protein-disulfide isomerase
PH	phlorizin hydrolase
Phe	phenylalanine
PMSF	phenylmethylsulfonyl fluoride
PPI	peptide-prolyle isomerase
Pro	proline
PSG	penicillin, streptomycin, glutamine
PSMA	prostate-specific membrane antigen
Q	glutamine
R	arginine
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	rounds per minute
s	supernatant
S	serine
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	second(s)
Ser	serine
SI	sucrase isomaltase

SNAP	soluble NSF attachment protein
SNARE	SNAP receptor
SRP	signal-recognition particle
SS	signal sequence
T	thymine
TAE	Tris acetate EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylendiamine
TGF	transforming growth factor
TGN	*trans*-Golgi network
Thr	threonine
Tris	Tris-hydroxymethyl-aminomethane
U	unit
UDP	uridine diphosphate
UK	United Kingdom
USA	United States of America
UV	ultraviolet
V	volt
v/v	volume by volume
VWF	von Willebrand factor
w/o	without
w/v	weight by volume
WT;wt	wild type
Y	tyrosine
YFP	yellow fluorescent protein
®	registered trademark

6.2 List of figures

6.3 List of tables

7. Curriculum vitae

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9. Declaration/ Erklärung

Hiermit versichere ich, dass die Dissertation

„Characterization of natural and artificial mutants of human intestinal lactase phlorizin hydrolase”

selbständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogenen Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den

_____

(Unterschrift Marc Behrendt)