# Routing

## of normal and mutant brush-border sucrase-isomaltase and lactase-phlorizin hydrolase

Joke Ouwendijk

## Erratum

At pages 54 - 55, figures 1 and 3 have been printed incorrectly
The correct figures are depicted below

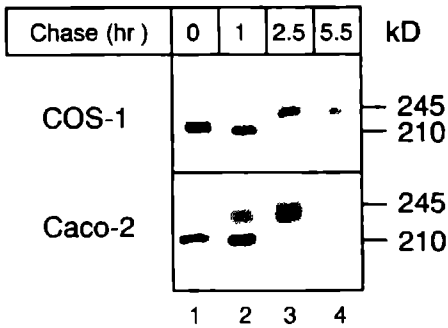


**Fig. 1** Processing of SI in COS-1 and Caco-2 cells Caco-2 and transfected COS-1 cells were biosynthetically labeled with [35S]methionine for 1 5 hours, and chased for the indicated times Homogenates of these specimens were immunoprecipitated with monoclonal anti-SI antibodies Immunoprecipitates were subjected to SDS-PAGE on 5% slab gels Gels were analyzed by fluorography

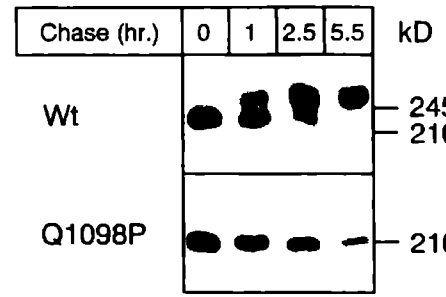


**Fig. 3** Processing of wild type (Wt) and mutant (Q1098P) SI in COS-1 cells Cells were biosynthetically labeled with [35S]methionine for 1 5 hours, and chased for the indicated times Homogenates of these specimens were immunoprecipitated with monoclonal anti-SI antibodies Immunoprecipitates were subjected to SDS-PAGE on 5% slab gels

Routing of normal and mutant brush-border sucrase-isomaltase and lactase-phlorizin hydrolase
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Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

PROEFSCHRIFT

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Chapter 1

# General introduction and scope of the thesis

## 1.1 Polarized epithelial cells

Epithelial cells form a layer that lines many cavities of the body, such as the lining of the respiratory, urinary and digestive systems. The most important function of an epithelium is to form a barrier against invasion of external organisms or chemicals, and to enable transport of selective substances, such as nutrients. To prevent leakage between the cells, a tight seal is formed by tight junctions. These junctions also separate the plasma membrane into a basolateral and a apical domain The apical membrane domain faces the lumen of a cavity whereas the basolateral domain faces neighboring cells and the underlying basal surface of connective tissue (reviewed in (6,7)). Both domains serve different functions and therefore have a very different protein and lipid composition. For instance, the apical membrane of intestinal absorptive epithelial cells contains enzymes that are involved in digestion of the food contents of the intestine, while the basolateral surface contains molecules that are involved in adhesion of the cell to substrate and to other cells.

## 1.2 Polarized sorting

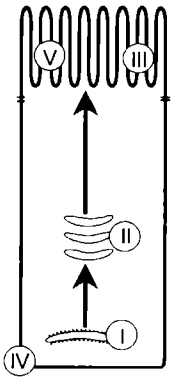
In recent years, the sorting mechanisms that accomplish the localization of proteins to the appropriate membrane domains have been extensively studied (3,6,7,10,11) As will be discussed in more detail in chapter 2, plasma membrane proteins are synthesized in the rough ER, and transported through the Golgi apparatus to the trans-Golgi network (TGN). In the TGN proteins are sorted and packed into clathrin-coated vesicles that deliver them directly to their final destination, the apical or basolateral surface Alternatively, the TGN derived vesicles first deliver the proteins to the basolateral membrane, after which they are endocytosed and transported to the endosomes (6). Most proteins are recycled from the endosomes back to the basolateral surface, but a few are transcytosed to the apical surface (6,7). In some polarized cells, such as hepatocytes, transcytosis is the most common pathway for apical delivery (7). A third mechanism for polarized delivery of proteins in epithelial cells is differential retention at one particular surface. For instance the $Na^+$, $K^+$-ATPase is targeted to both the apical and the basolateral surface. At the apical surface this protein disappears after a short time, probably because it is degraded, while it remains at the basolateral membrane for a much longer time (2)

## 1.3 Routing of brush-border proteins in polarized intestinal epithelial cells

In polarized intestinal epithelial cells, a large number of glycoproteins is synthesized and transported to a variety of destinations in the cell The apical membrane of these cells consists of a layer of microvilli that collectively form the brush-border. This brush-border results in an increase in the membrane surface at this side of the cell, resulting in an extensive increase of the absorptive capacity. At the brush border, enzymes are expressed that play a role in the digestion of food. Since these brush-border enzymes have to be specifically transported to the apical membrane, they form an interesting group for the study of the polarized transport mechanisms that are mentioned in the former paragraph. This thesis focuses on the routing and processing of two brush-border proteins: sucrase-isomaltase (SI) and lactase-phlorizin hydrolase (LPH). In chapter 3, the main features of both proteins will be reviewed.

## 1.4 Scope of the thesis

In previous studies it was shown that mutations in the gene that codes for SI can influence the intracellular transport, leading to congenital SI deficiencies (CSID) (1,8). The cases of CSID investigated so far can be subdivided into five phenotypes in which the enzyme is expressed, but is not functional, as described in more detail in fig 1 (1). Three of the phenotypes are caused by a disturbed




| Phenotype | Description |
|---|---|
| I | Transport block in ER |
| II | Accumulation in Golgi, degradation before complex glycosylation |
| III | Transport to brush-border, no SI-activity |
| IV | Basolateral missorting |
| V | Intracellular cleavage, Isomaltase transported to brush-border |

**Fig. 1** Five different phenotypes of congenital sucrase-isomaltase deficiency have been identified with respect to transport and processing of sucrase-isomaltase (1,8).

intracellular transport of the enzyme resulting in an intracellular accumulation in ER or Golgi apparatus (phenotypes I and II resp.) or in missorting to the basolateral membrane (phenotype IV). The latter phenotypes could provide more insight in transport mechanisms that are responsible for the correct delivery of proteins to their final destinations. Therefore biopsies from a patient with CSID were in this thesis analyzed not only at ultrastructural and biochemical level, but at genetical level as well. SI was shown to accumulate between the ER and the Golgi apparatus, and the SI cDNA was isolated and sequenced. A point mutation was identified that was shown to be responsible for the transport failure. The results of these experiments are described in chapter 4. This is the first report that characterizes a point mutation in the SI gene that is responsible for the transport incompetence of SI and hence for its retention between the ER and the Golgi.

The mutated protein was further analyzed with respect to its folding and localization, and the same mutation was introduced into a highly homologous region of α-glucosidase (4,9), a protein that is normally localized in lysosomes, but also localized and secreted apically (5,12). Mutated α-glucosidase accumulated in transfection experiments in a fashion similar to mutant SI. These experiments are described in chapter 5.

To further examine the influence of the identified amino acid substitution on the processing of SI, the mutation was replaced by mutations leading to other amino acids at this position. The results of transfection experiments with these mutants are described in chapter 6.

Chapter 7 describes another protein that is expressed at the brush-border of intestinal epithelial cells: lactase-phlorizin hydrolase (LPH). During its processing, LPH is cleaved into two regions One of these regions, mature LPH, is expressed at the brush-border, but the fate of the other region, the pro-fragment, is not clear In previous experiments it was shown that this pro-fragment is essential for the transport of LPH out of the ER and out of the Golgi apparatus. Whether it is important for transport beyond the Golgi is not known. Because the pro-fragment is relatively large, and because it is homologous to mature LPH, another function for the pro-fragment could be possible If the pro-fragment has another function, its isolation and localization could provide more information To perform these experiments we used antibodies against the pro-fragment and in addition a VSV-epitope tag was introduced into the pro-fragment The thesis is completed by a general discussion in chapter 8, and summarized in chapter 9.

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Chapter 2

# Protein synthesis and processing in the secretory pathway

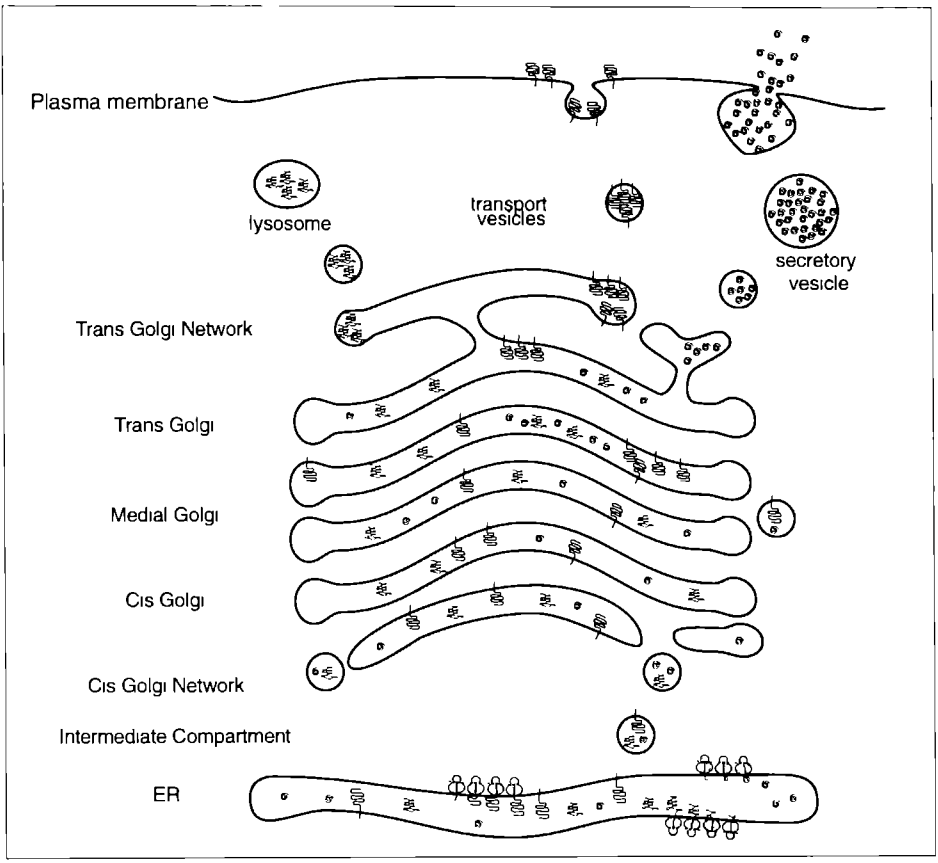


**Fig. 1**: Representation of the stations along the secretory pathway

## 2.1 Protein synthesis and processing in the secretory pathway

The pathway that is responsible for the delivery of proteins from the ER to intracellular organelles or the plasma membrane in eucaryotes was originally defined by Palade for the pancreatic exocrine cell (67), who named it the secretory pathway In fig 1 several stations in this pathway are represented the rough ER, the ER-Golgi intermediate compartment (ERGIC), the cis side of the Golgi apparatus (cis-Golgi network, CGN), the Golgi stack (divided into cis, medial and trans cisternae) and finally the trans-side of the Golgi apparatus (trans-Golgi network, TGN) It has been established that proteins traverse this pathway in individual vesicles in a vectorial fashion in a cis to trans direction rather then via tubular connections between ER and Golgi (76) The transport intermediates of the secretory pathway have been defined by a variety of experimental approaches (59,78) In these intermediates, various factors have been identified that play a role in the formation and the fusion of transport vesicles How all these components cooperate as a protein transport machine and how the endomembrane system is organized is very complex, but becoming more and more clear due to extensive studies in recent years (reviewed in (88))

Most likely, the mechanisms that are responsible for intracellular transport and

sorting operate in all eucaryotic cells with some variations. For instance, the sorting event in polarized cells (ranging from epithelial to neuronal cells) is more complex because of the functional difference between the two plasma membrane domains (34,98). Another variation is regulated secretion, in which the fusion of secretory vesicles with the plasma membrane is mediated by a stimulus. All these variations make the picture even more puzzling (15).

In the next paragraphs protein synthesis and maturation in the secretory pathway will be discussed in more detail from the translocation into the ER all the way to the final arrival at the plasma membrane. First the major organelles along this pathway will be discussed, then transport mechanisms and sorting events will be described.

## 2.2 Endoplasmic Reticulum

### 2.2.1 Translocation

Once a nascent peptide chain has reached a length of about 70 residues, a sequence emerges from the ribosome that can be recognized by the Signal Recognition Particle (SRP)(61) If SRP binds with high affinity, translocation is paused, and the whole complex is targeted to the ER (24,25). SRP interacts with its receptor, and the nascent chain is transferred to the translocon, that translocates it to the lumen cotranslationally. If the signal sequence is specific for the SRP-independent pathway, the binding to SRP is not efficient enough, and translation occurs at free ribosomes in the cytoplasm, where cytosolic chaperones conserve the nascent chain in an unfolded conformation. The complex is somehow targeted to the ER, where it is translocated post-translationally (63). The structural differences between signal sequences destined for the post- or the co-translational translocation pathway are not completely understood so far (63,116). If there is no signal sequence at all, no translocation into the ER takes place, and the protein maturates in the cytoplasm, under guidance of cytoplasmic chaperones.

In mammalian cells co-translational translocation predominates, but in yeast both routes are used, dependent on the protein to be transported (83) The cotranslational and post-translational pathways share common components and features, but also differ in fundamental aspects (reviewed in (39,83)). Both routes utilize a protein-conducting channel, the translocon, for the transfer of the polypeptide to the ER lumen (13,97). This channel must be gated in two dimensions, perpendicular to the plane of the membrane to transport proteins completely across the membrane, and in the plane of the membrane to integrate membrane proteins into the bilayer by releasing their hydrophobic anchors into the lipid environment (97,99). The cotranslational translocation of several types of membrane proteins is illustrated in fig. 2.

As the chain translocates across the translocon, several proteins interact. For instance the signal sequence protease removes signal sequences from the translocated chains. Several proteins however, possess a signal sequence that cannot be removed, and functions as a membrane anchor (fig. 2B). Another protein that interacts is BiP, which not only functions as a chaperone, but also as a molecular motor to reel the precursor out of the pore into the ER lumen (53,63). Other chaperones interact as well, some of these will be discussed in the next paragraphs, just as the proteins that are responsible for glycosylation and formation of disulfide bonds

### 2.2.2 *N*-Glycosylation

A crucial biosynthetic function of the ER is the covalent addition of sugars to nascent glycoproteins. A preformed oligosaccharide that consists of 14 sugar residues (see fig. 3 A) is transferred *en bloc* from a lipid carrier (dolichol phosphate) to the $NH_2$-group at the side chain of an asparagine residue in the protein (45) This

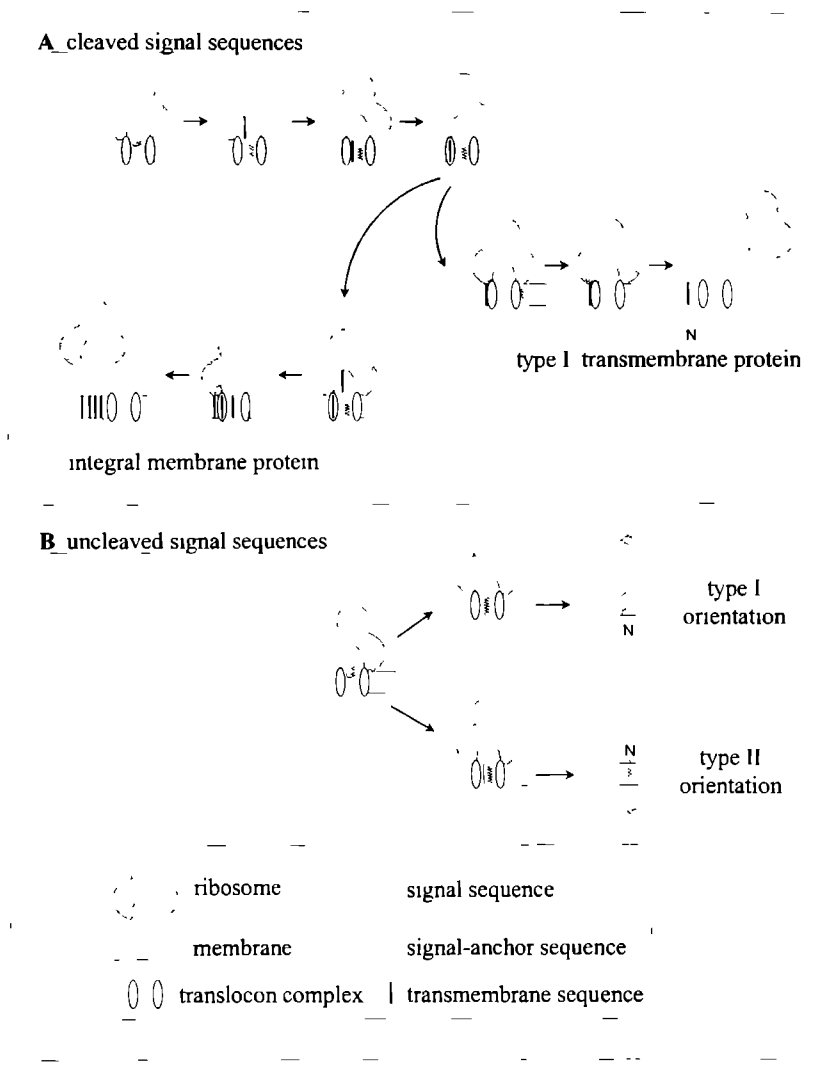


**Fig. 2:** Mechanisms of cotranslational translocation across the ER membrane of several classes of proteins

transfer is accomplished by a membrane-bound oligosaccharyltransferase (1,41,96), of which one molecule is associated with each translocation complex Only asparagine residues in the sequences Asn-X-Ser or Asn-X-Thr (X can be any amino acid except proline) can become *N*-glycosylated. Of the sugar side chains, one mannose and three glucose residues are rapidly removed by the interaction of another set of proteins, the glucosidases. More modifications of the oligosaccharide side chains occur in the Golgi apparatus.

### 2.2.3 Chaperones and folding enzymes

The highest concentration of molecular chaperones can be found in the lumen of the ER, although some reside in the cytoplasm. In the ER they are present as soluble proteins or as membrane-bound proteins that face the

lumen of the ER. They associate transiently with folding intermediates and unassembled subunits to assist in their translocation, promote efficient folding and prevent premature oligomerization or processing (28,29,32,40,58,108). In addition, the association plays a role in the quality control process that prevents export of premature proteins from the ER to the Golgi complex and beyond (27,37,81). Three chaperones in the ER have been extensively studied: calnexin, calreticulin and BiP (32,75,87).

The membrane protein calnexin and its soluble homologue calreticulin are two chaperones in the ER that interact with the oligosaccharide side chains of newly synthesized glycoproteins. Other interactions between substrate glycoproteins and these chaperones could occur as well. One theory describes the initial contact with the glycan, followed by a more stable interaction with hydrophobic peptide moieties on the surface of incompletely folded molecules (87,102,112). The association between the substrate protein and the chaperone is believed to last until the substrate has folded and lost the conformational features responsible for attachment. Both calnexin and calreticulin specifically bind to mono-glucosylated core glycans (28,32,75,102, 112), which are glucosylation intermediates that have been generated by oligosaccharyl transferases and α-glucosidases I and II (45). Another luminal ER enzyme, UDP-glucose:glycoprotein glucotransferase (UDP Glc-transferase), generates these mono-glucosylated glycans as well by specific reglucosylation of glucose-free, high mannose *N*-linked glycans present on incompletely folded glycoproteins (68,101,105). Calnexin, calreticulin and UDP Glc-transferase are thought to be involved in

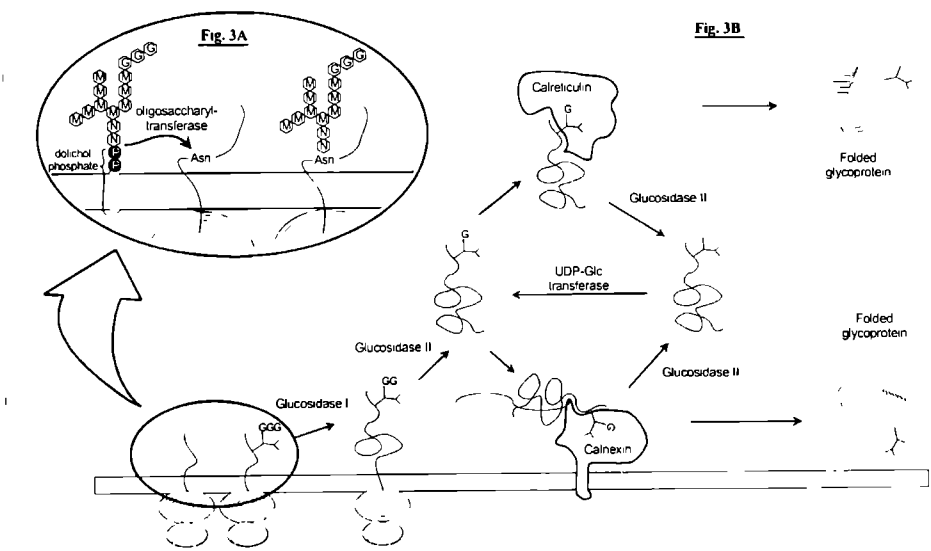


**Fig. 3:** Calreticulin and calnexin are involved in cycles of de and re- glucosylation that finally result in properly folded proteins that are allowed to leave the ER. A)A preformed oligosaccharide is transferred en bloc from a lipid carrier (dolicholphosphate) to the NH2-group of an Asparagine residue in the nascent polypeptide. B)Oligosaccharides are trimmed by Glucosidases I and II, and reglucosylated by UDP-Glc transferase. Calnexin and calreticulin bind to the monoglucosylated saccharides until the protein is folded properly. G: glucose; M: mannose; N: N-acetylglucosamine; P: phosphate

cycles of de- and reglucosylation (see fig 3B) that finally result in properly folded proteins (29,32,75,87)

BiP is the only member of the Hsp70 family of chaperones that can be found in the ER. Whether BiP plays an active role in protein folding has not been established so far, although its interaction with mis- or partially folded and unassembled proteins has been demonstrated. Overexpression of BiP results in retardation of proteins that interact with it, while other proteins are normally transported (17). Furthermore, the release of BiP from several substrate proteins coincides with their conformational maturation and release from the ER (7,44,54).

In addition to chaperones there are other enzymes that assist proteins to acquire a correct folding For instance protein disulfide isomerase (PDI) accelerates the refolding of proteins containing multiple disulfide bonds. Disulfide bonds are spontaneously formed by oxidation of two Cys-SH groups in the nascent chain. PDI catalyzes the breakage and reformation of these bonds, which results in the formation of the thermodynamically most stable conformation (20)

### 2.2.4 Quality control and degradation

Many proteins that are not folded properly accumulate in the ER and are finally degraded. A model for this ER-associated degradation has recently been proposed by Brodsky and McCracken (10): If a protein is not folded properly, chaperone complexes remain associated with the polypeptide and the translocation machinery 'decides' on the basis of the kinetics of the chaperone interaction to re-export the polypeptide to the cytosol. During the interaction with the lumenal chaperones, the nascent polypeptide might remain attached to the translocation complex, which could result in a prolonged opening of this complex (23,64,66). Soluble polypeptides may be re-exported through this open channel, whereas integral membrane proteins could re-enter the channel by a partial dissociation of the complex (30). When the proteins are delivered to the cytosol, they must be recognized by the proteasome. Some proteins were shown to be tagged first by ubiquitin (6,33,79,111). Other proteins were degraded independent of ubiquitin (57,113,114), possibly because they were unfolded or had specific chaperones attached to them. These specific chaperones could help to "feed" substrates to the proteasome. At the cytosolic face of the ER, both ubiquitin-conjugating enzymes (100) and proteasomes (84) are present, suggesting that the degradation machinery can be tightly coupled to retrotranslocation (10).

## 2.3 The intermediate compartment and the cis-Golgi network

Once a protein has passed all quality control machineries in the ER, it is packed into transport vesicles, and exported. The next compartments on its route are the intermediate compartment and the cis-Golgi network. The intermediate compartment, or ER-Golgi intermediate compartment (ERGIC, (94,95)) seems to consist of an extensive network of tubules that covers large areas of the cytoplasm. The ERGIC has been a subject of debate (2) since it is not clear whether it is a collection of ER outposts (31) that is involved in exchange of materials with the Golgi complex, or a distinct organelle (77).

The cis-Golgi network (CGN) appears to embody an array of tubules, associated with the *cis*-most cisternae of the Golgi complex (48,82). The CGN receives newly synthesized proteins from the ER that are sorted and transported to the Golgi apparatus, or back to the ER (16,70-72). In most cells it is most likely involved in the fatty acylation of membrane proteins (8,85), the phosphorylation of lysosomal enzymes (70), and the addition of the first *N*-acetylglucosamine residues of *O*-linked oligosaccharides (104).

## 2.4 Compartmental boundaries

The boundaries of compartments are in general defined by the presence of marker proteins and biochemical functions in combination with morphological features For example ERGIC-53 (31) and its rat homologue p58 (91) reside in the ERGIC, whereas the KDEL receptor (47,103) and gp74 (2) can be found in the CGN (21) Morphologically, compartments are defined by direct visualization of membrane boundaries However, biochemical and morphological boundaries do not completely overlap, for most resident proteins are confined to more than one morphologically defined compartment Rabouille and Nilsson proposed a model that explains how compartments are organized and maintained (80)

## 2.5 The Golgi complex

### 2.5.1 Structure

During their passage through the Golgi apparatus, newly synthesized proteins are exposed to several kinds of post-translational processing Most remarkable are the stepwise addition of *O*-linked glycans and the remodeling of *N*-linked oligosaccharide side chains of glycoproteins The processing of carbohydrates takes place in an ordered sequence of reactions, catalyzed by specific enzymes as will be discussed in the next paragraph This sequence necessitates transport through the Golgi to be directional Therefore, the Golgi apparatus is subdivided into compartments all of which possess distinct polarities and distinct sets of enzymes

Ultrastructurally, the most typical features of the Golgi complex can be distinguished (82) It has a characteristic stack of usually between 3 and 8 flattened cisternae At one side of this stack clathrin-coated vesicles and tubular endosomes are found (94) This trans-side faces secretion granules or centrioles The other side, the cis-side, is usually oriented towards the ER By immunogold labeling of resident enzymes, specific cisternae of the Golgi can be labeled (9,115), and the stacks can be subdivided into three regions cis, medial and trans In a simplified model, the cis-Golgi network is the receiving area of non-clathrin coated vesicles, containing newly synthesized proteins that were packaged in the ER, the trans-Golgi network is the sorting area, where proteins that were processed in the preceding cisternae are sorted and packed into clathrin-coated vesicles which are destined for other organelles and for the cell surface

### 2.5.2 Oligosaccharide processing

*N*-linked oligosaccharides are attached to asparagine residues and trimmed in the ER as described in one of the previous paragraphs In the Golgi apparatus, these groups are further modified into two broad classes complex oligosaccharides and high-mannose oligosaccharides High-mannose residues contain just two *N*-acetyl-glucosamines and many mannose groups, often near the number present in the lipid-bound precursor that was originally added in the ER Complex oligosaccharides can contain more than the original two *N*-acetylglucosamines, as well as a variable number of galactose and sialic acid residues, and sometimes fucose (3,45) These residues are a result of a combination of trimming and addition of sugars as exemplified in fig 4 A High-mannose residues can be removed by digestion with Endo-N-acetyl-glucosaminidase H (Endo H), whereas complex glycosylated residues cannot This sensitivity is often used to monitor the processing of proteins

Some proteins have sugars added to the OH groups of specific serine or threonine side chains This *O*-glycosylation is catalyzed by a sequence of glycosyl-transferase enzymes In most cases, *N*-acetylgalactosamine is added first, followed by a number of sugar residues that can vary from just a few to 10 or more (18,35,38,90, see fig 4 B)

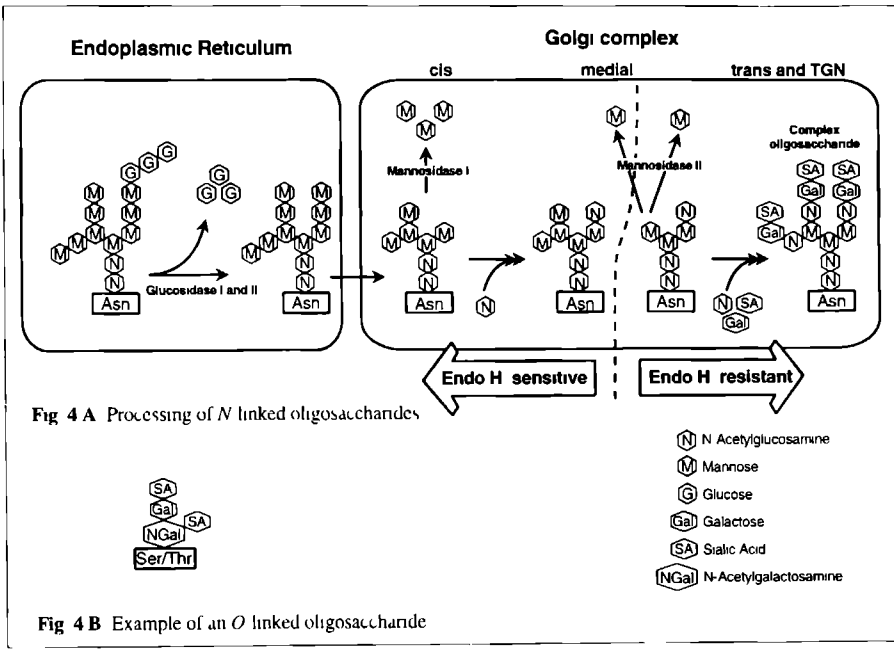


**Fig 4 A** Processing of *N* linked oligosaccharides

**Fig 4 B** Example of an *O* linked oligosaccharide

## 2.6 Vesicular transport and sorting

### 2.6.1 Vesicle transport

How every different compartment in the cell acquires its own specific set of proteins can be explained in general terms by machineries that are responsible for formation, targeting and fusion of transport vesicles Each separate vesicle allows transport between two membrane-bounded compartments the donor compartment that produces the vesicle and the acceptor compartment that receives the vesicle and its cargo The machinery that takes care of vesicle transport involves i) budding of the vesicle which is accomplished by coat proteins that mold a vesicle out of a donor membrane, ii) uncoating of the budded vesicle, thereby exposing target- and vesicle specific identifiers (t and v-SNAREs resp , SNARE − SNAP receptor) which bind to each other and thereby dock the vesicle to the acceptor membrane, iii) binding of NSF (*N*-ethylmaleimide sensitive fusion protein) and SNAP (soluble NSF attachment protein) to the SNARE complex, thereby initiating membrane fusion The sequence of events is regulated and controlled by various small GTP-binding proteins such as ARFs and Rabs (73,74) This 'SNARE-hypothesis' is reviewed in (89) and (93)

Depending on the route on which vesicles are travelling, the constituents of coats can vary, but the general principle of budding and fusion is the same For instance between ER and Golgi apparatus, transport is mediated by vesicles coated with two distinct coat complexes COPI and COPII (5,46) Vesicles that bud from the trans-Golgi network are coated with clathrin, another coat that also plays a role in receptor-mediated endocytosis (69) Apart from COP- and clathrin coats, several other coats have been found, reviewed in (93) and (86)

### 2.6.2 Sorting and transport signals

Whether or not a protein enters a transport vesicle is determined by structural features sorting signals Most sorting signals

are discrete peptide domains of 4 to 25 residues (9,11,43,49,106,115), but can also consist of conformationally determined epitopes (4,76) One protein can comprise multiple sorting signals, each specifying the fate of that protein at successive stages and collectively determining its itinerary (89) A sorting signal specifies movement or lack of movement, therefore there are three options for a protein 1) If a protein contains a signal that specifies movement, it is concentrated in a parting vesicle 2) If the signal specifies retention, the protein is excluded from the vesicle 3) If there is no signal at all, the protein enters the vesicle by bulk flow, and is transported 'by default' (89) Analysis of signals in proteins that should be transported in specific vesicles could lead to a more complete view on the whole vesicle transport machinery

The sorting that takes place in the TGN of polarized cells results in protein-containing vesicles with a basolateral or an apical destination (110) In the basolateral direction, sorting is mediated by signals in the cytoplasmic domains of basolateral transmembrane proteins (36,56,60) However, neither the cytoplasmic domains of apical transmembrane proteins nor their transmembrane regions seem to be important for their apical transport, because truncated forms are secreted apically Therefore, transport signals must be localized in the ectodomains (12,109) of these proteins The signals were until recently thought to consist of carbohydrates For instance *N*-glycosylated growth hormone is secreted apically, while the wild type, non-glycosylated form is secreted from both sides of polarized MDCK-cells (92) Furthermore, almost all proteins reported to be apically sorted contain *N*-glycans that can be critically involved in apical delivery (42,62,107) There are some exceptions that conflict with this hypothesis For instance, the hepatitis B surface antigen is apically delivered without being *N*-glycosylated (19,26,55), and the hepatitis B virus glycoprotein (52) and aminopeptidase N (a brush-border enzyme) (65) are normally transported in cells with a *N*-glycosylation defect

There is one group of proteins that has another apical sorting signal These proteins are not transmembrane, but are attached to the membrane via a glycosyl phosphatidyl inositol (GPI) anchor that links their C-terminus to the outer leaflet of the lipid bilayer (11,50,51) and is responsible for the apical delivery In Caco-2 cells, these proteins were found to be closely associated in membrane patches (22) that remain insoluble upon detergent treatment Interestingly, the transmembrane brush-border protein sucrase-isomaltase (SI) was found in these glycolipid 'rafts' as well (22) In the small intestinal brush-border membrane of the pig, not only SI, but also dipeptidyl peptidase IV, aminopeptidase N and aminopeptidase A were found in rafts, together with the GPI-anchored alkaline phosphatase (14) The newly synthesized brush border enzymes began to associate with detergent insoluble complexes while still in their transient, high mannose-glycosylated form, and their insolubility increased to that of the steady-state level soon after they achieved their mature, complex glycosylation, i e , after passage through the Golgi complex This shows that sorting of newly made brush-border membrane proteins into the glycolipid rafts takes place on their way through the Golgi apparatus (14) However, not all proteins destined for the apical membrane travel in rafts, for instance lactase-phlorizin hydrolase was essentially fully soluble in detergent (14) and might travel in separate vesicles Why some apical transmembrane proteins travel in rafts and others do not is still unclear, just as the exact mechanisms behind this apical transport

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Chapter 3

# Sucrase-isomaltase and lactase-phlorizin hydrolase[1]



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## 3.1 Introduction

Carbohydrates are an essential component of the human diet They consist of monosaccharides such as glucose and fructose, disaccharides like sucrose and lactose and complex polymers of saccharides such as glycogen and starch Because only monosaccharides can be absorbed by the intestine, di- and polysaccharides first have to be degraded For this degradation a number of enzymes exists each of which possess specificity towards certain chemical bonds between the monosaccharide constituents (37) Most of the chemical bonds in food-based carbohydrates are of the α-configuration The family of α-glucosidases is specialized in the hydrolysis of these bonds

This chapter will not only concentrate on a member of the α-glucosidase family, sucrase-isomaltase (SI), but also on lactase-phlorizin hydrolase (LPH), an enzyme that is capable of hydrolyzing β-linked disaccharides of galactose and glucose Both enzymes are expressed at the brush-border of epithelial cells in mammalian small intestine, but in most species not at the same time at comparable levels LPH is responsible for hydrolysis of lactose, the main carbohydrate in milk, and the expression levels of LPH are therefore highest in newborns This expression decreases during the period of weaning, when the carbohydrate source switches The expression of SI, that is responsible for the hydrolysis of sugar and some degradation products of starch, increases markedly to adult levels in this period (22) Caucasian people and some isolated populations form an exception on this expression pattern because their LPH expression remains high throughout life

SI and LPH share a number of similarities, but are at the same time very different Both LPH and SI are transmembrane glycoproteins possessing two active sites, and both are cleaved during their processing in the intestine LPH possesses both sites at one mature polypeptide chain, whereas SI activity is divided over two chains that are non-covalently attached SI is a type II transmembrane protein with an uncleaved signal sequence, LPH possesses a C-terminal membrane anchor Both proteins are transported via the secretory pathway directly to the apical membrane SI is probably transported in glycolipid 'rafts', LPH not (8) Because of these similarities and differences, LPH and SI form an interesting couple for the study of several aspects of apical transport All these features will be discussed in the following paragraphs, and more First the main characteristics of SI will be described, after which the major features of LPH will be dealt with

## 3.2 Sucrase-isomaltase

### 3.2.1 Protein structure

The 2D- and 3D-structure of SI is not completely known yet In figure 1, features of the protein structure are depicted that are known or that can be predicted from homologies to other proteins At the amino terminus of the SI polypeptide, a number of positively charged amino acids are followed by a hydrophobic stretch of 20 amino acids (Fig 1) This sequence functions both as membrane anchor and as a signal sequence for translocation into the ER (15) The polypeptide is not released from this signal peptide because it is not susceptible to signal peptidases (15) (see also fig 2 B, chapter 2) It remains attached to the membrane as a type II transmembrane protein

After the signal anchor, a serine threonine rich sequence of 17 residues forms the stalk on which the globular, catalytic domains are directed into the intestinal lumen (19) Such a stalk has been found in many brush-border proteins (38), and is responsible for the 3-3 5 nm distance between the membrane and the isomaltase domain as has been determined by electron microscopy (3) Danielsen et al (1989) proposed that the distance between the membrane and the bulk

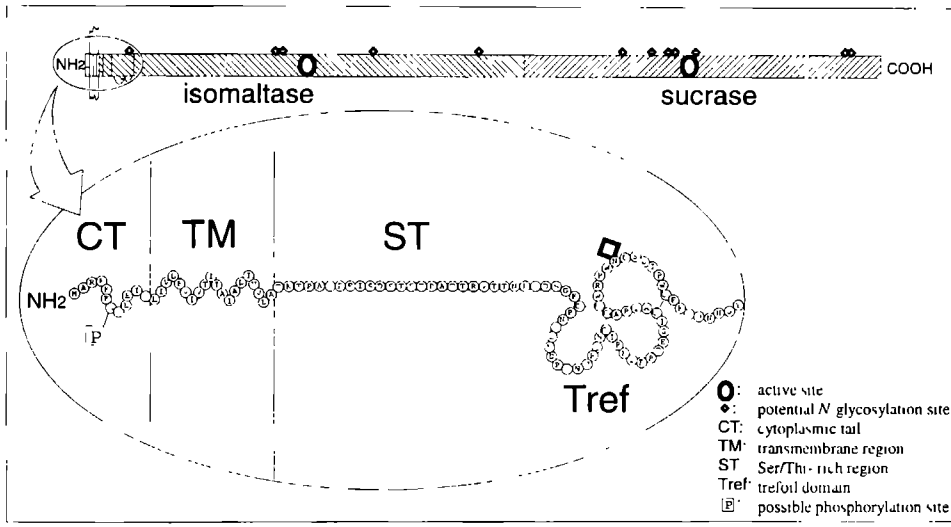


**Fig. 1:** Representation of the structural features of sucrase-isomaltase. Potential N-glycosylation sites can be predicted on NXT or NXS sequence motifs, where X represents any amino acid except P. The trefoil domain and the possible phosphorylation site were predicted according sequence homologies.

protein provides space to the external parts of molecules buried deeper in the membrane (e.g. different types of transport molecules) (4). In analogy with comparable Ser/Thr rich regions in other proteins (36,43,44,47), the stalk is *O*-glycosylated, probably for protection against pancreatic proteases (19).

The stalk is followed by a domain in which three internal disulfide bonds can be predicted that cause a folding of the domain into a trefoil conformation (18). The exact function of such a so-called P-domain, that has been found on several intestinal peptides (18), is not known yet, but a role in the maintenance of surface integrity and pathology of mucus epithelia has been suggested (14,18), possibly by interacting with the *O*-linked carbohydrates of mucins (34).

Following the P-domain, there are two globular subunits: isomaltase and sucrase. The first possesses activity towards 1,6-α-D-glucosidic linkages in isomaltose and dextrins produced from starch and glycogen digestion by α-amylase. The second subunit is responsible for sucrose and maltose digestion by an α-D-glycosidase type action. The active sites are localized around Asp 505 in the isomaltase and Asp 1394 in the sucrase subunit. Both Asp residues play an important role in the catalytic mechanism (2).

### 3.2.2 Biosynthesis and apical transport

Human SI is synthesized as a single polypeptide of ~185 kDa (27). In the ER, *N*-linked high-mannose oligosaccharides are attached to the polypeptide. The potential glycosylation sites are depicted in fig. 1. When the polypeptide passes the Golgi cisternae, the high mannose, oligosaccharides are trimmed and converted to a complex type of sugar chains, and *O*-linked sugars are attached. After these modifications, the enzymatically inactive high-mannose SI (41) becomes active (10). The apparent molecular weight of this complex glycosylated SI is 215 kDa (27). After sorting in the trans Golgi network, SI is transported to the apical membrane. In the intestinal lumen, pancreatic proteases cleave the precursor into its subunits (5,11,12,25,39,40), which remain non-covalently attached (1,6,19).

One or more of the *N*-glycosylated residues at the ectodomain of SI could be

important for its apical sorting. However, which of the potential *N*-glycosylation sites that are encoded by Asn-X-(Ser/Thr) is indeed glycosylated still has to be established, just as the role each site plays in the apical delivery

### 3.2.3 Congenital Sucrase Isomaltase Deficiency (CSID)

A deficiency of SI results in osmotic diarrhea when sugar or starch is ingested, because only the component monosaccharides that are products of hydrolysis can be absorbed. Hauri et al (13) isolated SI from biopsy specimens from a child with congenital SI deficiency. The molecule had the same size as the high-mannose form in control biopsies. Furthermore, SI could not be identified at the brush-border membrane by immuno electron microscopy, whereas control proteins (LPH, aminopeptidase N) were normally expressed Therefore, it was suggested that the deficiency was a consequence of a defect in intracellular transport of SI

Until now five different phenotypes of sucrase-isomaltase deficiency have been identified (7,13,33). Phenotypes I and II are characterized by an intracellular accumulation of mannose-rich SI in the ER and the Golgi respectively In phenotype III a transport-competent, but enzymatically inactive SI is expressed. Phenotype IV expresses a partially folded, mannose-rich SI molecule that is missorted to the basolateral membrane. Finally, phenotype V reveals a SI species that undergoes intracellular degradation leaving behind the isomaltase subunit that is correctly targeted to the brush border membrane (see also fig. 1, chapter 1)

In chapter 4 of this thesis the analysis of a biopsy specimen from a patient with CSID is described in which SI is located in the ER, the intermediate compartment and the cis-Golgi In this case of Phenotype II CSID a single point mutation is responsible for the impaired transport behavior of SI. This mutation, A-to-C at nucleotide 3298 in the coding region of the sucrase subunit of the enzyme complex, leads to a substitution of proline for glutamine-1098 (Q1098P) (33).

More detailed analysis showed that the Q1098P substitution is not only functional in intestinal epithelial cells but produces a similar phenotype when expressed in non-polarized COS-1 cells. Furthermore, position 1098 in the amino acid sequence is situated in a highly homologous region that shares homology to many other members of the glucosidase family (17,28) In one of these members, human lysosomal α-glucosidase, the same Q→P substitution was introduced at the corresponding position. This mutant α-glucosidase showed a similar phenotype when expressed in COS-1 cells. Therefore, (i) the Q1098P mutation in SI is responsible for the generation of the CSID phenotype, (ii) cellular factors specific for epithelial cells are not responsible for this CSID phenotype and (iii) the subdomain in which the mutation is localized is critical for the intracellular transport of not only SI, but of lysosomal α-glucosidase as well (26). Altogether, not only this mutation, but also mutations causing other cases of CSID could provide more insights in general transport and/or control mechanisms.

### 3.2.4 Additional functions

Recently, it was shown that rabbit sucrase isomaltase contains binding sites for *Clostridium difficile* Toxin A (35). Binding of this toxin to α-galactosyl residues on SI resulted in biological effects in the ileum and in CHO cells expressing rabbit SI. The latter cells showed an increase in the intracellular calcium levels as a response to the binding of the toxin (35). Such an increase is usually found for membrane receptors of the class of G-protein associated receptors with seven membrane-spanning domains. SI is not related to this family, but shares structural features with a group of G-protein associated receptors with single membrane-spanning domains (23,31,42). One member of this family is the sperm receptor protein ß-1,4

galactosyltransferase that binds *O*-linked oligosaccharides on the glycoprotein ZP3 on the egg zona pellucida (9). This binding induces a signal transduction cascade that results in the allowance for the sperm to entrance into the zona pellucida. The cytoplasmic domain of this galactosyltransferase consists of 24 amino acids with serine and threonine residues that are phosphorylated as a response to ligand binding (9). Interestingly, the 12-amino acid cytoplasmic tail of SI contains a serine at position 6 that can be phosphorylated by cAMP-dependent protein kinase in vivo and in vitro (21). This serine is present not only in rabbit SI, but also at the same position in human and rat SI. If this serine indeed becomes phosphorylated upon Toxin A binding has not been investigated yet, but remains an interesting point in the quest for interacting heterotrimeric G-proteins and putative cellular signalling functions of SI.

## 3.3 Lactase-phlorizin hydrolase

### 3.3.1 Protein structure

LPH is a type I transmembrane glycoprotein that contains both β-galactosidase and β-glucosidase activities within one polypeptide. At the C-terminal end LPH has a hydrophobic membrane anchor followed by a short hydrophilic stretch. This stretch was presumed to be cytoplasmic, but Keller et al have shown by phosphorylation experiments that this region is presumably directed into the lumen, and therefore in the same direction as the N-terminal region (20). Although these experiments were performed on rabbit LPH, both tails could have the same orientation since rabbit and human LPH are highly homologous.

Between the N-terminal signal sequence and the C-terminus in LPH four homologous regions are present that have probably evolved by two cycles of partial gene duplication (24). The first two regions, I and II, do not possess the Glu residue that was shown to be essential for the active sites in mature LPH. Region III and IV both do contain this residue ($Glu_{1273}$ and $Glu_{1749}$, resp.), embedded in the glycosyl hydrolase consensus sequence (16), and possess catalytic activity. Region III was shown to be responsible for the degradation of lactose, while region IV possesses the phlorizin hydrolase activity (45). In fig. 2, the structural features of LPH are depicted.

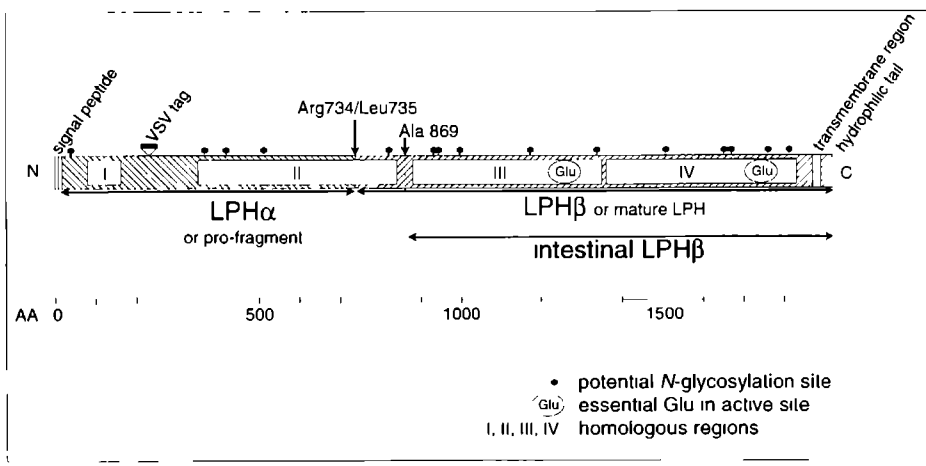


**Fig. 2** Representation of the structural features of lactase-phlorizin hydrolase Potential N-glycosylation sites can be predicted on NXT or NXS sequence motifs, where X represents any amino acid except P Arg734/Leu735 represents the cleavage site between LPHα and LPHβ Ala869 represents the N-terminal amino acid of intestinal LPHβ

### 3.3.2 Biosynthesis and processing

LPH is synthesized as a 1927 amino acid precursor, prepro-LPH. The first 19 amino acids of this precursor form the signal sequence that is cleaved off in the ER by signal peptidases (similar to fig. 2 A in chapter 2). The remaining 1908 amino acids form pro-LPH, which is complex- and *O*-glycosylated on its way through the Golgi apparatus. A subsequent cleavage takes place after export from the trans-Golgi (29) and results in the 160 kDa LPHβ and a stretch that is called the pro-fragment or LPHα (30). This cleavage site was assumed to be localized between $Arg_{868}$ and $Ala_{869}$ (24), but recently Wütrich et al (46) showed that proLPH is cleaved between $Arg_{734}$ and $Leu_{735}$. LPHβ, that contains both regions III and IV, is expressed at the apical membrane, where it is trimmed by luminal trypsin in the small intestine to the intestinal form of LPHβ (LPHβ), that has $Ala_{869}$ at its N-terminus (46). In contrast to LPHβ, the fate of LPHα after cleavage is not clear.

LPHα has previously been isolated from intestinal biopsy samples using an antibody directed against the 12 amino acids directly after the signal peptide (S20-T31) (30). The molecular mass of the immunoprecipitated pro-fragment in these experiments was ~100 kDa (30). Because none of the used glucosaminidases (endoH and endoF/GF) influenced the apparent molecular weight, it was concluded that none of the 5 consensus *N*-glycosylation sites were glycosylated (30). It was shown that LPHα does not form a stable complex with LPHβ after cleavage in intestinal biopsy specimens (30). Where exactly the cleavage takes places as well as what happens to LPHα after cleavage is still obscure.

Individual expression of LPHβ in COS-cells resulted in a protein that did not reach the cell surface and accumulated intracellularly (30,32). Co-expression of separate LPHα with the LPHβ construct, did not improve the transport competence of LPHβ, but resulted in a comparable accumulation as observed when LPHβ was expressed individually (32). These results proposed a role for LPHα as an intramolecular chaperon (30,32). Both reports however are based on experiments using a truncated cDNA construct coding for only the intestinal LPHβ (from $Ala_{869}$ to the C-terminus). It could be that expressing the complete LPHβ (from $Leu_{735}$ to the C-terminus) would lead to different results, for instance to a transport-competent molecule.

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# Congenital sucrase-isomaltase deficiency:

## Identification of a glutamine to proline substitution that leads to a transport block of sucrase-isomaltase in a pre-Golgi compartment.[1]

**Abstract**

Congenital sucrase-isomaltase deficiency (CSID) is an example of a disease in which mutant phenotypes generate transport-incompetent molecules Here, we analyze at the molecular level a phenotype of CSID in which sucrase-isomaltase (SI) is not transported to the brush border membrane but accumulates as a mannose-rich precursor in the ER, ERGIC and the cis-Golgi, where it is finally degraded A 6 kb clone containing the full length cDNA encoding SI was isolated from the patient's intestinal tissue and from normal controls Sequencing of the cDNA revealed a single mutation, A/C at nt 3298 in the coding region of the sucrase subunit of the enzyme complex The mutation leads to a substitution of the glutamine residue by a proline at amino acid 1098 (Q1098P) The Q1098P mutation lies in a region that is highly conserved between sucrase and isomaltase from different species and several other structurally and functionally related proteins

This is the first report that characterizes a point mutation in the SI gene that is responsible for the transport incompetence of SI and for its retention between the ER and the Golgi

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## 4.1 Introduction

The highly organized cascade of processing and modification events along the biosynthetic pathway of secretory and membrane proteins has lead to the general concept of compartimentalization of the cell into structurally and functionally distinct organelles (1, 2, 3, 4). Conformational modifications of membrane and secretory proteins commence already during their translocation across the ER membrane and continue in the ER lumen (5, 6, 4). Among the most important modifications are those elicited by cotranslational glycosylation, inter- or intramolecular disulfide bond formation, and subunit assembly known as oligomerization (7, 8, 5) These events are considered to be rate-limiting along the exocytic pathway. However, modifications in the Golgi, such as acquisition of complex type of oligosaccharides, have been frequently reported to confer also effects on the transport kinetics of proteins to the cell surface and secretion into the external milieu (9, 10, 11, 12). Analysis and identification of the various steps of the secretory pathway and dissection of the molecular mechanisms adequate for efficient transport of membrane and secretory proteins to the cell surface has been successfully investigated in naturally-occurring mutant phenotypes of proteins with impaired targeting (for a review see: 13).

Congenital sucrase-isomaltase deficiency (CSID) has been used to characterize various steps in the biosynthesis, transport and sorting of sucrase-isomaltase as a model for cell surface membrane proteins (14, 15, 16, 17). CSID is an autosomal recessive disorder of the small intestine that is clinically manifested as a watery osmotic-fermentative diarrhoea upon ingestion of di- and oligosaccharides. Epithelial cells of patients with this disease lack the sucrase activity of the enzyme SI, while the isomaltase activity can vary from absent to practically normal.

SI is a type II integral membrane glycoprotein that is exclusively expressed in the small intestinal microvillar membrane (18) and is responsible for the terminal digestion of dietary sucrose and starch (for a review see 19). The glycoprotein comprises two subunits that are highly homologous and are thought to be derived from the same ancestral gene (20). These two subunits are associated with each other by strong non-covalent, ionic interactions (19, 21). SI is synthesized in the rough endoplasmic reticulum as a single chain mannose-rich precursor comprising both subunits (pro-SIh, 210 kDa) (22) It is transported to the Golgi apparatus at a relatively slow rate and does not form homodimers before ER exit (11). The strong homologies between the two main domains suggest that quasi-dimers or pseudo dimers (8) are formed, which are presumably sufficient for acquisition of transport-competence After modification of the N-linked glycans and O-glycosylation, SI is sorted to the apical membrane and cleaved *in situ* by lumenal pancreatic proteases to its two active subunits, sucrase and isomaltase (23, 22).

The molecular basis of CSID is still not elucidated However, it has been proposed that different molecular defects or mutations in the SI gene are responsible for CSID (24) This hypothesis has been strongly supported by the analysis of several cases of CSID, which has lead to the identification of five different phenotypes of SI (17, 14). Phenotypes I and II, for instance, are characterized by an intracellular accumulation of mannose-rich SI in the ER and the Golgi respectively. In phenotype III an enzymatically inactive, but transport-competent SI is expressed. Phenotype IV expresses a partially folded, mannose-rich SI molecule that is missorted to the basolateral membrane. Finally, phenotype V reveals a SI species that undergoes intracellular degradation leaving behind the isomaltase subunit that is correctly targeted to the brush border membrane. In this paper we analyzed

a biopsy specimen of CSID phenotype II and demonstrated that SI is located in the ER, the intermediate compartment and the cis-Golgi We show for the first time that a single point mutation is responsible for the impaired transport behavior of SI

## 4.2 Methods

*4 2 1 Antibodies*

Four epitope-specific monoclonal antibodies (Mabs) directed against sucrase, isomaltase or sucrase-isomaltase were used (25) These antibodies were products of the following hybridomas HBB 1/691, HBB 2/614, HBB 2/219, HBB 3/705 Mab anti-human lactase-phlorizin hydrolase (Mab anti-LPH) and Mab anti-human aminopeptidase N (Mab anti-ApN) were products of hybridomas HBB 1/909 and HBB 3/153 respectively (25) Mab anti-ERGIC-53 was a product of hybridoma G1/93 (26) All Mabs were generously provided by Dr H P Hauri, Biocenter, Basel, and Dr E E Sterchi, University of Berne, Switzerland

*4 2 2 Cell lines*

Caco 2 cells (ATCC HTB-37) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% heat inactivated Fetal Call Serum (FCS), 1% non-essential amino acids and antibiotics (all from Gibco BRL) Monkey Kidney COS-1 (ATCC CRL-1650) cells were cultured in DMEM supplemented with 10% FCS and antibiotics All cell lines were cultured at 37°C in a humidified 5% $CO_2$ incubator

*4 2 3 Processing of biopsy samples*

The patient with CSID had a lifelong history of abdominal pain, cramps and osmotic diarrhea upon ingestion of sucrose CSID was assessed by the breath hydrogen test and by sucrose tolerance test CSID was further confirmed by enzyme activity measurements of sucrase and isomaltase in intestinal biopsy homogenates (27) Control intestinal tissue was taken from patients screened for diagnostic purposes other than CSID Peroral suction intestinal biopsy samples from CSID and controls were taken from the upper jejunum and were histologically normal Five biopsy samples were obtained from each patient and immediately processed as follows One sample was cut into small pieces and fixed for immuno-electronmicroscopy (see later) One sample was frozen in liquid nitrogen for RNA preparation Three samples were biosynthetically labeled in organ tissue culture dishes (Falcon Division, Becton, Dickinson and Co ) on stainless-steel grids according to Naim *et al* (28) Here, the tissue was labeled continuously with 150 µCi of [$^{35}$S]methionine (trans-[$^{35}$S]methionine, >1000 µCi/mmol, ICN) for 0 5h, 4h or 18h After the labeling periods, the specimens were washed three times in RPMI 1640 (Gibco BRL) and homogenized at 4°C with a Teflon-glass homogenizer in 1 ml of homogenization buffer (25 mM Tris-HCl 50 mM NaCl, pH 8 1), and a cocktail of protease inhibitors containing 1 mM PMSF (phenylmethylsulfonylfluoride), 1 µg/ml pepstatin, 5 µg/ml leupeptin, 17 4 µg/ml benzamidine and 1 µg/ml aprotinin (all from Sigma) The homogenates were either further processed directly for immunoprecipitation or kept frozen at -20°C until use

*4 2 4 Synthesis and construction of cDNA encoding Pro SI from Caco 2 cells and from tissue of control individuals and CSID patients*

Reagents and methods for routine recombinant DNA techniques and polymerase chain reactions were as described previously (29 30) Cloning of the SI cDNA from the patient's mucosal cells, control biopsies and Caco-2 cells followed a similar strategy Total RNA isolation and cDNA synthesis were performed as described by Moolenaar *et al* (31) Total RNA was isolated from postconfluent Caco-2 cells using the guanidinium-isothiocyanate method (32) Randomly primed cDNA was synthesized from 10 µg total RNA by avian myeloblastosis virus reverse transcriptase (AMV-RT, Boehringer, Mannheim) mRNA was incubated for 60 min at 37°C in 50 mM Tris/HCl (pH 8 3), 40 mM KCl 6 mM $MgCl_2$, 2 5 mM DTT 1 mM of dNTP (Boehringer, Mannheim) in the presence of 200 ng random primers and 25 units of AMV-RT The negative control underwent the same procedure, only AMV-RT was excluded from the reaction mixture For each PCR reaction 1/10 (2 µl) of the reaction mixture was used as a template PCR reactions were performed as described by Innis *et al* (33) with minor

modifications. Briefly, cDNA templates were amplified in a total volume of 100 µl containing, 10 mM Tris/HCl (pH 8.3), 25 mM KCl, 1.5 mM $MgCl_2$, 140 mM of each dNTP, 700 nM of primer and 2 units of Taq polymerase (Boehringer, Mannheim) Double stranded DNA was dissociated at 94°C for 45 sec, the annealing step was at 55°C for 1 min and the extension step at 70°C for 1.5 min. The reactions were subjected to 30 cycles of amplification Some of the primers used in the PCR reactions were designed based on the published sequences of the rabbit and human SI cDNA (34, 35) Table 1 shows the position of these primers within the SI cDNA. The products of two independent PCR reactions were directly or after cloning into pGEM4Z sequenced with a DNA sequencing kit according to the instruction of the manufacturer (Sequenase V. 2 0, United States Biochemical Corporation, Cleveland, OH).

The various DNA fragments of wild type SI cDNA were ligated together and cloned into the expression vector pCB7 (36) to generate the plasmid phSI encoding wild type pro-SI Sequencing of the DNA fragments obtained by PCR from the CSID-tissue revealed a single mutation A/C at nucleotide 3298. This mutation was found in the PCR product encompassing nucleotides 2967-4201. A 619 bp (NcoI-ApaI) fragment containing this mutation was purified and exchanged with its counterpart in the wild type phSI plasmid The generated plasmid was denoted phSIA/C

*4 2 5 Transfection and metabolic labeling of COS-1 cells*

COS-1 cells were either transfected by the DEAE-dextran method as described by Naim *et al* (29) or via electroporation in a Biorad gene pulser a 0.4 cm cuvet containing 200 µl ice-cold PBS with 1 5 million cells and 10 µg DNA was exposed to 0 3 kV and 125 µF. Transiently transfected COS-1 cells were metabolically labeled with 80 µCi L-[$^{35}$S]methionine (ICN) as described by Naim *et al* (29) After the labeling period, the cells were washed and stored at -70°C until use

*4.2.6 Immunoprecipitation*

Homogenates of biosynthetically-labeled biopsy specimens were lysed on ice by addition of Triton X-100 and sodium deoxy cholate to final concentrations of 0 5% each (28). Biosynthetically labeled transfected COS-1 cells were lysed at 4°C for 1 h in lysis buffer (25 mM Tris-HCl, pH 8 0, 50 mM NaCl, 0.5% Triton X-100, 0 5% sodium deoxy cholate and a mixture of protease inhibitors containing 1 mM PMSF, 1 µg/ml pepstatin, 5 µg/ml leupeptin, 17.4 µg/ml benzamidine and 1 µg/ml aprotinin). Usually 1 ml ice cold lysis buffer was used for each 100-mm culture dish (about 2-4 x $10^6$ cells). Detergent extracts of cells or biopsy samples were centrifuged for 1h at 100,000 x g at 4°C and the supernatants were immunoprecipitated as described by Naim *et al* (17, 22, 28, 29) Four epitope-specific monoclonal antibodies directed against sucrase, isomaltase or sucrase-isomaltase were used (25). These antibodies were used in the form of ascites Usually, 0 5 - 1 µl ascites was used for each immunoprecipitation For epitope mapping studies with four Mabs directed against SI the extracts of biopsy samples were divided into equal aliquots and each aliquot was immunoprecipitated with a different antibody.

**Table 1**

*Oligonucleotides utilized to synthesize various cDNAs by polymerase chain reaction*

| DNA-fragment (bp)* | primers (5'-3' direction) |
|---|---|
| -29 - 695 | CCGGGTACCAGCCTTATCCAAGTCTGTGAGATCTGTAAGTACTGGTCA |
| 652 - 1366 | GCATTGGTCCCTTAGTGTACATCCACACATGTTGTGTGTT |
| 1299 - 2066 | ATAGGTCGACGTGCCAATGGCTGCCTTGATGATTTAACCA |
| 1952 - 3139 | GATGCAACTTGGGGCATTTGGTACTTCATATCTCTTCTT |
| 2967 - 4201 | GCTCGCTATTCATCCATGGGGATGGCTCATTCATATCAAT |
| 4071 - 4923 | TCCAGAGCTCATGTAGCTTTCGTACTGGGGTAACCATAAATGCT |
| 4876 - 5555 | TATTCAAGCAGTTCTTATGGTGTAAGTGCTGTGAAACTT |

*bp numbering according to Green *et al* (33).

*4 2 7 Immunofluorescence*

Cellular localization of expressed proteins in COS-1 cells was studied with cells grown on coverslips. Cells were fixed with 3% paraformaldehyde and immunolabeling was carried out after 0 1% Triton X-100 permeabilization, using Mab anti-SI (HBB1/614) antibody (1 1000), Mab anti-ERGIC 53 (G1/93) (1 100) as the primary antibodies The secondary antibodies employed fluorescein-isothiocyanate-conjugated goat anti-mouse or anti-rabbit IgG (1 100), Texas Red conjugated goat anti-mouse or anti-rabbit IgG (1.200) (all were from Boehringer, Mannheim) Cell surface localization of proteins was assessed in transfected cells that were incubated on ice with the primary antibody prior to fixation and without incubation with Triton X-100. The cells were visualized with a confocal laser microscope using a double channel for fluorescein isothiocyanate and Texas Red and/or with a routine fluorescence microscope.

*4.2 8 Immunoelectron Microscopy*

Biopsy samples were cut into small pieces and fixed in a mixture of 2% formaldehyde and 0.1% glutaraldehyde for 1h at room temperature They were then stored in 2% formaldehyde until use Cryosectioning and immunolabeling with anti-SI, anti-ERGIC 53 and protein A-gold were performed as described previously (26, 37)

*4 2.9 Other Procedures*

SDS-PAGE was performed according to the method of Laemmli (38). High molecular weight markers from Sigma were used as a reference. Treatment of immunoprecipitates with Endo-N-acetylglucosaminidase H (Endo H), Endo-N-acetylglucosaminidase F/glycopeptidase F (Endo F/GF, also known as PNGase F) (both from Boehringer Mannheim) was done as described before (28)

## 4.3 Results

Initial assessment of CSID in the patient was achieved by the breath hydrogen test. Subsequently a biopsy specimen was taken from the upper jejunum and enzymatic activity measurements of the disaccharidases, SI and lactase-phlorizin hydrolase (LPH) were performed. Here, no activity of sucrase or isomaltase could be detected, while the activity of LPH was in the normal range of the control tissue (16-32 IU/mg protein). Likewise, normal activity of another control brush border enzyme, aminopeptidase N (ApN) was revealed in the patient's biopsy sample. The results therefore pointed to an enzymatic defect that is restricted to SI Previous analyses of several cases of SI deficiency have demonstrated that SI is synthesized as in normal controls, but is not correctly processed or transported along the secretory pathway (14, 17). We therefore set out to analyze the biosynthesis and processing of SI in the patient's tissue. Metabolic labeling of biopsy samples and immunoprecipitation of the detergent extracts with four different epitope-specific monoclonal anti-SI antibodies revealed after 30 min of labeling an Endo H-sensitive 210-kDa polypeptide that corresponds to mannose-rich pro-SI (Fig. 1, lanes 1, 2 and 3). This polypeptide remained the predominantly labeled species after 4 h and 18 h of labeling (Fig. 1, lanes 4-9). In the control biopsy specimen, by contrast, the Endo H-sensitive mannose-rich pro-SI (Fig. 1, lanes 10, 11 and 12) was processed to a complex glycosylated Endo H-resistent form after 4 h of labeling (Fig. 1, lanes 13-15). When comparing the metabolic labeling intensity of the patients material after 4 h and 18 h of labeling no increase is observed as compared to the control (Fig. 1 lanes 4-6, 7-9 versus lanes 10-12, 13-15) From these data we conclude that no processing of the patient's SI in the Golgi apparatus has occurred, and that the protein is finally degraded.

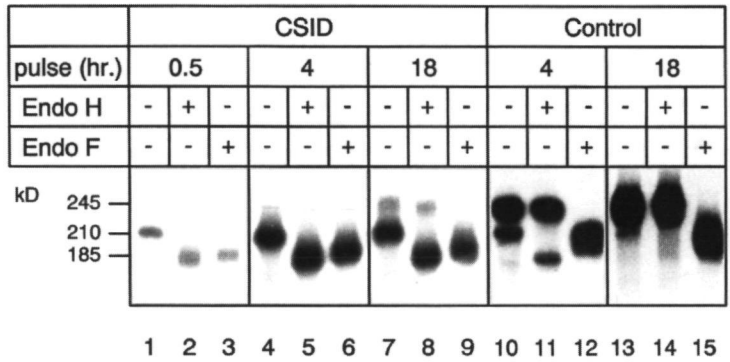


**Fig. 1** *Molecular forms of SI in CSID and in normal control biopsies.*
Biopsy samples from patient with CSID (lanes 1-9) and normal control (lanes 10-15) were biosynthetically labeled for the indicated times with $^{35}$Smethionine. The specimens were homogenized, solubilized and immunoprecipitated with monoclonal anti-SI antibodies. The immuno-precipitates were subjected to SDS-PAGE on 5% slab gels without treatment (lanes 1, 4, 7, 10, 13) or after Endo H (lanes 2, 5, 8, 11, 14) or Endo F/GF (lanes 3, 6, 9, 12, 15) treatment.

To determine whether the impaired processing is the consequence of general cellular defect or is restricted to SI, the transport of other brush border proteins was investigated. ApN and LPH were synthesized and processed in the patient's intestinal cells in a fashion similar to control cells (Fig. 2, lanes 1-8). Thus, within 1 h of chase ApN was detected in the patient's biopsy sample as an Endo H-sensitive mannose-rich 130-kDa polypeptide, which was converted into an Endo H-resistant 160-kDa polypeptide after 20 h of chase (Fig. 2, lanes 3 and 4). Similarly no changes in the biosynthetic pattern of LPH could be observed. Here, LPH was synthesized as a mannose-rich species of Mr = 215,000 that was cleaved intracellularly to the mature 160-kDa polypeptide (Fig. 2, lanes 7-8). Therefore the failure of mannose-rich SI to mature to an Endo H-resistant is indeed restricted to this molecule and is not the result of a general cellular defect since two control proteins, ApN and LPH, were

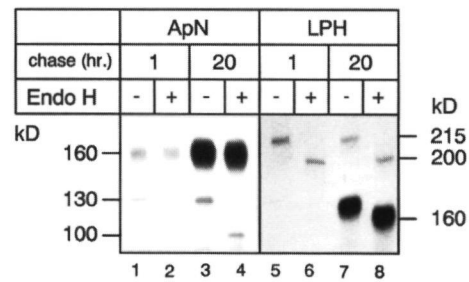


**Fig. 2:** *Biosynthesis of control brush border glycoproteins in CSID biopsies.*
Lysates of biopsy samples were immuno-precipitated with mAb anti-ApN (lanes 1-4) or mAb anti-LPH (lanes 5-8). The immuno-precipitates were analyzed by SDS-PAGE on 5% slab gels without treatment (lanes 1, 3, 5, 7) or after Endo H (lanes 2, 4, 6, 8) treatment.

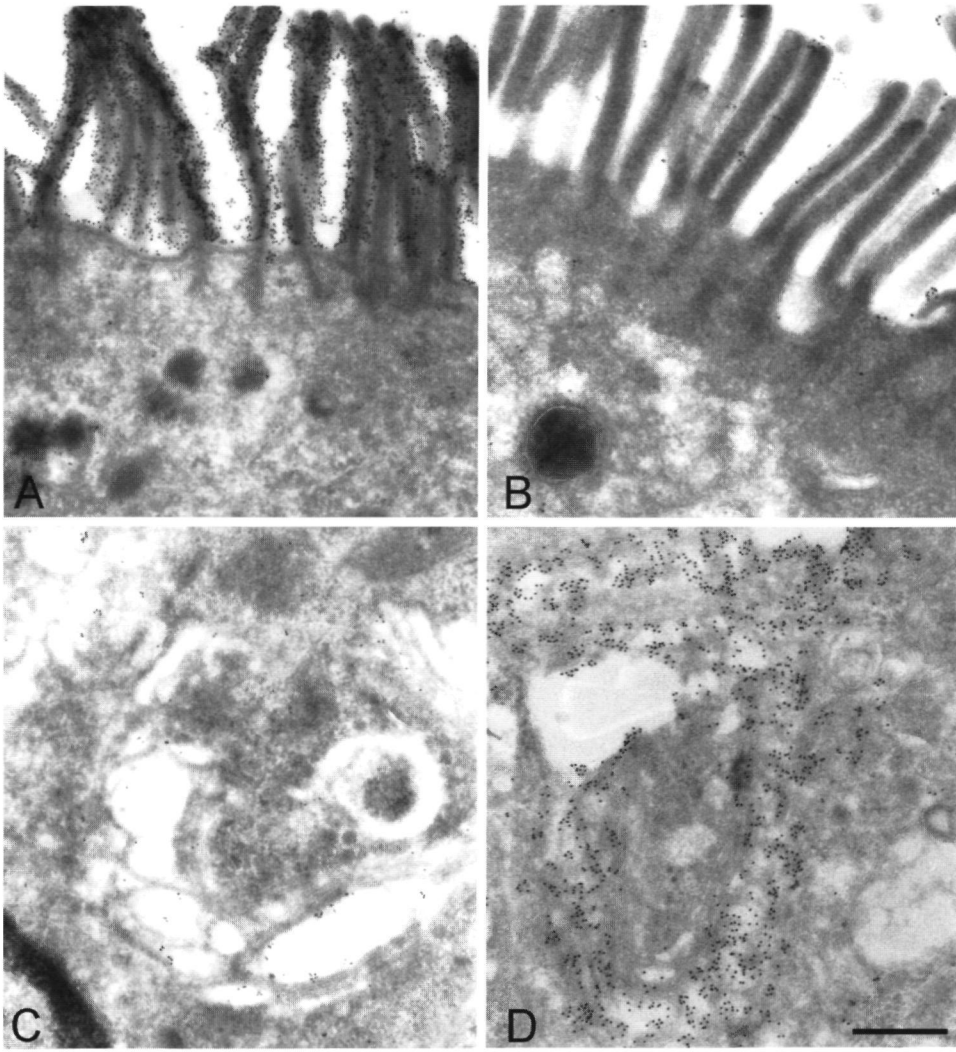


**Fig. 3** *Immunocytochemical localization of SI in CSID and normal control.*
Sucrase-isomaltase was localized in ultrathin cryosections from a control biopsy (A and C), and from the patients biopsy (B and D), using antibody HBB 2/614, followed by rabbit anti-mouse and protein A-10 nm gold staining. **A**. Brush border staining observed in a control biopsy; **B**. brush border staining observed in the patients biopsy; **C**. Golgi staining observed in a control biopsy; **D**. Golgi staining observed in the patients biopsy. Note the strong accumulation of SI in this compartment. Also note that always a few cisternae on one side of the Golgi apparatus (arrows) are devoid of label.
Bar = 0.5 μm.

processed to their mature forms and transported normally in the patient's tissue.

Since SI is synthesized at almost normal levels in the patient's tissue, but does not mature we wanted to determine the subcellular localization of SI in the patient's epithelial cells. Here, ultrathin cryosections of the patient's tissue were incubated with monoclonal anti-SI antibodies followed by protein A-gold. The subcellular localization

was finally assessed by electron microscopy (Fig. 3). While most of the labeling in the control intestinal cells was confined to the brush border membrane, no labeling of the brush border membrane in the patient's tissue could be detected (Figs. 3A and 3B). Instead, intense labeling of the ER and Golgi cisternae was observed in the patient's tissue (Fig. 3D), while the Golgi apparatus and the ER of the control were weakly labeled (Fig. 3C). The latter case reflects the normal steady state situation in biopsy samples (14). Finally, both specimen, the patient's as well as the control, did not show any labeling of the basolateral membrane

The SI labeling in the patient's cells was visible on one side of the Golgi (Fig. 3D arrows) and corresponds most likely to the cis-Golgi and the ER-Golgi intermediate compartment (ERGIC). This is based on the labeling pattern obtained when biopsy samples are labeled with antibodies against ERGIC-53, a marker of the cis-Golgi as well as ERGIC (Fig. 4). It should be noted that in the patients biopsy material more label was observed for ERGIC-53 then in the control biopsies (compare Figs. 4A and 4B). In addition ERGIC-53 was also observed in the cisternae on one side of the Golgi apparatus in contrast to control biopsies where only a tubulo-vesicular structure on one side of the Golgi was labeled (Fig. 4A; 26). Based on morphological criteria we conclude that the labeling of ERGIC-53 in the patients biopsy coincides with the label of SI (compare Figs. 3D and 4B), although a direct double labeling of SI and ERGIC-53 proteins could not be performed. The antibodies used to detect these proteins are both Mabs and these are difficult to combine in immuno-gold double staining experiments. Control experiments using antibodies against the brush border glycoproteins lactase-phlorizin hydrolase and aminopeptidase-N demonstrated localization of these proteins in the apical membrane (not shown).

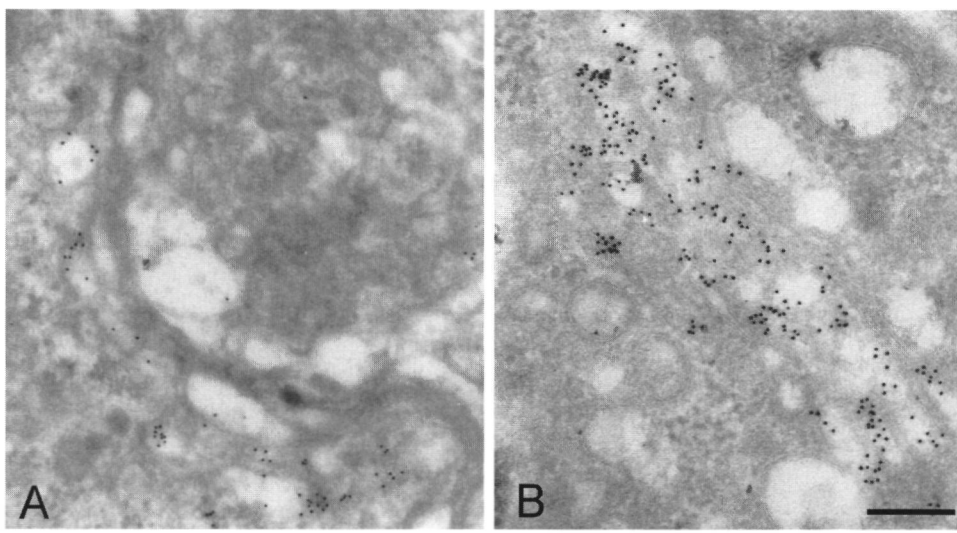


**Fig. 4:** *Immunocytochemical localization of ERGIC-53 in CSID and normal control.*
Localization of ERGIC-53 was performed on ultrathin cryosections from a control biopsy (A) and from the patients biopsy (B) using antibody G1/93, followed by rabbit anti-mouse and protein A-10 nm gold staining. **A**. In the control biopsy vesicular structures on the cis-side of the Golgi apparatus are stained; **B**. In contrast, in the patients biopsy the labeling is not only stronger, but also additional staining over cisternae on one side of the Golgi is observed. Morphologically the label coincides with that observed for sucrase-isomaltase (compare with fig. 3D). Bar = 0.25 µm.

Altogether, the data demonstrate that in the patient's biopsy the SI molecule has egressed the ER and is arrested in an ERGIC-53 positive, cis-Golgi compartment where it is finally degraded. The phenotype therefore resembles phenotype II of CSID described previously (17).

It is likely that the transport block of SI in the ERGIC, cis-Golgi and ER as well as the persistence as a mannose-rich polypeptide are the consequence of structural alterations in the SI molecule. To examine this possibility, we performed epitope mapping using four different epitope-specific monoclonal anti-SI antibodies, HBB 2/614, HBB 2/219, HBB 1/691 and HBB 3/705. Except for Mab HBB1/691 all other antibodies were able to recognize epitopes on mutant SI (not shown). The results imply that the epitopes of HBB 1/691 and 2/219 did not mature in the patient's SI and/or was altered. Drastic alterations in the protein folding of SI did not presumably take place, since three out of four antibodies have recognized the mutant SI.

To analyze the molecular defects in this phenotype we cloned the full length cDNA encoding wild type SI from Caco-2 cells and from the patient's biopsy sample. Construction of full length cDNA encoding wild type SI was performed by combining two approaches. The first employed conventional screening of a lgt-11 expression library made from the colon carcinoma cell line, Caco-2. Here, a clone comprising 1486 bp from nt. 550-2036 (bp numbering according to Green *et al* (35)) and covering almost 50% of the isomaltase subunit was isolated. This clone was denoted IM21.1 (Lacey, unpublished). In the second approach we made use of the published sequences of rabbit and human SI (20, 34). Several oligonucleotides were designed and used to amplify SI DNA fragments using RT-PCR on RNA extracted from Caco-2 cells or biopsy samples. These fragments and the IM21.1 clone were ligated together to generate the full length pro-SI cDNA Sequencing of this clone revealed two nucleotide differences versus the published sequence (34). These differences are not due to PCR artifacts, as assessed by sequencing of PCR products of two independent PCR reactions. One difference is A/G at nucleotide 4001 which represents a silent mutation. The second variation, a C/G at nucleotide 3612, leads to a glutamic acid instead of glutamine at amino acid 1203 in the sucrase subunit. Comparison with the amino acid sequences of rat, rabbit and our patients (see below) sucrase subunits revealed also a glutamic acid residue at this particular position. Moreover, the human and rabbit isomaltase also contained a glutamic acid in the corresponding homologous stretch Therefore, it is likely that the glutamine/glutamic acid exchange represents a polymorphism in the published sequence

To test the full length cDNA, it was

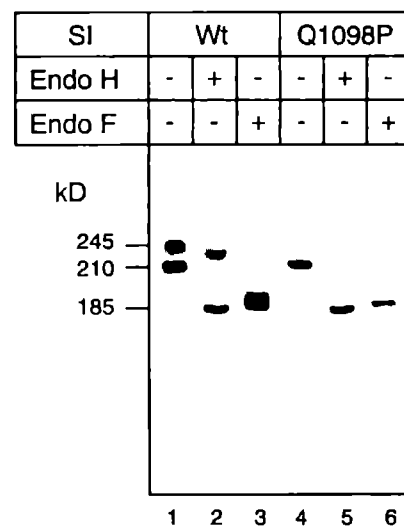


**Fig. 5:** *Expression of wild type and mutant SI cDNA in COS-1 cells*
COS-1 cells were transfected with phSI (lanes 1-3) or phSIa/c (lanes 4-6) plasmids and biosynthetically labeled with [$^{35}$S]methionine for 6h The cell extracts were immunoprecipitated with Mab anti-SI The immunoprecipitates were analyzed by SDS-PAGE on 5% gels without treatment (lanes 1, 4) or after Endo H (lanes 2, 5) or Endo F treatment (lanes 3, 6)

cloned into the pCB7 expression vector and transiently expressed in COS-1 cells. Metabolic labeling of these cells showed that SI is processed as in normal control samples (Fig. 5, lanes 1-3, compare with Fig. 1 lanes 10-15). Also when expressed in COS-1 cells, cell surface expression could be observed (Figs. 6A and B).

To isolate a full length SI cDNA encoding SI in the CSID tissue a similar strategy was followed as for wild type SI. The PCR products derived from two independent reactions were cloned and sequenced. The sequence analysis revealed one alteration relative to the SI cDNA cloned from Caco-2 cells. This alteration, A/C at nucleotide 3298, was found in the sequence encoding the S of the SI complex and resulted in a substitution of the glutamine 1098 by a proline (Q1098P). The mutation was confirmed by direct sequencing of several independent PCR products. The wild type sequence could never be identified, suggesting that both alleles of the SI-gene contain this mutation or, more likely, that one allele is not expressed.

The patient's cDNA was also cloned into the expression vector pCB7 and transiently expressed into COS-1 cells. Again metabolically labeled COS-1 cells showed that mutant cDNA is processed as in the patient's biopsy (Fig. 5, lanes 4-6, compare with Fig. 1, lanes 1-9). In addition,

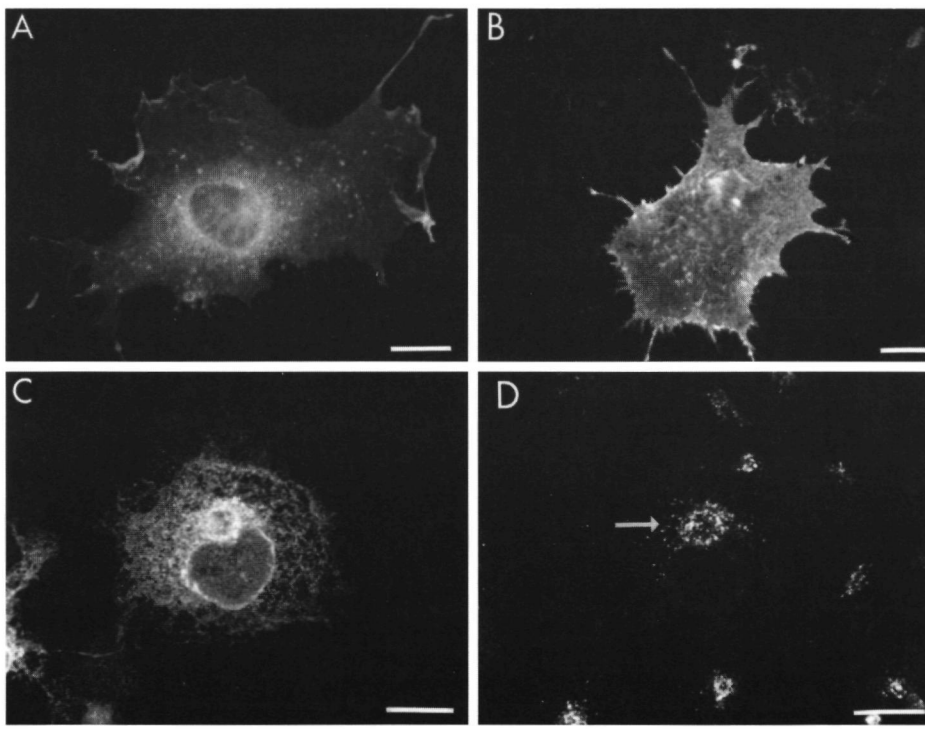


**Fig. 6:** *Immunofluorescence of wild type and mutant SI cDNA in COS-1 cells.*
COS-1 cells were transfected with control sucrase-isomaltase cDNA (A and B), and Q1098P mutant sucrase-isomaltase cDNA (C and D). **A**. control SI can be observed in the rER, Golgi and cell surface of COS-cells; **B**. cell surface staining of control SI, see materials and methods for details of the procedure. **C** and **D**. immuno-double labeling of SI and ERGIC-53 in a COS-cell transfected with mutant SI cDNA and observed using a confocal scanning laser microscope equipped with a Kr/Ar laser; **C**. immunostaining with HBB 2/614 anti-SI antibody in the ER and a ERGIC-53 positive compartment, note the absence of surface label (compare with fig. A); **D**. immunostaining with G1/93 anti-ERGIC-53 antibody, note the structure (arrow) that is labeled both for SI (C) and ERGIC-53 (D). Bar = 15 μm

localization studies show that the patient's SI is not expressed at the cell surface of COS-1 cells, but instead accumulates intracellularly in the ER and ERGIC, as shown by immuno double-labeling on transiently transfected cells (Figs 6C and D).

Upon comparison, lysosomal α-glucosidase, *Sch. occidentalis* glucoamylase, sucrase and isomaltase from various species share striking similarities in their amino acid sequences. This has lead to the hypothesis that these molecules are derived from a common ancestral gene (39). Sequence comparison revealed that the mutation Q1098P in the S subunit is located in a a stretch of 10 amino acids that is highly homologous among sucrase (aa 1093-1116), isomaltase (aa 219-243), lysosomal α-glucosidase (aa 239-262) and *Sch. occidentalis* glucoamylase (aa 196-220) (Fig. 7).

## 4.4 Discussion

Congenital sucrase-isomaltase deficiency (CSID) has been used to characterize various steps in the biosynthesis, transport and sorting of sucrase-isomaltase as a model for cell surface membrane proteins. Several different phenotypes of CSID have been meanwhile described (14, 15, 16, 17) In all these CSID-phenotypes the transport-incompetence, aberrant enzymatic function or missorting to the basolateral membrane are exclusively restricted to the SI molecule ruling out a possible general cellular defect.

While analyses of the current and previous phenotypes at the subcellular and protein levels lent strong support to the hypothesis that a point mutation in the cDNA of SI is responsible for the generation of CSID, the present data provide the first definite evidence that this is in fact the case Here, we could characterize a point mutation in the region encoding the sucrase subunit of the SI complex which converts a glutamine to a proline (Q1098P).

The CSID malabsorption is therefore another example of a clinical disorder or disease that ensues by single amino acid mutation in the polypeptide chain leading ultimately to dramatic alterations in the structural features, biosynthesis, posttranslational processing, transport and function of cell surface and secretory proteins. While the CSID phenotype usually ends up in mild malabsorption of sugars and starch, the symptoms of which are readily identifiable, other disorders, such as cystic fibrosis (40), collagen-based diseases, familial hypercholesteremia (41), just to name a few, are more severe (review: (13)).

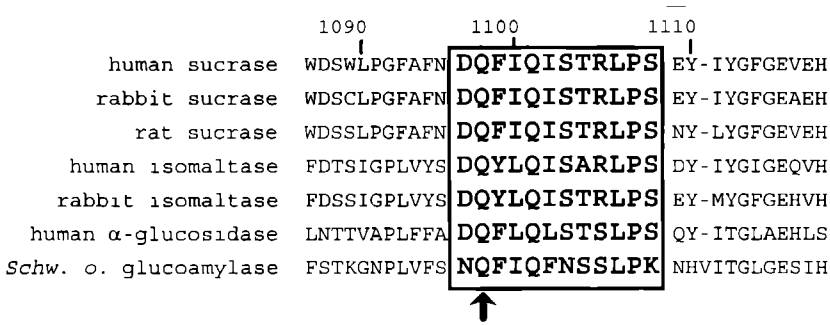


**Fig. 7:.** *Amino acid homology around the mutated region in the patient's SI*
The amino acid sequences of normal human sucrase (32), rabbit sucrase (20), rat sucrase (45), human isomaltase (32), rabbit isomaltase (20), human lysosomal α-glucosidase (43) and *Schwanniomyces occidentalis* glucoamylase (39) are aligned The numbering and arrow indicate the position in this highly conserved region where in the patients SI the Q1098P mutation is located.

A rather unusual mutant form of SI is generated in CSID phenotype II Here, SI exits the ER and is transported to the medial and trans-Golgi where it undergoes degradation (17) To our knowledge, no mammalian cell surface membrane glycoprotein has been so far identified that has efficiently egressed the ER and arrived in the Golgi cisternae where its transport is blocked This strongly suggests that there are other criteria, in addition to the acquisition of correct folding and quaternary structure in the ER, that may modulate and control protein transport within the Golgi and to the cell surface

The phenotype of SI described in this paper is very much similar to phenotype II (17) Analyses of the structural features of SI in this phenotype by epitope mapping and biosynthetic labeling reveal that incomplete initial folding of the SI precursor protein is responsible for the observed inefficient transport of the molecule along the secretory pathway An effect of the oligomeric state of SI could be excluded since human SI does not dimerise and exits the ER efficiently as a monomeric molecule (8)

The most interesting aspect of phenotype II is the ability of SI to egress the ER to the Golgi, while still in an incompletely or partially folded state The subsequent arrest and final degradation of SI in a cis-Golgi compartment or in the Golgi is unusual and, except for SI, has not been demonstrated for an endogenous protein destined for the cell surface This is surprising since membrane and secretory proteins are believed to acquire transport-competence in the ER and leave this organelle en route to their final destination

It is generally accepted that a quality control mechanism exists in the ER that sorts correctly folded from malfolded proteins and retains the latter in the ER (5, 6, 7, 42) How could phenotype II of CSID be accommodated within these general views and concepts?

Human lysosomal α-glucosidase (43) and the yeast *Schwanniomyces occidentalis* glucoamylase (39) share striking homologies with human, rat and rabbit S and I species and have been suggested to be evolved from a common ancestral gene (39) Interestingly the Q1098P mutation is located within a stretch of the S subunit that is homologous to lysosomal α-glucosidase and *Sch occidentalis* glucoamylase (Fig 7) These proteins are all synthesized and translocated into the ER where they are processed to transport-competent forms and are then transported along the secretory pathway to their final destinations While SI is sorted in the TGN to the apical membrane, lysosomal α-glucosidase is mainly transported to the lysosomes after phosphorylation in the cis Golgi and maturation in the medial and trans-Golgi *Sch occidentalis* glucoamylase is transported to the periplasmic space and partially secreted into the exterior milieu in yeast Obviously all three proteins share a common pathway from the ER to the Golgi and from there diverge to different destinations It is therefore conceivable that homologies in the polypeptide sequences should be implicated in the biological function or transport mechanisms common to all of these proteins Consistent with this is the observation that the regions containing the active catalytic centers of these proteins have almost identical amino acid sequences (34) If the mutation Q1098P does not initially induce gross conformational change in the S subunit, but rather alters a subdomain around the mutation that is critical for the intracellular transport of the SI protein or creates a retention signal, then a similar effect would be also expected if the mutation is introduced into the corresponding homologous regions of lysosomal α-glucosidase or *Sch occidentalis* glucoamylase

It is possible that in the particular case of proteins with multi-domain structure, such as SI, complete maturation of all protein domains is not absolutely required for

egression from the ER, whereby the folding of some domains may be more essential or critical than others in the context of the overall conformation SI is composed of two large homologous domains that may fold independently (unpublished) and it is reasonable to assume that the presence of the mutation in the sucrase subunit leads to alterations in its structure while the entire isomaltase subunit remains unaffected While these alterations could be tolerated by the quality control mechanism in the ER, they could be crucial for the further secretory pathway of SI via the Golgi to the cell surface Recent data with a temperature-sensitive mutant of VSV G protein have proposed a quality control mechanism outside the ER that prevents immature VSV G protein from traversing the Golgi (44) Mutant SI-phenotype II may be subject to a similar mechanism that operates via a putative retention signal created by the Q1098P mutation

We conclude that the simplest and most straight forward explanation for the phenotype described in this paper, is that the mutation Q1098P has created a signal or conformational change in the sucrase subunit which retains the enzyme complex in a cis-Golgi compartment, where it is finally degraded by an unknown quality control mechanism operating at a level beyond the ER

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# A mutation in a highly conserved region in brush-border sucrase-isomaltase and lysosomal α-glucosidase results in Golgi retention.[1]

**Abstract**

A point mutation in the cDNA of human intestinal sucrase isomaltase has been recently identified in phenotype II of congenital sucrase isomaltase deficiency (Ouwendijk et al, 1996, *J Clin. Invest* 97.633-641). The mutation results in a substitution of glutamine by proline at position 1098 (Q1098P) in the sucrase subunit. Expression of this mutant sucrase isomaltase cDNA in COS-1 cells results in an accumulation of sucrase isomaltase in the ER, intermediate compartment and the cis-Golgi cisternae similar to the accumulation in phenotype II intestinal cells An interesting feature of the Q1098P substitution is its location in a region of the sucrase subunit that shares striking similarities with the isomaltase subunit and other functionally related enzymes, such as human lysosomal acid α–glucosidase and *Schw occidentalis* glucoamylase. We speculated that the Q P substitution in these highly conserved regions may result in a comparable accumulation Here we examined this hypothesis using lysosomal α–glucosidase as a reporter gene Mutagenesis of the glutamine residue at position 244 in the homologous region of α–glucosidase to proline results in a protein that is neither transported to the lysosomes nor secreted extracellularly but accumulates in the ER, intermediate compartment and cis-Golgi as a mannose-rich polypeptide similar to mutant sucrase isomaltase in phenotype II

We propose that the Q1098P and Q244P mutations (in sucrase isomaltase and α–glucosidase respectively) generate structural alterations that are recognized by a control mechanism, operating beyond the ER in the intermediate compartment or cis-Golgi

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## 5.1 Introduction

Transport of proteins along the secretory pathway is a complicated multistep process involving numerous checkpoints that define the ultimate fate of a newly synthesized protein During recent years our understanding of the molecular events that are implicated in and that control the trafficking of secretory and membrane proteins from one cellular organelle to the other has evolved greatly (19,39,44,45) For instance proteins destined for the cell surface or for secretion into the exterior milieu are extensively modified in the ER before their further transport to the Golgi (for reviews see, (4,40)) The chemical structure of these proteins as well as their conformation become altered after they have been synthesized and translocated across the ER membrane (8) The transport-competent conformation that proteins acquire prior to their transport from the ER to the Golgi and beyond is generally dependent on the attainment of a correct folding and assembly into a proper quaternary structure (38) The observed variations in the transport rates of proteins from the ER to the Golgi are presumably linked to different kinetics of protein folding and oligomerization, for example, via chaperon-mediated events (9,20,49) Consequently, the ER-to-Golgi transport could be considered as being rate limiting along the secretory pathway Nevertheless, conformational maturation in the Golgi (21,26), for instance due to carbohydrate modification (29), or oligomerization (21,25), may also affect the transport rates from the Golgi to the cell surface This suggests that a rate-limiting step also exists at the level of the Golgi and may at least in part be responsible for the asynchronous intracellular transport of brush border enzymes (14,29,33,34,48) In general, proteins leaving the ER are subjected to a "quality control" mechanism that is responsible for sorting correctly folded from conformationally immature proteins, whereby misfolded proteins are retained and properly folded molecules leave the ER and traverse the Golgi on the way to their final destinations (12,20,36)

Several observations suggest that not all proteins conform to this general scheme Firstly, in congenital sucrase-isomaltase deficiency (CSID) two naturally occurring mutants of sucrase-isomaltase (SI) reveal folding patterns different from wild type SI, while both are able to leave the ER (6,31) In one case, the mutant SI undergoes intracellular arrest as an unprocessed mannose-rich precursor in the medial and trans Golgi (31), and the other phenotype is sorted to the basolateral, rather than the apical membrane (6) Secondly, several chimeric mutants of the hemagglutinin glycoprotein, which persist as monomers in contrast to the trimeric wild type molecule, reveal different folding patterns, and yet are able to egress the ER, in many cases with wild type kinetics (24) Finally, a temperature sensitive mutant of the vesicular stomatitis virus (VSV) glycoprotein (G protein) has been shown to recycle as a malfolded species between the ER, the intermediate compartment and the Golgi (13)

These findings raise questions as to whether "minimal folding requirements" exist, which should be fulfilled by secretory and membrane proteins before egress from the ER is tolerated Additionally, there could exist a quality control mechanism acting beyond the ER Studies on the transport of mutant glycoproteins could give insight into these questions

We set out to investigate the molecular characteristics and the fate of a naturally occurring mutant form of sucrase-isomaltase in which the substitution Q1098P causes arrest of SI in the cis-Golgi and intermediate compartment The same Q->P mutation was introduced by site-directed mutagenesis into the structurally homologous lysosomal α–glucosidase at position 244 in the evolutionary conserved

region Expression studies demonstrate that the Q->P mutation in both proteins has a similar effect. The structural alteration resulting from this mutation seems to be either recognized by a quality control mechanism operating beyond the ER or to have altered the recognition site for the hypothetical 'transport receptors' as described by Rothman and Wieland (45). Both scenarios result in a retention of the proteins in the intermediate compartment or cis-Golgi, where they are finally degraded.

## 5.2 Materials and Methods

*5 2 1 Cell lines*

Monkey kidney COS cells (ATCC CRL-1650) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics Caco-2 cells (ATCC HTB-37) were cultured in DMEM supplemented with 20% heat inactivated FCS, 1% non-essential amino acids and antibiotics (all from Gibco BRL Life Technologies). All cell lines were cultured at $37^0$C in a humidified 5% $CO_2$ incubator.

*5 2 2 Antibodies*

Four epitope-specific monoclonal antibodies directed against sucrase, isomaltase or sucrase-isomaltase were used (14). These antibodies were products of the following hybridomas. HBB 1/691, HBB 2/614, HBB 2/219, HBB 3/705 mAb anti-ERGIC-53 was a product of hybridoma G1/93 (47) Antibodies against human acid α–glucosidase comprised a product of hybridoma 43D1 (16) and a rabbit polyclonal antiserum which was generously provided by Dr. A.J. Reuser (42) Polyclonal anti-protein disulphide isomerase (PDI, (7) was kindly provided by Dr. N J. Bulleid, Manchester, UK, and polyclonal anti-SI was kindly provided by Dr. A. Zweibaum, INSERM U178, Paris, France

**Table 1**

| oligo | bp (17) | sequence 5'->3' |
|---|---|---|
| Glu1 | 217-234 (coding) | ACCATGGGAGTGAGGCAC |
| Glu2 | 960-940(non-coding, T950G) | CTGAAGGAACGGATCCGC AAA |
| Glu3 | 937-957 (coding, A950C) | TTCTTTGCGGATCCGTTC CTT |
| Glu4 | 1239-1222 (non-coding) | GTAGACATCCAGGATCCC |

*5 2 3 cDNA probes*

Full length wild-type and Q1098P mutant SI cDNA's (37) were subcloned into the mammalian expression vectors pCB7 (derived from pCB6 (1,11)) and PSG5 (10)) Wild type α–glucosidase cDNA was cloned into the pSG5 expression vector as described before (18) to generate pSHAG2.

A mutation A/C at nucleotide 950 (17) of α–glucosidase generates a substitution of glutamine by a proline at amino acid residue 244 This substitution corresponds with the one found at position 1098 in mutant sucrase-isomaltase

Mutagenesis of the α–glucosidase cDNA was performed by PCR Here, the primer Glu2 directed against the non-coding strand was designed to contain the A/C mutation at nucleotide 950 (see Table I). This primer together with Glu1 in the 5'-3' direction (Table I) were used to amplify a 743 bp fragment (denoted a-Glu I) (nucleotides 217 to 960). Another pair of primers, Glu3 in the 5'-3' direction and Glu4 for the complementary strand (see Table I) were used to amplify a 302 bp DNA fragment encompassing nucleotides 937 to 1239 (denoted a-Glu II). Glu3 contained also the A/C mutation at nucleotide 950. For cloning purposes another mutation was introduced at position 948. This C/T mutation is silent but creates an additional BamHI-site. The DNA fragments a-Glu I and a-Glu II contained 18 overlapping bp between nucleotides 940 and 957. These two fragments were assembled by assembly PCR using the primers Glu1 and Glu4 to produce a 1022 bp DNA fragment (nucleotides 217 to 1239). This PCR product was digested with SacI/StuI to generate a 348 bp fragment This fragment replaced the SacI-StuI fragment in the wild type α–glucosidase cDNA Sequence analysis of the 348 bp exchanged fragment demonstrated that only the mutations A950C and C948T were present (not shown) Sequencing was performed with a DNA sequencing kit according to the

manufacturer's instructions (Sequenase 2 0, United States Biochemical Corporation, Cleveland, OH) No other sequence alterations were found The mutant α glucosidase cDNA was subcloned into the pSG5 expression vector to generate the plasmid pSHAG2A/C which was subsequently used in transfection experiments

*5 2 4 Transfection and metabolic labeling*

COS-1 cells were either transfected by the DEAE-dextran method as described by Naim et al (28) or via electroporation as described before (37) Caco 2 cells or transiently transfected COS-1 cells were metabolically labeled with 80 μCi L-[$^{35}$S]methionine (ICN Biomedicals, Meckenheim, Germany) as described by Naim et al (28) After the labeling period, the cells were washed and stored at -70$^{0}$C until use

*5 2 5 Immunoprecipitation and SDS-PAGE*

Metabolically-labeled Caco-2 cells or transfected COS-1 cells were lysed at 4$^{0}$C for 1 hour in lysis buffer (25 mM Tris-HC l, pH 8 0, 50 mM NaCl, 0 5% Triton X-100 0 5% sodium deoxy cholate and a mixture of protease inhibitors containing 1 mM PMSF, 1 μg/ml pepstatin, 5 μg/ml leupeptin, 80 μg/ml soybean trypsin inhibitor and 1 μg/ml aprotinin, all from Sigma, Deisenhofen Germany) Usually 1 ml ice cold lysis buffer was used for each 100-mm culture dish (about 2-4 x 10$^{6}$ cells) The mixture of protease inhibitors was omitted from the lysis buffer when the sensitivity of SI towards trypsin was assessed (see later) Detergent extracts of cells were centrifuged for 1 hour at 100,000 x g at 4$^{0}$C and the supernatants were immunoprecipitated as described by Naim et al (28) For epitope mapping studies with four epitope-specific monoclonal antibodies directed against sucrase, isomaltase or sucrase-isomaltase the extracts of Caco 2 cells or transfected COS-1 cells were divided into equal aliquots and each aliquot was immunoprecipitated with a different antibody Usually, 0 2 μl mAb in the form of ascites was used for each immunoprecipitation Human α–glucosidase was immunoprecipitated using 0 5 μl of a rabbit polyclonal anti-human α–glucosidase serum (42)

SDS-PAGE was performed according to Laemmli (23) and the apparent molecular weights were assessed by comparison with high molecular weight markers (Bio-Rad Laboratories GmbH Munchen, Germany) run on the same gel In some experiments, deglycosylation of the immunoprecipitates with Endo-N-acetylglucosaminidase H (Endo H), Endo-N-acetylglucosaminidase F/glycopeptidase F (Endo F/GF, also known as PNGase F) (both from New England Biolabs GmbH, Schwalbach/Taunus, Germany) was performed prior to SDS-PAGE analysis as described before (32)

*5 2 6 Trypsin treatments*

Metabolically labeled cells were solubilized in lysis buffer without protease inhibitors and subsequently treated with trypsin (500 μg/ml, Sigma, Deisenhofen, Germany) for various time periods or with various trypsin concentrations for 30 min at 37 C The reaction was stopped by cooling on ice and the addition of soybean trypsin inhibitor (Sigma, Deisenhofen, Germany) and protease inhibitors

*5 2 7 Enzyme activities*

Disaccharidase activities were measured according to Dahlqvist (3), using sucrose and isomaltose as substrates Protein quantities were determined by Western blotting and densitometric scanning of fluorograms

*5 2 8 Immunofluorescence*

Cellular localization of expressed proteins in COS-1 cells was studied with cells grown on coverslips Cells were fixed with 3% paraformaldehyde and permeabilized with 0 1% Triton X 100 Immunolabeling was carried out using mAb anti-SI (HBB1/614), mAb anti-ERGIC-53 polyclonal anti-SI polyclonal anti-PDI, mAb anti α–glucosidase (43D1) and polyclonal anti-human α–glucosidase as the primary antibodies The secondary antibodies employed FITC-conjugated goat anti-mouse or swine anti rabbit IgG, Texas Red conjugated goat anti-mouse or anti-rabbit IgG (all were from Boehringer Mannheim BV Almere the Netherlands) Surface localization of proteins was assessed in transfected cells that were not fixed nor permeabilized, labeling was carried out at 4 C Label was visualized with a BioRad MRC1000 confocal scanning laser microscope using a double channel for fluorescein isothiocyanate and Texas Red or on a routine fluorescence microscope

*5.2.9 Immuno electron microscopy*

Ultrastructural localization studies were performed on transfected COS-1 cells. Cells were fixed with 1% paraformaldehyde (PFA) and 0.1% glutaraldehyde in phosphate buffer (pH 7.3) for 1 hour, scraped and spun down in 10% gelatin. Pellets were fixed and stored until use in 1% PFA. Ultrathin cryosectioning was performed as described before (5,47). Sections were incubated with mAbs against SI (HBB 2/614/88) or α-glucosidase (43D1), followed by a rabbit polyclonal serum against mouse IgG (Dako A/S, Glostrup, Denmark) and protein A complexed with 10 nm gold (5). Electron microscopy was performed with a JEOL 1010 electron microscope.

## 5.3 Results

*5.3.1 Human sucrase-isomaltase expressed in COS-1 cells reveals similar structural and biosynthetic features as the naturally expressed enzyme in intestinal cells.*

Wild type SI produced in transfected COS-1 cells is indistinguishable in its behavior on SDS-PAGE from the SI naturally produced in intestinal epithelial cells (37). Furthermore, the wild type enzyme expressed in COS-1 cells was processed with the same rate as in intestinal cells and showed comparable enzymatic activities (not shown) and glycosylation patterns: the 245 kDa complex glycosylated and the high mannose 210 kDa forms appear resp. disappear after approximately the same times of chase (Fig. 1). It can therefore be assumed that the folding pattern of the wild-type SI expressed in COS-1 cells is normal.

The enzyme expressed in COS-1 cells was further investigated with respect to trypsin sensitivity, an indicator for protein folding. For this purpose Caco-2 cells and transfected COS-1 cells were biosynthetically labeled for 5 hours and the detergent extracts were treated with trypsin. Under these conditions the mannose-rich SI precursor (pro-SI) as well as the complex glycosylated form become accessible to the protease. Cell lysis without protease

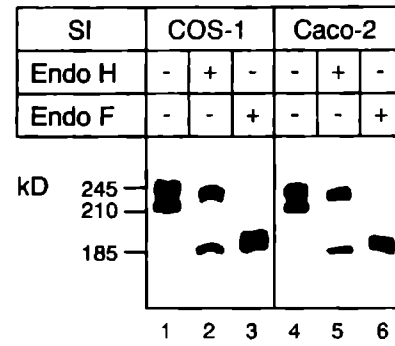



**Fig. 1** Processing of SI in COS-1 and Caco-2 cells. Caco-2 and transfected COS-1 cells were biosynthetically labeled with [$^{35}$S]methionine for 1.5 hours, and chased for the indicated times. Homogenates of these specimens were immunoprecipitated with monoclonal anti-SI antibodies. Immunoprecipitates were subjected to SDS-PAGE on 5% slab gels. Gels were analyzed by fluorography.

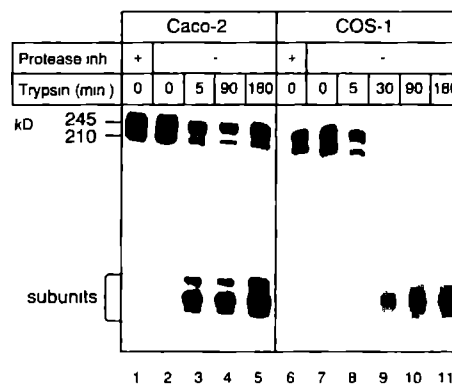



**Fig. 2** Trypsin sensitivity of SI in Caco-2 cells and COS-1 cells. Caco-2 and transfected COS-1 cells were biosynthetically labeled with $^{35}$S methionine for 5 hours. The specimens were homogenized, solubilized and treated with 500 μg/ml trypsin for the indicated times. After the reaction was stopped with protease inhibitors the samples were immunoprecipitated with monoclonal anti-SI antibodies. The immunoprecipitates were subjected to SDS-PAGE on 5% slab gels. Gels were analyzed by fluorography.

inhibitors did not result in proteolytic cleavage of pro-SI (Fig. 2, lane 2 and 7). Likewise both cell types behaved similarly when trypsin was added to the lysates. The pattern obtained with trypsin in both cell types is comparable and was maintained at 500 μg of trypsin for at least 3 hours (Fig. 2, lanes 5 and 11). The differences in intensity between the Caco-2 and the COS-lanes that are visible in Fig.2 (compare lanes 1-5 with 6-11) are due to differences in labeling efficiency. Since differently folded molecules are expected to behave differently towards the same protease, in this case trypsin, the results lend strong support to the notion that the folding of pro-SI in Caco-2 cells and COS-1 cells is similar

To corroborate these data by another approach, we probed the folding of pro-SI with epitope-specific monoclonal antibodies These mAbs recognize specific conformations or different epitopes of SI (14) Therefore, the binding of these antibodies to pro-SI in COS-1 cells would be consistent with the notion that the corresponding epitopes on pro-SI in COS-1 cells and in intestinal cells display comparable folding patterns. All antibodies reacted with pro-SI from both cell types and with comparable affinities (not shown). Since the epitopes recognized by these antibodies are randomly distributed over the pro-SI molecule, it is reasonable to assume that the overall folding pattern of pro-SI in both cell types is comparable or similar.

Altogether, the protease sensitivity measurements and the epitope mappings with four monoclonal antibodies indicate that no gross structural differences exist between pro-SI in COS-1 and Caco-2 cells.

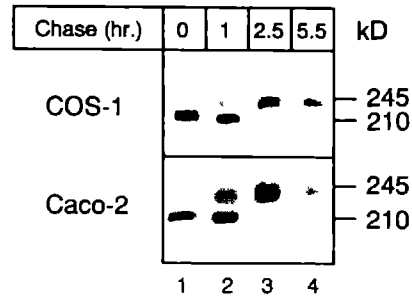



**Fig. 3** Processing of wild type (Wt) and mutant (Q1098P) SI in COS-1 cells. Cells were biosynthetically labeled with [$^{35}$S]methionine for 1 5 hours, and chased for the indicated times Homogenates of these specimens were immunoprecipitated with monoclonal anti-SI antibodies Immunoprecipitates were subjected to SDS-PAGE on 5% slab gels

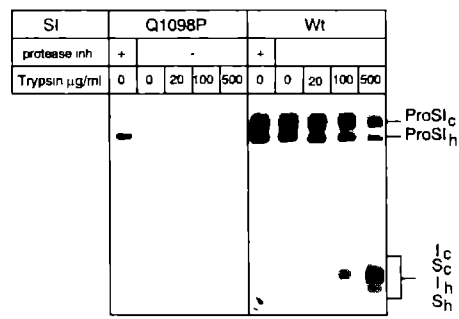



**Fig. 4** Trypsin sensitivity of mutant Q1098P and wild type (Wt) SI Transfected COS-1 cells were biosynthetically labeled with [$^{35}$S]methionine for 5 hours The specimens were homogenized, solubilized and treated for 30 minutes at 37$^0$C with the indicated trypsin concentrations After the reaction was stopped with protease inhibitors the samples were immunoprecipitated with monoclonal anti-SI antibodies The immunoprecipitates were subjected to SDS-PAGE on 5% slab gels In these experiments, mutant SI transfected cells were less efficiently labeled (compare 1st lane 'Q1098P' with 1st lane 'Wt') $S_c$, $I_c$, $proSI_c$ complex glycosylated sucrase subunit, isomaltase subunit, proSI (resp ), $S_h$, $I_h$, $proSI_h$ high mannose sucrase subunit, isomaltase subunit, proSI (resp )

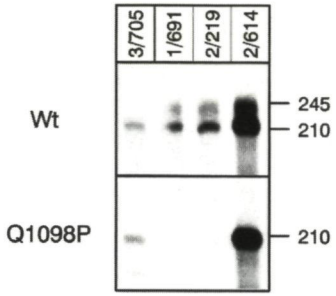



**Fig. 5** Epitope mapping of mutant Q1098P and wild type (Wt) SI. Transfected COS-1 cells were biosynthetically labeled with [$^{35}$S]methionine for 4 hours. Cell homogenates were immunoprecipitated with four different monoclonal anti-SI antibodies (HBB 3/**705**, HBB 1/**691**, HBB 2/**219**, HBB 2/**614**). Immunoprecipitates were subjected to SDS-PAGE on a 5% slab gel.

*5.3.2 Analysis of the folding pattern of mutant human sucrase-isomaltase expressed in COS-1 cells.*

The mutation Q1098P elicits similar effects on the posttranslational processing of SI in transfected COS-1 cells as in intestinal epithelial cells (37). In both celltypes the mutant protein is not complex glycosylated, but accumulates as a high-mannose precursor in the ER, intermediate compartment and the cis-Golgi. We investigated the biosynthesis of mutant SI in transfected COS-1 cells by pulse-chase analysis. As shown in Fig. 3, mutant SI persists as a high-mannose polypeptide during its entire life cycle. The intensity of this form becomes weaker with increasing chase periods indicating that the protein undergoes intracellular degradation, most likely in the ER. Degradation in the

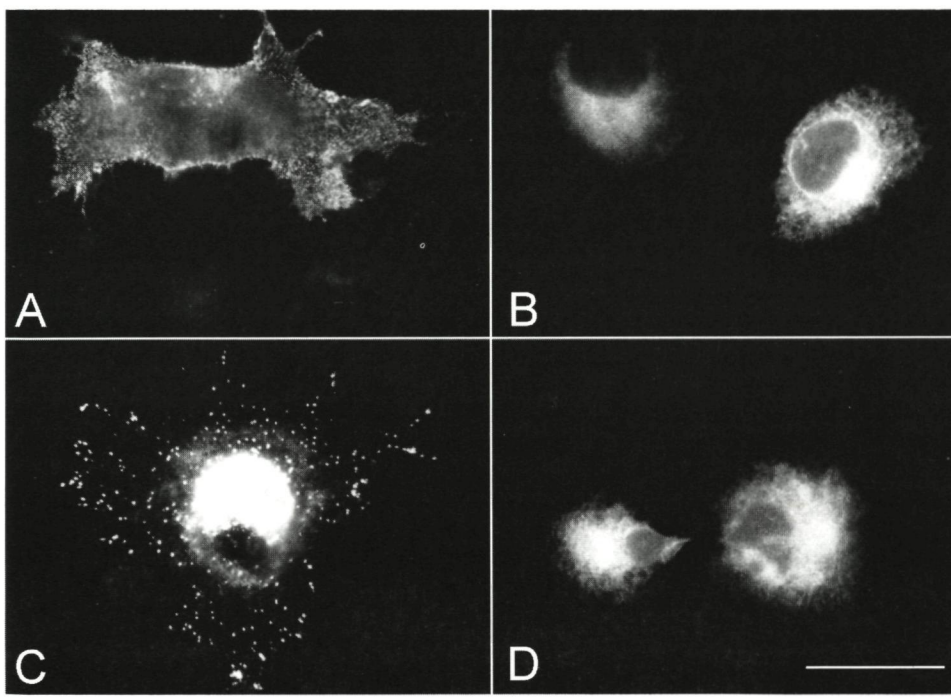



**Fig. 6** Immunofluorescence localization of wild type SI, mutant Q1098P SI, wild type α–glucosidase and mutant Q244P α–glucosidase in transfected COS-1 cells. All cells except those in (A) were fixed with paraformaldehyde, permeabilized with Triton X-100 and labeled with anti-SI mAbs (A, B) or anti- α–glucosidase (C, D) antiserum. (A) Cell surface staining of wild type SI on non-permeabilized cells. (B) Intracellular labeling of mutant Q1098P SI. (C) Intracellular, lysosomal labeling of wild type α–glucosidase. (D) Intracellular, non-lysosomal labeling of mutant Q244P α–glucosidase. Bar, 20 μm.

lysosomes could be excluded, since the mutant SI protein is not tranported beyond the ER, ERGIC and the cis-Golgi (see later Figs 7 and 8)

To investigate whether the mutant enzyme would acquire additional trypsin sensitive sites when expressed in COS-1 cells the experiments described in Fig 2 were repeated with transfected COS-1 cells expressing wild type or mutant SI Fig 4 shows that neither mutant pro-SI nor the anticipated sucrase or isomaltase subunits were visible after lysis of the cells without protease inhibitors (Fig 4, 2nd lane) indicating that the mutant pro-SI underwent complete degradation after exposure to endogenous proteases Wild type SI was processed correctly within 30 min of trypsin treatment (Fig 4, 'Wt') The high-mannose as well as the complex-glycosylated species of sucrase (Sh, Sc) and isomaltase (Ih, Ic) were generated by the trypsin treatment

The mutant SI was also different from the wild type enzyme with respect to epitope exposure, and failed to react with mAbs HBB 2/219 and HBB 1/691 (Fig 5) Together, the protease-sensitivity assays as well as the epitope mapping indicate that the Q1098P mutation has altered the folding of the SI molecule

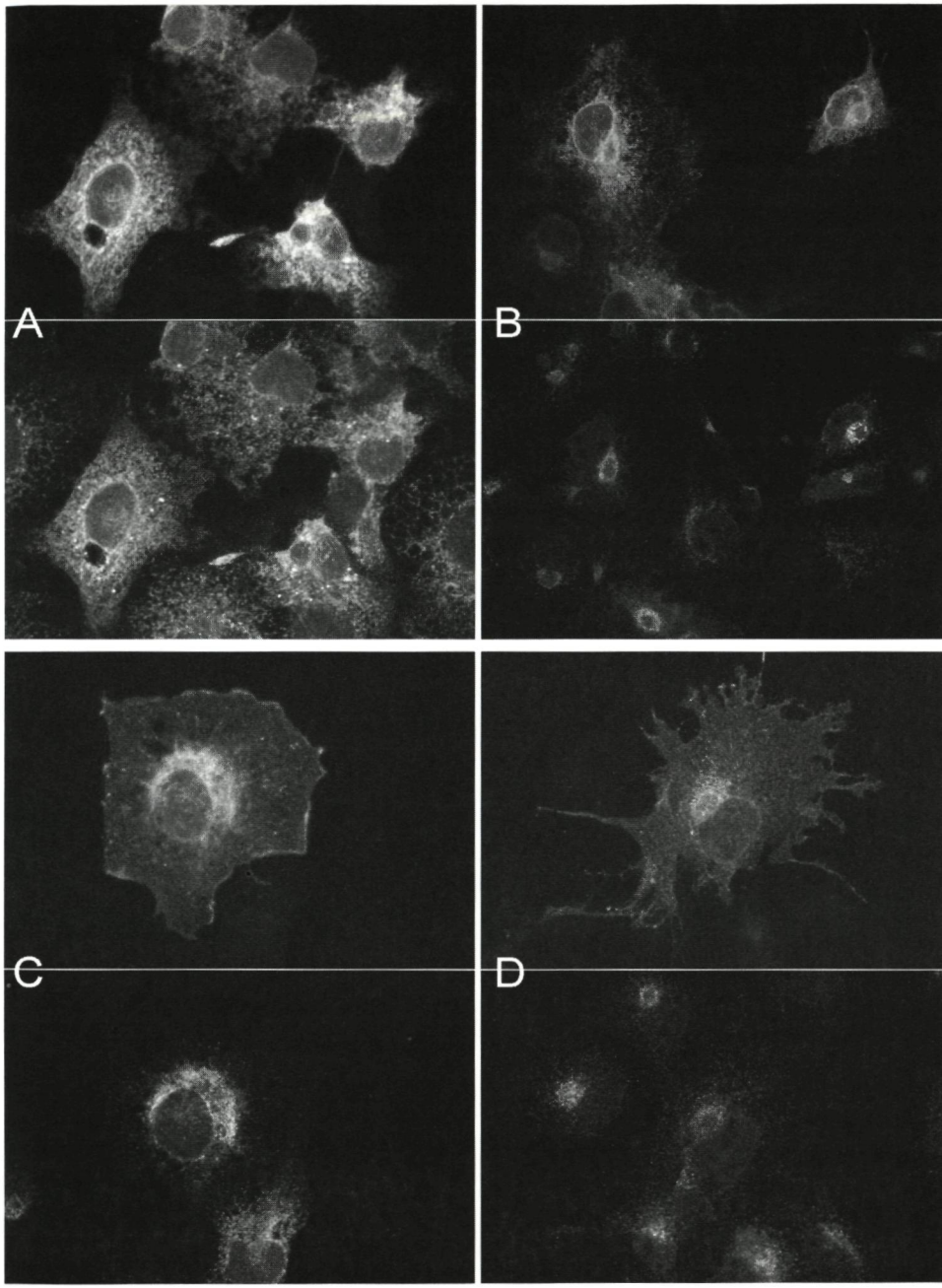
*5 3 3 Subcellular localization of wild-type and mutant SI expressed in COS 1 cells*

The intracellular transport of the wild type and mutant SI protein in transfected COS-1 cells was examined by immunofluorescence using mAb anti-SI HBB 2/614 and FITC-conjugated anti-mouse-IgG on permeabilized or non-permeabilized cells Bright staining was revealed at the surface of non-permeabilized cells expressing wild type pro-SI (Fig 6 A) By contrast, mutant pro-SI was not stained at the cell surface and could only be visualized when the cells were permeabilized with Triton X-100 (Fig 6 B)

To characterize more precisely the subcellular localization of mutant SI we used confocal scanning laser microscopy and double labeling with antibodies to SI and to markers for the ER, i e PDI (7), or the ER-Golgi intermediate compartment (ERGIC), i e ERGIC-53 (47) Fig 7 shows that mutant SI colocalizes with PDI (panel A), and with ERGIC-53 (panel B) These results indicate that mutant SI is not only localized in the ER, but also in the ERGIC It has been demonstrated that some ERGIC-53 resides also in the cis-Golgi (15) Wild-type SI showed not only the same labeling patterns as observed with mutant SI, but also an additional, strong cell surface staining could be observed (Fig 7, panel C and D), indicating that the only difference in localization between wild-type and mutant SI is the cell-surface expression

The exact localization of mutant SI was determined by immuno-electron microscopy Wild type SI was localized at the cell surface and in the ER Non-transfected cells did not show labeling (not shown) Mutant Q1098P SI showed labeling in the ER and in vesicles and membrane structures at one side of the Golgi apparatus (Fig 8) resembling the ERGIC-structures at the cis-side of the Golgi as described by Schweizer et al (47) This pattern exactly matches the pattern observed in the CSID biopsy samples (37) Unfortunaltely, the antibodies used against ERGIC or PDI could not be used for electron microscopy on COS-cells However, double labeling was already established by confocal laser scanning microscopy

Collectively, a) the double-labeling experiments showing co-localization with ERGIC-53, b) the protein not being complex glycosylated, which excludes the possibility that it has reached the trans-Golgi, and c) the EM-localization demonstrate that the Q1098P mutation has induced in a non-epithelial heterologous transfection system a similar phenotype of SI as that identified in the biopsy sample


A
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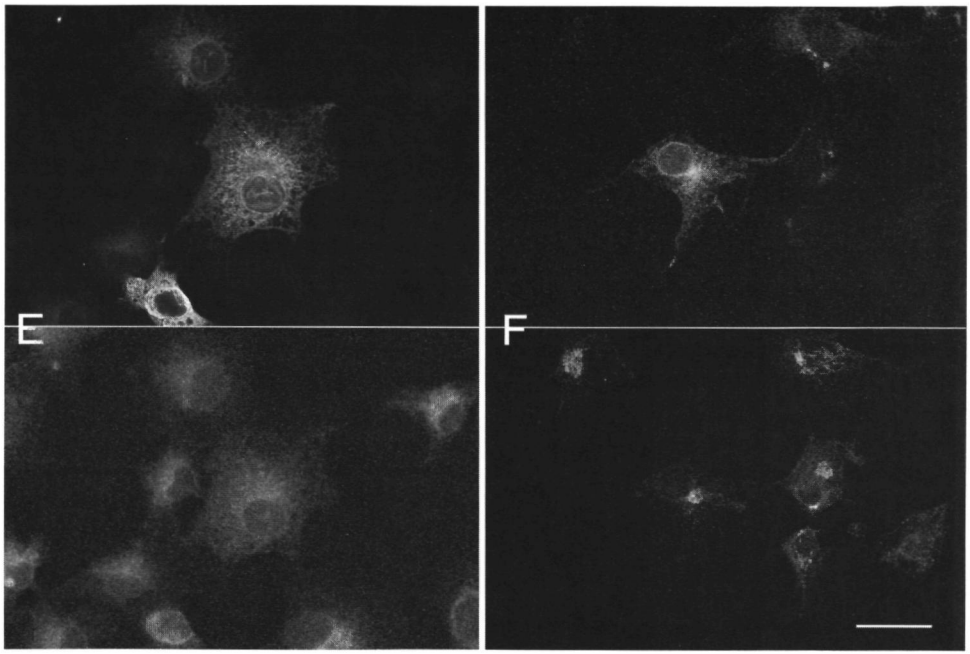


**Fig. 7** Immuno double-labeling of mutant Q1098P SI, wild type SI and mutant Q244P α–glucosidase with PDI and ERGIC-53 in transfected COS-1 cells. Cells were fixed with paraformaldehyde, permeabilized with Triton X-100 and labeled with anti-SI (A, B, C, D) or anti- α–glucosidase (E, F) antibodies, combined with antibodies against PDI (A, C, E) or against ERGIC-53 (B, D, F). PDI and ERGIC-53 are stained red, SI and α–glucosidase are stained green. When these colors coincide, a yellow staining appears. Mutant Q1098P SI shows colocalization (yellow) with PDI (A) and ERGIC-53 (B). Wild type SI shows the same colocalizations (C, D) with additional surface staining. Mutant α–glucosidase shows colocalization with PDI (E) and ERGIC-53 (F). Cells were analyzed on a confocal scanning laser microscope equipped with a Kr/Ar laser. Bar, 25 μm.

*5.3.4 Analysis of mutant lysosomal acid α–glucosidase expressed in COS-1 cells.*

In order to investigate the influence of the Q1098P mutation in a different protein the Q at position 244 in α–glucosidase was mutated to P. COS-1 cells were transfected with wild type and Q244P α–glucosidase cDNA and labeled for 3 hours. The detergent extracts and the culture media were immunoprecipitated with anti- α–glucosidase antibodies and subjected to SDS-PAGE with or without Endo H and Endo F/GF treatments. As shown in Fig. 9, wild type α–glucosidase appeared as a polypeptide of Mr = 110,000 (lane 1) consistent with previous reports (42,43,50). Treatment of this polypeptide with Endo H revealed a broad band of Mr 95,000-105,000 (Fig. 9, lane 2). This pattern was not due to incomplete digestion of the mannose-rich chains, since higher concentrations of the Endo H did not affect it (not shown). The diffuse pattern of the Endo H product indicates that the Mr = 110,000 species comprises Endo H-sensitive as well as Endo H-resistant or partially Endo H-resistant carbohydrate side chains as has been also observed in Caco-2 cells (22). In fact, Endo F/GF, which cleaves mannose-rich as well as complex type N-linked oligosaccharides, shifted the Mr = 110,000 species to a Mr =

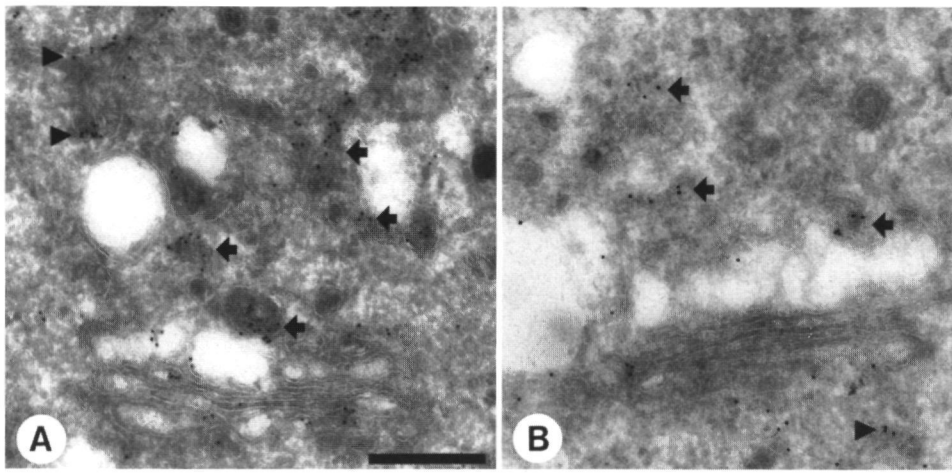


**Fig. 8** Subcellular localization of mutant SI and mutant α–glucosidase expressed in COS-1 cells. Ultrathin cryosections of fixed transfected COS-1 cells were labeled with monoclonals against SI (2/614/88) or against α–glucosidase (43D1). Mutant Q1098P SI is localized in ER (arrowheads) and in vesicles and tubules at one side of the Golgi apparatus (arrows) resembling ERGIC (conform Schweizer et al (47)) and occasionally in some cisternae of the Golgi apparatus (A). Mutant Q244P α–glucosidase shows a comparable localization (B). Bar, 300 nm.

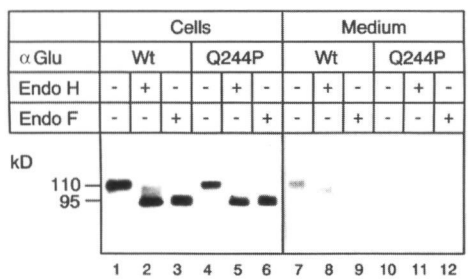


**Fig. 9** Glycosylation of mutant Q244P and wild type (Wt) α–glucosidase. Transfected COS-1 cells were biosynthetically labeled with $^{35}S$ methionine for 1.5 hours. The cells were homogenized and solubilized. Homogenates and medium were immunoprecipitated with polyclonal anti- α–glucosidase antibodies. The immuno-precipitates were treated with Endo H (lanes 2, 5, 8, 11), Endo F/GF (lanes 3, 6, 9, 12) or left untreated (lanes 1, 4, 7, 10). They were subjected to SDS-PAGE on 7% slab gels. Gels were analyzed by fluorography.

95,000 band (Fig. 9, lane 3). This result therefore indicates that α–glucosidase arrived in the Golgi, where it acquired complex type of glycans. By contrast, the mutant Mr = 110,000 α–glucosidase species persisted completely as a mannose-rich polypeptide, since both Endo H and Endo F/GF generated a similar digestion product of Mr = 95,000 (Fig. 9, lanes 4-6). Furthermore, wild type lysosomal α–glucosidase was partially secreted by COS-1 cells (Fig. 9, lanes 7-9) consistent with previous reports (18). Mutant α–glucosidase however was not discerned in the culture medium (Fig. 9, lanes 10-12). Altogether, the results clearly demonstrate that the mutant α–glucosidase has neither been processed in the Golgi apparatus nor secreted into the culture medium.

It is known that lysosomal α–glucosidase undergoes phosphorylation in the cis-Golgi at some of its mannose-residues and is then transported to the lysosomes via binding to the mannose 6-phosphate receptor. On its way to, and in the lysosomes

proteolytic processing to a Mr = 95,000 intermediate and finally to the Mr = 76,000 enzymatically mature protein occurs (42,43,50)

To determine whether mutant α–glucosidase reaches the lysosomes and undergoes cleavage, transfected COS-1 cells were metabolically labeled for 1 5 hours and chased for the indicated time periods (Fig 10) As a control mock-transfected COS-1 cells underwent the same procedure Fig 10 demonstrates that the wild type protein was intracellularly processed into the Mr = 95,000 intermediate and to the Mr = 76,000 protein ('cells', upper panel) These two bands were partially sensitive to Endo H treatment, while treatment with Endo F/GF resulted in a further shift of the two species, comparable to the digestion pattern of wild type α–glucosidase in Fig 9, lane 1-3 (not shown) The Endo H sensitivity indicates that some mannose-rich chains have not been processed in the Golgi to complex glycosylated glycans This is mainly due to phosphorylated mannose residues of the carbohydrate chains, which do not undergo further processing in the Golgi apparatus and are required for correct sorting of α–glucosidase to the lysosomes (42,43,50)

A proportion of the Mr = 110,000 precursor, but neither the Mr = 95,000 nor the Mr = 76,000 processed forms were secreted by COS-1 cells expressing wild type α–glucosidase (Fig 10, 'medium', upper panel) The faint bands in the control ('Cells', lower panel) are a result of a weak reaction of endogenous α–glucosidase with the antibodies Mutant Q244P α–glucosidase persisted as a high-mannose precursor polypeptide, was not processed to its mature form as the wild-type molecule and was not secreted into the medium (Fig 10, middle panels) The results indicate that mutant α–glucosidase is neither transported to the cell surface, nor to the lysosomes

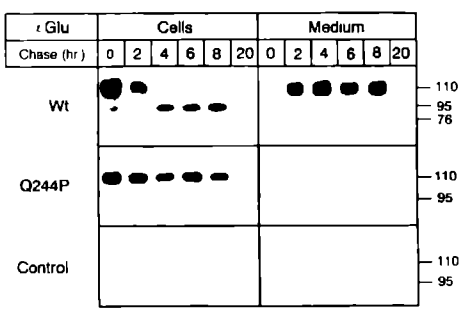


**Fig. 10** Processing of mutant Q244P and wild type (Wt) α–glucosidase COS 1 cells were transfected with cDNA coding for mutant Q244P α–glucosidase, wild type α–glucosidase (Wt) or mock-transfected (control) The cells were biosynthetically labeled with [$^{35}$S]methionine for 1 5 hours, chased for the indicated times, homogenized and solubilized Homogenates and medium were immunoprecipitated with polyclonal anti- α–glucosidase antibodies Immuno precipitates were subjected to SDS-PAGE on 7% slab gels

Altogether, the results demonstrate that wild type α–glucosidase is transport-competent since it has been processed in the Golgi, sorted to the lysosomes and partially secreted into the medium (18) By contrast, the mutant α–glucosidase protein persisted as a Mr = 110,000, mannose-rich polypeptide and was not processed to the Mr = 95,000 or Mr = 76,000 forms (Fig 10, 'cells', middle panel) Furthermore, no secreted Mr = 110,000 precursor forms could be detected even after a chase period of 20 hours (Fig 10, middle panel, 'medium') and longer exposure of the gel (not shown) Together, mutant α–glucosidase is a transport-incompetent molecule that persists as a mannose-rich precursor and is finally degraded, most likely in the ER

*5 3 5 Subcellular localization of wild type and mutant lysosomal α–glucosidase expressed in COS-1 cells.*

Having assessed the biosynthetic features of mutant α–glucosidase we wanted to determine its subcellular localization and see whether the mutation Q244P has also induced a similar transport block as that

observed with mutant SI Fig 6, panel C shows a punctate staining pattern typical of the lysosomes as well as ER and Golgi staining in COS-1 cells expressing wild type α-glucosidase The punctate pattern was completely absent in cells expressing mutant α-glucosidase, and instead immunofluorescence images corresponding predominantly to ER structures were observed (panel D)

Double-labeling experiments of mutant α-glucosidase with PDI and ERGIC-53 (Fig 7, panel E and F) resulted in co-localization of mutant α-glucosidase with PDI (panel E), and ERGIC-53 (panel F) in regions around the nucleus

Immuno EM analysis of wild-type α-glucosidase expressed in COS-1 cells revealed labeling in the ER, in the lysosomes and at the cell surface conform Hoefsloot et al (18) Non-transfected cells were devoid of label (not shown) Mutant Q244P α-glucosidase however did not show label in the lysosomes nor at the cell surface (not shown), but was localized in the ER and in vesicles and cisternae at one side of the Golgi apparatus (Fig 8 B), comparable to mutant Q1098P SI (Fig 8 A)

The results indicate that the mutation Q244P in α-glucosidase has produced a similar phenotype as Q1098P in SI, which is characterized by a block in the transport at the level of ERGIC and cis-Golgi It is therefore concluded that this highly conserved region of the molecules is important for the correct processing and transport of both enzymes

## 5.4 Discussion

Analysis of congenital sucrase-isomaltase deficiencies has resulted in the identification of different phenotypes showing transport incompetence of the SI protein In a recent paper we analyzed the cDNA of a phenotype II patient and found a point mutation which generates a Q1098P substitution responsible for a block of the molecule in the ER-Golgi intermediate compartment and the cis-Golgi (37) From these studies the question arose about the possible existence of a quality control mechanism operating at a level beyond the ER

Here we analyze the Q1098P substitution in more detail and demonstrate that it is not only functional in intestinal epithelial cells but also produces a similar phenotype when expressed in non-polarized COS-1 cells This supports the view that (i) the Q1098P mutation per se is responsible for the generation of the SI phenotype and (ii) cellular factors specific for epithelial cells are not implicated in the onset of this CSID phenotype

The expression of a similar CSID phenotype in COS-1 cells using the patients cDNA enabled us to analyze the processing of the mutant SI protein One important observation was the folding state of the mutant SI protein Here protease sensitivity assays using trypsin demonstrate that the mutant SI is not as stable as its wild type counterpart and is degraded within a relatively short period of time Interestingly, the sucrase as well as the isomaltase subunits are degraded While a malfolded sucrase subunit as a result of the mutation Q1098P is expected to exploit a different pattern of trypsin sensitivity as compared to wild type sucrase, the concomitant degradation of isomaltase can be explained as follows

Wild type SI does not have its protease resistent conformation directly after translation It reaches this conformation after a certain 'lag-period' during which both

subunit are susceptible for degradation (26). Probably mutant SI cannot reach this conformation because: a) The protein is not transported into the compartment with the proper 'folding machinery'; b) The mutation makes it energetically impossible to attain the proper folding; or c) Because of the improper folding of sucrase the protein is not transported further so isomaltase is not folded properly either and remains protease sensitive as well.

Alternatively, associations between the sucrase and isomaltase subunits could mask putative trypsin cleavage sites in either subunit preventing further cleavage. One type of association could take place after the independent folding of each subunit These are ultimately assembled as pseudodimers (21) which efficiently leave the ER. Another type of association could take place very early in the ER, where the subunits possibly assist each other to fold properly and in this way function as "intramolecular chaperons" (27,35,46). In mutant SI, these protective associations might be absent, resulting in exposed protease sensitive sites.

Another interesting feature of the Q1098P mutation is its presence in a region that shares striking homologies between human, rat and rabbit sucrase and isomaltase variants as well as human lysosomal α–glucosidase and *Schw. occidentalis* glucoamylase (30) These proteins, which have been suggested to have evolved from a common ancestral gene, are all synthesized and translocated into the ER where they are processed to transport-competent forms and then transported along the secretory pathway to their final destinations. SI is sorted, most likely in the TGN, to the apical membrane Lysosomal α–glucosidase is after phosphorylation in the cis-Golgi and maturation in the medial and trans-Golgi mainly transported to the lysosomes, but is also to a lesser extent secreted at the apical membrane in polarized epithelial cells. *Schw. occidentalis* glucoamylase is transported to the periplasmic space and partially secreted into the exterior milieu in yeast. Obviously all three proteins share a common pathway from the ER to the Golgi and from there diverge to different destinations It is therefore conceivable that homologies in the polypeptide sequences should be implicated in common functions needed for proper transport or catalytic activity. If the mutation Q1098P does not induce gross conformational changes in the sucrase subunit, but rather alters a subdomain that is critical for the intracellular transport of SI, then a similar effect might be expected if the same mutation is introduced into the corresponding homologous regions of lysosomal α–glucosidase or *Schw occidentalis* glucoamylase.

Our results show indeed that the Q1098P mutation of sucrase elicits a similar effect when introduced at the corresponding amino acid position 244 in lysosomal α–glucosidase The mutated lysosomal α–glucosidase precursor as well as the mutant SI precursor remain mannose-rich polypeptides that do not undergo maturation in the Golgi, are not cleaved into mature enzyme and are not transported through the Golgi cisternae. It is conceivable that the homologous domain of α–glucosidase, sucrase and isomaltase harbor a structural motive with a potential role in transport signaling or quality control acting at the level of ERGIC-cis-Golgi at which level the mutant proteins are blocked. The observations described here can be explained as follows:

1) the mutation has introduced a retention signal for the cis-Golgi, 2) the mutation has lead to the recognition of the protein by the ER - Golgi recycling machinery, or 3) the mutation has lead to a structural alteration that functions as a recognition site for a quality control machinery operating in the intermediate compartment or cis-Golgi.

The first possibility suggests that a single point mutation alters a highly conserved region into a region with a novel

function Although it can not be excluded, we think this is very unlikely

The second possibility is that mutant SI and lysosomal α-glucosidase exit the ER, are transported to ERGIC or cis-Golgi and then are recycled back to the ER Recent observations with a temperature sensitive mutant of the VSV G protein have shown that at the non permissive temperature the G protein leaves the ER, arrives in the ERGIC and the cis Golgi and recycles back to the ER Presumably several cycles of ER ERGIC-ER occur during which the G protein is always found associated with the luminal binding protein (BiP) and calnexin in the ER and with BiP in ERGIC (13) In view of these findings the hypothesis has emerged that misfolded proteins that have escaped the ER are retrieved from ERGIC or cis Golgi back to the ER presumably to be reexposed to the folding machinery and thus have additional opportunities to acquire correct folding A similar recycling mechanism between the ER and ERGIC or cis Golgi is potentially possible for mutant SI and lysosomal α-glucosidase In the case of the VSV G protein a retrieval mechanism to the ER has been proposed that implicates the binding of the BiP/VSV G complex to the BiP receptor in the ER Although we do not know whether SI or lysosomal α-glucosidase bind BiP or calnexin, a retrieval mechanism of mutant SI or lysosomal α-glucosidase complexes with ER-resident proteins cannot be excluded

The third possibility is that a quality control mechanism operates at a level beyond the ER and prevents misfolded proteins from being further transported along the secretory pathway to the cell surface After longer periods the mutant proteins are finally degraded We suggest therefore that the Q1098P mutation in SI and the Q244P mutation in α-glucosidase have generated structural alterations in the proteins that cause an arrest by a control mechanism operating in the intermediate compartment or cis-Golgi A comparable situation has been reported for misfolded major histocompatibility complex (MHC) classI molecules in mice that are deficient for TAP1, the transporter associated with antigen presentation (41) In these mice the MHC can not bind to peptides, which prevents the complexes to fold properly As a result they accumulate in an expanded ER Golgi intermediate compartment Although we did not find exactly the same expansion, we did report an enlargement of the Golgi complex and a change in the localization of ERGIC-53 in the patient's intestinal cells (37)

In essence, the analysis of CSID has resulted in the identification of a mutation that is responsible for a block of the molecule in the ERGIC or the cis-Golgi Although our results favor a possible quality control mechanism in these compartments, analysis of the binding kinetics of mutant SI and lysosomal α-glucosidase with BiP or calnexin is required to determine whether a recycling mechanism is possible for these proteins The observations regarding the role of a highly conserved region of SI in proper transport of the molecule could be an interesting contribution to the identification of novel control steps involved in proper transport along the secretory pathway

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# Analysis of a naturally occurring mutation in sucrase-isomaltase: glutamine 1098 is not essential for transport to the surface of COS-1 cells[1].

**Abstract**

A glutamine for proline substitution at position 1098 was previously shown to result in accumulation of brush-border sucrase-isomaltase in the Golgi apparatus The substitution is present in a highly homologous region of the protein, and results in a comparable accumulation when introduced into the same region in lysosomal α-glucosidase To study the importance of the glutamine-1098, we analyzed the transport compatibility of two mutants in which glutamine-1098 is substituted by lysine or alanine. Both mutants were transported to the cell surface and processed like wild type. We concluded that glutamine-1098 is not essential for transport to the cell surface

[1] Submitted for publication

## 6.1 Introduction

Sucrase-isomaltase (SI) is a type II transmembrane glycoprotein that is expressed at the brush-border of intestinal epithelial cells, where it is responsible for the degradation of sugar and some products of starch digestion (4,19) After synthesis in the ER and processing in the Golgi apparatus, SI is directly transported to the apical membrane (19) Along this pathway there are several general checkpoints that regulate transport of only properly folded proteins (17,18) The most prominent checkpoint is situated in the ER, where several chaperones cooperate with modification enzymes such as protein disulfide isomerase and glycosyl transferases to generate properly folded molecules (6,8,15,16) Improperly folded molecules are retained until they have acquired a proper folding or they are degraded in a proteasome-dependent fashion (1) There are some proteins however, that are not folded properly, but still exit the ER For instance in some phenotypes of congenital sucrase-isomaltase deficiency (CSID), SI is transported out of the ER but is retained in the Golgi apparatus (3,13) These phenotypes are a useful candidate to study quality control mechanisms beyond the ER

Previously we reported the identification of a point mutation in the cDNA from a CSID patient that is responsible for the accumulation of the molecule in the Golgi apparatus (11,14) The mutation results in a substitution of a glutamine for a proline at position 1098 in the amino acid sequence (Q1098P) This mutation is localized in a region that is highly homologous between several proteins of the family of glycosyl hydrolases (fig 1) (12) Substitution of the glutamine residue for proline at the corresponding position of another member of the family, human lysosomal α-glucosidase, resulted in a comparable accumulation (11)

What exactly causes the accumulation remains to be established It could be that the glutamine residue in the conserved region is important for proper transport of the proteins along the secretory pathway In this case, introduction of other amino acids at the same position would lead to transport defects as well Alternatively, the introduction of the proline residue into the polypeptide could have lead to such alterations in the folding of the molecule, that it is recognized by some kind of control mechanism, and retained In this case, other amino acids at the same position would lead to transport competent molecules

To study both possibilities, a mutagenesis protocol was designed that can result in several other amino acids at position 1098 in the SI amino acid sequence (Q1098X) The transport compatibility of the mutants in COS-1 cells was studied by immunoprecipitation and immuno-fluorescence microscopy

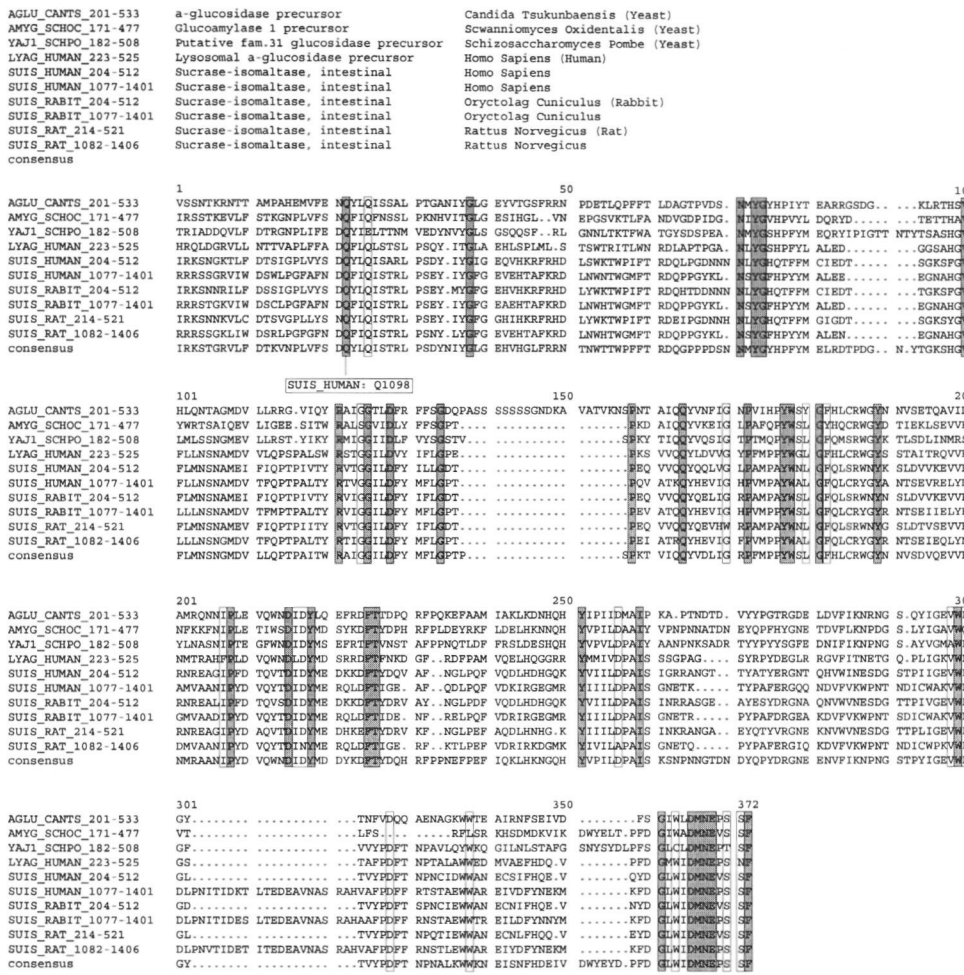



**Fig. 1** Alignment of 10 parts of amino acid sequences of related proteins. All proteins are α-glucosidases, and possess the consensus sequence of the family of glycosyl hydrolases. The amino acids that are 100% homologous are marked by a grey box, the 16 amino acids with 90% homology by a white box. The position of Q1098 in human SI is indicated below the sequence.

## 6.2 Experimental

*6.2.1 Cell line*

Monkey kidney COS cells (ATCC CRL-1650) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics (all from Gibco BRL Life Technologies) Cells were cultured at 37°C in a humidified 5% $CO_2$ incubator

*6.2.2 Antibodies*

Four epitope-specific monoclonal antibodies directed against sucrase, isomaltase or sucrase-isomaltase were used (7) These antibodies were products of the following hybridomas HBB 1/691/79, HBB 2/614/88, HBB 2/219/20, HBB 3/705/60, and will be referred to as HBB1/691, HBB2/614, HBB2/219 and HBB3/705 (resp )

*6.2.3 cDNA probes*

For the substitution of Q1098 in the SI amino acid sequence, a two-step PCR protocol was used A *Bgl*II fragment (from position 2756 to 3377) of wild type SI cDNA was subcloned into the *Bgl*II site of a modified pGEM4 vector, resulting in the pSIBF construct The partially *Bgl*II-digested SI cDNA was self-ligated, which resulted in the pSIΔ*Bgl* The pSIBF construct was used as a template for the first PCR reaction with 100 ng of SP6 primer (5'GA TTTAGGTGACACTATAG3') and 100 ng of the degenerate oligonucleotide SIMUT (5'CGATATTIGAAIGAA[A/T][G/T][C/T]GT CATTAAAAGCAAA3') that is complementary to position 3281-3313 of the SI cDNA, with the exception of the nucleotides placed in brackets The second PCR reaction was performed using 12 pg of a 1850 bp *Sca*I-fragment of pSIBF and 20 ng of the 558 bp PCR-product obtained from the previous amplification round Samples were taken after 5, 10, 15, 20 and 25 cycles of the PCR protocol The products of the second PCR in the respective samples were amplified by PCR using primers T7 (5'TAATACGA CTCACTATAGGG3') and SP6 (both 100 ng). The final 731 bp products were isolated and digested with *Bgl*II, cloned into a modified pGEM4 vector, and sequenced Those inserts that were mutated at the desired position were digested with *Bgl*II, and cloned into the *Bgl*II digested pSIΔ*Bgl* vector, which resulted in Q1098X mutant SI cDNA Full-length wild-type and Q1098X mutant SI cDNAs as well as the Q1098P mutant (14) were subcloned into the mammalian expression vector PSG5 (5)

pGEM4 and the SP6 and T7 primers were from Promega (Madison, USA). All PCR reactions were performed using the following conditions 5 µl 10x PCR buffer (Gibco BRL), 2 mM $MgCl_2$, 2.5 µl W1-detergent (Gibco BRL), 250 µM dNTPs, 2 units Taq polymerase (Gibco BRL), primers and template DNA in a final volume of 50 µl The mixture was incubated at 94°C for 5 min , followed by 25 cycles of 1 min 94°C, 1 min 45°C, 1 min. 72°C After the last cycle and 10 min at 72°C, the mixture was stored at 4°C

*6.2.4 Transfection, metabolic labeling, immunoprecipitation and SDS-PAGE*

COS-1 cells were transiently transfected via electroporation as described before (14) Transfected cells were washed once with methionine/cysteine-free medium, and incubated in this medium for 1 hour After this starvation period, 100 µCi Tran$^{35}$S label (ICN Biomedicals) was added to the medium. After the labeling period (followed by an optional chase period in normal medium), the cells were washed with PBS and scraped in lysis buffer (1% Triton X-100, 0 2% BSA in 100 mM phosphate buffer pH 8.0 containing 1 tablet complete protease inhibitor cocktail (Boehringer Mannheim BV, Almere, the Netherlands) per 25 ml) and lysed at 4°C for 1 h. Usually 1 ml ice cold lysis buffer was used for each 100-mm culture dish (about 2-4 x $10^6$ cells) Lysates were stored at -135 °C until use Detergent extracts of cells were centrifuged for 1h at 100,000 x g at 4°C and the supernatants were immunoprecipitated as described by Schweizer et al (3), using a mixture of the four monoclonal antibodies against SI in a dilution of 1/1000 each

SDS-PAGE was performed according to Laemmli (9) and the apparent molecular weights were assessed by comparison with high molecular weight markers (Bio-Rad Laboratories) run on the same gel

### 6.2.5 *Immunofluorescence*

Cellular localization of expressed proteins in COS-1 cells was studied with cells grown on coverslips. Cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. Immunolabeling was carried out using each of the four monoclonal antibodies against SI separately as primary antibody in a 1/1000 dilution. The secondary antibodies employed FITC-conjugated goat anti-mouse IgG (Boehringer Mannheim BV, Almere, the Netherlands). Label was visualized with a BioRad MRC1000 confocal scanning laser microscope or on a routine fluorescence microscope.

## 6.3 Results

### 6.3.1 *Mutagenesis*

To introduce mutations at amino acid 1098 of the SI sequence, a PCR strategy was designed with a degenerate oligonucleotide containing a triplet of mismatches at position 3296 to 3298 of the SI cDNA. This strategy (for details see the methods section) resulted in two mutant SI cDNAs: Q1098A and Q1098K. The constructs were expressed in COS-1 cells and the transport compatibility of the mutants was assessed by immunoprecipitation and immuno-localization studies.

### 6.3.2 *Immunoprecipitation*

The processing of wild-type SI and the SI mutants in COS-1 cells was studied by a pulse-chase labeling experiment followed by immunoprecipitation of SI. After 30 min. pulse and 0 hours chase, only the high-mannose, 210 kDa band of both wild type SI and the mutants is visible (fig. 2 lanes 1, 4, 7, 10). The complex glycosylated band of about 245 kDa appears after 4 hours of chase in the wild type, Q1098A and Q1098K lanes. The high-mannose band has completely disappeared after 24 hours of chase in lanes 3, 6, and 9. In contrast, in the Q1098P lanes there is no complex glycosylated band visible after 4 or 24 hours chase (fig. 2, lane 11, 12), after 24 hours there is no band visible at all, the protein is completely degraded.

### 6.3.3 *Immunofluorescence*

The localization of wild type and mutant proteins in COS-1 cells was assessed by immunofluorescence using four monoclonal antibodies against SI. In fig. 3, the localization of four proteins (Q1098A, Q1098K, wtSI, Q1098P) was determined in permeabilized cells. Overall is visible that the localization of the Q1098A and Q1098K mutants exactly matches the pattern visible in the wild-type panels. All three molecules can be found in an intracellular network and at the cell surface. The Q1098P mutant however, can not be found at the cell surface, but only in an intracellular network. This network was shown to consist of ER and cis-Golgi structures (11).

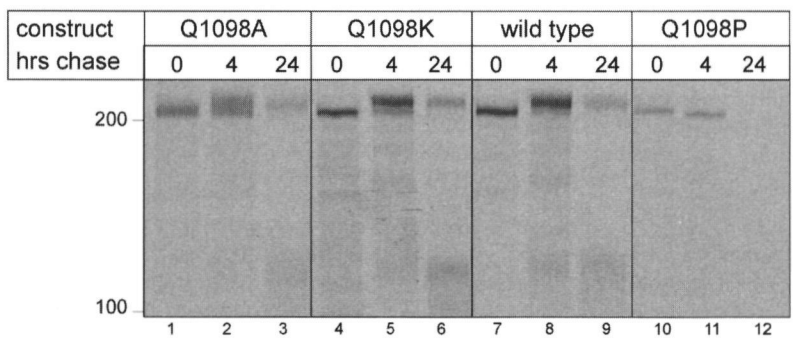



**Fig. 2** Transfected COS-1 cells were metabolically labeled for 30 minutes and chased for 0, 4 and 24 hours. After immuno-precipitation using a mixture of the HBB 1/691, HBB 2/614, HBB 2/219 and HBB 3/705 monoclonal antibodies against SI, the precipitates were analyzed on a 8% SDS-PAGE gel.

The four monoclonal antibodies against SI have epitopes that do not overlap, and some are maturation dependent It has previously been established that HBB3/705 only has affinity for high-mannose glycosylated SI (7) Therefore, it is not surprising that it only labels an intracellular network, probably the ER, in all panels HBB2/614 was shown to have equal affinity for high-mannose and complex glycosylated SI On wild type, Q1098A and Q1098K transfected cells a surface labeling and an intracellular network is visible, whereas in Q1098P transfected cells, only the network can be seen

In immunoprecipitation studies on Q1098P transfected COS cells, no protein could be precipitated using antibodies HBB2/219 and HBB1/691 (11) The localization studies confirm these data, there were no Q1098P transfected cells labeled by these monoclonals (see fig 4) Cells transfected with wild type, Q1098A and Q1098K SI were all positive for both HBB1/691 and HBB2/219 Apparently the epitopes differ in maturation sensibility, because HBB2/219 showed more affinity for an intracellular perinuclear network, whereas HBB1/691 mainly labeled the cell surface Studies using a mixture of the four monoclonals on non permeabilized cells revealed a strong surface labeling of the wild type, Q1098A and Q1098K transfected cells A few of the Q1098P transfected cells showed a faint labeling at their surface, but the majority was negative (data not shown)

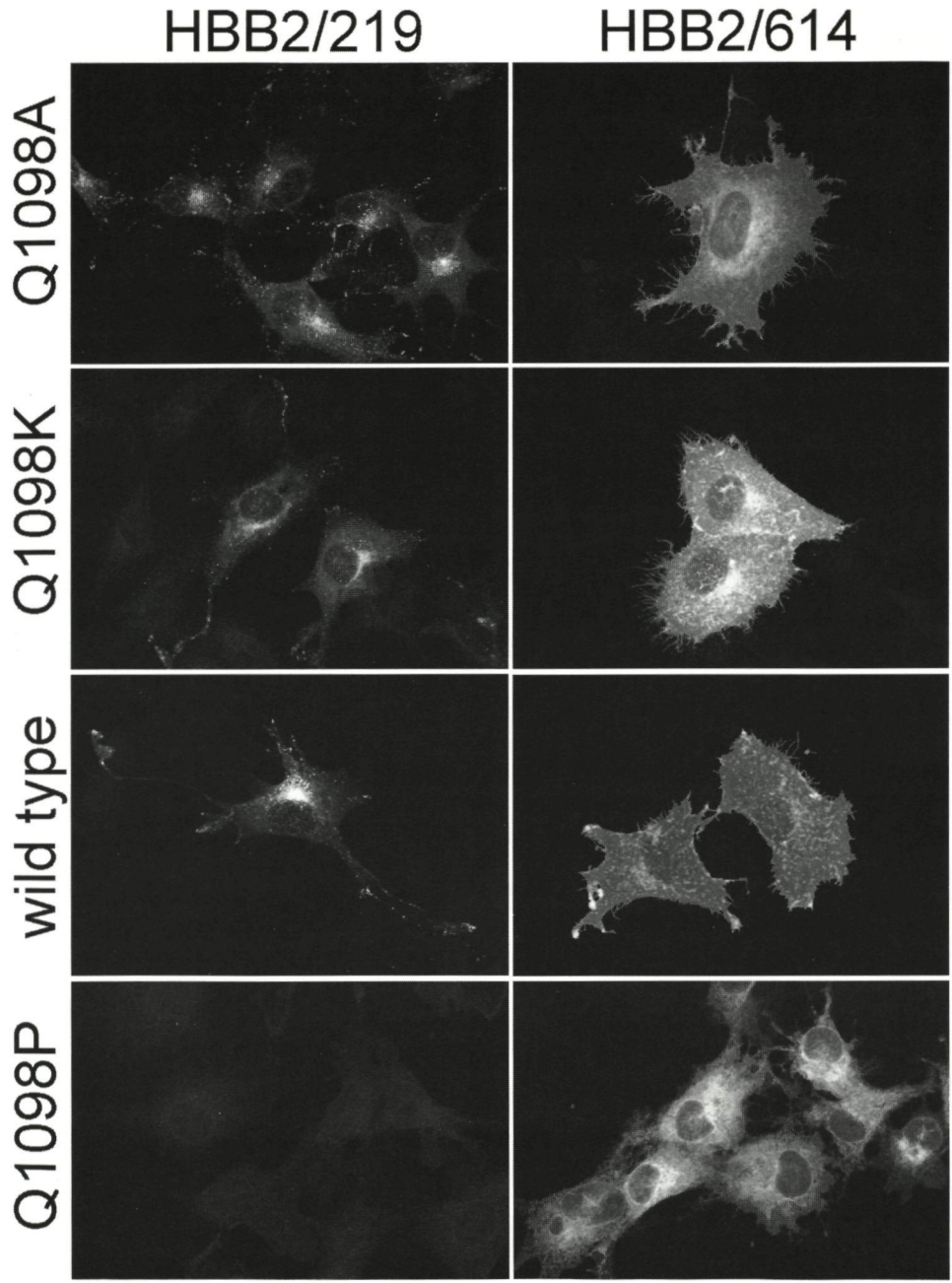
Overall these results indicate that substitution of glutamine at position 1098 by alanine or lysine does not alter the transport compatibility of SI Substitution by proline has more consequences in this regard, as was shown before (11,14)

## 6.4 Discussion

Amino acid substitutions can disturb the transport of a protein along the secretory pathway Previously we reported a glutamine for proline substitution at position 1098 (Q1098P) that resulted in the accumulation in the Golgi apparatus of the brush-border enzyme sucrase-isomaltase in intestinal epithelial cells from a patient with a phenotype II congenital sucrase-isomaltase deficiency (11,14) The substitution is present in a highly homologous region of the protein, and results in a comparable accumulation when introduced into the same region in a related protein with another intracellular destination, lysosomal α-glucosidase (11) To study the importance of the presence of a glutamine residue at position 1098 (Q1098), we analyzed the transport compatibility of two mutants in which Q1098 is substituted by lysine (Q1098K) or alanine (Q1098A)

The pulse-chase experiments clearly show that both Q1098A and Q1098K are processed at the same rate as wild type sucrase-isomaltase, whereas the processing of the Q1098P mutant is severely delayed The immunofluorescence data confirm this notion both Q1098A and Q1098K are expressed at the cell surface, while the bulk of Q1098P accumulates intracellularly (except for some overexpressing cells) Altogether can be concluded that both Q1098A and Q1098K are fully transport competent

The mutagenesis strategy was designed to generate up to 6 different amino acids (Lys, Ala, Asn, Thr, Glu, Asp) at position 1098 Lysine and alanine were the first two mutants from the pool of mutants that were identified and analyzed Because both these mutants were fully transport competent, we concluded that the presence of glutamine at position 1098 is not essential for proper transport, therefore we did not undertake more efforts to analyze other mutants


HBB2/219
HBB2/614
Q1098A
Q1098K
wild type
Q1098P

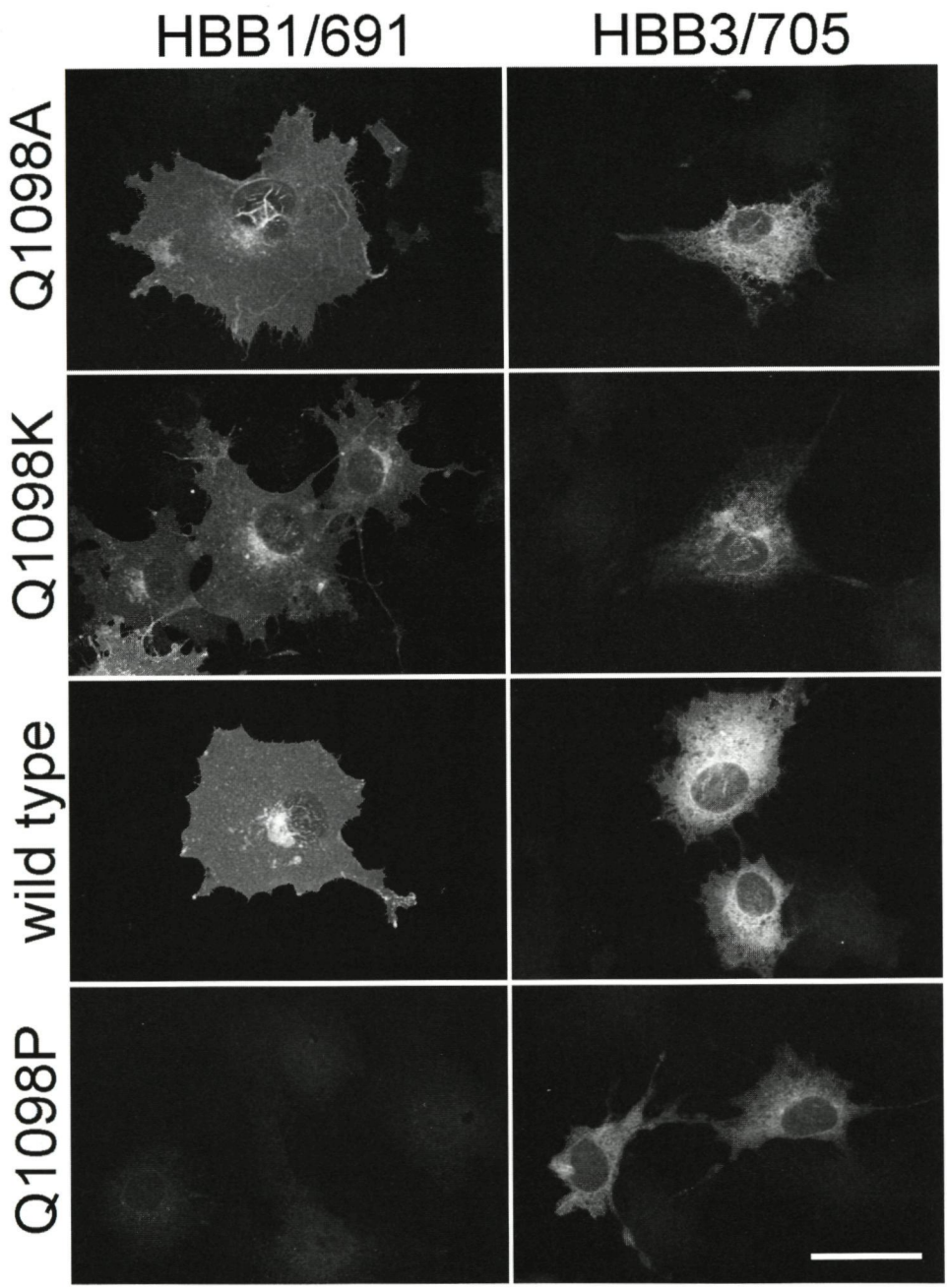



**Fig. 3** Immunofluorescence microscopy on transfected COS-1 cells. Cells were grown on coverslips, fixed and labeled by four monoclonal antibodies against SI with non-overlapping epitopes (HBB1/691, HBB2/614, HBB2/219 and HBB3/705), followed by FITC- conjugated second antibodies.

If the conserved glutamine residue in the highly homologous region at position 1098 is not essential for proper transport, what could have caused the transport defect of the Q1098P mutant? Most likely introduction of a proline at this position has lead to such alterations in the 3D structure of the protein that it is recognized by some kind of control mechanism, and as a result retained in the Golgi. To study gross structural alterations, a panel of monoclonals was used that recognize different non-overlapping epitopes of sucrase-isomaltase, of which some depend on the processing of the protein. The Q1098P mutant is not recognized by the HBB1/691 and HBB2/219 monoclonals against SI in the immuno-fluorescence localization study. This could be a result of incomplete processing by which the epitopes remain immature and are not recognized. Alternatively, it can indeed be due to an aberrant folding of the mutant, by which the epitopes have disappeared Due to this improper folding the mutant could be retained and finally degraded.

Proline is known to influence strongly the folding and 3D structure of a protein. It has been reported to introduce kinks in α-helices (20,21). Furthermore, several mutant proteins have been described that lost their function and/or transport properties after introduction of a proline (see for instance (2,10,22)).

Concluding, although the glutamine at position 1098 is highly conserved, it is not essential for the transport compatibility of SI because substitution of this residue by lysine or alanine does not affect transport compatibility. We therefore conclude that proline introduced a structural alteration that resulted in the retention and final degradation in the Golgi apparatus of the Q1098P mutant.

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# Routing and processing of lactase-phlorizin hydrolase in transfected Caco-2 cells[1].

**Summary**

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

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

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Submitted for publication

## 7.1 Introduction

Human lactase-phlorizin hydrolase (LPH)[3]) is a disaccharidase that is localized at the microvillar membrane of epithelial cells in the small intestine. It is responsible for the hydrolysis of lactose, the main carbohydrate in mammalian milk.

LPH is synthesized as a 1927 amino acid precursor, prepro-LPH. The first 19 amino acids of this precursor form the signal sequence that is cleaved off in the ER. The remaining 1908 amino acids form the pro-LPH, which is complex glycosylated on its way through the Golgi apparatus. A subsequent cleavage between Arg734 and Leu735 (26) takes place after export from the trans-Golgi and results in the 160 kDa LPHβ and a stretch that is called the pro-fragment or LPHα (15). LPHβ is expressed at the apical membrane, where it is trimmed by luminal trypsin in the small intestine to the intestinal form of LPHβ. In contrast to LPHβ, the fate of LPHα after cleavage is not clear. In fig. 1, the processing of LPH is illustrated.

Prepro-LPH consists of four homologous regions between the signal peptide and the hydrophobic stretch at the C-terminus. In fig 2 in chapter 3, prepro-LPH is schematically drawn The first two regions, I and II, are localized in LPHα. None of them possess the Glu residue that was shown to be essential for the active sites (25) Indeed, LPHα is enzymatically not active towards lactose when expressed separately in COS-cells or isolated after trypsin cleavage of the pro-LPH expressed in COS-cells (16). Regions III and IV both contain the Glu residue and are responsible

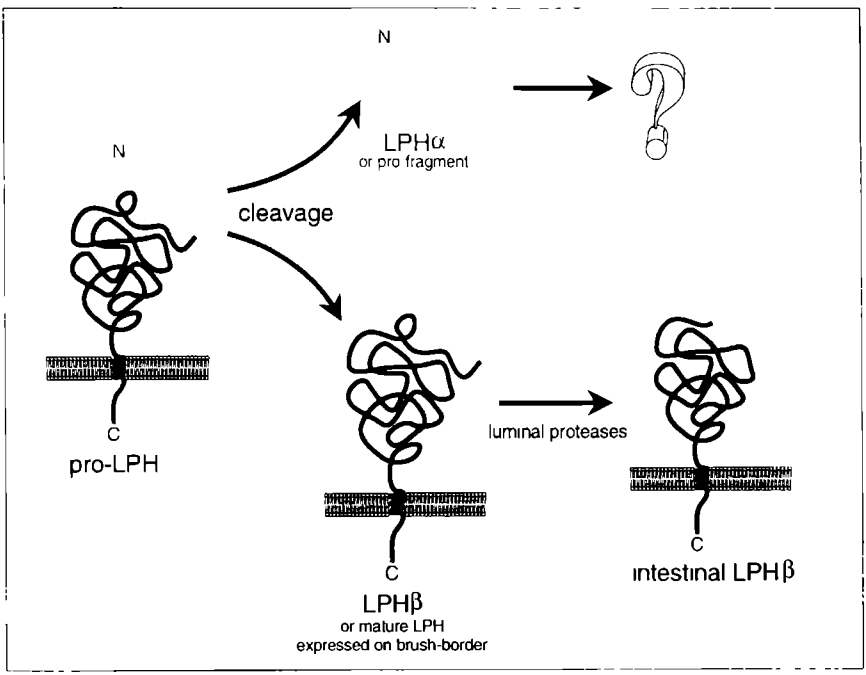


**Fig. 1** Cartoon of the processing of LPH LPH is synthesized as prepro-LPH Removal of the signal sequence results in pro-LPH. After complex glycosylation in the Golgi apparatus, pro-LPH is cleaved into LPHα (pro-fragment) and LPHβ (mature LPH). LPHβ is expressed at the brush-border, but the fate of LPHα remains unknown In intestinal cells, LPHβ is further trimmed by luminal proteases to intestinal LPHβ

for the disaccharidase activity of LPHβ (25). Because of the homology between LPHα and LPHβ, and because LPHα comprises 40% of the primary translation product, various functions have been suggested for LPHα (15,17).

LPHα has previously been isolated from intestinal biopsy samples using an antibody directed against the 12 amino acids directly after the signal peptide (S20-T31) (15). The molecular mass of the immunoprecipitated pro-fragment in these experiments was ~100 kDa (15). Because none of the used glucosaminidases (endo H and endo F/GF) influenced the apparent molecular weight, it was concluded that the 5 consensus *N*-glycosylation sites were not glycosylated (15) although some conflicting data exist (26). In addition it was shown that LPHα does not form a stable complex with LPHβ after cleavage in intestinal biopsy specimens (15). These observations and further expression studies of the intestinal form of LPHβ (from Ala869 to the C-terminus) in COS-1 cells lead to the hypothesis that LPHα functions as an intramolecular chaperone. Where exactly in the cell the cleavage takes places as well as what happens to LPHα after cleavage is still uncertain, despite extensive published data (15,17).

To track down LPHα after cleavage, we decided to express and analyze LPH in Caco-2 cells. These cells are able to express LPH endogenously, and to perform the cleavage between the a and b domain of pro-LPH (7). Furthermore, we introduced a VSV-tag into LPHα to generate additional recognition possibilities. Immuno-precipitation and localization studies were performed in Caco-2 cells that were stably transfected with wild type and VSV-tagged LPH cDNA. The results strongly suggest that the LPHα pro-fragment is immediately degraded after cleavage in Caco-2 cells, and therefore argue in favor of the intramolecular chaperon function.

## 7.2 Experimental procedures

### *7.2.1 Antibodies*

HBB1/909 is an epitope-specific monoclonal antibody directed against LPH (7). This product of the hybridoma HBB1/909/34/74 was provided by Hans-Peter Hauri, Biozentrum der Universität Basel, Switzerland. V496 is a polyclonal antiserum, directed against the first 12 amino acids following the signal sequence of pre-pro-LPH (15). P5D4 is a monoclonal antibody against a specific epitope in VSV-G-protein (9), the hybridoma was provided by Thomas Kreis, Department of Cell Biology, University of Geneva, Switzerland.

### *7.2.2 DNA constructs*

To insert a VSV epitope tag into the LPH cDNA, the LPH cDNA (14) was cloned into a pBluescript KS+ vector in which the SstI site was removed. This construct was digested with SstI, blunted with T4 DNA polymerase (New England Biolabs, Beverly, MA 01915, USA) and treated with calf intestinal phosphatase (Boehringer Mannheim BV, Almere, the Netherlands). For the construction of a 43 bp VSV-tag insert, coding for 11 amino acids of the epitope, two oligonucleotides (5'GGAGATCTTATACAGAC ATAGAGATGAA3' and 5'GGGGGATCCCTTT CCAAGTCGGTTCATCTCTATGTCTGTA3') were annealed and treated with klenow (Boehringer Mannheim BV, Almere, the Netherlands) in the presence of dNTPs in order to fill in the 5' overhangs. The double-stranded product was digested with BamHI and BglII, and the 5' overhangs were filled in by klenow treatment. This blunted BamHI/BglII insert was ligated into the blunted, SstI digested LPH cDNA so that the reading frame remained intact. The orientation of the insert was determined by sequencing. Fig. 2 depicts the sequence of the insert and the flanking LPH sequences, as well as the amino acid sequence of the wild type LPH and the tag-insert in this region. This construct, denoted LPHST, was cloned into a modified pSG5 (4) expression vector containing a puromycin resistance cassette (pSGpuro) which resulted in the pLPHST plasmid. Wild type LPH cDNA was cloned into pSGpuro as well (pLPHwt).

To generate the pSGpuro vector the SalI-fragment of the pKSpuro vector which was kindly provided by Peter Laird (University of Southern California School of Medicine, Norris Comprehensive Cancer Center Los Angeles, CA

90033, USA) (23) was inserted into the partially SalI digested pSG8 (a pSG5 vector (4) with a multiple cloning site)

*7 2 3 Cell lines*

Monkey kidney COS cells (ATCC CRL-1650) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Calf Serum and antibiotics. Caco-2 TC7 and PD10 cells (a gift from Dr. Monique Rousset, (1)) were cultured in DMEM supplemented with 20% heat inactivated FCS, 1% non-essential amino acids and antibiotics (all from Gibco BRL Life Technologies). All cell lines were cultured at 37°C in a humidified 5% $CO_2$ incubator

*7 2 4 Transfection and selection*

COS-1 cells were transfected via electroporation as described before (18) Caco-2 cells were transfected by lipofectamine treatment One or two days prior to transfection, 0.2 * $10^6$ Caco-2 TC7 cells were seeded per 35 mm culture dish, so that on the day of transfection the culture was 60-80% confluent, but not polarized During the whole transfection procedure, no antibiotics were used. On the day of transfection, cells were washed twice with OptiMEM (Gibco, BRL). Solution A, consisting of 2 µg linearized DNA in 200 µl OptiMEM, was gently mixed with solution B, consisting of 6µl lipofectamine (Life Technologies) and 200 µl OptiMEM The mixture was incubated at room temperature for 30 min, and 1600 µl OptiMEM was added. The resulting 2 ml. was added to the cells, and incubated at 37 °C and 5% $CO_2$ for 6 hours Cells were washed with normal Caco-2 medium and incubated in this medium for 24 hours, refreshed, and incubated for another 24 hours. Cells were trypsinized and seeded into 96 wells plates at a density of 5*$10^3$ cells per well After 24 hours, selection medium was added, which consisted of normal Caco-2 medium with an (empirically determined) puromycin concentration of 13 µg/ml After 1 week, wells with only one colony were selected. After another week, these colonies were trypsinized and cultured further in selection medium.

*7 2 5 Metabolic labeling and immunoprecipitation*

Caco-2 cells or transiently transfected COS-1 cells were metabolically labeled with 100 mCi Tran$^{35}$S label (ICN Biomedicals) as described by Naim et al (14) After the labeling period, the cells were scraped in lysis buffer (1% Triton X-100, 0.2% BSA in 100mM phosphate buffer pH 8 0 containing 1 tablet complete protease inhibitor cocktail (Boehringer Mannheim BV, Almere, the Netherlands) per 25 ml) and lysed at 4°C for 1 h. For COS-cells usually 1 ml ice cold lysis buffer was used for each 100-mm culture dish (about 2-4 x $10^6$ cells) For Caco-2 cells, 0.5 ml was used per filter Lysates were stored at -135°C until use. Detergent extracts of cells were centrifuged for 1h at 100,000 x g at 4°C and the supernatants were immunoprecipitated as described by Schweizer et al, (20).

*7 2 6 SDS-PAGE*

SDS-PAGE was performed according to Laemmli (10) and the apparent molecular weights were assessed by comparison with high molecular weight markers (Bio-Rad Laboratories) run on the same gel. In some experiments, deglycosylation of the immunoprecipitates with Endo-N-acetylglucosaminidase H (endo H), Endo-N-acetylglucosaminidase F/glycopeptidase F (endo F/GF, also known as PNGase F) (both from New England Biolabs) was performed prior to SDS-PAGE analysis as described before (13)

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          668
wtLPH     GTC CTG CGA GCT GAA GAT ATC CCG GAG CTC CTG CTA GAA CCA CCC ATA TCT GCG
          Val Leu Arg Ala Glu Asp Ile Pro Glu Leu Leu Leu Glu Pro Pro Ile Ser Ala
          220

          686
LPHST     ATC CCG GGA TCT TAT ACA GAC ATA GAG ATG AAC CGA CTT GGA AAG GGA TCC CTG CTA
          Ile Pro Gly Ser Tyr Thr Asp Ile Glu Met Asn Arg Leu Gly Lys Gly Ser Leu Leu
          226
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**Fig. 2** Alignment of the amino acid and DNA sequences of wtLPH and LPHST around the introduction site of the VSV-tag Lines connect the corresponding amino acids. The box is drawn around the VSV-tag sequence, that is typed in boldface Basepair numbering is given above the sequence, amino acid numbering below

*7 2 7 Trypsin sensitivity assay*

The sensitivity for proteatic digestion of LPH and LPHST was compared using a trypsin sensitivity assay. Transfected COS-1 cells were metabolically labelled and lysed. LPH and LPHST were immunoprecipitated (see above) using the HBB1/909 antibody. Precipitates were divided into several fractions, and treated with 50 µg/ml trypsin at 37°C for the indicated incubation times. Samples were subjected to SDS-PAGE on a 8% gel, which was analyzed by fluorography.

*7 2.8 Enzyme activities*

Disaccharidase activities of immunoprecipitated LPHs were measured according to Dahlqvist (2), using lactose as a substrate. The method was essentially the same as described by Naim et al (14). LPH was immunoprecipitated using the HBB1/909 antibody from lysates of 165 $cm^2$ culture flasks of (transfected) Caco-2 cells The precipitates were dissolved in 2ml PBS containing 0.3% Triton X-100, of which 25 µl aliquots were used for the enzyme activity assay. The amount of precipitated LPH was estimated in 500, 300, 200 and 100 µl aliquots on an SDS-PAGE gel with a BSA concentration standard series run on the same gel, the gel was stained with coomassie brilliant blue.

*7 2 9 Immunofluorescence and confocal microscopy*

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

*7 2 10 Immuno electron microscopy*

Ultrastructural localization studies were performed on transfected Caco-2 TC7 clones grown on filter that had been confluent for 5 days They were fixed with 1% paraformaldehyde (PFA) and 0.1% glutaraldehyde in phosphate buffer (pH 7.3) for 1 hour, and stored until use in 1% PFA Filters were stacked in 10% gelatin and fixed in 1% PFA for 24 hours Ultrathin cryosectioning was performed as described before (3,20). Sections were incubated with the mAbs HBB1/909 against human LPH or P5D4 against the VSV-epitope tag, followed by a rabbit polyclonal serum against mouse IgG (Dako A/S, Glostrup, Denmark) and protein A complexed with 10 nm gold (3) Electron microscopy was performed with a JEOL 1010 electron microscope

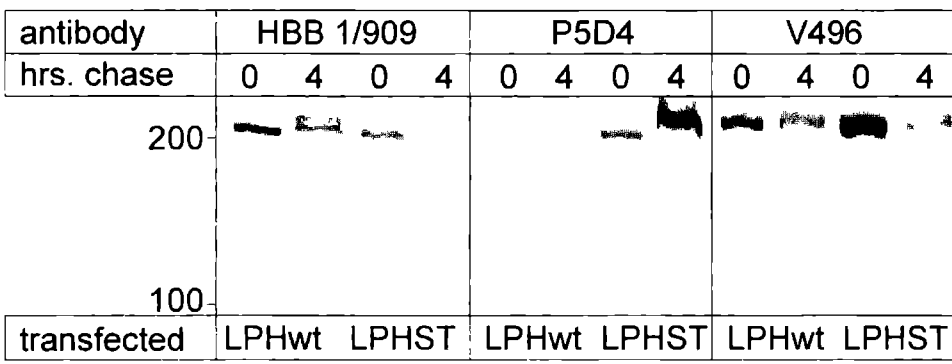


**Fig. 3** Immunoprecipitation of wtLPH and LPHST from transfected COS-1 cells using the antibodies HBB1/909, P5D4, and V496. Transfected COS-1 cells were labeled for 1 hour, and chased for 0 or 4 hours. The aliquots were divided and precipitated with the indicated antibodies. In the P5D4 '0' lane, a faint background band is visible. Neither pro-LPHwt nor pro-LPHST are cleaved into LPHα and LPHβ

## 7.3 Results and discussion

### 7.3.1 Processing of wtLPH and LPHST in COS-1

To examine the influence of the introduction of the VSV-tag into LPH, the processing, localization and protease sensitivity of both wtLPH and LPHST were compared in COS-1 cells. Immunoprecipitations using the HBB1/909 antibody directed against pro-LPH and mature LPH resulted in precipitation of high-mannose (~215 kDa) and complex glycosylated (~230 kDa) LPH (fig.3, first 4 lanes). No mature form (~160 kDa) could be observed, due to the absence in these cells of the protease that is responsible for the α/β cleavage (11,14,15,26). However, both LPHST and wtLPH were present at the cell surface as shown by immunofluorescence labeling (see later). The results clearly show that introduction of the VSV-tag did not affect the transport competence of the mutant.

P5D4, the antibody against the VSV-epitope tag, did not recognize wild type pro-LPH, but did precipitate the tagged LPH construct LPHST (fig. 3, middle lanes). The V496 antibody directed against an epitope of LPHα precipitated both constructs (fig. 3, last four lanes). The pro-LPH species precipitated by the V496 antibody corresponds to the mannose-rich precursor as was determined by endo H treatment of the precipitates (not shown).

### 7.3.2 Trypsin sensitivity assay

To compare sensitivities towards protease treatment of both LPH and LPHST proteins, they were precipitated from transfected COS-1 cells by the HBB1/909 antibody against LPH. The precipitates were treated for several time intervals with trypsin. In fig. 4 is shown that insertion of the tag did not alter the sensitivity towards trypsin, since both proteins show exactly the same pattern. Trypsin treatment results already after 75 sec in a complete cleavage of the high-mannose and complex forms of wtLPH and LPHST into two bands of around 140 kDa that remain trypsin insensitive for at least one hour.

After 75 sec., a band of about 100 kDa ca be observed that gradually disappears after 15 min. In the same time a band of about 69 kDa appears. Most likely these bands comprise LPHα as proposed earlier (15,26).

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

### 7.3.3 Processing and enzymatic activity of wtLPH and LPHST in Caco-2

A Caco-2 clone with an undetectable endogenous LPH expression (TC7, (1)) was transfected with the pLPHST and pLPHwt constructs. Stable transfectants were selected with puromycin and screened using immunofluorescence microscopy with antibodies directed against LPHβ. From

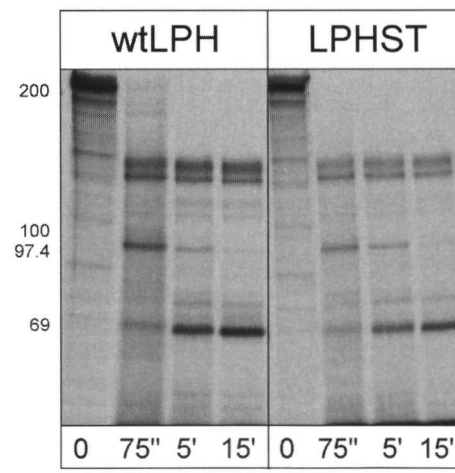


**Fig. 4** Trypsin sensitivity assay of LPH and LPHST expressed in COS-1 cells. Transfected COS-1 cells were labeled with Tran$^{35}$S label for 4 hours. LPH was precipitated using the HBB1/909 antibody. Precipitates were treated with 50 μg/ml trypsin for the indicated time intervals, and analyzed by SDS-PAGE

each transfection, the clone with the highest expression was chosen for further studies. For the LPHST construct this clone is called ST54, the wtLPH clone is WT2. As a control, a clone with an endogenous LPH expression (PD10, (1)) was used.

Immunoprecipitation of pro-LPH and mature LPH from pulse-chased WT2 cells shows that LPH is synthesized as a 215 kDa single-chain polypeptide (fig. 5, lanes 1 and 2) which becomes complex glycosylated to 230 kDa and therefore endo H insensitive after 4 hours of chase (lanes 3 and 4). At this point, LPHβ appears as a faint band of 160 kDa. This band is more intense after 24 hours of chase, when the high mannose form has disappeared (lanes 5 and 6). This processing pattern matches the pattern observed in PD10 cells (lanes 13-18). In intestinal explants, the processing appears to be somewhat faster (15), but is essentially the same.

Introduction of a VSV tag in LPH did not influence the processing since LPHST was processed in ST54 cells (fig. 5, lanes 7-12) in a comparable fashion to wild type LPH in both WT2 and PD10 cells. Sometimes a band of about 180 kDa is observed in fig. 5 (lanes 1, 3, 7, 9, 11, 13). This is a non-specific background band that can be observed in other immunoprecipitations as well, like with anti-sucrase-isomaltase antibodies from not only ST54, but from other Caco-2 cells as well (data not shown).

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

### 7.3.4 Immunoprecipitation of LPHα

V496 is an antibody directed against the 12 amino acids following the signal sequence of pro-LPH (15). In previous studies, a 100 kDa band could be observed after immunoprecipitations from intestinal explants using this antibody, that was suspected to be LPHα (15). A band of this size was never observed in immunoprecipitation studies from Caco-2 clones in this study. Only the high mannose pro-LPH could be precipitated by the V496 antibody

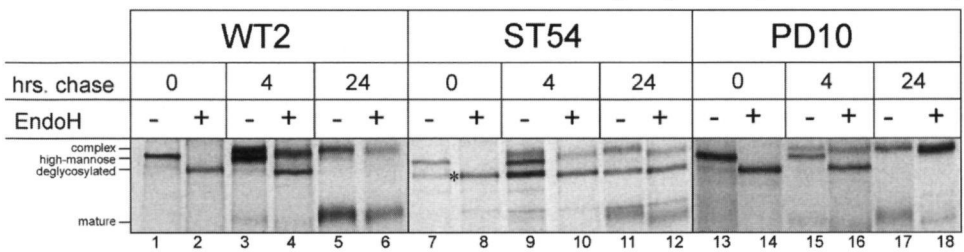


**Fig. 5** Immunoprecipitation of LPH and LPHST from three Caco-2 clones: WT2 and ST54, two clones tranfected with wtLPH and LPHST (resp.), and PD10, that expresses LPH endogenously. Confluent Caco-2 clones were labeled for 1 hour, and chased for 0, 4 and 24 hours. LPH was immunoprecipitated from lysates using the HBB1/909 mAb. Precipitates were treated with endo H (+lanes) or left untreated (-lanes). The processing pattern of transfected wtLPH and LPHST is essentially the same as the pattern of endogenous LPH. In the ST54-lanes, a persistent, endo H insensitive background band is marked with an asterix. This band is also observed in immunoprecipitations of other proteins (sucrase-isomaltase, DPPIV) from Caco-2 clones, and is also faintly visible in lanes 1and 7.

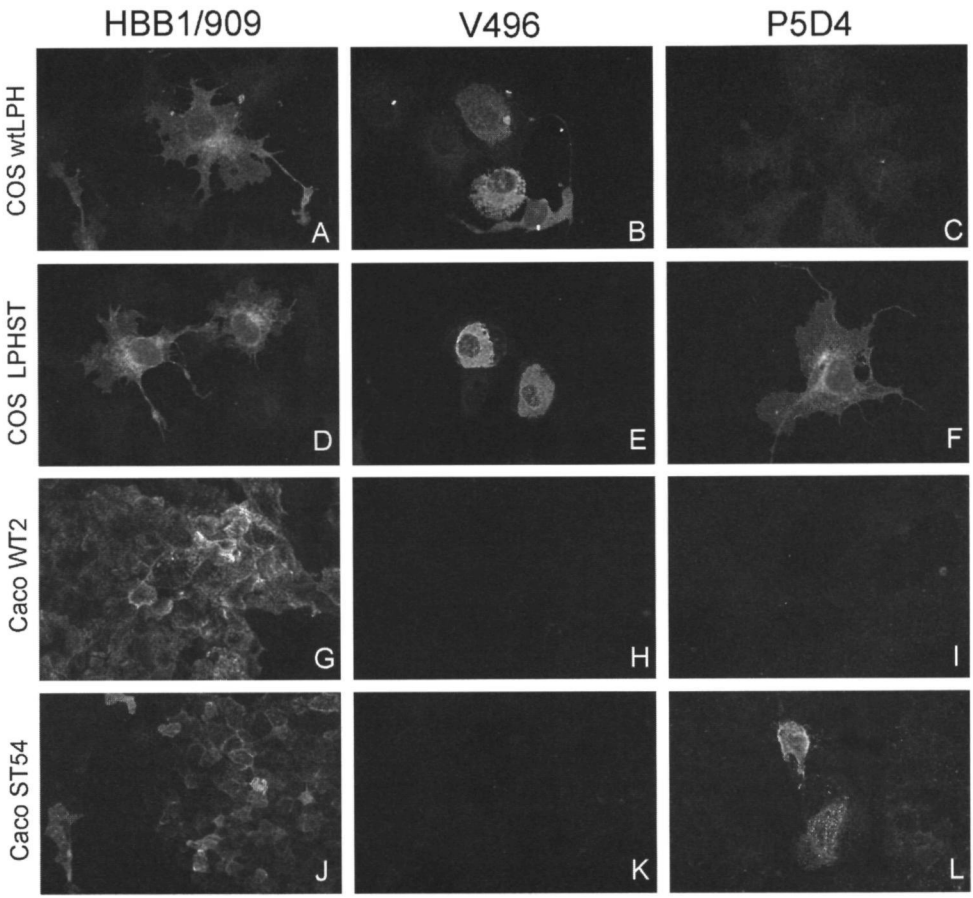


**Fig. 6** Immunofluorescence microscopy of permeabilized COS-1 (panels A-F) and Caco-2 cells (panel G-L), transfected with wtLPH (A, B, C, G, H, I) or LPHST (D, E, F, J, K, L) using antibodies against pro-LPH and mature LPH (HBB1/909), LPHα (V496), or the VSV epitope tag (P5D4).

from both Caco-2 WT2 and ST54 cells. No complex glycosylated bands were observed, even after a chase period of 24 hours (not shown). Apart from this high-mannose band, no other specific band was visible. This lower affinity of V496 for complex glycosylated LPH was found in COS-1 cells as well.

P5D4 did not precipitate any specific protein from WT2 cells, but precipitated both high-mannose and complex glycosylated form from ST54 cells (not shown). No other specific band could be found. Overall no specific LPHα band could be precipitated by the different antibodies and from the different cellular models.

### 7.3.5 Localization of wtLPH and LPHST in COS-1 cells

HBB1/909 is a monoclonal antibody (mAb) that recognizes pro-LPH and mature LPH (7). In immunofluorescence studies on permeabilized wtLPH and LPHST transfected COS-1 cells, this antibody labelled the cell surface and an intracellular network, probably the ER and the Golgi apparatus (fig 6, panel A and D). On non-permeabilized cells, only the surface was labelled (not shown). The V496 antibody labeled an intracellular network, probably the ER and ERGIC (fig. 6, panel B and E). On non-permeabilized cells, no labeling could be observed (not shown), indicating that V496 was not able to label LPH at the cell surface. By contrast, P5D4 did not only show labeling of an intracellular network, but also surface labeling on permeabilized cells that were transfected with LPHST (fig. 6, panel F). P5D4 was also able to label the cell surface of non-permeabilized LPHST cells (not shown). P5D4, but not V496 recognizes complex glycosylated pro-

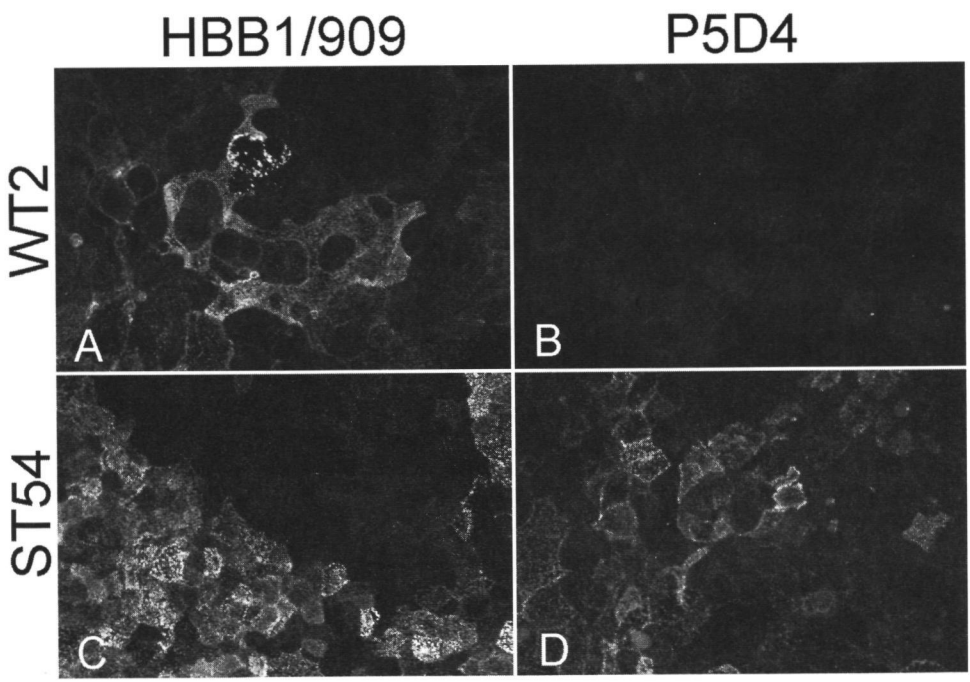


**Fig.7** Immunofluorescence microscopy of non permeabilized Caco-2 WT2 (A, B) and ST54 (C, D) cells labeled with HBB1/909 (A, C) or P5D4 (B, D).Bar, 25 μm

LPHST, indicating that only complex glycosylated LPHST reaches the cell surface, as was already shown by Sterchi et al (26). Cells transfected with wtLPH were not labeled by P5D4 (fig. 6 panel C).

### 7.3.6 Localization of wtLPH and LPHST in Caco-2 cells

In immunofluorescence studies using HBB1/909 on permeabilized 5 day confluent Caco-2 WT2 and ST54 cells, a patchy labeling pattern was visible on some clusters of cells, whereas others did not show label (fig. 6, panel G and J). After subcloning using a limiting dilution protocol, the same distribution was observed (not shown). A comparable mosaic expression has been described for *in vivo* expression of LPH and for other transfections in Caco-2 cells before (1,24). Labeling of non-permeabilized cells also showed a patchy labeling pattern, indicating that mainly the cell surface was labeled (fig.7, panel A and D). This surface was shown to be the apical membrane by confocal microscopy (fig. 8).

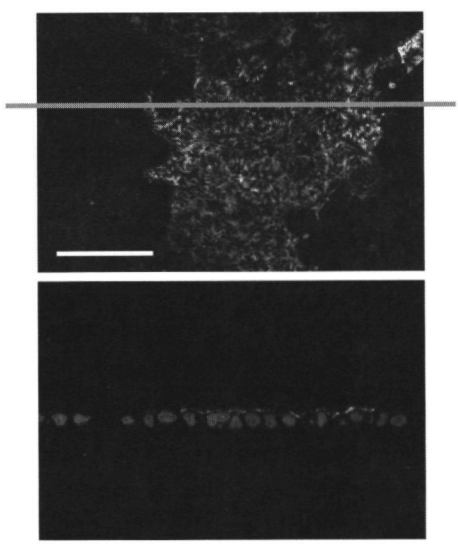

**Fig. 8** Confocal microscopy on permeabilized Caco-2 LPHST cells showed that only the apical surface of the cells was labeled. LPH was labeled with HBB1/909 and RAM-FITC, and the nuclei were stained using Propidium Iodide. On the line across panel A, a vertical section was made, that is represented in panel B.

Immuno-EM studies on WT2 and ST54 cells confirmed the immuno-fluorescence data. Some cells did not have any label, while others were clearly labeled the brush-border (fig. 9). At the basolateral membrane (fig. 9, arrow), no labeling was observed. Some gold particles could be found in the ER. The labeling pattern of WT2 (fig. 9, panel A) and ST54 (panel B) were essentially the same.

Despite the fact that V496 labeled some intracellular structures in transfected COS-1 cells in immunolocalization studies, no labeling could be found under the same conditions in transfected Caco-2 cells (fig 7 panel H, K). This is a consequence of the differences in the steady-state in COS-1 and Caco-2 cells. In transiently transfected COS-1 cells, the protein synthesis level is much higher then in stably transfected Caco-2. Therefore relatively more LPH in COS-1 cells is present in its high-mannose glycosylated form, and recognized by V496. P5D4 against the VSV tag was able to show a faint label at the surface of a few (less then 0.1%) Caco-2 cells expressing tagged LPH (fig. 6 panel L; fig. 7 panel D). This surface staining is most likely labeling of complex glycosylated pro-LPH, that could be precipitated in surface immunoprecipitation experiments on Caco-2 ST54 and WT2 cells using the HBB1/909 antibody (fig. 10). From the apical membrane, LPHβ and complex glycosylated pro-LPHST could be precipitated (lane 1). From the basolateral membrane, some pro-LPHST but not LPHβ could be precipitated (lane 2). Expression of pro-LPH at the cell surface has been found before in biopsy samples (12). In transfected MDCK cells, it was found at both membrane domains as well (5,8). The ultimate fate of pro-LPH on the basolateral membrane, degradation or transport to the brush-border needs further analysis.

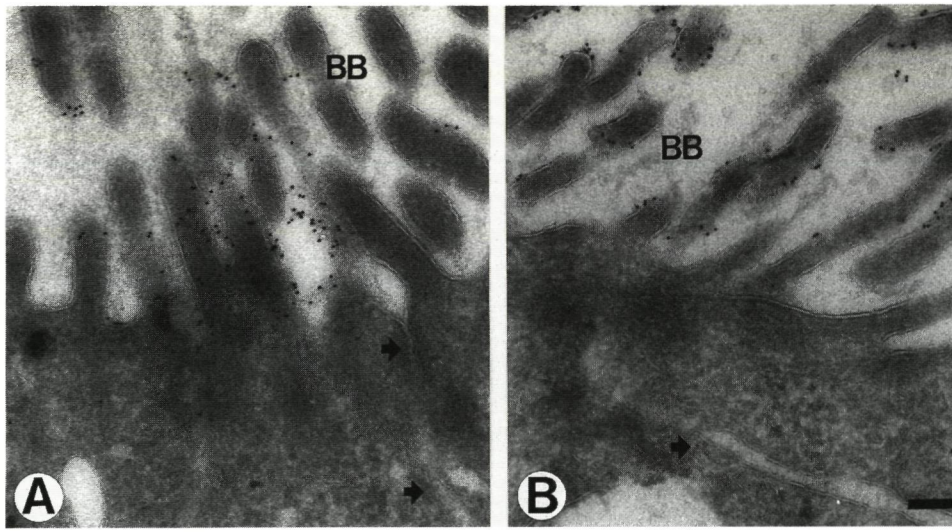


**Fig. 9** Electron microscopy on Caco-2 LPHwt (A) and LPHST (B) cells shows immunogold staining at the brush-border (BB). No labeling could be detected at the basolateral membrane (arrows). Bar, 200 nm.

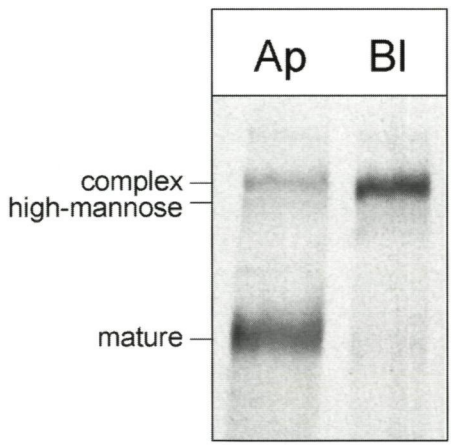


**Fig. 10** Surface immunoprecipitation of Caco-2 ST54 cells. At the apical membrane (Ap), complex glycosylated pro-LPH can be found, as well as LPHβ (mature). At the basolateral side (Bl), only complex glycosylated pro-LPH is present. WT2 cells showed essentially the same pattern (not shown).

## 7.4 Concluding remarks

Many proteins undergo proteolytic processing after translation. Among these proteins are lysosomal (6), secretory (19,22) and some plasma membrane proteins. Most disaccharidases are plasma membrane proteins that are cleaved into their subunits (21). LPH is an example of a disaccharidase that undergoes proteolytic cleavage. Unlike other examples LPH has a C-terminal instead of a N-terminal transmembrane region and both its active sites are located on one cleavage product. Furthermore, it consists of four homologous regions instead of two, which are probably derived from a double gene duplication (12). Two of these regions, III and IV, contain the active sites, and are both localized on the same cleavage product, LPHβ, that is expressed on the brush-border membrane. The second cleavage product, LPHα, possesses the other two homologous regions, I and II, but does not show any activity towards disaccharides (15,16).

Two separate studies have reported a role for LPHα as an intramolecular chaperon (15,17). An additional function could be hypothesized because of the internal homologies and because LPHα is relatively large (714 residues). Therefore we developed a model in which we could study LPHα in more detail. This model consists of Caco-2 cells, expressing wild type or modified LPH containing a VSV-epitope tag in its LPHα domain for additional recognition possibilities. Immunoprecipitation studies from these cells using both an antibody against LPHα and an antibody against an in LPHα inserted tag did not result in a specific pro-fragment band. Furthermore, localization studies in Caco-2 cells did not result in specific labeling in any LPH-positive cell. We suggest that in Caco-2 cells LPHα is degraded soon after cleavage since no intra- or extracellular accumulation could be observed Our results do not ascertain whether degradation of LPHα occurs on its way to the brush-border or at the brush-border itself.

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Chapter 8

# General discussion

**Routing and modifications of brush-border sucrase-isomaltase and lactase-phlorizin hydrolase.**

At the brush border of intestinal epithelial cells, enzymes are expressed that play a role in the digestion of food Since these brush-border enzymes have to be specifically transported to the apical membrane, they form an interesting group for the study of polarized transport mechanisms This thesis is focused on the routing and processing of two brush-border proteins sucrase-isomaltase (SI) and lactase-phlorizin hydrolase (LPH) To study transport mechanisms responsible for proper transport of SI, naturally occurring mutations in SI were analyzed that caused transport defects of the enzyme To study transport and processing of LPH, the fate of the profragment (a large part of the protein that is cleaved off during processing) and of the mature protein was studied in intestinal epithelial cells after introduction of an epitope tag in the profragment

**Naturally occurring mutations in SI**

To characterize various steps in the biosynthesis, transport and sorting of sucrase-isomaltase as a model for cell surface membrane proteins, naturally occurring mutations in SI have been studied in cases of congenital SI deficiency (CSID) Several different phenotypes of CSID have been meanwhile described (4,9,13,17) In all these CSID-phenotypes the transport incompetence, aberrant enzymatic function or missorting to the basolateral membrane are exclusively restricted to the SI molecule and not a general cellular defect Analyses of the phenotypes at the subcellular and protein levels lent strong support to the hypothesis that a point mutation in the cDNA of SI is responsible for the generation of CSID Indeed, the data described in chapter 4 of this thesis provide the first definite evidence that this is the case, a point mutation was characterized in the region of the cDNA encoding the sucrase subunit of the SI complex which results in conversion of a glutamine to a proline (Q1098P)

The phenotype of SI described in chapter 4 is very much similar to phenotype II (17) SI exits the ER and is transported to the medial and trans-Golgi where it undergoes degradation Analyses of the structural features of SI in this phenotype by epitope mapping and biosynthetic labeling revealed that incomplete initial folding of the SI precursor protein is responsible for the observed inefficient transport of the molecule along the secretory pathway An effect of the oligomeric state of SI could be excluded since human SI does not dimerise and exits the ER efficiently as a monomeric molecule (12)

The most interesting aspect of phenotype II is the ability of SI to egress the ER to the Golgi, while still in an incompletely or partially folded state This is surprising since membrane and secretory proteins are believed to acquire transport-competence in the ER and leave this organelle en route to their final destination It is generally accepted that a quality control mechanism exists in the ER that sorts correctly folded from malfolded proteins and retains the latter in the ER (1,5,23,24) How could phenotype II of CSID be accommodated within these general views and concepts?

It is possible that in the particular case of proteins with multi-domain structure, such as SI, complete maturation of all protein domains is not absolutely required for egression from the ER, whereby the folding of some domains may be more essential or critical than others in the context of the overall conformation SI is composed of two large homologous domains that may fold independently and it is reasonable to assume that the presence of the mutation in the sucrase subunit leads to alterations in its structure while the entire isomaltase subunit remains unaffected While these alterations could be tolerated by the quality control mechanism in the ER, they could be crucial

for the further secretory pathway of SI via the Golgi to the cell surface. Data from a temperature-sensitive mutant of VSV G protein have proposed a quality control mechanism outside the ER that prevents immature VSV G protein from traversing the Golgi (7). Mutant SI-phenotype II may be subject to a similar mechanism that operates via a putative retention signal created by the Q1098P mutation.

In chapter 5, the Q1098P substitution is analyzed in more detail and it is demonstrated to be not only functional in intestinal epithelial cells but also to produce a similar phenotype when expressed in non-polarized COS-1 cells. This supports the view that (i) the Q1098P mutation per se is responsible for the generation of the SI phenotype and (ii) cellular factors specific for epithelial cells are not implicated in the onset of this CSID phenotype.

An important observation during studies of the protein in COS-1 cells was the folding state of the mutant SI protein. Protease sensitivity assays using trypsin demonstrated that mutant SI is not as stable as its wild type counterpart and is degraded within a relatively short period of time. Interestingly, the sucrase as well as the isomaltase subunits are degraded. A malfolded sucrase subunit as a result of the mutation Q1098P is expected to exploit a different pattern of trypsin sensitivity as compared to wild type sucrase, but the concomitant degradation of isomaltase needs more explanation.

Wild type SI reaches its protease resistant conformation after a certain 'lag-period' during which both subunit are susceptible for degradation (16) Presumably mutant SI cannot reach this conformation because: a) The protein is not transported into the compartment with the proper 'folding machinery', b) The mutation makes it energetically impossible to attain the proper folding, or c) Because of the improper folding of sucrase the protein is not transported further so isomaltase is not folded properly either and remains protease sensitive as well.

Alternatively, further cleavage could be prevented by associations between the sucrase and isomaltase subunits that could mask putative trypsin cleavage sites in either subunit. One type of association could take place after the independent folding of each subunit. These are ultimately assembled as pseudodimers (12) which efficiently leave the ER. Another type of association could take place very early in the ER, where the subunits possibly assist each other to fold properly and in this way function as "intramolecular chaperons" (19,21,27). In mutant SI, these protective associations might be absent, resulting in exposed protease sensitive sites.

**Artificial mutations: analysis of the Q1098P mutation in SI**

An interesting feature of the Q1098P mutation is its presence in a region that shares striking homologies between human, rat and rabbit sucrase and isomaltase variants as well as human lysosomal α-glucosidase and *Schw occidentalis* glucoamylase (18), (chapter 6, fig 1) These proteins, which have been suggested to have evolved from a common ancestral gene, are all synthesized and translocated into the ER where they are processed to transport-competent forms and then transported along the secretory pathway to their final destinations. SI is sorted, most likely in the TGN, to the apical membrane. Lysosomal α-glucosidase is after phosphorylation in the cis-Golgi and maturation in the medial and trans-Golgi mainly transported to the lysosomes, but is also to a lesser extent secreted at the apical membrane in polarized epithelial cells *Schw occidentalis* glucoamylase is transported to the periplasmic space and partially secreted into the exterior milieu in yeast. Obviously all three proteins share a common pathway from the ER to the Golgi and from there

diverge to different destinations. It is therefore conceivable that homologies in the polypeptide sequences should be implicated in common functions needed for proper transport or catalytic activity. If the mutation Q1098P does not induce gross conformational changes in the sucrase subunit, but rather alters a subdomain that is critical for the intracellular transport of SI, then a similar effect might be expected if the same mutation is introduced into the corresponding homologous regions of lysosomal α-glucosidase or *Schw. occidentalis* glucoamylase.

The results described in chapter 5 show indeed that the Q1098P mutation identified in the sucrase subunit elicits a similar effect when introduced at the corresponding amino acid position 244 in lysosomal α-glucosidase. The mutated lysosomal α-glucosidase precursor as well as the mutant SI precursor remain mannose-rich polypeptides that do not undergo maturation in the Golgi, are not cleaved into mature enzyme and are not transported through the Golgi cisternae. It is conceivable that the homologous domain of α-glucosidase, sucrase and isomaltase harbor a structural motive with a potential role in transport signaling or quality control acting at the level of ERGIC-cis-Golgi at which the mutant proteins are blocked. These observations can be explained as follows:
1) the mutation has introduced a retention signal for the cis-Golgi, 2) the mutation has lead to the recognition of the protein by the ER - Golgi recycling machinery, 3) the mutation has lead to a structural alteration that functions as a recognition site for a quality control machinery operating in the intermediate compartment or cis-Golgi, or 4) the mutation has altered a region that is of crucial importance for transport through the Golgi apparatus.

The first possibility suggests that a single point mutation alters a highly conserved region into a region with a novel function. Although it can not be excluded, this is very unlikely.

The second possibility is that mutant SI and lysosomal α-glucosidase exit the ER, are transported to ERGIC or cis-Golgi and then are recycled back to the ER. Observations with a temperature sensitive mutant of the VSV G protein have shown that at the non permissive temperature the G protein leaves the ER, arrives in the ERGIC and the cis Golgi and recycles back to the ER. Presumably several cycles of ER-ERGIC-ER occur during which the G protein is always found associated with the luminal binding protein (BiP) and calnexin in the ER and with BiP in ERGIC (7). In view of these findings the hypothesis has emerged that misfolded proteins that have escaped the ER are retrieved from ERGIC or cis Golgi back to the ER presumably to be reexposed to the folding machinery and thus have additional opportunities to acquire correct folding A similar recycling mechanism between the ER and ERGIC or cis Golgi is potentially possible for mutant SI and lysosomal α-glucosidase. In the case of the VSV G protein a retrieval mechanism to the ER has been proposed that implicates the binding of the BiP/VSV G complex to the BiP receptor in the ER. Although we do not know whether SI or lysosomal α-glucosidase bind BiP or calnexin, a retrieval mechanism of mutant SI or lysosomal α-glucosidase complexes with ER-resident proteins cannot be excluded.

The third possibility is that a quality control mechanism operates at a level beyond the ER and prevents misfolded proteins from being further transported along the secretory pathway to the cell surface. After longer periods the mutant proteins are finally degraded. Therefore the Q1098P mutation in SI and the Q244P mutation in α-glucosidase could have generated structural alterations in the proteins that cause an arrest by a control mechanism operating in the intermediate compartment or cis-Golgi. A comparable

situation has been reported for misfolded major histocompatibility complex (MHC) class I molecules in mice that are deficient for TAP1, the transporter associated with antigen presentation (25) In these mice the MHC can not bind to peptides, which prevents the complexes to fold properly As a result they accumulate in an expanded ER-Golgi intermediate compartment Although not exactly the same expansion was found, an enlargement of the Golgi complex and a change in the localization of ERGIC-53 in the patient's intestinal cells is described in chapter 4

The fourth possibility suggests that the mutation has altered a subdomain that is critical for the intracellular transport of both SI and α-glucosidase If the glutamine residue in the conserved region is important for proper transport of the proteins along the secretory pathway, introduction of other amino acids at the same position would lead to transport defects as well On the other hand, if introduction of the proline residue into the polypeptide has lead to such structural alterations in the folding of the molecule that it is recognized by some kind of control mechanism and retained, then other amino acids at the same position would lead to transport competent molecules Both possibilities were investigated in chapter 6 Q1098 was substituted by alanine and lysine and the transport compatibility of the mutants was studied in COS-1 cells by immuno-precipitation and immunofluorescence microscopy Both mutant proteins were shown to be fully transport competent

Concluding, although the glutamine at position 1098 is highly conserved, it is not essential for the transport compatibility of SI We therefore conclude that proline introduced a structural alteration that resulted in the retention and final degradation in the Golgi apparatus of the Q1098P mutant Proline is in this regard probably the most infamous example of an amino acid that can severely alter the 3D structure of a protein It is known to introduce kinks in α-helices (31,32) Furthermore, several mutant proteins have been reported that lost their function and/or transport properties after introduction of a proline (see for instance (14,22,24))

In essence, the analysis of CSID has resulted in the identification of a mutation that is responsible for a block of the molecule in the ERGIC or the cis-Golgi Although the results favor a possible quality control mechanism in these compartments, analysis of the binding kinetics of mutant SI and lysosomal α-glucosidase with BiP or calnexin is required to determine whether a recycling mechanism is conceivable for these proteins The observations regarding the role of a highly conserved region of SI in proper transport of the molecule could be an interesting contribution to the identification of novel control steps involved in proper transport along the secretory pathway

**The fate of the pro-fragment of LPH**

Many proteins undergo more or less extensive proteolytic processing after translation Among these proteins there are lysosomal (8) and secretory (26,29) proteins, but also some plasma membrane proteins Most disaccharidases are plasma membrane proteins that are cleaved into their subunits (28) LPH is a special example of such a disaccharidase, because it has a C-terminal instead of a N-terminal transmembrane region and because both its active sites are located at one cleavage product Furthermore, it consists of four homologous regions instead of two, which are probably derived from a double gene duplication (15) Two of these regions, III and IV contain the active sites, and are both localized on the same cleavage product, LPHβ, that is expressed at the brush-border The second cleavage product is called LPHα Although it possesses the other two homologous regions, I and II, it did not show any activity towards disaccharides (19,20) In contrast, most other cleaved disaccharidases possess enzyme activity at both products of the cleavage Because of this, and because LPHα is relatively large (714 residues) it can be hypothesized that LPHα has an additional function apart from the 'intramolecular chaperon' properties that have been described before (19,21) Therefore it could be interesting to study LPHα in more detail

In order to develop a system in which LPHα could be studied separately from pro-LPH, transfected Caco-2 cells without endogenous LPH expression (TC7, (3)) were stably transfected with wild type LPH cDNA or with a construct in which a VSV-epitope tag was introduced into LPHα (LPHST) Immunolocalization studies and immunoprecipitations were performed using antibodies against LPHα or against the VSV-tag Overall, LPH was expressed in both transfected cell lines similar to Caco-2 cells with a endogenous LPH expression (PD10, (3)) The processing, monitored by the HBB1/909 antibody against pro-LPH and mature LPH (10), was, although somewhat slower, essentially the same as described for intestinal biopsy samples (19) In immunofluorescence and immuno electron microscopy, HBB1/909 mainly labeled the apical cell surface

V496 is an antibody directed against the 12 amino acids following the signal sequence of pro-LPH (19) In previous studies, a 100 kDa band could be observed after immunoprecipitations from intestinal explants using this antibody (19) A band of this size could never be observed in immunoprecipitation studies from Caco-2 clones in this study Only the high mannose, uncleaved pro-LPH form was recognized This lower affinity of V496 for complex glycosylated LPH was found in COS-1 cells as well Possibly complex glycosylation of a putative *N*-glycosylation site adjacent to the V496 recognition sequence masks this sequence In intestinal biopsy samples, LPHα was not glycosylated at all (19) Because Caco-2 cells show a different glycosylation pattern then biopsy samples (10), LPHα could be (complex) glycosylated in Caco-2, and therefore not recognized by V496

Insertion of a VSV-epitope tag into LPHα did not result in an improved recognition of LPHα the antibody against this tag was not capable either to precipitate a pro-fragment, although it was, in contrast with V496, able to precipitate the complex glycosylated pro-LPH indicating that the VSV-epitope was intact

Despite the fact that V496 labeled some intracellular structures in transfected COS-1 cells in immunolocalization studies, no labeling could be found under the same conditions in transfected Caco-2 cells P5D4 against the VSV tag however, was able to show a faint label at the surface of Caco-2 cells expressing tagged LPH This surface staining is most likely labeling of complex

glycosylated pro-LPH, that was precipitated in surface immunoprecipitation experiments on transfected Caco-2 cells Expression of this form at the cell surface has been found before in biopsy samples (15) and in transfected MDCK cells (6,11)

This surface expression can be explained by overexpression of LPH, and therefore saturation of the processing machinery, but there is another explanation possible Many endogenous and transfected proteins show a mosaic expression pattern in Caco-2 cells (2,3,30) Also after a limiting dilution subcloning protocol, the proteins remain expressed in a heterogeneous fashion The number of cells that were positive for the anti-VSV-tag antibody was markedly lower then for the anti-LPH-antibody This could be due to mosaic expression of the protease that is responsible for the α/β cleavage The cells that can be labeled with the anti-tag antibody presumably do not express this protease, so pro-LPHST is expressed at the cell surface of these cells

Immunoprecipitation studies using both an antibody against LPHα and an antibody against an in LPHα inserted tag did not result in a specific pro-fragment band We suggest that in Caco-2 cells LPHα is degraded soon after cleavage, either on its way to the brush border or on the brush border itself

**Concluding remarks and future perspectives**

Altogether, modification of the glutamine residue to proline at position 1098 of the sucrase subunit of SI has lead to defects in the routing of sucrase-isomaltase in a patient with a SI deficiency Not the substitution of the glutamine residue was the cause of this defect, but the introduction of the proline residue Most likely proline has introduced such structural alterations that the protein is retained by some kind of post-ER control mechanism To study such mechanisms, it could be determined what proteins are associated with the mutant protein and what degradation machinery is responsible for the final degradation of the mutant

Modification of the pro-fragment of LPH by introduction of a VSV-epitope tag did not alter the enzyme activity or transport compatibility of LPH The mature protein was normally expressed at the brush-border Since the profragment could not be retrieved from lysates nor localized apart from the uncleaved pro-LPH, we suppose that it is degraded soon after cleavage Just as with the mutant SI, it could be determined what degradation mechanisms play a role This could be accomplished by inhibition of specific degradation processes, for instance by addition of inhibitors of proteasome or lysosome dependent degradation Furthermore, the transfected Caco-2 cells form an excellent subject for the identification of the protease that is responsible for the cleavage of pro-LPH into LPHα and LPHβ

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# Chapter 9

# Summary
# &
# Samenvatting

## SUMMARY

Epithelial cells form a barrier between two spaces· cells of the body at one side and liquid (blood, intestinal contents, urine) or air at the other Epithelial cells are attached firmly by so called tight junctions. Tight junctions divide the membrane of these cells into two domains: the apical and the basolateral membrane In intestinal epithelial cells, proteins destined for the apical membrane (for instance digestive enzymes) can be transported either directly from the Golgi apparatus to the apical membrane, or indirectly via the basolateral membrane, followed by transcytosis. Both proteins investigated in this project are transported via the direct route, the apical pathway. Both are expressed in epithelial cells in the small intestine, and both are involved in the degradation of specific sugars During this project the importance of protein domains in intracellular transport was studied: 1) a naturally occurring mutant sucrase-isomaltase has been identified and analyzed and 2) the routing of the pro-fragment of lactase-phlorizin hydrolase (LPH) has been studied by introduction of an epitope tag

**Sucrase-isomaltase**

A point mutation in the cDNA of human intestinal sucrase-isomaltase has been identified in phenotype II of congenital sucrase-isomaltase deficiency. This mutation results in a substitution of glutamine by proline at position 1098 (Q1098P) in the sucrase subunit. Expression of this mutant sucrase-isomaltase cDNA in COS-1 cells results in an accumulation of sucrase-isomaltase in the cis-Golgi cisternae similar to the accumulation in phenotype II intestinal cells. The glutamine at position 1098 is highly conserved, and is located in a highly conserved region of the sucrase subunit that shares striking similarities with the isomaltase subunit and other functionally related enzymes, such as human lysosomal acid α-glucosidase and *Schw occidentalis* glucoamylase. Because of this homology, it was suspected that introduction of the mutation into the corresponding region of another protein would result in a comparable accumulation Indeed, mutation of glutamine 244 to proline in α-glucosidase resulted in a protein that was not transported beyond the Golgi apparatus.

Although the glutamine residue at position 1098 is highly conserved, it is not essential for proper transport since substitution of this residue by alanine or lysine resulted in fully transport competent mutant proteins.

Most likely the proline that was discovered in the sucrase isomaltase in the patient and that was introduced into α-glucosidase has generated structural alterations that are recognized by a control mechanism operating beyond the ER, probably in the cis-Golgi.

**Lactase-phlorizin hydrolase**

After terminal glycosylation in the Golgi apparatus, the 215 kD pro-LPH is cleaved into the 160 kD mature brush-border LPH (LPHβ) and the pro-fragment (LPHα) Because LPHα is essential for LPHβ to pass the ER and Golgi apparatus, it has been suggested that it functions as an intramolecular chaperon for the transport all the way from the ER through the Golgi apparatus What the fate of LPHα is after the cleavage has never been established Because it is relatively large (100 kD), and because it shares homology with LPHβ, it could have additional functions

To investigate LPHα in more detail, a VSV epitope tag was inserted into LPHα Wild type and tagged LPH cDNAs were stably transfected in Caco-2 cells Both proteins were processed at similar rates The mature LPHs were expressed at the apical cell surface By immunoprecipitation and localization studies with antibodies against the pro-fragment epitope or the VSV-tag no specific pro-fragment could be observed Most likely LPHα is degraded immediately after detachment from LPHβ, and therefore only functions as a intramolecular chaperone

## SAMENVATTING

Epitheelcellen vormen een barrière tussen twee milieus aan een kant cellen van het lichaam, aan de andere kant vloeistof (bloed, darminhoud, urine) of lucht. Deze epitheelcellen zijn stevig met elkaar verbonden door zogenaamde tight junctions De celmembraan van epitheelcellen wordt door die tight junctions verdeeld in twee domeinen: apicaal en basolateraal. De apicale membraan staat in het algemeen in direct contact met de buitenwereld, terwijl de basolaterale membraan contact met andere cellen onderhoudt. Eiwitten die in epitheelcellen bestemd zijn voor de apicale membraan kunnen direct vanaf het Golgi naar de apicale membraan getransporteerd worden, maar ook indirect. eerst naar de basolaterale membraan, waar ze weer worden opgenomen en via transcytose de apicale membraan bereiken. In dit proefschrift worden twee eiwitten beschreven die de directe weg volgen: sucrase-isomaltase en lactase-phlorizin hydrolase. Beide eiwitten komen tot expressie in darmepitheelcellen, en beide zijn verantwoordelijk voor de afbraak van specifieke suikers.

**Sucrase-isomaltase**

Gedurende dit promotieonderzoek is een mutatie aangetoond in het cDNA coderend voor sucrase-isomaltase die verantwoordelijk is voor een ophoping van het eiwit in het Golgi apparaat. Dit cDNA is geïsoleerd uit darmbiopten van een patiënt met een congenitale sucrase-isomaltase deficiëntie. Expressie van het mutante cDNA in COS-cellen liet een ophoping zien die vergelijkbaar was met de ophoping in darmbiopten van de patiënt; het sucrase-isomaltase werd niet verder getransporteerd dan de eerste cisternae van het Golgi apparaat, en werd niet complex geglycosyleerd. De mutatie veroorzaakt een substitutie van een glutamine op positie 1098 in de aminozuursequentie door een proline in de sucrase subunit van het complex.

De glutamine op positie 1098 is zeer geconserveerd, en bevindt zich in een gebied dat veel homologie vertoont met andere eiwitten, bijvoorbeeld met glucoamylase uit de gistsoort *Schw. occidentalis*, met een humaan lysosomaal eiwit, α-glucosidase, en met de isomaltase subunit van het sucrase-isomaltase van verscheidene diersoorten zelf. Vanwege deze homologie bestond het vermoeden dat dezelfde mutatie, geïntroduceerd in een ander eiwit op dezelfde plaats, een vergelijkbare ophoping te zien zou geven. Dit bleek inderdaad het geval; mutatie van glutamine 244 naar proline in de aminozuursequentie van lysosomaal α-glucosidase resulteerde in een eiwit dat ook niet verder werd getransporteerd dan het Golgi apparaat.

Hoewel de glutamine op positie 1098 zeer geconserveerd is, is zij niet essentieel voor het juiste transport van sucrase-isomaltase· substitutie van dit aminozuur door alanine of lysine veroorzaakte geen transportdefecten, beide mutante eiwitten werden normaal naar het oppervlak van COS-cellen getransporteerd. Hoogstwaarschijnlijk heeft de proline die in het sucrase isomaltase van de patiënt op positie 1098 werd aangetroffen de ruimtelijke structuur van het eiwit dusdanig veranderd, dat het door een of ander controle mechanisme onderweg wordt tegengehouden Dergelijke controle mechanismen worden meestal verondersteld te functioneren in het ER, en niet daarna Deze mutatie zou dan ook een bijdrage kunnen leveren aan de identificatie van een controle mechanisme in het begin van het Golgi apparaat en van de factoren die daarin een rol spelen.

**Lactase-phlorizin hydrolase**

Nadat lactase-phlorizin hydrolase (LPH) in het Golgi apparaat de laatste glycosylering heeft ondergaan, wordt het 215 kD grote pro-LPH gekliefd in de 160 kD mature brush-border vorm of LPHβ en het pro-fragment of LPHα LPHβ komt op de apicale membraan tot expressie Omdat het voor het transport door het ER en het Golgi apparaat essentieel is dat beide domeinen aan elkaar vastzitten, wordt gesuggereerd dat LPHα functioneert als intramoleculaire chaperone voor LPHβ Wat er echter gebeurt met het LPHα als het eenmaal is losgeknipt is tot nu toe onduidelijk Omdat het relatief groot is, en omdat het homologie vertoont met LPHβ, zou het nog een extra functie kunnen hebben

Om meer te weten te komen over het lot van LPHα nadat het is losgeknipt van LPHβ is een zogenaamde epitoop tag ingebouwd in LPHα Deze tag kan worden herkend door een specifiek antilichaam Het cDNA coderend voor dit 'getagde' LPH en voor het wild type LPH zijn apart in Caco-2 cellen tot expressie gebracht Deze humane colon carcinoma cellijn fungeert als model voor darm-epitheelcellen Beide eiwitten werden in deze cellen op dezelfde manier en even snel gesynthetiseerd en gekliefd, en hadden bovendien een vergelijkbare enzymactiviteit In beide cellijnen werd LPHβ op de brush-border tot expressie gebracht Met het antilichaam tegen de tag werd uitsluitend het ongeknipte pro-LPH geprecipiteerd Waarschijnlijk wordt LPHα onmiddelijk nadat het van LPHβ is losgeknipt afgebroken, en heeft het alleen een functie als intramoleculaire chaperone

## Abbreviations

| | |
|---|---|
| AMV-RT | Avian Myeloblastosis Virus-Reverse Transcriptase |
| ApN | Aminopeptidase N |
| ARF | ADP Ribosylation Factor |
| BiP | Luminal Binding Protein |
| CGN | Cis-Golgi Network |
| COP | Coat Protein |
| CSID | Congenital Sucrase-Isomaltase Deficiency |
| DMEM | Dulbecco's Modified Eagle's Medium |
| EM | Electron Microscopy |
| Endo F/GF | Endo-N-acetylglucosaminidase F/glycopeptidase F |
| Endo H | Endo-N-acetyl-glucosaminidase H |
| ER | Endoplasmic Reticulum |
| ERGIC | ER-Golgi Intermediate Compartment |
| GPI | Glycosyl Phosphatidyl Inositol |
| kDa | Kilodalton |
| LPH | Lactase-Phlorizin Hydrolase |
| LPHST | LPH with a VSV epitope tag |
| mAb | Monoclonal Antibody |
| MHC | Major Histocompatibility Complex |
| Mr | Relative Molecular Weight |
| NSF | *N*-ethylmaleimide Sensitive Fusion Protein |
| PCR | Polymerase Chain Reaction |
| PDI | Protein Disulphide Isomerase |
| prepro-LPH | LPH, translated mRNA |
| pro-LPH | prepro-LPH without signal sequence |
| pro-SI | SI, translated mRNA |
| Q1098A;Q1098K;Q1098X | Substitution of glutamine by alanine, lysine or any amino acid (resp ) at position 1098 |
| Q1098P; Q244P | Substitution of glutamine by proline at amino acid position 1098; 244 (resp.) |
| Rab | Ras-like Protein from rat brain |
| SI | Sucrase-Isomaltase |
| SNAP | Soluble NSF Attachment Protein |
| SNARE | SNAP Receptor |
| SRP | Signal Recognition Particle |
| TGN | Trans-Golgi Network |
| UDP Glc Transferase | UDP-glucose.glycoprotein Transferase |
| VSV G-protein | Vesicular Stomatitis Virus Glycoprotein |
| Wt; wt | Wild Type |

## Publications

J Ouwendijk, C E C Moolenaar, W J M Peters, C P Hollenberg, L A Ginsel, J A M Fransen, and H Y Naim Congenital sucrase-isomaltase deficiency Identification of a glutamine to proline substitution that leads to a transport block of sucrase-isomaltase in a pre-Golgi compartment *Journal of Clinical Investigation* (1996) 97 633-641

C E C Moolenaar, J Ouwendijk, M Wittpoth, H Wisselaar, H P Hauri, L A Ginsel, H Y Naim and J A M Fransen A mutation in a highly conserved region in brush-border sucrase-isomaltase and lysosomal a-glucosidase results in Golgi retention *Journal of Cell Science* (1997) 110 557-567

J Ouwendijk, L A Ginsel, J A M Fransen Sucrase-isomaltase a unique model to study specific cell-biological processes *Trends in Glycoscience and Glycotechnology* (1997) Vol 9, 46 223-232

J Ouwendijk, W J M Peters, R H M te Morsche, R A van de Vorstenbosch, L A Ginsel and J A M Fransen Analysis of a naturally occuring mutation in sucrase-isomaltase presence of a glutamine at position 1098 is not essential for transport to the surface of COS-1 cells *Submitted for publication*

J Ouwendijk, W J M Peters, R A van de Vorstenbosch, L A Ginsel, H Y Naim and J A M Fransen Routing and processing of lactase-phlorizin hydrolase in transfected Caco-2 cells *Submitted for publication*

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## Curriculum Vitae

Joke Ouwendijk werd op 23 december 1965 in Vierpolders geboren. In 1984 behaalde zij het Atheneum-B diploma aan de Rijksscholengemeenschap Brielle. In datzelfde jaar begon ze aan het Van Leeuwenhoek Instituut te Delft (tegenwoordig de polytechnische faculteit van de Hogeschool Rotterdam en omstreken) met de Hogere Laboratorium Opleiding Na een stage bij de afdeling experimentele chemotherapie van het Nederlands Kanker Instituut behaalde ze in 1988 haar HLO diploma in de richting biochemie, waarna ze meteen met de studie Biologie aan de Universiteit van Utrecht begon Deze studie werd in 1992 afgerond na een tweetal stages Tijdens de eerste stage bij de vakgroep Veterinaire Immunologie werd in samenwerking met het RIVM de toepassing van heat-shock eiwitten als immuun-stimulerende carriers bestudeerd onder supervisie van dr Ruurd van der Zee De tweede stage bij de vakgroep electronen-microscopie en structuur analyse (EMSA) had als onderwerp de lokalisatie van een kernmatrixeiwit en werd begeleid door dr Arjan de Graaf.

In 1992 begon een periode als OIO bij de vakgroep Celbiologie en Histologie van de Katholieke Universiteit Nijmegen op het projekt 'Routing of brush-border proteins in polarized intestinal epithelial cells' onder begeleiding van dr Jack Fransen en prof. dr Leo Ginsel In het kader van dit projekt is drie maanden in Dusseldorf gewerkt bij de groep van dr Hassan Naim in het Institut für Mikrobiologie om een gedeelte van het Sucrase-Isomaltase (SI) cDNA te kloneren en te sequencen van een patient met een deficientie van dit eiwit Het in dit proefschrift beschreven werk werd tijdens een aantal internationale bijeenkomsten gepresenteerd (o a tijdens de Fourth European Congress of Cell Biology in Praag, 1994 en het LX Cold Spring Harbor Symposium 'Protein Kinesis the Dynamics of Protein Trafficking and Stability' in Cold Spring Harbor, 1995)

Binnenkort zal een nieuwe periode beginnen als post-doc bij de groep van dr Tommy Nilsson bij het European Molecular Biology Laboratory in Heidelberg, Duitsland