Signals and mechanisms implicated in the apical sorting of small intestinal proteins

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Chapter 1

General Introduction and scope of the thesis

Introduction

Cells of eucaryotic organisms are far more complicated structurally and functionally than prokaryotic cells and differ radically in their organisation. Whereas prokaryotic cells generally consists of a single intracellular compartment enclosed by the plasma membrane, eucaryotic cells are characterized by membrane enclosed subcellular compartments that compartmentalize specific functions and confine different specific metabolic reactions and the proteins required to carry them out within distinct structurally and functionally specialized membrane-bound compartments called organelles, and uses the membrane trafficking system as a device for segregating, controlling their chemical reactions and organize the flow of materials and signal transduction(1;2). In addition to the difference in the membrane structure, each of these compartment serves different purposes and contains its own characteristic set of proteins and lipids constituents. These internal membranes allow the eucaryotic cells to compartmentalize functions that in prokaryotic cells take place within the cytoplasm or on the plasma membranes. Important examples of such complex distributed internal membrane systems which transport proteins from one organelle to another includes the endoplasmic reticulum, Golgi apparatus, vacuoles as well as different kinds of transport vesicles.

Polarized epithelial cell

Each tissue or organ in the mammalian body has a specialised function, reflected in its anatomy and metabolic activity. Polarized epithelial cells for example are organized into sheets occurring on the body surface and forming a layer that covers most of internal body cavities of mammalian, (e.g. respiratory, urinary, reproduction, and digestive systems) called epithelium. The most important function of epithelial cells is to provide protection and

regionalization to the body, and regulate the molecular composition of and exchange between, the compartments that they separate. (3-6). Many functions of epithelial cells,

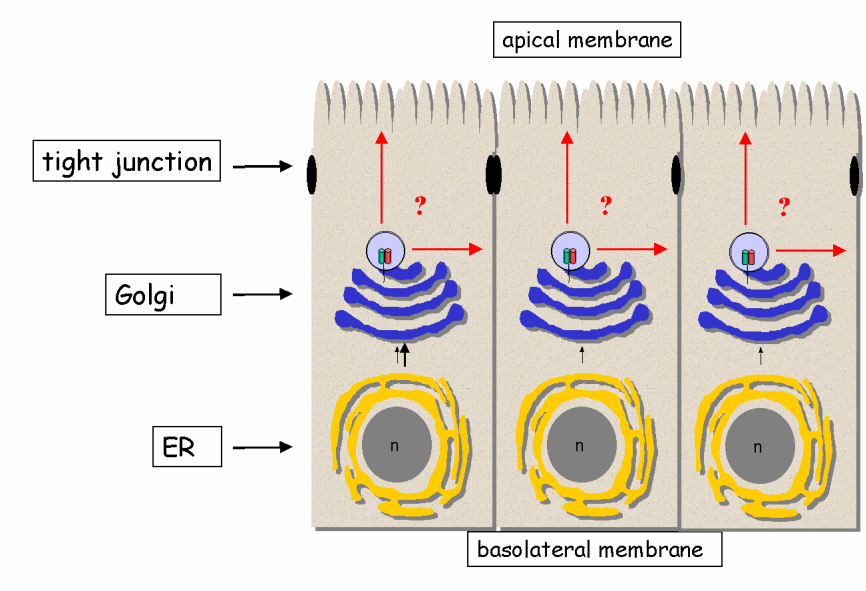

Fig.1 . Schematic diagram of polarized epithelial cell

neuronal cells and certain cells of the immune system depend on the maintenance of characteristically polarized phenotype, polarity also is fundamental to understanding tissue morphogenesis, neural transmission and aspects of the immune response (7;8). These highly specialised cellular functions require a unique structural und functional organization of the cell. First, specialized cell adhesion complexes and cytoskeleton organization are required to maintain cell-to-cell adhesion and cell attachment to extracellular matrix (ECM) (4;9-11). Second, the plasma membrane of these highly differentiated cells is divided into two structurally and functionally distinct membrane domains commonly called apical and basolateral domains. Each domain is comprises of a distinct subset of proteins and lipids composition whose biological function may critically depend upon their localization in the correct surface domain (12) (5;8;13;14). The apical membrane domain, often covered with finger like projections called microvilli which faces the external milieu of the organism, whereas the basolateral domain is in contact with neighbouring cell and the internal milieu, facing the mesenchymal space and blood supply (15-18). Both domains are separated by tight junctions that encircle the apex of the cell and seal neighbouring cells to each other. Neuronal cells also have distinct membrane domains, axonal matodendritic. Certain polarity feature are

shared between epithelial cells and neurons (19;20), which derive embryologically from epithelial cells during embryonic development, epithelial and neuronal cell phenotypes are acquired de novo, by expression of new genes (e.g. of neuron from epithelial cells and trophoplast epithelium from unpolarized morula blastomeres)

Polarized sorting

The understanding of protein synthesis and how plasma membrane proteins are sorted and transported to distinct organelles or to the same plasma domains in different polarized epithelial cells, is still the most fascinating process and one of the greatest and exciting challenges in biochemistry. This process requires that the delivered proteins have to have intrinsic information in their structure which is interpreted by the sorting machinery of the cell which is and responsible for sorting to the correct final destination (7;21). In recent years sorting signals which defined the target destination and the mechanisms, that guide proteins to proper membrane domains have been extensively studied and a series of signal sequences and/or structures are characterised (22-26). In a typical mammalian cell we find a great number of different types of proteins. In general, most of these proteins are synthesised on ribosomes in the cytosol, a few proteins are synthesized on the ribosomes of mitochondria and chloroplast (in plants), but the transport from this point diverges into two main routes. In the first one, the nascent protein is released into cytosol after their synthesis has been completed. Some of these proteins have specific uptake signal sequences which direct them by delivering from cytosol to the final destination (e.g. mitochondria, peroxysome, or nucleus) (27-29), while others with out specific sorting signals remain in the cytosol as permanent cytosolic proteins (30;31). The great part of nascent proteins which are destined for lysosome, secretion or integration in the plasma membrane, take the second main route which was defined by Palade called the secertory pathway (32). In this pathway all nascent proteins possess a signal sequence, a stretch of at least 6 hydrophobic amino acids, that mediates the intergration of nascent protein into the ER (33;34). Some of these proteins either retained there, or the majority are transported onward by vesicular transport (13) or by tubular structure (35), to the cis-Golgi network (CGN) up to trans–Golgi network (TGN). Secretory and membrane-bound proteins undergo several structurally and posttranslationally modification reactions in the different cellular compartments before being delivered to their final intended destination. This includes signal sequence cleavage and attachment of

mannose-rich carbohydrate chains in ER (34;36-40), converting and modification of N-glycans and addition of O-glycans as well as proteolytic cleavage in Golgi complex (39;41;42). In TGN where apical and basolateral proteins in polarized cells, as in contrast to non polarised cells are sorted into different cellular membrane domains or secreted into exterior milieu by virtue of specific sorting signals or motifs contained in their primary, secondary, or tertiary structures (21;43-47) and selectively incorporated into structurally distinct apical and basolateral vesicular carriers for delivery to their specific final destination (34;48;49). Targeting and delivery of proteins to the basolateral membrane occurs directly from TGN to that domain in most epithelial cell types like the LDL receptor and lysosomal membrane glycoprotein (Igp120) (50-53). On the other hand, delivery of proteins to the apical membrane domain varies. According to cell and protein type they are transported to their final destination along at least two different routes, i.e. a direct route from TGN (e.g. membrane proteins responsible for catalysing dietary carbohydrates, like sucrase-isomaltase (SI) which is delivered directly from TGN to the apical plasma membrane domain of intestinal epithelial cells, (13;18;54-62) and an indirect route from TGN to basolateral surface followed by transcytosis to the apical domain, like IgA and its polymeric immunoglobulin receptor pIgR as well as dipeptidyl peptidase IV (DPPIV) (34;55;56;63-66). In some polarized cells, such as hepatocytes transcytosis is the most common pathway for apical delivery(49;67;68).These finding suggest that sorting of plasma membrane proteins in polarized epithelial cells occurs from two sorting centres. The TGN and the endosomes. It has been proposed that both centres may share a similar sorting mechanism(64;65;69-71)

Intestinal epithelial cells as cell model

The epithelial cells that line our small intestine are a highly polarized cells with regard to the function, structure and biochemical composition of their surface membrane domain (5;21;39;72-75). These kinds of cells are an excellent model to study and analyse for various reasons. Firstly, to study the biogenesis and trafficking of membrane proteins and lipids to the appropriate cell surface domain (39;55;76;77). Secondly, they also provide a good experimental system to study endocytosis and transepithelial transport from basolateral to apical membranes for some proteins like polymeric immunoglobulin receptors pIgR (69;78;79) and DPP IV (55;66). Thirdly, the major constituents of the apical microvillar membrane of the intestinal epithelium are digestive hydrolases, including peptidases and disaccharidases, like sucrase-isomaltase, lactase-phlorizin

hydrolase and dipeptidyl peptidase IV(18;39;42;55;59;73;75;80;81). The presence of these proteins make the intestinal epithelium a valuable model to study the biosynthesis of brush border hydrolases as well as the structure and function of these highly interesting membrane proteins Finally, the dramatic change during differentiation from relatively non polar progenitor to high polarized cell is extremely useful for studying the cell differentiation and the development of intestinal cell polarity, and the various mechanisms which contribute to generating and maintaining their characteristically polarized feature (82;83).

Scope of the thesis

In previous studies it was shown that selective targeting of proteins requires that they carry sorting determinants which can be recognized by a specific sorting machinery in the TGN or in basolateral endosomes. Apical targeting has been attributed to a number of different types of sorting signals, including glycosylphosphatidylinositol (GPI) anchor, N-and O-linked carbohydrates, or specific transmembrane domains(84-86). The cellular components that interact with apical targeting signals have not been identified yet and are probably as diverse as the signals themselves. The mechanisms that control this simple process are in fact rather complicated. It involves many different steps from the recognition event between sorting signal and sorting receptor(s), to the formation of the vesicle, their budding and the docking to the right plasma membrane domain. The central aim of the present work is to study the role of glycosylation, in particular O-glycosylation in the trafficking and polarized sorting of the heavily N- and O-glycosylated intestinal proteins such as sucrase-isomaltase (SI), DPPIV and Aminopeptidase N (APN) as well as to analyse the role of sphingolipid-cholesterol rich membrane microdomains (rafts) in the sorting behaviour of these proteins.The role of O-glycans and the relationship between the apical sorting of SI and its association with rafts and the mechanism underlying this process will be analysed in Chapter 3, by inhibition of O-glycosylation and the blocking of rafts synthesis with chemical inhibitors. In Chapter 4, the structural determinants and the motifs which are thought to play an important role in the sorting behaviour of SI will be characterised by preparing a series of mutations which lead to a lack of the membrane anchor and the Ser/Thr rich stalk domain (potential O-glycan domain) of SI. The temporal association between O-glycosylation and processing of N-linked glycans in the Golgi apparatus and the implications of these events in the polarized sorting of three brush border proteins will be discussed in Chapter 5. In Chapter 6, the location and hierarchy of sorting signals

in lactase-phlorizin hydrolase (LPH) an apical protein in intestinal cells, will be studied by preparing a series of mutations that lacked the LPH cytopasmic tail or had the cytoplasmic tail of LPH replaced by sequences which comprised basolateral sorting signals and overlapping internalisation signals of various potency. These signals are mutants of the cytoplasmic domain of the influenza hemagglutinin (HA), which have been shown to be dominant in targeting HA to the basolateral membrane. This thesis is completed by a general discussion in Chapter 7 and summarized in Chapter 8.

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Chapter 2

Protein Synthesis and Processing in the Secretory Pathway

Introduction

Proteins are the most abundant biological macromolecules. They are designed to bind conceivable molecules from simple ions to large complex molecules like fats, sugars, nucleic acids and other proteins. They catalyse an extraordinary range of chemical reactions, provide structural rigidity to the cell, control flow the of material through membranes regulate the concentrations of metabolites, act as sensors and switches, cause motion, and control gene function. Proteins containing covalently bound carbohydrate are called glycoproteins. These proteins are ubiquitous in nature (1-4). They are the most common and most versatile product of post-translation modification of proteins in all living organisms, from archaebacteria to humans, although they are rare in eubacteria. In fact, most of the proteins are glycoproteins. They include all kinds of biologically active substances such as enzymes, antibodies, hormones, cytokines and receptors as well as structure proteins like collagens. Glycoproteins are found in cytoplasm and the subcellular organelles in the cell, in cell membranes and in extracellular fluids. A eucaryotic cell has a great number of different types of proteins and must ensure that a protein destined for one of the several distinct subcellular compartments within the cell arrives at the correct membrane system. In most cases the protein precursor possesses a signal of some kind, often a partially conserved series of amino acid residue which marks the protein as being destined for a appropriate compartment (5).

the Secretory Pathway

Several organelles in this pathway are represented: the ER, the ER-Golgi intermediate compartment (ERGIC), cis-Golgi network (CGN) trans–Golgi network (TGN). Proteins destined for secretion, integration in the plasma membrane, or inclusion in lysosomes generally share the first few steps of the secretory pathway. This begins with the translocation in the endoplasmic reticulum via a special short sequence of amino acids called signal sequence(5;6), proteins that leave the ER are funnelled through the Golgi complex

before being sorted for transport to their different final destinations (7;8). The sorting and delivering of newly synthesized proteins and lipids from TGN to the correct final subcellular compartment or cell surface domains of polarized cells, is not a simple bulk flow process but requires multiple sorting signals, various transport machinery, and several sorting events (9-13) . How all these components cooperate as a protein delivering machine, and how the endomembrane system is organized is very complex, but is become clearer in recent years due to extensive studies. Most likely, the mechanisms that are responsible for intercellular targeting and deliver operate in all eucaryotic cells with some variation. For instance, the sorting events in polarized cells from epithelial to neuronal cells are more complicated because these cells have two differently structured and functioning membrane domains (14-16). In the next section of this chapter protein synthesis and maturation modifications, as well as the transport mechanism and the targeting signals will be discussed .

Endoplasmic reticulum (ER)

In eucaryotic cells, the first step in the biogenesis of proteins destined to be secreted and lumenal proteins that are residents of the secretory pathway is the targeting and translocation of these proteins across the membrane of the ER. The ER also is the starting point for the synthesis of all secreted proteins in eucaryotic cells, the lumen of ER provides an exclusive, highly specialized environment for the controlled folding, oligomeric assembly, quality control and maturation of membrane and soluble proteins, most of which are destined for export to other organelles such as Golgi complex and lysosomes, as well as to reside on different membrane domains in the cell or for secretion (17-19).

Translocation and ER targeting signal

Proteins in the secretory pathway are initially synthesized on ribosomes attached to the the rough endoplasmic reticulum (RER) membrane in the cytosol of the cell and are selectively targeted to the ER. The basis of an ER targeting signal is a continuous stretch of six to twenty hydrophobic amino acids with one or more basic residues flanking the hydrophobic core, usually found at the amino terminus of the protein in mammalian cells (6). The majority of proteins which possess a signal sequence, are targeted to the ER in a obligatory cotranslational, ribososme dependent manner (20;21). A cytoplasmic ribonucleoprotein,

termed signal recognation partical (SRP), binds to the signal sequence as it emerges from the ribosome causing an arrest or pause in the elongation of the nascent protein (20;22;23). This pause may extend the time in which the nascent protein can be productively targeted to the ER membrane. Targeting of the ribosome-nascent-chain complex to the ER membrane is mediated by the specific interaction of SRP with ER membrane hetrodomainic protein complex, the SRP receptor (24;25). Once SRP interacts with its receptor, the signal sequence dissociated from SRP and the elongation arrest is released. Upon release from SRP, the nascent protein inserts into ER becomes tightly associated with the ER membrane via interaction with components of the machinery that mediate the translocation of protein across the membrane, collectively referred to as the translocon. (26-28). Following nascent chain insertion, translocation of the nascent chain proceeds through a protein conducting channel across the ER membrane and into the lumen. In the lumen several proteins interact with the nascent chain for instance the signal sequences protease removes the signal sequences from some but not all translocated chain

Membrane Topology

Different proteins assume different orientation within the membrane. Integral membrane proteins that span the membrane once can expose either the amino(type I) or carboxyl (type II) terminus on the exoplasmic side of the membrane. Proteins that span the membrane several time are referred to as multiple spanning (29;30). The ER targeting signal will define the orientation which a protein assumes in the ER membrane, this topology will then be maintained as the protein transits the various membrane enclosed compartments of the secretory pathway up to and including the plasma membrane. The portion of the protein which is trans-located into the lumen of the ER will ultimately be located on the outside of the cell (31). Upon arrival at the ER membrane, an ER targeting signal can either be cleaved from the protein or remain attached and function as a transmembrane anchor. Membrane proteins which span the bilayer once and have a cleavable signal sequence invariable always have a type I orientation. Most of type I proteins have a second signal i.e. the stop transfer sequence on the carboxyl-terminal side, which functions as the transmembrane anchor (20;23;32). In the absence of the stop transfer sequence, the protein is completely translocated across the membrane and enter the ER lumen. Cleavable signal sequences are present at the N-terminus of a protein, and following initial cleavage by signal peptidase further processing of the cleaved signal sequence takes place (33).

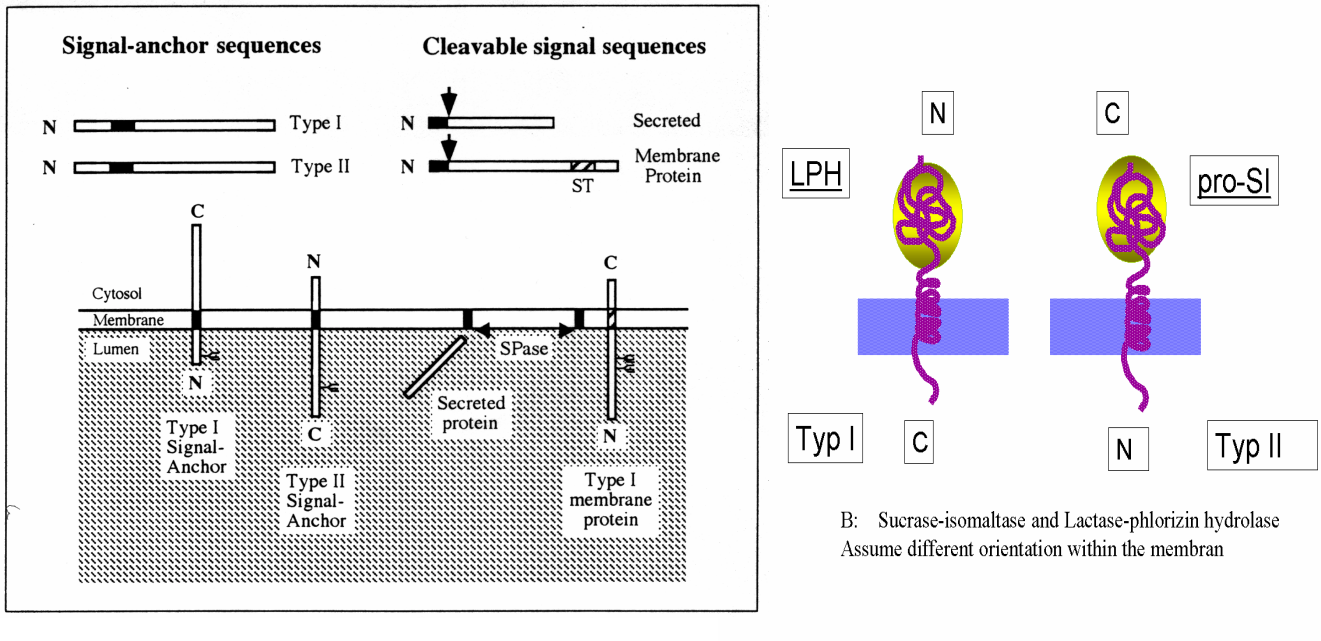

Fig. 1 **A.** Types of ER targetingsignals. Filled region represent the hydrophobic regions of signal-anchor or cleavable signal sequences as indicated. ST denotes a stop transfer sequence present in a membrane protein. Arrow heads indicate signal peptidase(Spase) cleavage sites. The branched chain indicate asparagine linked carbohydrate side chain added on the luminal side of ER membrane *(G.von Heijne; Membrane protein assembly 1997)*

If the signal sequence is not cleaved from the protein, it is usually referred to as a signal anchor sequence. Membrane proteins which have a uncleavable signal sequence can assume type I orientation (N-terminus translocated with signal anchor sequence) or type II orientation (C-terminus translocated, type II membrane protein) (29;34;35), in order to distinguish them from type I membrane proteins with a cleavable signal sequence, type I orientation proteins with signal anchor sequence are usually referred to either as type I signal anchor or type III membrane proteins. The actual topology is influenced by the distribution of the charged amino acid residues flanking the hydrophobic core of the signal anchor sequence (35;36). It has been shown that the folding behaviour of N-terminal of the signal anchor protein can influence the topology which the protein assumes in the membrane (37).

N-Glycosylation

N-glycosylation is now established as an important post-translational modification reaction of proteins in eucaryotic cells and plays a variety of functions including: modulatory roles in proteins stabilization, affinity of receptor-ligand interaction protection of the polypeptide backbone against proteolytic degradation, influencing the folding of the polypeptide chain (38-41) modulation of biological activity, and targeting of glycoproteins to various subcellular compartments and to the cell surface (35;42-44).

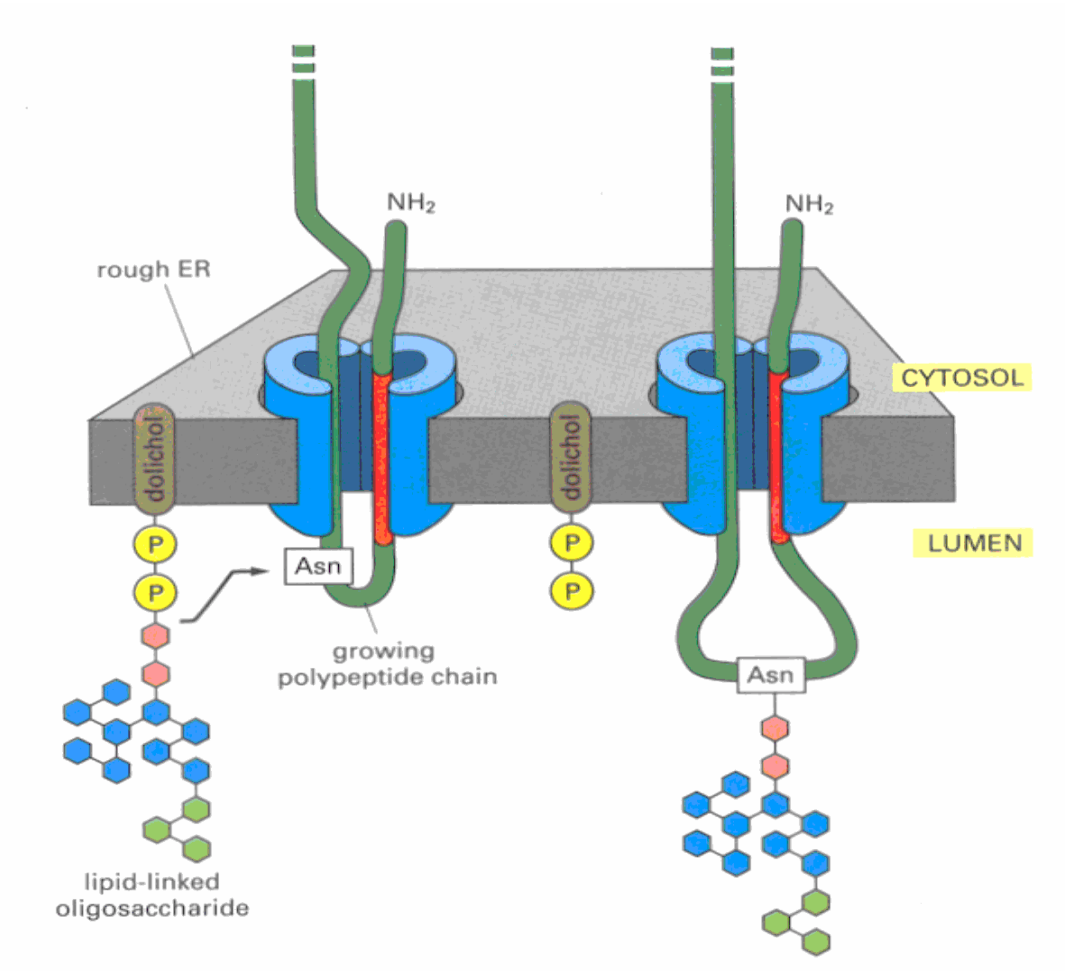

Fig 2. Protein glycosylation starts in the lumen of the ER with the transfer of 14-residue oligosaccharides ($Glc_3man_9glcNAc_2$) from a dolichol-pyrophosphat-linked oligosaccharides en bloc to the NH_2 group on the side chain of distinct asparagine residues of the nascent polypeptide chains *(B. Alberts; Molecular biology of the cell, third edition).*

In contrast, the removal of carbohydrate moieties from certain glycoproteins is known to cause structural and physiochemical changes in their core proteins, thereby leading to significant effects on the functional properties of such glycoproteins. The biosynthesis of N-glycosidically linked glycans of glycoproteins is a multistep process (3;45), which starts in the lumen of the ER with the transfer of 14-residue oligosaccharides ($Glc_3man_9glcNAc_2$) from a dolichol-pyrophosphat-linked oligosaccharides en bloc to the NH_2 group on the side chain of distinct asparagine residues of the nascent polypeptide chains (46-48) this transfer is accomplished by a membrane-bound oligosaccharyl transferase complex. Only asparagine residues in the seqonce Asn-Xaa-Ser/Thr (where X is any amino acid except proline and perhaps aspartic acid) can become N-glycosylated (49;50). Transfer of the oligosaccharide generally occurs co-translationally as soon as the acceptor site enters the lumen of ER. However, the presence of the sequon does not ensure core glycosylation, as many proteins contain sequence that remain either unglycosylated or glycosylated to a variable extent (51). The oligosaccharide chains are subject to trimming by ER glucosidases. Glucosidase I which removes the terminal α1,2- linked glucose followed by the elimination of the two remaining α1,3- linked glucose by glucosidase II (43;52-54). Further trimming by cleavage of 2-4 mannose residues by the ER mannosidase, occurs at least in part, post-translationally before the regularly folded and possibly oligomerized protein is exported out of the ER to the Golgi apparatus and to further destinations (55-57).

Molecular chaperones and folding enzymes

In addition to the proteins that are directly involved in the membrane integration process, a number of other ER proteins are intimately associated with the insertion site but are not required for the actual integration process (58;59). These accessory soluble or membrane-bound proteins often catalyse modifications of the nascent chain as it emerges on the luminal side of the ER membrane. A number of molecular chaperones or folding factors associate transiently with folding intermediates and unassembled proteins to assist in their translocation, promote efficient folding and prevent premature oligomerization or processing, on the luminal side of the ER either during or immediately after insertion (18;44;60;61).

Components such as heavy chain binding protein (Bip), which is the unique member of the of Hsp70 family of

chaperones that can be found in the ER, (62;63) , and protein disulfide isomerase (PDI), promote the correct folding and disulfide bond formation of many proteins (64-66). Newly synthesized polypeptides with N-linked glycan interact transiently and specifically with two homologous lectin-like chaperones that are unique to the ER. Calnexin, is a non glycosylated type I trans membrane protein of 64 kD, which interacts transiently and specifically with a large number of different glycoproteins during their folding and maturation (67;68), and its soluble homologe Calreticulin 46kD, interacts with an overlapping but not identical group of proteins (69-71). Both bind specifically, to partially trimmed monoglcosylated forms of the N-linked core glycans (Glc1 man5-9 GlcNAc2) present on the folding intermediates, wherears they do not bind to glycans with two or three or no glucose residues. Both the affinity for the glycosaccharides and the number of binding sites are unknown. 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 this attachment. The roles of Bip, calnexin and calreticulin seem to be two fold. Firstly, to promote an efficient correct protein folding and to assemble the oligomeric complexes from individual subunit, and secondly, remain bound to misfolded or unassembled proteins and to exert a quality control function (68;72).

Quality control and degradation

It has been proposed that there is a highly developed quality control system in the ER made up of molecular chaperones and folding enzymes that strictly regulate the transport of newly synthesized proteins from the ER to Golgi apparatus and beyond (7;73;74). As a rule these proteins, soluble or membrane-bound, are transported only when they acquired a fully folded, native conformation. Most misfolded and incompletely assembled proteins probably accumulate in the ER and if they fail to reach the proper conformation they eventually undergo degradation without reaching the Golgi complex (73;75). A model for this ER-associated degradation has recently been proposed by (76). Typically, misfolded proteins, unassembled subunits, and incompletely assembled oligomers remain in the ER, prorerly the chaperone complex remains associated to the polypeptide and the translocation machinery

“decides” on the basis of the kinetics of the chaperone interaction to reexport the polypeptide to the cytosol. During the interaction with the lumenal chaperones, the nascent protein might remain attached to the translocation complex, which could result in a prolonged opening of this

complex (77-79). Soluble polypeptide may be re-exported through this open channel, whereas integral membrane proteins could re-enter the channel by a partial dissociation of the complex (80). When the proteins are re-transported from ER to the cytosol, they are degraded if they are recognized by the proteosome. Some proteins were shown to be degraded by ubiquitin-proteasome mediated proteolytic pathway (81-83). Other proteins were degraded independently of ubiquitin,. possibly because they were unfolded or had specific chaperone associated with them (84-86). These chaperones could help to feed substrate to the proteosome. At the cytosolic face of the ER, both ubiquitin-conjugated enzyme and the proteosome(87;88) are present, suggesting that degradation machinery can be tightly coupled with retrotranslation (76)

Golgi apparatus

Normally, all eucaryotic cells have Golgi complexes i.e. a system of membranous stacks or cisternae arranged as flattened stacks (89). Each cisterna consists of a closed structure bounded by a single continuous membrane. A stack usually consists of 10 cisternae (90), but the number of stacks varies considerably among different type of cells. The cisternae appear to be separate structures each containing its own membrane and distinct sets of enzymes. The Golgi apparatus is polarized with the cis side facing RER and the nucleus, and the trans side facing the plasma membrane (91;92) the medial elements are located between this sides. Proteins during their synthesis on ribosome bound to RER are inserted into the interior of ER cisternae. Once a protein has passed all quality control mechanisms in the ER it is packed into small membrane cargo vesicles containing the newly synthesized proteins budding from the ER and moved to next compartments on its transport route through the intermediate compartment, or ER-Golgi intermediate compartment (ERGIC) (90), which is seen to consist of an extensive network of tubules that cover large areas of cytoplasm. The ERGIC has been a subject of

debate (93) since it is not clear whether it is a collection of ER outposts that is involved in exchange of materials with the Golgi complex or a distinct organelle (55;94). The cis-Golgi network (CGN) appears to embody an area of tubules, associated with cis-most cisternae of the
Golgi complex (95). The CGN receives newly synthesized proteins and lipids from ER which are destined for plasma membrane, lysosomes and secretion, or backed to ER (96;97). In most
cells the CGN is most likely involved in the fatty acid acylation of membrane protein (98;99),

the phosphorylation of lysosome enzymes(97), and the addition of the first N-acetylglucosamine residues of O-linked oligosaccharides (100) the protein passes through the Golgi complex to the trans side, different types of Golgi enzymes modify the delivered protein molecules. The most remarkable modification is the stepwise addition of O-linked glycan and the remodelling of N-linked oligosaccharide side chains of glycoprotein and the addition of sulfate or lipid moieties to side chains of certain amino acids. This kind of modification could act as a specific molecular address in the nascent proteins in addressing to their final intended destinations as they leave the TGN in coated cargo vesicle budding from the trans element. Certain proteins are enclosed in secretory granules, eventually to be released from the cell by exocytosis. Others are targeted to intercellular organelles like lysosomes or incorpration into plasma membrane during cell growth. The TGN is also the main sorting and delivering port in the cells.

The roads which lead to complex N-glycans

As described previously the synthesis of N-glycans begins in the ER with the transfer of $Glc_3Man_9GlcNAc_2$ from dolicholpyrophosphate to an asparagine residue of the nascent proteins. Oligosaccharides undergo different processing steps and these modifications are highly orderd so that each step shown is dependent on the previous reaction in the series.

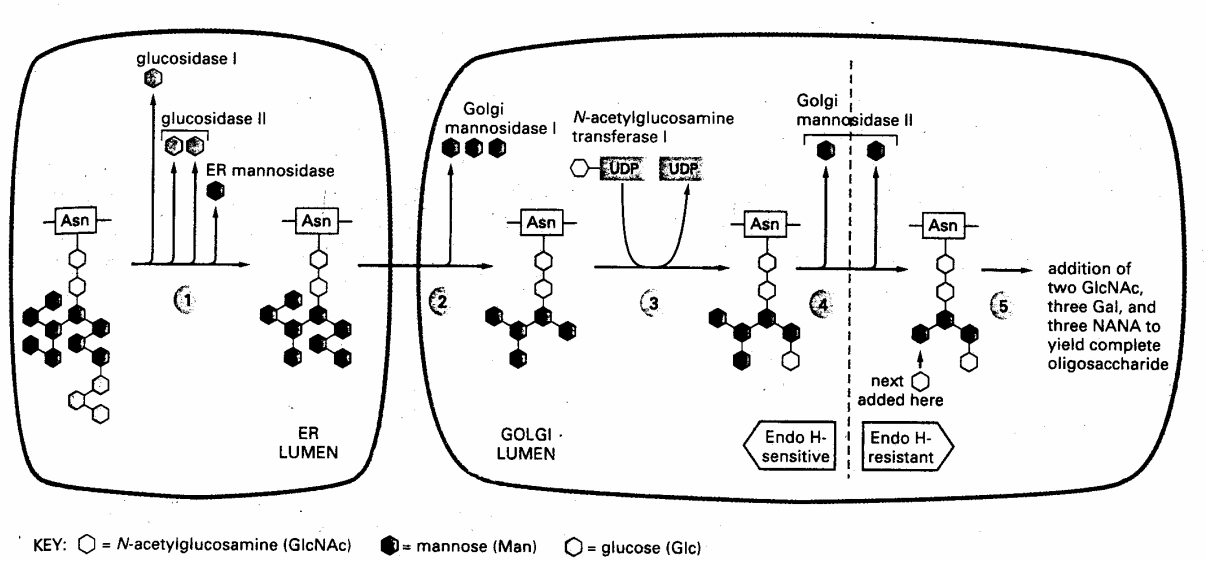

Fig. 3 . Oligosaccharide Processing begins in the ER with removal of the three glucoses by ER glucosidase I und II, and the ER mannosidase removes one mannose. These modifications continue in theGolgi complex (*B. Alberts; Molecular biology of the cell, therd edition 1994):*

N-glycosylation modification continues after glycoconjugates have been exported out of the ER and then been transported across ERGIC to cisternae of the Golgi complex (101;102) where the high mannose oligosaccharide becomes converted into complex type and hybrid type glycans (103;104). In the Golgi stack, mannosidase I removes three more mannose residues and from ($Man_5GlcNAc_2$-Asn-x) and N-acetyl-glucosamine transferase I add a residue of GlcNAc, which enables mannosidase II to remove two additional mannose residue (105). This yields the final core of three mannose residues that are present in a complex type of oligosacchride. At this point, the bond between the two GlcNAc residues becomes resistant to degradation by a highly specific enzyme endoglycosidase H (Endo H). Treatment of this enzyme is widely used to distinguish high mannose oligosaccharide (ER form) from the complex oligosaccharide form (Golgi form). Finally, additional GlcNAc, galactose, and sialic acid residues and some times fucose are added (3;103). The extent of processing depends on the protein and on the location of the asparagine residues within the protein to which the oligosaccharide is attached.

O- Glycosylation

Another glycosylation event of many but not all glycoproteins is O- linked glycosylation. Most O-linked oligosaccharides exhibit a much greater hetrogenecity in their structure than N-linked ones (106;107). The biosynthesis of these O-linked glycans appears to be a late posttranslational event (2;108-110), which starts mainly in cis-Golgi and therefore does not

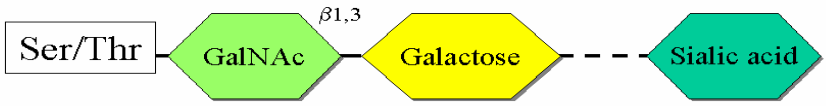

Fig 4. Biosynthesis of O-Glycan begins in Goli complex by the transfer of N-acetylgalactose-amine GalNAc to a glycosidic link at a –OH of Threonin or to Serine residues

involve dolichol derivatives which are restricted to the ER. There is no processing by glycosidases and a consensus acceptor sequence such as the tripeptide Asn-Xaa-Ser/Thr in N-linked glycosylation, does not exist. It has been suggested that stretches of threonine and serine residues in high glycosylated protein, such as sucrase-isomaltase, aminopeptidase N or LDL-receptor (109;111;112) are potential sites for O-glycosylation. The first step in the synthesis of O-linked glycan is catalysed by polypeptide GalNAc transferase (113;114). This enzyme is probably expressed in all mammalian cells and acts in the cis-Golgi, thus after the addition of N-glycan, (115)

In vitro the enzyme efficiently transfers N-acetylgalactosamine (GalNAc) to a glycosidic link at a –OH of threonine but or to serine residues, and requires Mn^{2+}(O-linked glycan) (116-119). The initial insertion of GalNAc is followed by consecutive step by step addition of further sugar residues, such as galactose, fucose, and sialic acid. O-glycosylation`s role in the biological function of glycoproteins, and in conferring stability and protection against

proteolytic degradation has been proposed (108-110) Also a direct implication of O-glycosylation in the intercellular transport of proteins has been proposed (120).

Cargo vesicle

In general, proteins are transported in vesicles that bud from one membrane compartment and specifically target to and fuse with the next one (121-125). Each separate vesicle allows transport between two membrane-bound compartments, the donor compartment that produces the vesicle and the receptor that receives the vesicle and its special cargo.

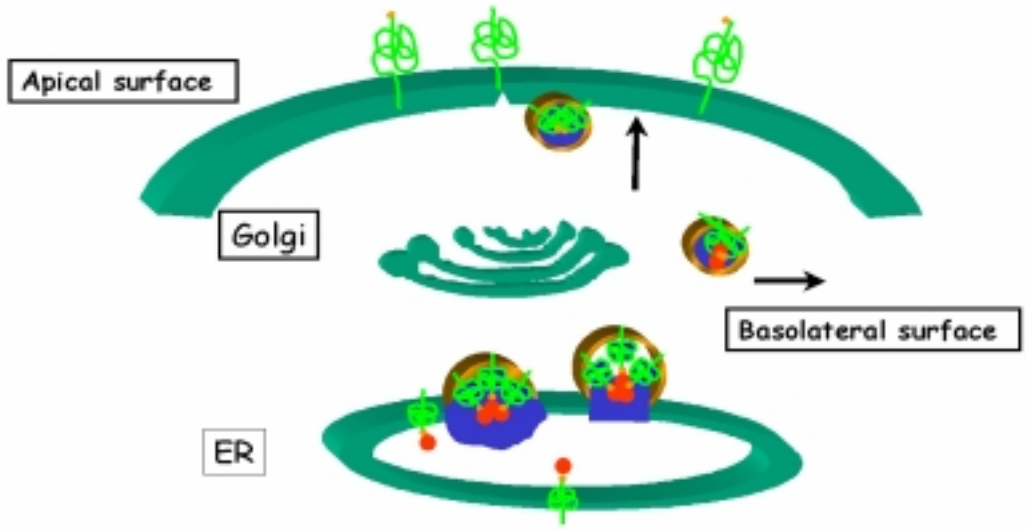

Fig. 5. Vesicular sorting in the TGN of polarized epithelial cells

Depending on the route on which the transport vesicles are following, the coating constituents can vary (126;127). The transport vesicles “Tracks” from TGN to apical or basolateral membrane may be different, and these differences may be recognized by domain specific receptors in the cytoplasmic aspect of the vesicles But the general principle of budding and fusion is almost the same (128-130). For instance, between the ER and the

Golgi apparatus, transport is mediated by vesicles coated with two different coating complexes i.e. COPI and COPII (131-133), vesicles that bud from TNG are coated with clathrin, and another coating also plays a role in receptor- mediated endocytosis (134)

Delivering and sorting signal

Polarized expression of most epithelial plasma membrane proteins is achieved by highly selective sorting and transport from Golgi apparatus or basolateral endosome as sorting centres to specific cell surface domains.(135-139). The mechanisms that control this simple process are in fact rather complicated. They involve many different steps, from the recognition event between sorting signals and sorting receptor(s), to the formation of the vesicles, their budding and the docking to the right plasma membrane domain. In both sorting centres apical and basolateral proteins are packed into different vesicular carriers for delivery to their specific final destination. Although apical and basolateral transport vesicles may share elements of the protein-sorting machinery they must certainly differ in some components or in their structure in order to specifically carry out the processes of cargo recruitment and targeting to the appropriate surface domain (140;141). Basolateral targeting signals have been so far identified. Recruitment of integral proteins for basolateral transport appears to be mediated by the recognition of distinct sorting signals in their cytoplasmic domain by sorting machinery (142). This probably includes some elements related to proteins capable of recognizing either tyrosine motif (143;144) dileucine motif (145) or hydrophobic/aromatic amino acid(137;146)

Basolateral targeting determinants can be divided into those that are co-linear with a clathrine-coated pit domain, and those that are not (144;147). It is not known whether these different types are recognized by the same sorting machinery. Unlike basolateral sorting, apical sorting appears to implicate signals at least of different types, There are some structures and motifs that are thought to act as sorting signals to the apical surface domain, including signals located in the ectodomains or in their transmembrane domain. In contrast, the cytoplasmic domain does not seem to play any role in apical targeting (14). Glycosylphosphatidylinositol (GPI) anchored proteins are expressed exclusively on the apical membrane surface of several polarized epithelial cell lines GPI anchorage appears

sufficient to direct different associated proteins to apical surface domain in a polarized system (42;148-152).

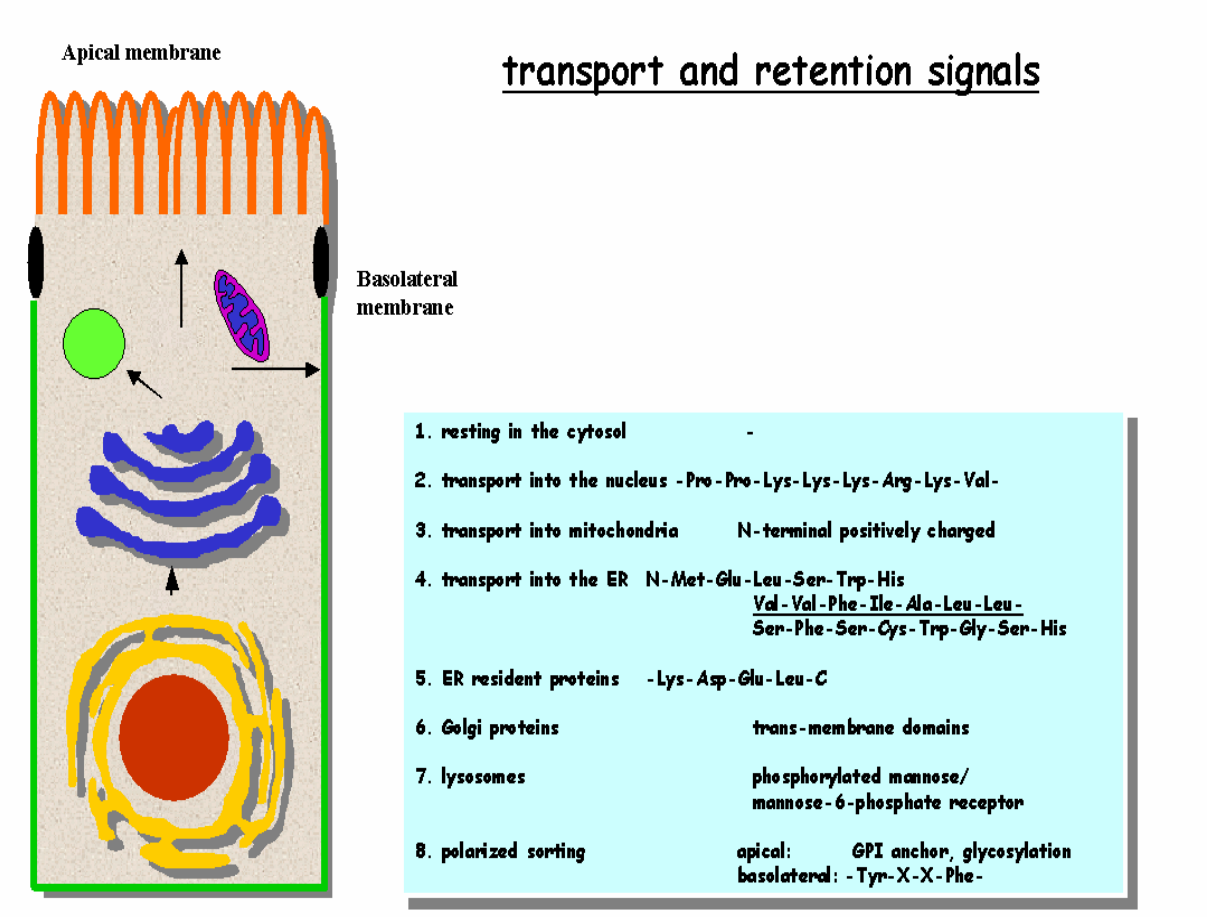

Fig 6. Protein transport and retention signals in polarized epithelial cells.

It has been suggested that both N- and O-linked oligosaccharides act as apical sorting signals (42;56;120;150;153-155). The addition of oligosaccharide to certain proteins, such as the growth hormone or a GPI-anchored form of growth hormone, can confer apical targeting. However, there are numerous exceptions, for example, the hepatitis B surface antigen is apically delivered without being N-glycosylated. The hepatitis B virus glycoprotein as well as APN and LPH (56;153;156;157), are normally transported to the apical membrane with glycans independent sorting mechanisms. This exception suggests that oligosaccharide targeting is only one of a number of mechanisms for apical targeting. A novel mechanism for

the apical pathway seems to involve a new process, using sphingolipid-cholesterol membrane microdomain rafts (152;158;159). These microdomains contain apical cargo and several proteins with the potential function of apical sorting delivery. This model is supported by evidence obtained from the Madin-Darby canine kidney (MDCK) cell system showing that influenza virus hemagglutinin (HA) and GPI anchor proteins are included into rafts during biosynthetic transport to the apical surface (160;161), whereas basolateral proteins are excluded (162;163). In the small intestinal brush border membrane of the big SI, APN, and DPPIV were found in rafts, together with the GPI anchor (10;56;153;164;165). However, not all proteins destined for apical membranes are associated with rafts. For instance LPH is a highly polarized apically sorted protein that does not associate with lipid rafts on its way to the apical membrane and might be delivered in raft independent vesicles(10;56;165).

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Chapter 3

Sucrase-Isomaltase and Lactase-Phlorizin-Hydrolase

Introduction

Carbohydrates are an essential component of the mammalian diet. They occur as disaccharides such as sucrose in plant and lactose in mammalian and as complex polymers of saccharides such starch and cellulose in plant and glycogen in animals. Only monosaccharides can be absorbed by the intestine, di-and polysaccharides have to be degraded. These carbohydrates are hydrolysed in the intestinal lumen by specific enzymes to monosaccharides before being transported across the brush border membrane of epithelial cells into the cell interior. These enzymes are specified by the chemical structure of the carbohydrates (1). The monosaccharide components of most known carbohydrates are linked to each other in an α–orientation, such as in starch, glycogen, sucrose, or maltose. In comparison, ß-glycosidic linkages are minor. This chapter will not only concentrate on a member of the α-glucosidase family, such as sucrase-isomaltase (SI), but also on lactase-phlorizin hydrolase (LPH), an enzyme that is responsible for hydrolysis ß-linked disaccharides. This type of covalent bond is present in lactose which is one of the major essential carbohydrates of mammalian milk (2-4). SI and LPH share a number of similarities, but are at the same time very different. Both enzymes implicated in the digestion of carbohydrates in the intestinal lumen are membrane-bound glycoproteins that are transported via the secretory pathway directly to the apical microvillus membrane of the entrocytes (2;5;6), SI is probably transported in glycolipid membrane microdomains rafts whereas LPH is not (7-10). These similarities and differences make the SI and LPH an interesting couple for studying several aspects. The structural and biosynthetic features of SI and LPH will be discussed in this chapter.

Structure features of sucrase-isomaltase (SI)

SI is a type II integral membrane glycoprotein that is synthesized with uncleavable signal sequence (11-13). The signal sequence domain has a dual function. Firstly, it is necessary for the translocation of nascent proteins to the ER (14) and secondly, it acts as a membrane anchor and has therefore an N-in/C- out orientation (13). In addition to the 20 amino acids transmembrane domains, SI contains three autonomous major domains. The cytoplasmic tail which contains 12 amino acid residues and may be phosphorylated in vivo through a conserved serine residue (Ser6). This raises the possibility that phosphorylation of the cytoplasmic domain may be implicated in the post-translation regulatory processes. The membrane anchoring region is directly followed by a Ser/Thr rich domain, also known as stalk domain (1) that is considered to be part of isomaltase domain and is (13) heavily O-glycosylated (7;9;15;16). This subunit ends with the amino acid residue Arg1107, and the sucrase domain starts directly with Ile 1008. The Arg/Ile peptide sequence between both subunits is a trypsin site where the mature large pro SI is split in the intestinal lumen upon exposure to pancreatic secretion. These two domains remain associated with each other by non covalent strong ionic interactions (6;12;15).

Biosynthesis and expressing of sucrase-isomaltase

The sucrase-isomaltase enzyme complex is an intrinsic glycoprotein that is exclusively synthesized and expressed in the small intestinal microvillus membrane (15;17) and is responsible for the final hydrolysis of dietary carbohydrates such as starch and sucrose into their monosaccharides components of glucose and fructose (18;19). A deficiency, miss sorting or drastic reduction of enzyme activity leads to sucrose intolerance and is associates with clinical diarrhea (11;20-24). Brush border SI is synthesized as a two active domains, single chain polypeptide precursor which is post-translationally N-glycosylated in the ER. The first detectable form of SI is a large high mannose precursor with a molecular mass of 210kD. Unlike other intestinal hydrolases peptidases the SI molecule is transported at a fairly slow rate from the ER to the Golgy cisternae ($t_{1/2}$ 75 min) while other intestinal proteins like dipeptidyl peptidase IV(DPPIV) and aminopeptidase N(APN) have a higher rate ($t_{1/2}$ 15-20 min)(6;15). In Golgi apparatus the mannose–rich N-linked glycans of SI are modified by Golgi mannosidases

and different types of sugar residues are added resulting in a complex type of N-glycosylated molecule. Concomitant with the processing of N-glycan in the Golgi complex, pro-SI acquires O-glycosidically linked carbohydrates. The modification of mannose- rich chains and the start of the O-glycosylation event in the Golgi are temporally associated. O-glycosylation of pro-SI does not commence before the first outermost mannose residues of mannose-rich chains are trimmed by mannosidase I in the cis-Golgi.

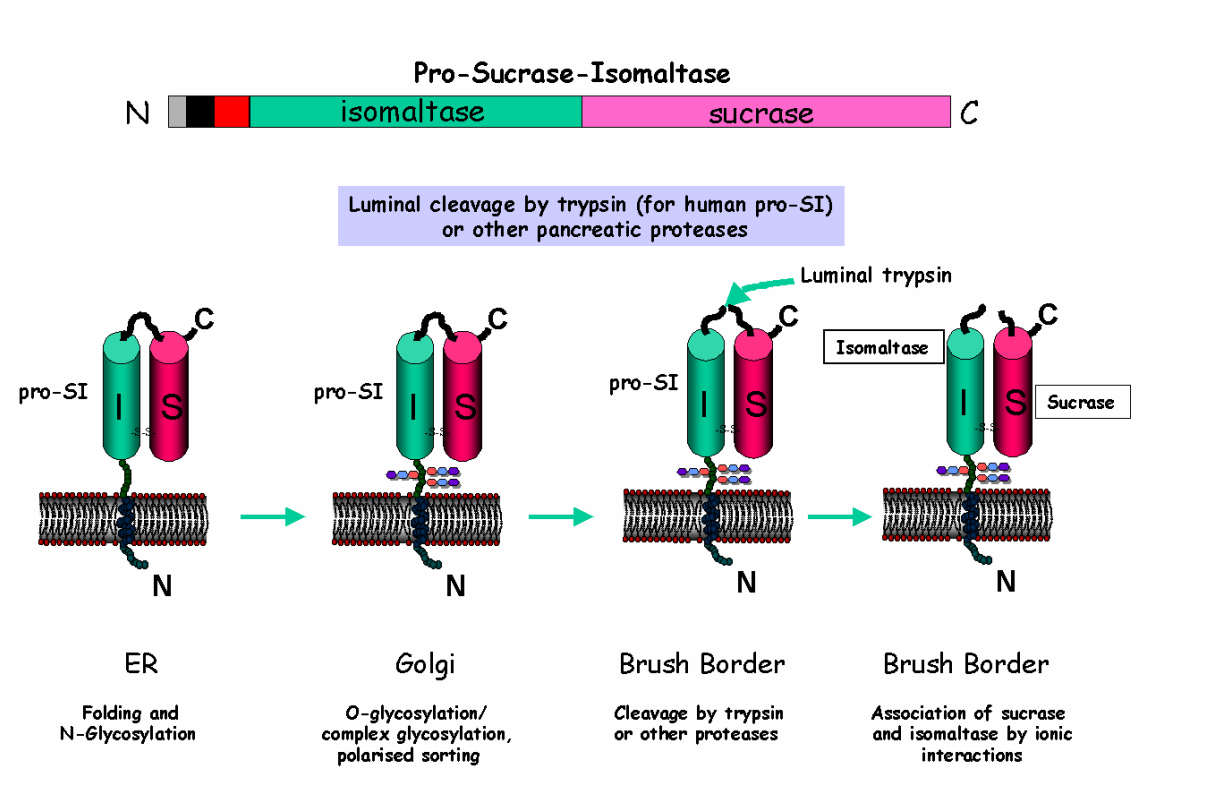

Fig. 1: Schematic representation of the structure and biosynthesis of pro –sucrase-isomaltase in human small intestinal cells

Complete processing of N-linked chains is essential for the function of SI. Complex glycosylated SI is most likely sorted in the TGN and packed into transport vesicles that deliver it directly to the microvilliuos apical membrane by a new mechanism involving the O-glycosylation of SI in association with sphingolipid-cholecterol membrane microdomains rafts (7-10;25;26). Once SI reaches the apical cell surface it is split by trypsin or other pancreatic proteases into two enzymatically active subunits of unequal size (i.e. sucrase with

an apparent molecular weight of 145 kD and the isomaltase subunit which is larger than sucrase with a molecular weight of 151 kD). This difference is primarily due to an increased of O-glycosylation of isomaltase (the isomaltase subunit is responsible for anchoring to the membrane bilayer), while the sucrase subunit remains noncovalenty associated with the isomaltase subunit. Both subunits of SI are N- and O-glycosylated. In human small intestinal O-glycosylation of the sucrase subunit is heterogeneous by virtue of the existence of at least four glycoforms of different O-glycans. On the other hand, the O-glycosylation form of the isomaltase is unique suggesting a more efficient and consistent glycosylation event of isomaltase in comparison to sucrase. It has been suggested that the variation in the O-glycosylation pattern of the subunits has been proposed to correlate with the position of each domain within the protein complex. The isomaltase domain is readily O-glycosylated since it contains a Ser/ Thr stalk region (13) which is located in direct proximity to the membrane, while the sucrase domain is more distal.

Lactase-phlorizin hydrolase (LPH)

Lactase-phlorizin hydrolase (LPH) is an integral membrane glycoprotein that is synthesized by the small intestinal epithelial cells of virtually all mammals (27;28). LPH belongs to the well characterized group of intestinal microvillar disacharidases which includes sucrase-isomaltase and maltase-glucamylase (29). Unlike other intestinal membrane hydrolases LPH is unique in its pattern of developmental expression (30-33). LPH activity of humans and of most mammals is only high during the newborn and suckling period, when the milk is the sole nutrient and decreases strongly to low levels upon weaning (34-36), LPH reveals two hydrolytic activities on the same polypeptide chain. The lactase (ß-D-galactoside galactohydrolase) activity which is responsible for the conversion of dietary disaccharide lactose (the main carbohydrate in mammalian milk) into its two monosaccharides components (glucose and galactose) and the phlorizin hydrolase (glycosyl-N-acylsphingosine glucohydrolase (2-4;37-39), that digests ß-glycosylceramide, which are part of the diet of most vertebrates. In the rat lactase, activity is well developed in the latter part of gestation and reaches a maximum at or shortly after birth, but declines markedly after weaning to a low level that persists through adult life. (34;40). Exceptions to this development pattern are North Europeans and their descendants as well as some African and Arabic tribes (41;42), where lactase expression remain high throughout the adult life. In the absence of or drastic reduction

in the lactase activities, absorption of sugars through the microvillus membrane does not occur, milk conssumption in newborns and adults in such case is associated with diarrhea, flatus, and abdominal cramps (43). A physiological role of phlorizin hydrolase activity is still unknownn(36;44).

Structure of LPH

LPH is a type I integral membrane glycoprotein, with an extracellular NH2-terminus and COOH-terminus in cytoplasm. The studies of biochemical data, cDNA cloning and the analysis of the structure, biosynthesis, and processing of pre-pro LPH in the human small intestine (36;45) have established that the LPH molecule is comprised of five main domains, 1: Cleavable signal sequence peptide of 19 amino acids necessary for translocation into ER located at the N terminus (Met^{1-} Gly^{19}), 2: A large pro-region of 849 amino acids(Ser^{20}-Arg^{868}), 3: An extracellular domain of 1014 amino acids, carrying both active sites of the enzyme, 4: Transmembrane domain comprising 19 hyrophobic amino acids (Ala^{1883}-Leu^{1901}), 5: A short cytoplasmic domain of 26 hydrophilic amino acids that lies (Ser^{1902}-Phe^{1927}) on the cytoplasmic side of the membrane COOH-terminal. The gene coding for human LPH is located on chromosome 2 and contains 17 exons (36;44). The cDNA consists of 6274 base pairs, coding for 1927 amino acids between the NH2-terminal signal sequence and the COOH-terminus. The DNA was found to be internally repeated four times in an all cloned LPH species. These domains are called I-IV and reveal 38% -55% identity to each other. The catalytic activity of lactase is localized to glutamine 1273 in the homologous region III and phlorizin-hydrolase to glutamine 1749 in the homologous region IV (46). The LPH molecule is highly glycosylated. The primary sequence of the human enzyme contains 15 potential N-glycosylation sites, the rabbit and rat enzymes possess 14 and 15, respectively (47).

Biosynthesis and processing of LPH

In epithelial cells of the small intestinel LPH is synthesized as 1927 amino acid precursor, prepro -LPH (27;28;48;49) and undergoes two sequential interacellular cleavages. In the first step, the first 19 amino acids (Met^{1-} Gly^{19}) of this precursor mediating the integration of pro-LPH into the ER is cleaved off in ER by signal peptidases. The remaining 1908 amino acids form the mannose-rich precursor pro LPH (215kDa) undergo core mannose-rich N-

glycosylation during translocation into the ER. Beside the signal sequence cleavage, and the attachment of mannose-rich carbohydrate chains, several other modification reactions are

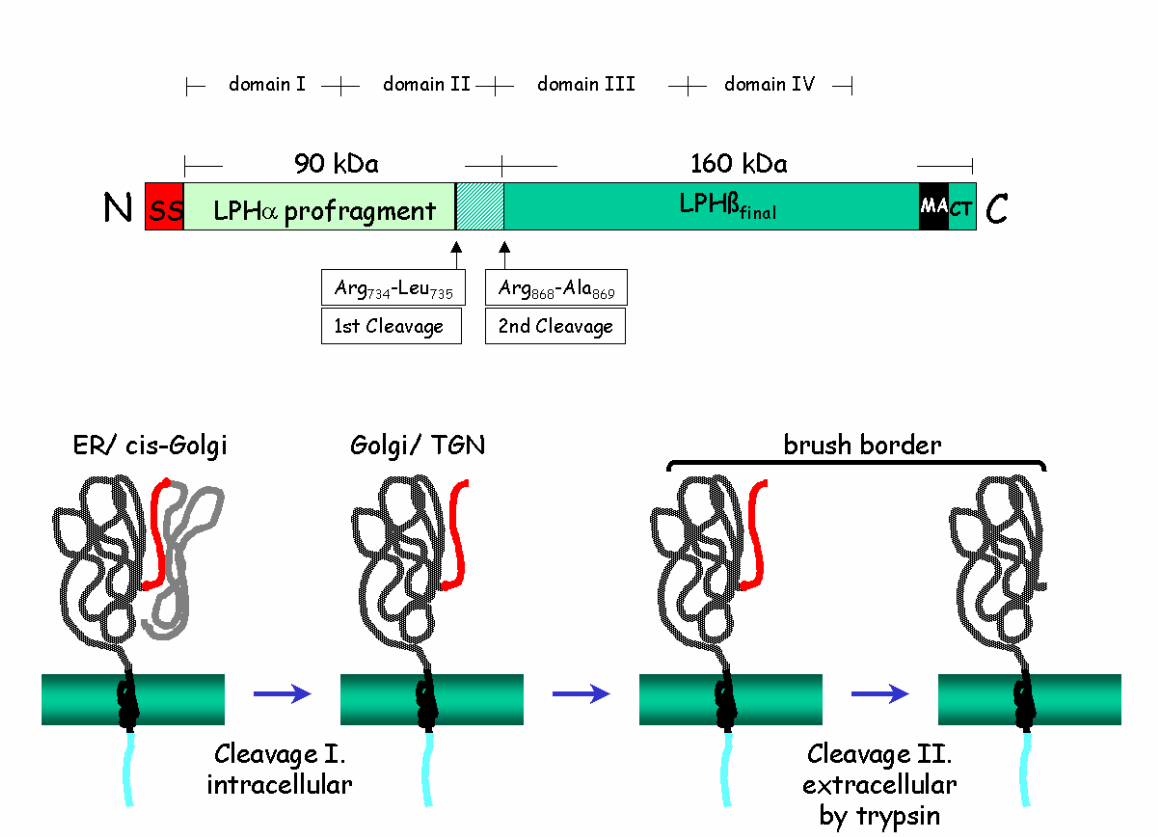

Fig. 2: Schematic representation of the structure and biosynthesis of pro–lactase-phlorizin hydrolase in human small in testinal cells.

initiated during the entry of pro-LPH into the ER lumen and may facilitate the folding of this protein into native conformation. Correct folding and dimerisation is not only important for efficient transport from the ER, it is also crucial for the acquisition of the enzymatic activity of LPH (50). During the pathway through the Golgi apparatus en route to the cell surface, the mannose-rich N-linked sugar chains are modified by Golgi mannosidases and several types of sugar are added resulting in a complex glycoprotein of an approximate apparent MW (230-kDa). In the cis-Golgi this modified molecule acquires its O-linked glycosylation.

The O-glycosylation of LPH molecule at Ser or Thr is a post-translation event which is involved, or at least may cooperate with other mechanisms in the regulation of LPH activity (5;30). Complex glycosylated pro-LPH of human and pig small intestinal cells as well as in the colon carcinoma cell line Caco-2 undergoes the second intercellular proteolytic cleavage steps to the final brush border form (5;6;27;28;51-54). Cleavage of rat pro-LPH is reported to

take place either intracellularly or at the cell surface in the brush border membrane. The large profragment LPHα is intracellulrarly cleaved at Arg734 /Leu 735 leaving the membrane-bound LPH which extends from Leu 735 to Tyr 1927. This form is transported to the apical membrane where it is cleaved by luminal pancreatic trypsin in the small intestine at Arg A868/ Ala 869 to $LPH_{ß}$ (Arg 868/Tyr 1927), which is known as mature brush border $LPH_{ß}$ of 160-kDa and comprises the functional domain of the enzyme (5;27;28). The pro LPHα domain (100kDa) has a significantly shorter half-life than $LPH_{ß.}$ $LPH_{α}$ is neither N- nor O-glycosylyted, despite the presence of 5 potential N-glycosylation sites and it may play a crucial role as an intramolecular chaperone during folding of pro-LPH in ER (17;55-58)

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Chapter 4:

O-linked glycans mediate apical sorting of human intestinal sucrase-isomaltase through association with lipid rafts.

Abstract

The plasma membrane of polarised epithelial cells is characterised by two structurally and functionally different domains, the apical and basolateral. These domains contain distinct protein and lipid constituents which are sorted by specific signals to the correct surface domain [1]. Missorting of proteins often results in pathological conditions [2]. The best characterised apical sorting signal is that of glycophosphatidyl inositol (GPI) membrane anchors [3]. N-linked glycans on some secreted proteins [4] and O-glycans [5] function also as apical signals. In the latter cases, however, the underlying sorting mechanisms remain obscure.

Here, we analyse the role of O-glycosylation in the apical sorting of sucrase-isomaltase (SI), a highly polarised N- and O-glycosylated intestinal enzyme and the mechanism underlying this process. Inhibition of O-glycosylation by benzyl-N-acetyl-α-D-galactosaminide (benzyl-GalNAc) is accompanied by a dramatic shift in the sorting of SI from the apical to both membranes. The sorting mechanism of SI implicates its association with sphingolipid-cholesterol-rich membrane rafts. This association is eliminated when O-glycosylation is inhibited by benzyl-GalNAc. The results demonstrate for the first time that O-linked glycans mediate apical sorting through association with lipid rafts.

RESULTS AND DISCUSSION

Pro-SI is a highly polarized apically-sorted and heavily O-glycosylated intestinal membrane glycoprotein [6]. The role of O-linked glycans on the structure, transport and sorting of pro-SI in colon carcinoma Caco-2 cells was evaluated by employing benzyl-N-acetyl-α-D-galactoseaminide (benzyl-GalNAc)[1], a potent inhibitor of O-linked glycosylation [7,8].

Pro-SI was immunoprecipitated from detergent extracts of cells labelled with [^{35}S]methionine for 6 h in the presence or absence of 4 mM of the inhibitor. Control Caco-2 cells contained mannose-rich 210-kDa pro-SI_h and complex N- and O-glycosylated 245-kDa pro-SI_c (Fig. 1A) [6]. In the presence of benzyl-GalNAc a substantial shift in the size of the complex glycosylated species (denoted thereafter pro-$SI_{c/benzyl}$) was observed, while pro-SI_h remained unaffected. To determine whether indeed O-linked glycans were affected, enzymatic deglycosylations were performed. Endoglycosidase F/N-glycopeptidase F (endo F/GF) generates a 205-kDa O-glycosylated protein from complex glycosylated pro-SI_c (Fig. 1B) [6]. A similar band was not found in endo F/GF-treated pro-$SI_{c/benzyl}$ and a smaller product was detected. This indicates that pro-$SI_{c/benzyl}$ has a substantially reduced or no O-linked glycans, while its N-glycosylation pattern was not affected. We corroborated this result by treating pro-SI_c and pro-$SI_{c/benzyl}$ glycoforms with neuraminidase/galactosidase (Fig. 1C). Neuraminidase generated smaller glycoforms of pro-SI_c and pro-$SI_{c/benzyl}$ and neuraminidase/galactosidase shifted pro-SI_c and pro-$SI_{c/benzyl}$ to the same apparent molecular weight. The reduced size of pro-$SI_{c/benzyl}$ is therefore the consequence of inhibited or impaired O-glycosylation of this glycoform.

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[1] **Abbreviations**

SI, sucrase-isomaltase (all forms); pro-SI, uncleaved precursor (all forms); pro-SI_h, mannose-rich pro-SI; pro-SI_c, complex glycosylated mature pro-SI; pro-$SI_{c/benzyl}$, complex glycosylated pro-SI in the presence of benzyl-GalNAc; benzyl-GalNAc, benzyl-N-acetyl-α-D-galactosaminide; GPI, glycophosphatidyl inositol; TGN, trans Golgi network; MDCK cells, Madin-Darby canine kidney cells; LPH, lactase-phlorizin hydrolase.

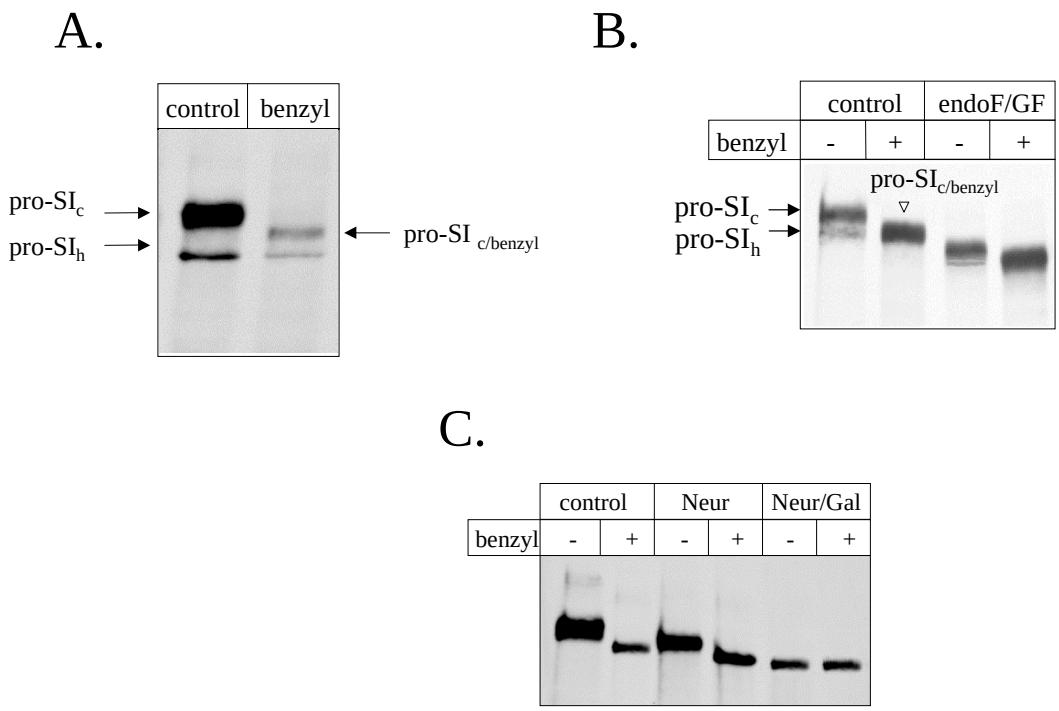

Fig. 1: Effect of benzyl-GalNAc on the O-glycosylation of intestinal sucrase-isomaltase.

A. Pro-SI was immunoprecipitated from Caco-2 cells biosynthetically labelled in the presence or absence of benzyl-GalNAc (indicated benzyl) and analyzed by SDS-PAGE.

B. Similar experiment as in A. Following immunoprecipitation the proteins were treated or not treated with endoglycosidase F/N-glycopeptidase F (endo F/GF).

C. Caco-2 cells were labelled in the presence or absence of 4 mM benzyl-GalNAc for 1 h with [^{35}S]methionine and chased for 10 h to enrich in mature forms of pro-SI_c and pro-$SI_{c/benzyl.}$ These forms were immunoprecipitated and treated or not treated with neuraminidase or neuraminidase/galactosidase and subjected to SDS-PAGE.

Next we examined the sorting of pro-$SI_{c/benzyl}$ to gain insights into the role of O-glycans in this event. For this, cell surface immunoprecipitations of pro-SI were performed with Caco-2 cells that have been grown on transmembrane filters and biosynthetically labelled in the presence

or absence of 4 mM of benzyl-GalNAc. In non-treated Caco-2 cells pro-SI_c appeared after 3 h and 6 h of chase predominantly at the apical membrane (Fig. 2A,B) [9]. By contrast, in the presence of benzyl-GalNAc a significant shift in the sorting of pro-$SI_{c/benzyl}$ to the basolateral membrane could be discerned (Fig. 2A,B) due to reduced or inhibited O-glycosylation.

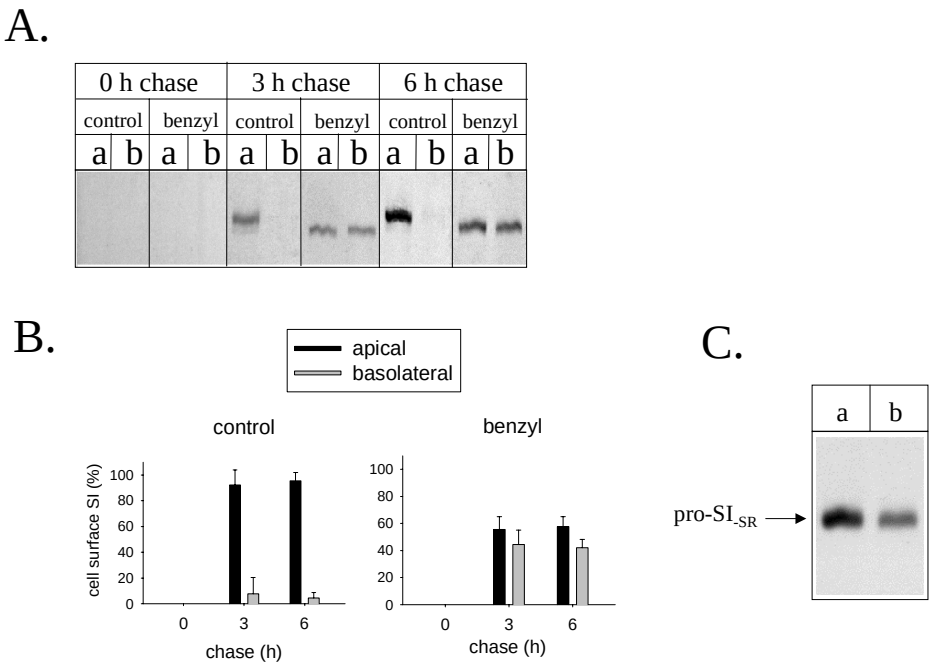

Fig. 2: Implication of O-linked glycans in the sorting of pro-SI.

A. Cell surface immunoprecipitation of pro-SI from the apical (a) or basolateral (b) membranes was performed with Caco-2 cells that were grown on transmembrane filters and subjected to pulse-chase labelling in the presence or absence of benzyl-GalNAc.

B. Quantitation of the proportions of pro-SI appearing at the apical and basolateral membranes in the presence or absence of benzyl-GalNAc.

C. Analysis of the role of the Ser/Thr-rich stalk region of pro-SI in its apical sorting. A stalk-free mutant of the cDNA encoding pro-SI was stably expressed in MDCK cells. Cell surface immunoprecipitation of pro-SI from the apical (a) or basolateral (b) membranes and SDS-PAGE were performed as above.

Almost 40% of pro-$SI_{c/benzyl}$ was found at the basolateral membrane as compared to about 5% in pro-SI_c. This result points to a direct implication of O-linked glycans as an apical sorting signal within pro-SI. This alteration in the sorting of pro-$SI_{c/benzyl}$ is not the consequence of a disrupted sorting machinery in the presence of benzyl-GalNAc, since the sorting patterns of control proteins such as intestinal LPH were not affected (not shown). The data further indicate that impaired O-glycosylation has no effects on the transport kinetics of pro-$SI_{c/benzyl}$, since this glycoform reached the cell surface (apical/basolateral) at the same time as pro-SI_c. O-linked glycans have been proposed to be involved in sorting of the neurotrophin receptor heterologously expressed in MDCK cells [5]. Our findings are the first, however, to describe a direct role of O-linked glycans in the sorting of an endogenous glycoprotein in a polarised cell line. Except for properly processed O-linked glycan units, no other motifs or signals contribute to the high fidelity of the apical sorting of pro-SI. Also repressed basolateral sorting elements are not located in pro-SI. This type of signals exists in the neurotrophin receptor and acquires functionality after elimination of the apical signal [5]. The question arises as to the possible location of the O-glycan sorting signal within the pro-SI molecule. Pro-SI is composed of two N- and O-glycosylated species, sucrase and isomaltase, and it is likely that O-glycan units in both species participate, perhaps with variable impact, in the sorting event. However, observations with pro-SI mutants in congenital sucrase-isomaltase deficiency [2] suggest that isomaltase plays the predominant role in the sorting process of pro-SI. Isomaltase possesses a potentially O-glycosylated Ser/Thr-rich stalk region that is located juxtapose the membrane [10]. To delineate the role of the stalk region in the sorting event, a stalk-free pro-SI mutant was constructed (denoted pro-SI_{-SR}), expressed in MDCK cells and its sorting assessed. Fig. 2C shows that the stalk-free pro-SI_{-SR} mutant is randomly delivered

to both membranes demonstrating a direct role of the Ser/Thr-rich domain in apical sorting of pro-SI. Evidently isomaltase contains adequate sorting information. However, this does not fully exclude a possible contribution of sucrase to the sorting of pro-SI, perhaps by tuning the sorting fidelity.

We next sought to elucidate the mechanism by which the sorting of SI occurs. One type of signals for sorting proteins to the apical surface of epithelial cells, or axon of neurons, is that of a glycolipid anchor [3]. The sorting mechanism implicates association of these anchors with detergent-insoluble membrane microdomains enriched in glycosphingolipids and cholesterol, known as lipid rafts [11,12]. Although initially observed with GPI-anchored proteins, this mechanism appears also to occur with proteins that possess a transmembrane domain. Localisation of pro-SI to membrane microdomains has been morphologically demonstrated in Caco-2 cells [13]. We investigated here the association of pro-SI with lipid rafts, the implication of these structures in apical sorting of pro-SI, and the role possibly played by O-linked glycans in these events.

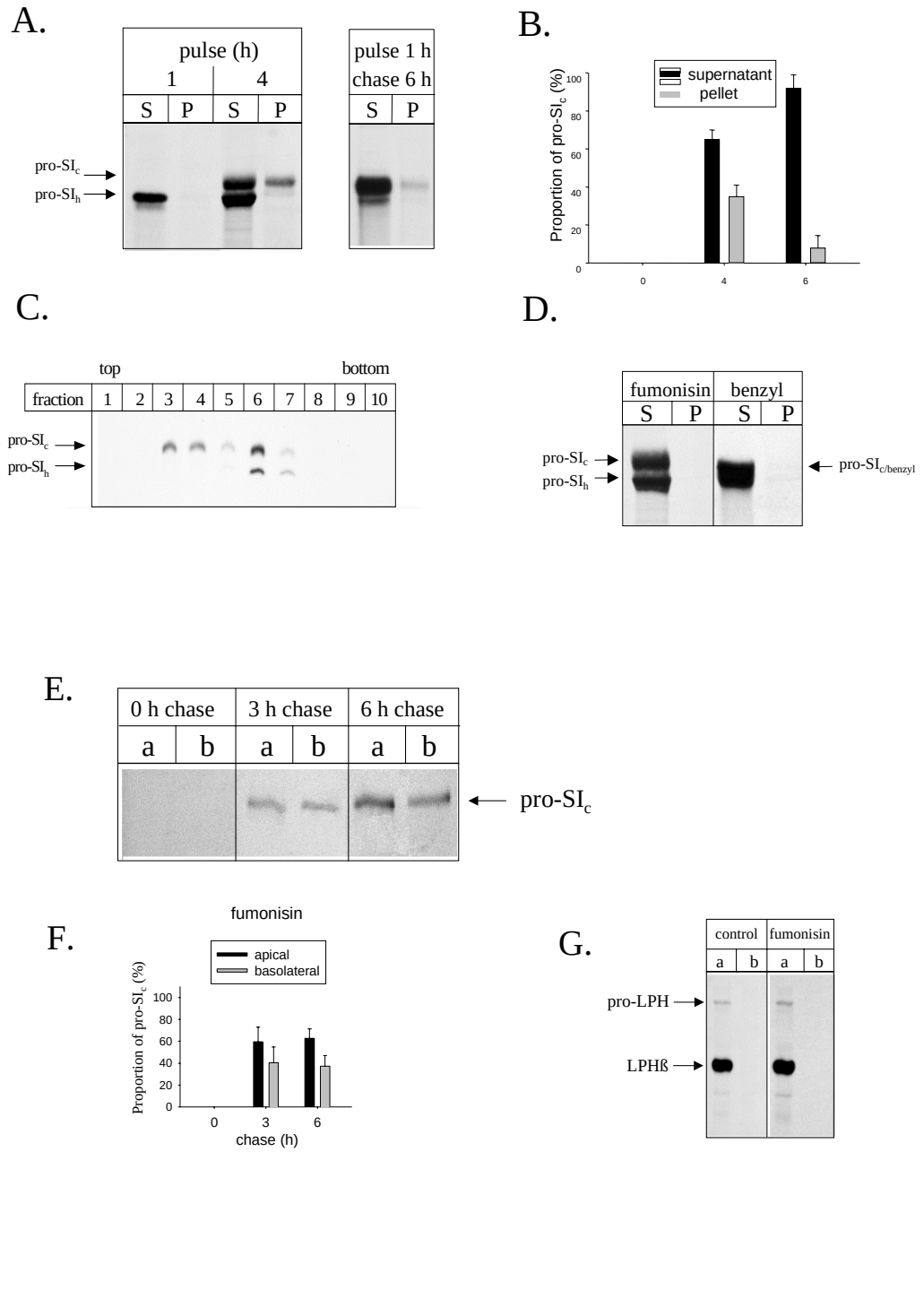

Fig. 3: Apical sorting of pro-SI implicates its association with lipid rafts through O-linked glycans.

A. Pro-SI was immunoprecipitated from the detergent-soluble (S) and isoluble pellet (P) of Triton X-100 solubilized labelled Caco-2 cells.

B. Quantitation of the proportions of complex glycosylated pro-SI_c in the supernatants and pellets of Triton X-100 solubilised Caco-2 cells.

C. Sucrose gradient centrifugation of Triton X-100 solubilized Caco-2 cells. Microdomains were prepared by loading the detergent extracts of 4 h-labelled Caco-2 cells on a 5-35% sucrose gradient as described [15]. Pro-SI was immunoprecipitated from the individual gradient fractions.

D. Pro-SI was immunoprecipitated from the supernatant (S) or pellet (P) of Triton X-100 solubilized Caco-2 cells that were biosynthetically labelled the presence or absence of fumonisin or benzyl-GalNAc.

E. Cell surface immunoprecipitation of pro-SI was performed with Caco-2 cells that were subjected to a pulse-chase protcol in the presence or absence of fumonisin.

F. Quantitation of the proportions of pro-SI appearing at the apical and basolateral membranes in the presence of fumonisin.

G. Cell surface immunoprecipitation of LPH from cells labelled in the presence or absence of of fumonisin.

One approach to assess the specific association of a membrane protein with microdomains is to eliminate or reduce sphingolipids or cholesterol and examine the detergent extractability of the protein of consideration. For this purpose, we performed pulse labelling experiments of Caco-2 cells in the presence or absence of 10 µM fumonisin, an inhibitor of sphingolipid synthesis [14]. Cells were solubilised with 1% Triton X-100 and the supernatants and pellets were further processed for immunoprecipitation. After 1 h of pulse mannose-rich pro-SI_h was exclusively found in the supernatant (S) (Fig. 3A). Within 3 h of chase complex pro-SI_c in addition to pro-SI_h appeared in the supernatant fraction, while the insoluble pellet contained exclusively pro-SI_c (Fig. 3A). At this stage almost 35% of pro-SI_c was found associated with the detergent insoluble pellet (Fig. 3B). When pro-SI_c was chased for 6 h, at which time a large proportion of pro-SI_c reaches the cell surface [6], only about 12% of pro-SI_c were

present in the insoluble pellet indicating a temporal association of pro-SI_c with microdomains. The results indicate that pro-SI_c associates with lipid rafts prior to further transport to the cell surface and that this association occurs after pro-SI has exit the ER and passed through the Golgi apparatus. To exclude the possibility that the detergent insolubility of pro-SI_c is due to its interactions with the cytoskeleton we examined the association of pro-SI_c with lipid rafts in sucrose gradients [15] of Triton X-100 detergent extracts of Caco-2 cells that have been continuously labelled for 4 h with [^{35}S]methionine. Fig. 3C demonstrates that pro-SI_c, but not pro-SI_h, was found in the floating fraction at low buoyent density of the gradient characteristic of detergent insoluble lipid rafts. Pro-SI_c as well as pro-SI_h were also found in higher density fractions indicating a temporal association of pro-SI_c with microdomains along the secretory pathway. In the presence of fumonisin, no pro-SI_c species could be recovered in the detergent insoluble pellet (Fig. 3D) demonstrating that inhibition of sphingolipid synthesis had dramatic effects on association of pro-SI_c into lipid rafts. On the other hand, pro-SI_h as well as pro-SI_c could be identified in the soluble detergent extracts of fumonisin-treated cells indicating that the drug has no effects on the biosynthesis and processing of pro-SI glycoforms.

To determine whether the different detergent extractability of pro-SI_c is associated with variations in its polarised sorting, Caco-2 cells grown on transmembrane filters were pulsed and chased in the presence or absence of 10μM fumonisin. Here, cell surface immunoprecipitation of pro-SI_c from the apical or basolateral membranes revealed a drastic reduction in the proportion of pro-SI_c to about 60% at the apical and an increase of up to 40% at the basolateral membrane. This points to a sorting of pro-SI_c by default when its association with lipid rafts has been disrupted (Figs. 3E & 3F). As a control we investigated the sorting of pro-LPH, an apically-sorted protein not associated with lipid rafts [16,17]. Pro-LPH expressed in MDCK rather than Caco-2 cells was used, since (i) the sorting pathways of pro-LPH in MDCK and Caco-2 cells are similar [17] and (ii) the expression level of endogenous pro-LPH

in Caco-2 cells is low. In the presence or absence of fumonisin no change in the polarity of pro-LPH could be observed (Fig. 3G). The effect of fumonisin is therefore restricted to those proteins that are associated with lipid rafts.

What proceeded points to a direct involvement of O-linked glycans in the association of pro-SI with lipid rafts, which in turn constitutes the driving mechanism for an efficient apical sorting of pro-SI. In fact, pro-$SI_{c/benzyl}$ could not be detected in the pellet of Triton X-100 lysed Caco-2 cells that were biosynthetically labelled in the presence of benzyl-GalNAc. By contrast this glycoform was retained exclusively in the supernatant of the detergent extracts.

In essence, the data demonstrate that the sorting of pro-SI occurs through an association of this molecule with lipid rafts. This association implicates O-linked glycans, the modification of which by benzyl-GalNAc results in a completely detergent-soluble glycoform, pro-$SI_{c/benzyl}$. Importantly, the disruption of the lipid rafts alters the sorting behaviour of pro-SI from a highly polarised to a randomly delivered molecule to either membrane. This is the first evidence of a relation occurring between the glycosylation pattern of an apical protein and its association with lipid rafts. Although the sites and mode of action of fumonisin and benzyl-GalNAc are different, there is a synergy in their effects with respect to detergent extractability and sorting of pro-SI. It is evident that apical sorting of pro-SI implicates its association with lipid rafts through O-linked glycans. This association may occur through binding of O-linked glycans of pro-SI, and probably the sugar moiety of a GPI-anchor, to a common sorting protein in the TGN, a lectin-like protein. A possible candidate is VIP36 [18], a member of leguminous-lectin-like proteins that are implicated in various steps of the secretory pathway.

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Chapter 5

Structural Determinants Required for Apical Sorting of an Intestinal Brush Border Membrane Protein

Abstract

The distinct protein and lipid constituents of the apical and basolateral membranes in polarized cells are sorted by specific signals. O-glycosylation of a highly polarized intestinal brush border protein sucrase-isomaltase is implicated in its apical sorting through interaction with sphingolipid-cholesterol microdomains. We characterized the structural determinants required for this mechanism by focussing on two major domains in pro-SI, the membrane anchor and the Ser/Thr rich stalk domain. Deletion mutants lacking either domain, pro-SI$_{\Delta ST}$ (stalk-free) and pro-SI$_{\Delta MA}$ (membrane anchor-free), were constructed and expressed in polarized MDCK cells. In the absence of the membrane anchoring domain pro-SI$_{\Delta MA}$ does not associate with lipid rafts and the mutant is randomly delivered to both membranes. Therefore, the O-glycosylated stalk region is not sufficient *per se* for the high fidelity of apical sorting of pro-SI. pro-SI$_{\Delta ST}$ does not associate either with lipid rafts and its targeting behavior is similar to that of pro-SI$_{\Delta MA}$. Only wild type pro-SI containing both determinants, the stalk region and membrane anchor, associates with lipid microdomains and is targeted correctly to the apical membrane. We conclude that the stalk-region and the membrane anchor act synergetically in the apical sorting of pro-SI.

INTRODUCTION

Biological membranes of polarized cells contain an asymmetrical distribution of lipid and protein components (1-4). Both kinds of biomolecules are sorted in the *trans* Golgi Network Complex (TGN)[2] into different types of vesicles for apical or basolateral delivery (5,6). Whereas all basolateral sorting signals of membrane proteins described to date reside in the cytoplasmic domains (7,8), apical signals appear to be luminal (7,9-11). One criterion for apical delivery could be the presence of asparagine-linked carbohydrates, since their removal by tunicamycin treatment or site directed mutagenesis results in nonpolar secretion (12,13). Additionally, insertion of novel N-glycosylation sites into the normally randomly secreted rat growth hormone leads to apical secretion (14). Further evidence has accumulated that some apical membrane proteins accumulate in sphingolipid- and cholesterol-rich microdomains (15), which have been termed sphingolipid-cholesterol rafts. GPI-anchored membrane proteins as well as transmembrane proteins, like the haemagglutinin of influenza virus (16) or intestinal brush border proteins like sucrase-isomaltase and dipeptidyl peptidase (17) are associated with lipid rafts. Rafts can be discriminated from other membraneous components based on their insolubility in nonionic detergents like TX-100 or CHAPS at low temperature.
By virtue of its structural features and sorting behavior sucrase-isomaltase (SI, EC 3.2.1.48-10) constitutes an exquisite model protein to identify apical sorting signals and to unravel molecular mechanisms underlying apical targeting. SI is an intestinal transmembrane protein that is apically targeted in a highly polarized manner in association with lipid rafts (17,18). It is a heavily N- and O-glycosylated protein that is composed of two homologous subunits, sucrase and isomaltase. Inhibition or drastic reduction of O-glycosylation in Caco-2 cells by using benzyl-N-acetylgalactosaminide substantially affects the high sorting fidelity of pro-SI ending with a random delivery of the protein to both membranes (19). Importantly, O-linked glycans mediate apical sorting through association with lipid rafts. pro-SI contains a stretch of a Ser/Thr-rich domain in immediate proximity of the membrane (20). Similar domains, referred to as stalk regions, have been also described for glycophorin (21), the neurotrophin-receptor (22) or the LDL-receptor (23) and are thought to be the site of heavy O-glycosylation. In pro-SI this stretch serves as a link between the globular protein and the plasma membrane by forming a rigid unfolded structure. Based on its membrane proximity this domain constitutes a sterically suitable site for interaction with other cellular factors. For the neurotrophin receptor it has been shown that the O-glycosylated stalk domain is required

[2] The Abbreviations used are: TGN, trans-Golgi network; GPI, glycophosphatidyl inositol ; ER, endoplasmic reticulum; SI, sucrase-isomaltase (all forms); pro-SI, uncleaved sucrase-isomaltase; Triton X 100, TX-100; mAb, monoclonal antibody; benzyl-GalNAc, benzyl-N-acetyl- -D-galactosaminide; endo H, endoglyosidase H; endo F/GF, endoglycosidase F/N-glycopeptidase F; LPH, lactase-phlorizin hydrolase; MDCK, Madin-Darby canine kidney cells

for apical targeting (22). In addition this requirement could only be demonstrated for membrane anchored receptors, whereas the soluble form expressed in Caco-2 cells was secreted into the basolateral medium in presence of the stalk domain (24). These findings suggest that O-glycosyalted apically-sorted proteins interact via O-linked carbohydrates with a lectin-like cellular protein.

In this paper we describe the characterization of the structures implicated in the interaction of pro-SI with lipid microdomains along the polarized transport of pro-SI. We demonstrate that deletion of the stalk region of pro-SI leads to default targeting of the normally apically transported pro-SI to both membrane domains and to a disruption of the association of pro-SI with lipid rafts. The same sorting behavior was also observed with the soluble form of pro-SI, indicating that the presence of the membrane-proximal O-glycosylated stalk determines apical targeting and raft association. Furthermore, membrane-association of pro-SI is a necessary requirement for the stalk domain to fulfill this role in pro-SI-transport.

EXPERIMENTAL PROCEDURES

Materials and Reagents - Transwell filters (24 mm) were obtained from Falcon. L-[^{35}S] Redivue™ PRO-MIX™ and protein A-Sepharose were obtained from Amersham/Pharmacia. Streptomycin, penicillin, geneticin-418, Dulbecco's modified Eagle's medium (DMEM), methionine-free DMEM (denoted met-free medium) and fetal calf serum (FCS) were purchased from Gibco. Benzyl-N-acetyl-α-D-galactosaminide (benzyl-GalNAc) Endo-*ß-N*-acetylglucosaminidase F/glycopeptidase F (endo F/GF), Polybrene, pepstatin, leupeptin, aprotinin and molecular weight standards for SDS-PAGE were purchased from Sigma. Acrylamide and N,N'-methylenebisacrylamide were obtained from Carl Roth GmbH & Co, Karlsruhe, Germany. Sodium dodecyl sulfate (SDS), *N,N,N´,N´,* tetramethylethylenediamine (TEMED), ammonium persulfate, dithiothreitol and Triton X-100 were obtained from Merck, Darmstadt, Germany. Endo-*ß-N*-acetylglucosaminidase H (endo H), restriction enzymes, Taq-polymerase and ligase were purchased from Roche Biochemicals, Mannheim, FRG. All other reagents were of superior analytical grade.

Immunochemical Reagents - Monoclonal antibodies (mAbs) were a generous gift from Dr. H.-P. Hauri, Biocenter, Basel and Dr. E.E. Sterchi, University of Bern, Switzerland (25). For immunoprecipitation of pro-SI a mixture of four different monoclonal antibodies was used (HBB 1/691, HBB 2/614, HBB 3/705, HBB 2/219).

Construction of the deletion mutants - Deletion of the stalk-domain of pro-SI (pro- $SI_{\Delta ST}$, ST stands for stalk-region) and subdomains of the stalk-domain was performed by oligonucleotide directed looping out mutagenesis with the Quick Change™ *in vitro* Mutagenesis System from Stratagene. The template constituted a full length cDNA encoding

pro-SI cloned into pSG8-vector (26). The following oligonucleotides were used in this context:

$\Delta stalk_{upstream}$: 5´- GCC TTA ATT GTT GTT TTA GCA GGA AAA TGT CCA AAT GTG T - 3´

$\Delta stalk_{downstream}$: 5´- ACA CAT TTG GAC ATT TTC CTG CTA AAA CAA CAA TTA AGG C - 3´

$\Delta stalk37\text{-}48_{upstream}$: GTT TTA GCA ACT AAG ACA CCT GCT ACT ACT CGT GTG ACT ACA

$\Delta stalk37\text{-}48_{downstream}$: TGT AGT CAC ACG AGT AGT AGC AGG TGT CTT AGT TGC TAA AAC

$\Delta stalk49\text{-}57_{upstream}$: AGT GAT TCT ACT TCA ACT CCA TCT GAT TCA GGA AAA TGT CCA

$\Delta stalk49\text{-}57_{downstream}$: TGG ACA TTT TCC TGA ATC AGA TGG AGT TGA AGT AGA ATC ACT

$\Delta stalk37\text{-}57_{upstream}$: GTT TTA GCA ACT AAG ACA CCT TCT GAT TCA GGA AAA TGT CCA

$\Delta stalk37\text{-}57_{downstream}$: TGG ACA TTT TCC TGA ATC AGA AGG TGT CTT AGT TGC TAA AAC

Deletion of the sequences was confirmed by sequencing and the plasmids obtained were denoted pSG8-$SI_{\Delta ST}$, pSG8-$SI_{\Delta ST37\text{-}48}$, pSG8-$SI_{\Delta ST49\text{-}57}$ and pSG8-$SI_{\Delta ST37\text{-}57}$.

For the generation of an anchoreless, soluble form of pro-SI the signal sequence of lactase-phlorizin hydrolase (LPH) (27) was fused with the N-terminus of the stalk domain by ”PCR-soeing”. In this procedure four different oligonucleotides were used:

*Eco*RI-LPH:5´- GAA TTC GTT CCT AGA AAA TGG AGC TGT CTT GGC ATG TAG –3´

cLPH-signal: 5´- TGA CCC CCA GCA TGA AAA ACT – 3´

SI-st (st stands for stalk-region): 5´- ATC AGT GAT TCT ACT TC – 3´

cLPH-ma (ma stands for membrane anchor): 5´- GAG AAA AGA GAA CGT ACA AAG CTT GAA CAC TAA AGT TTC TTC C – 3´

In the first two PCR-reactions the LPH signal sequence and the isomaltase domain were amplified with the primer pairs *Eco*RI-LPH/ cLPH-signal and SI-st/ cLPH-ma. The resulting fragments were annealed by PCR-soeing, and finally an 853 bp *Eco*RI/*Xho*I-fragment of this construct was used to replace the N-terminus of pro-SI encoded on pSG8-SI (26). The resulting sequence was confirmed by sequencing and the plasmid obtained was denoted pSG8-$SI_{\Delta MA}$.

Transfection and Generation of stable MDCK Cell Lines - MDCK cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Eggenstein/Germany) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin and streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were transfected

with 5 µg of the appropriate recombinant DNA using polybrene (28). Stably transfected MDCK cells were selected in the presence of 0.25 mg/ml active G418 (Gibco Life Technologies) and after 18-23 days, surviving colonies were isolated with cloning rings. Stable transformants expressing pro-$SI_{WT,}$ pro-$SI_{\Delta ST}$ and pro-$SI_{\Delta MA}$were screened by immunoprecipitation. For transient transfection experiments MDCK cells were transfected with pSG8-SI $_{\Delta ST37-48}$, pSG8-SI $_{\Delta ST49-57}$ and pSG8-SI $_{\Delta ST37-57}$ using the calcium phosphate procedure as described before (29).

Biosynthetic labeling of cells, immunoprecipitation and SDS-PAGE - Metabolic labeling of MDCK cells grown on filters or plated in six-well culture dishes was performed as described previously (28). For higher transfection efficiency on filters transiently transfected MDCK cells were treated with trypsin prior to transfection to dissociate the cells and achieve an optimal exposure of cells to DNA. Transiently transfected cells and MDCK clones expressing pro-$SI_{WT,}$ pro-$SI_{\Delta ST}$ and pro-$SI_{\Delta MA}$ were labeled for 1 h with 100 µCi [^{35}S]methionine and chased for different times with unlabeled methionine. The medium of MDCK cells expressing the anchorless mutant pro-$SI_{\Delta MA}$was collected, set to pH 8.0 by Hepes (30 mM) and protease inhibitors were added. The medium and cell lysates were immunoprecipitated with the mAb anti-SI mixture as described by Naim et al. (18) and cell surface antigens were immunoprecipitated from intact cells on filters by addition mAb anti-SI to either the apical or basolateral compartments. The immunoprecipitates were analyzed by SDS-PAGE according to Laemmli (30). After electrophoresis the gels were fixed, soaked in 16% salicylic acid for signal amplification and subjected to fluorography.

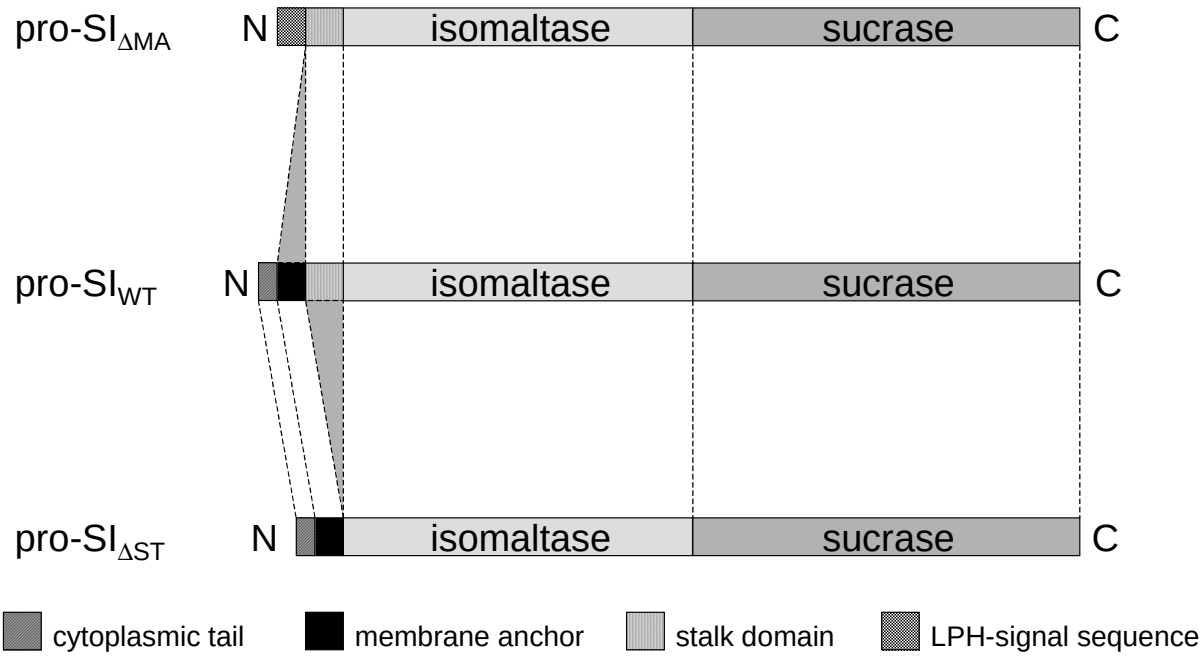
Sucrose gradient centrifugation of Triton X-100 solubilized MDCK cells – Transiently transfected and stable MDCK cells expressing wildtype pro-SI, pro-$SI_{\Delta ST}$ and pro-$SI_{\Delta MA}$ were biosynthetically labelled with [^{35}S]methionine for 4 h and solubilized as described above. Microdomains preparation was performed by loading the detergent extracts on 5-35% sucrose gradients, followed by ultracentrifugation as described (31). The gradients were fractionated into 12 fractions, immunoprecipitated with mAb anti-SI and the isolated proteins subjected to SDS-PAGE.

Other Procedures - Digestion of ^{35}S-labeled immunoprecipitates with endo-*ß-N*-acetylglucosaminidase H (endo H) and endo-*ß-N*-acetylglucosaminidase F/glycopeptidase F (endo F/GF) was performed as previously described (32).

RESULTS

Expression of pro-$SI_{WT,}$ pro-$SI_{\Delta ST}$ and pro-$SI_{\Delta MA}$ in MDCK cells - To investigate the influence of the stalk and the membrane anchoring domain on the sorting of pro-SI in polarized cells two constructs of pro-SI were generated. In the first construct only the stalk region (Thr_{33} - Ser_{60}) was deleted from wild type pro-SI (denoted pro-$SI_{\Delta ST}$). The second construct contained the stalk region, but lacked the entire transmembrane domain (denoted pro-$SI_{\Delta MA}$). pro-SI is a type II glycoprotein that is synthesized with an uncleavable signal sequence residing in its membrane domain. The signal sequence is therefore eliminated upon deletion of the membrane anchor. We therefore fused the cleavable signal sequence of the type I glycoprotein, human intestinal brush border lactase-phlorizin hydrolase, to the N-terminal end immediately in front of the stalk region of pro-SI (Fig. 1).

Fig. 1: Schematic representation of the structure of pro-SI in small intestinal cells.
Structural features of pro-SI deduced from biosynthetic studies (18) and cDNA cloning (20,42). pro-SI is a type II membrane glycoprotein (N_{in}/C_{out}) that is synthesized with an



uncleavable signal sequence which also serves as a membrane anchoring domain (20). The cytoplasmic tail contains 12 amino acid residues and is followed by a membrane anchor of 20 amino acids and a Ser/Thr rich stalk domain /region of 28 amino acids that is considered to be part of the isomaltase subunit. The stalk region is suggested to be heavily O-glycosylated (42). Isomaltase ends with amino acid residue Arg_{1007} and sucrase starts immediately thereafter with Ile_{1008}. The Arg/Ile peptide sequence between isomaltase and sucrase is a

trypsin site where the mature large precursor pro-SI is cleaved in the intestinal lumen by pancreatic trypsin (25).

Both constructs, pro-SI$_{\Delta ST}$, pro-SI$_{\Delta MA}$ as well as wild type pro-SI were stably transfected in MDCK cells and the positive clones were selected by immunoprecipitation of detergent extracts of biosynthetically labeled cells with mAb anti-SI.

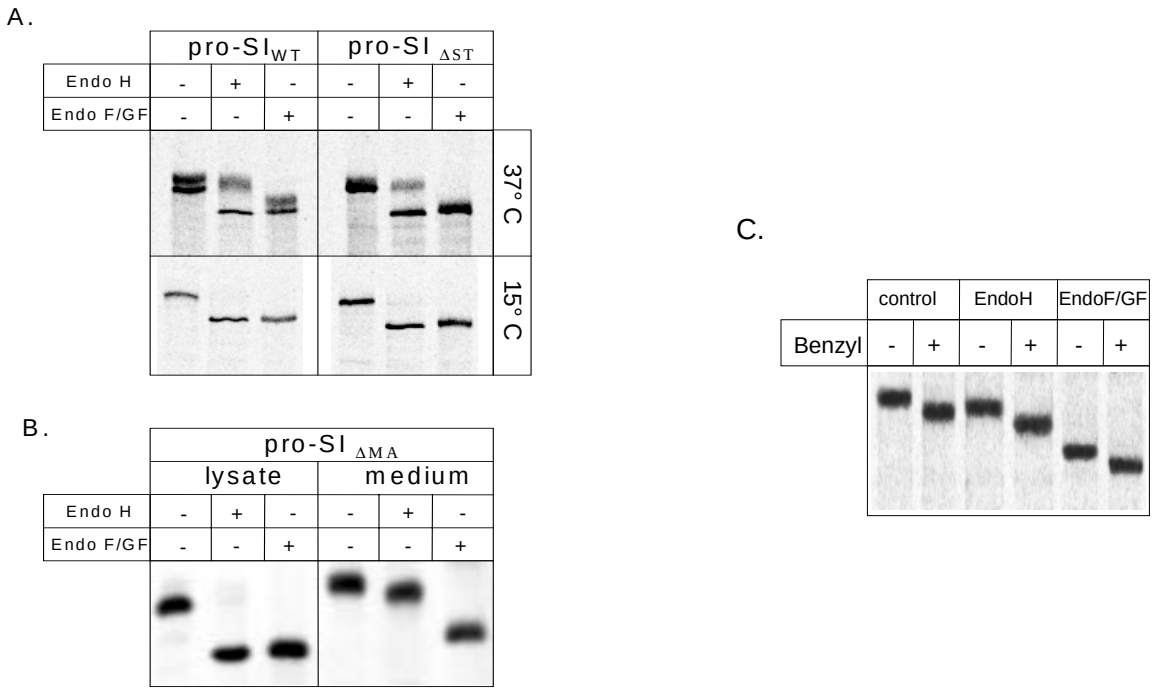


Fig. 2: Identification of molecular forms of wild type pro-SI, pro-SIΔ_{ST} and pro-SIΔ_{MA} in MDCK cells.

A. MDCK cells stably expressing wild type pro-SI or pro-SI$_{\Delta ST}$ were biosynthetically labeled for 6 h at 37°C (upper panel) or 10 h at 15°C (lower panel) with [^{35}S]methionine. Detergent extracts were immunoprecipitated with mAb anti-SI antibodies. The immunoprecipitates were divided into equal parts and treated with endo H, endo F/GF or not treated. The proteins were submitted to SDS-PAGE on 6% slab gels followed by fluorography.
B. MDCK stably expressing pro-SI$_{\Delta MA}$ cells were biosynthetically labeled for 6 h with [^{35}S]methionine at 37° C. Cellular lysates and culture medium were immunoprecipitated with mAb anti-SI antibodies. Further treatment of the immunoprecipitates was as in A).
C. MDCK stably expressing pro-SI$_{\Delta MA}$ cells were biosynthetically labeled for 6 h with [^{35}S]methionine in the presence or absence of benzyl-GalNAc (denoted Benzyl). Secreted

pro-$SI_{\Delta MA}$ was immunoprecipitated from the culture medium and treated or not treated with endo H and endo F/GF. The samples were analyzed by SDS-PAGE and fluorography.

Fig. 2A shows that biosynthetic labeling of MDCK-SI_{WT} for 6 h revealed two polypeptides, an endo H-resistant and an endo H-sensitive band. In analogy with pro-SI isolated from human intestinal biopsy samples (18) and Caco-2 cells (25), these polypeptides correspond to the 210-kDa mannose-rich pro-SI (pro-SI_h) and the 245-kDa complex glycosylated pro-SI (pro-SI_c). Similar to pro-SI in intestinal cells (18), N-deglycosylation of pro-SI revealed two polypeptides, one which contains O-linked glycans (a 205-kDa polypeptide) and is derived from mature pro-SI_c and the other is derived from mannose-rich pro-SI_h and is devoid of O-glycosylation. A similar double band pattern was not revealed upon endo F/GF treatment of the pro-$SI_{\Delta ST}$ mutant from which the stalk region was truncated. Instead one slightly diffuse band of apparent molecular weight approximately similar to that of the N-deglycosylated mannose-rich species of pro-$SI_{\Delta ST}$ was discerned. Obviously a substantial reduction in the size of the O-glycans has occurred due to the deletion of the stalk region. On the other hand, N-linked glycosylation and maturation in the Golgi apparatus of pro-$SI_{\Delta ST}$ has occurred normally (see later Fig. 3). As shown in Fig. 2A pro- $SI_{\Delta ST}$ mutant revealed two biosynthetic forms during the same labeling period as wild type pro-SI. These polypeptides could be distinguished from each other when endo H treatment was performed. Similar to wild type pro-SI, a mannose-rich endo H-sensitive form and a mature endo H-resistant form could be discerned indicating that truncation of the stalk domain had no influence on the transport of pro-SI from the ER to the site of maturation in the Golgi apparatus. Another evidence for the drastic reduction in the amount of O-glycosylation due to the deletion of the stalk region is obtained when cells were biosynthetically labeled at 15°C. At this temperature transport of proteins is arrested in the ER preventing thus O-glycosylation which commences in the cis-Golgi (33). Under these conditions, pro-$SI_{\Delta ST}$ revealed a mannose-rich form which was N-deglycosylated with endo H or endo F/GF to polypeptides with similar apparent molecular weights. The diffused band pattern of the endo F/GF product of pro-$SI_{\Delta ST}$ at 37°C as compared to its counterpart at 15°C indicates that some O-glycosylation has occurred. Nevertheless, the extent of O-glycans is substantially less than that in wild type pro-SI. Together the results demonstrate that the stalk domain of pro-SI is the major site of O-glycosylation in the pro-SI-molecule.

Next the structural features and biosynthesis of the anchorless mutant pro-$SI_{\Delta MA}$ were investigated. This mutant was secreted into the medium in biosynthetically labeled MDCK cells indicating that deletion of the membrane anchoring domain had no consequences on the intracellular transport of pro-$SI_{\Delta MA}$ (Fig. 2B). The soluble pro-$SI_{\Delta MA}$ form had an apparent molecular weight of 240 kDa and is resistant to treatment with endo H indicating that it is complex glycosylated. The cellular form of pro-$SI_{\Delta MA}$ in the cell lysates revealed the

mannose-rich form which was sensitive to treatment with endo H. Endo F/GF treatment of secreted complex glycosylated pro-SI$_{\Delta MA}$ generated a polypeptide that was larger than the N-deglycosylated cellular counterpart indicating that the secreted form contained endo F/GF-resistant O-glycans. O-glycosylation of the pro-SI$_{\Delta MA}$ was further confirmed by treatment of cells with benzyl-GalNAc, a potent inhibitor of O-glycosylation. Fig. 2C demonstrates that pro-SI$_{\Delta MA}$ was transport-competent in the presence of benzyl-GalNAc and it was secreted into the medium. However, a marked shift in the apparent molecular weight was revealed as compared to the control pro-SI$_{\Delta MA}$ in the absence of benzyl-GalNAc indicating a decrease in the extent of O-linked glycosylation. This view was confirmed by N-deglycosylation of the secreted pro-SI$_{\Delta MA}$ glycorms with endo F/GF. The deglycosylated control pro-SI$_{\Delta MA}$ was larger than its counterpart from cells treated with benzyl-GalNAc. This demonstrates that the shift in the size of secreted pro-SI$_{\Delta MA}$ generated upon benzyl-GalNAc treatment is not due to N-linked glycosylation otherwise a similar product of N-deglycosylation would have been obtained and this was not the case.

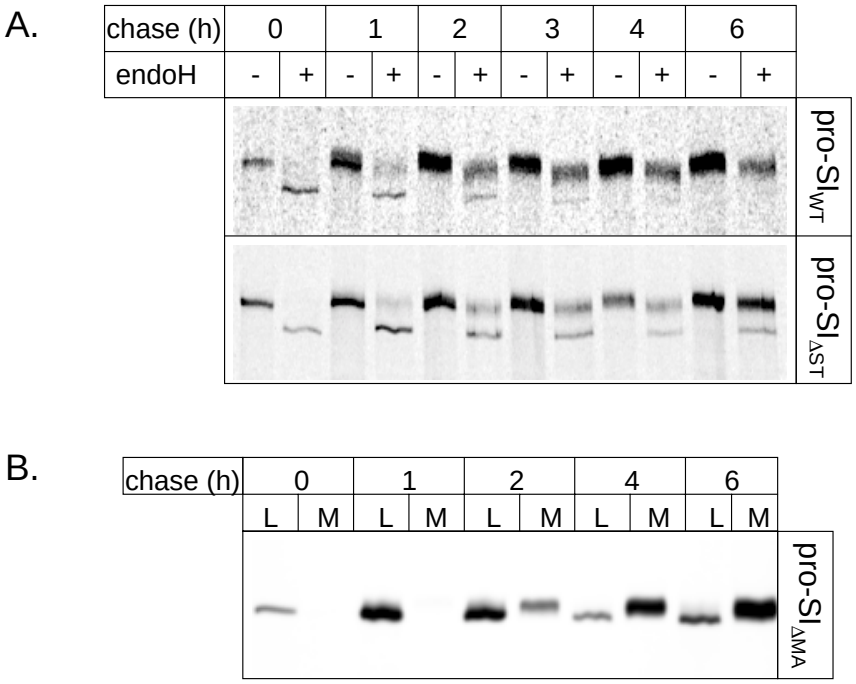


Fig. 3: Transport kinetics of wild type pro-SI, pro-SI$_{\Delta ST}$ and pro-SI$_{\Delta MA}$ in MDCK cells. *A*. MDCK cells stably expressing pro-SI and pro-SI$_{\Delta ST}$ were pulse labeled for 1 h with [^{35}S]methionine and chased for the indicated times with 2.5 mM unlabeled methionine. Wild

type pro-SI and pro-SI$_{\Delta ST}$ were immunoprecipitated from the cell lysates with mAb anti-SI and the immunoprecipitates were divided into equal parts, one of which was treated with endo H and the other was not treated. The samples were analyzed by SDS-PAGE on 6% gels and fluorography.

B. MDCK cells stably expressing pro-SI$_{\Delta MA}$ were subjected to pulse-chase labeling as in A). pro-SI$_{\Delta MA}$ was immunoprecipitated from the cell lysates and the medium and the immunoprecipitates were analyzed by DS-PAGE on 6% SDS-gel followed by fluorography.

The results shown above demonstrated that the pro-SI mutants were transport-competent. To determine, however, whether truncation of the stalk region and the transmembrane domains have affected the transport kinetics of these mutants as compared to wild type pro-SI, pulse-chase experiments with [^{35}S]methionine were performed. Fig. 3A shows that within 1 h of pulse, only the mannose-rich glycosylated forms of wild type pro-SI, pro-SI$_{\Delta MA}$ and pro-SI$_{\Delta ST}$ appeared which were sensitive to endo H treatment. After 1 h of chase endo H-resistant complex glycosylated forms of pro-SI$_{WT}$ and pro-SI$_{\Delta ST}$ could be detected, which became the predominant bands with increasing chase periods. The fact that complex glycosylated species were detected within the same period of chase indicates that wild type pro-SI and pro-SI$_{\Delta ST}$ have been transported to the Golgi apparatus at almost similar rates. However, complete processing of the mannose-rich species to the complex glycosylated form was achieved only with wild type pro-SI after 6 h of chase, while almost 15% pro-SI$_{\Delta ST}$ were still present in the mannose-rich form indicating that the processing kinetics of the pro-SI$_{\Delta ST}$ mutant are slightly slower than those of wild type. The transport kinetics of pro-SI$_{\Delta MA}$ were assessed by comparing the proportions of pro-SI$_{\Delta MA}$ in the cell lysates with those in the medium (Fig. 3B). A faint band corresponding to the complex glycosylated secreted form of pro-SI$_{\Delta MA}$ was found in the medium already after 1 h of chase and the intensity of this band increased substantially within the next chase time points. At 6 h of chase the secreted form constituted almost 90% of total pro-SI$_{\Delta MA}$. Here again, pro-SI$_{\Delta MA}$ is transported within the cell at almost similar rates as wild type pro-SI and comparable to the transport of pro-SI in the small intestine (18).

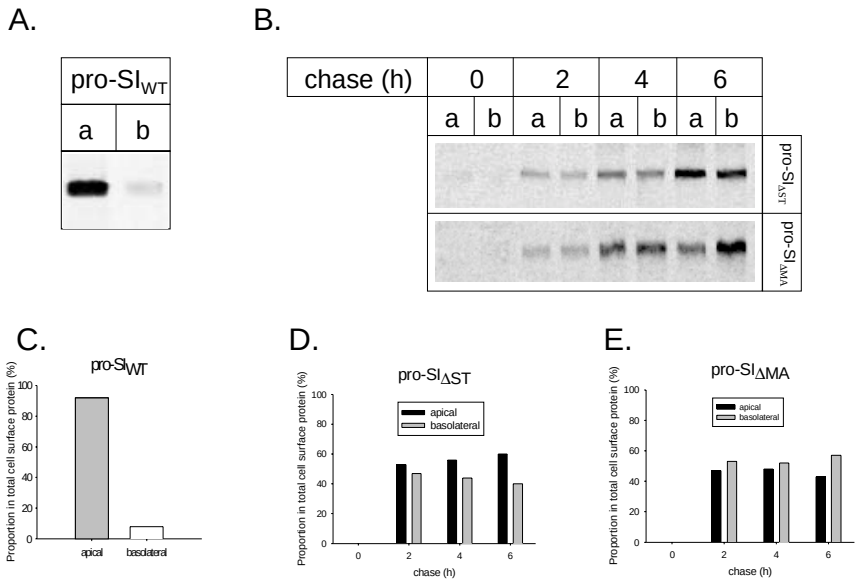


Fig. 4: Polarized delivery of wild type pro-SI, pro-SIΔ_{ST} and pro-SIΔ_{MA} to the cell surface of MDCK cells.

A. MDCK cells expressing wild type pro-SI were grown on filters and labeled with [^{35}S]methionine for 6 h. SI was immunoprecipitated either from the apical (a) or the basolateral (b) membrane and subjected to SDS-PAGE (6%) and fluorography.

B. MDCK cells expressing pro-SIΔ_{ST} or pro-SIΔ_{MA} were grown on filters, pulse-labeled with [^{35}S]methionine for 1 h and chased in medium containing 2.5 mM unlabeled methionine for the indicated times. pro-SIΔ_{ST} was immunoprecipitated from the apical or basolateral membranes. pro-SIΔ_{MA} is a secreted form and was immunoprecipiated from the culture media that were collected from the apical or basolateral compartments. Samples were analyzed by SDS-PAGE on 6% slab gels and fluorography.

C, *D* and *E*: The fluorogrammes in *A*) and *B*) were scanned and the proportions of wild type pro-SI (panel *C*), pro-SIΔ_{ST} (panel *D*) and pro-SIΔ_{MA} (panel *E*) in the apical or basolateral membranes (*C* and *D*) or media (*E*) were calculated.

Cell surface expression of wild type pro-SI, pro-SI$_{\Delta ST}$ and SI$_{\Delta MA}$ in MDCK cells – Next we wanted to determine how the deletions of the transmembrane domain and the stalk region have affected the polarized sorting of the pro-SI mutants. For this purpose, MDCK cells expressing wild type pro-SI and the mutants were grown on transparent polyester membranes in multiwell tissue culture plates, which allow separate access to both surface domains, the apical and the basolateral. The cells were labeled 5-8 days after confluency with [^{35}S]methionine for 6 h and cell surface immunoprecipitation of pro-SI was performed with mAb anti-SI. Fig. 4A shows that approximately 95% of pro-SI$_{WT}$ were immunoprecipitated from the apical membrane in line with previous data obtained in Caco-2 cells. By contrast,

deletion of the transmembrane domain was associated with a dramatic shift in the sorting behavior of the pro-SI mutants. In pulse-chase experiments pro-$SI_{\Delta ST}$ was immunoprecipitated from the apical and basolateral membranes at all chase time points. Scanning of the fluorogrammes revealed about 60% of pro-$SI_{\Delta ST}$ at the apical membrane as compared to 40% at the basolateral (Fig. 4D). These values did not change with increasing chase times demonstrating a random delivery of this mutant to the cell surface. The deletion of the 28 amino acid Ser/Thr-rich stalk region has therefore substantial effects on the polarized sorting of pro-$SI_{\Delta ST}$ but not on its transport-competence (see Fig. 3A). Consequently, the stalk domain plays an important role in apical targeting of pro-SI in MDCK cells.

We next asked whether the stalk domain *per se* is capable of targeting pro-$SI\Delta_{MA}$ to the apical membrane. MDCK cells expressing this form were grown on filters and subjected to a pulse-chase protocol. Since pro-$SI_{\Delta MA}$ is a secreted form, the media were collected from the apical and basolateral sides of the filter and immunoprecipitated with mAb anti-SI. At 2 h of chase pro-$SI\Delta_{ST}$ was almost equally secreted into both compartments. This pattern did not change with increasing chase times. Densitometric scanning demonstrated that almost 50% of pro-$SI_{\Delta MA}$ were secreted through either membrane (Fig. 4E). It is clear that the stalk-region of pro-SI is a necessary, but not a sufficient component of the apical sorting signal of pro-SI. To accomplish its task in directing pro-SI to the apical membrane it requires additionally the membrane anchoring domain.

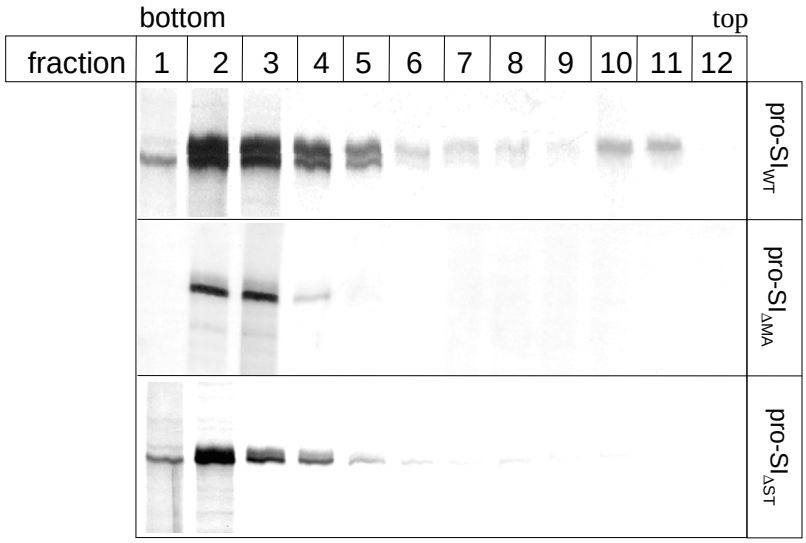


Fig. 5: Sucrose gradient centrifugation of Triton X-100 solubilized MDCK cells stably expressing wild type pro-SI, pro-$SI_{\Delta ST}$, pro-$SI_{\Delta MA}$.

MDCK cells expressing wild type pro-SI, pro-$SI_{\Delta ST}$, pro-$SI_{\Delta MA}$ were biosynthetically labeled for 4 h with [^{35}S]methionine. The cells were solubilized with Triton X-100 at 4°C and the detergent extracts were loaded on a 5-35% sucrose gradient as described (34). Each gradient

was divided into 12 fractions. Wild type pro-SI and the mutants pro-$SI_{\Delta ST}$ and pro-$SI_{\Delta MA}$ were immunoprecipitated and subjected to SDS-PAGE on 6% slab gels followed by fluorography.

pro-$SI_{\Delta ST}$ and pro-$SI_{\Delta MA}$ are not associated with lipid rafts in MDCK cells - Many membrane proteins with a glycolipid anchor and also some transmembrane proteins are transported to the apical surface of epithelial cells in association with detergent-insoluble membrane microdomains enriched in glycosphingolipids and cholesterol (DIGs), known as lipid rafts (3,10,15,34). Pro-SI belongs to this class of proteins that associate with lipid rafts through O-linked glycans prior to apical sorting. We wanted therefore to determine whether or not the deletion mutants are associated with lipid rafts. Here, pro-$SI_{\Delta ST}$ and pro-$SI_{\Delta MA}$ and wild type pro-SI were analyzed in sucrose gradients of Triton X-100 detergent extracts of biosynthetically-labeled cells. In line with previous data (19,35), Fig. 5 demonstrates that the complex glycosylated mature wild type pro-SI, but not the mannose-rich polypeptide, was associated with lipid rafts, since it was found in the floating fractions (9 and 10) at low buoyant density (10,36). By contrast to wild type pro-SI, the gradients corresponding to pro-$SI_{\Delta ST}$ and pro-$SI_{\Delta MA}$ did not contain pro-SI forms in the floating fractions indicating that the mutants are not associated with lipid rafts. The results indicate that association of pro-SI with membrane microdomains requires the presence of both protein domains, the stalk region and the membrane anchor.

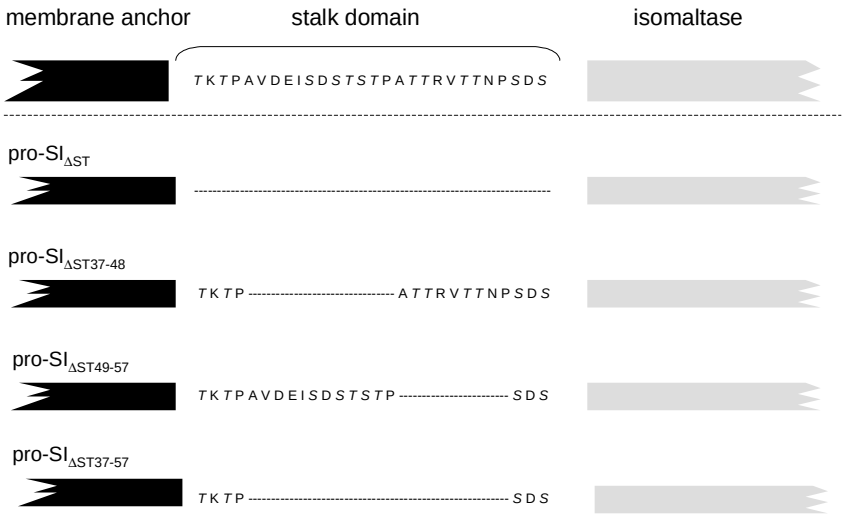


Fig. 6: Schematic representation of the deletion mutants, pro-$SI_{\Delta ST37-48}$, pro-$SI_{\Delta ST49-57}$ and pro-$SI_{\Delta ST37-57}$.

The orientation of the membrane anchor, the stalk domain and the start of the isomaltase subunit are indicated. The sequence of the stalk is shown in single letter code.

Characterization of a stretch within the stalk region that is required for sorting and association of pro-SI with lipid rafts – We wanted further to identify the sequences within the stalk region responsible for polarized targeting of pro-SI and its association with lipid rafts. For this purpose three different pro-SI mutants were generated from which short stretches within the stalk region were deleted. The mutant pro-$SI_{\Delta ST37-48}$ lacked 12 amino acids (Ala_{37}-Pro_{48}) from the N-terminal half of the stalk whereas 9 residues (Ala_{49}-Pro_{57}) at the C-terminal half were deleted in the pro-$SI_{\Delta ST49-57}$. Finally, the mutant pro-$SI_{\Delta ST37-57}$ comprised sequences around the center of the stalk domain (Fig. 6). These mutants were transiently transfected in MDCK cells followed by metabolic labeling and immunoprecipitation with mAb anti-SI. Each of the mutants was characterized by a double band, which corresponded to the mannose-rich and complex glycosylated forms as assessed by endo H treatment (Fig. 7).

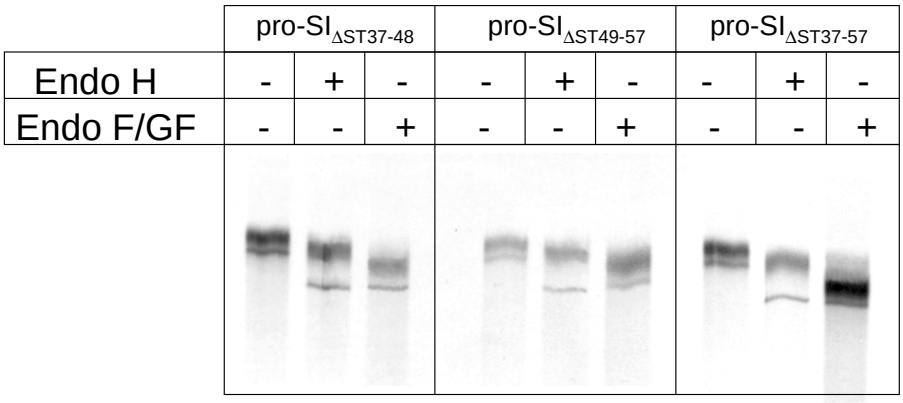


Fig. 7: Identification of molecular forms of pro-$SI_{\Delta ST37-48}$, pro-$SI_{\Delta ST49-57}$ and pro-$SI_{\Delta ST37-57}$ in transiently transfected MDCK cells.
MDCK cells were transfected with pSG8-$SI_{\Delta ST37-48}$, pSG8-$SI_{\Delta ST49-57}$ and pSG8-$SI_{\Delta ST37-57}$ and biosynthetically labeled for 6 h at 37°C with [^{35}S]methionine. Detergent extracts were immunoprecipitated and treated with endo H, endo F/GF or not treated as indicated for Fig. 2.

Deglycosylation of the mutants with endo F/GF revealed in each case two bands. In analogy with previous deglycosylation data (see Fig. 2) the upper bands contain endo F/GF resistant O-glycans. The higher apparent molecular weight of the upper bands of N-deglycosylated pro-$SI_{\Delta ST37-48}$ and pro-$SI_{\Delta ST49-57}$ as compared to that of pro-$SI_{\Delta ST37-57}$ suggests that the latter mutant is less O-glycosylated than the former two mutants. This is supported by the fact that 9 Ser/Thr putative O-glycan sites were deleted in this mutant, while pro-$SI_{\Delta ST37-48}$ and pro-$SI_{\Delta ST49-57}$ lack 5 and 4 potential O-glycosylation sites respectively. The comparable size of the O-glycosylated pro-$SI_{\Delta ST37-48}$ and pro-$SI_{\Delta ST49-57}$ glycofroms suggest that these mutants had no significant differences in the number of O-glycosyl sugar chains.

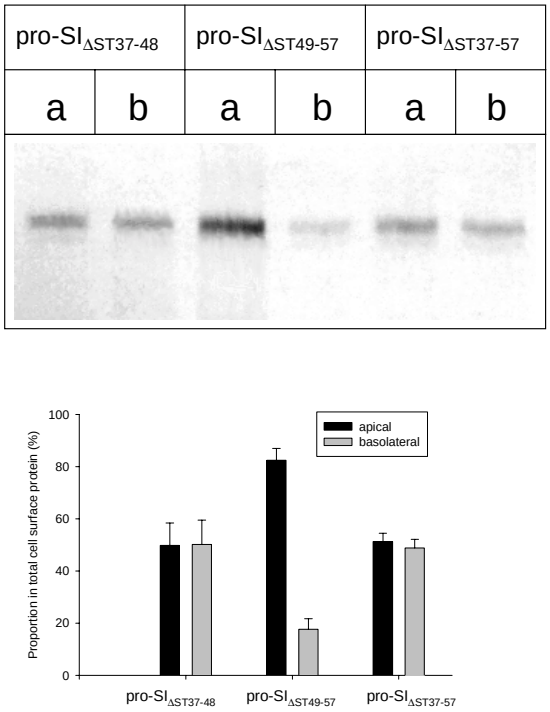


Fig. 8: Polarized delivery of pro-$SI_{\Delta ST37-48}$, pro-$SI_{\Delta ST49-57}$ and pro-$SI_{\Delta ST37-57}$ to the cell surface of MDCK cells.

A. Transiently transfected MDCK cells were grown on filters and labeled with [^{35}S]methionine for 6 h. SI was immunoprecipitated either from the apical (a) or the basolateral (b) membrane and subjected to SDS-PAGE (6%) and fluorography.

B. The fluorogrammes were scanned and the proportions of pro-SI in the apical or basolateral membranes were calculated.

Next the polarized transport of the mutants was investigated in transiently transfected MDCK cells that have been grown on membrane filters. Fig. 8 demonstrates, that pro-$SI_{\Delta ST49-57}$ was transported predominantly to the apical cell surface, whereas pro-$SI_{\Delta ST37-48}$ and pro-$SI_{\Delta ST37-57}$ are equally segregated to both membranes, the apical and basolateral. This demonstrates that only O-glycosylation of the membrane proximal half of the stalk encompassing the sequences Ala_{37}-Pro_{48} is absolutely required for apical delivery of pro-SI. In view of these findings it was necessary to examine the association of these mutants with membrane microdomains. Fig. 9 demonstrates that the pro-$SI_{\Delta ST49-57}$ mutant was found in the Triton X-100 insoluble floating fractions, while pro-$SI_{\Delta ST37-48}$ and pro-$SI_{\Delta ST37-57}$ were not detected in similar fractions of sucrose gradients.

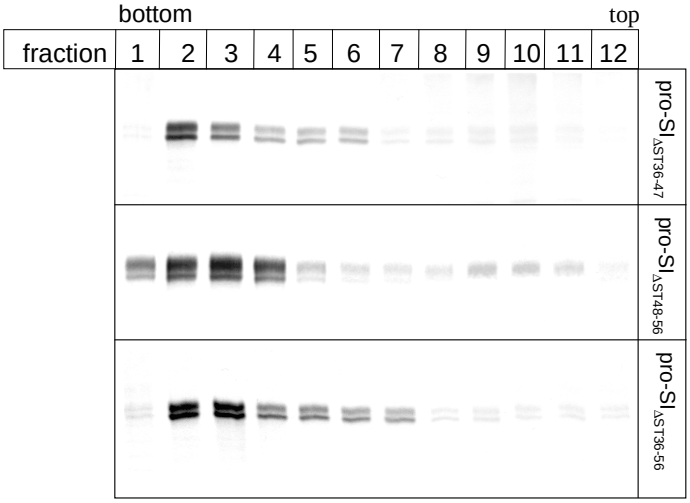


Fig. 9: Analysis of the association of wild type pro-SI and its mutants with lipid rafts.
MDCK cells transiently expressing pro-$SI_{\Delta ST37-48}$, pro-SI $_{\Delta ST49-57}$ and pro-SI $_{\Delta ST37-57}$ were biosynthetically labeled, solubilized and the extracts were loaded on a sucrose gradient as described for Fig. 5. From each gradient 12 fractions were immunoprecipitated with mAb anti-SI and subjected to SDS-PAGE followed by fluorography.

It is obvious therefore that association of pro-SI with membrane microdomains is the decisive step along the apical sorting of this protein. This association takes place through O-glycans located in the proximal half of pro-SI. O-glycosylation in the distal half of the stalk does not constitute an efficient signal for microdomain association and subsequently apical transport of pro-SI.

DISCUSSION

The high fidelity of sorting of pro-SI to the apical membrane is dramatically lost when O-glycosylation is affected resulting in random delivery of pro-SI to both membranes (19,37). Another highly O-glycosylated membrane protein, dipeptidyl peptidase IV, is also sorted by a default mechanism when O-glycosylation is affected (37). An impaired processing of the N-glycans of pro-SI and DPPIV, which are heavily N-glycosylated, is not necessary for correct sorting, provided that O-glycans are properly processed. These observations underline the key role played by O-glycans in the segregation mechanism of pro-SI (and also DPPIV) into vesicles destined for the apical plasma membrane. The question that arises is that of the structural determinants within pro-SI required for its high sorting fidelity. Is the presence of O-glycans alone sufficient for an efficient sorting and what is the location of the O-glycans involved in the sorting event? It has been always proposed, but never shown, that the stalk region of pro-SI is heavily O-glycosylated due to the presence of a Ser/Thr-rich domain

(18,19,37). Our data demonstrate that this is indeed the case, since deletion of this domain results in a significant reduction of O-glycosylation of pro-SI. The stalk region belongs structurally to the isomaltase subunit. Here we could demonstrate that the O-glycosylated stalk region of pro-SI plays a central role in the sorting event. The polarized transport of pro-$SI_{\Delta ST}$ in MDCK cells is dramatically altered upon deletion of this domain and this finding indicates that other O-glycans, most notably those located in the sucrase-subunit, do not constitute an essential part of the apical sorting signal. Importantly, the deletion of the stalk region neither affects the overall folding of pro-SI nor its transport-competence which occurs within the cell and to the cell surface, apical or basolateral, with wild type kinetics. This lends support to the notion that one important role of the stalk region in the overall structure of pro-SI is to serve as a link between the globular protein and the membrane. An association of O-glycans with lipid rafts has been demonstrated to be the driving sorting mechanism of pro-SI to the apical membrane (17,35) whereby this association is disrupted in the absence of O-glycans (19). The data presented here define structural determinants required for this association and demonstrate that O-glycans alone are neither sufficient for an association of pro-SI with lipid rafts nor for its apical targeting. A mutant that contains the O-glycosylated stalk domain, but lacks the membrane anchoring domain, is not detected in membrane microdomains and is secreted randomly from both sides of the membrane. It is important to note that this deletion does not affect the transport rate of pro-SI, since the mutant is transported intracellularly with wild type kinetics. Likewise, the membrane anchor alone without the O-linked glycans is not capable of promoting the interaction of pro-SI with lipid rafts and, as in the anchorless mutant, also here sorting by default takes place with normal transport kinetics. Obviously the two determinants, the O-glycosylated stalk region and the membrane anchor of pro-SI are not absolutely required for efficient transport of pro-SI intracellularly and to the cell surface in non-polarized fashion. Nevertheless, these structures are indispensable components of the sorting mechanism of pro-SI. It is clear, however, that the stalk region *per se* without or with impaired O-glycosylation does not constitute the sorting signal, since impaired or inhibited O-glycosylation of the stalk in full length pro-SI leads to random delivery of the molecule to both membranes.

Our data could further define a subdomain within the stalk region that is necessary and sufficient for the association of pro-SI with membrane microdomains and subsequent apical sorting. This domain comprises a stretch of 12 amino acids located juxtapose the membrane anchoring domain of pro-SI. In fact, a panel of deletion mutants of the stalk domain reveal that only a pro-$SI_{\Delta ST49\text{-}57}$ mutant containing this stretch is transported in a polarized fashion, while pro-$SI_{\Delta ST37\text{-}48}$ and pro-$SI_{\Delta ST49\text{-}57}$ are not. Importantly all these mutants are O-glycosylated, membrane anchored and transport-competent, but display different detergent extractabilities with Triton X-100. Here again, the apically-sorted pro-$SI_{\Delta ST49\text{-}57}$ is the only mutant that associates with membrane microdomains. A direct implication of these data is

that O-glycosylation *per se* is not sufficient for pro-SI to enter into an interaction with micordomains even in the presence of the membrane anchoring domain. It is clear that the signal for apical sorting and for micordomain association constitutes the membrane anchoring domain and the immediate upstream O-glycosylated stretch of the stalk region. Algorithmic analyses of the Ala_{37}-Pro_{48} subdomain reveal a high O-glycosylation potential for the quartet Ser_{44}-Thr_{45}-Ser_{46}-Thr_{47} suggesting the presence of a bulky O-glycan structure that could be efficiently recognized and more avidly bound by a putative sorting receptor. A similar motif is not present in the other deletion mutants in which the potential O-glycosylation sites are distributed over the sequence.

An example of an apically sorted protein that utilizes O-glycans as a sorting signal is the neurotrophin receptor ($p75^{NTR}$). In MDCK cells and in contrast to pro-SI, $p75^{NTR}$ does not require the membrane anchoring domain as an auxiliary component, since a secretory mutant of this protein that contains an O-glycosylated stalk region is correctly sorted (22). However, it is not known yet whether the sorting mechanism of $p75^{NTR}$ is similar to that of pro-SI and occurs through an interaction of O-linked glycans with lipid rafts. Apical sorting signals contained in the membrane anchoring domain have been described for the hemagglutinin of the influenza virus (HA), which associates with membrane microdomains prior to apical delivery (16). Mutations in the critical residues Gly_{520} and Ser_{521} of the membrane anchor reduce substantially the interaction of the mutants with lipid rafts with subsequent alteration in the apical sorting pattern (38). However, it is still unclear whether other determinants in HA, such as N-linked glycans, are primarily required as a recognition site before association of HA with microdomains takes place. In light of growing knowledge with endogenous and engineered apical proteins the consensus is now emerging that one major sorting mechanism to the apical membrane constitutes the interaction of proteins with membrane microdomains (15). How and when does this interaction ensue? The pro-SI model suggests that an interaction between O-glycans and a putative component in the TGN triggers the sorting events with lipid rafts marking the final step. Neither the presence of the membrane anchor nor an unglycosylated stalk region are sufficient for lipid rafts association and apical sorting. Until present only a few studies are known which allude to a putative role of O-linked glycans in apical sorting. These involve proteins of the brush border membrane and the neurotrophin receptor, $p75^{NTR}$ (22,37). For example, the sorting of the brush border proteins pro-SI, dipeptidyl peptidase IV (DPPIV) and $p75^{NTR}$ largely depends on the presence of O-glycosylated carbohydrates. By contrast, two other heavily O-glycosylated proteins, aminopeptidase N and lactase-phlorizin hydrolase are sorted to the apical membrane through non-glycan signals located in their ectodomains and do not associate with rafts before sorting (17,39). In the particular case of aminopeptidase N, it has been shown that the wild type protein associates with rafts (17). However, deletion of the potentially O-glycosylated stalk region and the membrane anchor of aminopeptidase N remains without marked effects on the

apical sorting of a secretory form of this protein (40,41) suggesting that the sorting of aminopeptidase N occurs through a mechanism independent of association with lipid rafts.
In view of the increasing body of data on the role of N- and O-linked glycosylation in the context of apical sorting, a comparison of the sorting pathways that utilize these two types of carbohydrates as recognition signals is worthwhile. For example, are the N- and O-linked glycan signals recognized by similar or different cellular elements and is recognition the primary step in a cascade of events. Of the few common structural features between N- and O-linked glycans are galactose and sialic acid residues. It is reasonable to assume that a common cellular, lectin-like protein binds these residues, whereby the binding capacity and the kinetics of this binding vary depending on the location of the carbohydrate residues within the protein and probably on the extent of glycosylation. Glycans found in the vicinity of the membrane are likely to interact more readily with a putative sorting factor than residues in more distal positions. O-glycans in the stalk region of pro-SI or in the heavily O-glycosylated DPPIV would conform to this pattern. Along this it is important to determine whether deletion of particular Ser/Thr residues in the stalk region of pro-SI is associated with reduction in the sorting fidelity. Drastic elevation in apical sorting of a glycosylated mutant of the growth hormone occurs when the number of N-glycosylated sites is increased (14) supporting the view that a higher level of glycosylation may be critical in the sorting event. However, the idea should not be excluded that the engineered N-linked glycans in the growth hormone do not constitute the apical signal *per se*, but are implicated in the generation of a particular epitope in the protein that acts as a signal. Such a putative role of glycosylation is unlikely to apply for the heavily O-glycosylated and rigid stalk region of pro-SI indicating that O-linked glycans act directly as an apical signal.

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Chapter 6

Temporal Association of the N- and O-linked Glycosylation Events and Their Implication in the Polarized Sorting of Intestinal Brush Border Sucrase-Isomaltase, Aminopeptidase N and Dipeptidyl peptidase IV

Abstract

The temporal association between O-glycosylation and processing of N-linked glycans in the Golgi apparatus as well as the implication of these events in the polarized sorting of three brush border proteins has been the subject of the current investigation. O-glycosylation of pro-sucrase-isomaltase (pro-SI), aminopeptidase N (ApN) and dipeptidyl peptidase IV (DPPIV) is drastically reduced when processing of the mannose-rich N-linked glycans is blocked by deoxymannojirimycin (dMM), an inhibitor of the Golgi-located mannosidase I. By contrast, O-glycosylation is not affected in the presence of swainsonine, an inhibitor of Golgi mannosidase II. The results indicate that removal of the outermost mannose residues by mannosidase I from the mannose-rich N-linked glycans is required before O-glycosylation can ensue. On the other hand, subsequent mannose residues in the core chain impose no sterical constraints on the progression of O-glycosylation. Reduction or modification of N- and O-glycosylation do not affect the transport of pro-SI, ApN or DPPIV to the cell surface per se. However, the polarized sorting of two of these proteins, pro-SI and DPPIV, to the apical membrane is substantially altered when O-glycans are not completely processed, while the sorting of ApN is not affected. The processing of N-linked glycans, on the other hand, has no influence on sorting of all three proteins. The results indicate that O-linked carbohydrates are at least a part of the sorting mechanism of pro-SI and DPPIV. The sorting of ApN implicates neither O-linked nor N-linked glycans and is driven most likely by carbohydrate-independent mechanisms.

INTRODUCTION

Glycosylation of membrane and secretory glycoproteins comprises a cascade of steps that implicate a number of oligo- and monosaccharide transferases and commences in the lumen of the endoplasmic reticulum (ER)[3] concomitant with protein translation and translocation (1-3). This initial glycosylation event, known as N-linked core glycosylation, involves the transfer of $Glc_3Man_9GlcNAc_2$ unit to acceptor asparagine residues in the tripeptide sequon Asn-Xaa-Ser/Thr and is catalyzed by the enzyme oligosaccharyltransferase (1-3). N-linked glycosylation is essential for the function, stability, folding, intracellular transport and secretion of glycoproteins (4-7). Upon arrival to the cis-Golgi network the initial mannose-rich core chains are trimmed by mannosidases I and II of the cis–Golgi. In the medial and trans-Golgi formation of complex type of glycans ensues and is terminated by the addition of sialic acid in the trans-Golgi network (TGN) (1-3, 8, 9).

Another glycosylation event of many, but not all, membrane and secretory glycoproteins is O-linked glycosylation at particular serine or threonine residues. In contrast to N-linked glycosylation, O-glycosylation is a posttranslational event and occurs in the cis-Golgi (4, 10, 11). A consensus acceptor sequence, as the tripeptide Asn-Xaa-Ser/Thr in N-linked glycosylation, does not exist. However, comparison of amino acid sequences around a large number of O-glycosylation sites of several glycoproteins revealed a significantly increased frequency of proline residues at positions -1 and +3 relative to the glycosylated residues and a marked increase of serine, threonine and alanine residues (12). It has been also suggested that stretches of serine and threonine residues in glycoproteins, such as in sucrase-isomaltase (SI, EC 3.2.1.48-10) (13), aminopeptidase N (ApN, EC 3.4.15.1) (14), glycophorin (15) or the LDL-receptor (16), are potential sites for O-glycosylation.

A role of O-glycosylation in the biological function of glycoproteins and in conferring stability and protection against proteolytic degradation has been proposed (16-18). A direct implication of O-glycosylation in the intracellular transport of proteins is unlikely, since acquisition of transport-competence implicates a cascade of steps that occur in the ER and O-glycosylation is a cis-Golgi event. Consistent with this is the finding that truncation of the highly O-glycosylated stalk region of ApN does not affect the transport and targeting of this enzyme (19).

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[3] The Abbreviations used are: ER, endoplasmic reticulum; TGN, trans-Golgi network; SI, sucrase-isomaltase (all forms); pro-SI, uncleaved sucrase-isomaltase; ApN, aminopeptidase N; LPH, lactase-phlorizin hydrolase; DPPIV, dipeptidyl peptidase IV; dMM, deoxymannojirimycin; mAb, monoclonal antibody; endo H, endoglyosidase H; endo F/GF, endoglycosidase F/N-glycopeptidase F; TFMS, trifluoromethanesulfonic acid; MDCK, Madin-Darby canine kidney cells; VIP36, 36kDa vesicular integral membrane protein

At first glance N- and O-linked glycosylation could be considered as independent events by virtue of the different structural requirements of the glycosylation sites, the temporal aspect - cotranslational versus posttranslational - and the different monosaccharide composition of the individual chains. Recent observations, however, suggested that at least an indirect association between the two events does exist. Here, inhibition of trimming of the mannose-rich chains of brush border lactase-phlorizin hydrolase (LPH, EC 3.2.1.23-62) (20) by deoxymannojirimycin (dMM), an inhibitor of cis-Golgi α-mannosidase I (21), was accompanied by generation of only N- rather than N- and O-glycosylated forms of LPH (17). In this very example it was proposed that conformational alterations in LPH due to persisting mannose-rich type of glycosylation in the presence of dMM may render potential O-glycosylation sites in LPH inaccessible to Golgi transferases. It is not clear, however, whether the observed effect with LPH may be generalized to other O-glycosylated proteins.

In an effort to analyse the role of carbohydrate modification on the sorting of the brush border hydrolases, pro-SI, ApN and dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5), we used inhibitors of trimming of carbohydrate chains in the cis-Golgi and surprisingly found that dMM has generated similar effects on three different proteins as those observed with LPH. The O-linked glycosylation of pro-SI, ApN and DPPIV was substantially affected. Moreover, the polarized sorting of SI and DPPIV, but not of ApN, was drastically altered from high sorting fidelity to the apical membrane to random distribution on both sides.

EXPERIMENTAL PROCEDURES

Biological material and cell lines - Human intestinal biopsies (5-10 mg) were obtained for routine diagnosis by suction with a pediatric Watson capsule and processed as described elsewhere (22). The colon carcinoma Caco-2 cells (23) were cultured in Dulbecco´s modified Eagle´s medium containing 0.45% glucose and supplemented with 20% heat-inactivated fetal calf serum, 1% nonessential amino acids and antibiotics (Life Technologies, Inc.).

Immunochemical Reagents - Monoclonal antibodies (mAbs) against human small intestinal brush border membrane hydrolases were a generous gift from Dr. H.-P. Hauri, Biocenter, Basel and Dr. E.E. Sterchi, University of Bern, Switzerland (24). The mouse mAb anti-ApN and anti-DPPIV were products of hybridomas HBB 3/775 and HBB 3/153 respectively. For immunoprecipitation of pro-SI a mixture of four different monoclonal antibodies was used (HBB 1/691, HBB 2/614, HBB 3/705, HBB 2/219).

Biosynthetic labeling - Intestinal biopsy specimens were either continuously labeled with [^{35}S]methionine or subjected to a pulse-chase protocol essentially as described elsewhere (18, 22). Caco-2 cells were cultured on membrane filters (Falcon) according to the procedure

described previously for LPH in MDCK cells (25) and were usually labeled at day 6 post confluency. When used, dMM, an inhibitor of mannosidase I (21) and swainsonine, an inhibitor of mannosidase II (26) were present in the culture medium during preincubation of the tissue or cells in methionine-deficient medium and during the pulse and chase periods essentially as described before (20). The final concentration of dMM was 5 mM and that of swainsonine was 4µg/ml (both reagents were obtained from Boehringer Mannheim, Mannheim). Immunoprecipitations and SDS-PAGE - The labeled biopsy specimens were homogenized with a Teflon-glass homogenizer in 1 ml of 25 mM Tris-HCl, pH 8.1, supplemented with 50 mM NaCl and protease-inhibitors (1mM PMSF, pepstatin (1µg/ml), leupeptin (5µg/ml), benzamidine (17,4µg/ml) and aprotinin (1µg/ml)) (all were from Sigma). Cell debris and nuclei were removed by centrifugation at 1000 g for 30 min. The supernatant was recovered and solubilized with 0,5% Triton X-100 and 0,5% sodium deoxycholate (final concentrations) by stirring on ice for 30 min. Caco-2 cells were lysed at 4° C for 1 hour in lysis buffer (25mM Tris-HCl, pH 8.0, 50 mM NaCl, 0,5% Triton X-100, 0,5% sodium deoxycholate and the mixture of protease inhibitors mentioned above). The solubilized cells or biopsy homogenates were spun for 1 hour at 100.000 g at 4° C and the supernatants were immunoprecipitated as described by Naim et al. (18, 22). SDS-PAGE was performed according to Laemmli (27) and the apparent molecular masses were assessed by comparison with high molecular mass markers (Bio-Rad Laboratories, Munich, Germany) run on the same gel. Cell surface immunoprecipitations of proteins from biosynthetically-labeled Caco-2 cells on membrane filters were performed by adding the appropriate antibody to either the apical or basolateral compartment. The cells were extensively washed to remove excessive antibody and lysed as described above. Protein A-Sepharose (Pharmacia) was finally added to capture the antigen-antibody complexes. Deglycosylation experiments - Treatments with endoglycosidase H (endo H) (Boehringer-Mannheim, Mannheim) and endogylcosidase F/N-glycopeptidase F (endo F/GF) (Sigma) were performed according to Naim et al. (22). For elimination of O-linked sugars, the immunoprecipitated glycoproteins were first N-deglycosylated with endo F/GF and the digestion products were precipitated with ice cold acetone. The protein pellets were subjected to treatment with trifluoromethanesulfonic acid (TFMS) that cleaves O-glycosidically linked sugar chains (28) as described (18).

RESULTS

O-glycosylation of aminopeptidase N (ApN) and dipeptidyl peptidase IV (DPPIV) - We have shown previously that pro-SI is an O-glycosylated molecule, whose O-glycosylation pattern is heterogenous and correlates with the position of the individual subunits, sucrase and isomaltase that comprize this enzyme complex (18). These data were obtained by employing a number of approaches, including enzymatic and chemical deglycosylation and lectin binding studies. Here, we extended these studies to ApN and DPPIV. First we demonstrate that ApN and DPPIV are O-glycosylated and that as in the case of pro-SI and LPH this glycosylation is also heterogenous.

Enzymatic and chemical deglycosylation of ApN and DPPIV –

1. ApN - Human ApN is synthesized in intestinal cells as a 130-kDa mannose-rich glycosylated precursor which matures with a half time of 30-40 min to a complex glycosylated species of apparent molecular weight of 160-kDa (24, 29). The biosynthesis and processing of ApN in other species has been also studied (30). The full length cDNA of the human enzyme has been cloned (14) and the amino acid sequence of the protein deduced from cDNA revealed a Ser/Thr-rich domain, which as in SI, may provide potential O-glycosylation sites. However, no direct evidence from biosynthetic or structural analyses has been sofar described.

We first assessed the O-glycosylation pattern by employing a strategy that combines enzymatic and chemical deglycosylation of precursor and mature forms of ApN and compared the apparent molecular weights of the deglycosylation products. First the glycoprotein was enzymatically deglycosylated of its N-linked sugars by using the combination endoglycosidase F/N-glycopeptidase F (endo F/GF), which removes complex and mannose-rich N-linked carbohydrates. Endo H, which cleaves mannose-rich glycans was used when mannose-rich glycoproteins were analysed. In either case, the resulting molecular form(s) were further treated with TFMS, a chemical reagent that cleaves O-glycosidic linkages (28). A further reduction in the apparent molecular weight of an enzymatically N-deglycosylated species provides an ample evidence for the presence of O-linked sugars in the treated protein. The results are shown in Fig. 1.

Immunoprecipitation of biopsy samples with mAb anti-ApN revealed a 130-kDa labeled protein after 30 min of chase. This band corresponded to the mannose-rich precursor of ApN (denoted ApN_h) as assessed by its sensitivity to treatment with endo H and its conversion to a 100-kDa polypeptide (Fig. 1A, lanes 1 and 2). A similar digestion product was obtained with endo F/GF (Fig. 1A, lane 3) supporting the endo H data that the 130-kDa polypeptide is N-glycosylated of the mannose-rich type. Treatment of the endo F/GF product with TFMS did not produce a further shift in the size of the 100-kDa band (Fig. 1A, lane 4) indicating that the 130-kDa protein is not O-glycosylated. After 4 h of chase a predominantly labeled endo H-resistant 160-kDa ApN band could be detected which corresponded to the complex glycosylated mature form of ApN (Fig. 1A, lanes 5 and 6) (this form will be referred to

throughout as ApN_c). When this immunoprecipitate was treated with endo F/GF a diffuse band pattern was obtained that, however, could be resolved into at least two bands (Fig. 1A, lane 7, see also Fig. 1B). Prolonged treatment of mature ApN_c with endo F/GF by using higher concentrations of the endoglycosidase did not eliminate the blurred band pattern (not shown) indicating the existence of endo F/GF-resistant oligosaccharides.

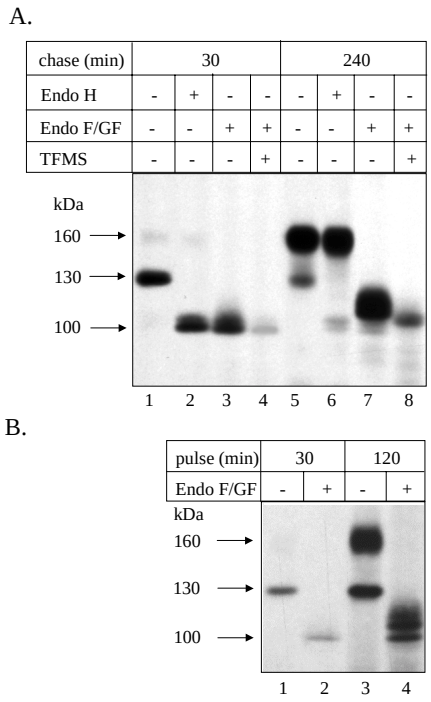

Fig. 1: N- and O-glycosylation of ApN

A. Biopsy speciemens were pulse labeled with [^{35}S]methionine for 15 min and chased for the indicated times with cold methionine. Detergent extracts of the labeled tissues were immunoprecipitated with mAb anti-ApN. The immunoprecipitates were treated or not treated with endo H, endo F/GF, and endo F/GF followed by TFMS. The proteins were subjected to SDS-PAGE on 6% slab gels and fluorography.

B. Biopsy specimens were continuously labeled with [^{35}S]methionine for 30 min or 120 min followed by solublization and immunoprecipitation with mAb anti-ApN. The isolated proteins were treated or not treated with endo F/GF and subjected to SDS-PAGE on 6% slab gels and fluorography.

Treatment with TFMS eliminated the band heterogeneity and generated one single band, which was slightly smaller than the lowest periphery of the diffuse band pattern. Due to the specificity of TFMS in cleaving O-glycosidic bonds the result demonstrates the existence of

O-linked glycans in mature ApN_c. Fig. 1B provides a clearer evidence for the existence of several products of N-deglycosylated ApN and compares further in the same experimental sample the endo F/GF-products of the 130-kDa mannose-rich ApN_h and the 160-kDa complex glycosylated ApN_c species (Fig. 1B, lane 4). In this experiment, ApN was immunoprecipitated from a biopsy sample that was continuously labeled for 2 h, during which time the proportion of ApN_h relative to ApN_c is still high enough allowing thus a direct comparison of the N-deglycosylated forms of the two species in the same experimental sample (Fig. 1B, lane 3). Mannose-rich ApN_h immunoprecipitated from a biopsy sample labeled for 30 min served as a control (Fig. 1B, lane 1). Endo F/GF-treated ApN from the 2 h-labeled biopsy sample revealed three major bands (Fig. 1B, lane 4). The lowest of these is the N-deglycosylation product of the 130-kDa, since a similar product was obtained upon deglycosylation of the 130-kDa ApN_h alone (compare with lane 2). The other two larger products corresponded hence to the endo F/GF-treated 160-kDa ApN_c species. The smaller size of the N-deglycosylated mannose-rich 130-kDa ApN_h (lanes 2 and 4) as compared to the N-deglycosylated mature 160-kDa ApN_c provides another evidence for the existence of sugar residues in mature ApN other than the N-linked ones.

2. DPPIV - The earliest detectable form of DPPIV synthesized in intestinal epithelial cells is the mannose-rich precursor of apparent molecular weight around 100-kDa ($DPPIV_h$) (Fig. 2, lane 1 and see ref. 24, 31). This form is rapidly converted to a complex glycosylated mature form ($DPPIV_c$) (Fig. 2, lane 5) with a half time of 15-25 min (24, 31). Mature $DDPIV_c$ appears as a diffuse band of an average apparent molecular weight of 124-kDa (this molecular weight will be referred to throughout). Deglycosylation of the $DPPIV_h$ mannose-rich polypeptide with endo H and endo F/GF revealed essentially similar molecular species of an apparent molecular weight around 85 kDa (Fig. 2, lanes 2 and 3). TFMS treatment of the N-deglycosylated $DPPIV_h$ did not generate a further shift in its size indicating that the 85-kDa species is devoid of O-glycosidically linked glycans. By contrast, the mature 124-kDa $DPPIV_c$ form obtained within 4 h of chase (Fig. 2, lane 5) is heavily O-glycosylated as assessed by the combined endo F/GF and TFMS treatments. N-deglycosylation generated a diffuse band of an apparent molecular weight ranging between 90-kDa and 110-kDa (Fig. 2, lane 7). TFMS treatment converted this heterogenous form to a band of an apparent molecular weight smaller than the lowest band periphery of the N-deglycosylated product (Fig. 2, lane 8 and compare this band with the band in lane 7). The result demonstrates that the N-deglycosylated protein contains O-glycosidically linked oligosaccharides, which have been eliminated by TFMS.

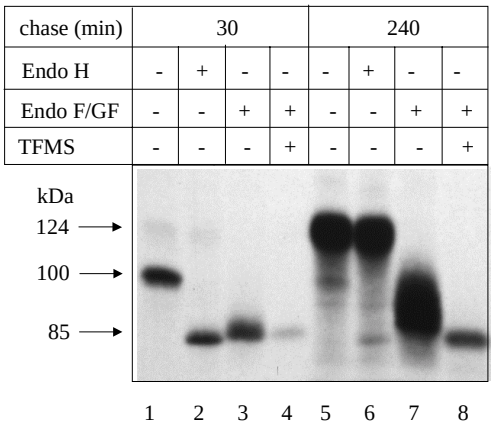

Fig. 2: N- and O-glycosylation of DPPIV
Biosynthetic labeling, immunoprecipitation and deglycosylations were perforemd as described in Fig. 1A, except that mAb anti-DPPIV was used in the immunoprecipitations instead of mAb anti-ApN.

Altogether, the chemical and enzymatic treatments provide an evidence for O-glycosylation of ApN and DPPIV. Further, the observed multiple and heterogenous band patterns in N-deglycosylated ApN and DPPIV suggest the presence of several populations of differently O-glycosylated species of these glycoproteins, which could be converted to one unglycosylated species upon TFMS treatment. The existence of several populations of N- and O-glycosylated glycoproteins has been also reported for two other brush border enzymes, LPH (17, 20) and SI (18). In all these cases a possible association between the differentiation state of intestinal cells and the various glycoforms is favored.

Association between processing of mannose-rich N-linked carbohydrates and O-glycosylation- We have demonstrated before that impaired processing of cotranslationally added mannose-rich N-linked sugars in the cis- and medial Golgi by inhibitors of mannosidases I and II is associated with drastic reduction, if not complete blocking, of the O-linked glycosylation of human intestinal LPH (20). A possible conformational interference of unprocessed N-linked sugar chains with potential O-glycosylation sites lying in their vicinity was proposed. This has insighted us to further examine other heavily O-glycosylated proteins, such as ApN, DPPIV and pro-SI with the ultimate goal of analysing the role of N-linked and more importantly O-linked glycosylation on the sorting behavior of proteins in polarized cells. We studied therefore the effects of deoxymannojirimycin (dMM), an inhibitor of mannosidase I (21), and swainsonine, an inhibitor of mannosidase II (26), on the processing of pro-SI, ApN and DPPIV in intestinal cells. The results are depicted in Fig. 3 and a survey of the glycoforms of the proteins analysed is provided in Table 1.

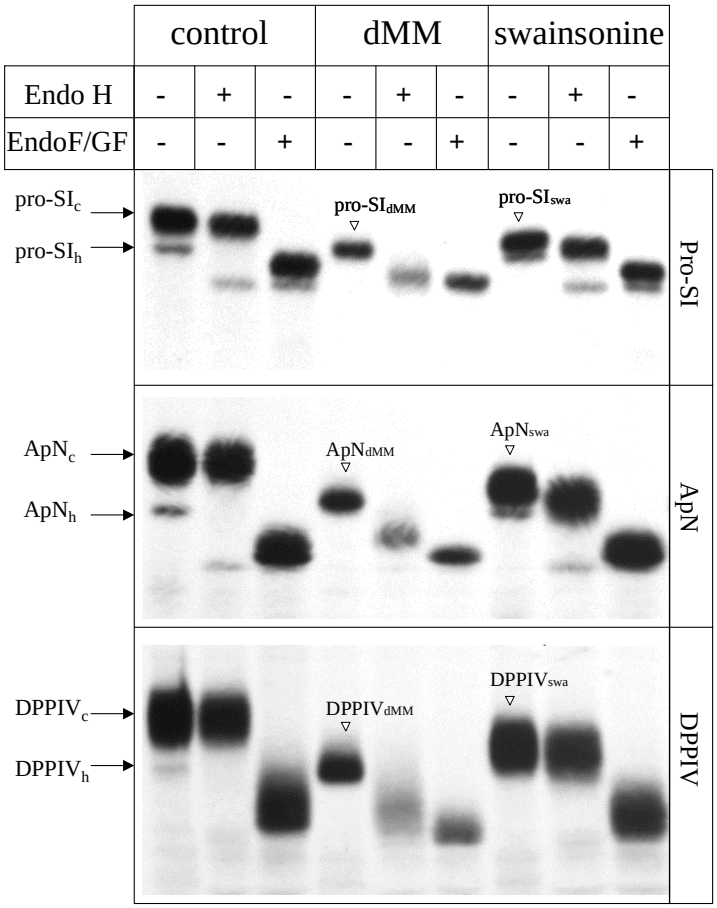

Fig. 3: Effects of dMM and swainsonine on the glycosylation patterns of pro-SI, ApN and DPPIV.

Biopsy samples were biosynthetically labeled with [^{35}S]methionine for 6 h in the presence or absence of 5 mM dMM and 4µg/ml swainsonine. Detergent extracts of the labeled tissues were immunoprecipitated with mAbs directed against pro-SI (upper panel), ApN (middle panel) and DPPIV (lower panel). Part of the immunoprecipitates was treated with endo H, another part with endo F/GF and one part was untreated. Treated and non-treated samples were subjected to SDS-PAGE on 5% (pro-SI), and 6% (ApN and DPPIV) followed by fluorography.

Effects of dMM on pro-SI, ApN and DPPIV - Fig. 3 shows the molecular forms of pro-SI, ApN and DPPIV immunoprecipitated from a normal intestinal biopsy specimen that has been labeled for 6 h. In each case, a mannose-rich and a complex glycosylated polypeptides were detected, as has been shown previously for pro-SI (18) and above for ApN and DPPIV. For pro-SI these forms are denoted pro-SI$_h$ (mannose-rich 210-kDa) and pro-SI$_c$ (complex glycosylated 245-kDa) (18). For ApN and DPPIV these forms have been described above (ApN$_h$, ApN$_c$ and DPPIV$_h$ and DPPIV$_c$; see Figs. 1 and 2). Treatment of the biopsy specimen

with 5 mM of dMM during the labeling period has resulted in the generation of single glycosylated forms of pro-SI (pro-SI_{dMM}), ApN (ApN_{dMM}) and DPPIV ($DPPIV_{dMM}$). Pro-SI_{dMM} and ApN_{dMM} had apparent molecular weights almost similar to their mannose-rich pro-SI_h and ApN_h counterparts respectively. The glycoform of DPPIV was a diffuse band of an average apparent molecular around 100-kDa (denoted $DDPIV_{dMM}$) and is therefore significantly smaller than complex glycosylated $DPPIV_c$ (124-kDa). All three glycoforms were sensitive to endo H indicating their predominant mannose-rich type glycosylation. The endo H-resistant proportions of the sugar content in pro-SI_{dMM}, ApN_{dMM} and $DPPIV_{dMM}$ were eliminated by endo F/GF as assessed by the shift in the apparent molecular weight upon treatment with the endoglycosidase (in the case of pro-SI_{dMM} this shift was slight). N-deglycoylation of pro-SI_{dMM} and ApN_{dMM} revealed in each case a single band pattern that corresponded to the lowest band of the endo F/GF-forms of the control glycoforms pro-SI_c and ApN_c. For $DPPIV_{dMM}$, endo F/GF treatment resulted in the generation of a substantially less diffuse band than that of $DPPIV_c$ and which had also a reduced apparent molecular weight. Since the endo F/GF-forms of pro-SI_c, ApN_c and $DPPIV_c$, which are O-glycosylated (18, and see above), are substantially larger than those of their pro-SI_{dMM}, ApN_{dMM}, and $DPPIV_{dMM}$ counterparts our results demonstrate a drastic reduction in O-linked glycosylation of pro-SI, ApN and DPPIV in the presence of dMM.

Table I. Glycoforms of the brush border proteins, pro-SI, ApN and DPPIV

Protein	mannose-rich	complex	dMM	swainsonine
pro-SI	pro-SI_h 210-kDa	pro-SI_c 245-kDa	pro-SI_{dMM} ~210-kDa	pro-SI_{swa} ~235-kDa
ApN	ApN_h 130-kDa	ApN_c 160-kDa	ApN_{dMM} ~130-kDa	ApN_{swa} ~145-kDa
DPPIV	$DPPIV_h$ 100-kDa	$DPPIV_c$ 124-kDa	$DPPIV_{dMM}$ ~105-kDa	$DPPIV_{swa}$ ~115-kDa

Effects of swainsonine on pro-SI, ApN and DPPIV - The same type of analysis was performed with swainsonine. A marked effect on the glycosylation patterns of pro-SI, ApN and DPPIV was obtained with this inhibitor albeit to a lesser extent than that revealed with dMM (Fig. 3). Two glycoforms of pro-SI and ApN were revealed, the mannose-rich pro-SI_h and ApN_h and the slightly endo H-sensitive species, a 235-kDa pro-SI (denoted thereafter pro-SI_{swa}) and a 145-kDa ApN (denoted ApN_{swa}). In the case of DPPIV a diffuse band was obtained, the lower periphery of which run at the same apparent molecular weight as

mannose-rich $DPPIV_h$. $DPPIV_{swa}$ was also slightly sensitive to endo H and had an apparent molecular weight of approximately 115-kDa (denoted $DPPIV_{swa}$). That all three glycoforms were only partially sensitivite to endo H is indicative of a processing of the mannose-rich N-linked glycans to complex type. However, the marked difference in the apparent molecular weight of pro-SI_{swa}, ApN_{swa} and $DPPIV_{swa}$ as compared to the corresponding control complex glycosylated pro-SI_c, ApN_c and $DPPIV_c$ indicates that complete processing of the N-linked glycan units has not occurred. Treatment of the 235-kDa pro-SI_{swa}, the 145-kDa ApN_{swa} and the 115-kDa $DPPIV_{swa}$ with endo F/GF generated N-deglycosylation products similar in size to the completely N-deglycosylated products of wild type pro-SI_c, ApN_c and $DPPIV_c$ and these products are O-glycosylated (18 and see above). The result indicates therefore that, in contrast to dMM, swainsonine treatment of intestinal cells had no effect on the processing of O-linked chains in pro-SI, ApN and DPPIV.

In summary, the data shown here with three different proteins demonstrate that inhibition of mannose-rich chains processing by dMM has induced substantial effects on the O-linked glycosylation, while swainsonine effects are restricted to N-linked glycosylation. These results are supported by the data obtained with LPH (20).

Effects of variations in the O- and N-linked glycosylation patterns on the polarized sorting of pro-SI, ApN and DPPIV – Pro-SI, ApN and DPPIV are apically sorted proteins in intestinal cells. In Caco-2 cells pro-SI and ApN follow predominantly the direct sorting pathway to the apical membrane, while DPPIV follows both the direct and the transcytotic pathways (32). The significant effects of dMM on the O-glycosylation and swainsonine on the N-linked complex glycosylation patterns of pro-SI, ApN and DPPIV have insighted us to investigate these events in the context of polarized sorting of these proteins.

For this purpose, we performed cell surface immunoprecipitations of pro-SI, ApN and DPPIV using Caco-2 cells that have been cultured on membrane filters and biosynthetically labeled for 6 h in the presence or absence of dMM and swainsonine. Immunoprecipitation of pro-SI, ApN and DPPIV was performed with antibodies added to either the apical or basolateral compartments. As expected (32), Fig. 4A shows that pro-SI_c, ApN_c and $DPPIV_c$ purified from non-treated Caco-2 cells were mainly immunoprecipitated from the apical surface (left panels). However, the sorting behavior of the corresponding glycoforms of these proteins generated in the presence of dMM was different. While the sorting of ApN was not affected, the dMM-glycoforms of pro-SI and DPPIV were found on both sides of the cell, the apical and basolateral (Fig. 4A, middle vertical panels).

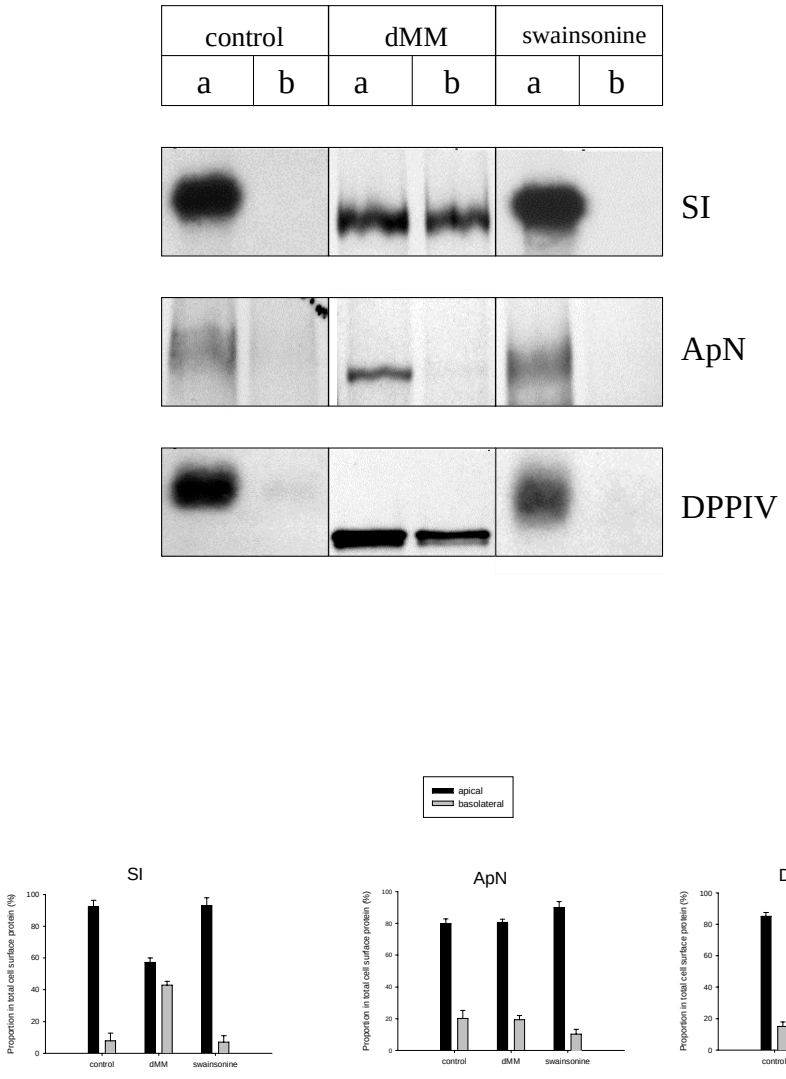

Fig. 4: Polarized sorting of pro-SI, ApN and DPPIV in the presence or absence of dMM and swainsonine.

Caco-2 cells were cultured on membrane filters and labeled 6 days post confluency with [^{35}S]methionine for 6 h in the presence of 5 mM dMM (middle vertical panels) or 4µg/ml swainsonine (right vertical panels) or in their absence (left vertical panels). Cell surface immunoprecipitations were performed by adding mAbs directed against pro-SI (upper horizontal panel), ApN (middle horizontal panel) and DPPIV (lower horizontal panel) to the apical (denoted a) or basolateral (denoted b) compartments. The immunoprecipitates were then analysed by SDS-PAGE on 5% (pro-SI) and 6% (ApN and DPPIV) slab gels followed by fluorography. a = apical surface; b = basolateral surface.

B. The proportions of pro-SI, ApN and DPPIV appearing at the apical and basolateral membranes in the presence or absence of dMM and swainsonine were calculated from densitometric scans of the fluorogram shown in A) and those of two more experiments.

Almost 42% of pro-SI_{dMM} was found at the basolateral membrane as compared to 7% of pro-SI_c and the delivery of DPPIV to the basolateral membrane shifted from 8% to almost 40% in the presence of dMM (Fig. 4B). This indicates that elimination or reduction of O-glycosylation has resulted in abolishment of the high fidelity of polarized sorting of pro-SI_{dMM} and $DPPIV_{dMM}$ to the apical surface. On the other hand, modulation of N-linked glycosylation in the presence of swainsonine remained without effect on the sorting behavior of pro-SI_{swa}, ApN_{swa} and $DPPIV_{swa}$, since these glyoforms were predominantly transported to the apical membrane as their wild type counterparts (Figs. 4A, right panels and 4B). The results clearly demonstrate that alterations in the O-glycosylation patterns of pro-SI and DPPIV had strong influence on their polarized sorting in epithelial cells, while modification of N-linked glycosylation in the Golgi apparatus is not an essential event in the sorting process. The sorting of ApN is neither affected by O-linked nor N-linked glycosylation.

DISCUSSION

N- and O-Glycosylations exert various fundamental roles that relate to biological function, processing, folding, trafficking and sorting of membrane and secertory proteins. A large number of these proteins is both N-glycosylated at particular Asn residues and O-glycosylated at either Ser or Thr residues (1-4). These glycosylation types are the outcome of distinct temporal events, and are structurally different (3, 4, 10-12). A direct or an indirect linkage between these processes has not been so far demonstrated, except for brush border LPH (20). Here it could be demonstrated that O-glycosylation does not proceed to completion, or is perhaps blocked, as a result of impaired processing or complete inhibition of the processing of the first mannose residues in the cis-Golgi by dMM, an inhibitor of cis-Golgi α-mannosidase I (21). This rather unexpected finding appeared initially to represent a special case of LPH. We postulated then that the unprocessed mannose-rich chains in the presence of dMM may sterically hinder the addition of glycan chains to neighbouring potential O-glycosylation Ser or Thr residues. The present paper, however, clearly documents that this phenomenon is not restricted to LPH alone. Three structurally and functionally different proteins of the brush border membrane have been examined with inhibitors of the processing of mannose-rich chains. In all these cases inhibition by dMM of the processing of the first four mannose residues of the mannose-rich sugar chains results in a substantial impairment of O-glycosylation. How do these results accommodate with the role of glycosylation in general and with our knowledge of the structure of the analysed brush border proteins in particular?

All the proteins analysed are extensively N- and O-glycosylated. The potential N-glycosylation sites vary between sixteen for pro-SI (1827 amino acids) (13, 18) and LPH (1927 amino acids) (34), to ten for ApN (966 amino acids) (14) and nine for DPPIV (766

amino acids) (35) and are almost evenly spread all over the ectodomains of these proteins. Many of the potential O-glycosylation sites are found in stalked regions, which are located in close proximity to the membranes and also among potential N-glycosylation sites (13, 14, 19, 34, 35). By virtue of the large number of N-linked glycosylation sites it is likely that a particular structure of the mannose-rich N-linked glycans, which exist earlier in the biosynthesis than O-glycosidically linked sugars, elicit specific constraints on the processing of the O-linked sites. The results obtained with two different inhibitors of mannosidases I and II of the cis-Golgi compartment strongly suggest that the accessibility of potential O-glycosylation sites to galactosyl- or N-acetyl-galactoseaminyl-transferases requires a reduced number of mannose residues of neighbouring N-linked chains. A completely unprocessed N-linked mannose-rich chain in the presence of dMM may sterically reduce or hinder the glycosylation of a neighboring potential O-glycan site. Processing of the mannose-rich chains by mannosidase I appears to be sufficient for O-glycosylation to ensue, since inhibition of mannosidase II and subsequently further processing steps by swainsonine remains without marked effects on normal O-glycosylation of the four brush border proteins. Since the drastic impairment in O-linked glycosylation is only observed when mannosidase I, but not mannosidase II, is inhibited, our data suggest that the outermost four mannose residues cleaved by mannosidase I (21) are critically important or may be rate-limiting in determining the extent of O-glycosylation. Furthermore, the results suggest that O-glycosylation does not commence before processing of the mannose-rich chains by mannosidase I is achieved.

One main aspect of the present work is that related to the implication of glycosylation, in particular O-glycosylation in the trafficking and polarized sorting of the brush border proteins pro-SI, ApN and DPPIV. The observation that O-glycosylation could be substantially affected in the presence of dMM, i.e. when particular mannose residues are not cleaved, opens up the possibility to investigate in a simple and straightforward set up the role of O-glycosylation in sorting. Recent studies have demonstrated that carbohydrates, N- or O-linked, carry sorting information to the apical membrane (36-38). However, a general concept on the significance of the glycosylation events in polarized sorting cannot be drawn, in particular since only a few studies have addressed the role of N- and O-glycosylation of proteins naturally-occurring in epithelial cells. In addition, it is not obvious how glycosylation correlates with sorting and what carbohydrate structures are implicated in the sorting event. It is clear that modifications in the N-linked carbohydrate structure of glycoproteins from mannose-rich to complex type precede polarized sorting in the trans-Golgi network. Also many proteins acquire O-linked glycosylation prior to transport to the TGN. Should carbohydrates play a role in the sorting at all, one may expect that the complex type as well as O-glycosylated may be involved. Our paper shows that modification of the N-linked glycosylation is not effective in the context of apical sorting of a number of heavily N-glycosylated brush border enzymes. By contrast, elimination or reduction of O-glycan units in the presence of dMM affects the sorting

behavior drastically. In their predominantly mannose-rich type of glycosylation, pro-SI_{dMM} and $DPPIV_{dMM}$ follow a random pattern of delivery to the apical and basolateral membranes. It is obvious that O-linked glycans are directly implicated in apical sorting. On the other hand, the sorting signal of ApN appears not to involve sugar chains, since ApN_{dMM} and ApN_{swa} are delivered with similar fidelity to the apical membrane as their fully glycosylated wild type ApN_c counterpart. This result supports data, in which deletion of the potentially O-glycosylated Ser/Thr-rich stalk domain of ApN has no effects on the sorting behavior of the mutant (19) and indicates that sorting motifs other than O-linked glycans are resposnible for apical targetting of ApN. The strong role of O-glycans in the sorting of pro-SI and DPPIV does not exclude, however, that the sorting of these proteins may implicate additional structural motifs that may function to tune up the apical sorting of these proteins. This may explain why a slightly more apical than basolateral delivery of pro-SI_{dMM} and $DPPIV_{dMM}$ still occurs despite the removal of O-glycans. One possible mechanism of apical sorting has been described for the pig species of pro-SI, ApN and DPPIV (39) which involves association of these proteins with glycolipid microdomains or rafts. If O-glycosylation is implicated in this mechanism, then at least the apical delivery of ApN would implicate different sorting signals and mechanism, since ApN_{dMM}, which is largely devoid of O-linked glycans, is targetted correctly to the apical membrane.

Unlike basolateral sorting, apical sorting appear to implicate signals of different types, structure and location emphasizing the diversity of mechanisms responsible for this process. It has been proposed that the heavily O-glycosylated stalked region of the neurotrophin receptor is implicated in its sorting to the apical membrane, since deletion of this region leads to a mistargetting of the mutant protein to the basolateral membrane and suggesting the existence of a suppressed basolateral signal in this protein (38). A similar situation does not prevail with pro-SI_{dMM} and $DPPIV_{dMM}$ suggesting that except for apical sorting signals no other motifs are present in these proteins. Due to the presence of GalNAc residues in O-linked carbohydrates, these chains could be considered as an exquisite candidate for binding specific sorting elements. The leguminous lectin-like VIP 36 protein, for example, is a resident of the Golgi apparatus that has been located to glycolipid rafts and is presumably involved in the sorting of some GPI-anchored proteins to the apical membrane (40).

Our results do not exclude a partial O-glycosylation occuring at Ser or Thr residues in SI, ApN and DPPIV in the presence of dMM. The slight differences in the electrophoretic patterns obtained upon endo F/GF treatment of $DPPIV_{dMM}$ as compared to DPPIV (and to a lesser extent those of SI_{dMM} and ApN_{dMM}) suggest that some O-glycosylation still occurs. Nevertheless, an interaction with potential sorting elements, may not be as avid as with completely processed O-chains to ensure a high sorting fidelity.

While O-glycans are strongly implicated in apical sorting of pro-SI and DDPIV, partial modification of the N-glycan units to a hybrid type in the presence of swainsonine remains

without significant effect on the sorting of these proteins. This is direct evidence that N-linked complex type of glycosylation per se is not an essential factor for correct sorting to the apical membrane. Moreover, it is not clear and also experimentally not established yet, how N-linked mannose-rich residues would mediate polarized sorting. Particulary since many proteins are targetted to the apical membrane regardless of their N-glycosylation pattern, such as those shown in this report. One possible explanation is that these residues participate in early sorting processes between the ER and Golgi, for instance via binding leguminous lectin-like proteins, such as ERGIC-53 (41-43), whereby later sorting of these proteins from proteins with strong basolateral signals constitutes an indirect discriminatory process. This mechanism may apply only to a limited class with no or preferentially weak apical sorting signals. Meanwhile the sorting of many apical proteins, most notably those of the intestinal brush border membrane, appears to take place via specific sorting elements (19, 25, 44-46). Perhaps the most important role of the N-linked glycans in these proteins is to achieve correct folding (4, 5) and presumably to impose and stabilize particular secondary structures that may act as sorting motifs and interact with sorting receptors (47). Since the initial and most critical folding of glycoproteins occurs in the ER, the type of N-glycosylation, mannose-rich or complex, plays an indirect role, if any, in the sorting event as our results clearly demonstrate.

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Chapter 7

Hierarchy of Sorting Signals in Chimeras of Intestinal Lactase-Phlorizin Hydrolase and the Influenza Virus Hemagglutinin

Abstract

Lactase-phlorizin hydrolase (LPH) is an apical protein in intestinal cells. The location of sorting signals in LPH was investigated by preparing a series of mutants that lacked the LPH cytoplasmic domain or had the cytoplasmic domain of LPH replaced by sequences which comprised basolateral targeting signals and overlapping internalization signals of various potency. These signals are mutants of the cytoplasmic domain of the influenza hemagglutinin (HA), which have been shown to be dominant in targeting HA to the basolateral membrane. The LPH-HA chimeras were expressed in Madin-Darby canine kidney (MDCK) and colon carcinoma (Caco-2) cells and their transport to the cell surface was analyzed. All of the LPH mutants were targeted correctly to the apical membrane. Furthermore, the LPH-HA chimeras were internalized, indicating that the HA tails were available to interact with the cytoplasmic components of clathrin-coated pits. The introduction of a strong basolateral sorting signal into LPH was not sufficient to override the strong apical signals of the LPH external domain or transmembrane domains. These results show that basolateral sorting signals are not always dominant over apical sorting signals in proteins that contain each, and suggest that sorting of basolateral from apical proteins occurs within a common compartment where competition for sorting signals can occur.

INTRODUCTION

Polarized cells such as neurons and epithelial cells maintain separate plasma membrane domains, each with a distinct protein and lipid composition, through intracellular sorting mechanisms that recognize classes of proteins and deliver them into separate vesicles for transport to the correct surface domain (1,2). Sorting to the correct membrane is essential for the proteins to exhibit their biological functions, whereas missorting often results in pathological conditions (3, 4). The recognition event responsible for sorting has been under intense investigation for two decades and a number of peptide sequences capable of specifying transport to the basolateral surface of epithelial cells (5-11), or cell body of

neurons (12-16), have been characterized. All of these signals are located in the cytoplasmic domains of transmembrane glycoproteins. In addition to basolateral signals, three types of signal for sorting proteins to the apical surface of epithelial cells, or axon of neurons, are known. Glycolipid anchors direct proteins to the apical surface of several types of epithelial cells (17, 18), apparently by associating in the trans Golgi network[4] (TGN) (19, 20) with detergent-insoluble membrane domains enriched in glycosphingolipids and cholesterol (21). Oligosaccharides on some secreted proteins appear to specify apical transport (22), although this mechanism does not apply to all secreted proteins (23-26).

For many transmembrane glycoproteins, deletion of cytoplasmic sequences containing a basolateral sorting signal results in efficient transport of the protein to the apical surface, rather than the random transport expected for the deletion of specific sorting information (10, 27, 28, 29, 30). For other proteins, deletion of cytoplasmic sequences caused randomized transport, proving that transport to the apical surface does not occur by default (9, 31). In the reverse approach, introducing basolateral sorting signals into the cytoplasmic domain of the influenza hemagglutinin (HA) was shown to have dominant effect over apical sorting information (8, 31, 32) that has been recently localized to the transmembrane domain (8). These observations implied that some proteins carry apical sorting information that is recessive to cytoplasmic basolateral sorting signals. Basolateral signals could dominate over apical signals simply by being recognized earlier in the biosynthetic pathway, or sorting could occur in a common compartment where basolateral signals might bind tighter to the sorting machinery than apical signals. To investigate these questions, we attached a series of basolateral sorting signals to the strictly polarized membrane protein of small intestinal epithelial cells, lactase-phlorizin hydrolase (LPH, EC 3.21.23-3.2.1.62) and determined their effect on the sorting of LPH.

LPH, an integral type I membrane glycoprotein, is 1927 amino acids long containing a membrane anchor of 19 contiguous hydrophobic amino acids and a cytoplasmic domain of 26 amino acids. It is synthesized as a precursor with apparent molecular masses of 215 and 230 KDa, representing the mannose-rich (pro-LPH_h) and complex (pro-LPH_c) glycosylated forms. Maturation of LPH involves proteolytic cleavage after complex glycosylation of the precursor to yield the brush border form of 160 KDa (33-37). LPH is targeted strictly to the apical membrane of intestinal epithelial cells and Madin-Darby canine kidney (MDCK) cells (38). To investigate the position and relative strength of apical sorting signal of LPH, sorting of a

[4] The abbreviations used are: TGN, trans Golgi network; LPH, Lactase-phlorizin hydrolase; MDCK cells, Madin-Darby canine kidney cells; PAGE, polyacrylamide gel electrophoresis; HA, influenza virus hemagglutinin; mAb, monoclonal antibody; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate

tailless LPH mutant (LPH_{-ct}) (39) and chimeric proteins made by fusing LPH external and transmembrane sequences to the short, 12 amino acid long cytoplasmic domain of several HA mutants was studied in MDCK cells. Wild type HA lacks basolateral sorting signals and is transported to the apical surface (40), but point mutations in the cytoplasmic domain of HA were identified that created both internalization signals and dominant basolateral sorting signals (8, 32, 41-43). In contrast to their function in HA, these basolateral sorting signals did not affect strict apical delivery of LPH. However, the chimeric LPH proteins gained the internalization capacity similar to those of the HA counterparts.

EXPERIMENTAL PROCEDURES

Construction of cDNA encoding mutant LPH_{-ct} and chimeras of intestinal LPH and influenza virus HA - Standard recombinant DNA-techniques were employed according to Sambrook et al., (44). The LPH mutant lacking the cytoplasmic tail, LPH_{-ct}, has been reported previously (39). cDNAs encoding the external and transmembrane domain of LPH (amino acids 1-1901) (45) and the short, 12 amino acid long, cytoplasmic domain of HA (amino acids 536-547) (36) denoted as LPH-HA), were generated by PCR SOEing (46). The PCR product encoding $LPH\text{-}HA_{wt}$ was cloned into the expression vector pJB20 (32). A similar strategy was utilized to construct the LPH-HA chimeras which contain a single mutation ($LPH\text{-}HA_{Y543}$) or a double mutation ($LPH\text{-}HA_{Y543/Y546}$ and $LPH\text{-}HA_{Y543/R546}$) in the HA cytoplasmic domain. The sequence of LPH_{-ct} and each LPH-HA chimera was determined by sequencing with a Sequenase kit according to the instructions of the manufacturer (U. S. Biochemical Corp.).
Transfection and generation of stable cell lines - MDCK cells and COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Eggenstein/Germany) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin and streptomycin at 37°C in a 5% CO2 atmosphere. Cells were transfected with 5 µg of the appropriate recombinant DNA using DEAE-dextran (for COS-1 cells) as described (47) or polybrene (for MDCK cells) (38). Stably transfected MDCK cells were selected in the presence of 0.25 mg/ml active G418 (Gibco Life Technologies) and after 18-23 days, surviving colonies were isolated with cloning rings. Stable transformants expressing LPH_{-ct} or LPH-HA chimeras were screened by immunoprecipitation and by immunofluorescence staining. Expression of LPH-HA chimeras in intestinal Caco-2 cells was preformed transiently on membrane filters using the calcium phosphate procedure (48). Here, the cells were grown to confluency and the corresponding DNA was added at 2µg/ml. For higher transfection efficiency on filters, the cells were treated prior to transfection with trypsin to dissociate the cells and to achieve an optimal exposure of cells to DNA. Three days posttransfection the cells were processed for cell surface immunoprecipitation with mAb anti-LPH (see below) after biosynthetic labeling for 18 h. We found that a 3-days period was

sufficient for the cell layer to achieve complete polarity. This was biochemically assessed by cell surface immunoprecipitation of sucrase-isomaltase, which is targetted in Caco-2 cells to the apical membrane. Infection of Caco-2 cells with HA cDNA was performed as decsribed by Naim and Roth (42) for MDCK cells.

Biosynthetic labeling of cells, immunoprecipitation and SDS-PAGE - Metabolic labeling of MDCK cells grown on filters or plated in six-well culture dishes was performed as described previously (38). MDCK clones expressing LPH_{-ct} or LPH-HA chimeras were labeled for 1 h with 100 µCi [^{35}S]methionine (10 mCi/ml L-[^{35}S] Redivue^{TM} PRO-MIX^{TM} Amersham, Braunschweig/Germany) and chased for different times with unlabeled methionine. Caco-2 cells expressing transiently transfected LPH-HA_{wt} or LPH-$HA_{Y543/F546}$ were labeled continuously for 18 h to ensure a maximum labeling of the expressed recombinant proteins. Caco-2 cells infected with HA_{wt} or $HA_{Y543/F546}$ were continuously labeled for 2 h. Cell lysates were immunoprecipitated with mouse mAb anti-LPH (from hybridoma HBB 1/900/34/74) (34) as described by Naim et al., (47) and cell surface antigens were immunoprecipitated from intact cells on filters by addition of anti-LPH or anti-HA (42) antibody to either the apical or basolateral compartments. The immunoprecipitates were analyzed by SDS-PAGE according to the method of Laemmli (49). After electrophoresis the gels were fixed, soaked in 16% salicylic acid for signal amplification and subjected to fluorography.

Detergent extractability of LPH and sucrase-isomaltase - MDCK-ML cells expressing LPH were biosynthetically-labeled for 1 h with [^{35}S]methionine and chased over several time points. The cells were solubilized in the cold for 2 h with 1% Triton X-100 in 25 mM Tris-HCl, pH 8.0, 50 mM NaCl. The detergent extracts were centrifuged and the supernatant was immunoprecipitated with mAb anti-LPH. The pellet was dissolved by boiling in 1% SDS for 10 min. Thereafter 10 fold volume of buffer containing 1% Triton X-100 was added. These extracts were centrifuged and the supernatant was immunoprecipitated with a mixture of two monoclonal antibodies, MLac 6 and MLac 10, that recognize denatured and native forms of LPH (39). Similar experimental procedure was followed to assess the detergent solubility of intestinal brush border sucrase-isomaltase by using the colon carcinoma Caco-2 cells. Immunoprecipitation of native and denatured forms of sucrase-isomaltase was performed with a mixture of monoclonal antibodies (34). The immunoprecipitates were analyzed by SDS-PAGE on 5% or 6% gels.

Internalization assays - COS-1 cells transiently transfected with pJB20 encoding LPH_{-ct} or the LPH-HA chimeras were grown in duplicates on coverslips. 48 h post-transfection, cells were rinsed in ice-cold DMEM and placed on ice. The cells were incubated for 2 h with mAb anti-LPH diluted 1:200 in 5% BSA/PBS. Unbound antibody was removed by 3 washes with DMEM containing 10% FCS. Control samples were retained at 4°C and for a second set of samples the temperature was raised to 37°C with DMEM for 10 min in a circulating water bath. The cells were chilled on ice, were rinsed in ice-cold PBS, then fixed with 2%

paraformaldehyde for 20 min at room temperature. After two washes with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse antibody (Boehringer, Mannheim, Germany), diluted 1:100 in 5%BSA/PBS for 30 min at room temperature. Intracellular localization of proteins was assessed in transfected cells that were permeabilized with 0.1% Triton X-100 after fixing. The cells were examined with an Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with a 100x immersion objective.

RESULTS

The cytoplasmic tail of LPH is not required for its sorting to the apical membrane - To evaluate the role of the cytoplasmic tail of LPH in its transport and sorting, we constructed a mutant cDNA lacking the entire sequence encoding the cytoplasmic tail of LPH (denoted pro-LPH_{-ct}, Table I) and found that in COS-1 cells this deletion did not affect the transport-competence of the molecule (39). Biosynthetic processing of LPH_{-ct} in a MDCK cell line continuously expressing the protein was similar to that of wild type pro-LPH in intestinal and MDCK cells (35, 38). The first detectable biosynthetic form, the 215-kDa mannose-rich pro-LPH_{-ct} species, chased into the complex glycosylated 230-kDa pro- LPH_{-ct} polypeptide after 4-6 h and a cleaved form of LPH_{-ct} (approximate apparent molecular mass 160 kDa) appeared (Figure 1). The cleaved 160-kDa form is the tailless analogue of LPHß previously characterized in intestinal biopsy specimens (35), in transfected MDCK and CHO cells (38, 50), and will be therefore denoted $LPHß_{-ct}$.

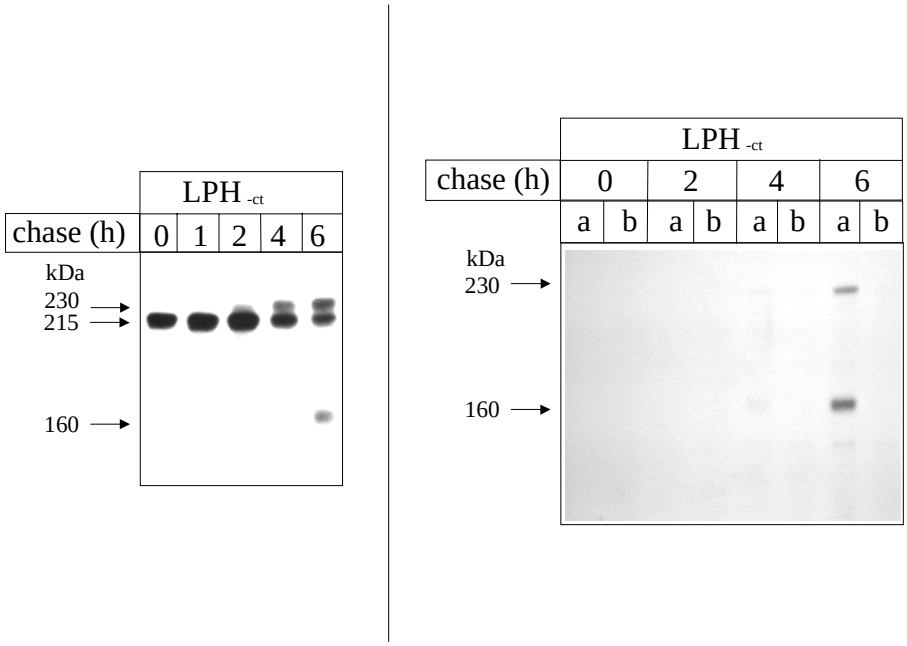

Fig. 1: Transport kinetics and polarized delivery of newly synthesized LPH$_{-ct}$ to the surface of MDCK cells. A. MDCK cells stably expressing LPH$_{-ct}$ were pulse labeled for 1 h with [^{35}S]methionine and chased for the indicated times with 2.5 mM unlabeled methionine. LPH$_{-ct}$ was purified by immunoprecipitation and analyzed by electrophoresis on a 6% SDS-gel and fluorography. B. MDCK cells stably transfected with LPH$_{-ct}$ were grown on filters, pulse-labeled with [^{35}S]methionine for 1 h and chased in medium with 2.5 mM unlabeled methionine for the indicated times. LPH$_{-ct}$ was immunoprecipitated either from the apical (a) or the basolateral (b) surface and analyzed as in Figure A.

The kinetics of appearance of pro-LPH$_{-ct}$ in the apical or basolateral domains were investigated by cell surface immunoprecipitation of pro-LPH$_{-ct}$ and its derivative LPHß$_{-ct}$ from cells grown on transparent polyester membrane filters as described previously for wild type LPH (38). Figure 2 shows that the complex glycosylated 230-kDa pro-LPH$_{-ct}$ precursor and LPHß$_{-ct}$ appeared after 4 h of chase at the apical surface. The intensity of the bands isolated from the apical domain became stronger at the 6 h-chase point. No significant bands corresponding to these two LPH-species were detected at the basolateral surface. Together, the pulse-chase and sorting analyses indicate that intracellular processing and targeting of pro-LPH$_{-ct}$ in MDCK cells is similar to its wild type pro-LPH counterpart and that the cytosolic portion of pro-LPH is devoid of apical sorting signals.

Apical transport of LPH is independent of its association with sphingolipid-cholesterol rafts - A number of apically-sorted proteins, such as influenza virus neuraminidase, HA and some intestinal proteins have been shown to be selectively associated with sphingolipid-cholesterol

rafts (21, 51, 52). One of the characteristics of these protein-membrane structures is their insolubility in detergents such as Triton X-100 at 4°C. We therefore examined whether pro-LPH is associated with rafts in the polarized cell line MDCK-ML that expresses pro-LPH (38). Cells were pulse-labeled with ^{35}S-methionine for 1 h and chased for various time points. The cells were extracted with Triton X-100 and detergent solubility of pro-LPH biosynthetic forms versus insolubility was examined. Figure 3 shows two representative chase time points. The 215-kDa mannose-rich pro-LPH appeared in the supernatant fraction (denoted S) at the earliest chase time point (1 h pulse, 0 h chase) and the pellet (P) was devoid of this form. The complex glycosylated 230-kDa pro-LPH was also found exclusively in the supernatant after 4 h of chase together with the mannose-rich 215-kDa species. Similar results were obtained with chase points earlier and later than 4 h. The absence of pro-LPH in the detergent insoluble fraction (P) indicates that pro-LPH is not associated with sphigolipid-cholesterol rafts. These data agree with previous observations that pro-LPH was only found in the detergent soluble form in biosynthetically-labeled explants from the pig small intestine (52). By contrast to pro-LPH, the 245-kDa complex glycosylated mature form of another brush border protein, sucrase-isomaltase (SI) (34, 53), could be found in the Triton X-100 insoluble pellet (P) after 3 h of chase in biosynthetically-labeled intestinal Caco-2 cells (Fig. 3). The mannose-rich form (210-kDa), on the other hand, was found only in the supernatant (Fig.3). This result indicates that the 245-kDa mature form of sucrase-isomaltase is associated with sphigolipid-cholesterol rafts and suggests a role of these structures in the targeting of this glycoprotein to the apical membrane, but not in the sorting of LPH.

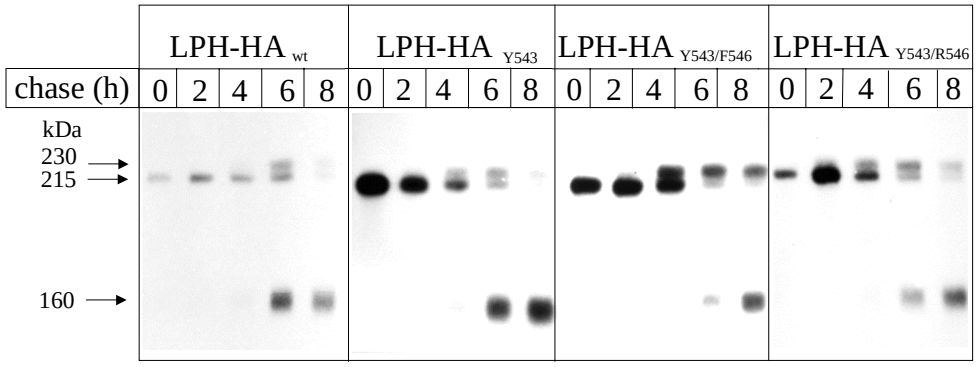

Fig. 3: LPH is not associated with sphigolipid-cholesterol rafts. MDCK-ML and intestinal Caco-2 cells expressing LPH and sucrase-isomaltase (SI) respectively were biosynthetically-labeled for 1 h with [^{35}S]methionine and chased over several time points. Triton X-100 detergent extracts were centrifuged and the supernatants were immunoprecipitated with mAb anti-LPH or mAb anti-SI. The cellular pellets were extracted by boiling in 1% SDS followed by dilution with 10 fold volume of buffer containing 1% Triton X-100. These extracts were centrifuged and the supernatants were immunoprecipitated with antibodies that recognize denatured and native forms of LPH or SI. The immunoprecipitates were analyzed by SDS-PAGE on 6% (LPH) or 5% (SI) gels and fluorography. Representative chase time points are shown.

The chimeric LPH-HA mutants - The high fidelity of sorting of LPH (more than 90% was apically targeted) and its cleaved product (exclusively apically-located) strongly suggest that this protein contains strong apical sorting signals. The strength and efficiency of the basolateral sorting machinery has lead to the notion that basolateral signals are dominant over apical signals in MDCK cells. Consistent with this concept, HA mutants constructed to contain basolateral signals were sorted to the basolateral rather than the apical membrane (8). To examine the strength of potential apical sorting signal within the LPH molecule, the same cytosolic sequences that completely reversed the polarity of HA were fused to the ectodomain and transmembrane domain of pro-LPH (the chimeras are indicated LPH-HA). The cytosolic tails of the HA mutants used in this study were derived from mutant HA_{Y543}, $HA_{Y543/F546}$ and $HA_{Y543/R546}$. The corresponding chimeras are referred to as LPH-HA_{Y543}, LPH-$HA_{Y543/F546}$ and LPH-$HA_{Y543/R546}$. As a control, the pro-LPH tail was replaced by the cytosolic tail of wild type HA (LPH-HA_{wt}). These chimeric proteins were stably expressed in MDCK cells and the biosynthesis, processing, transport and sorting of the chimeric proteins were investigated.

LPH-HA chimeras containing basolateral sorting signals are sorted to the apical membrane - Exchanging the cytosolic tails of pro-LPH with mutants of the HA tail had no significant effects on the biosynthesis, processing and transport rate of pro-LPH. In a fashion similar to wild type pro-LPH (38), the mutants were processed from the mannose-rich 215-kDa species to the compelx glycosylated mature 230-kDa and proteolytically cleaved to the 160-kDa LPHß analogues (Fig. 4A). To examine whether the transplanted cytosolic tails in pro-LPH had affected its sorting to the apical in a fashion similar to the effects observed with mutant HA molecules, monolayers of MDCK cells expressing LPH-HA_{Y543}, LPH-$HA_{Y543/F546}$, LPH-$HA_{Y543/R546}$ or LPH-HA_{wt} were grown on filters and were pulse labeled with [^{35}S]methionine for 1 h and chased for several intervals. Cell surface immunoprecipitation with mAb anti-LPH was performed followed by SDS-PAGE. Figure 4B demonstrates that all the biosynthetic forms of the LPH-chimeras, i.e. the uncleaved complex glycosylated pro-LPH and the corresponding cleaved LPHß analogues, appeared exclusively in the apical membrane. Very little, if any, was found at the basolateral membrane. Even the chimera containing the double mutation (Y_{543}/F_{546}) in the cytosolic domain was not effective in the context of the pro-LPH species. By contrast, this mutation completely reversed the sorting of HA from an apically to a basolaterally sorted molecule (8). Since the proportion of the labeled species located in the basolateral membrane was minor through out the chase periods and since this proportion did not change in relation to the apical proportion, we conclude that the transport of the chimeras to the apical surface was direct, as it was the case with wild type pro-LPH (38). We demonstrate that not even a minor effect on the polarized sorting of pro-LPH to the apical membrane could be discerned when basolateral signals in the cytosolic tail of HA were examined in the pro-LPH species.

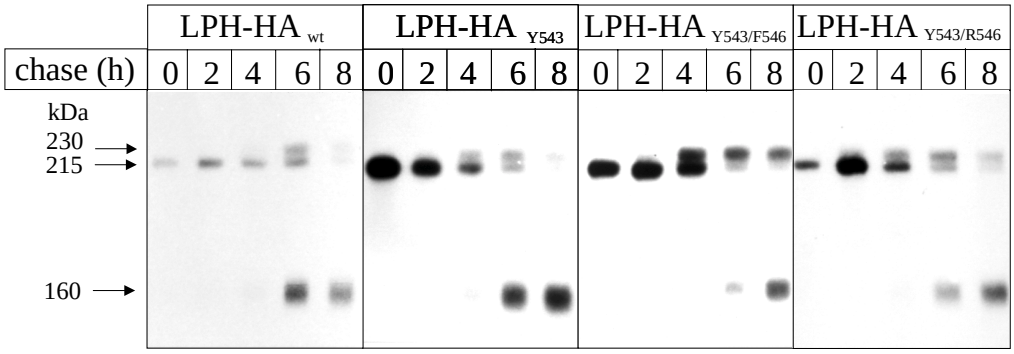

Fig. 4A: Transport kinetics of LPH-HA chimeras. MDCK cells expressing the chimeras LPH-HA$_{wt}$, LPH-HA$_{Y543}$, LPH-HA$_{Y543/F546}$ or LPH-HA$_{Y543/R546}$ were biosynthetically labeled for 1 h with [^{35}S]methionine and were chased over a period of 6 h. Samples were analyzed by SDS-PAGE on 6% slab gels and fluorography.

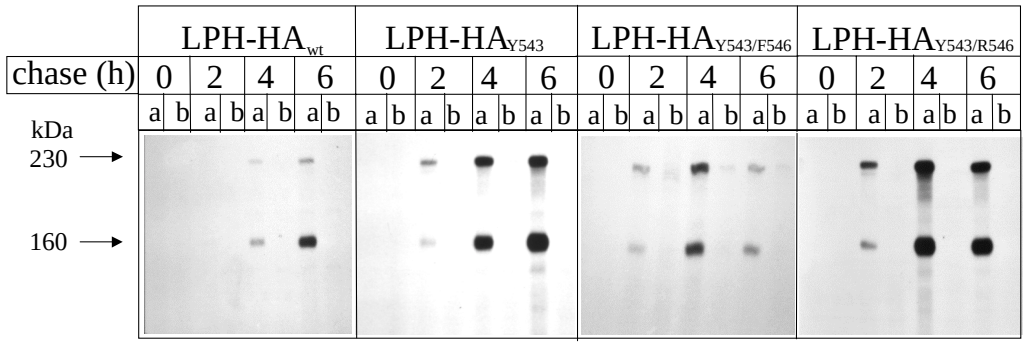

Fig. 4B: Polarized expression of LPH-chimeras in MDCK cells. Monolayers of MDCK cells expressing LPH-HA$_{wt}$, LPH-HA$_{Y543}$, LPH-HA$_{Y543/F546}$ or LPH-HA$_{Y543/R546}$ were grown on filters. 6 days after confluence, the cells were labeled with [^{35}S]methionine for 1 h and chased for the indicated times. Chimeras were immunoprecipitated either from the apical (a) or basolateral (b) surface with mAb anti-LPH, and analyzed by SDS-PAGE on 6% gels and fluorogrpahy.

To examine whether a similar or a different sorting behavior of these mutants occurs in an enterocytic cell line, we used colon carcinoma Caco-2 cells. In these cells endogenous or recombinant LPH is processed in a similar fashion to its counterparts in the intestinal mucosa and is targetted to the apical membrane (54). For this purpose the LPH-HA$_{wt}$ and LPH-HA$_{Y543/F546}$ chimeras as well as the full length HA cDNA analogues, i.e. HA$_{wt}$ and HA$_{Y543/F546}$

were expressed in Caco-2 cells. Fig. 4C demonstrates that the LPH-HA chimeras examined, LPH-HA_{wt} and LPH- $HA_{Y543/F546}$, behaved in a fashion similar to their analogues in MDCK cells. Here, pro-LPH (230-kDa) as well as the cleaved 160-kDa LPHß species were sorted to the apical membrane. Likewise, the biosynthesis and sorting of HA_{wt} and $HA_{Y543/Y546}$ Caco-2 cells were essentially similar to their counterparts in MDCK cells (Fig. 5D). Thus, HA_{wt} was predominantly found at the apical and $HA_{Y543/F546}$ at the basolateral membrane. In essence, the data demonstrate that the sorting pathways for the LPH-HA chimeras and also for the HA molecule are similar in MDCK and Caco-2 cells.

Internalization of LPH-HA chimeras is dependent on mutations in the cytoplasmic domain - Two possibilities could explain the apical sorting of the LPH-HA chimeras. The apical sorting signal of pro-LPH might be dominant over the basolateral signals or the introduced signals might not function in the context of pro-LPH and were not accessible to the sorting machinery. One way to examine the latter possibility is to determine if these sequences function as internalization signals in the context of the pro-LPH sequences, as they do in the context of HA (55). Normally, LPH does not contain an internalization signal; it escapes coated pits and remains exclusively at the cell surface. To determine if the LPH-HA mutants were internalized, expression plasmids encoding each protein were transfected into COS-1 cells. Wild type pro-LPH and the tailless pro-LPH_{-ct} mutant were included as controls, since neither should be internalized. Transfected COS-1 cells were grown on coverslips for 48 h post-transfection and were assayed for endocytosis by indirect immunofluorescence procedures (Figure 5).

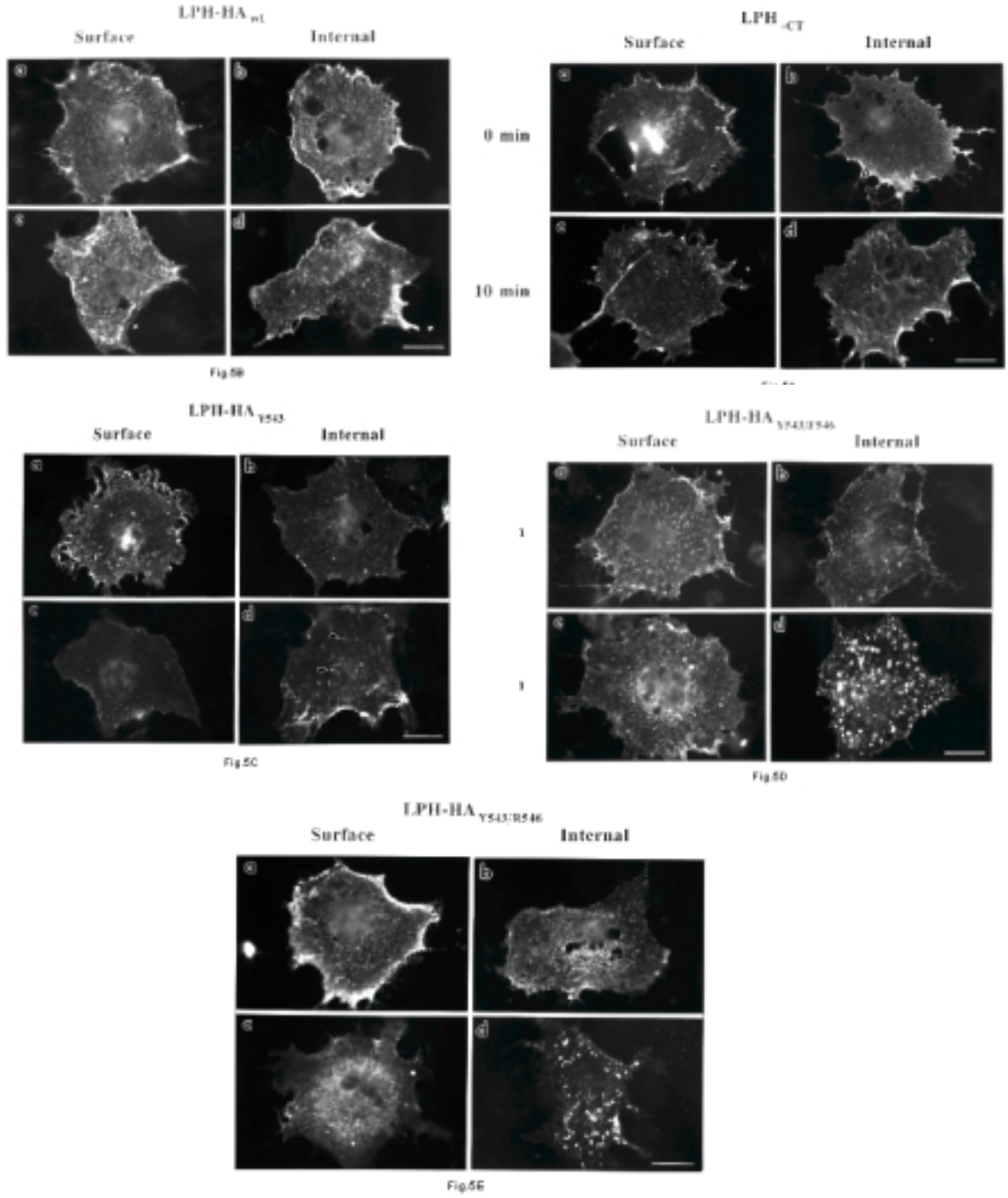

Fig. 5: Internalization of LPH$_{-ct}$ and LPH-HA chimeras. Duplicates of COS-1 cells expressing (A) LPH$_{-ct}$, (B) LPH-HA$_{wt}$, (C) LPH-HA$_{Y543}$, (D) LPH-HA$_{Y543/F546}$ and (E) LPH-HA$_{Y543/F546}$ were treated with mAb anti-LPH antibody at 0°C. One set of transfected cells were kept on ice as controls (indicated as 0 min), whereas the other set of cells were chased for 10 min at 37°C to induce internalization (indicated as 10 min). All cells were fixed with 2% paraformaldehyde and either permeabilized with Triton X-100 (denoted as internal, b and d) or non-permeabilized (denoted as surface, a and c). The cells were stained with a second FITC-conjugated antibody to determine the location of anti-LPH antibodies bound to the expressed proteins.

Cells expressing either tailless pro-LPH$_{-ct}$ or LPH-HA$_{wt}$ that were incubated with monoclonal anti-LPH antibody at 0°C (5A, a) or at 37°C to allow endocytosis to proceed, had predominantly bright staining at the cell surface. By contrast, all three chimeric proteins containing internalization signals were internalized. Most notably, cells expressing the LPH chimeras with HA tails containing the double mutations, Y_{543}, F_{546} and Y_{543}, R_{546} revealed punctate fluorescence staining after incubation at 37°C (Fig. 5D and E, panel d in each case) but fluorescence that was restricted to the cell surface at 0°C (Fig. 5D and E, panel a in each case). LPH-HA$_{Y543}$ revealed punctate pattern to a lesser extent (Fig. 5C), as would be expected from its slower rate of internalization (43, 55). These data demonstrate that the cytoplasmic sequences of the LPH-HA chimeras were available to be bound by components of the internalization apparatus and therefore must be exposed to the cytosol.

DISCUSSION

In MDCK cells basolateral signals have been observed to dominate over apical signals when both are present in the same protein. Many basolateral proteins become apical proteins when the cytoplasmic basolateral sorting signal is mutated or removed. Basolateral sorting signals, when transferred to an apical protein, direct the chimeric protein to the basolateral surface. A single exception to this generality has been reported recently. When a portion of the ectodomain of the normally apically expressed neurotropin receptor is removed, the mutated protein is routed to the basolateral surface (56). However, it is not yet clear whether the neurotropin receptor contains a recessive basolateral signal, or whether the deletion in the ectodomain causes conformational changes in the protein that expose a cryptic basolateral signal that is dominant when available to interact with the sorting machinery. To address the question of whether basolateral signals are always dominant over apical signals, we made a series of mutants of LPH that contain overlapping basolateral and internalization signals that differed in the efficiency with which each specified either internalization or basolateral sorting (8, 42). Because these signals had dual function, we could determine if the signals were available to interact with cellular sorting machinery by monitoring the capacity of the internalization signals to allow endocytosis of LPH, which normally lacks that capacity. Our results show clearly that a series of basolateral sorting signals capable of directing the influenza virus HA to the basolateral surface could not redirect the LPH, although the internalization signals in those sequences did cause LPH to be internalized. These results demonstrate that basolateral sorting signals are not always dominant over apical signals. This eliminates the possibility that basolateral signals are simply recognized earlier in the biosynthetic pathway and suggests that sorting is determined by the relative affinity of various sorting signals for the sorting machinery.

The nature of the signal or signals in LPH that allow it to be sorted so efficiently to the apical surface of epithelial cells remains to be determined. In contrast to basolateral signals, which all appear to be short amino acid motifs located in the cytosolic domain, three quite different features have been proposed to be important in apical targeting of transmembrane and secreted proteins in polarized epithelial cells (18, 21, 57). Recent data indicate that oligosaccharides can mediate protein sorting to the apical membrane. For instance, engineering two N-linked glycosylation sites into the normally unglycosylated growth hormone leads to a polarized targeting to the apical membrane of the otherwise unsorted protein (22). Another type of glycosylation, O-glycosylation, may constitute a targeting signal to the apical membrane (56). As yet unidentified apical sorting signals have been proposed to reside in the ectodomain of a number of proteins, such as intestinal brush border proteins (58), but whether these signals depend upon glycosylation or not is currently unclear. Finally, protein association with sphingolipid-cholesterol rafts has been proposed as a potential mechanism in targeting proteins to the apical surface (22). This association occurs in the TGN and results in detergent-insoluble membranes. Three examples are known of apical signals residing in transmembrane segments of proteins that associate with detergent insoluble membranes (59-61), although detergent insolubility has been shown not to be sufficient for apical sorting in one of these cases (60).

For LPH, a number of observations strongly support the idea that apical sorting signals are located in a specific portion of the ectodomain extending from Ala_{869} to Ile_{1646}. The LPH precursor, pro-LPH, is cleaved intracellularly to LPHα(Ser_{20}-Arg_{868}) and LPHß, which is targeted to the brush border membrane. Cleavage is not required for sorting, since the uncleaved pro-LPH precursor is also sorted almost exclusively to the apical membrane (38). The LPHα is apparently not necessary for sorting pro-LPH, since LPHß expressed individually in MDCK cells is correctly sorted in a fashion similar to wild type pro-LPH (50). Deletion of 236 amino acids in the homologous region IV (45) of the ectodomain that is juxtaposed to the membrane generates a transport-competent and correctly sorted mutant protein (62). Importantly, the deletion of this stretch of 236 amino acids eliminates 4 potential N-glycosylation sites and the entire O-glycosylated domain of pro-LPH. Since these substantial changes in the glycosylation pattern of this mutant do not affect its sorting behavior, it is likely that neither N- nor O-linked glycosylation are directly responsible for the sorting of LPH. The cytosolic portion of LPH does not contain targeting signals, since a tailless LPH-mutant is sorted to the apical membrane with similar fidelity to wild type LPH. Finally, the membrane anchoring domain of LPH does not have the characteristics of other transmembrane domains that contain apical sorting signals. LPH is completely soluble in Triton X-100 at 4°C and is therefore not associated with lipid rafts (this study and (52)). As there are other apically sorted proteins which could not be demonstrated to associate with detergent insoluble membrane domains (52, 63), a separate mechanism for sorting these

proteins into a parallel pathway to the apical surface is possible, or these proteins might associate with detergent-insoluble membranes with an affinity too weak to be observed experimentally.

Currently, we do not understand why the LPH has stronger apical sorting signals than does the HA. At least 90% of wild type LPH expressed in MDCK cells is delivered to the apical membrane compared to 75% apical sorting of HA expressed in continuous MDCK cell lines (11, 60). Since there are a number of different ways in which proteins can interact with apical sorting machinery, it is possible that apical sorting of LPH is regulated by several signals, which then act cooperatively to ensure strong association with apical sorting machinery.

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TABLE I

Transmembrane and carboxy-terminal sequences of wild type LPH, LPH_{-ct} and LPH-HA chimeras

Protein	Transmembrane sequence	Cytoplasmic sequence
LPH_{wt}	ALYVLFSLVLLGVCGLAFL	**SYKYCKRSKQGKTQRSQQELSPVSSF**
LPH_{-ct}	ALYVLFSLVLLGVCGLAFL	
$LPH\text{-}HA_{wt}$	ALYVLFSLVLLGVCGLAFL	**C S N G S L Q C R I C I**
$LPH\text{-}HA_{Y543}$	ALYVLFSLVLLGVCGLAFL	**C S N G S L Q <u>Y</u> R I C I**
$LPH\text{-}HA_{Y543/F546}$	ALYVLFSLVLLGVCGLAFL	**C S N G S L Q <u>Y</u> R I <u>F</u> I**
$LPH\text{-}HA_{Y543/R546}$	ALYVLFSLVLLGVCGLAFL	**C S N G S L Q <u>Y</u> R I <u>R</u> I**

All sequences are shown in single letter code. The cytoplasmic domains are illustrated in bold letters. The first depicted sequence is that of the transmembrane domain (19 amino acids) and the cytoplasmic domain (26 amino acids) of wild type LPH (45). LPH_{-ct} is a mutant form of LPH lacking the cytoplasmic domain (39). Chimeras containing the external and transmembrane domain of LPH and the cytoplasmic domain of HA are indicated as LPH-HA. The cytoplasmic domain of HA contains 12 amino acids. This sequence is predicted to be the maximum length of the A/Japan HA cytoplasmic domain (Naim *et al.*, 1992). Single or double mutations in the cytoplasmic tail of HA are indicated by underlined lette.

Chapter 8

General Discussion

The absorptive cells of the small intestine are characterized by the brush border structure of their apical plasma membrane domain. The brush border of intestinal epithelial cells, contain a number of common glycoproteins, in particular, the digestive enzymes (1-8). Some of these hydrolases, such as sucrase-isomaltase, aminopeptidase N, dipeptidylpeptidase IV and lactase-phlorizin hydrolase are typically apical constituents and have to be specifically sorted and transported to the apical membrane domain. Delivery of newly synthesized membrane spanning proteins to the apical membrane domain of polarized epithelial cells is dependent on sorting signals or motifs present in the luminal domains of these proteins. This thesis focuses on the role of O-linked glycans in the structure, trafficking and sorting of two intestinal membrane glycoproteins in colon carcinoma Caco-2 cells, such as the high polarized apical sorted sucrase-isomaltase which delivers a direct route from the Golgi apparatus to the apical membrane and dipeptidylpeptidase IV which uses the indirect route from Golgi apparatus to the basolateral membrane and is then endocytosed and sent to the apical surface (3;9) and analyses the role of sphingolipids-cholesterol-rich membrane microdomains in the apical sorting of these proteins.

The role of glycan in polarized trafficking

The potential role of glycosylation in intracellular trafficking in polarized cells has been highlighted by several recent data. N-linked glycosylation was shown to be involved in the apical targeting of several secretory, transmembrane or GPI-anchor model proteins in Madin-Darby canine kidney (MDCK) cells (3;9-13). The presence of O-glycosylation sites is also important and directly involved in the apical delivery of the human neurotrophic receptor when the latter is stably expressed in MDCK cells (3;9;14). The current work is the first to describe the potential role of O-linked glycans in the sorting of an endogenously expressed membrane glycoprotein in a highly polarized cell line. Except for properly and fully processed O-linked glycan units, no other motifs or signals may contribute to the high fidelity of the apical sorting of pro-SI and DPPIV. This is in line with the observation that a random transport to both membranes, the apical and basolateral, is the consequence of diminished or impaired O-linked glycans. Repressed basolateral sorting elements are also not located in both of our model proteins. This type of signal exists in the neurotrophic receptor and acquires functionality as soon as the apical signal is eliminated, thus leading to a predominant targeting of the mutant protein to the basolateral membrane. Several lines of evidence led to the

concludsion that the O-linked glycans of some but not all intestinal glycoproteins are required for efficient apical sorting.

N-glycosyIation modification is required for apical sorting

Aminopeptidase N, DPPIV and SI are three structurally and functionally different apical intestinal brush border glycoproteins. They are sorted to the microvillar membrane at different rates in the differentiated intestinal cell line Caco2, in which both direct and indirect pathways are used: SI follows the direct pathway whereas APN and DPPIV partially follow the indirect one (3). The apical targeting of these proteins seems to be signal mediated by still ill-defined part(s) of the extracellular domain (15). The present work analyses the effect of two N-linked glycan trimming inhibitors of the intracellular trafficking of these proteins, and demonstrates that the O-glycosylation of these proteins does not proceed to completion, or is perhaps blocked, as a result of impaired processing or complete inhibition of the processing of the first mannose residues in the cis-Golgi by dMM, an inhibitor of cis-Golgi α-mannosidase I. The effects of swainsonine an inhibitor of cis-Golgi α-mannosidase II effects are restricted to N-linked glycosylation (9;16-20). These data clearly show that the unprocessed mannose-rich chains in the presence of dMM may sterically hinder the addition of glycan chains to neighbouring potential O-glycosylation Ser or Thr residues. Not only the glycosylation in the particular site is important in directing proteins but the precise structure of the glycan may be also critical. These results are supported by the data obtained with LPH (21). However, reduction of O-glycan event in the presence of dMM is accompanied by a dramatic alteration in the sorting behavior of SI and DPPIV from a highly polarized to a randomly delivered molecule in epithelial cells. While the sorting of ApN was not affected, on the other hand modulation of N-linked glycosylation in the presence of swainsonine had no effect on the sorting behavior. Modulation of N-and O-linked glycan does not affect the overall folding or the transport-competence of these proteins, which occurs within the cell and to the cell surface, apical or basolateral, with wild type kinetics. Also the sorting behavior of ApN is neither affected by O-linked nor N-linked glycosylation. This result supports data, in which deletion of the potentially O-glycosylated Ser/Thr-rich stalk domain of ApN has no effects on the sorting behavior of this mutant (2) and indicates that the apical targeting of APN appears to employ glycan-independent sorting signals. It is obvious that O-linked glycans are directly implicated in the polarized trafficking of SI and DPPIV. The question is if the presence of O-glycans alone is sufficient for an efficient apical sorting, and if the location of the O-glycans involved is in the sorting event. To address these questions an important tool is used in the present work. Benzyl GalNAc is a potent inhibitor of the extension of O-linked glycans (22;23), It functions most likely through the competition with GalNAc in binding to potential

O-glycosylated Ser/Thr sites, which thus prevents the addition of Gal to GalNAc-O-Ser/Thr by galactosyltransferase (24). It has been shown that the optimal substrate of galactosyltransferase is GalNAc-O-Ser/Thr as compared to benzyl GalNac-O-Ser/Thr(25) . The data presented are in line with this mode of action since the pro-SIc/benzyl glycoform contains no O-linked Gal residues. Another possible function of benzyl GalNAc implicates competitive inhibition of α-2,3-sialyltransferase by the disaccharide benzyl GalNAc that is metabolised in the cell instead of GalNAc (22). This mechanism applies particularly to mucins in HT-29 cells and some proteins in Caco-2 cells, which do not express α-2,3-sialyltransferase, are also affected by benzyl GalNAc (this work) (26). This indicates therefore that not sialylation, but rather an earlier glycosylation step is effected in the cells. Nevertheless, disregarding the mechanism of action which applies to benzyl GalNAc, the common aspect that emerges is that benzyl GalNAc significantly affects the O-glycosylation of proteins. Moreover, this effect is restricted to O-glycans since N-linked carbohydrates of HA, an exclusively N-glycosylated protein (27) are not affected. Is there another sorting motif or structural determinant within pro-SI required for its high sorting fidelity? And which mechanisms are invoIved in the sorting of these proteins?.

O-linked glycan is directly implicated in the apical sorting of SI and DPPIV

To address whether alteration in the glycosylation process through the competitive inhibition of O-glycosylation by benzyl-N-acetyl-α-D-galactosaminide(benzyl-GalNAc), could affect the intracellular transport and sorting in intestenal epithelial cells, the present work here analyses the role of O-glycosylation in the apical sorting of sucrase-isomaltase SI (4).

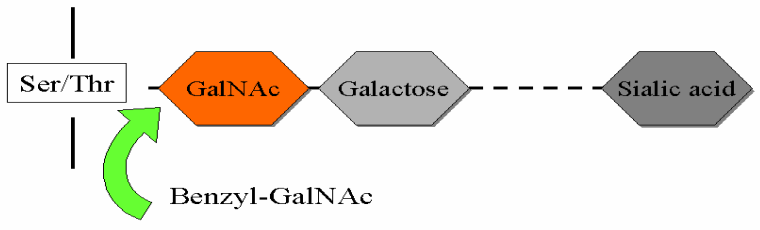


Fig.1 : Benzyl-GalNAc, an inhibitor of O-glycosylation

It demonstrates that inhibition of O-glycosylation by (benzyl-GalNAc) is accompanied by a dramatic shift in the sorting of SI from the apical to both membranes, the apical and basolateral. The sorting mechanism of SI implicates its association with sphingolipid-cholesterol-rich membrane rafts. This association is eliminated when O-glycosylation is inhibited by benzyl-GalNAc(28). These results demonstrate for the first time that O-linked glycans mediate apical sorting through association with lipid rafts. Is there another structural determinant or motif(s) within these proteins which could be implicated in the sorting behaviour of these proteins?.

To address the question of whether O-glycan alone is sufficient for an efficient apical sorting and to characterize if other structure determinantes implicated in this apical sorting process, a series of mutants of SI were made that lack the stalk region subunit or the membrane anchor domain.

The O-glycosylated stalk domain and transmembrane domain as determinants for raft association

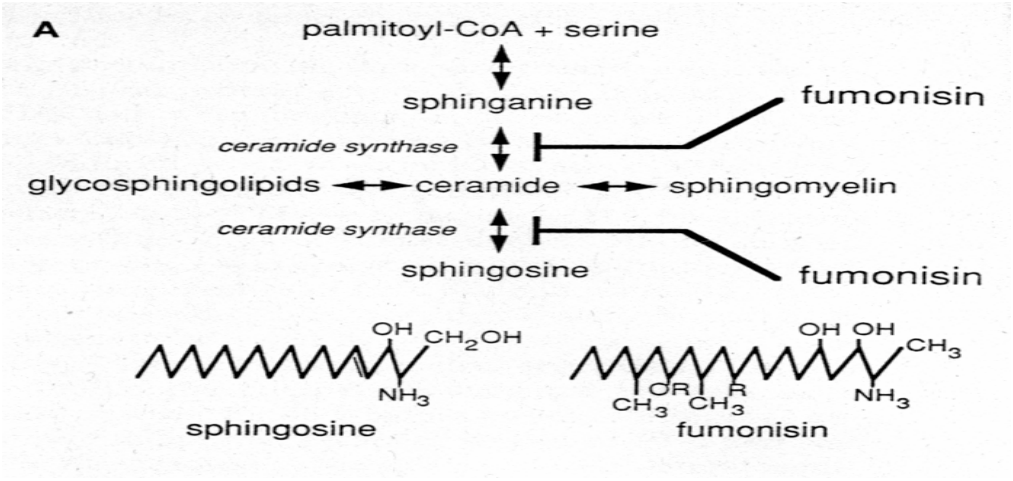
It is now widely accepted that the distribution of lipid molecules in the bilayer is non-random and asymmetrical, molecules such as cholesterol and sphingolipid can form membrane microdomains or rafts (29-33). These membrane microdomains have been thought to metdiate apical transport in polarized epithelial cells (28;34;35). This work identified determinants required for raft association of transmembrane proteins and demonstrates that raft association is an intrinsic property encoded in the protein (36-40). As the GPI anchor has been shown to have a dual function, linking proteins to rafts and functioning as apical targeting signals, this work proposes that determinants for raft association of SI also function in apical transport. Two apical signals have been found in GPI-anchored proteins: beside the lipid anchor, the ectodomain also contains apical targeting information most likely the N-glycans (10;41;42) Similarly, apical transmembrane proteins like SI appear to contain two signals, one is the stalk region that is located juxtaposed with the membrane of pro-SI and is heavily O- glycosylated due to the presence of a Ser/Thr-rich domain (4;28;37;43). The fact that deletion of the stalk region domain is accompanied by a significant reduction of O-glycosylation and demonstrates that the O-glycosylated stalk region of pro-SI plays a central role in the apical sorting behaviour of SI (44). The polarized transport of this mutant in MDCK cells is dramatically changed. Another mutant which contains the O-glycosylated stalk domain, but lacks the membrane anchoring domain, is not detected in membrane microdomains and is transported randomly to both sides of the membrane. The transport of both mutants within the cell and to the cell surface, apical or basolateral, occurs with the kinetics of the wild type protein. These structures are indispensable components of the sorting mechanism of pro-SI. It is possible that one or more mechanisms act individually or probably act cooperatively. The O-glycosylated

stalk region in the ectodomain and the transmembrane domain which could function together to ensure strong association with the apical sorting machinery (10). However, the presence of both signals at the same time appears not to be strictly required for apical sorting, because there are apical proteins which are not detergent insoluble and others which are not glycosylated, Also detergent insolubility alone has been shown not to be sufficient for apical sorting in many cases (35;39;45;46). This may explain why some brush border proteins with extensive O-glycosylation, such as aminopeptidase N are associated with lipid rafts in an O-glycan-independent way (9) and lactase-phlorizin hydrolase, which is not associated in rafts (17;28;47). These protein can exhibit a weak but significant raft interaction which is not detectable by detergent insolubility studies. In these cases the sorting signals have been located to the ectodomains and do not involve N-or O-linked glycosylation. Also different sorting mechanisms are operating to ensure high fidelity of sorting that characterized these proteins. Analysis of detergent insolubility and non glycosylated apical transmembrane proteins will allow separation of the two potential apical sorting determinants. SI has been considered to be a paradigm of transmembrane apical protein. However, whereas SI becomes insoluble after biosynthesis, some of the apical integral membrane proteins analyzed do not. The fact that O-glycan depletion in polarized epithelial cells affects apical transport of transmembrane protein including SI and DPPIV or excluding LPH from rafts might be interpreted in two ways 1) two types of apical vesicles exist for transmembrane protein targeting, one enriched with rafts-associated proteins and the other enriched with proteins excluded from rafts (48-51). or only one type of apical vesicles exists with both rafts-associated and non rafts associated transmembrane proteins (44). Alternatively, it is possible that functional rafts are required as a barrier to exclude specific proteins that use a different apical route.

Involvement of glycosphingolipid and cholesetrol in the apical targeting

The data in the present work provide here direct evidence for the functional significance of sphingolipid as a component of sphingolipid-cholesterol rich membrane microdomain rafts in membrane trafficking. They clearly demonstrate that pro-SI (2;28) and DPPIV associate during their transport to the apical membrane with rafts. This is a specific interaction since inhibition of sphingolipid synthesis in living cells by fumonisin B1 (52) leads to complete detergent-solubility of pro-SI and DPPIV. The disruption of this association is accompanied by a dramatic alteration in the sorting behaviour of both of these proteins from a highly polarized to a randomly delivered molecule to both membranes. Importantly, the association of these proteins with lipid rafts implicates O-linked glycans, the modification of which by benzyl GalNAc or by deletion of Ser/Thr rich stalk domain as well as by deletion of the membane domain results in a detergent-soluble glycoform of pro-SI and DPPIV. This

provides is the first evidence of a relation occurring between the glycosylation pattern of an apical protein and its association with lipid rafts. Although the sites and mode of action of fumonisin and benzyl GalNAc are different, there is a synergy in their effects according to studies which have localized pro-SI to vesicular structures derived from the TGN and in the case of DPPIV from endosomes and containing predominantly GPI-anchored proteins.



B

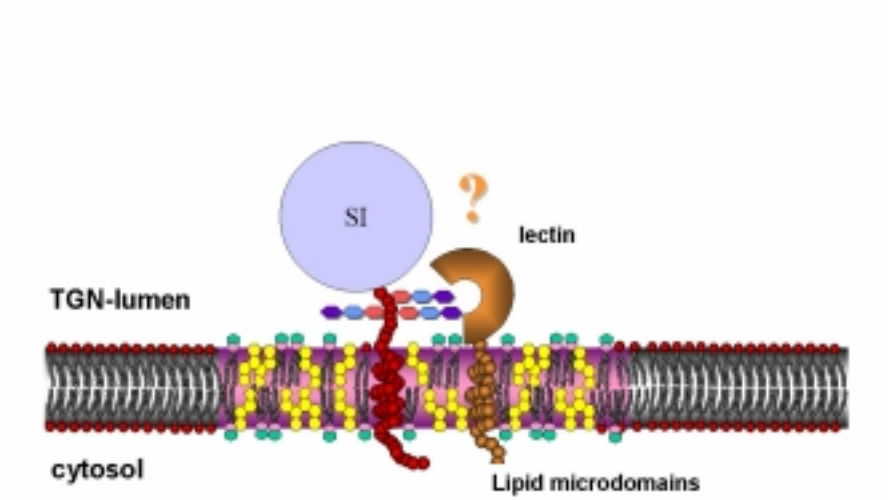


Fig 2. (A) : Schematic representation of the structure of Fumonisin B 1. (*R:Mays et al 1995*)

(B) : A lectin as putative model for sorting receptor in transe Golgi network

The modulation or blocking of the specific association between O-glycans of SI and DPPIV and lipid rafts is accompanied by a dramatic alteration in the sorting behaviour of both proteins. This association could be interpreted for instance, as the binding of O-linked glycans of pro-SI and the sugar moiety of a GPI-anchor to a common lectin-like sorting protein in the TGN. A possible candidate for these events was VIP36, a member of a recently characterized leguminous-lectin-like proteins, which is implicated in various steps of the secretory pathway (53). Alternatively, it is also possible that the association between lipid rafts and the transmembrane proteins is mediated by direct lipid-protein interaction and requires high affinity to specific lipid molecule in the rafts. Not only the composition of lipid but also the distribution of lipid molecules in the bilayer may be critical for this association. PLAP (12) and HA are found associated with lipid rafts in epithelial cells (39). Delivering of PLAP to the apical surface is sensitive to treatment with fumonisin B1 but resistanct to cholesterol sequestration with methel-ß-cyclodextrin (54). Apical transport of HA on the other hand is sensitive to cholesterol depletion (33;51). Thus it appears that at least two different pathways of transport exist for apical proteins in lipid rafts. One sensitive for fumonisin B1 and the other dependent on normal level of cholesterol. The data in this work provides concrete evidence for the functional significance of rafts in membrane trafficking, and demonstrates that one of the main roles of sphingolipids in mammalian cells is to function as a co-organizer of lipid rafts. A challenge for the future will now be to integrate the lipid organization of membranes into rapidly progressing research on protein involvement in membrane structure and function.

A hierarchy of sorting signals determines the polarized distribution of LPH in polarized epithelial cells

The existing data demonstrate that apical and basolateral signals operate hierarchically in the sorting of cell surface proteins in the epithelial cells (55;56). This complexity probably evolved to allow regulation of protein sorting in different cell types as they terminally differentiate during development. Generally basolateral targeting appears to be dominant over apical sorting when both signals are present in the same protein (57;58). But this is not always the case. To support these data a series of mutants of LPH were made that contain overlapping basolateral and internalization signals that differed in the efficiency with which each specified either internalization or basolateral sorting (59;60). Because these signals had dual function, it could be determined whether the signals were available to interact with the cellular sorting machinery by monitoring the capacity of the internalization signals to allow endocytosis of LPH, which normally lacks that capacity. The results show clearly that a series of basolateral sorting signals capable of directing the influenza virus HA to the basolateral

surface could not redirect the LPH, although the internalization signals in those sequences did cause LPH to be internalized. The data presented in this thesis address this issue and demonstrate for the first time that putative apical sorting signals of the brush border glycoprotein lactase-phlorizin hydrolase (LPH) are dominant over basolateral signals fused into the membrane anchor and ectodomain of LPH. Many basolateral membrane proteins are sorted efficiently to apical membrane when their basolateral targeting determinant is inactivated. Even influenza virus HA, an apically sorted N-glycosylated protein that possesses a transmembrane domain able to associate with rafts (10) is sorted to the basolateral membrane when a tyrosine-dependent basolateral targeting determinant is interoduced into its cytoplasmic domain (61). One possibile explanation of this dominance would be to assume that basolateral determinants have a higher affinity than apical targeting signals for their respective sorting machineries (11;13). A simpler mechanism would be the spatial separation of the sites for apical and basolateral sorting so that proteins encounter the site of basolateral sorting before that of apical sorting. But this is not always the case the first exception to this common case has been reported in this thesis. The dominance of LPH apical sorting signals over the basolateral eliminates the possibility that basolateral signals are simply recognized earlier in the biosynthetic pathway and suggests that sorting is determined by the relative affinity of various sorting signals for the sorting machinery. The structure, motif(s), signal or signals in LPH that allow it to be sorted so efficiently to the apical surface independent of sphingolipid-cholesterol rich membrane microdomain rafts remain to be determined. It is clear that further studies are required to address this issue

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Chapter 9

Summary

The epithelial cells that line our small intestine are highly polarized cells as far as function, structure and biochemical composition of their surface membrane domain are concerned. The plasma membrane of intestinal epithelial is divided into structurally and functionally distinct domains, the apical and basolateral domain which are separated by tight junctions. These domains subserve different purposes and localise different proteins and lipids at their apical and basolateral surface, which are sorted by specific signals to their final intended destination in order to carry out their proper functions. This kinds of cells serve as an excellent model to study and analyse the intercellular sorting and polarized delivering in polarized systems. For instance, the apical sorting of highly polarized N-and O-glycosylated proteins that are exclusively expressed in the small intestinal apical membrane, such as dipeptidyl peptidase IV (DPPIV). sucrase-isomaltase (SI) lactase-phlorizin-hydrolase(LPH) aminopeptidaseN(APN), the recent work demonstrates that the high fidelity sorting of SI and DPPIV to the apical membrane domain in high polarized intestinal cells implicates O-linked sugars as a direct molecular address, in association with sphingolipids-cholesterol-rich membrane microdomains. A modulation of the O-glyosylation event in SI and DPPIV by benzyl-GalNAc was accompanied by a dramatic shift in the sorting behaviour of SI and DPPIV from the apical to both membrane domains. This alteration in the sorting behaviour occurred when the formation of lipid raft was inhibited by fumonisin, or inhibition of O-glycosylation eliminated the association with rafts. To support these data deletion mutants lacking either the Ser/Thr rich stalk domain (strong O-glycan potential) or membrane anchor domain of SI were constructed and expressed in polarized cell lines. In the absence of the membrane anchor domain as well as in the absence of the stalk domain, SI fails to associate with rafts and both mutants are randomly delivered ,which indicates that the recognition signal for apical delivery comprises O-glycosylation and requires the plasma membrane anchoring domain. Also the temporal association between O-glycosylation and processing of N-linked glycan in Golgi complex as well as the implication of these events in the polarized sorting of different microvillus proteins like SI, APN, and DPPIV was studied. O-glycosylation of these proteins was strongly reduced when the mannose rich N-linked glycan trimming was blocked by

deoxymannojirimycine, an inhibitor of Golgi mannosidase I. In contrast, it was not affected by inhibition of mannosidase II with swainsonine. The removal of the outermost mannose residues by mannosidase I from the mannose rich N-linked glycan is recquired before O-glycosylation can occur. The modification of N-and O-glycosylation does not affect the transport. The apical sorting of SI and DPPIV is substantially changed, while the sorting of APN is not affected. These data indicate that O-glycans are at least part of the sorting mechanism of SI and DPPIV. The sorting of APN implicates neither O-glycan nor N-glycan and most likely follows the carbohydrate independent sorting mechanism. In the last part of this work, the location of sorting signals in LPH was analysed. This was carried out by preparing a series of mutants that lacked the LPH cytoplasmic domain or had the cytoplasmic domain of LPH replaced by sequences comprising basolateral targeting signals and overlapping internalization signals of various potency. These signals were mutants of the cytoplasmic domain of the influenza hemagglutinin (HA), which have been shown to be dominant in targeting HA to the basolateral membrane. The LPH-HA chimeras were expressed in MDCK and Caco-2 cells and their transport to the cell surface was analyzed. All of the LPH mutants were targeted correctly to the apical membrane. Furthermore, the LPH-HA chimeras were internalized, indicating that the HA tails were available to interact with the cytoplasmic components of clathrin-coated pits. The introduction of a strong basolateral sorting signal into LPH was not sufficient to override the strong apical signals of the LPH external domain or transmembrane domains. These results demonstrate that basolateral sorting signals are not always dominant over apical signals. This eliminates the possibility that basolateral signals are simply recognized earlier in the biosynthetic pathway and suggests that sorting is determined by the relative affinity of various sorting signals for the sorting machinery.

Polarized cells, Proteinsorting, O-Glycosylation, Rafts.

Zusammenfassung

Der Transport von sekretorischen und membranständigen Proteinen der Zelle erstreckt sich über mehrere Zellkompartimente und enthält eine Vielzahl von modifikationen und kontrollpunkte, an denen ein Weitertransport zum folgenden Kompartiment beeinflußt werden kann. Unsere Untersuchungen konzentrieren sich auf die post-translationalen Modifikationen wie die O-Glycosylierung, und deren Rolle bei dem gerichteten Transport von Membranproteinen in hoch polarisierten Bürstensaummembranezellen des Dünndarms zu analysieren. Die Fähigkeit dieser Zellen, neu synthetisierte Proteine spezifisch auf zwei verschiedene Membrankompartimente, ein Apikales und ein Basolaterales, zu verteilen stellt ein interessantes Modell für den gerichteten Proteintransport dar. Als Modellproteine für diesen Sortiervorgang untersuchen wir zwei Hydorlasen, die humane Saccharase-Isomaltase (SI), welche Sacchrose und Isomaltase hydrolysiert, und die humane Lactase-Phlorizin-Hayrolase (LPH), die die Lactose der Milch spaltete. Die bis heute einzige Signale für einen apical Transport von Proteinen ist deren Verankerung über einen Glykosylphosphtidylinositol (GPI)-Ancher. Interessanterweise wird die SI als nicht GPI-verankertes Membranprotein zusammen mit GPI-verankerten Proteinen in sogenannten ´´Rafts´´ transportiert. Dabei handelt es sich um sphingolipidreiche, Triton X100 unlösliche Membranbereiche des sekretorischen Transportwegs. Durch Inhibierung der Sphingolipidsynthese mit dem spezifischen Syntheseinhibitor Fumonisin konnten wir einen Einfluß auf die Sortierung der SI sehen. Das vornehmlich apikal sortierte Protein wird unter Einwrikung von Fumonisin in CaCo2 verstärkt in basolateral Membranen eingelagert.
Unsere Untersuchungen gaben Hinweise darauf, das die O-Glykosylierung von SI einen starkeN Einfluß auf das apikale Sortieren ausüben kann. In Anwesenheit des Inhibitor der O-Glycosylierung Benzyl-GalNAc wird der basolateral, transportierte Anteil im vergleich zu der normalerweise O-glycosylierten SI deutlich erhöht.
Diese Untersuchungen geben einen Hinweis darauf , dass zumindest zwei Prozesse an dem apikal Transport der SI beteiligt sind.

Hoch polarisiertenzellen, Membranproteinen, Gerichteten Transport, O-Glycosylierung.

Abbreviations

ApN	Aminopeptidase N
ATP	Adenosintriphosphat
bp	Basenpaare
cDNA	Complementary deoxyribo-nucleic acid
CSID	congenitale Saccharase-Isomaltase Defizienz
DPPIV	Dipeptidylpeptidase IV
dMM	Desoxymannojirimycin
DNA	Deoxyribo nucleic acid
DTT	Dithiothreithol
ER	endoplasmatisches Retikulum
ERGIC	ER-Golgi intermediäres Kompartiment
GPI	Glykosyl-Phosphatidyl-Inositol
GFP	green fluorescent Protein
HA	Influenza Virus Haemagglutinin
kb	Kilobasen (-paare)
HIV	Human Immunodeficiency Virus
LPH	Laktase-Phlorizin Hydrolase
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Natrium-Dodecylsulfat
SDS-PAGE	SDS-Polyacrylamidgelelektrophorese
SI	Saccharase-Isomaltase
VSV	Vesikuläres Stomatitis Virus

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Eidesstattliche Erklärunung

Ich versichere an Eides statt, dass ich die vorliegende arbeit selbständig verfasst und keine andre als die angegebene Quellen und Hilfsmittel verwendet habe.Diese Arbeit wurdebisher nicht an einer anderen Universität oder einem anderen Fachbereich als Dissertation eingereicht.

Hannover, 20 November 2001

Erklärung über den eigenen Anteil an den wissenschaftlichen Publikationen

Publikation 1:

M. Alfalah, R. Jacob, U. Preuss, K.-P. Zimmer, H. Naim und H.Y. Naim (1999):
O-linked glycans mediate apical sorting of human intestinal sucrase-isomaltase through association with lipid rafts
***Current Biology,* 9, 593-596**

Eigene Beiträge: Selbständige Planung, Durchführung and Auswertung aller dargestellten Untersuchungen, bis auf das Kontrollexperiment mit LPH-Filter-Analysen.

Publikation 2:

R. Jacob, **M. Alfalah**, J. Grünberg, M. Obendorf und H.Y. Naim (2000):
Structural Determinants required for apical sorting of an intestinal brush border membrane protein
***The Journal of Biological Chemistry,* 275, 6566-6572**

Eigene Beiträge: Planung, Durchführung der Experimente, in denen die Wildtyp SI analysiert wurde (Teile der Fig. 2C, 3A, 5 und Fig. 4A).

Publikation 3:

H.Y. Naim, G. Joberty**, M. Alfalah** und R. Jacob (1999):
Temporal Association of the N- and O-linked Glycosylation Events and Their Implication in the Polarized Sorting of Intestinal Brush Border Sucrase-Isomaltase, Aminopeptidase N and Dipeptidyl peptidase IV
***The Journal of Biological Chemistry,* 274, 17961-17967**

Eigene Beiträge: Planung, Durchführung und Auswertung aller Experimente mit Membranfiltern zur Analyse des Sortierverhaltens der Bürstensaumproteine in An-und Abwesenheit von Inhibitoren der Glykosylierung (Fig. 4)

Publikation 4:

R. Jacob, U. Preuss, P. Panzer, **M. Alfalah,** S. Quack, M.G. Roth, H. Naim und H.Y.Naim (1999):
Hierarchy of Sorting Signals in Chimeras of Intestinal Lactase-Phlorizin Hydrolase and the Influenza Virus Hemagglutinin
***The Journal of Biological Chemistry,* 274, 8061-8092**

Eigene Beiträge: Planung und Durchführung das Rafts-Analyse (Fig. 3) und Teile der Endocytose-Analyse (fig 5).