Synthesis and transport of glycoproteins and proteoglycans in the apical and basolateral secretory pathways of epithelial MDCK cells

Thesis submitted for the degree of Philosophiae Doctor

By

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Tilahun Tolesa Hafte

ABBREVIATIONS

AP	Adaptor-protein complex
COP	Coat protein complex
CS	Chondroitin sulfate
DS	Dermatan sulfate
ER	Endoplasmatic reticulum
ERGIC	ER-to-Golgi intermediate compartment
GAG	Glycosaminoglycan
Gal	Galactose
GalNAc	N-acetylgalactosamine
GalNAcT	N-acetylgalactosaminyltransferase
Gal T	Galactosyltransferase
GFP	Green fluorescent protein
GPI-Aps	Glycosylphosphatydylinositol-anchored proteins
GlcA	Glucuronic acid
GlcNAc	N-acetylglucosamine
GnT I/II	N-acetylglucosaminyltransferase I/II
HS	Heparan sulfate
IdoA	Iduronic acid
KS	Keratan sulfate

LDLR	Low density lipoprotein receptor
ManI/II	Mannosidase I/II
MDCK	Madin-Darby canine kidney
MS	Mass spectrometry
NDST	N-deacetylase-N-sulfotransferase
NeuAc	N-acetylneuraminic acid
NeuGc	N-glycolylneuraminic acid
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PAPST1	PAPS transporter 1
PC	Procollagen
PG	Proteoglycan
rGH	Rat growth hormone
Ser	Serine
SG	Serglycin
TfR	Transferrin receptor
TGN	*Trans*-Golgi network
Thr	Threonine
UDP	Uridine diphosphate
VIP36	Vesicular integral membrane protein of 36 kDa
Xyl	Xylose

LIST OF PAPERS

Paper I

Protein core dependent glycosaminoglycan modification and glycosaminoglycan dependent polarized sorting in epithelial MDCK cells.

Hafte TT, Fagereng GL, Prydz K, Grøndahl F, and Tveit H.

Glycobiology vol. 21 no. 4 pp. 457–466, 2011

Paper II

N-glycan synthesis in the apical and basolateral pathway of epithelial MDCK cells on a model protein core and the influence of an additional glycosaminoglycan domain.

Moen A, Hafte TT, Tveit H, Egge-Jacobsen W, Prydz K.

Accepted manuscript (Glycobiology) with minor revisions

Paper III

The protein conformation of the proteoglycan Serglycin influences the length and sulfation of the GAG chains.

Fagereng GL, Hafte TT , Tveit H, Prydz K

Manuscript

Summary

Sorting of newly synthesized proteins in the secretory pathway of polarized cells is governed by signals of proteinacous nature or post-translationally added structures such as glycans. Among all polarized cell types, epithelial cells are the most studied when it comes to polarized protein sorting, due to the differential access to the apical and basolateral membrane domains obtained when cells are grown on permeable supports. The most studied epithelial cell line in culture is the Madin-Darby canine kidney (MDCK) cell line, isolated from a dog kidney cortex in 1958.

In the textbook view, proteins destined for the apical and basolateral membrane domains travel a common route through the secretory pathway to the *trans*-Golgi network, the exit site of the Golgi apparatus, from where further transport is mediated by specialized apical and basolateral transport containers. Some data obtained from studies of the soluble proteoglycan (PG) serglycin (Tveit et al. 2005; Vuong et al. 2006) and N-linked glycoproteins (Alfalah et al. 2005), however, indicate that sorting may take place early in the secretory pathway. Serglycin was modified differently in the apical and basolateral secretory routes, where basolateral passage resulted in secreted serglycin which was much more intensely sulfated than the apically secreted variant, the latter accounting for 85 % of the secreted molecules (Tveit et al. 2005). Based on these data, we wanted to study further the apical sorting capability of the serglycin molecule and the differential post-translational modification in the two pathways.

Serglycin had been expressed with a green fluorescent protein (GFP) tag at the C-terminus, and for comparison, we chose to make all new variants and model proteins as GFP fusion proteins when expressed in MDCK cells. The glycosaminoglycan (GAG) attachment domain of serglycin was transferred to the junction between GFP and rat growth hormone (rGH), a non-glycosylated, non-sorted secretory protein. A variant of rGH carrying two N-glycosylation sites in addition was also expressed, since these have been shown to mediate apical sorting in MDCK cells (Scheiffele et al. 1995). The serglycin GAG domain was mainly modified with chondroitin sulfate (CS) chains and mediated apical sorting in the rGH

context, but the higher sulfation intensity in the basolateral route was lost (Paper I). In fact, this capability seems to be coded by structures outside the GAG binding domain of serglycin, although not the internal disulfide bridge of the protein core (Paper III). The protein core also harbours a second type of apical sorting information of yet unidentified nature, since serglycin molecules from which all GAG attachment sites had been removed were also secreted mainly to the apical medium (paper III).

We also wanted to address whether N-glycans also could be differentially processed in the apical and basolateral secretory routes of MDCK cells. To this end, we expressed rGH with two sites for N-glycosylation and a C-terminal GFP tag, and a variant with the GAG binding domain of serglycin fused between rGH and GFP in addition. The N-glycan modifications were analysed by mass spectrometry. Only marginal differences in N-glycan processing and site occupancy were observed for the model proteins after their respective apical and basolateral secretion. Insertion of the GAG attachment domain, however, influenced the synthesis of the N-glycan structures in a more acidic direction. Thus in PGs that also carry N-glycans, the GAG modification may have an impact on N-glycan structure.

1. Introduction

1.1 Epithelial cells

The plasma membrane of epithelial cells is constituted by two domains, the apical and the basolateral, which are functionally distinct and exposed to different environments. Each domain has a characteristic lipid and protein composition. The apical surface is in contact with the external environment through invaginations of the body cavities, such as the lumen of the intestine, whereas the basolateral side faces the blood circulation system. These two domains are separated by “tight junctions”, a protein complex that seals adjacent epithelial cells and contributes to the maintenance of epithelial polarity by preventing lateral diffusion of molecules between the apical and basolateral membrane domains. Epithelial polarity is preserved by intracellular sorting mechanisms that maintain the different composition of lipids and proteins in the apical and basolateral surface domains (Matter 2000; Nelson and Yeaman 2001). Epithelial cells are easily grown on filters with differential access to the apical and basolateral domains, due to the fence function of the tight junctions. In this respect, cell polarity aspects have been much more extensively studied in epithelial cells, than in other polarized cells, like for instance neurons.

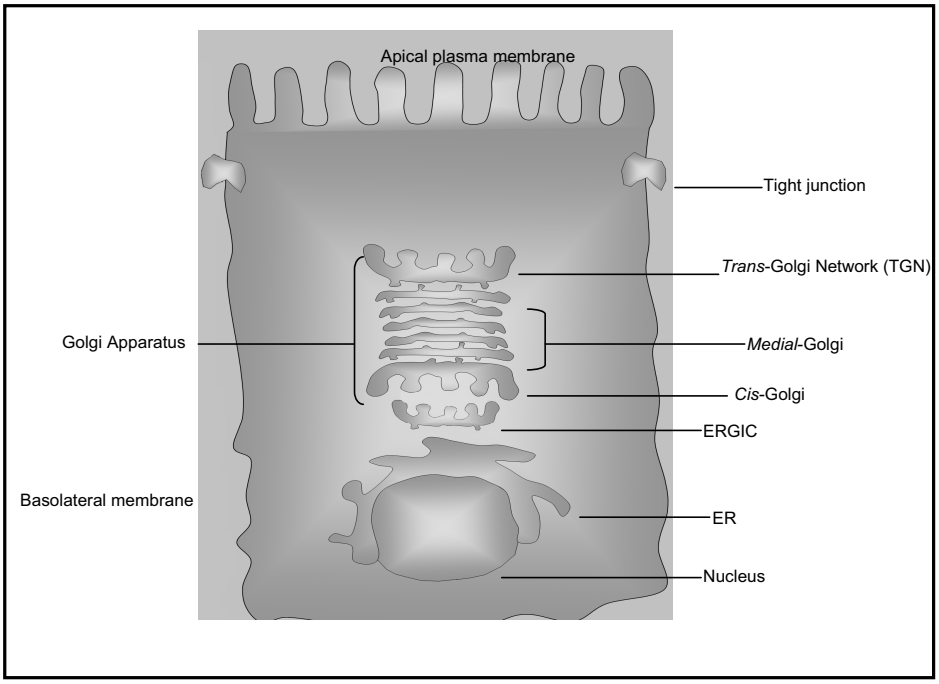

Fig. 1 A schematic representation of an epithelial cell with tight junctions.

1.1.1 MDCK cells

The Madin-Darby canine kidney (MDCK) cell line is an epithelial cell line isolated from dog kidney cortex and provides an excellent model system to study epithelial cell polarity. In fact, it is the most commonly studied epithelial cell line which, when grown on permeable supports, forms well polarized monolayers that can be accessed from both the apical and basolateral surfaces (Zegers and Hoekstra 1998). The MDCK cell line has been shown to retain many of the differentiated properties associated with kidney tubule epithelial cells (Herzlinger et al. 1982; Rindler et al. 1979; Valentich 1981). There are two main strains of MDCK cells termed MDCK I and MDCK II with the former resembling distal tubule epithelial cells and the latter sharing some characteristics with proximal tubule cells. Another physiological difference between the two strains is the trans-epithelial electrical resistance. The MDCK I strain is characterized by having a higher electrical resistance

across filter-grown cell monolayers as compared to the MDCK II strain, and thus, MDCK I cells form tighter epithelia (Balcarova-Stander et al. 1984; Richardson et al. 1981).

1.2 The secretory pathway

1.2.1 Transport from the ER to the Golgi apparatus

Newly synthesized proteins destined for cellular compartments such as the plasma membrane, endosomes and lysosomes, utilize the secretory pathway to reach their final destinations. In this pathway, proteins travel from the endoplasmic reticulum (ER) through the Golgi apparatus to the plasma membrane, or to endosomes and lysosomes. The transport of proteins between membrane compartments of the secretory pathway takes place in a sequential manner involving both coated vesicular and tubular carriers. Secretory proteins must be sorted from residential ones and become enriched in transport carriers before undergoing the next step. All proteins in this pathway, whether resident or in transit, originate in the ER lumen. Secretory proteins are transported to their destination, either constitutively or in a regulated manner after translocation to the lumen of the ER. Such translocation is normally guided by a signal peptide at the N-terminus of the protein, but internal signals also exist, particularly for membrane proteins. The ER possesses quality control mechanisms that ensure the correct folding and assembly of both its own resident proteins, and proteins destined for other cellular locations. The protein folding process is aided by several chaperone proteins that assist malfolded proteins through several cycles of quality control. Finally, irreversibly misfolded proteins are targeted for degradation. Protein transport in the secretory pathway is a multistep process involving the generation of transport carriers loaded with enrichment of defined sets of cargo molecules, the shipment of cargo-loaded transport carriers between compartments, and the specific fusion of these carriers with a target membrane (Derby and Gleeson 2007). The process of protein transport between the ER and the Golgi apparatus involves events like the collection of cargo molecules from the lumen of the ER followed by the formation of transport carriers that bud from the ER membrane and are transported in the direction of the Golgi apparatus. Protein trafficking in the secretory pathway can occur both via vesicle budding with subsequent

fusion with a target membrane and via passage along tubules that might connect to acceptor compartments (Vitale and Denecke 1999). ER exit sites (ERES), also known as transitional ER (tER), are specialized domains of ER membranes responsible for the directed export of secretory cargo (Palade 1975). The formation and budding of cargo vesicles at these ER sites involve the COPII coat protein complex that binds receptor protein tails at the cytoplasmic side of the membrane. The COPII complex thus mediates indirectly the selection of soluble cargo within the ER lumen; and transport from the ER towards the Golgi apparatus is initially mediated by vesicles coated with COPII coat proteins (Blazquez and Shennan 2000; Watson and Stephens 2005). Proteins moving from the ER to the Golgi apparatus face another sorting station called the ER-Golgi intermediate compartment (ERGIC). ERGIC was originally defined by the mannose-binding transmembrane protein lectin ERGIC-53 (Appenzeller-Herzog and Hauri 2006) that cycles between the ER and the ERGIC, serving as a cargo receptor (Fiedler and Simons 1994; Itin et al. 1996). Glycoproteins, including cathepsin Z, cathepsin C, and the blood coagulation factors V and VIII were inefficiently secreted when ERGIC-53 was either mislocated to the ER or non-functional (Nichols et al. 1998; Vollenweider et al. 1998). *In vitro* analysis has demonstrated that the short cytoplasmic domain of ERGIC-53 contains binding sites for both COPI and COPII coat proteins, whereas the lumenal domain binds to immobilized mannose in a Ca^{2+} dependent manner (Itin et al. 1996). ERGIC is composed of several tubulovesicular membrane clusters proposed to constitute an obligatory membrane entity (Hauri et al. 2000) which is involved in both anterograde and retrograde transport between the Golgi apparatus and the ER. ERGIC employs the COPI protein complex for its retrograde transport (Letourneur et al. 1994) activity and vesicles derived by COPII protein complex mediated budding (Appenzeller et al. 1999) to receive cargo vesicles from the ER, thereby making the ERGIC the first post- ER sorting station for both anterograde transport and retrograde return of proteins. Further anterograde transport from the ERGIC to the *cis*-Golgi and through the Golgi apparatus does not seem to directly require COP proteins, but little is known about anterograde sorting from the ERGIC in the direction of the Golgi apparatus (Appenzeller-Herzog and Hauri 2006). Proper tethering of COPI vesicles to Golgi *cisternae* is, however, required for normal Golgi glycosylation (Smith and Lupashin 2008).

1.2.2 The Golgi apparatus

The Golgi apparatus or Golgi complex was discovered in 1898 by the Italian scientist Camillo Golgi, but was not until the 1970-ies shown to play a central role in the secretory pathway. It is primarily involved in the concentration, modification and sorting of newly synthesized protein and lipid molecules underway from the ER to the cell surface and endomembrane organelles. The Golgi complex consists of a series of membrane limited compartments through which proteins destined for the plasma membrane, secretory vesicles, endosomes and lysosomes move sequentially (Griffiths and Simons 1986). The central part of the Golgi apparatus may be observed as stacks of flattened *cisternae* which are classified as *cis, medial*, and *trans*, based on their functional proximity to the ER and plasma membranes. The *cis cisternae* are enriched in the Golgi enzymes first encountered by cargo molecules (Bejarano et al. 2006; Prydz et al. 2008). The appearance of the Golgi apparatus differs significantly among cell type. In plants and lower animals, the Golgi apparatus exists as several copies of discrete stacks dispersed throughout the cytoplasm, while in vertebrate cells the Golgi stacks are normally localized in proximity to the nucleus (Shorter and Warren 2002). During protein trafficking, the Golgi apparatus, which is both dynamic and polar, receives cargo molecules from the ER via ERGIC at its *cis*-end and transports these from the *cis* end of the Golgi, through the *medial*-Golgi to the *trans*-Golgi region. The *cis*-Golgi functions to receive most of the biosynthetic output from the ERGIC, whereas the *trans*-Golgi network (TGN) sorts completed post-translationally modified products to their final destination (Shorter and Warren 2002).

1.2.3 Protein modifications in the Golgi apparatus

The Golgi apparatus is involved in post-translational protein modification mechanisms like glycosylation and proteolytic cleavage. Protein modification by glycans is initiated in the ER and proteins that arrive at the Golgi complex may be subjected to further post-translational modifications, such as removal of glycan units by glycosidases, additional glycosylation mechanisms, sulfation and proteolysis by the action of various Golgi resident enzymes in different *cisternae*. Golgi enzymes and associated substrate transporters are non-uniformly distributed within a Golgi stack, allowing sequential modification of cargo molecules in

transit (Smith and Lupashin 2008). Those Golgi processing enzymes which act early in glycan processing are usually concentrated in *cis*-Golgi *cisternae*, while those which act at later stages are confined to the *trans*-Golgi network (Farquhar and Palade 1981; Kweon et al. 2004; Martinez-Menarguez et al. 2001). N-acetylglucosaminyltransferase I (GlcNAcTI) and mannosidase II (ManII) are preferentially localized in *cis/medial cisternae*, while galactosyltransferases (GalT) and sialyltransferases (SiaT) are mostly found in the *trans*-Golgi region (Nilsson et al. 1993; Rabouille et al. 1995).

1.2.4 Protein journey through the Golgi apparatus

Most proteins arriving at the Golgi apparatus from the ER are glycosylated (N-glycosylation) and are subjected to sequential modifications as they pass through the *cis*-, *medial*, and *trans*-Golgi *cisternae*. However, the mechanisms by which proteins travel through the Golgi stacks remain controversial among scientists in the field. Because of the structural complexity and highly dynamic nature of the Golgi apparatus, understanding the mechanisms that regulate cargo modification and trafficking through the *cisternae* has been elusive (Jackson 2009). The anterograde vesicular transport model and the cisternal maturation model are two alternative descriptions of intra-Golgi transport (Pelham and Rothman 2000). Previously, the Golgi apparatus was assumed to be a rather static membrane system, and based on this view, a vesicular transport model was proposed. According to this model, intra-Golgi trafficking is mediated through vesicles that bud from one cisternal compartment, which has its own defined environment and enzymatic content, followed by targeting to and fusion with the next acceptor compartment with cargo being transported from one face of the Golgi apparatus to the other in a series of vesicular transport steps (Jackson 2009; Mironov et al. 2005; Palade 1975; Simon 2008) in a COPI coat mediated manner (Lee et al. 2004). However, the vesicular transport model was challenged when it failed to explain the intra-Golgi transport of large proteins like procollagen-I (PC) which can move through the Golgi apparatus without entering COPI vesicles (Bonfanti et al. 1998; Mironov et al. 2001). PC folds in the ER into rod-like trimers that further assemble in the Golgi into150 nm stable, cylindrical aggregates (Beck et al. 1996; Leblond 1989). PC- I aggregates, which are larger than COPI vesicles (50-60 nm diameter) traverse the Golgi stacks without ever leaving the lumen of Golgi *cisternae* (Bonfanti et al. 1998), supporting a

cisternal maturation model, which views the Golgi apparatus as a dynamic structure where the *cisternae* progress through the stack in the *cis* to *trans* direction with Golgi enzymes being recycled in a COPI vesicle dependent manner (Glick et al. 1997; Glick and Malhotra 1998). Thus, in both models, COPI vesicles are involved in intra-Golgi trafficking and maintenance of the normal structure of Golgi complex (Duden 2003). In the cisternal maturation model, *cis*-Golgi cisternal membranes are formed continuously by the fusion of pre-Golgi intermediates, an existing *cis-cisterna* becomes a *medial* one, and an existing *medial cisterna* becomes a *trans cisterna* (Glick 2000; Warren and Malhotra 1998). A basic difference between these two models lies in the differential movement of Golgi resident and cargo proteins, as indicated above. The vesicular transport model implies that the Golgi apparatus is a stable entity, where resident proteins are retained in the compartment while small vesicles mediate transport of cargo between subsequent *cisternae* along the Golgi stacks. Whereas, the cisternal maturation model predicts that cargo molecules remain within the lumen of the *cisternae* and move passively as *cisternae* mature and progress through the stack, while resident proteins are recycled by retrograde transport to a less mature *cisterna*, to maintain differential concentration across the stack in a COP I coated vesicle manner (Mironov et al. 2005; Smith and Lupashin 2008). Since the cisternal maturation model implies that Golgi enzymes are concentrated in COPI coated vesicles, has this possibility been investigated (Lanoix et al. 1999; Lanoix et al. 2001; Martinez-Menarguez et al. 2001). Although data supporting the view that Golgi enzymes are enriched in COPI vesicles was provided, this model was challenged by the finding of mannnosidase II deficient Golgi associated COPI vesicles (Cosson et al. 2002; Orci et al. 2000). The mannosidase II depleted COPI vesicles were enriched in KDEL receptors, which bind the ER retention sequence KDEL, suggesting the function of COPI coat vesicles to be restricted to transport from Golgi *cisternae* and ERGIC back to the ER. The role of COPI in retrograde transport in the maturation model was also questioned when COPI vesicles containing anterograde cargo proteins like pro-insulin (Orci et al. 1997) were identified, opposed to the assumption of the maturation model that cargo molecules do not leave the lumen of the *cisternae*. The contents and directionality of COPI vesicles are thus not entirely clear, but at present the maturation model gains most support from authorities in the field (Emr et al. 2009). The observation of tubular connections between *cisternae* of the Golgi complex (Marsh et al. 2004; Trucco et al. 2004) led to the suggestion of a third model called the continuity- based model. This model

predicts that different Golgi *cisternae* are connected with each other through tubular structures which could facilitate the flow of large cargo like PC in a *cis*-to the-*trans* direction, as well as the retrograde transport of Golgi enzymes in a COPI vesicles independent manner (Mironov et al. 2005). The fourth alternative model that has emerged, the rapid-partitioning model, postulates the movement of both Golgi resident enzymes and cargo molecules in both the anterograde and retrograde directions through the Golgi apparatus (Jackson 2009; Patterson et al. 2008) indicating a clear departure from the cisternal maturation model, which asserts actively sorted trafficking of Golgi enzymes only in the *trans*-to-*cis* direction. However, a unification of the anterograde vesicle transport model and the cisternal maturation model, the bidirectional trafficking of cargo molecules within the Golgi apparatus has been suggested previously (Pelham and Rothman 2000). Another interesting feature of the rapid partitioning model is the inclusion of a lipid sorting concept in the trafficking pathways, where cargo and glycosylation enzymes have their own lipids of preference to associate with in different Golgi domains. Golgi domains enriched in glycerophospholipids (GPLs) usually accommodate Golgi processing enzymes while Golgi domains with high content of sphingolipids (SLs) harbour cargo molecules (Jackson 2009).

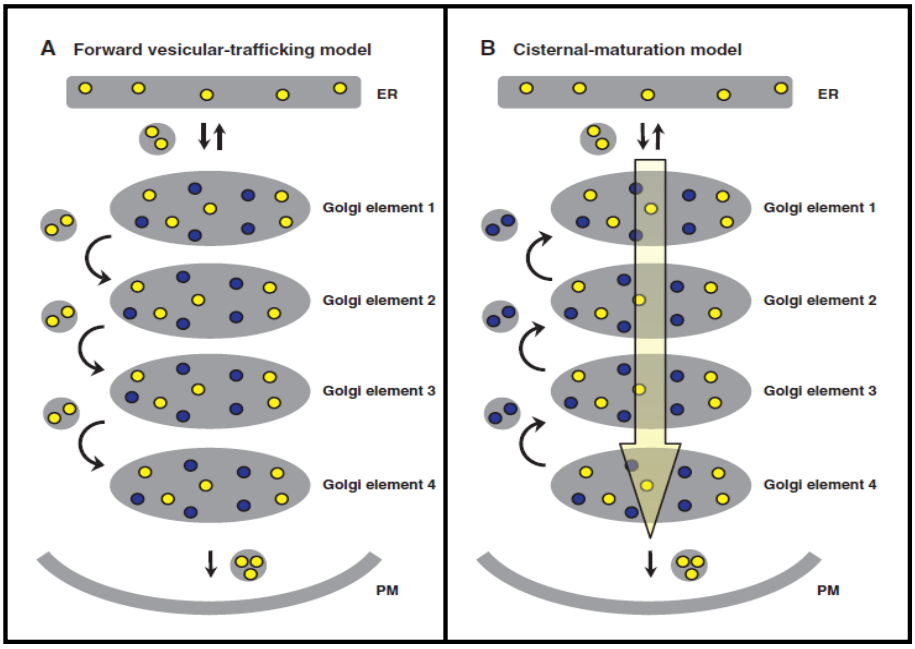

Fig.2. **Two main models for intra-Golgi trafficking**. Cargo synthesized in the ER and transported through the secretory pathway is shown in yellow; Golgi processing enzymes are shown in blue. Golgi element 1 is at the *cis* side and receives material from the ER, and Golgi element 4 is at the *trans* side and is involved in packaging of cargo for delivery to the plasma membrane (PM). Arrows indicate the direction of trafficking. (A) Forward vesicular-trafficking model. Vesicles carrying cargo bud from a donor compartment, and are then targeted to and fuse with the following compartment in the secretory pathway (acceptor compartment). (B) Cisternal-maturation model. Vesicles carrying Golgi processing enzymes bud from a later compartment in the secretory pathway (donor compartment), then fuse with an earlier compartment (acceptor compartment). The cargo progresses as a result of maturation of an earlier compartment into a later one. Adapted from Jackson, C.L.(2009).

1.2.5 Sorting of proteins in the secretory pathway of epithelial cells

In the classical view of the Golgi apparatus, the TGN plays an important role in directing secretory proteins to their appropriate destinations, by serving as a site for the sorting of proteins to various cellular components such as endosomes, lysosomes, and different domains of the plasma membrane. The TGN is not only involved in the sorting of secretory proteins to different destinations, but also in receiving extracellular material and recycled molecules from endocytic compartments (Gu et al. 2001). In addition, proteins can be routed from the TGN to their final target site through different pathways. Direct pathways deliver proteins to the cell surface, while indirect pathways sort proteins to endosomes for later transport to the plasma membrane (Ang et al. 2004). In epithelial MDCK cells, pathways for direct delivery of newly synthesized proteins exist to both the apical and basolateral surfaces (Jacob and Naim 2001; Kreitzer et al. 2003), ensuring the asymmetric distribution of proteins that require sorting (Traub and Kornfeld 1997). In other epithelia, indirect pathways are common, where proteins are first transported to the basolateral surface domain from where they are endocytosed and delivered to the opposite surface via early and recycling endosomes (Huet et al. 2003; Mostov et al. 2000). However, the mechanisms behind the sorting of proteins directly from the TGN to the PM or into the indirect transendosomal route to the PM are not fully elucidated (Gravotta et al. 2007).

1.3 Sorting signals for the journey beyond the Golgi apparatus

The sorting machinery in the TGN controls multiple divergent pathways directed to spatially segregated acceptor compartments, such as the apical and basolateral plasma membranes of epithelial cells, early/sorting endosomes or late endosomes, recycling endosomes, secretory granules or Golgi stacks accepting retrogradely transported molecules (De Matteis and Luini 2008). A wide variety of signals located within the cytoplasmic domains of transmembrane proteins can mediate exit from the Golgi complex, including signals (DXXLL motif based) for sorting of mannose-6-phosphate receptors (M6PRs) which target newly synthesized acidic hydrolases modified with mannose-6-phosphate to the lysosomal compartment (Bonifacino and Traub 2003), and signals targeting proteins to the apical and basolateral domains of the plasma membrane in epithelial cells (Rodriguez-Boulan et al. 2005). Knowledge concerning the sorting signals directing secretory proteins in the apical and basolateral biosynthetic pathways is limited. However, N- and O- glycans, GPI-anchors, and glycosaminoglycan (GAG) chains, have been suggested to contain sorting information that drives secretory proteins in the apical direction (Alfalah et al. 1999; Benting et al. 1999; Kolset et al. 1999b; Scheiffele et al. 1995).

1.3.1 Basolateral sorting signals

Polarized epithelial cells maintain their polarity by ensuring asymmetric distribution of newly synthesized proteins, transporting distinct populations of proteins to their apical and basolateral membrane domains in a signal- mediated manner. Much work has been carried out to identify the sorting signals that drive proteins along the secretory pathway. The first basolateral sorting signal to be proposed was a signal in the cytoplasmic tail of the polymeric immunoglobulin A receptor (pIgR) (Mostov et al. 1986). Basolateral sorting of transmembrane protein is now generally known to depend on cytoplasmic peptide sequences, some of which conform to tyrosine (NPXY or YxxΦ) consensus motifs and others to dileucine (DXXLL or [DE]XXXL[LI]) based motifs (Nelson and Yeaman 2001). Additional discoveries, like a basolateral sorting signal for Stem Cell Factor (SCF) comprising a single leucine motif with a cluster of acidic amino acids at its N-terminal

(Wehrle-Haller and Imhof 2001) further substantiated the evidence that basolateral sorting of transmembrane proteins can be mediated by a variety of signals, and that the signal is located at the cytoplasmic domain. A glycosylphosphatidyl-inositol (GPI) linked heparan sulfate proteoglycan (HSPG), glypican, which when expressed in MDCK cells, appeared at the basolateral membrane was detected mostly at the apical membrane after removal of the site for HS GAG chain attachment (Mertens et al. 1996), suggesting a role of HS GAG chains in basolateral sorting of glypican. Basolateral signals with tyrosine and dileucine based motifs interact with adaptor protein (AP) complexes and clathrin to direct proteins to the basolateral cell surface (Bonifacino and Traub 2003; Potter et al. 2006b; Rodriguez-Boulan and Musch 2005). Several adaptor protein family members like AP1B, AP3 and AP4 seem to be involved in sorting of basolateral membrane proteins (Folsch et al. 1999; Simmen et al. 2002). AP1B and AP1A with common β, γ, and σ subunits are involved in endosomal targeting from the TGN in a clathrin dependent manner (Ohno et al. 1999). However, AP1B, having an epithelial-specific μ1B subunit (Ohno et al. 1999) is also involved in promoting the sorting of LDL and transferrin receptors (LDLR and TfR) at recycling endosomes (Gan et al. 2002). The sorting of the Mannose-6-phosphate receptor (M6PR) at the TGN to endosomes, is facilitated by the cooperative activity of AP1 and GGAs (Golgi localized, γ-ear-containing, Arf binding proteins) which require the involvement of clathrin (Bonifacino and Lippincott-Schwartz 2003; Ghosh et al. 2003; Puertollano et al. 2001). AP3 has been shown to regulate the exit of vesicular stomatitis virus glycoprotein (VSVG) from the Golgi complex in non-polarized cells (Nishimura et al. 2002), while AP4 has been proposed to participate in the basolateral sorting of LDLR, TfR and M6PR (Simmen et al. 2002). In contrast to other adaptor proteins like AP1, AP2 and AP3 which have a predicted clathrin binding site, AP4 is devoid of such a clathrin binding site and might therefore participate in the formation of tubules directed to the basolateral membrane (Rodriguez-Boulan and Musch 2005). Clathrin has been implicated to have a role in the transport of PM protein to the basolateral membrane of epithelial MDCK cells. The depletion of clathrin in MDCK cells by siRNA led to loss of polarized transport of basolateral PM proteins including TfR, VSVG, E-cadherin, and neural cell adhesion molecule (NCAM) (Deborde et al. 2008). In contrast to the sorting signals of TM proteins in the basolateral pathway, sorting signals directing secretory proteins in the basolateral direction have not been determined to date. However, for secretory proteins such as apoA-I and apoA-II, it has been suggested that

preferential basolateral transport is facilitated by cell-dependent default pathways and not by sorting signals in both Caco-2 and MDCK cells (Remaley and Hoeg 1995; Rindler and Traber 1988).

1.3.2 Apical sorting signals

In contrast to sorting signals discovered for basolateral transmembrane proteins, which are mainly confined to their cytoplasmic domain, apical sorting signals known to date are more diverse. Different types of apical sorting signals, including those located in extracellular, transmembrane, and cytoplasmic protein domains (Lin et al. 1998; Mostov et al. 2000) drive apically destined proteins along the biosynthetic route. In addition, apical sorting signals have also been postulated to reside in the glycosylphosphatidylinositol (GPI) lipid modification and N- and O-glycan moities of proteins (Potter et al. 2006). Apical sorting information might also be localized to the glycan moieties of chondroitin sulfate (CS) proteoglycans (Kolset et al. 1999). The utilization of diverse sorting signals for apical proteins makes the elucidation of the mechanisms behind the delivery in this direction difficult. The observation of the presence of glycosylphosphatidylinositol-anchored proteins (GPI-Aps) at the apical surface of MDCK cells (Lisanti et al. 1988) led to the postulation of the GPI-anchor as an apical sorting signal, because recombinant addition of a GPI-anchor to a secretory protein (Lisanti et al. 1989) resulted in apical surface localization of the chimeric plasma membrane protein (Rodriguez-Boulan et al. 2005). However, a selective point mutation resulting in removal of the GPI-anchor from naturally N-glycosylated and GPI-anchored membrane dipeptidase (MDP) did not affect apical sorting (Pang et al. 2004) and the coupling of the secretory protein rGH with a GPI- anchor did not increase apical transport of the protein (Benting et al. 1999). These data indicated that a GPI-anchor is not always sufficient to route a protein to the apical surface. The apical sorting of GPI-anchored proteins was suggested to be the result of the ability of such proteins to segregate into glycolipid- and cholesterol-enriched microdomains or lipid “rafts” (Brown and London 1998). The introduction of N-glycans in the secretory protein rat growth hormone (rGH) (Scheiffele et al. 1995) and the GPI-anchored version of rGH (Benting et al. 1999) in MDCK cells changed its surface transport pattern, triggering more apical secretion as compared to secretory and GPI-linked unglycosylated rGH. N-glycans on several secretory

proteins like erythropoetin (Epo) (Kitagawa et al. 1994) and endolyn (Potter et al. 2006), and on naturally N-glycosylated and GPI anchored membrane dipeptidase (MDP) (Pang et al. 2004) confer apical sorting in MDCK cells, N-glycans are, however, not universal signals for apical sorting. The complete removal of N-glycans from the soluble secretory protein hepatitis B surface antigen (HBsAg) (Marzolo et al. 1997), and from the voltage- and Ca^{2+}-activated K^{+} channel alpha -subunit endogenous plasma membrane protein in MDCK cells (Bravo-Zehnder et al. 2000) did not affect their apical transport, suggesting that oligosaccharide targeting is one of multiple mechanisms that can promote apical sorting (Mostov et al. 2000). Apical sorting can also be mediated by O-glycosylation (Weisz and Rodriguez-Boulan 2009). The addition of an O-glycosylated stalk region, which is important for apical sorting of the small intestinal membrane glycoprotein sucrase-isomaltase (SI), to the secretory protein rGH by converting it to a membrane protein, resulted in strong sorting preference to the apical surface (Alfalah et al. 1999; Spodsberg et al. 2001). O-glycosylation mediated the association of rGH with lipid rafts (Weisz and Rodriguez-Boulan 2009). Apical targeting information may also be constituted by proteinaceous motifs (Rodriguez-Boulan and Musch 2005). Sorting signals in cytoplasmic tail sequences of apical proteins, like the multiligand receptor megalin (Takeda et al. 2003), the light sensitive protein rhodopsin (Chuang and Sung 1998), and receptor guanylyl cyclase (GC) (Hodson et al. 2006) target these proteins to the apical membrane. Apical sorting signals can also reside within transmembrane domains of apically sorted proteins (Folsch et al. 2009) as shown for influenza haemagglutnin (HA) and neuraminidase (NA) (Kundu et al. 1996; Lin et al. 1998). The identity of apical sorting signals is, however, a matter of continued debate (Ihrke et al. 2001), and the sorting mechanisms utilized by apical signals are still poorly understood (Rodriguez-Boulan and Musch 2005). Inhibition of glycosylation of the tight junction protein occludin resulted in accumulation of the protein in the Golgi complex of MDCK cells (Gut et al. 1998), implying the involvement of oligosaccharides in protein sorting. A suggested sorting lectin for apical selection of cargo was the vesicular integral protein (VIP) 36 (Fiedler and Simons 1996), proposed to recognize carbohydrates, such as N-glycans in the TGN. However, the discovery that VIP 36 is predominantly localized to the early secretory pathway (Fullekrug et al. 1999) and recognizes high-mannose glycans (Yamaguchi et al. 2007) , put the candidacy of this vesicular integral membrane protein as an apical sorting receptor to rest. Interestingly, the proposal that sorting of cargo in the biosynthetic

pathway of polarized cells could occur earlier than previously thought (Alfalah et al. 2005; Tveit et al. 2005) might reinstate VIP 36 as a potential sorting receptor for apical secretion, but VIP36 might actually be involved in a quality control cycle between the ER and the Golgi apparatus (Reiterer et al. 2010). The importance of oligomerization for proper targeting and stabilization of GPI-Aps into rafts was also proposed from the observation that GPI-anchored green fluorescent protein (GPI-GFP) was misrouted to the basolateral surface when oligomerization/cluster formation was impaired by the introduction of a mutation in the GFP gene (Paladino et al. 2004).The lipid raft hypothesis which postulates that certain proteins, among these GPI-linked proteins, are sorted apically, because of their affinity for glycosphingolipid and cholesterol rich domains was challenged, however, when it failed to account for the presence of some GPI-Aps on the basolateral surface of MDCK cells (Paladino et al. 2004). However, further investigation revealed that the level of cholesterol was a driving factor for the proper oligomerization, which in turn was necessary for the proper apical sorting of GPI-APs. This was verified when addition of cholesterol resulted in oligomerization and more apical transport of GFP-PrP which is sorted basolaterally as monomers (Paladino et al. 2008), indicating the importance of the lipid environment in apical sorting.

2. Glycoproteins and proteoglycans

Glycoproteins are proteins to which carbohydrates are covalently attached and modified in a stepwise manner by a process called glycosylation. The attachment of oligosaccharides to a protein can occur either by co-translational or posttranslational mechanisms and is catalyzed by the action of a variety of glycosyltransferases. Glycans play important functional and structural roles in both membrane-associated and secreted proteins and can be classified according to their attachment site in a protein. Most glycoproteins carry N-glycans, where the carbohydrate is attached to a polypeptide through the amide group of an asparagine (Asn) residue forming N-glycosidic bonds. O-glycans are linked to a protein through the hydroxyl group of serine, threonine or hydroxylysine residues of polypeptides which result in the formation of O-glycosidic bonds. Proteoglycans (PGs) are glycoproteins consisting of a protein core to which chains of repeating disaccharide units called glycosaminoglycans (GAGs) are attached via a linker tetrasaccharide to the hydroxyl group of a serine residue. GAG disaccharides are most often composed of either N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc), and a uronic acid such as glucuronate or iduronate. GAGs are unbranched polysaccharides with high negative charge, due to modification of the disaccharide units by sulfate and the charge of the uronic acid groups. PGs can be classified as chondroitin sulfate (CS)/ dermatan sulfate (DS), heparin/heparan sulfate (HS), and keratan sulfate (KS) based on the type of GAGs they contain. The only GAG with no bound core protein is hyaluronic acid, which contains GlcNAc and GlcA sugars.

2.1 Synthesis of N- and O -linked Glycans

The synthesis of N-linked glycoproteins is initiated co-translationally in the endoplasmic reticulum (ER) and is completed in the Golgi apparatus by post-translational modifications involving a number of glycosidases and glycosyltransferases. An N-linked oligosaccharide arises when a core oligosaccharide ($Glc_3Man_9GlcNAc_2$) assembled on the lipid carrier dolicholpyrophosphate in the ER membrane, is transferred cotranslationally to nascent polypeptide chains with the acceptor sequon Asn-X-Ser/Thr, where X can be any amino acid except proline (Freeze and Aebi 2005; Gavel and Vonheijne 1990; Helenius and Aebi 2001),

by the action of oligosaccharyltransferase (OST), localized to the ER membrane (Silberstein and Gilmore 1996). In the ER, protein-bound N-linked oligosaccharides have specific functions in protein folding and quality control, and in forward transport.

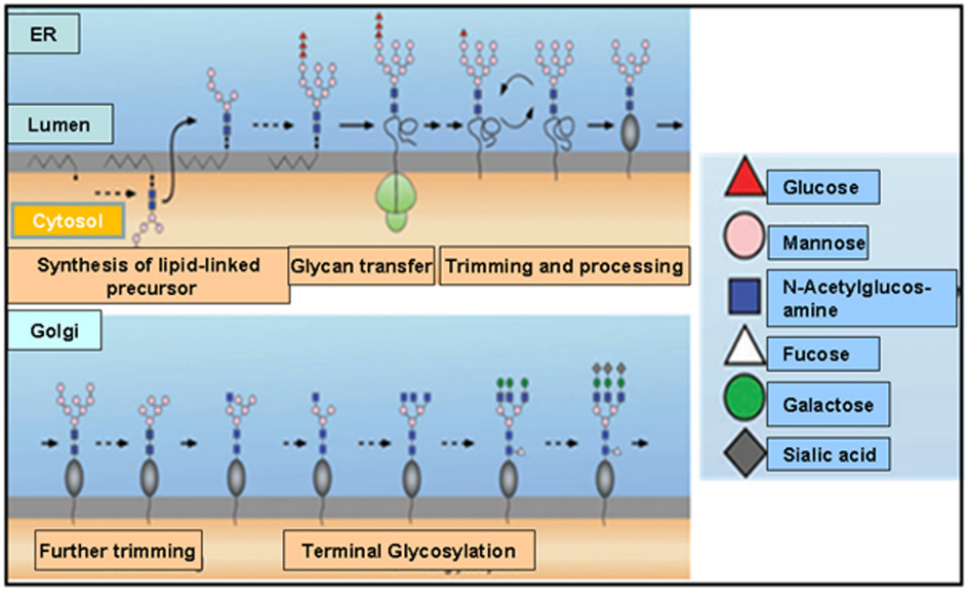

Fig. 3. Biosynthesis of N-linked glycans. Adapted from (Helenius and Aebi 2001)

The overall structure of N-glycans can be classified as complex, hybrid, or high mannoses (oligomannose), which share a common core sugar structure, Man α-(1–6) - Man α-(1–3)-Man β-(1–4)-GlcNAc β-(1–4)-GlcNAc β1-Asn-X-Ser/Thr. The three major stages in the pathway for synthesis of N-glycans include the assembly of the core oligosaccharide on the lipid carrier dolichol through a pyrophosphate linkage by the action of enzymes on both the cytoplasmic and the lumenal side of the ER (Gahmberg and Tolvanen 1996; Helenius and Aebi 2001; Kornfeld and Kornfeld 1985). The synthesis of the lipid-linked precursor starts at the cytoplasmic face of the ER membrane by the addition of two GlcNAc and five mannose residues provided by nucleotide sugar donors, UDP-GlcNAc and GDP-Man, respectively. When this is completed, the lipid carrier with bound sugars is flipped to the ER lumen, where four more mannose and three glucose residues are provided as dolichol linked sugars which are added one sugar at the time to form a complete core oligosaccharide. The transfer of the completed lipid-linked core oligosaccharide to a growing nascent polypeptide

acceptor also takes place in the lumen of the ER and the core oligosaccharide is coupled to the asparagin residue in an Asn-X-Ser/Thr acceptor sequence catalyzed by oligosaccharyltransferase, a complex enzyme with its active site in the lumen of the ER (Silberstein and Gilmore 1996), marking the second stage in the synthesis of N-glycans. The second phase of the glycosylation process involves both the removal of selected monosaccharides in the ER and the rebuilding by addition of other monosaccharides in the Golgi apparatus. This introduces structural diversity to the newly synthesized N-glycans, due to non-uniform modifications, especially terminal glycosylation in the Golgi complex as a result of the variation in the expression and organization of modifying enzymes in the Golgi apparatus as well as the availability of substrates (Freeze and Aebi 2005). N-glycans have a common role in events like protein folding, quality control, and in sorting events early in the secretory pathway, whereas further modification in the Golgi gives rise to diverse mature glycoproteins on the cell surface (Helenius and Aebi 2001). N-glycans transferred to the nascent polypeptide are subjected to further trimming, where terminal glucose and mannose residues are removed sequentially by the action of glucosidases I and II, and mannosidase I in the ER (Kornfeld and Kornfeld 1985). Glucosidase I is responsible for the removal of the terminal glucose residue, whereas glucosidase II removes the remaining two inner glucose residues, signalling that the newly synthesized glycoprotein is ready to transit from the ER to the Golgi apparatus, provided that it has passed the quality control regime, which ensures the correct folding of newly synthesized glycoproteins, assisted by calnexin and calreticulin lectin chaperones in the ER. The removal of three terminal glucose residues is imperative in order that further processing to the mature carbohydrate unit can take place (Spiro 2000). ER and *cis*-Golgi resident mannosidases together remove four additional mannose residues from the core oligosaccharide, generating a series of oligomannose-type N-glycans in the ER and the Golgi apparatus (Tomiya et al. 2004) and these high mannose variants move through various compartments to the cell surface, escaping further modifications constituting the third stage in the synthesis of N-glycans. This stage primarily occurs in the Golgi apparatus, where synthesis of other complex glycan structures is initiated by the action of Golgi resident transferases. It starts when N-acetylglucosaminyltransferase I (GnT I), which is located in the medial portion of the Golgi, transfers one GlcNAc residue donated by UDP-GlcNAc to the Man α-(1-3) arm of the core oligosaccharide, followed by the subsequent removal of two mannose residues by

medial-Golgi mannosidase II from the (1-6) arm of the core oligosaccharide. N-acetylglucosaminyltransferase II (GnT II) then adds the second GlcNAc residue to the (1-6) arm of the core, resulting in the synthesis of a bi-antennary complex structure (Schachter 2000). Further in the secretory pathway, the complex structures are extended by the addition of a single galactose unit donated by UDP-Gal and a sialic residue from cytidine-monophosphate-NeuAc (CMP-NeuAc) to each terminal GlcNAc residue in the core oligosaccharide, by the action of galactosyltransferases (GalTs) and sialyltransferases (SiaTs), respectively in the *trans*-Golgi compartment. The activated donor nucleotide sugars are made in the cytoplasm and must be transported into the lumenal compartment of the ER, facilitated by nucleotide sugar transporter molecules (Freeze and Aebi 2005). N-glycans with hybrid structure are formed when α-mannosidase no longer acts after the introduction of the first GlcNAc residue to the core oligosaccharide by GnT I, due to the competition by other glycosyltransferases for the common substrate (Schachter 1991). Different cell types can generate a variety of biosynthetic enzymes which can transfer different sugar residues to a growing oligosaccharide group, thereby synthesizing glycans of variable structure on a given protein in a cell type and tissue specific manner. These structures can undergo time-dependent changes during development and metastatic progression (Schachter 1991). Altered expression of GnT-V was suggested to affect the glycan structure and function of epidermal growth factor receptor (EGFR) in hepatocarcinoma cells (Guo et al. 2004). Mouse embryos lacking GnT I, producing only the high-mannose type, was shown to have failure in neural tube formation, vascular system malformation, and lack of bronchial epithelium (Fukuda and Akama 2003). Altered glycosylation in terms of alteration in sialylation and fucosylation of the cell surface exposed glycans have been observed in MDCK cells after transformation by murine sarcoma virus (Bruyneel et al. 1990). Altered glycosylation might also be caused by a change in the pH of the Golgi apparatus lumen which impairs glycosylation by interfering in processes including nucleotide sugar transport, activity of glycosyltransferases in either reducing their activity or mislocalizing of Golgi glycosyltransferases into post-Golgi organelles (Axelsson et al. 2001; Gawlitzek et al. 2000; Waldman and Rudnick 1990). Elevated Golgi pH affected the status of terminal N-glycosylation in African green monkey kidney cells (COS-7) (Rivinoja et al. 2009). However, the mechanism by which neutralization of the slightly acidic pH in the Golgi affects glycosylation is not known. Most of Aberrant glycosylations are results of altered

SiT expression in human cancers (Sotiropoulou et al. 2002; Wang et al. 2005). Increased fucosylation and /or decreased sialylation has been observed in cystic fibrosis airway epithelial cells as compared with the non-cystic fibrosis, due to a change in the activity of fucosyltransferases (Glick et al. 2001). Increased expressions of α-(2-6)-linked sialic acids on N-glycans have been usually associated with human cancer progression, metastatic spread (Hedlund et al. 2008). Mass spectrometric analysis of glycan structures in Swine Respiratory Epithelial cells (SRECs), a primary target cell type of influenza virus, revealed the presence of a variety of glycans terminating in sialic acid, where α-(2-6)-linked sialylation was more abundant than α-(2-3)-linked sialylation, and N-acetylneuraminic acid (NeuAc) was more abundant than N-glycolylneuraminic acid (NeuGc) (Bateman et al. 2010). Both human respiratory tract and chicken intestine express NeuAc, whereas both NeuAc and NeuGc are present in swine trachea (Guo et al. 2007; Suzuki et al. 1997). Neural Cell Adhesion Molecules (NCAMs), which play a role in neural development, are highly polysialylated in embryonic tissues, as compared with adult tissues (Angata et al. 2000; Kiss and Rougon 1997; Rutishauser and Landmesser 1996) indicating the variation of the N-glycan structure with time, tissue and developmental stage. The abundance of polysialylated NCAM molecules is dependent on the expression level of different polysialtransferases during different developmental stages (Angata and Fukuda 2003; Muhlenhoff et al. 1998). The structure of N-glycans, both secretory and transmembrane, derived from MDCK cell is not well studied. The structural analysis of N-glycans, both secretory and transmembrane variants, derived from the apical and basolateral membrane domains of polarized MDCK cells has revealed no significant difference in the overall structure of N-glycans between the two domains. However, the secretory proteins from both the apical and basolateral domains were predominantly composed of N-glycans with Sia α-(2-6)-GalNAc β-(1- 4)-GlcNAc sequence (Ohkura et al. 2002). N-glycans with high-mannose structure have also been observed to appear at the cell surface, possibly utilizing a secretory transport route bypassing the Golgi apparatus. For instance, protein-tyrosine phosphatase CD45 in T lymphoma cells (Baldwin and Ostergaard 2002), the AE1 anion exchanger (Ghosh et al. 1999), and the subunits of the sodium channel (ENaC) at the apical surface of epithelial cells (Weisz and Johnson 2003), predominantly contain high-mannose sugars which are endo-H sensitive. Defects in the N-glycan synthesis pathway have been implicated in different types of congenital disorders of glycosylation (CDG) which result from deficiencies in either the

biosynthesis of oligosaccharide precursors or steps in N-glycan assembly, affecting especially normal brain development and functions of the nervous, hepatic, gastrointestinal and immune systems (Freeze and Aebi 2005; Jaeken 2003; Marquardt and Denecke 2003).

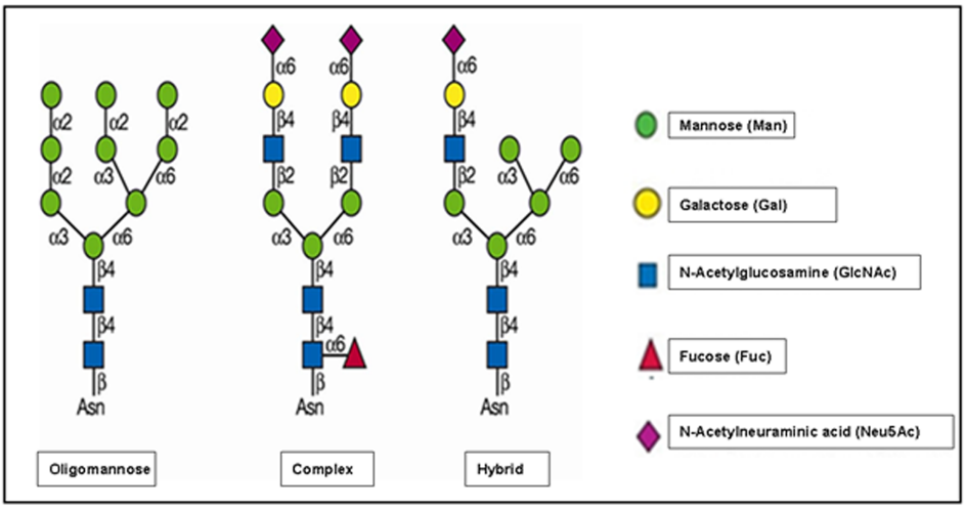

Fig. 4 Main types of N-glycans. Modified from "Essentials of Glycobiology" second edition (2009).

O-linked glycans are usually added to a protein core through the hydroxyl group of Ser or Thr residues on the polypeptide in the Golgi apparatus. In contrast to the synthesis of N-glycans, addition of O-glycans to a polypeptide does not require a lipid carrier in eukaryotes O-linked glycoproteins are synthesized by the stepwise addition of one sugar at the time, donated from nucleotide sugars imported into the secretory pathway, directly onto the glycoprotein by a glycosyltransferase. The first step in the O-glycosylation pathway is transfer of GalNAc to either a serine or threonine residue of a protein (Potapenko et al. 2010), by the action of a N-acetylgalactosaminyltransferase (Ten Hagen et al. 2003). There is no known consensus amino acid sequence or core structure identified for O-glycosylation, it can occur on several adjacent Ser or Thr residues, especially in mucins (Gill et al. 2010). O-linked glycans have been implicated in a variety of biological activities, including as

recognition markers for different blood antigens, as adhesive ligands, and as modulators of cell-cell signalling (Aoki et al. 2008).

2.2 Synthesis of proteoglycans (PGs) and glycosaminoglycans (GAGs)

PGs are found at the cell surface, in the extracellular matrix (ECM) or intracellularly in secretory granules (Kjellen and Lindahl 1991; Kolset et al. 2004).The biological functions of GAGs range from functions such as modulating signal transduction pathways, cell proliferation and angiogenesis, adhesion, cell migration (Souza-Fernandes et al. 2006), wound healing and blood clotting (Kuberan et al. 2008). PGs can also be used by many pathogens for entry into cells (Handel et al. 2005). GAGs interact with specific proteins depending on their sulfation pattern. Particular sulfation patterns in heparan sulfate GAG chains allow interactions of ionic nature with growth factors (Prydz and Dalen 2000; Schaefer and Schaefer 2010). The secretory pathway is the site of initiation, formation of the GAG linker region. Further elongation/polymerization and modification of the GAG chains involves various biosynthetic enzymes. Activated sugar and sulfate donors like UDP-sugars and 3'-phosphoadenosine 5'-phosphosulpate (PAPS) are required for the biosynthesis of GAGs. These activated sugars and sulfate donors are then translocated from the cytoplasm to the lumen of the ER and the Golgi apparatus by specific sugar and sulfate transporters (Hirschberg et al. 1998; Mandon et al. 1994). The formation of GAGs occurs through a sequence of events. The first step in GAG biosynthesis is chain initiation by the transfer of a xylose (xyl) residue to a serine amino acid adjacent to a glycine in the core protein, followed by the assembly of a tetrasaccharide linkage serving as acceptor for the chain elongation by the alternate addition of D-gluronic acid and aminosugars (Garud et al. 2008; Kuberan et al. 2008; Prydz and Dalen 2000; Victor et al. 2009). In the linker tetrasaccharide the xyl is followed by two galactoses (Gal) and finally glucuronic acid (GlcA) forming a common linkage [GlcA β-(1–3)-Gal β-(1–3) Gal β-(1–4)Xyl β-(1-O-Ser)] between the core protein and the GAG chain (Lindahl and Roden 1966). The synthesis of the linker tetrasaccharide is probably initiated in the ER or a subsequent compartment by the addition of xylose to a core protein, followed by addition of the two galactoses in the *cis/medial* Golgi region and the

linker is completed by the addition of the glucuronic acid unit in the *medial/trans* Golgi (Silbert and Sugumaran 2002). The linker region can be modified by phosphorylation of xylose at C-2 for both CS and HS GAGs (Fransson et al. 1985; Oegema et al. 1984). Sulfation of one or both galactose residues in the linker region of CS/DS has been found in various tissues, but never in HS/heparin (Sugahara and Kitagawa 2000). It is the addition of the fifth sugar that decides whether a GAG chain becomes glucosaminoglycan (HS/heparin) or galactosaminoglycan (CS/DS), since the same enzymes are involved in the biosynthesis of the linker tetrasaccharide for both types of GAG chains (Kjellen and Lindahl 1991; Prydz and Dalen 2000). The mechanism that commits the process towards the synthesis of CS/DS or HS/heparin PGs is not fully understood, and a single consensus sequence for chain initiation is not clearly defined yet. However, the GAG binding domain serving as an acceptor site for xyl transfer has a repetitive Ser-Gly consensus sequence flanked by a cluster of acidic amino acid residues for the synthesis of HS in betaglycan and the substitution of these acidic residues resulted in the synthesis of more CS (Zhang and Esko 1994). This might indicate the importance of the GAG binding domain and the acidic residues nearby in determining the outcome of the GAG synthesis process. The mutation of an adjacent tryptophan residue resulted in the synthesis of less HS and a shift towards the synthesis of more CS, while the introduction of a tryptophan closer to a CS site triggered the production of more HS (Zhang and Esko 1994), again implying the influence a globular residue might have on the type of GAG chains to be synthesized. Recent studies of alignment of amino acid sequences of several CS attachment sites from various core proteins resulted in the generation of the consensus sequence a-a-a-a-Gly-Ser-Gly-a-b-a (a =Glu/Asp and b = Gly, Glu or Asp) (Silbert and Sugumaran 2002). Serglycin isolated from the rat yolk sac carcinoma cell line L2, on the other hand contains 24 consecutive Ser-Gly repeats where only one of these repeats is flanked by acidic amino acid residues (Avraham et al. 1989; Bourdon et al. 1986; Kjellen et al. 1989; Stevens et al. 1988). All these findings, however, do not indicate the existence of a universal consensus sequence for GAG attachment onto the core protein. The type of GAG chain synthesized might also be affected by a domain at a distance from the GAG binding sites in the protein core. Insertion of an N-terminal globular domain of the HSPG glypican-1 at a significant distance from the GAG binding sites in another PG enhanced the assembly of HS chains, while removal of the same globular domain from glypican-1 converted this PG into a PG with predominantly CS chains (Chen

and Lander 2001). Cellular mechanisms underlying GAG biosynthesis have also been studied using different exogenous xylosides called click-xylosides, containing various hydrophobic aglycone moieties in Chinese hamster ovary (CHO) cells. The study revealed that the introduction of external click-xylosides generated free GAG chains of variable CS composition and quantity, as well as GAG chains with different sulfation intensity, sulfation pattern and chain length (Victor et al. 2009). The synthesis of GAG chains with different modifications after the admnistration of xylosides to CHO cells can be explained in light of the GAGosome model. In this model, each GAGosome is composed of different set of modifying enzymes which, when recruited, generate heterogeneous GAG chains with variable structure, sulfation pattern as well as length. In contrast to heparin that occurs exclusively in the connective tissue mast cells, HS is produced by most cells in the body (Lindahl and Li 2009). CS/DS PGs, on the other hand, are synthesized mostly in vertebrate cells as major components of connective tissue matrix (Kjellen and Lindahl 1991b; Lindahl and Li 2009).

Keratan sulfate (KS) is another sulfated GAG and is abundant in cornea, and is also present in cartilage and brain. KS does not contain the same linker tetrasaccharide structure as CS- and HSPGs. The core structure of KS consists of repeating -[3Gal-1-4GlcNAc-1]- disaccharide units (N-acetyllactosamine) attached to a complex type biantennary oligosaccharide N-linked on Asn residue in KS type I in cornea, whereas the core structure of KS II in cartilage is attached to protein through GalNAc-O-Ser/Thr (Funderburgh 2000). KS is synthesized via the action of glycosyltransferases that alternately add Gal and GlcNAc residues to the growing polymer. In humans, β4GalT-IV and β3Gn-T7 have been shown to activate the polymerization of oligosaccharides in corneal KS (Kitayama et al. 2007). The core oligosaccharide polymer in KS can be modified by sulfation occuring on C-6 of both Gal and GlcNAc residues (Lauder et al. 1997). Sulfate modification of KS GAGs varies between tissues and is carried out by a variety of sulfotransferases (STs). For instance, 6-O-sulfation of GlcNAc residues is carried out by GlcNAc-6ST-5 in cornea (Akama et al. 2000; Hayashida et al. 2006), whereas GlcNAc-6ST-1 catalyzes the sulfation of KS GAGs in brain (Zhang et al. 2006).

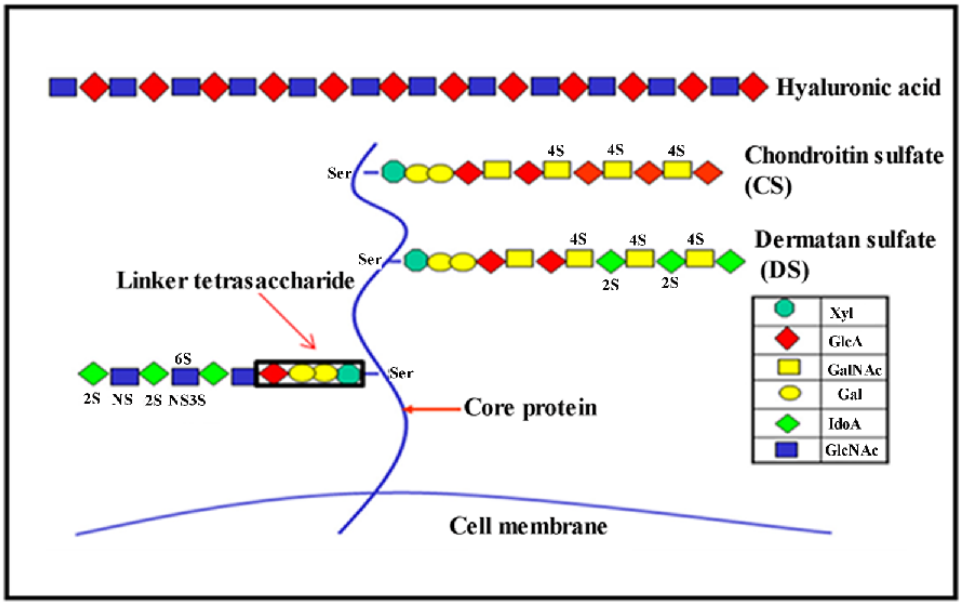

Fig. 5. Schematic structure of glycosaminoglycan chains attached to a proteoglycan and a free hyaluronic acid chain. Adapted from: (Souza-Fernandes et al. 2006)

2.2.1 Chondroitin sulfate synthesis

Chondroitin/dermatan sulfate GAGs are composed of chains of alternating N-acetylgalactosamine (GalNAc) and glucuronic acid (GlcA) or iduronic acid (IdoA) residues, and can consist as many as 100 repeating disaccharide units (Silbert and Sugumaran 2002). Chondroitin sulfates serve as an important structural component of cartilage, providing resistance to compression. The specificity for the synthesis of CS/DS GAGs is provided by the transfer of the first GalNAc residue after completion of the linker tetrasaccharide. The basis for this commitment, and as a result, the emergence of cell-specific GAG chains with a variety of distinct sulfation patterns is not yet fully understood. However, an enzyme that is involved in the transfer of the first GalNAc residue is different from the enzymes that transfer GalNAc in the polymerization process (Ogawa et al. 2010; Sugahara and Kitagawa 2000). Peptide sequence motifs close to GAG bound serine residues could influence the addition of a GalNAc or GlcNAc units to initiate the polymerization of a particular type of GAG chains (Esko and Zhang 1996). CS/DS chain elongation or polymerization is carried

out by the action of two distinct transferases involved in the alternating transfer of GalNAc and GlcA (Kitagawa et al. 2001; Silbert and Sugumaran 2002). Six glycosyltransferases are known to be involved in CS synthesis: chondroitin sulfate synthase 1-3 (CSS)1-3, chondroitin sulfate glucuronyltransferase , and chondroitin N-acetylgalactosaminyltransferase-1 and-2 (Kitagawa et al. 2001; Sato et al. 2003; Yada et al. 2003a; Yada et al. 2003b), all containing an N-terminal transmembrane domain. (CSS)1-3 exhibit both N-acetylgalactosaminyltransferase-II (GalNAcT-II) and glucuronyltransferase-II (GlcAT-II) dual enzymatic activities (Ogawa et al. 2010). Chondroitin sulfate glucronyltransferase, on the contrary, displays only GlcAT-1 activity despite having two glycosyltransferase domains (Gotoh et al. 2002). CS GAG chains acquire a variety of modification patterns by the transfer of sulfate groups to hydroxyl groups of both GalNAc and GlcA residues. These modifications are mediated by the action of different STs, giving rise to non-, mono-, di-, or tri- sulfated disaccharides (Kusche-Gullberg and Kjellen 2003). For example, CS can be sulfated as GalNAc 4-sulfate, as GalNAc 6-sulfate, with both GalNAc 4-sulfate and GalNAc 6-sulfate in the same GAG chains, and occasionally as GalNAc 4,6-disulfate, and also as GlcA 2- or 3- sulfate in combination with GalNAc 4-sulfate or GalNAc 6-sulfate (Silbert and Sugumaran 2002). Epimerization is another modification that occurs to GAG chains as a result of the conversion of GlcA residues at C-5 by chondroitin glucuronate C-5 epimerase (Maccarana et al. 2006) to iduronic acid (IdoA) residue at the polymer level, constituting the formation of dermatan sulfate (DS) (Kjellen and Lindahl 1991; Silbert and Sugumaran 2002; Victor et al. 2009). The hydroxyl groups of GalNAc and IdoA in dermatan sulfate (DS) disaccharides may be substituted by sulfate groups as follows: GalNAc at C-4 and IdoA at C-2 in combination with both non-sulfated GalNAc and IdoA and some sulfated GalNAc at C-6 (Ernst et al. 1995; Saito et al. 1968).

2.2.2 Heparan sulfate synthesis

Heparan sulfate (HS)/heparin contains a carbohydrate backbone constituted by alternating GlcNAc and GlcA or IdoA sugars. As already discussed above, after the completion of the tetrasaccharide linker region synthesis, the polymerization of HS chains is initiated by the addition of a GlcNAc residue by GlcNAc transferase I (GlcNAc-TI) before polymerization proceeds through the alternate transfer of GlcA and GlcNAc sugars, catalyzed by two

complexed glycosyl transferases, EXT1 and EXT2 (Hereditary multiple exostoses gene products) (Busse et al. 2007; Carlsson et al. 2008; Sasisekharan and Venkataraman 2000). Modification of HS chains occurs while the chain is being elongated. The nascent chain is modified concomitantly and sequentially by the action of a series of enzymes (Sasisekharan and Venkataraman 2000). The first modification of the HS chain is initiated by N-deacetylation and N-sulfation of GlcNAc units by the action of the bifunctional enzyme N-deacetylase/N-sulfotransferase (NDST), requiring PAPS as the sulfate donor (Busse et al. 2007; Carlsson et al. 2008; Salmivirta et al. 1996). Four NDST enzymes (NDST1-4) have been identified (Kusche-Gullberg and Kjellen 2003), with NDST1 and NDST2 being the most widely expressed (Grobe et al. 2002). Subsequent HS chain modifications include epimerization of GlcA to IdoA carried out by HS glucuronyl C-5 epimerase (Li et al. 1997), and 2-O-sulfation of iduronic acid and 6-O and 3-O -sulfation of N-acetylated and N-sulfated GlcN residues (Busse et al. 2007), involving several STs. These modifications are largely confined to N-sulfated regions of the HS chains, indicating the importance of bifunctional NDST enzymes in determining the final structure of HS chains (Carlsson et al. 2008). The magnitude of these modifications varies and results in the generation of heterogeneous HS domains. In comparison with HS GAG chains, heparin is more intensely sulfated and contains a larger proportion of N-sulfated GlcN and IdoA (Kusche-Gullberg et al. 1998). Heparin is defined by the 3-O-sulfate that allows binding of antithrombin, thus the anticoagulant activity.

3. Model protein in this study

The model protein used in this study was rat growth hormone (rGH), fused to green fluorescent protein (GFP) at the C-terminus. rGH is a non-glycosylated single polypeptide having a molecular mass of approximately 22 kDa. The model protein was further mutated by the introduction of eight GAG binding sites transferred from the serglycin (SG) GAG attachment domain and by the introduction of two N-glycosylation sites into the polypeptide. Four different mutants with or without two N-glycosylation sites and with or without GAG binding sites have been generated and used in this study. These mutants are: 1) rGH-GFP, 2) rGH-2N-GFP, 3) rGH-2N-GAG-GFP, and 4) rGH-GAG-GFP. Previously it has been demonstrated that when stably expressed in MDCK cells, the wild type rGH was randomly secreted to both the apical and basolateral sides (Gottlieb et al. 1986) and hence can effectively be used as a reporter protein for the epithelial cell polarity studies. In addition, MDCK cells have been shown to synthesize and secrete both CS and HS proteoglycans (Svennevig et al. 1995).

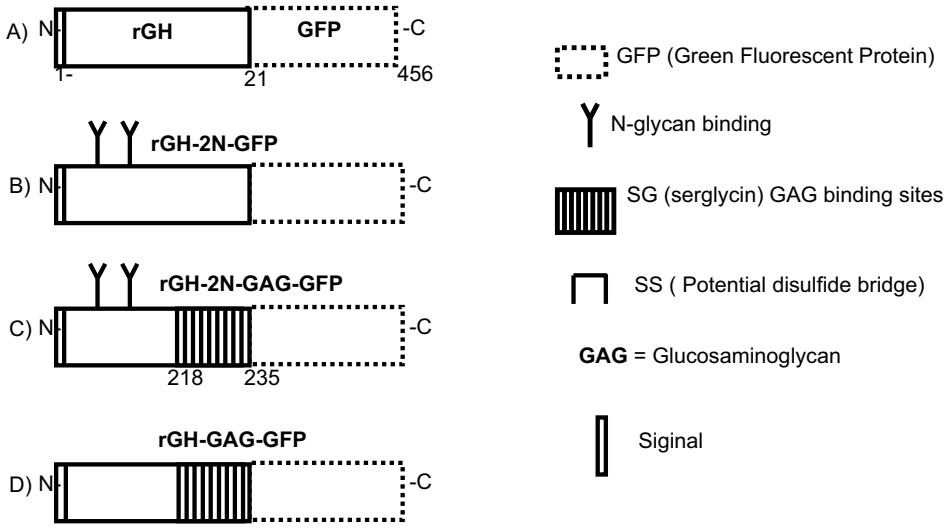

Fig. 6 Schematic of different mutants of the rGH model protein. A) rGH with bound GFP at C-terminus. B) rGH with two N-glycosylation sites. C) rGH with both N-glycosylation and GAG binding sites. D) rGH with GAG binding sites.

4. Aims of study

We have previously shown that a recombinant serglycin (SG) proteoglycan fused to green fluorescent protein modified mainly with CS GAG chains which were recovered mostly (85%) from the apical media of filter grown MDCK cells. Surprisingly, the remaining 15% recovered from the basolateral side were more intensely sulfated and contained shorter GAG chains as compared to their apical counterpart (Tveit et al. 2005; Vuong et al. 2006) constituting differential modifications of one and the same protein along the secretory pathway. The major aim of the study was to investigate whether the GAG binding domain of SG (with eight GAG binding sites), which was inserted into the rGH, would encode the synthesis of differentially modified GAG chains similar to the GAG chains of the parental SG. The second aim of the study was to investigate possible structural differences of N-glycans on rGH with and without GAG binding sites, secreted in the apical and basolateral directions. The third aim of the study was to investigate the influence of the protein conformation of the proteoglycan serglycin on the polymerization as well as sulfation of the GAG chains.

5. Summary of Papers

Paper I

Protein core dependent glycosaminoglycan modification and glycosaminoglycan dependent polarized sorting in epithelial MDCK cells.

Hafte TT, Fagereng GL, Prydz K, Grøndahl F, and Tveit H

Glycobiology vol. 21 no. 4 pp. 457–466, 2011

In this work, we have transplanted the GAG binding domain of the secretory protein SG into rGH and investigated the effect of this mutation on sorting and modification, specially the intensity and pattern of sulfation as well as the length of GAG chains attached to the rGH in the apical and basolateral secretory pathways in MDCK cells. Previously we have shown that GAG chains attached to SG and recovered from the apical and basolateral domains of MDCK cells are differentially modified interms of the intensity of sulfation and the length of GAG chains (Tveit et al. 2005; Vuong et al. 2006). GFP-fused rGH mutants were stably transfected in MDCK cells and MDCK cells expressing our mutants were radioactively labelled by ^{35}S-sulfate and ^{3}H-GlcN and sorting and modification investigated in both pathways. Our investigation revealed no difference in the intensity and pattern of sulfation as well as the length of GAG chains attached to rGH as compared to the differential modification achieved by the GAG chains of SG in the apical and basolateral domains of MDCK cells. The GAG binding domain of SG was not able to encode the synthesis of differentially modified GAG chains in rGH. Interestingly, the introduction of GAG chains to rGH clearly triggered more secretion in the apical direction demonstrating the involvement of GAG chains in the polarized sorting in MDCK cells. However, the introduction of both N-glycan and GAG binding sites to the rGH did not exhibit a synergetic effect by increasing the secretion in the apical direction as it was assumed.

Paper II

N-glycan synthesis in the apical and basolateral secretory pathways of epithelial MDCK cells on a model protein core and the influence of a glycosaminoglycan domain

Moen A, Hafte TT, Tveit H, Egge-Jacobsen W, Prydz K.

Accepted manuscript with minor revisions (Glycobiology)

N-glycans are implicated to play a role in apical sorting of both secretory and GPI-anchored glycoproteins (Benting et al. 1999; Pang et al. 2004; Scheiffele et al. 1995). Also other classes of glycans have been suggested to harbor apical sorting information (Kolset et al. 1999a; Yeaman et al. 1997). The *trans*-Golgi network has been regarded as the major site of divergence for the apical and basolateral secretory pathways in epithelial MDCK cells, thus sorting has been thought to take place after the completion of glycan synthesis. More recently, however, the possibility of segregation of proteins in the secretory pathway prior to completion of glycan structures has been suggested (Alfalah et al 2005; Tveit et al 2005; Prydz et al. 2008). We have previously shown that chondroitin sulfate GAG chains attached to the secretory proteoglycan serglycin (SG) were differentially modified in the apical and basolateral secretory pathways of epithelial MDCK cells (Tveit et al. 2005; Vuong et al. 2006). Thus, we wanted to perform similar investigations with an N-glycanated model protein. In this work, we have investigated whether N-glycans bound to the secretory model protein rat growth hormone (rGH) fused to green fluorescent protein (GFP) with or without additional GAG binding sites (rGH-2N and rGH-2N-GAG) would undergo differential N-glycan modification in the apical and basolateral secretory pathways of filter-grown MDCK cells. We established methods for enrichment of the GFP-tagged glycoproteins by immune precipitation from apical and basolateral media followed by structural and site occupancy analysis of N-glycans by mass spectrometry for both of the N-glycosylation sites. Our analysis revealed no prominent differences in the N-glycan structures or relative N-glycosylation site occupancy of apically and basolaterally secreted rGH-2N and rGH-2N-GAG. However, a difference in site occupancy for the two N-glycan binding sites, NFT (average 94-97 %) and NAS (average 65-75 %), was observed. The microheterogeneity observed for the N-glycans synthesized onto rGH-2N and rGH-2N-GAG (8-9 glycoforms)

was influenced by the presence of GAG chains which resulted in N-glycans with more acidic terminal sugar residues.

Paper III

The protein conformation of the proteoglycan Serglycin influences the length and sulfation of the GAG chains.

Fagereng GL, Hafte TT , Tveit H, Prydz K

Manuscript

We have previously shown that the PG Serglycin (SG) fused to green fluorescent protein is mainly modified with CS chains and secreted from the apical membrane in polarized MDCK cells (Tveit et al 2005). We have also shown that transfer of the GAG attachment domain of SG to the non-glycosylated model protein rat growth hormone (rGH-GAG), also fused to green flourescent protein, induced a preferential secretion of this protein to the apical medium (Hafte et al. 2011). The GAG chains on SG and rGH-GAG were of similar type (mostly CS) and length, but the sulfation intensity observed basolaterally for SG was not observed for rGH-GAG. Previously, it has been shown that neither amyloid precursor-like protein 2, nor the asyloglycoprotein H1 subunit were directed apically in MDCK cells by a single CS chain (Kobialka et al. 2009; Lo et al. 1995). Therefore, we reduced the number of GAG attachment sites of the SG to one to investigate whether this would affect the apical targeting. This variant (SG-1GAG) was also secreted mainly apically, and sulfation was still more efficient in the basolateral secretory pathway. A difference, however, was that a fraction of the GAG chains attached to SG-1GAG protein cores were significantly longer than those of SG, while another fraction was shorter. To further adress the apical secretion of SG, a variant completely without GAG attachement sites (SG-0GAG) was expressed in MDCK cells. Surprisingly, this GAG-free variant was also mainly secreted apically. Thus, both the GAG attachment sites (Hafte et al. 2011) and the protein core (this paper) of SG contain apical sorting information, but the GAG attachment domain does not alone encode the higher sulfation efficiency of SG in the basolateral secretory pathway of MDCK cells (Hafte et al. 2011). Thus, structures outside the GAG attachment domain could be of

importance. We therefore adressed whether the disulfide bridge of SG could play a role in this respect by expressing a variant unable to form disulfide bridges (SG-ΔSS), but this variant was modified and secreted similarly to the parental molecule SG. Thus, other features of the SG protein core are responsible for the high sulfation efficiency of SG in the basolateral secretory pathway of MDCK cells.

6. Discussion

A hall mark of epithelial cells is a polarized secretion of newly synthesized proteins in the direction of the apical and basolateral domains of their plasma membrane. This asymmetric secretion of proteins is a prerequisite to maintain their polarized architecture. Previously, MDCK cells have been shown to synthesize and secrete both CS and HS proteoglycans (PGs), with the former mainly secreted from the apical surface, while the latter is mostly secreted in the basolateral direction (Kolset et al. 1999; Svennevig et al. 1995). These initial studies in our research group were addressing the synthesis and sorting of the bulk of endogenous PGs produced in epithelial MDCK cells. To study these processes in more detail, the next step was to address the modification and sorting of individual PGs. However, no applicable antibodies were available, thus the PG serglycin (SG) fused to green fluorescent protein (GFP) was expressed in MDCK cells. Synthesis and polarised secretion was studied by precipitating SG-GFP via the GFP moiety from the apical and basolateral medium of filter-grown epithelial MDCK cells. SG-GFP was secreted mainly apically (85 %) and carried mainly CS chains, although a minority of HS chains was observed, slightly more abundant after basolateral secretion. Surprisingly, the GAG chains of SG-GFP secreted in the basolateral direction were more intensily sulfated than their apical counterpart (Tveit et al. 2005; Vuong et al. 2006), suggesting differential modification of GAG chains attached to the same core protein in the two seceretory routes to the apical and basolateral surface domains of MDCK cells. This was the first report of a protein core being differentially modified in the apical and basolateral secretory pathway. With this background, we wanted to investigate whether transfer of the GAG modification domain of the SG-GFP into another secretory protein would maintain or abolish the observed difference. For this purpose we chose the secretory protein rat growth hormone (rGH), a normally un-glycosylated protein with no apical or basolateral sorting information in the protein core when expressed in MDCK cells, which was sorted strictly apically when modified with two N-glycosylation sites (Scheiffele et al. 1995). A GAG binding domain consisting of the eight potential GAG attachment sites of SG was inserted at a position between the rGH and GFP, generating a novel rGH-GAG variant. Additional variants of rGH were generated, also fused to GFP, with and without N-glycans and GAGs (rGH-2N and rGH-2N-GAG) and stably expressed

and used for studies of synthesis and sorting in MDCK cells (Paper I). We also wanted to investigate whether the N-glycans attached to rGH variants were processed similarly or differently in the apical and basolateral secretory route of filter-grown epithelial MDCK cells (paper II). For all the rGH-GFP variants we did not observe differences in the apical and basolateral processing of neither GAG chains, nor N-glycans. However, the GAG chains of chondroitin sulfate (CS) type promoted apical sorting equally well as the attachment sites for two N-glycan groups and the concomitant presence of GAG attachment sites in rGH altered the structure of the N-glycans when present in the same protein core. The data obtained in paper I and II pointed to structures outside the GAG attachment domain in the SG protein core as determinants of the GAG modification difference observed in the apical and basolateral secretory pathways of epithelial MDCK cells. To this end, various variants of SG-GFP were expressed in MDCK cells and their synthesis and sorting were studied (paper III).

6.1 GAG modifications obtained in the apical and basolateral secretory pathways of epithelial MDCK cells, and their effect on polarized sorting

Different metabolic labels may be employed in order to label PGs. The protein core can be labelled with ^{35}S-cys/met, since most proteins possess these amino acids. GAG chains can be labelled with ^{35}S-sulfate and ^{3}H-GlcN. ^{35}S-sulfate labelling is the most specific label for PGs, since other proteins are sulfated to a lesser extent. The incorporation of ^{35}S-sulfate has been used for the determination the amount of PG secreted in the apical and basolateral directions in MDCK cell (Kolset et al. 1999; Mertens et al. 1996). When the incorporation of sulfate in a cell is inhibited, an alternative is labelling with ^{3}HGlcN (Fjeldstad et al. 2002). ^{35}S-sulfate labelling must, however, be used with caution for quantitation purposes, after the discovery that the apical and basolateral secretory pathways in polarized MDCK cells sulfate PGs with different intensities (Tveit et al. 2005; Dick et al. 2008).

The model protein rGH has previously been used as a reporter protein in studies of the effect of glycans in polarized protein sorting in MDCK cells. The introduction of N-glycosylation sites to rGH, both secretory and GPI-anchored variants, promoted a preferential apical

transport (Benting et al. 1999; Scheiffele et al. 1995) as compared to non-glycosylated variants, when stably expressed in MDCK cells. Since SG-GFP was used in previous studies and would be used for comparison in our studies, all the rGH variants constructed were GFP fusion proteins. We transferred the GAG binding domain of SG-GFP into native rGH without glycoslylation sites fused with GFP at the C-terminal and generated a novel rGH-GAG variant. In addition, another model protein was made, where we fused rGH with two N-glycosylation sites (Scheiffele et al. 1995) to GFP, while a third variant of rGH-GFP, rGH-2N-GAG contained both two N-glycosylation sites and a the SG GAG attachment domain. All variants were stably expressed in MDCK cells and studied.

The knowledge available on the effect of transfer of a GAG attachment domain to a non-PG protein core on synthesis, sorting, and transport is limited. GAG chains, both of CS and HS types, have been suggested to mediate polarized sorting in MDCK cells, CS in the apical direction and HS towards the basolateral surface (Kolset et al. 1999; Mertens et al. 1996). MDCK cells expressing the novel construct rGH-GAG and the variant rGH-2N-GAG acquired mainly CS GAG chains (Fig. 3A, lane 3 and 6 and 3B, Paper I) and were secreted mostly in the apical direction (Fig. 4A). The fact that addition of CS GAG chains redirected rGH in the apical direction shows that CS chains can mediate apical sorting independently. This has been suggested previously, since both core protein bound and xyloside based CS chains are preferentially secreted apically in MDCK cells (Kolset et al. 1999a). However, the insertion of a single GAG binding site into the asialoglycoprotein receptor H1 subunit (ASGP-R H1), which was mainly modified by CS (Kobialka et al. 2009) did not redirect the receptor in the apical direction. This could be due to the prescence of a dominant basolateral sorting signal in the cytoplasmic tail of the receptor as previously shown for the LDL receptor, for which the basolateral sorting signal in the cytoplasmic tail dominated over the apical sorting information localized to the N-glycans (Hunziker et al. 1991). Another explanation could be that a single CS chain is not sufficient to re-route a protein towards the apical surface, since a single CS GAG chain was not able to mediate the apical secretion of amyloid precursor-like protein 2 (Lo et al. 1995). The GAG modification domain of human SG has eight potential GAG sites, which might allow the amount of CS GAGs to exceed a level required to mediate apical sorting, since the preferential secretion to the apical medium of filter-grown MDCK cells was maintained after transfer to rGH. These results are to our

knowledge the first example where a GAG modification domain of a PG, has been shown to redirect a non-PG protein core after transfer to this novel context and expression in epithelial MDCK cells. The data obtained expand the current knowledge of the role of GAG chains in polarized sorting and could help in the elucidation of the mechanism of protein sorting in the apical and basolateral secretory pathways. Further investigations are, nevertheless, needed to verify whether this is also the case for GAG binding domains of other PGs and what the sorting mechanism could be.

We have also investigated whether differential sulfate modification of CS GAG chains attached to SG observed in the apical and basolateral secretory routes of filter-grown MDCK cells (Tveit et al. 2005) was maintained after transfer of the SG GAG binding domain to rGH. SG-GFP bound CS GAG chains secreted basolaterally were several times more intensely sulfated than their apical counterpart (Tveit et al. 2005). Surprisingly, the higher sulfation intensity observed for CS GAGs synthesized on SG-GFP in the basolateral pathway of filter-grown MDCK cells was lost after transfer of the GAG binding domain into rGH (Fig.5C, paper I). Our results from HPLC-based disaccharide analysis confirmed that there was no difference in the sulfation pattern and intensity of the GAG chains on rGH-GAG, recovered from apical and basolateral media after secretion (Fig.6, Paper I), supporting the SDS-PAGE and Western blot data. Taken together, our data suggest that the GAG attachment domain of SG is alone unable to mediate the synthesis of differentially sulfated GAGs of rGH-GAG and rGH-2N-GAG in the apical and basolateral secretory pathways.

The reason for the observed difference outlined above is not evident. How protein passage through the Golgi apparatus is regulated is still a matter of debate (Emr et al. 2009; Jackson 2009). The differences occurring to SG-GFP in the apical and basolateral secretory pathways of epithelial MDCK cells (Tveit et al. 2005; Vuong et al. 2006) is a similar phenomenon to what is observed after detergent extraction of apical and basolateral glycoproteins from MDCK cells (Alfalah et al. 2005), supporting segregation of apical and basolateral secretory cargo earlier than the *trans*-Golgi network, possibly already in the ER, ERGIC or the *cis*-Golgi *cisternae* (Alfalah et al. 2005; Prydz et al. 2008; Tveit et al. 2005). An undersulfation in the apical route, compared to the basolateral counterpart, was also observed for endogenous PGs of the CS type (Dick et al. 2008), which could be counteracted by over-

expression of PAPS transporter I (Dick et al. 2008). The difference could also be abolished by neutralization of acidic intracellular compartments (Grøndahl et al. 2009), in all likelihood including the apical secretory route, by treatment with Bafilomycin A_1. In both of these studies manipulation of the secretory pathway indicates that the apical and basolateral routes for PG synthesis are segregated - a possibility also suggested by the studies of SG-GFP secretion. Analysis of secreted rGH-GAG and rGH-2N-GAG did, however, not give any indications that these two model proteins were modified differently in the apical and basolateral routes. Thus, these proteins could instead be transported along the same route until their modifications were completed and be sorted into the apical and basolateral directions late in the Golgi apparatus, or even later – in endosomes (Schneider et al. 2010). It is interesting to note that the sulfation intensity of the rGH-GAG variants in the apical and basolateral pathway is similar to that of SG-GFP in the apical pathway, making the basolateral route for SG-GFP the "specialized" route.

Another possible explanation for the observed difference in sulfate modification intensity of GAG chains attached to SG, might be that this PG spends a prolonged time in the basolateral pathway, which could result in more intense sulfation. In this case, the apical and basolateral secretory routes are not necessarily segregated, but the apical cargo PG would depart from the GAG synthesis machinery in the Golgi apparatus before the fraction transported basolaterally. We have recently investigated this possibility using ^{35}S-sulfate pulse-labelled MDCK cells stably expressing SG-GFP and rGH and chased for times up to 24 hrs. Our preliminary results from this investigation, however, do not support this possibility (Fagereng et al. unpublished observation).

It has been suggested that heparin sulfate (HS) synthesis is mediated by enzyme complexes recruited onto the GAG polymerization sites of the PGs. The term GAG some has been suggested for such complexes (Eskom and Select, 2002; Presto et al. 2008). The enzymatic composition of each GAGosome may differ stoichiometrically, and based on this view, different GAG chains may be synthesized in the same area of the Golgi apparatus. GAGosomes have also been suggested to play a role in CS synthesis, but only indirect evidence exists. Chinese hamster ovary (CHO) cells were challenged with a large variety of xylosides which gave rise to CS GAG chains of different characteristics (Victor et al. 2009). The observed differences in CS structure were proposed to result from recruitment of

GAGosomes of different enzymatic composition onto the GAG chains emanating from the different xylosides. The difference in the sulfation intensity and pattern observed for CS GAG chains attached to SG-GFP in the apical and basolateral secretory pathways of epithelial MDCK cells could be a result of recruitment of different GAGosomes in the two pathways. Still, however, it remains to be explained why different GAGosomes could be recruited onto seemingly identical protein cores without physical segregation of the pathways. In this model, rGH-GAG and rGH-2N-GAG would contrary to SG-GFP recruit identical GAGosomes in the apical and basolateral secretory routes.

In a recent publication secretion of endogenous serglycin was studied in polarized endothelial cells from the umbilical cord. In this study, the apical fraction of was seemingly carrying most of the incorporated ^{35}S-Sulfate, but a thorough quantification was not performed (Meen et al. 2011). Still PG modification might be both protein core and cell type dependent. Different treatment of the same molecules in endothelial cells and epithelial MDCK cells has previously been observed for tissue factor (Camerer et al. 1996). It has also been previously shown that treatment of MDCK cells with Brefeldin A (BFA) resulted in the lower sulfation of PGs, specially PGs secreted from the apical medium, suggesting differential modification (Fjeldstad et al. 2002). However, it was not investigated whether this effect was due to the reduction in sulfation or reduced apical secretion or a combination of both effects.

The fact that the sulfation intensity of CS GAG chains from rGH-GAG and SG is similar in the apical pathway (Fig. 5C), but different in the basolateral direction suggests that sulfation in the basolateral pathway could be dependent on the protein core structure outside the GAG modification domain. In order to investigate the possibility that parts of the protein core outside the GAG binding domain in SG-GFP could be responsible for the observed differential modifications, we made variants of the protein core of SG-GFP and expressed these in MDCK cells. Studies addressing the effect of alterations in the protein core flanking the GAG attachment sites on the synthesis and modification of GAG chains are scarce. GAG chain polymerization might, however, be affected by protein domains outside the GAG binding site (Chen and Lander 2001). The synthesis and sorting of the variants, SG-1GAG (SG variant with only one GAG binding site), and SG-ΔSS (SG with intact GAG binding domain, but no disulfide bridge) (Fig.1, Paper III), were compared with SG-GFP in

filter-grown MDCK cells after metabolic labelling with ^{35}S-Sulfate or ^{3}H-GlcN. Our experiments demonstrated that none of the changes introduced to the SG-GFP protein core were able to abolish the previously observed higher sulfation intensity for SG-GFP in the basolateral secretory pathway (Tveit et al. 2005), as measured by the ratio of ^{35}S-Sulfate/^{3}H-GlcN incorporation (Fig.4C and Fig.5, Paper III) suggesting that the differential modification of GAG chains bound to SG-GFP in the apical and basolateral pathways does not require the disulfide bridge and is observable with only one GAG modification site. However, it is worth to note that even if the apical to basolateral sulfation intensity ratio is maintained for the novel variants, there is a clear reduction in the absolute amount of sulfate incorporated into the GAG chains attached to the mutant proteins as compared to SG-GFP. The reduction in the amount of sulfate incorporated might of course be due to the reduced number of GAG binding sites as in the case of SG-1GAG and possibly also changes in the protein structure in the case of SG-ΔSS, but no final quantification of the expression levels has been carried out. Further investigations are required in order to determine which part of the core protein is indeed responsible for the high sulfation intensity of SG in the basolateral direction.

An interesting difference between the two model proteins rGH and SG is that while unglycosylated rGH is secreted with an unpolarized pattern, the SG variant devoid of GAG attachment sites was still secreted predominantly from the apical membrane. This indicates that the SG protein core has two features that diverge from that of rGH – apical sorting information and information coding sulfation intensity in the basolateral pathway.

6.2 Any possible physiological role of differential sulfation in epithelial cells?

The physiological role of differential sulfation in polarized MDCK cells is not yet clearly defined. In fact certain sulfate groups on HS PGs have been shown to mediate the binding of basic fibroblast growth factor (bFGF) (Turnbull et al. 1992) to growth factor receptor in the basolateral side of polarized epithelial cells (Murphy et al. 2004). As compared to HS PGs, the importance of CS PGs in signalling pathways has not received considerable attention. However, recently emerging data show the functional role of CS PGs in signalling pathway.

CS PGs have been shown to be essential in the growth factor signaling during cartilage morphogenesis (Kluppel et al. 2005). It has also been demonstrated that CS PGs in cooperation with HS PGs bind to growth factors in epithelial cells (Deepa et al. 2004). Undersulfation of CS PGs was shown to be the cause of reduced signaling activities, for instance in Indian hedgehog (Ihh) signaling (Cortes et al. 2009), whereas oversulfated CS PGs were shown to bind heparin-binding proteins (Ueoka et al. 2000). Extensive sulfation of mucins and PGs has been shown from the apical side of epithelial cell line from cystic fibrosis trachea (Mendicino and Sangadala 1999). Results showing the expression of different sulfotransferases mediating the sulfation of HS PGs in tissue specific and time dependent manner (Sedita et al. 2004) and that the sulfation intensity and pattern of CS PGs are tightly regulated by the temporal and spatial expression of chondroitin sulfotransferases (Kluppel 2010) suggest possible physiological need for epithelial cells to regulate the sulfation of the GAG chains.

6.3 Further implications of early segregation of the apical and basolateral secretory pathways

Classically the TGN has been considered as the major site for the sorting of secretory cargo molecules into the apical and basolateral routes of the secretory pathway of epithelial MDCK cells (Griffiths et al. 1985; Shorter and Warren 2002). With time also endosomal compartments have been established as important sorting sites in the secretory direction (Polishchuk et al. 2000). However, several studies promote the view that segregation of cargo molecules destined for the apical and basolateral surfaces of epithelial cells also can take place at an early stage in the secretory pathway. Studies of synthesis and sorting of the recombinant SG-GFP which even demonstrated differential modification of the linker tetrasaccharide of the CS GAGs polymerized onto SG-GFP in the apical and basolateral directions favours the view that segregation takes place early in the pathway (Tveit et al. 2005; Vuong et al. 2006). Cargo molecules destined for the plasma membrane have been shown to bud from the Golgi apparatus at cisternal rims prior to the TGN (Ladinsky et al. 1999), also supporting the view of early segregation in the secretory pathway. Additional studies conducted in both CaCo-2 and MDCK cells also promoted the view of early

segregation, since detergent extraction studies supported the concept of cargo segregation into different lipid microdomains early in the secretory pathway (Alfalah et al. 2005). For the observed difference in the intensity as well as pattern of sulfation on CS GAGs of the recombinant SG-GFP protein in the apical and basolateral pathway, an early segregation into different domains of the secretory pathway is plausible, even though the exact location of this early segregation is not yet elucidated. The synthesis of HS has been shown to be completed before the TGN, in the Gogi *cisternae*, as observed after challenging cells with the Golgi inhibitor Brefeldin A (BFA) (Fransson et al. 1992; Spiro et al. 1991), whereas the synthesis of CS normally is completed in the TGN (reviewed in: Prydz and Dalen 2000), suggesting that segregation of the two pathways would occur prior to the TGN.

An interesting observation has been made with *Drosophila* imaginal disc cells, where Golgi stacks dispersed throughout the cytoplasm displayed differential content of Golgi proteins, like a sulfotransferase, a protease and a nucleotide-sugar transporter, indicating that all Golgi stacks are not alike (Yano et al. 2005). In mammalian cells, this possibility is difficult to investigate, since the Golgi stacks normally are localized together in the perinuclear area. In some mammalian cell types, like polynuclear muscle cells, are Golgi elements observed as distinct units (Nevalainen et al. 2010; Percival and Froehner 2007), but the possibility that their processing capacity is different has not been investigated.

6.4 Analysisis of N-glycanated rGH with and without GAG binding domain

N- glycans on both secretory and GPI-anchored variants of rGH have been implicated as drivers of apical sorting in filter-grown epithelial MDCK cells (Benting et al. 1999; Scheiffele et al. 1995). However, no structural requirements for N-glycans involved in the mediation of apical sorting have been well studied so far. Previous studies for the gross determination of N-glycan structure on all the N-glycanated proteins in the apical and basolateral media of filter-grown MDCK cells have not revealed any significant structural difference in the structures obtained in the two pathways (Ohkura et al. 2002). The fact that the core protein of serglycin acquired GAG chains with different structure in the apical and basolateral secretory pathways of filter-grown MDCK cells (Tveit et al. 2005), made us

investigate whether N-glycans on rGH variants with two N-glycan modification would obtain N-glycans with different structure in the apical and the basolateral route. To this end, we performed IP of the GFP-tagged proteins secreted to the apical and basolateral media of filter-grown MDCK cells, in order to analyse N-glycan structure and site occupancy by mass spectrometry (MS). We could not detect any difference in N-glycan structure after apical and basolateral secretion of rGH-2N or rGH-2N-GAG, but our MS analysis revealed a difference in the relative site occupancy of the NFT and NAS N-glycosylation sites in both rGH-2N and rGH-2N-GAG (Fig. 8, paper II), with a higher NFT site occupation as compared to the NAS binding site, in agreement with previous observations. NXT sites are glycosylated 2-3 times more frequently than NXS sites (Kasturi et al. 1995; Petrescu et al. 2004). However, no significant difference in the relative site occupancy was observed for rGH-2N or rGH-2N-GAG secreted from the apical and basolateral domains of filter-grown MDCK cells (Fig. 8C, paper II). Furthermore, the NFT and NAS glycosylation sites in each of rGH-2N and rGH-2N-GAG carried similar N-glycan isoforms. However, a significant difference in N-glycan structure was observed when the glycans of rGH-2N-GFP and rGH-2N-GAG were compared. The rGH-2N-GAG variants carried more N-glycans with terminal sialic acid, as compared to the N-glycans of rGH-2N (Fig. 7B, and 7C paper II). Furthermore, the majority of N-glycans bound to rGH-2N were modified with distal fucose residues in addition to the core fucose residue bound to the first HexNAc as compared to what was observed for the N-glycans attached to rGH-2N-GAG (Fig. 7A, paper II). Altogether, this might imply that the GAG binding domain of SG inserted into rGH in rGH-2N-GAG influences the structure of the N-glycans (microheterogeneity), but not the relative site occupancy. Further investigations are required to elucidate how the presence of GAG modification sites led to more acidic N-glycans on rGH-2N-GAG than on rGH-2N.

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I

II

N-glycan synthesis in the apical and basolateral secretory pathway of epithelial MDCK cells and the influence of a glycosaminoglycan domain

Running title
N-glycan analysis of a polarized secreted glycoprotein.

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One supplementary table (Table SI)

Key words
Epithelial cells, MDCK, N-glycan, glycosaminoglycan (GAG), secretory pathway, Golgi

Abstract

Different classes of glycans are implicated as mediators of apical protein sorting in the secretory pathway of epithelial cells, but recent research indicates that sorting to the apical and basolateral surfaces may occur before completion of glycan synthesis. We have previously shown that a proteoglycan (PG) core protein can obtain different glycosaminoglycan (GAG) structures in the apical and basolateral secretory routes (Tveit, H., Dick, G., et al. 2005) of epithelial Madin-Darby canine kidney (MDCK) cells. We have now also determined the detailed N-glycan structures acquired by a single glycoprotein species in the same apical and basolateral secretory pathways. For this purpose, rat growth hormone (rGH) with two N-glycan sites (rGH-2N) inserted into the rGH portion (NAS and NFT) was fused to green fluorescent protein (GFP) and expressed in MDCK cells. Immunoisolated rGH variants were analyzed for site occupancy and N-glycan structure by mass spectrometry. The extent of NAS and NFT site occupancy was different, but comparable for rGH-2N secreted apically and basolaterally.

Microheterogeneity existed for the glycans attached to each N-glycan site, but no major differences were observed in the apical and basolateral pathways. Transfer of the GAG modification domain from the PG Serglycin to the fusion site of rGH-2N and GFP, allowed polymerization of GAG chains onto the novel protein variant, and influenced the microheterogeneity of the N-glycans towards more acidic glycans, but did not alter the relative site occupancy.

Introduction

The biological functions of glycoproteins are strongly influenced by their glycans, but the details of the carbohydrate structure is difficult to elaborate, since post-translationally added glycans can not be deduced directly from genomic information, may be branched, and are variable with tissue and developmental stage, even for the same protein core. The immature N-glycan structures are important to quality control procedures for glycoprotein folding in the endoplasmic reticulum (ER) (Anelli, T. and Sitia, R. 2008, Ellgaard, L. and Helenius, A. 2003). The mature structure of N-glycans eventually obtained during glycoprotein passage through the Golgi apparatus can influence the residence time at the cell surface and the signalling potential of trans-membrane receptors (Lau, K.S. and Dennis, J.W. 2008, Partridge, E.A., Le Roy, C., et al. 2004). Although less abundant than N-glycans, O-glycans of the mucin and glycosaminoglycan (GAG) types are important contributors to signalling processes, and are in some instances essential to development and growth (Schaefer, L. and Schaefer, R.M. 2010, Singh, P.K. and Hollingsworth, M.A. 2006). N-linked and O-linked glycans have been shown to contribute to polarized sorting of glycoproteins to the apical surface of MDCK epithelial cells (Benting, J.H., Rietveld, A.G., et al. 1999, Gut, A., Kappeler, F., et al. 1998, Kolset, S.O., Vuong, T.T., et al. 1999, Scheiffele, P., Peranen, J., et al. 1995, Yeaman, C., Le Gall, A.H., et al. 1997), but not all glycan modification sites have shown the same potential in this respect (Su, T., Cariappa, R., et al. 1999), even when localised to the same protein (Kitagawa, Y., Sano, Y., et al. 1994, Potter, B.A., Ihrke, G., et al. 2004). An important question is whether particular details of the glycan structures mediate apical sorting, or whether the glycan structures produced merely result from the glycosylation

regimes encountered in the secretory pathway. In the classical view, apical and basolateral proteins pass through the same Golgi environment, to the *trans*-Golgi network, the Golgi terminus at the *trans* side, before segregation for exit in direction of the apical or basolateral surfaces, or towards endosomes. According to this model, apical and basolateral proteins are subjected to the same processing machinery prior to their disjunction and departure from the *trans*-Golgi network. During the last few years, however, this view has been challenged (Alfalah, M., Wetzel, G., et al. 2005, Prydz, K., Dick, G., et al. 2008, Tveit, H., Dick, G., et al. 2005, Vuong, T.T., Prydz, K., et al. 2006) by indications of segregation early in the secretory pathway of apically and basolaterally destined proteins in epithelial MDCK cells. In *Drosophila* imaginal disk cells, where individual Golgi stacks are more easily resolved in the fluorescence microscope, due to their dispersal throughout the cytoplasm, there are indications of specialized processing environments in different Golgi stacks (Yano, H., Yamamoto-Hino, M., et al. 2005). Since Golgi stacks are mostly joined in a Golgi ribbon in the perinuclear region of vertebrate cells, resolution of individual stacks in the same manner is more difficult. Thus, analysis of the outcome of glycoprotein modification is one plausible strategy to investigate possible diversity in Golgi processing of glycoproteins. Although secretory glycoproteins and PGs in general are sorted predominantly towards one pole of an epithelium, a fraction of each protein species is always transported to the opposite surface domain, making analysis of the modifications that have occurred to the same protein in the apical and basolateral secretory routes feasible. We have previously discovered that a recombinant PG, Serglycin (SG) coupled to GFP, is modified differently in the apical and basolateral secretory pathways of epithelial MDCK cells (Tveit, H., Dick, G., et al. 2005,

Vuong, T.T., Prydz, K., et al. 2006). Thus, we wanted to carry out similar investigations with a model protein carrying sites for N-glycan modification. We took advantage of the previously studied non-glycosylated model protein rGH, where insertion of two N-glycan sites mediated apical sorting in MDCK cells (Scheiffele, P., Peranen, J., et al. 1995). We expressed the variant with two N-glycosylation sites (rGH-2N) as a GFP fusion protein in MDCK cells, and in addition, we also expressed a variant with the GAG modification domain of SG at the junction of rGH and GFP (rGH-2N-GAG). We then carried out immunoisolation of the rGH variants with anti-GFP antibodies from apical and basolateral media, followed by mass spectrometry (MS) analysis of the N-glycans that had been acquired by the rGH variants.

Results

Expression of rGH variants in polarized epithelial MDCK cells

How secretory proteins are transported through the complex membrane system of the Golgi apparatus remains controversial (Emr, S., Glick, B.S., et al. 2009). In polarized epithelial cells, secretory proteins partition into apical and basolateral pathways towards opposite plasma membrane domains. Segregation of apical and basolateral cargo has been observed at stages prior to established divergence points in the *trans*-Golgi network and endosomes (Prydz, K., Dick, G., et al. 2008). One such observation was that recombinant PG SG obtained characteristically different GAG chains underway to the apical and basolateral medium reservoirs of polarized MDCK cells (Tveit, H., Dick, G., et al. 2005, Vuong, T.T., Prydz, K., et al. 2006). A corresponding investigation to study the details of N-glycan modification of a similar recombinant protein after secretion to the apical and basolateral media of filter-grown epithelial MDCK cells has not yet been undertaken. Thus, rGH was constructed with two sites for N-glycosylation previously designed for studies of polarized glycoprotein sorting (Scheiffele, P., Peranen, J., et al. 1995) with and without an additional GAG attachment domain (Hafte, T.T., Fagereng, G.L., et al. 2010) and expressed in MDCK cells (Figure 1A). Stably transfected cell lines were subsequently plated on polycarbonate filters and maintained for 3 days to allow formation of polarized epithelial monolayers that efficiently segregate apical and basolateral medium compartments. After harvesting apical and basolateral medium samples, rGH variants were subjected to IP via their GFP domains and processed for MS as indicated in Figure 1B.

The rGH variants (Figure 1A) consist of rGH and rGH-2N with protein core masses of 50.1 kDa; rGH (21.7 kDa), GFP (26.9 kDa), and a linker between these two domains (1.4 kDa). rGH-2N carries additional mass corresponding to N-glycan modifications. rGH-2N-GAG has a protein core mass of 52.4 kDa; rGH (50.1 kDa) and 8 GAG attachment sites (25 amino acids more; 2.3 kDa), beside the masses contributed by N-glycans and GAGs. The constructs and the sequences flanking the N-glycan sites are shown in Figure 1A.

Verification of protein glycosylation

Apical and basolateral medium samples from polarized MDCK cells expressing the three constructs, rGH, rGH-2N or rGH-2N-GAG, were treated with PNGase F, while rGH-2N-GAG was in addition treated with cABC to digest CS GAG chains attached to the protein core (Hafte, T.T., Fagereng, G.L., et al. 2010). The recombinant proteins were detected by WB with anti-GFP antibodies, and only rGH-2N and rGH-2N-GAG were sensitive to mass shifts upon PNGase F treatment and thus contained N-glycans, while rGH-2N-GAG also carried CS GAGs (Figure 2).

SDS-PAGE and protein excision

The recombinant rGH variants were first isolated by IP from apical and basolateral medium samples via the GFP moiety to enrich the protein of interest relatively to other proteins. Following IP, SDS-PAGE was performed of the eluted proteins from apical and basolateral samples of rGH-2N and rGH-2N-GAG to further enrich the proteins prior to

MS. Bands of proper molecular mass stained with Coomassie brilliant blue were sliced and digested with trypsin.

Peptide prediction of rGH peptide digests

The rGH-2N, and rGH-2N-GAG tryptic peptide digest mixtures were analyzed with the Orbitrap XL mass spectrometer (ESI-MS/MS) in the precursor ion scan mode, using the HCD and collision induced dissociation (CID) mode for peptide fragmentation (FT mode, accuracy <7 ppm, resolution 7.500 till 30.000 R). Using the MS results, database searches against a human and a dog (in house) protein database were performed. Apart from rGH and GFP as the major protein search hits, no other significant matches were identified, except human keratins, porcine trypsin and immunoglobulins. In order to detect peptide fragments related to unspecific cleavage of rGH by trypsin, a database SEQUEST^{TM} search accepting also unspecific, non-enzymatic proteolytic cleavage was also carried out.

The rGH-2N peptide was detected first, and the LPAMPLSSLFANASLR (Figure 1A) peptide was detected as the most abundant peptide carrying the NAS glycosylation site after digestion of rGH-2N and was also the expected tryptic peptide after removal of the signal sequence. Several other less abundant rGH peptides, including the NAS glycosylation site, were also predicted *in silico*, PAMPLSSLFANASLR, PLSSLFANASLR and LSSLFANASLR. Their presence is either due to atypical cleavage by the signal sequence peptidase or by trypsin (cleavage after leucine, alanine, methionine or proline). The predicted tryptic peptide harboring the NFT glycosylation site (NFTNSLMFGTSDR) was detected in small amounts in the glycan-free form.

To generate a complete peptide map, and to detect all generated peptides with N-glycosylation sites in their completely glycan-free form, peptide mixtures after release of N-glycans by PNGase F treatment were analyzed. Upon enzymatic deglycosylation, we detected increased amounts of two major tryptic and semi-tryptic peptides with the expected sequences LPAMPLSSLFANASLR and NFTNSLMFGTSDR, respectively. Their amino acid sequences were confirmed by the fragmentation pattern (Figure 3A and 3B; e.g. for LPAMoxPLSSLFANASLR: fragment ions b_3 and b_4 at m/z 282.182 and 429.218, the y_3 to y_{15} ions at m/z 375.236 and 1590.842) and secondly the accurate peptide mass of 1703.916 Da (+2.5 ppm) and 1505.674 Da (+2.3 ppm)). The major detected peptides LPAMPLSSLFANASLR and NFTNSLMFGTSDR contained one of the two possible N-glycosylation sites of rGH-2N each, NAS and NFT, which match the N–X–S/T consensus sequence. The same peptides were detected for rGH-2N-GAG. The complete overview of all detected rGH-2N peptides is shown in Table SI (supplementary material).

Mass analysis of rGH peptide digests

The glycan structures were first determined for rGH-2N. To comprehensively characterize the glycosylation status, two strategies were used: First the MS/MS spectra were inspected manually for typical glycopeptide fragment patterns and searched for loss of either 203.08 or 162.05 Da, to identify glycopeptides with *N*-acetylglucosamine (GlcNAc) or hexose (hex) residues, or a search for the appearance of specific reporter ions at m/z 204.086 was carried out, since glycopeptides containing GlcNAc give rise to

an intense diagnostic oxonium ion by HCD fragmentation (Carr, S.A., Huddleston, M.J., et al. 1993).

Spectra with a signal at m/z 204.086 were subjected to further evaluation. Since the LC-MS run could be contaminated with glycopeptides from tryptic digestion of antibody molecules which may be glycosylated, we examined the MS/MS spectra specifically for fragment ion masses related to the two detected main peptides from rGH-2N with the sequences LPAMoxPLSSLFANASLR or NFTNSLMoxFGTSDR in addition to the glycan core structure for *N*-glycosylation (peptide + 2 GlcNAc + 1-3 mannose).

A representative base peak chromatogram (BPC) is shown in the top panel of Figure 4, while the lower panel shows the selected ion chromatograms (SIC) of precursor masses at *m/z* 1262.5059 and 1328.5886, respectively, which are the two potential rGH-2N glycopeptides we investigated further. The peak with the retention time of 68.2 min and a signal at *m/z* of 1328.5886 was chosen for a more detailed analysis. HCD fragmentation of the triply charged species at *m/z* 1328.5886^{3+} generated several major oxonium ions of *m*/*z* 204.086, 366.139, 512.198, 528.193 and 674.253, along with a series of ions in the high *m*/*z* region, consistent with either sequential losses of 203.079 or 162.053 Da (Figure 5A).

The doubly charged fragment signals of this MS/MS spectrum at *m/z* 954.003^{2+} and 1055.541^{2+} (monoisotopic peak for C_{12}) suggest to be fragments of a doubly charged rGH-2N peptide with the sequence of LPAMPLSSLFANASLR in the oxidized form, which carries a glycan epitope with the dimension of one and two GlcNAc residues (those glycan fragments generated in the MS by collision). The theoretical *m/z* ratios of these fragments are 1055.5416^{2+} and 954.0021^{2+} Da, respectively.

The MS/MS spectrum was required in the HCD mode, where the fragment ions were also detected with higher resolution (R=7500). The detected *m/z* values were acquired with an accuracy of 2 ppm or below. To further support the mass identification, HCD fragmentation of the ion species at *m/z* 1328.5886^{3+} with higher activation energy (55 eV instead of 35eV) was performed. Several signals related to expected y-ions from the peptide LPAMoxPLSSLFANASLR were detected and confirmed (data not shown). Taking into account that the complete glycopeptide mass was 3985.763 Da in the singly protonated form, these data provided strong evidence that the peptide LPAMoxPLSSLFANASLR (signal of the peptide backbone at *m*/*z* 1702.908) carried an *N*-linked oligosaccharide with the dimension (2 x GlcNAc, 3 x mannose, 2 x fucose and further 3 x GlcNAc and 3 x hexose; size 2281.845 Da).

Since the second peak with a retention time of 50.5 min and a signal at *m/z* of 1262.5059, in all likelihood represented another rGH-2N glycopeptide, this peak was also selected for more detailed analysis:

HCD fragmentation of that triply charged species at *m/z* 1263.176^{3+} generated corresponding major oxonium ions of *m*/*z* 204.086, 366.139, 528.193, and 674.253, along with a series of ions in the high *m*/*z* region, consistent with either sequential losses of 203.079 or 146.057Da (Figure 5B). Again, singly charged fragment signals of this MS/MS spectrum at *m/z* $1708,748^{1+}$ and $1911,818^{1+}$ (monoisotopic peak for C_{12}) for example suggest fragments of a singly charged rGH-2N peptide with the sequence of NFTNSLMFGTSDR in its oxidized form which carries a glycan epitope with the dimension of one and two GlcNAc residues.

The complete glycopeptide mass was determined to be 3785.5336 Da in the singly protonated form, suggesting that peptide NFTNSLMoxFGTSDR (mass of the peptide backbone 1505.672 Da) bearing a similar N-linked oligosaccharide with the dimension of 2 x GlcNAc, 3 x mannose, 2 x fucose and further 3 x GlcNAc and 3 x hexose (size 2281.845 Da), as described previously for the first N-glycosylation site (Figure 5B).

N-glycan analysis

Accurate mass determination alone was not sufficient to discriminate between potential isobaric glycan structures. Therefore, further evaluation of MS/MS fragmentation patterns was performed. In addition, putative structures were obtained using the GlycoMod software, which allows determination of possible glycan composition/structures from experimentally determined glycopeptide fragment masses (http://au.expasy.org/tools/glycomod/). Spectrum analysis suggested that the glycan structures attached to the NAS and NFT sites consisted of complex/hybrid glycan structures with bisecting bi-antennary composition with a core fucose and a distal fucose moiety attached (see Figure 5A for detailed structural analysis of the NAS site). The rGH-2N-GAG glycan structures were determined to be qualitatively identical as for rGH-2N.

Characterization of N-glycosylation heterogeneity

After characterization of the precursor ions at *m/z* 1262.5059 and 1328.5886^{3+}, the heterogeneity of N-glycosylation on the NAS and NFT sites was further investigated. First we screened the full survey scan for ion signals which would correspond to

precursor masses with (glycan intervals) one glycan molecule more or less (hexose, GlcNAc, fucose, sialic acid) than the detected masses at *m/z* 1262.5059^{3+} and 1328.5886^{3+}.These potential glycopeptides were structurally evaluated by MS/MS analysis. For example, Figure 5C shows the MS/MS spectrum of a glycopeptide with a signal at *m/z* 1376.9323. Due to an almost equal fragmentation pattern of the previously observed glycopeptide with *m/z* signal at 1328.5886, we concluded that this glycopeptide is also based on the peptide LPAMoxPLSSLFNASLR, but with a slightly different glycan moiety. The observation of new specific fragment ions at *m/z* 274.092, 657.236 (both oxonium ions) and 1801.303, together with the disappearance of specific fragment ions (e.g. *m/z* of 512.198, 674.253, and 1810.330) and a total glycopeptide mass increase of 145.125 Da, indicated a glycopeptide species carrying a distal *N*-acetylneuraminic acid molecule instead of a fucose unit. The detected glycopeptide population (glyco-profiling) for each site is summarized in Tables I and II and partly visualized for the NAS site at rGH-2N in Figure 6. The glycoform with distal fucose at m/z 1328.5886^{3+} was more abundant on the NAS site of rGH-2N than the glycoform with distal sialic acid and that with neither distal fucose nor sialic acid. All together, 8-9 main glycoforms were detected for both N-glycosylation sites in rGH-2N and rGH-2N-GAG, but their relative representation was variable. The difference in detected amounts of three of the most abundant glycopeptides in rGH and rGH-2N-GAG is visualized in Figure 7A, where the peak areas of major glycoforms attached to NAS glycopeptides are compared. The comparison shows that the N-glycans of rGH-2N-GAG carry more distal sialic acid than those of rGH-2N. The relative monoisotope intensities of each of the detected glycoforms were calculated and summarized in Figure 7B and 7C. No major differences were

detected between the *N*-linked glycan structures of glycosylated rGH molecules secreted to the apical and basolateral media (Figure 7B and 7C). When comparing the relative percentages for the monoisotope peak intensities of all glycoforms derived from rGH-2N and rGH-2N-GAG in more detail (Figure 7B and 7C), a number of differences become evident. While rGH-2N-GAG carries more distal sialic acid, rGH-2N carries more distal fucose than rGH-2N-GAG, while glycoforms with neither distal fucose nor sialic acid are attached to both glycosylation sites in approximately the same ratio for both rGH-2N and rGH-2N-GAG. The largest glycan groups were detected predominantly on rGH-2N, especially attached to the NFT site (Figure 7B and 7C). Figure 7B and 7C show further that all the detected glycoforms are found on both N-glycosylation sites, NAS and NFT, in rGH-2N but to a different extent for the two sites. Both N-glycan sites carry much of the glycoforms with one terminal fucose or one terminal sialic acid, while the NFT site carries more of the largest glycoform with one additional antenna and both fucose and sialic acid terminally.

For rGH-2N-GAG, glycoforms with terminal fucose are much less abundant, particularly for the NAS site, thus glycoforms with one or two distal sialic acids predominate for both the NAS and the NFT sites in rGH-2N-GAG.

Determination of glycosylation site occupancy

To determine more precisely the glycosylation site occupancy of the two *N*-glycosylation sites, aliquots of tryptic peptide samples were incubated with PNGase F in the presence of H_2O^{18}. The O^{18}-labeled water contributed to 75% of the reaction mixture volume (15

µl H_2O^{18} to a total of 20 µl). Enzymatic removal of *N*-glycan moieties was accompanied by conversion of asparagine to aspartate with a corresponding mass shift of +0.984 Da for the asparagine-aspartate conversion and +2.984 Da for conversion in the presence of H_2O^{18} (Figure 8A).

The intensity ratios of modified and unglycosylated peptides were used to calculate the respective site occupancy percentages. The NFT site was in the range of 94-97 % occupied by N-glycan groups, while the NAS site was 65-75 % occupied. N-glycan site occupancy was similar for apically and basolaterally secreted proteins and was not affected by the insertion of additional GAG sites (rGH-2N-GAG). The occupancy percentages are summarized in Figure 8B.

Discussion

We have established methods for analysis of GAGs (Grondahl, F., Tveit, H., et al. 2011) and N-glycans (the present work) attached to single proteins secreted to the apical and basolateral media of polarized epithelial cells. This requires that an antibody is available to differentially precipitate the protein in question from the two separate medium reservoirs. While GAGs are analyzed by HPLC, N-glycan structures are determined by MS. These methods may also be applied for cell membrane proteins, after differential surface labeling, but this remains to be tested. We have carried out our analyses with epithelial MDCK cells transfected to express GFP-tagged recombinant proteins. This allows the use of the same anti-GFP antibody for isolation of the proteins of interest, but endogenous proteins may very well be studied, as long as a proper antibody is available. GFP is an advantageous tag, since it makes selection of positive cell clones easy after transfection, and it has rarely been reported to interfere with the activities of the tagged protein. For the studies reported here, we expressed GFP-tagged rGH with two inserted N-glycoslylation sites (Scheiffele, P., Peranen, J., et al. 1995) and a variant with the GAG attachment domain of SG in addition (rGH-2N and rGH-2N-GAG, respectively). Although the total N-glycans attached to apical and basolateral secreted and membrane proteins synthesized by MDCK cells have been studied thoroughly (Ohkura, T., Seko, A., et al. 2002), this study is to our knowledge the first to compare N-glycans on a single protein species isolated from apical and basolateral medium reservoirs of epithelial cells. We have studied the glycans attached to the two sites, NAS and NFT, separately. For rGH-2N, the glycan structures were of similar types for the two sites, but quantitatively different. The NFT site carried more of the glycan structure with both fucose and sialic

acid terminally (Figure 7B and 7C). The N-glycans of rGH-2N are comparable to those observed by Ohkura et al. 2002, but the most abundant N-glycan structures, which accounted for 60-70 % of the N-glycans in their study (complex glycans with two terminal fucose residues and high-mannose glycans) were not observed on rGH-2N. This indicates that there is some selectivity in the synthesis of the N-glycans onto this recombinant glycoprotein. The fact that approximately 20 % of the N-glycans synthesized by epithelial MDCK cells reach the cell surface as high-mannose glycans (Ohkura, T., Seko, A., et al. 2002), supports the possibility of secretory routes bypassing the Golgi apparatus (Marie, M., Sannerud, R., et al. 2008, Prydz, K., Dick, G., et al. 2008). Further support for selectivity in N-glycan synthesis in MDCK cells was obtained when the glycans attached to rGH-2N-GAG were compared to those of rGH-2N (Figure 7B and 7C). The former protein carried significantly more N-glycans with one or two terminal sialic acids at the expense of structures with terminal fucose residues in rGH-2N. The relative site occupancy was similar for both the NAS and the NFT site in the two secreted proteins (Figure 8), thus the selectivity seems to be confined to glycan processing, not transfer. Structural microheterogeneity is observed in the addition of two or three GlcNAc residues to one of the mannose branches, and by the addition of either fucose or sialic acid distally to galactose. The other mannose branch shows microheterogeneity in the number of mannose residues (1 to 3), indicating variability in the action of mannosidases.

While micro- and macroheterogeneity are well known phenomena for glycoproteins (Marino, K., Bones, J., et al. 2010), altered glycan structures are related to congenital (genetic) disorders of glycosylation (Freeze, H.H. and Aebi, M. 2005) and cancer

(Peracaula, R., Barrabes, S., et al. 2008). For cells in culture, however, alterations in N-glycan processing do not seem to play an important role for cell growth or survival (Brandli, A.W., Hansson, G.C., et al. 1988, Le Bivic, A., Garcia, M., et al. 1993, North, S.J., Huang, H.H., et al. 2010).

N-glycans are required for apical sorting of several glycoproteins to the apical surface of epithelial MDCK cells (Kitagawa, Y., Sano, Y., et al. 1994, Pang, S., Urquhart, P., et al. 2004, Potter, B.A., Weixel, K.M., et al. 2006, Urquhart, P., Pang, S., et al. 2005), including secretory and GPI-linked versions of rGH (Benting, J.H., Rietveld, A.G., et al. 1999, Scheiffele, P., Peranen, J., et al. 1995), although not for all glycoproteins (Larsen, J.E., Avvakumov, G.V., et al. 1999, Trischler, M., Koch-Brandt, C., et al. 2001, Vogel, L.K. and Larsen, J.E. 2000). We have previously shown that rGH tagged with GFP C-terminally is directed to the apical surface either by the two N-glycosylation sites introduced by Scheiffele et al. 1995, or by the GAG modification domain from SG (Hafte, T.T., Fagereng, G.L., et al. 2010). Since the SG GAG chains were shown to be differentially modified in the apical and basolateral secretory pathways of epithelial MDCK cells (Tveit, H., Dick, G., et al. 2005, Vuong, T.T., Prydz, K., et al. 2006), it was of interest to find out whether differential modification of N-glycans also could occur. We did not, however, obtain any evidence for differential glycan processing in the apical and basolateral pathway of epithelial MDCK cells for the rGH variants we studied. This does not exclude the possibility that apically and basolaterally targeted glycoproteins may segregate early in the secretory pathway (Alfalah, M., Wetzel, G., et al. 2005) and that the apical and basolateral routes provide different environments for proteoglycan synthesis (Dick, G., Grondahl, F., et al. 2008, Grondahl, F., Tveit, H., et al. 2009). But

again, such segregation must not imply that the glycans formed in the apical and basolateral pathway must differ structurally. Further investigation is required to determine the extent of variation in N-glycan synthesis in the two routes. Important sorting decisions might also take place after glycan synthesis. Although the mechanisms have not been completely outlined, galectin-3 has been suggested as a cross-linking apical glycoprotein receptor (Delacour, D., Greb, C., et al. 2007). Since several galectins are expressed in MDCK cells, the contribution from the other variants remains to be established (Poland, P.A., Rondanino, C., et al. 2011).

In summary, we have expressed two rGH variants, rGH-2N, and rGH-2N-GAG, in polarized MDCK cells and observed that additional GAG chains shift the microheterogeneity of the N-glycan structures towards more acidic terminal sugars. Whether this is a result of differential partitioning of rGH-2N and rGH-2N-GAG in the Golgi apparatus, or differential efficiency as substrates for the glycosyltransferases in question, is a matter of further investigation.

Materials and Methods

Cell culture and transfection

MDCK cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza/BioWhittaker, Basel, Switzerland), complete with 5 % fetal calf serum (FCS; PAA Labratories, Brisbane, Australia), 1 % penicillin/streptomycin (P/S) and 1 % L-Glutamine (Lonza/BioWhittaker), at 37°C in humidified air with 5 % CO_2. Transfection of MDCK cells was performed by growing MDCK cells to 50-70 % confluency in 100 mm x 20 mm cell culture dishes (Corning incorporated, Corning, NY, USA) before addition of 4 µg of each rGH plasmid (Hafte, T.T., Fagereng, G.L., et al. 2010)and 12 µl of FuGENE 6 (Roche Diagnostics, Basel, Switzerland), as recommended by the manufacturer. After prolonged culture in the presence of the antibiotic G-418 (Duchefa Biochemie, Haarlem, The Netherlands) resistant colonies were screened by live confocal imaging (Confocal IX81 Olympus Fluorview FV1000, Olympus, Hamburg, Germany). Transfected and control MDCK cells were seeded onto Transwell polycarbonate filters (pore size 0.4 µm, diameter 75 mm, Costar 3419, Corning Inc., Corning, NY, USA) at a density of 10^7 cells per filter and left to grow for 3 days in 145 ml complete DMEM in holders placed in glass dishes with a diameter of 150 mm. The filters were moved to 100 mm diameter dishes, and 10 ml DMEM with 2 % FCS was added to the apical and basolateral sides of the filter. Apical and basolateral media from two filters were pooled individually and transferred to two separate 50 ml sterile centrifuge tubes after 24 and 48 h of incubation (replacement of medium after 24 h), amounting to 40 ml of sample for both apical and basolateral media.

Enzymatic digestion of GAG and N-glycans before SDS-PAGE and Western blotting

Apical and basolateral medium samples from filter-grown MDCK cells expressing rGH-2N or rGH-2N-GAG were subjected to enzymatic digestion. rGH-2N-GAG samples were either left untreated (control), or were treated with heparitinase I, II and III (Grampian enzymes, Orkney, UK) for heparan sulfate digestion, chondroitinase ABC (cABC), Seikagaku Corp., Tokyo, Japan) for chondroitin sulfate (CS) degradation, and/or PNGase F (Roche Diagnostics) for N-glycan degradation. CS degradation was carried out by the addition of 20 mU cABC to each sample, followed by incubation at 37°C for 2 h. Heparan sulfate chains were removed from the protein core after incubation of samples with 4 mU of each of heparitinase I, II and III at 28°C for 2 h. Removal of N-glycans was carried out by the incubation of samples with 1 U PNGase F at 37°C for 2 h.

Western blotting

Western blotting (WB) was performed by transferring proteins from SDS-PAGE gels onto Hybond-P PVDF transfer membranes (GE Healthcare, Fairfield, CT, USA), using polyclonal anti-GFP (Ab-6556, Abcam, Cambridge, UK) as primary antibody and goat anti-rabbit IgG; alkaline phosphatase linked whole antibody (NIF 1317, GE Healthcare) as secondary antibody. Membranes were visualized by ECF Western blotting Reagent Pack (GE Healthcare, Chalfont St Giles, UK) and blots were quantified with a Typhoon 9410 PhosphorImager (GE Healthcare).

Immune precipitation

300 µl of Dynabeads protein A (Invitrogen) per immune precipitation (IP) were precleared using 3 x 8 ml 0.1 M Na-phosphate buffer (pH 8.1), before the beads were incubated with 10 µl anti-GFP (Ab-290, Abcam) in 500 µl Na-phosphate buffer (pH 8.1) for 1 h with rotation at room temperature. After incubation, the beads were washed with 3 x 8 ml Na-phosphate buffer (pH 8.1) and incubated with 40 ml of either apical or basolateral medium samples with rotation at 4°C overnight (ON). The supernatants were discarded and the beads were washed with 3 x 25 ml IP wash buffer (50 mM Tris, 150 mM NaCl, 0.05 % Triton, pH 7.4) with 1 % BSA and 3 x 25 ml IP-wash buffer without BSA (pH 7.4). The beads were resuspended in 100 µl IP wash buffer (pH 7.4) per sample and transferred to microfuge tubes. The microfuge tubes were transferred to a magnetic rack, and the bead-free solutions were discarded. 80 µl of SDS-PAGE sample buffer (BioRad, Hercules, CA, USA) without reducing agent was added to the IP beads, boiled at 96°C for 5 min before loading samples on 4-12 % Criterion XT SDS-PAGE gels (BioRad).

SDS-PAGE

25 µl of each sample was loaded onto 4-12 % Criterion XT SDS-PAGE gels in MOPS buffer (pH 7.0, BioRad). The gels were run for approximately 2 h at 100 V, fixed for 30 min (50 % methanol/10 % acetic acid in water), stained for 30 min with Coomassie brilliant blue (Sigma-Aldrich, St. Louis, MO, USA), and finally destained ON with destaining solution (20 % methanol/5 % acetic acid in water).

In gel digestion

Protein bands of interest were cut from SDS-PAGE and sliced to smaller pieces. Pieces from the same bands were pooled in microfuge tubes with destaining solution (500 µl dH_2O/500 µl 2-propanol), and incubated at 55°C for 30 min, or longer, until all gel pieces were completely destained. 100 µl 2-propanol was added, followed by incubation at room temperature until all gel pieces had turned white. The solution was aspirated and the gel pieces were dried.

50 µl trypsin (16 ng/µl; Sigma-Aldrich) dissolved in trypsin buffer (50 mM NH_4HCO_3, pH 8.0) was added to each tube with gel pieces and incubated on ice for 30 min. Excess solution was then removed and trypsin buffer was added to each tube to cover the gel pieces (approximately 100 µl), followed by incubation ON at 37°C. Peptides were subsequently extracted twice with 30 µl formic acid (5 %)/30 µl acetonitrile, and then once with 60 µl acetonitrile. The extracts were combined and dried in a Speedvac concentrator (Thermo Fisher, Waltham, MA, USA).

Deglycosylation by N-glycosidase F (PNGase F) in the presence of H_20^{18}

Dried (glycol-)-peptide samples after trypsin digestion were dissolved in 20 µl 0.1 % formic acid, sonicated for 30 sec and centrifuged at 16,100 g for 10 min. The samples (10 µl of each) were used for tandem mass spectrometry (MS/MS) analysis of peptides with N-glycans, and the remaining samples were dried again prior to PNGase F treatment and dissolved in 2 µl Tris buffer (1.0 M, pH 7.8), 1 µl PNGase F (250 U/250 µl, Roche Diagnostics) and 15 µl H_20^{18} (Isotec, Sigma-Aldrich). All samples were incubated at 37°C ON and subseqeuntly dried in a Speedvac concentrator.

Nanoflow on-line liquid chromatography coupled MS analysis of proteolytic peptides

Dried samples after PNGase F digestion were dissolved in 20 µl 0.1 % formic acid, sonicated for 30 sec and centrifuged for 10 min at 16,100 g. 10 µl of each sample was subsequently transferred to MS vials.

Reverse phase (C_{18}) nano online liquid chromatography coupled MS/MS analysis of proteolytic peptides was performed using a system consisting of two Agilent 1200 HPLC binary pumps (nano and capillary) with autosampler, column heater and integrated switching valve. This LC system was coupled via a nano electrospray ion source to a LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen,Germany). For the analysis, 2 µl of peptide solution was injected into the 5 x 0.3-mm extraction column filled with Zorbax 300 SB-C18 of 5 µm (diameter) particles (Agilent, Santa Clara, CA, USA). Samples were washed with mobile phase (97 % MS grade water/0.1 % formic acid/3 % acetonitrile). A flow rate of 4 µl/min was provided by the capillary pump. After 7 min, the integrated switching valve was activated, and peptides were eluted in the back-flush mode from the extraction column onto a 150 × 0.075-mm C_{18}, 3 µm resin column (GlycproSIL C18–80Å, Glycpromass, Stove, Germany). The mobile phase consisted of acetonitrile and MS grade water, both containing 0.1 % formic acid. Chromatographic separation was achieved using a binary gradient from 5 to 55 % of acetonitrile in water for 210 min with a flow rate of 0.2 µl/min, provided by the nanoflow pump.

Mass spectra were acquired in the positive ion mode, applying a data-dependent automatic switch between survey scan and MS/MS acquisition. Peptide samples were

analyzed with higher energy collisional dissociation (HCD) fragmentation, acquiring one Orbitrap survey scan in the mass range of *m/z* 300–2000, followed by MS/MS of the three most intense ions in the Orbitrap. The target value in the LTQ-Orbitrap was 1,000,000 for survey scan at a resolution of 30,000 at *m/z* 400, using lock masses for recalibration to improve the mass accuracy of precursor ions. Fragmentation in the C-trap was performed by collision-induced dissociation, with a target value of 5,000 ions. Ion selection threshold was 500 counts. Selected sequenced ions were dynamically excluded for 180 sec.

Data analysis

MS data were analyzed with the in-house maintained human protein sequence database and a database of the constructs using SEQUEST™. The mass tolerances of a fragment ion and a parent ion were set as 0.05 Da and 5 ppm, respectively. Methionine oxidation and cysteine carbamidomethylation were selected as variable or fixed modifications. A false discovery rate of 0.01 was required for proteins and peptides with a minimum length of 6 amino acids. MS/MS spectra of rGH glycopeptides were manually searched by Qual Browser version 2.0.7.

Funding

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Abbreviations

cABC, chondroitinase ABC; CS, chondroitin sulfate; GAG, glycosaminoglycan; GFP, green fluorescent protein; HCD, high energy collisional dissociation; IP, immune precipitation; MDCK, Madin-Darby canine kidney,; MS, mass spectrometry; ON, overnight; PG, proteoglycan; rGH, rat growth hormone; SG, Serglycin.

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Figure legends

Figure 1. Constructs and overview of workflow.

A: A schematic presentation of the different rGH variants used: rGH, rat growth hormone (rGH) with green fluorescence protein (GFP) fused to the C-terminal part of the protein: rGH-2N, the same construct as rGH, but with two N-glycan sites inserted (Scheiffele et al., 1995): rGH-2N-GAG, the same construct as rGH-2N with the glycosaminoglycan (GAG) attachment domain (EDYSGSGFGSGSGSGSGSGSGFLTE) of Serglycin (SG) inserted between rGH and GFP. The two most abundant peptides during MS analysis carrying the N-glycan sites NAS and NFT are indicated by arrows. **B**: Epithelial MDCK cells expressing glycosylated rGH variants were grown for 3 days on polycarbonate filters to develop confluent and tight monolayers, comprising a barrier to mixing of the apical and basolateral medium reservoirs. The medium samples were processed as indicated in the figure and in Materials and Methods.

Figure 2. Polarized sorting and characterization of rGH variants in MDCK cells MDCK cells expressing rGH, rGH-2N, or rGH-2N-GAG were grown on polycarbonate filters until confluency (3 days), followed by replacement of media to DMEM with 2 % serum and medium harvest 24 h later. Medium aliquots were enzymatically treated as indicated to degrade N-glycans (PNGase F), heparan sulfate (H; heparitinase mix) and chondroitin sulfate (C; chondroitinase ABC), or left untreated, and were subsequently loaded onto 4-12 % Criterion XT SDS-PAGE gel. The gels were subjected to WB with an anti-GFP antibody, and the figure shows one representative experiment of four.

Figure 3. Identification of N-glycan modified peptides.

The rGH-2N and rGH-2N-GAG from polarized MDCK cells were IP and after SDS-PAGE. The collected proteins were sliced and digested with trypsin followed by removal of N-glycans by PNGase F. The two most abundant peptides detected using the Orbitrap XL mass spectrometer were LPAMoxPLSSLFANASLR and NFTNSLMoxFGTSDR. HCD MS/MS spectra of the doubly charged peptides at m/z 852.463^{2+} (**A**) and m/z 753.340^{2+} (**B**) confirmed the presence of the peptides.

Figure 4. Base peak and selected ion chromatograms of a tryptic digest of rGH-2N.

IP polarized secreted rGH-2N were after collection from SDS-PAGE trypsinated and analyzed by nano on-line-LC-MS/MS using the Orbitrap XL mass spectrometer in the HCD fragmentation mode, to search for peptides modified with a glycan moiety. The top panel represents the base peak ion chromatogram (BPC), and the lower panel shows the selected ion (SIC) chromatograms of precursor ions at m/z = 1262.5059 and m/z = 1328.5886, respectively from two selected glycopeptides.

Figure 5. Identification of the glycosylation status of the peptides by HCD MS/MS analysis using the Orbitrap XL mass spectrometer.

A: MS/MS spectrum of the triply charged peptide at m/z 1328.5886^{3+} confirms that the peptide LPAMoxPLSSLFANASLR carries an *N*-linked oligosaccharide with the dimension 2 x GlcNAc, 3 x mannose, 2 x fucose and 3 x GlcNAc and 3 x hexose. GlycoMod toll and MS/MS analysis suggest complex/hybrid glycan structures with bisecting bi-antennary composition with a distal fucose moiety. **B:** MS/MS spectrum of

the triply charged peptide at *m/z* 1262.5059^{3+} confirms that the peptide NFTNSLMoxFGTSDR carries an *N*-linked oligosaccharide with the dimension 2 x GlcNAc, 3 x mannose, 2 x fucose and 3 x GlcNAc and 2 x hexose. GlycoMod toll and MS/MS analysis suggest complex/hybrid glycan structures with bisecting bi-antennary composition with a distal fucose moiety. **C:** MS/MS spectrum of the triply charged peptide at *m/z* $1376{,}9323^{3+}$ confirms that the peptide LPAMoxPLSSLFANASLR carries an *N*-linked oligosaccharide with the dimension (2 x GlcNAc, 3 x mannose, 1 x fucose, 1 x sialic acid, 3 x GlcNAc and 3 x hexose). GlycoMod toll and MS/MS analysis suggest complex/hybrid glycan structures with bisecting bi-antennary composition, with a distal sialic acid moiety attached.

Figure 6. Characterization of protein glycosylation heterogeneity at the NAS site of rGH-2N.

Averaged LC-MS precursor ion spectrum between 1240 and 1450 *m/z* and 65 and 72 min are presented to visualize the relative intensities of the three major glycoforms detected for the peptide LPAMoxPLSSLFANASLR.

Figure 7. Characterization of protein glycosylation heterogeneity changes.

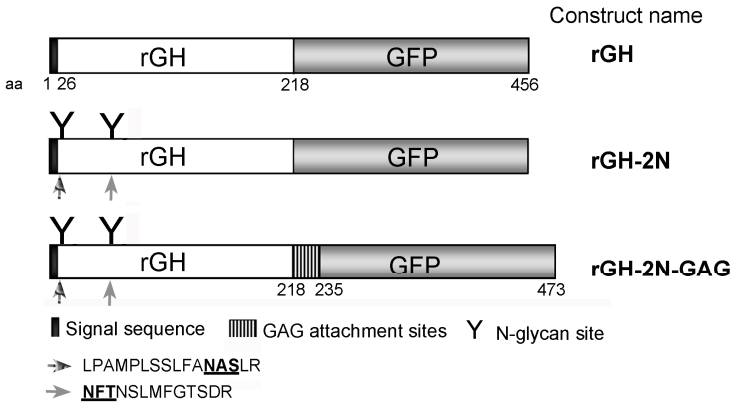
A: Characterization of protein glycosylation heterogeneity changes on the NAS glycosylation site in relation to the additional GAG modification domain in rGH-2N-GAG. Selected ion LC-MS precursor ion traces between 80 and 100 min visualize the relative intensity changes (peak area) of the three major glycoforms detected for the peptide LPAMoxPLSSLFANASLR. All glycoforms at the NAS **(B)** and NFT **(C)**

glycosylation sites were detected and their relative intensities determined by selected ion LC-MS precursor ion traces. The results are depicted as bar diagrams showing the relative amounts of the different glycoforms bound to the glycosylation sites in rGH-2N and rGH-2N-GAG isolated from apical and basolateral media of filter-grown, transfected epithelial MDCK cells. The bars with standard deviations are based on three independent experiments.

Figure 8. Determination of glycan occupancy at the NAS and NFT sites.

A: Middle panel: selected ion chromatogram of the glycan free peptide LPAMoxPLSSLFANASLR: top panel, total ion chromatogram of the same peptide: lower panel, selected ion chromatogram of the same peptide in the deglycosylated state. **B:** PNGase F treatment in the presence of 75 % H_2O^{18} (25 % H_2O^{16}) caused a mass shift of 2.98 (H_2O^{18}) and 0.98 Da (H_2O^{16}) when compared to the previously glycosylated peptides. **C:** Glycan site occupancy percentages were determined by relative comparison of the isotopic envelopes of those asparagine and aspartic acid containing peptides. The average percent site occupancy at NFT and NAS glycosylation sites for rGH-2N and rGH-2N-GAG secreted to the apical and the basolateral media of polarized epithelial MDCK cells was determined. Standard deviations are based on three independent experiments.

Figure 1A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rGH
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218
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rGH-2N
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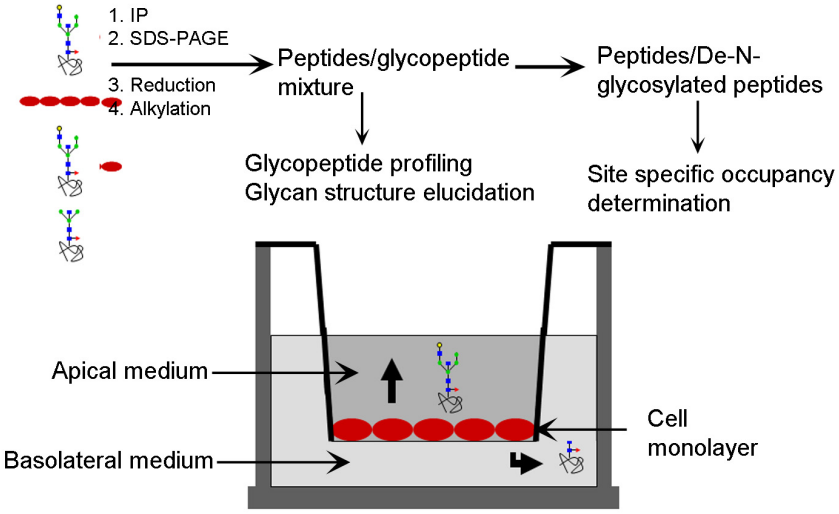
NFTNSLMFGTSDR

Figure 1B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2. SDS-PAGE
3. Reduction
4. Alkylation
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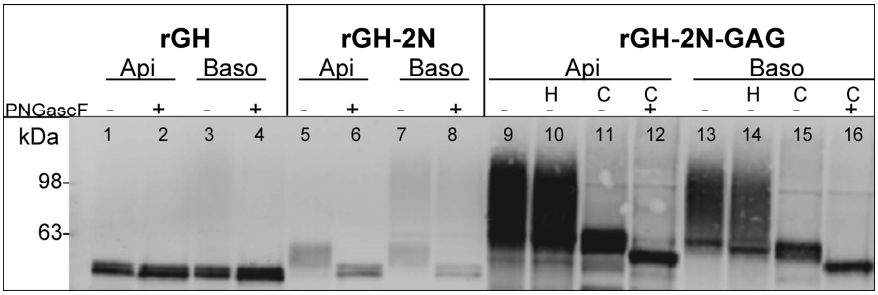
Site specific occupancy determination
Apical medium
Basolateral medium
Cell monolayer

Figure 2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Baso
Api
Baso
Api
Baso
PNGaseF
H
C
C
H
C
C
kDa
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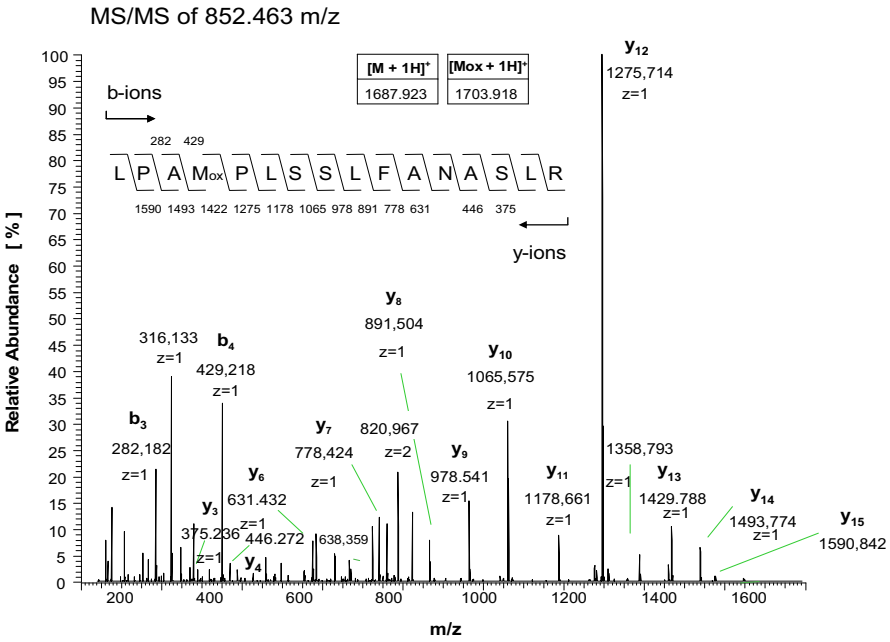
98-
63-

Figure 3A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y-ions
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1590 1493 1422 1275 1178 1065 978 891 778 631 446 375
Relative Abundance [%]
m/z
y12 1275,714 z=1
y8 891,504 z=1
y10 1065,575 z=1
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316,133 z=1
b3 282,182 z=1
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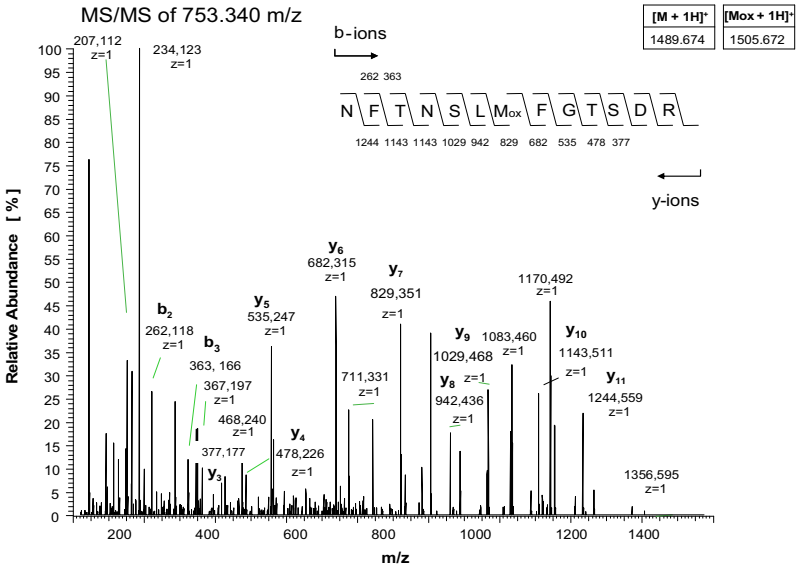
Figure 3B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y-ions
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Relative Abundance [%]
m/z
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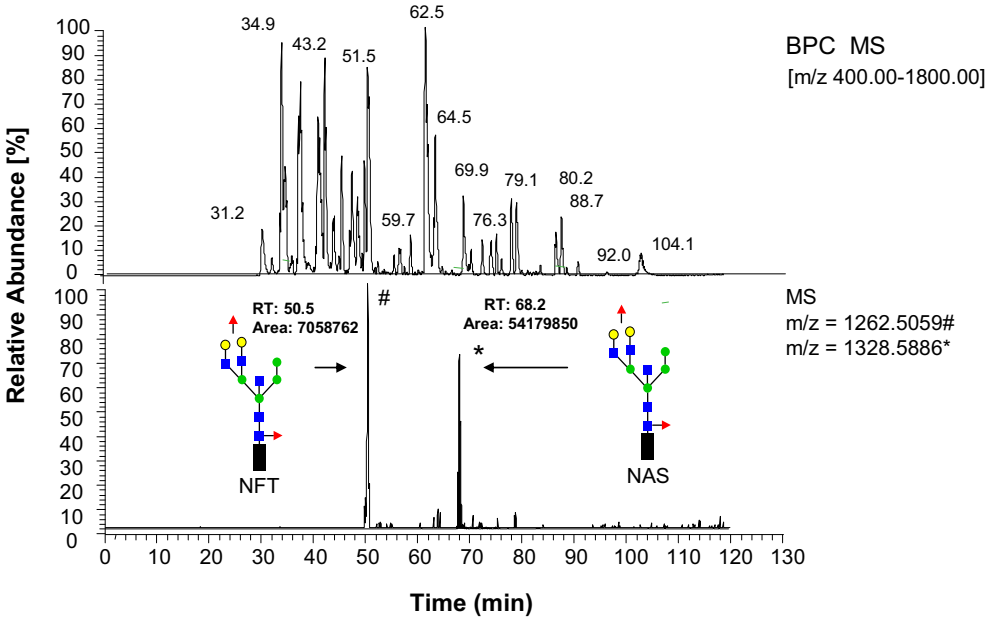
Figure 4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RT: 68.2
Area: 54179850
NFT
NAS
#
*
34.9
43.2
51.5
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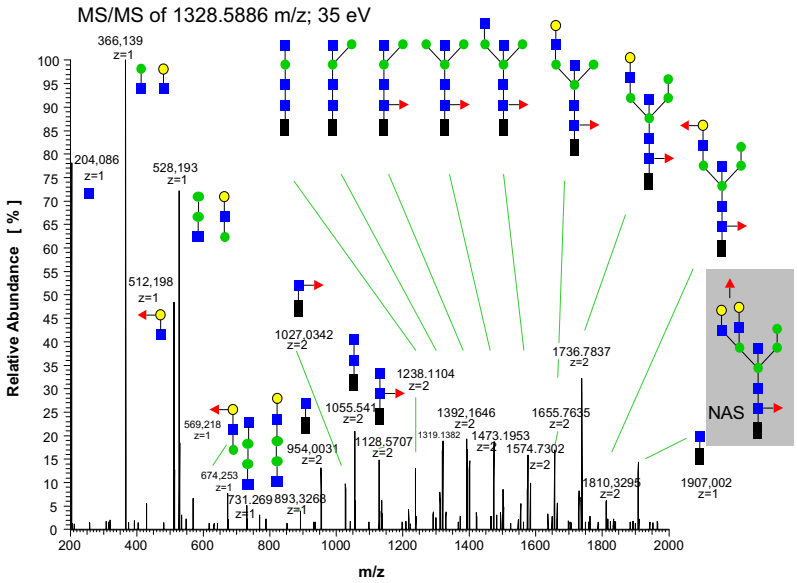
92.0
104.1

Figure 5A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Relative Abundance [%]
m/z
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NAS

Figure 5B

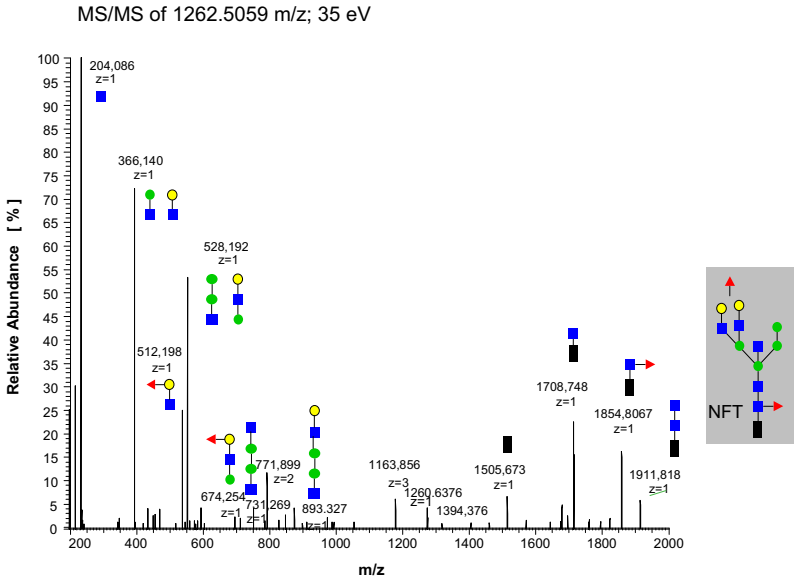

MS/MS of 1262.5059 m/z; 35 eV
Relative Abundance [%]
m/z
204,086
z=1
366,140
z=1
528,192
z=1
512,198
z=1
674,254
z=1
731,269
z=1
771,899
z=2
893,327
z=1
1163,856
z=3
1260,6376
z=1
1394,376
1505,673
z=1
1708,748
z=1
1854,8067
z=1
1911,818
z=1
NFT

Figure 5C

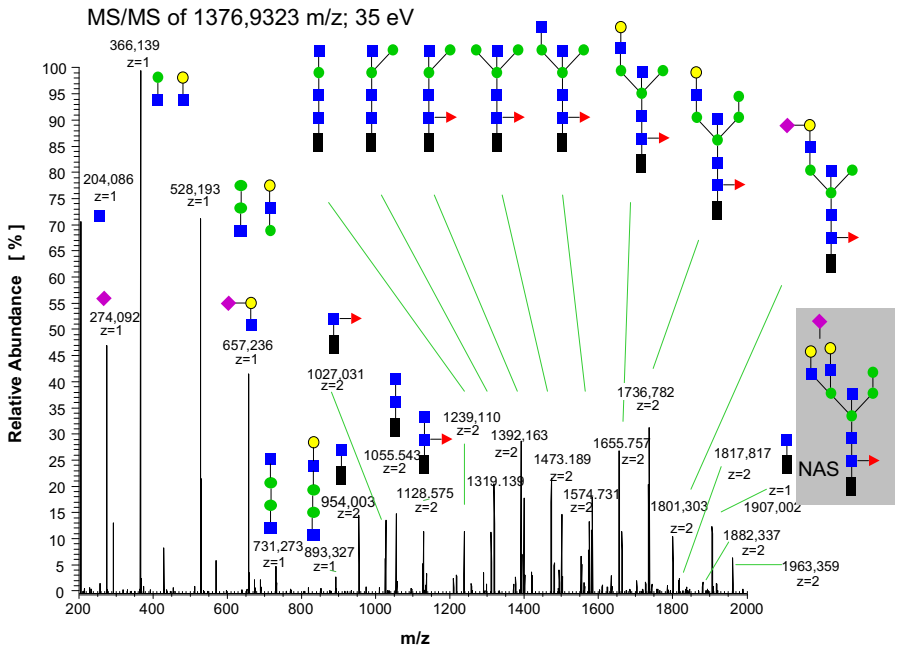

MS/MS of 1376,9323 m/z; 35 eV
Relative Abundance [%]
m/z
366,139 z=1
204,086 z=1
528,193 z=1
274,092 z=1
657,236 z=1
731,273 z=1
893,327 z=1
954,003 z=2
1027,031 z=2
1055,543 z=2
1128,575 z=2
1239,110 z=2
1319,139
1392,163 z=2
1473,189 z=2
1574,731 z=2
1655,757 z=2
1736,782 z=2
1801,303 z=2
1817,817 z=2
1882,337 z=2
1907,002 z=1
1963,359 z=2
NAS

Figure 6

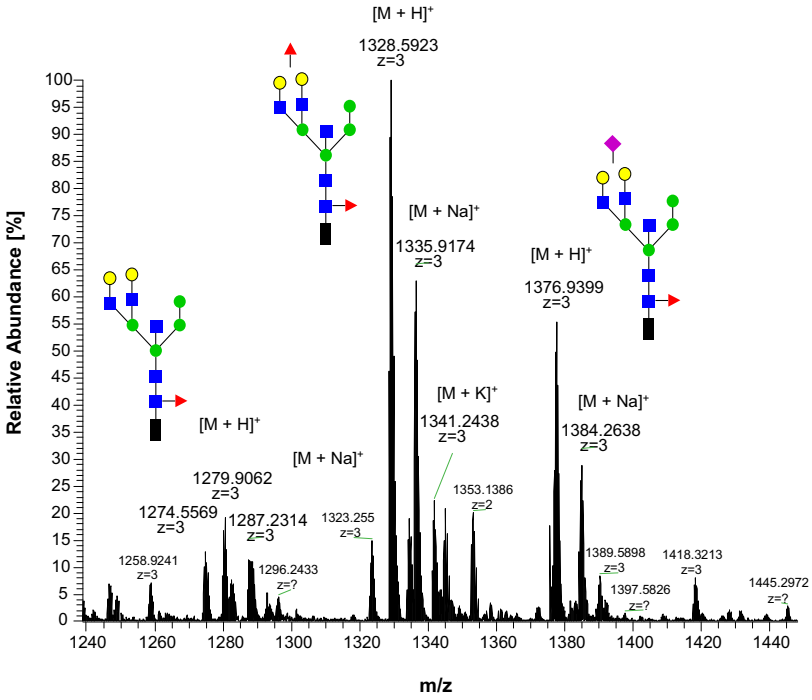

[M + H]+
1328.5923
z=3
[M + Na]+
1335.9174
z=3
[M + H]+
1376.9399
z=3
[M + K]+
1341.2438
z=3
[M + Na]+
1384.2638
z=3
[M + H]+
[M + Na]+
1279.9062
z=3
1274.5569
z=3
1287.2314
z=3
1323.255
z=3
1353.1386
z=2
1258.9241
z=3
1296.2433
z=?
1389.5898
z=3
1418.3213
z=3
1397.5826
z=?
1445.2972
z=?
Relative Abundance [%]
m/z

Figure 7A

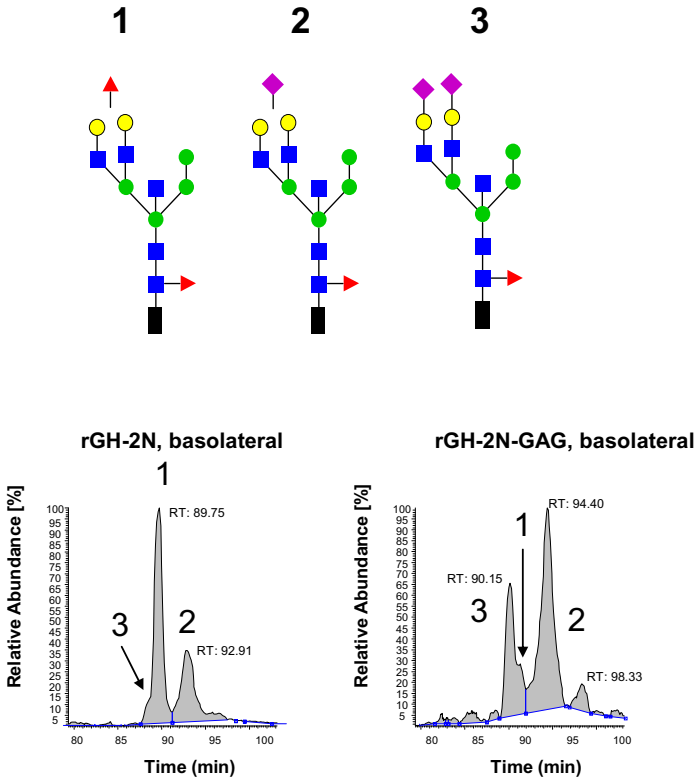

1
2
3
rGH-2N, basolateral
rGH-2N-GAG, basolateral
Relative Abundance [%]
Time (min)
RT: 89.75
RT: 92.91
RT: 94.40
RT: 90.15
RT: 98.33

Figure 7B

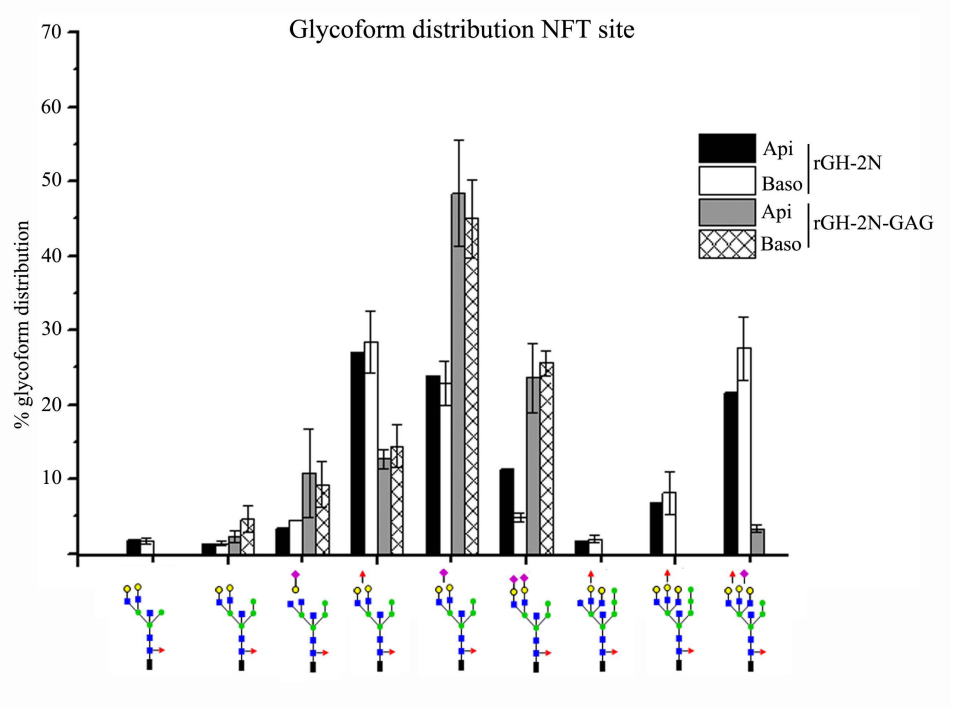

Glycoform distribution NFT site
% glycoform distribution
70
60
50
40
30
20
10
Api
Baso
rGH-2N
Api
Baso
rGH-2N-GAG

Figure 7C

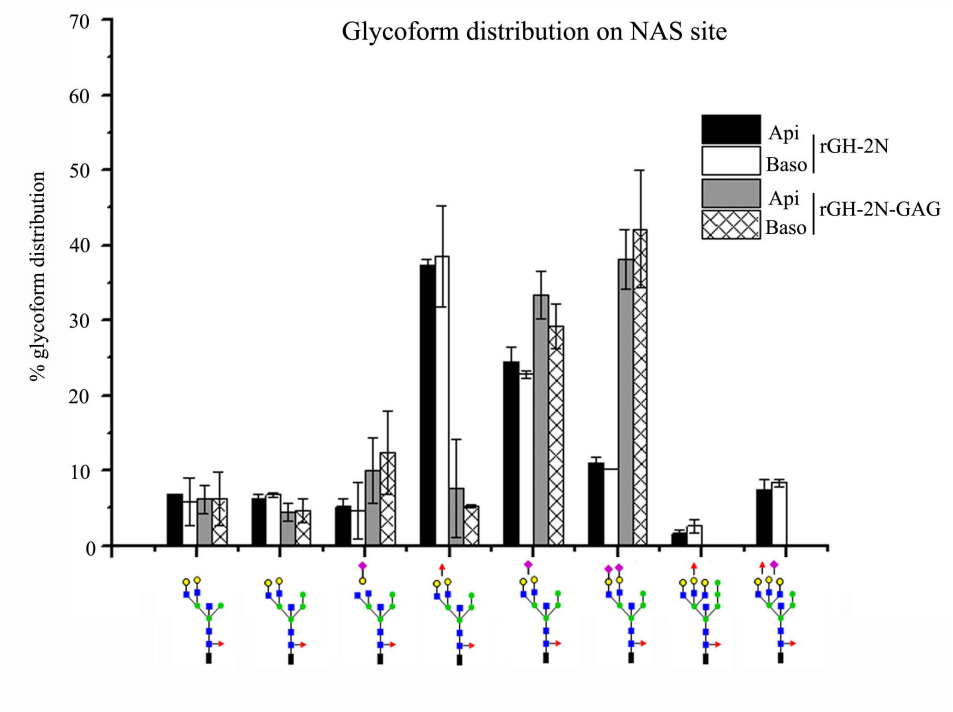

Glycoform distribution on NAS site
% glycoform distribution
70
60
50
40
30
20
10
0
Api
Baso
rGH-2N
Api
Baso
rGH-2N-GAG

Figure 8

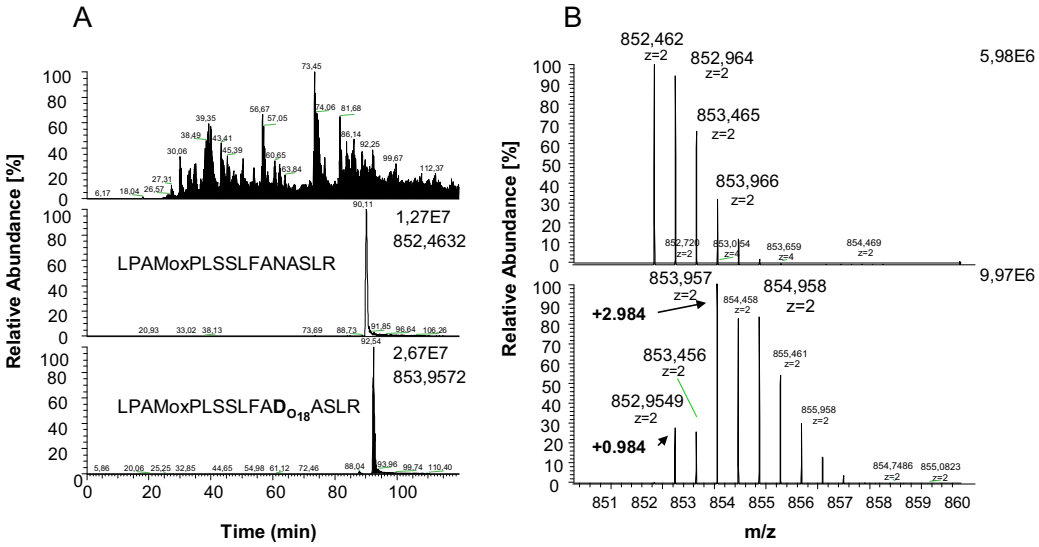
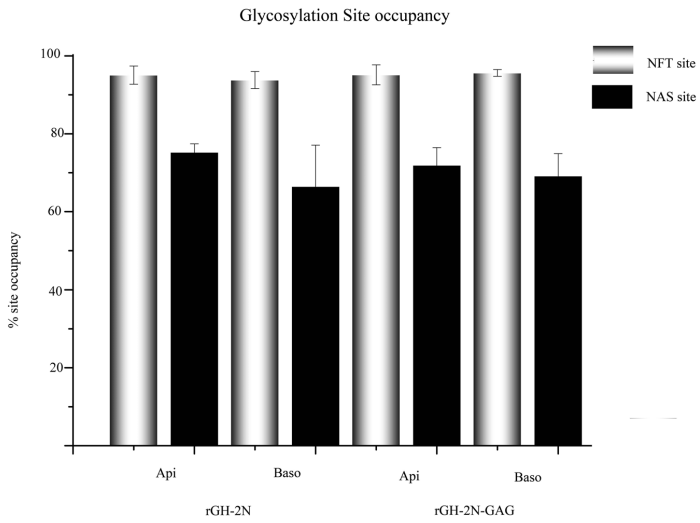

A
B
Relative Abundance [%]
Time (min)
m/z
LPAMoxPLSSLFANASLR
1,27E7
852,4632
LPAMoxPLSSLFAD$_{O18}$ASLR
2,67E7
853,9572
852,462
z=2
852,964
z=2
853,465
z=2
853,966
z=2
5,98E6
9,97E6
853,957
z=2
854,958
z=2
+2.984
853,456
z=2
852,9549
z=2
+0.984

Figure 8C


Glycosylation Site occupancy
NFT site
NAS site
% site occupancy
100
80
60
40
20
Api
Baso
Api
Baso
rGH-2N
rGH-2N-GAG

III