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Invited Review

Role of N- and O-glycans in polarized biosynthetic sorting

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Potter, Beth A., Rebecca P. Hughey, and Ora A. Weisz. Role of N- and O-glycans in polarized biosynthetic sorting. *Am J Physiol Cell Physiol* 290: C1–C10, 2006; doi:10.1152/ajpcell.00333.2005.—The maintenance of proper epithelial function requires efficient sorting of newly synthesized and recycling proteins to the apical and basolateral surfaces of differentiated cells. Whereas basolateral protein sorting signals are generally confined to their cytoplasmic regions, apical targeting signals have been identified that localize to luminal, transmembrane, and cytoplasmic aspects of proteins. In the past few years, both N- and O-linked glycans have been identified as apical sorting determinants. Glycan structures are extraordinarily diverse and have tremendous information potential. Moreover, because the oligosaccharides added to a given protein can change depending on cell type and developmental stage, the potential exists for altering sorting pathways by modulation of the expression pattern of enzymes involved in glycan synthesis. In this review, we discuss the evidence for glycan-mediated apical sorting along the biosynthetic pathway and present possible mechanisms by which these common and heterogeneous posttranslational modifications might function as specific sorting signals.

glycosylation; epithelia; polarity; kidney; intestine

EPITHELIA DISPLAY AN INTRINSIC polarity that is critical to their function in mediating interactions with and between two environments. For example, ions and small metabolites from the glomerular filtrate are transported vectorially across renal epithelial cells for reabsorption into the bloodstream. Efficient unidirectional transport requires the strict maintenance of a polarized distribution of plasma membrane components. Tight junctions divide the plasma membrane of epithelial cells into an apical surface that faces the external milieu and a basolateral domain that contacts neighboring cells and the basal lamina. The composition of these two membrane domains differs markedly and reflects the ability of epithelial cells to sort newly synthesized proteins and lipids to either cell surface. Cell polarity is also maintained by selective recycling of internalized proteins to the appropriate cell surface domain and by transcytosis of missorted molecules to the opposing cell surface.

Biosynthetic transport of newly synthesized proteins occurs via different routes and mechanisms in epithelial cells of distinct origin. Much of our understanding of how apical and basolateral proteins are differentially sorted and transported in renal cells comes from studies of Madin-Darby canine kidney (MDCK) cells, a well-characterized cell line with many characteristics resembling the distal nephron (66). In these cells, apical and basolateral proteins colocalize in the biosynthetic pathway until they reach the *trans*-Golgi network (TGN), where they are sorted into distinct transport carriers (19, 67). Transport from the TGN to the apical cell surface can occur directly or by way of an indirect (transcytotic) route via the basolateral cell surface (61). The transcytotic trafficking route appears to be a minor pathway in MDCK cells but is more significant in other types of epithelial cells, including hepatocytes and intestinal cells.

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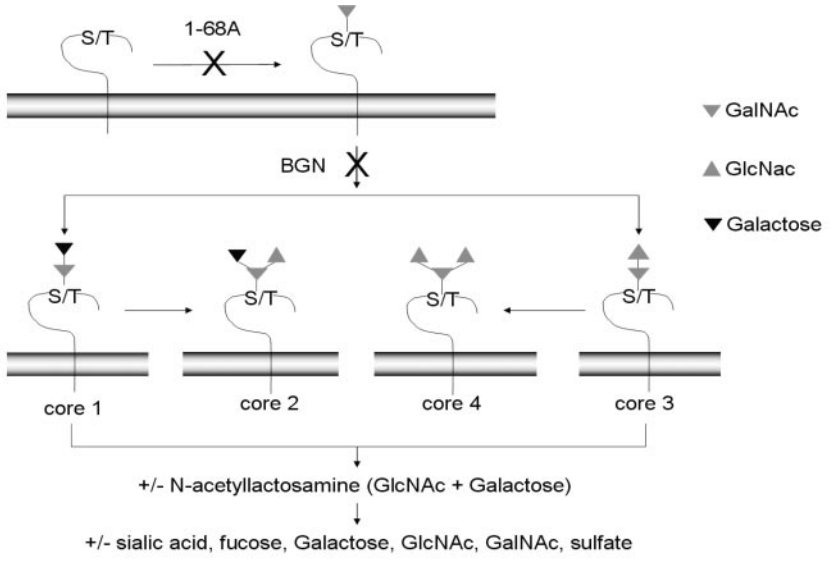
BIOSYNTHETIC SORTING OF CARGO IN POLARIZED CELLS

Distinct sorting signals are required to direct the export of newly synthesized proteins to either the apical or basolateral cell surface. In general, basolateral sorting is mediated by linear peptide sequences within the cytoplasmic portions of basolaterally directed proteins. One such signal found on many basolateral proteins is a tyrosine-based tetrapeptide motif that can bind to adaptor protein complexes (APs), although other AP-dependent and -independent targeting motifs have also been identified (for review, see Ref. 69). Tyrosine-based interactions with AP complexes are generally thought to be dominant over apical sorting signals (65), although a recent exception has emerged from our own work (33).

In contrast to basolateral proteins, biosynthetic sorting signals on apically destined cargo tend to be localized to transmembrane or luminal domains, although there are exceptions. The first apical sorting determinant to be identified was the glycosylphosphatidylinositol (GPI) lipid anchor found on some membrane-tethered proteins. These proteins are enriched at the apical plasma membrane of many polarized epithelial cells, although in Fischer rat thyroid (FRT) cells, the majority of GPI-anchored proteins are delivered to the basolateral domain (85). In contrast, apical transmembrane glycoproteins appear to be delivered normally in FRT cells (42, 48, 85). Biosynthetic sorting of GPI-anchored proteins has been suggested to involve their segregation into glycolipid-enriched microdomains or "rafts" in the TGN (11). In addition to GPI anchors, some transmembrane domains have been found to contain apical sorting information. The best studied example is influenza hemagglutinin (HA); apical delivery of this protein is impor-



Inhibitor	Mechanism	Effect
1-68A	This compound inhibits the transferases responsible for the initial addition of GalNAc to Ser/Thr.	O-glycosylation does not occur.
Benzyl-N-acetyl-α-galactosaminide (BGN)	This drug acts as an acceptor substrate for both galactosyl- and sialyltransferases and thus competes with endogenous glycoprotein substrates.	O-glycans cannot be elongated beyond the addition of the initial GalNAc resiudue; also affects N-glycosylation (see Fig. 2).

Fig. 1. Structure of O-glycans and effects of pharmacological perturbants. Mucin type O-glycosylation occurs in the Golgi complex with the addition of an *N*-acetylgalactosamine (GalNAc) residue to serine or threonine (S/T) residues. Because of the heterogeneity of mucin type O-glycans, they are characterized by their core structures. Shown in this diagram are 4 of the common core structures. All 4 structures can be processed further by the addition of *N*-acetylglucosamine (GlcNAc) and galactose (*N*-acetyllactosamine); however, only core 2 can be poly-*N*-acetyllactosaminylated. All core structures can be further modified by fucose, sialic acid, galactose, GlcNAc, GalNAc, and/or sulfate. *Bottom*, description of the two known inhibitors of O-glycan synthesis: 1-68A and benzyl-*N*-acetyl-α-galactosaminide.

tant for efficient delivery of HA to the site of influenza virus budding. Like GPI-anchored proteins, HA partitions into glycolipid-enriched membranes; however, association with lipid rafts is required but not sufficient for apical targeting of either protein (41). By contrast, targeting of the H^+-K^+-ATPase α-subunit is also mediated by sequences in one of its transmembrane domains, but this protein does not associate with lipid rafts (15).

More recently, a role for both N- and O-glycans as apical sorting signals has emerged from studies in both renal and intestinal epithelial cells. The remainder of this review briefly summarizes the pathways for assembling N- and O-linked glycans along the biosynthetic pathway, describes the evidence for glycosylation-dependent sorting, and discusses possible mechanisms for how these ubiquitous posttranslational modifications might function as selective apical targeting signals along the biosynthetic sorting pathway.

ASSEMBLY OF N- AND O-GLYCANS ALONG THE BIOSYNTHETIC PATHWAY

Unlike protein synthesis, in which amino acid sequence is dictated by a linear template, N- and O-glycosylation proceed via the stepwise addition or removal of individual glycosides to form linear or branched chains whose structures are determined by the specificities of the glycosyltransferases involved. Sugars can be added in either of two linkages (α or β) to different hydroxyl groups (typically on the second, third, fourth, or sixth carbon atom) on most saccharides, resulting in a vast array of possible glycan structures that can be generated in a given cell. Additional modifications, including phosphorylation and sulfation, also contribute to the vast array of structures found in mature glycoproteins. The diversity of glycoconjugates displayed by a particular cell is ultimately a reflection of the glycosyltransferases and glycosidases expressed in that cell. The levels and patterns of enzyme expression are cell type dependent and are differentially regulated during development. Moreover, both N- and O-linked oligosaccharide profiles are altered in many disease states. Thus, the structures of glycans on a given protein can vary, depending on the developmental stage, differentiation state, and type of cell in which it is expressed (25, 55, 84). Given the proposed requirements for distinct saccharides and glycan linkages in sorting of some proteins, it is intriguing to speculate that changes in the glycan structure on a given protein could function as a mechanism to regulate its targeting in a developmentally specific manner.

ASSEMBLY OF O-LINKED GLYCANS

Mucin-type O-linked glycosylation proceeds in a stepwise fashion with the addition of individual sugars to *N*-acetylgalactosamine (GalNAc)1α-Ser/Thr (Fig. 1). GalNAc is added to

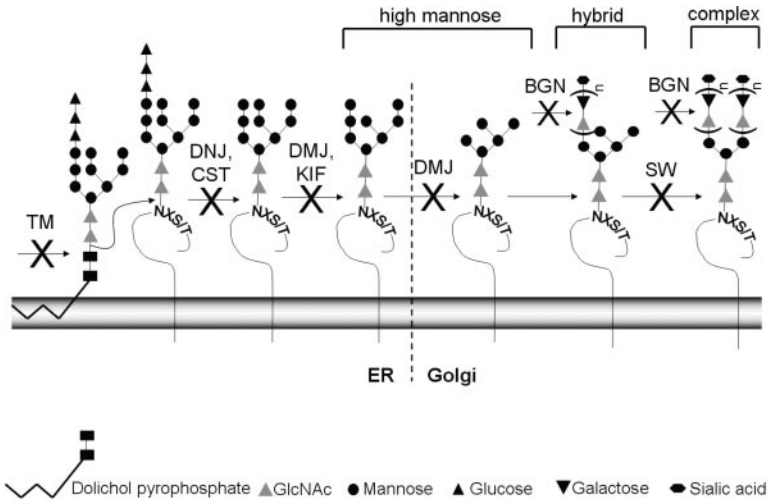
the protein by one of the many UDP-*N*-acetylgalactosaminopolypeptide *N*-acetylgalactosaminyl-transferases (ppGaNTases). Thirteen functional ppGaNTases have now been cloned and partially characterized, although 24 have been identified in the human genome (75). There is no obvious consensus site for O-GalNAc addition to proteins, although analysis of sequences utilized in native glycoproteins consistently indicates that flanking residues are enriched in proline, serine, threonine, and alanine. Systematic characterization of recombinant ppGaNTase activities in vitro using peptides and glycopeptide substrates representing well-characterized mucins indicates that these transferases can exhibit both redundant and hierarchical activities, which likely account for the full repertoire of sites that are utilized in vivo (64, 74, 75). Mature mucins exhibit hundreds of different linear and branched O-glycan structures that are likely dependent on a combination of developmentally, tissue-, and subcellularly specific expression (and dysregulation in cancers) of the numerous transferases for addition of galactose (Gal), GalNAc, GlcNAc, fucose, sialic acid, and sulfate to linear or branched oligosaccharides (8, 9). Interestingly, there is also evidence that glycan extension/maturation is also affected by the protein primary structure (e.g., hydroxyl amino acid density) (20). In addition to these mucin type O glycans, several less prevalent types of O-glycosylation have also been described, including O-linked fucose, glucose, GlcNAc, and mannose, which have diverse tissue-specific and developmental roles that are beyond the scope of this article (see Ref. 25 for an excellent review).

Synthesis of linear glycosaminoglycan (GAG) O-linked chains also proceeds in a stepwise fashion by addition of individual sugars to produce the tetrasaccharide GlcAβ1,3Gal β1,3Gal β1,4Xylβ-Ser core (where GlcA is glucuronic acid and Xyl is xylose). The addition of either GalNAc or GlcNAc alternating with GlcA to the nonreducing end of this core and subsequent sulfation produces either chondroitin/dermatan sulfate or heparan sulfate proteoglycans, respectively. Repeating sulfated disaccharides of Galβ1,4GlcNAc on the nonreducing ends of either N-glycans or mucin-type O-glycans produce keratin sulfate GAG chains. Because there is no evidence thus far that proteoglycans or GAG chains play a role in polarized targeting, these are not discussed further herein.

ASSEMBLY OF N-LINKED GLYCANS

In contrast to the synthesis of both the mucin-type and GAG-type O-linked glycans, oligosaccharides that are N-linked to asparagines are initially assembled as a common precursor of $Glc_3Man_9GlcNAc_2$ (where Glc is glucose and Man is mannose) on a dolichol-lipid anchor by stepwise addition. This core glycan structure is then transferred en bloc by the oligosaccharyltransferase multisubunit complex to a specific protein consensus site (Asn-X-Ser/Thr, where X is any amino acid, except Pro) during cotranslational translocation of proteins into the endoplasmic reticulum (ER; Fig. 2) (37). Not all Asn-X-Ser/Thr motifs receive N-glycans, but all N-glycans are attached at this motif. After transfer to the protein, three Glc and at least one mannose are removed in the ER, and additional mannose residues are sometimes removed in the early Golgi compartments before stepwise addition of individual sugars. The structures of N-glycans found on mature proteins are quite diverse, although all mature N-glycans contain at least the $Man_3GlcNAc_2$-Asn core. N-glycans with five to nine mannose ($Man_{5-9}GlcNAc_2$-Asn) are termed "high mannose," those with at least *N*-acetyllactosamine (Galβ1,4GlcNAc) on the two nonreducing mannose in $Man_3GlcNAc_2$-Asn are considered "complex," and those with at least five mannose residues and an *N*-acetyllactosamine on only one nonreducing mannose in the $Man_3GlcNAc_2$-Asn core are considered "hybrid." GlcNAc can also be added at more than one position on mannose, resulting in more branches or antennae, and addition of fucose, sialic acid (and polysialic acid), or repeating units of Gal and GlcNAc (poly *N*-acetyllactosamine), contribute to glycoprotein microheterogeneity. The remodeling of N-glycans is affected by the cell-specific expression of each sugar transferase, and site-specific modification of N-glycans within each protein is based on its accessibility to the processing enzymes. It should also be noted that terminal processing of N-glycans can sometimes result in glycan structures that are more commonly associated with glycolipids, mucin-type O-glycans, and glycosaminoglycan chains on proteoglycans, adding to the complexity of N-glycan function.

Up to one-half of all eukaryotic proteins are estimated to exhibit N-glycans (4), so it is no surprise to find that glycans exhibit diverse functions, including the regulation of protein folding and stability, enzymatic activity, membrane trafficking, and protein-protein interactions. For example, modification of high-mannose N-glycans to exhibit mannose 6-phosphate mediates trafficking of hydrolases to lysosomes by binding to mannose 6-phosphate receptors in either the biosynthetic pathway or at the cell surface (21). More recently, it has become clear that the core carbohydrate plays an equally important role in modulating glycoprotein folding and thus exit from the ER. Within the ER, the $GlcMan_9GlcNAc_2$-Asn structure is recognized by the lectin chaperones calnexin and calreticulin (16). Removal of Glc by the ER enzyme α-glucosidase II disrupts this binding, whereas reglucosylation by UDP-Glc:glycoprotein glucosyltransferase, which recognizes unfolded proteins, redirects the protein back into this unique folding pathway. Unfolded proteins can also be processed by mannosidase I, which removes a specific mannose from $Man_9GlcNAc_2$-Asn; the $Man_8GlcNAc_2$-Asn structure then becomes a ligand for the putative lectin EDEM (ER degradation enhancing α-mannosidase-like protein), which targets terminally unfolded glycoproteins for degradation (16, 28). Pharmacological inhibition of the ER glucosidases (Fig. 2) sometimes prevents proper folding of glycosylated proteins and results in their rapid degradation. In many instances, however, proteins synthesized in the presence of these inhibitors fold adequately and exit the ER with normal kinetics. In these cases, the subsequent processing of glycans in downstream compartments cannot proceed normally. Mutant cell lines can also be used to disrupt the processing of glycans. For example, the ricin-resistant MDCK cell line (MDCKII-RCA^r) is unable to transport UDP-galactose into the lumen of the Golgi for the elongation of both N- and O-glycans (6, 53). A less-well-characterized, concanavalin A-resistant MDCK cell line has also been described that exhibits alterations in glycoprotein core structures (53). Together, these cell lines and drugs provide useful tools with which to examine the effects of glycan processing on protein traffic and have widely been used to distinguish the role of N- and O-glycans in polarized protein sorting.



Inhibitor	Abr	Mechanism	Effect
Tunicamycin	TM	This analog of GlcNAc inhibits the first step in the synthesis of the core oligosaccharide by disrupting addition of the first GlcNAc residue to dolichol phosphate.	N-glycosylation does not occur.
Deoxynojirimycin	DNJ	Inhibits ER glucosidase I from removing the terminal glucose residue from the core oligosaccharide and thus prevents subsequent trimming by glucosidase II.	Proteins display glucosylated high mannose N-glycans.
Castanospermine	CST	Like DNJ, this drug inhibits ER glucosidases.	Proteins display glucosylated high mannose N-glycans.
Deoxymannojirimycin	DMJ	An analog of mannose, this drug inhibits the removal of core mannose residues by inhibiting ER α-mannosidase I and Golgi α-mannosidase I.	Only high mannose N-glycans are synthesized.
Kifunensine	KIF	Inhibits ER α-mannosidase I.	Only high mannose N-glycans are synthesized.
Swainsonine	SW	Inhibits Golgi α-mannosidase II.	Prevents the conversion of hybrid N-glycans to complex.
Benzyl-N-acetyl-α-galactosaminide	BGN	This drug acts as an acceptor substrate for both galactosyl- and sialyltransferases and thus competes with endogenous glycoprotein substrates.	Elongation and sialylation of hybrid and complex N-glycans is inhibited; also affects O-glycosylation (see Fig. 1).

Fig. 2. Structure of N-glycans and effects of pharmacological perturbants. The synthesis of N-glycans begins in the endoplasmic reticulum (ER) with the generation of the oligosaccharide core ($Glc_3Man_9GlcNAc_2$) on dolichol pyrophosphate. This core structure is cotranslationally transferred en bloc from dolichol pyrophosphate to an asparagine residue within the specific consensus sequence Asn-X-Ser/Thr (NXS/T). As the newly synthesized protein moves through the ER, the core oligosaccharide structure is processed. Initially, ER glucosidases I and II remove the three glucose residues. Before exiting the ER, α-mannosidase I removes one of the core mannose residues. Upon entering the Golgi complex, mannose residues can be further trimmed. Some glycans maintain a high-mannose form ($Man_{5-9}GlcNAc_2$), whereas others are trimmed further by Golgi α-mannosidases I and II, then modified by the addition of galactose and GlcNAc (*N*-acetyllactosamine) to one (hybrid) or two or more arms (complex) of the glycan. *N*-acetyllactosamine can be added repeatedly, resulting in poly-*N*-acetyllactosamine chains of varying length. Further terminal processing can occur with the addition of sialic acid or fucose. Pharmacological inhibitors of key processes in the synthesis of N-glycans have been important for understanding how N-glycans are involved in biosynthetic sorting. *Bottom*, pharmacological inhibitors that are commonly used and referred to in this review.

O-GLYCANS AS MEDIATORS OF POLARIZED BIOSYNTHETIC SORTING

Several mutagenesis studies have suggested a role for O-linked glycans in polarized protein sorting (see Table 1). Yeaman et al. (83) observed that deletion of an O-glycan-rich stalk region of the neurotrophin receptor p75 was necessary for accurate apical delivery of this protein in a heterologous MDCK expression system. Interestingly, this region was also required for apical secretion of a truncated, secreted mutant of p75 (83). Similarly, a combination of the transmembrane domain and adjacent O-glycosylated stalk of the apical membrane hydrolase sucrase-isomaltase (SI) are sufficient to direct apical delivery of this protein; in this case, lipid raft association of the protein may be important for polarized sorting (34). Moreover, this region of SI confers apical sorting when appended to rat growth hormone, a heterologously expressed secreted protein that is released without polarity from Caco-2 cells (72).

Other studies have used a pharmacological approach to examine the role of O-glycans in polarized biosynthetic sort-

Table 1. *Role of O-glycans in apical delivery of soluble and membrane proteins*

Protein	Methods Used	Cell Lines	Notes	References
Neurotrophin receptor ($p75^{NTR}$)	Glycosylation perturbants; mutagenesis	MDCK; Caco-2	O-glycan-rich stalk required for apical sorting of transmembrane protein; removal of membrane anchor results in apical secretion in MDCK but basolateral secretion in Caco-2 cells	54, 83
DPPIV, MUC1, carcinoembryonic antigen, CD44	Glycosylation perturbants	HT-29; Caco-2	Treatment with BGN results in intracellular accumulation of apical glycoproteins	22, 31
DPPIV	Glycosylation perturbants	Caco-2; HT-29	Apical delivery requires intact N-and O-glycans plus lipid raft association	1, 56
pro-SI	Glycosylation perturbants; mutagenesis	Caco-2	Apical delivery requires O-glycosylation of Ala37-Pro48 region of stalk in addition to the membrane anchoring domain	2, 56, 72
DPPIV (soluble mutant)	Glycosylation perturbants	MDCK	Sialylation of N- and O-glycans	71

NTR, neutrophin receptor; MDCK, Madin-Darby canine kidney; DPPIV, dipeptidyl peptidase IV; SI, sucrose-isomaltase; BGN, benzyl-*N*-acetyl-α-galactosaminide.

ing. However, a serious limitation to these studies is the lack of a selective inhibitor of O-glycosylation. Recently, a compound (1-68A; Fig. 1) was identified that inhibits ppGaNTases, which are responsible for the addition of the initial GalNAc residue to Ser/Thr residues (26). However, interference with this early step in O-glycan synthesis clearly has serious effects on the cell function because even short-term treatment with this drug induces apoptosis (76). A more commonly used drug to perturb O-glycan structure is benzyl-*N*-acetyl-α-galactosaminide [BGN; (29); related compounds such as phenyl-*N*-acetyl-α-galactosaminide behave similarly]. This membrane-permeant drug is recognized by galactosyltransferases and sialyltransferases as an efficient acceptor site and thus competitively inhibits the elongation of endogenous GalNAc residues on O-glycans. Thus incubation with this drug results in the expression of proteins with truncated O-glycans that contain a single GalNAc residue. However, in addition to inhibiting galactosyltransferase-mediated elongation of O-glycans, BGN can also affect the processing of N-glycans and glycolipids (see Ref. 30 for review). The benzyl-disaccharide formed on addition of GalNAc to BGN can be subsequently modified by additional glycosyltransferases common to both N- and O-glycan processing pathways and thereby compete with the processing of endogenous glycans. The resulting benzyl oligosaccharide products are eventually secreted into the medium (29). Thus an important caveat to the interpretation of studies using BGN is that the disruption in sorting observed with this drug could be due to alterations in multiple classes of glycoconjugates.

The effects of BGN on apical traffic are complex and cell-type dependent. Long-term (days) incubation of HT-29 cells with BGN resulted in decreased membrane delivery and secretion of mucins, consistent with a role for (N- or) O-glycosylation in efficient surface delivery of these proteins (14, 22, 23, 31). Moreover, in these cells, apical and at least some basolateral glycoproteins accumulated in intracellular vesicles (22, 31). In HT-29 cells, the major metabolite of BGN is Galβ1–3GalNAc-α-*O*-benzyl, which acts as a potent inhibitor of α2,3-sialyltransferases, and the effects of BGN on apical trafficking in these cells may result from this inhibition (32). These enzymes are responsible for the majority of sialyltransferase activity in these cells and add terminal sialic acid residues to both N- and O-linked glycans. Interestingly, lectin binding studies (51, 79) revealed that the α2,3 linkage predominates at the apical cell surface of polarized HT-29, Caco-2, and MDCK cells, whereas α2,6-linked sialic acids were more broadly distributed. Consistent with this finding, BGN selectively inhibited the sialylation of newly synthesized apical but not basolateral proteins in HT-29 cells (78).

The reported effects of BGN on polarized traffic in Caco-2 cells are less consistent. In independent studies, apical delivery of wild-type and a soluble mutant of dipeptidylpeptidase IV (DPPIV) was inhibited by BGN treatment of Caco-2 cells (1, 71); however, another group (22) found no effect of BGN on DPPIV polarity in Caco-2 cells, although the total amount of enzyme present at the cell surface was decreased. The latter finding was attributed to the low intrinsic α2,3-sialyltransferase activity of Caco-2 cells (22). The observation that apical transport occurs normally in cells that have low α2,3-sialyltransferase activity argues against a direct role for this linkage as an apical targeting signal. However, the mechanism by which apically destined proteins are selectively recognized and targeted for this modification may be a key to understanding the underlying sorting determinants that direct apical delivery of glycoproteins in polarized cells.

A recent study has provided a revised explanation for the effects of BGN on apical delivery in intestinal cell lines. Delacour et al. (13) reported that treatment of HT-29 cells with BGN causes a dramatic reduction in the association of galectin-4, a bivalent lectin that binds primarily to galactosylceramide and sulfatide lipids, with detergent-resistant rafts. The loss of galectin-4 binding to these rafts is due to BGN-mediated effects on glycosphingolipid synthesis rather than effects on N- or O-glycosylation. Importantly, siRNA-mediated knockdown of galectin-4 led to a block in cell surface delivery of several apical markers, similar to the effects observed previously on treatment with BGN. The authors therefore concluded that galectin-mediated organization of lipid rafts is an important step in the efficient delivery of apical proteins along the biosynthetic pathway (13). It should also be noted that some glycosylation-resistant cell lines, such as ricin-resistant MDCK cells, which are deficient in UDP-Gal transport into the Golgi, also alter cellular glycolipid profiles (6, 40). While these new studies on galectin-4 clearly shed light on the cellular effects of BGN and the role of glycolipids in polarized membrane traffic, they do not bring us closer to an understanding of how N- and O- linked glycans on some proteins can facilitate their apical sorting (see below).

Table 2. *Role of N-glycans in apical delivery of soluble and membrane proteins*

Protein	Methods Used	Cell Lines	Notes	References
Effects of disrupting N-glycosylation on polarized delivery of wild-type or chimeric glycoproteins				
gp80	Glycosylation perturbants; glycosylation-deficient cell lines	MDCK; ricin-resistant MDCK	Core oligosaccharides	60, 80, 82
Erythropoietin	Glycosylation perturbants; mutagenesis	MDCK	One of 3 N-glycans (position 38) required	36
gp114	Glycosylation perturbants; glycosylation-deficient cell lines	MDCK; ricin-resistant MDCK; ConA-resistant MDCK	Protein was mistargeted basolaterally in ricin-resistant cells; however, the effect could not be reproduced pharmacologically in wild-type MDCK cells.	40
Mouse Fc/LDL-receptor chimera	Glycosylation perturbants; Mutagenesis	MDCK	Apically targeted chimera is retained in the Golgi complex in the absence of N-glycans	24
Neuronal glycine transporter GLYT2	Mutagenesis	MDCK	Deletion of three of the four N-glycan sites disrupts polarity	49
DPPIV (soluble mutant)	Glycosylation perturbants	MDCK	Sialylation of N- and O-glycans	71
Endolyn	Glycosylation perturbants; mutagenesis	MDCK	Terminal sugars; two of eight N-glycans (positions 68 and 74) required	33, 62
DPPIV	Glycosylation perturbants	Caco-2; HT-29	Apical delivery requires intact N- and O-glycans plus lipid raft association	1, 56
Membrane dipeptidase (GPI-anchored protein)	Mutagenesis	MDCK; Caco-2	Removal of N glycans results in basolateral targeting	59
Effects on polarized delivery of adding N-linked glycans to nonglycosylated proteins				
Glycosylated rat growth hormone (gGH) and GPI- anchored gGH	Mutagenesis	MDCK	Addition of 2 N-glycans to rat GH results in efficient apical secretion	5, 70
Truncated occludin, ERGIC-53 chimera	Mutagenesis	MDCK	Addition of N-glycans to Golgi-retained proteins results in apical delivery	24
Glycosylated tail anchored construct	Mutagenesis	MDCK	Addition of short N-glycosylated lumenal sequence alters distribution from basolateral to nonpolarized.	12

LDL, low-density lipoprotein; GPI, glycosylphosphatidylinositol.

Dissection of the role of O-glycans in polarized traffic is further complicated by the interrelationship between N- and O-glycan processing during protein transport along the biosynthetic pathway. For example, Naim et al. (56) demonstrated that disruption of N-linked glycan processing by deoxymannojirimycin (DMJ; an inhibitor of mannosidase I) but not by the mannosidase II inhibitor swainsonine altered the biosynthetic elongation of O-glycans of DPPIV, SI, and aminopeptidase N. Apical polarity of DPPIV and SI was reduced in DMJ-treated Caco-2 cells, whereas aminopeptidase N delivery was normal (56). Because swainsonine did not alter the polarity of delivery of these proteins, the authors concluded that N-glycans are not involved in polarized sorting (56). However, the differences in the N-glycan structures that result as a consequence of mannosidase I vs. II inhibition are significant; thus a role for terminal N-glycan processing in the polarized sorting of these proteins cannot be ruled out. Indeed, subsequent studies from the same laboratory demonstrated that in the case of DPPIV, both N- and O-glycans are important for apical delivery in Caco-2 cells (1).

ROLE OF N-GLYCANS IN POLARIZED BIOSYNTHETIC TRAFFIC

The first observed requirement for N-glycans in polarized sorting was the demonstration in 1987 that treatment with tunicamycin, a potent inhibitor of N-glycan core synthesis, disrupted apically polarized secretion of gp80/clusterin, the major endogenous protein secreted by MDCK cells (80). Subsequently, it was demonstrated that one of the three N-glycan consensus sequences on erythropoietin was critical for its apical secretion when expressed in MDCK cells (36). Shortly thereafter, in an interesting twist, Scheiffele et al. (70) demonstrated that rat growth hormone, a nonglycosylated protein that is secreted equally into the apical and basolateral media upon expression in polarized MDCK cells, could be converted to an apically secreted protein by addition of two N-glycosylation consensus sequences. Table 2 summarizes published examples in which either the perturbation or removal or the addition of N-glycans has been demonstrated to alter the polarized distribution of membrane and secreted proteins.

Since these initial reports, several additional examples of N-glycan-dependent sorting of endogenous, heterologously expressed, and highly engineered chimeric membrane proteins have been reported. A concerted role for both N- and O-glycans in the apical sorting of dipeptidylpeptidase IV, an endogenous protein expressed in Caco-2 cells, has recently been described, and the glycan-dependent mechanism of sorting appears to involve DPPIV partitioning into detergent-insoluble lipid microdomains (1). In another case, mutagenesis of three of the four N-glycosylation consensus sequences from the neuronal glycine transporter GLYT2 resulted in the nonpolarized distribution of this partially active protein when expressed in polarized MDCK cells, whereas the wild-type protein was apically distributed (49). Removal of all four N-glycans abolished protein activity and cell surface delivery; however, the contribution of individual glycans to apical delivery was not tested (49). In a highly artificial scenario, the fusion of a 20-amino acid glycosylated luminal sequence

containing two N-linked glycosylation consensus sequences to a GFP-tagged, tail-anchored protein resulted in nonpolarized distribution of the glycosylated protein, whereas a nonglycosylated version was targeted primarily to the basolateral surface of polarized MDCK cells (12). Finally, apical delivery of newly synthesized endolyn, a sialomucin that cycles between the apical cell surface and lysosomes, was found to be dependent on N-glycosylation because treatment with tunicamycin or mutagenesis of N-glycan consensus sequences resulted in the loss of apical targeting (33, 62). Interestingly, in this case, the N-glycan-dependent sorting signal appears to be dominant over a tyrosine tetrapeptide sorting motif present in the cytoplasmic tail of the protein. This motif, in the absence of the glycosylated luminal domain, efficiently targeted newly synthesized chimeric proteins to the basolateral cell surface (33).

Whereas in most reported cases the addition or ablation of N-glycans alters the distribution of cell surface or secreted proteins between apical and basolateral domains without affecting the overall efficiency of surface delivery, one report (24) suggests that N-glycans are required instead for the export of some proteins from the Golgi complex to the cell surface. Addition of N-glycan consensus sequences to nonglycosylated, Golgi-retained mutants of occludin or ERGIC-53 resulted in their biosynthetic delivery primarily to the apical membrane of polarized MDCK cells. Moreover, mutagenesis of N-glycan consensus sequences in an apically targeted chimera encoding the mouse Fc receptor luminal and transmembrane domains fused to a portion of the low-density lipoprotein receptor resulted in retention of the nonglycosylated protein in the Golgi complex (24). Thus, in some cases, N-glycans may function as Golgi export signals in addition to conferring apical sorting information.

As noted above, GPI anchors were initially identified as apical sorting determinants in some polarized cell types, including MDCK. The role of glycans in apical sorting of GPI-anchored proteins has recently been examined in several studies. The premise for these experiments was the observation that removal of the GPI attachment signal from some normally GPI-anchored proteins resulted in efficient apical secretion of the resulting untethered protein, suggesting the existence of an alternate GPI-independent apical sorting signal within the luminal domain of these proteins (10, 44, 63). Moreover, apical delivery of some GPI-anchored proteins was disrupted when they were expressed in glycosylation-deficient mutant cell lines (45). In the most comprehensive study to date, Benting et al. (5) observed that addition of a GPI anchor attachment signal to nonglycosylated rat growth hormone did not result in apical delivery of this protein; however, a glycosylated version of this protein was efficiently targeted to the apical surface. Similarly, removal of N-glycans from GPI-anchored dipeptidase resulted in preferential localization of the protein to the basolateral cell surface of both MDCK and Caco-2 cells, whereas ablation of the GPI anchor attachment signal resulted in efficient apical secretion (59). In contrast, tunicamycin treatment of a GPI-anchored mutant of endolyn did not disrupt apical delivery of the protein in MDCK cells, suggesting that the GPI anchor conferred redundant apical sorting information that was able to target the protein in the absence of N-glycans (62). Thus the aggregate data are inconclusive with regard to whether GPI anchors in and of themselves are sufficient to mediate apical targeting in the absence of luminal N-glycans. In this regard, recent studies have demonstrated that clustering or aggregation of some GPI-anchored proteins, rather than their association with lipid rafts per se, is important for their apical delivery in both MDCK and FRT cells (43, 58). To date, however, there are no direct data that suggest the involvement of N- or O-glycans in GPI-protein clustering, and indeed, GPI-anchored proteins appear to be correctly sorted in ricin-resistant MDCK cells (86).

REQUIREMENTS FOR N-GLYCAN-DEPENDENT SORTING

Do specific sugars or linkages mediate glycan-mediated apical sorting? In a study of N-glycans in MDCK cells, Ohkura et al. (57) found no discernible differences between N-glycan structures isolated from apical vs. basolateral membrane proteins, although they were able to observe differences in the structure of glycans isolated from membrane vs. secretory proteins. Thus there does not appear to be a unique saccharide or glycan modification on apical proteins that confers apical targeting information. The lack of a universal glycan-dependent sorting determinant is borne out by other observations that demonstrate tremendous diversity in the structural and positional requirements for glycans in apical sorting of individual proteins. In the case of gp80/clusterin, studies using glycosidase inhibitors and the mutant cell line, MDCKII-RCA^r, demonstrated that the glycan core structures, rather than terminal or penultimate saccharides were critical for proper apical sorting (60, 82). In contrast, apical sorting of endolyn was sensitive to inhibitors of mannosidase I and II, but not to glucosidase inhibitors (62). It has also been shown that the apical trafficking of gp114 is disrupted in MDCKII-RCA^r cells. Thus these studies suggest that terminal processing of N-glycans is required for the apical sorting of these proteins. Similarly, terminal processing of the N-linked glycans of DPPIV appears to be important for efficient apical sorting of this protein in Caco-2 and HT-29 cells (1).

In some cases, glycans at specific positions are responsible for apical sorting. In the case of erythropoietin, mutagenesis of only one of the three N-glycan consensus sequences (at amino acid 38) resulted in nonpolarized secretion of the protein, whereas disruption of the other two sites individually or in common had little effect (36). Similarly, two of the eight N-glycans (at amino acids 68 and 74) of endolyn were found to be both necessary and sufficient for efficient apical delivery of the protein (62). These two glycans are localized to a globular and putative disulfide-linked loop present between the two mucin-like domains of endolyn and could therefore affect the conformation of this domain. In another case, no specific N-glycans could be identified that were critical for apical delivery of the Na^+-K^+-ATPase β-subunit. (81).

It is important to stress that N-glycans are not universal apical sorting signals, and indeed, nonglycosylated secretory and membrane proteins that are apically polarized have been identified (3, 38, 73). Moreover, there are many individual examples where disruption of N-glycosylation does not alter the polarized distribution of a protein in either MDCK or Caco-2 cells (7, 35, 39, 50, 52, 77). In addition, there are examples where N-glycans are interpreted as apical targeting signals in one cell line but not another (73). Finally, in studies utilizing pharmacological reagents to perturb glycosylation, the possibility exists that the role of glycosylation on protein

sorting is indirect. This is most vividly illustrated in cases where prolonged treatment with tunicamycin was found to inhibit the apical secretion of even nonglycosylated apical proteins (38, 39, 52). The loss of targeting could reflect the requirement for a glycosylated protein as part of the apical sorting or transport machinery, or a more general disruption in cell function.

POTENTIAL MECHANISMS FOR GLYCAN-MEDIATED PROTEIN SORTING

As described above, there is a wealth of evidence that both N- and O-glycans play important roles in the apical sorting and trafficking of many cargo molecules. How do these ubiquitous posttranslational modifications specify polarity? While no unifying hypothesis has emerged to explain the role of N- or O-glycans in polarized sorting, two models have been proposed to describe the mechanisms by which glycans may enable segregation of proteins into apically destined carriers (68). One proposed mechanism is that a family of receptors exist that sort proteins into apically destined vesicles. The receptors could recognize N- or O-glycans directly or, alternatively, could recognize a protein patch whose conformation is glycan dependent. As yet, however, no such receptor has been definitively identified. An early candidate was the protein VIP36, a lectin originally isolated from detergent-insoluble extracts of exocytic carrier vesicles (17). Subsequent analysis revealed that this protein is primarily localized to the early secretory pathway and is thus unlikely to function as a component of the TGN apical sorting machinery (18). However, the jury is still out on the role of this protein because another group has localized VIP36 preferentially to the apical surface and reported that overexpression of VIP36 stimulated secretion of gp80/clusterin but had no effect on apical release of a nonglycosylated protein (27). The MAL proteolipid has also been demonstrated to play a role in gp80/clusterin secretion, although whether this interaction is carbohydrate dependent has not been tested (47).

In the alternative model, N-glycans facilitate apical sorting by stabilizing a proteinaceous conformation required for TGN export, for example, by promoting oligomerization or association with apically destined lipid rafts. The observation that addition of N-glycan consensus sequences to mutant proteins that were retained in the Golgi complex resulted in their efficient transport to the apical surface has been cited in support of this model (24, 68). Similarly, the diversity in requirements for glycan-mediated sorting of different cargo proteins (e.g., N- vs. O-glycans, core vs. terminal sugars) might argue in favor of a conformational role for glycans in polarized sorting. While compelling evidence for receptor-mediated sorting of most glycan-dependent cargo molecules is lacking, some data argue against a purely conformation-dependent model for sorting. A recent study (12) demonstrated that N-glycosylation conferred increased apical delivery to a chimera that contains a very short luminal domain (29 amino acids). Such a short sequence is unlikely to contain conformation-sensitive sorting information and is most consistent with a receptor-based sorting mechanism in which the N-glycan itself is recognized directly.

In the absence of an identified candidate glycan-dependent sorting receptor, there are several possible ways to distinguish between these two mechanisms. A primary distinction between these models is that a receptor-mediated sorting mechanism should be saturable at high levels of protein expression. It has previously been demonstrated that apical targeting of other proteins whose sorting is not glycan dependent is saturable (46). Moreover, oligosaccharides or peptides that compete with the binding of proteins to a sorting receptor should selectively disrupt the polarized delivery of glycan-dependent apical cargo. In contrast, a conformation-dependent mechanism should be less sensitive to changes in protein expression or competitive inhibitors. Future studies are clearly needed to discriminate between these possible mechanisms.

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