Role of N-glycosylation in trafficking of apical membrane proteins in epithelia

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Submitted 2 June 2008; accepted in final form 27 October 2008

EPITHELIAL CELLS CARRY OUT vectorial transport that requires polarized distribution of transporters and receptors to apical or basolateral membrane domains. These domains are separated by tight junctions that connect neighboring cells in the epithelial monolayer and act as diffusion barriers to prevent mixing of apical and basolateral membrane components. Asymmetric distribution of plasma membrane proteins is accomplished by their sorting into apical and basolateral containers in the trans-Golgi network (TGN) and/or endosomes followed by their delivery to the appropriate plasma membrane domains. Sorting depends on the recognition of sorting signals in proteins by specific sorting machinery. In the present review, we summarize experimental evidence for and against the hypothesis that N-glycans attached to the membrane proteins can act as apical sorting signals. Furthermore, we discuss the roles of N-glycans in the apical sorting event per se and their contribution to folding and quality control of glycoproteins in the endoplasmic reticulum or retention of glycoproteins in the plasma membrane. Finally, we review existing hypotheses on the mechanism of apical sorting and discuss the potential roles of the lectins, VIP36 and galectin-3, as putative apical sorting receptors.

apical sorting; apical membrane retention; H-K-ATPase \( \beta \)-subunit; lectin

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Normal N-Glycosylation of Membrane Proteins

All membrane glycoproteins are synthesized by ribosomes attached to the ER and become inserted into the ER membrane via a protein-conducting channel, the translocon. Glycoproteins acquire the N-linked glycans during the process of trans-
location and elongation of the polypeptide chain. First, while the protein continues to be associated with the translocon, a 14-saccharide core is transferred from the dolichol phosphate precursor to the N-glycosylation site of the nascent membrane protein, an asparagine (Asn) residue within a consensus sequence of Asn-X-Thre/Ser (X is any amino acid residue except Pro). Next, terminal glucose residues of this core are trimmed by ER glucosidases. Then, after removal of one mannose residue by the ER α-mannosidase I, the glycoproteins are exported to the Golgi.

Some glycoproteins that are trafficked to the plasma membrane move through the Golgi and post-Golgi vesicular compartments to this destination without further processing. These glycans are high-mannose N-glycans (Fig. 1). However, the structure of the majority of N-glycans is altered further in the Golgi. After removal of one, two, or three mannose residues by Golgi mannosidases, various glycosyltransferases catalyze branching and elongation of the carbohydrate chains, producing hybrid or complex N-glycans (Fig. 1 and Table 1) (1, 39). This rebuilding of the N-glycans is initiated by addition of N-acetylglucosamine (GlcNAc) residues to the N-glycan core structure by one or more of the six different N-acetylglucosamine-glycosyltransferases (GnTs) located in the cis-Golgi and medial Golgi.

GnT-I initiates formation of hybrid N-glycans by attachment of GlcNAc to the α3 arm of the core at the stage when the core contains five mannose residues. Following the addition of GlcNAc and further mannose trimming, GnT-II catalyzes formation of the complex-type N-glycans in which GlcNAc is substituted for both α3- and α6-linked mannose residues. The actions of GnTs-III, -IV, -V, and -VI underlie the variability in the number of branches in N-glycans: GnTs-IV, -V, and -VI promote branching of N-glycans, whereas GnT-III stops branching. The latter enzyme adds the bisecting GlcNAc to hybrid or complex N-glycans that prevents the downstream action of the branching enzymes (Fig. 1 and Table 1). Many of the other glycosyltransferases, which compete with each other for the N-glycan substrate in the trans-Golgi, facilitate elongation of the GlcNAc-substituted chains by adding various monosaccharides. Thus individual branches of the mature N-glycan can vary in both their length and carbohydrate composition. Most frequently, individual branches are elongated by addition of galactose and sialic acid residues catalyzed by galactosyltransferases and sialyltransferases. Individual branches can also be elongated by addition of multiple sialic acid residues or polylactosamine sequences (series of repeated galactose-GlcNAc disaccharides).

The diversity of N-glycans in various glycoproteins is a result of the interplay of several factors. The particular set of glycosyltransferases expressed in a specific cell type is one of the most important. This difference in expression of glycosyltransferases likely explains the finding that N-glycans of...
the Na-K-ATPase β1-subunit and E-cadherin have a more complex structure in the rabbit intestinal cells than in rabbit renal cells (89).

Moreover, the expression of various glycosyltransferases can change during cell or tissue development, resulting in alterations of glycosylation of a particular protein. For example, downregulation of the enzymes GnT-IV and GnT-V, upregulation of GnT-III, and a decrease in the complexity of N-glycans of the Na-K-ATPase β1-subunit and E-cadherin are observed as dispersed Madin-Darby canine kidney (MDCK) cells in culture are transformed into a confluent monolayer (89).

In addition, the presence of certain amino acid motifs or residues in a glycoprotein can affect the nature of its glycosylation. For instance, scattered basic residues and a peptide loop adjacent to the glycosylation site of lysosome-resident acid hydrolases are recognized by a GlcNAc-phosphate transferase in the Golgi. This results in the formation of a mannose 6-phosphate on the termini of high-mannose N-linked glycans, binding to the mannose-6-phosphate receptor, and targeting to lysosomes (30, 45).

The spatial location of N-glycosylation sites in a protein tertiary structure can also affect the type of N-glycans formed. Certain N-glycans are more accessible to the Golgi glycosyltransferases and are therefore more likely to be processed to the complex N-glycans than others. As a result, particular glycosylation sites in a protein may be occupied by complex N-glycans and other sites by high-mannose or hybrid N-glycans. As an example, the mature Eag1 potassium channel has two N-glycans, but one of the N-glycans is complex while the other is high-mannose (61). Also, in the bovine lysosomal N-glycans, but one of the N-glycans is complex while the other is high-mannose.

Table 1. Roles of N-glycan-specific enzymes in processing and maturation of membrane glycoproteins

<table>
<thead>
<tr>
<th>Enzyme Description</th>
<th>Location</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosidases</td>
<td>ER</td>
<td>Removal of terminal glucose residues from oligosaccharide core and formation of high-mannose N-glycoproteins</td>
</tr>
<tr>
<td>α-Mannosidases I</td>
<td>ER and Golgi</td>
<td>Trimming of high-mannose N-glycans</td>
</tr>
<tr>
<td>GnT-I</td>
<td>ER and Golgi</td>
<td>Formation of hybrid-type glycoproteins</td>
</tr>
<tr>
<td>GnT-I, GnT-IV, and GnT-VI</td>
<td>ER and trans-Golgi</td>
<td>Formation of complex-type glycoproteins</td>
</tr>
<tr>
<td>GnT-II</td>
<td>Medial Golgi</td>
<td>Branching of N-glycans in hybrid-type glycoproteins</td>
</tr>
<tr>
<td>Sialyltransferase, galactosyltransferases, and other glycosyltransferases</td>
<td>Medial and trans-Golgi</td>
<td>Branching of N-glycans in complex-type glycoproteins</td>
</tr>
<tr>
<td></td>
<td>Medial and trans-Golgi</td>
<td>Prevention of branching of N-glycans</td>
</tr>
<tr>
<td></td>
<td>Medial and trans-Golgi</td>
<td>Elongation of branches in hybrid and complex N-glycans</td>
</tr>
</tbody>
</table>

Role of N-Glycans in Protein Folding, Stability, and Quality Control in the ER

Folding of proteins in the ER is initiated cotranslationally and is completed posttranslationally. Heat shock proteins, such as GRP78/BIP, act as chaperones facilitating the normal folding of proteins and their delivery from the ER to the Golgi. However, N-linked glycans also play a major role in folding and quality control of newly synthesized glycoproteins, helping to ensure that only properly folded proteins are delivered from the ER to the Golgi and thence to their final destination.

N-linked glycans can contribute to proper folding both directly and indirectly. Addition of bulky polar carbohydrate chains can modify the properties of the polypeptide (7, 70). Glycosylation can affect the local secondary structure of proteins by stabilizing the conformation of residues proximal to the glycosylation site (42). An interaction of N-glycan with the polypeptide chain can induce a β-turn structure (7). Also, glycoproteins are more stable and more resistant to proteolysis than their corresponding unglycosylated counterparts (42, 46). For example, the normally fully glycosylated wild-type β-subunit of the apical transport enzyme, the gastric H-K-ATPase, is more resistant to proteolysis by trypsin (13) or proteinase K (5) than its glycosylation-deficient mutants. Resistance to proteolysis has been attributed to restricted access of the protease to cleavage sites within the protein after it achieves its normal configuration.

N-glycans also can promote folding and quality control indirectly by serving as recognition “tugs,” or sorting signals, that allow glycoproteins to interact with a variety of lectins, glycosidases, and glycosyltransferases (37, 39, 40, 77) (Table 2). Immediately after coupling the oligosaccharide core to the asparagine of the N-glycosylation consensus site, the two outermost terminal glucose residues are removed sequentially by glucosidase I and glucosidase II. The monoglucosylated form of N-linked glycan binds to calnexin, the membrane-bound lectin, or to calreticulin, the homologous soluble lectin. Calnexin and calreticulin are associated with ERp57, a thiol-disulfide oxidoreductase that is involved in disulfide bond formation that influences protein folding (32, 33, 40). When glucosidase II removes the remaining glucose, the glycoprotein dissociates from calnexin and calreticulin.

The protein now meets one of three possible fates. If properly folded, it moves to the Golgi. Exit from the ER may be assisted by mannose-binding lectins, such as ERGIC-53, VIP36, and VIPL (2, 62, 83). If the glycoprotein is incompletely folded, it becomes a substrate for a luminal ER glucosyltransferase that reglucosylates the glycan located in improperly folded regions. Via these reformed glycans, the glycoprotein again binds to calnexin, calreticulin, and ERp57 and is completed posttranslationally. Heat shock proteins, such as GRP78/BIP, act as chaperones facilitating the normal folding of proteins and their delivery from the ER to the Golgi. However, N-linked glycans also play a major role in folding and quality control of newly synthesized glycoproteins, helping to ensure that only properly folded proteins are delivered from the ER to the Golgi and thence to their final destination.

...
ER-associated degradation (ERAD) is facilitated by lectin EDEM (57). ER retention and subsequent ERAD are critical for normal cellular function because they prevent accumulation of dysfunctional proteins in the cell (39, 40, 77). The contribution of N-glycans to the complex ER quality control system is emphasized by some of the functional abnormalities that arise in the presence of defective N-glycosylation (52, 64, 65).

In some glycoproteins with multiple N-glycosylation sites, individual N-glycans are not of equal importance in mediating the processes of folding, ER retention, and export from the ER to the Golgi. For example, only one of the six glycans of the apically located protein, influenza virus hemagglutinin, is essential for folding and exit from the ER (38).

Studies of the β-subunit of the gastric H-K-ATPase expressed in LLC-PK1 cells have also shown that mutation of some of its seven N-glycosylation sites affects its exit from the ER, while mutations of others have little impact. The normally glycosylated β-subunit is distributed between the plasma membrane and the intracellular compartments. At steady state, ~40% of the protein is detected in the ER, and the remaining 60% is distributed among the plasma membrane, Golgi, TGN, and endosomes (90). Removal of the seventh N-glycosylation site, N7, leads to virtually total retention of the subunit in the ER. Mutation of N1 increases the amount retained in the ER to 75%. Similarly, mutation of N3 and N5 also significantly increases the relative amount of the ER-resident fraction, but to a lesser extent. However, mutation of N2, N4, or N6 does not appreciably alter the quantity of protein retained in the ER.

On the other hand, for some glycoproteins, the number of N-glycans rather than a specific N-glycan is important for their normal folding and production of fully functional glycoproteins. Mutagenic removal of one or two of the four N-glycosylation sites from the apical bile salt export pump (ABCBl1) did not significantly affect its stability, trafficking, or function (56). However, removal of three or all four N-glycosylation sites dramatically increased the rate of proteasome-dependent degradation and resulted in significant intracellular retention of the protein. Comparison of mutants of ABCBl1 with various combinations of mutated glycosylation sites showed that presence of at least two N-glycans is required for normal folding and trafficking of the protein (56).

In oligomeric proteins, N-glycans can be important for subunit assembly and/or correct folding of the assembled complex. For example, studies using several different cell lines have shown that the catalytic α-subunits of either the Na-K-ATPase or the gastric H-K-ATPase are unable to exit the ER when expressed without their corresponding glycosylated β-subunits (29). These observations suggest that the β-subunit acts as a chaperone to ensure that the α-subunit is properly folded so it can exit the ER.

**Role of N-Glycans in Post-Golgi Trafficking and Retention in the Apical Membrane**

N-glycans are required for polarized apical distribution of many membrane proteins in epithelia. Removal of N-glycans from many apical proteins has been shown to decrease their abundance in the apical membrane. For example, mutagenic removal of N-glycosylation sites in the gastric H-K-ATPase β-subunit (90), bile salt export pump (56), and glycine transporter 2 (51, 94) significantly decreased their apical content and increased their intracellular accumulation (Table 3). Similarly, prevention of N-glycosylation by tunicamycin resulted in complete intracellular retention of the normally apically located membrane-bound enteropeptidase (95) (Table 3).

One explanation for these findings is that normal N-glycosylation is required for apical sorting of these glycoproteins. Alternatively, since N-glycosylation is necessary for proper folding of proteins for them to exit the ER, the lack of N-glycans could result in ER retention, which of course would prevent trafficking to any membrane. To account for this, it is necessary to subtract the fraction of the protein retained in the ER from the total cellular pool of the protein. The majority of mature glycoproteins that traverse the Golgi carry complex N-glycans, whereas those residing in the ER are of the high-mannose type. They can be distinguished from each other after treatment with EndoH, since high-mannose and hybrid glycans are sensitive to, whereas complex N-glycans are resistant to, cleavage by EndoH (Fig. 2). Only the quantity of mature, EndoH-resistant protein should be considered when the percentage of glycoprotein that is delivered to the apical membrane is calculated.

Using this approach, the abundance of glycosylation-deficient mutants and wild-type H-K-ATPase β-subunit present in the apical membrane, as determined using surface-selective biotinylation, was compared after normalization for the total cellular amount of the mature subunit (90). The relative quantity of the subunit in the apical membrane was greatly decreased after mutation of N1, N3, N5, or N7, but not after mutation of N2, N4, or N6. By contrast, these mutations had no effect on the relative abundance of this subunit in the basolateral membrane (90). These findings indicate that N-glycans at N1, N3, N5, and N7 are important for apical membrane localization of this β-subunit.

Similarly, comparison of a wild-type and a series of glycosylation-deficient mutants of the sialomucin endolyn expressed
in MDCK cells has shown that only two specific sites (N68 and N74) of the eight N-glycosylation sites are important for apical distribution of this protein (72). Disturbance of N-glycosylation of several other membrane proteins, such as the tail-anchored chimeric protein (10), glycine transporter 2 (51, 94), membrane dipeptidase (67), ecto-nucleotide pyrophosphatase/phosphodiesterase NPP3 (19, 53), and chimeric Fc receptor (31) reduced their apical expression compared with their basolateral expression (Table 3). However, the relative importance of specific N-glycans in apical localization of these proteins has not been determined.

Further evidence for the role of N-glycans in apical distribution of proteins has been derived from studies in which the effect of addition of N-glycans to various proteins on their localization was examined. A truncated ocludin and a chimeric ERGIC-53 residing in the Golgi in their nonglycosylated forms were redistributed to the apical membrane after addition of N-glycans (31) (Table 4). Similarly, addition of N-glycans to chimeric non-raft-associated transmembrane protein and tail-anchored chimeric protein, both residing on the basolateral membrane, caused them to be redistributed to the apical membrane (6, 10) (Table 4). Finally, the sequential addition of one to five N-glycans to the basolaterally located Na-K-ATPase β1-subunit, a protein with three N-glycans, caused an increasing fraction of this subunit to be distributed to the apical membrane of HGT-1 cells (91). The mutant of Na-K-ATPase β1 which contained eight N-glycans in a pattern identical to that of the homologous isoform, the Na-K-ATPase β2-subunit (which normally resides on the apical membrane in HGT-1 cells), was predominantly detected on the apical membrane (91).

These data are consistent with an important role for the N-glycans in promoting distribution of a number of proteins to the apical membrane of polarized cells. However, the presence of N-glycans on a protein does not ensure it will be distributed to the apical membrane. Several N-glycosylated proteins, including but not restricted to the transferrin receptor (63), liver sodium-dependent bile acid transporter (87), and E-cadherin (54, 55) are located only on the basolateral membrane of polarized cells.

### Table 3. Effect of impairment of N-glycosylation on localization of apical membrane proteins in polarized epithelia

<table>
<thead>
<tr>
<th>Apical Glycoprotein</th>
<th>Number of N-Glycans</th>
<th>Cell Type</th>
<th>Association With Lipid Rafts</th>
<th>Method Used to Impair N-Glycosylation</th>
<th>Effect on Protein Distribution</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enteropeptidase</td>
<td>3</td>
<td>MDCK</td>
<td>No</td>
<td>Exposure to tunicamycin</td>
<td>Intracellular retention*</td>
<td>95</td>
</tr>
<tr>
<td>Bile acid export pump (ABCB11)</td>
<td>4</td>
<td>MDCK</td>
<td>Not tested</td>
<td>Mutagenesis of 1 or 2 sites</td>
<td>No change</td>
<td>56</td>
</tr>
<tr>
<td>H-K-ATPase β-subunit</td>
<td>7</td>
<td>LLC-PK₁</td>
<td>Not tested</td>
<td>Mutagenesis of 3 or 4 sites</td>
<td>Intracellular retention*</td>
<td>90</td>
</tr>
<tr>
<td>Tail-anchored chimeric protein</td>
<td>2</td>
<td>MDCK</td>
<td>No</td>
<td>Mutagenesis of both sites</td>
<td>Decrease in ratio of apical to basolateral abundance</td>
<td>10</td>
</tr>
<tr>
<td>Glycine transporter 2 (GLYT2)</td>
<td>4</td>
<td>MDCK</td>
<td>No</td>
<td>Mutagenesis of all 3 sites</td>
<td>Decrease in ratio of apical to basolateral abundance</td>
<td>51, 94</td>
</tr>
<tr>
<td>Membrane dipeptidase (MDP)</td>
<td>2</td>
<td>MDCK, Caco-2</td>
<td>Yes</td>
<td>Exposure to tunicamycin; mutagenesis of 1-8 sites</td>
<td>Decrease in ratio of apical to basolateral abundance</td>
<td>67</td>
</tr>
<tr>
<td>Sialomucin endolyn</td>
<td>8</td>
<td>MDCK</td>
<td>Not tested</td>
<td>Exposure to tunicamycin; mutagenesis of 3 sites</td>
<td>Decrease in ratio of apical to basolateral abundance</td>
<td>72</td>
</tr>
<tr>
<td>NPP3</td>
<td>Not specified</td>
<td>MDCK, Caco-2</td>
<td>Yes</td>
<td>Exposure to tunicamycin; mutagenesis of all 3 sites</td>
<td>Intracellular retention*</td>
<td>19, 53</td>
</tr>
<tr>
<td>Chimeric Fc receptor</td>
<td>Not specified</td>
<td>MDCK</td>
<td>Not tested</td>
<td>Exposure to tunicamycin</td>
<td>Retention in Golgi</td>
<td>31</td>
</tr>
<tr>
<td>p75(NTR)</td>
<td>1</td>
<td>MDCK</td>
<td>No</td>
<td>Exposure to tunicamycin</td>
<td>No change</td>
<td>93</td>
</tr>
<tr>
<td>(SPNT)</td>
<td>3</td>
<td>MDCK</td>
<td>Not tested</td>
<td>Mutagenesis of all 3 sites</td>
<td>No change</td>
<td>50</td>
</tr>
<tr>
<td>Sodium-sulfate cotransporter (NaSi-1)</td>
<td>1</td>
<td>OK, LLC-PK₁, and MDCK</td>
<td>No</td>
<td>Exposure to tunicamycin; mutagenesis of a single site</td>
<td>No change</td>
<td>74</td>
</tr>
<tr>
<td>M2 muscarinic acetylcholine receptor</td>
<td>3</td>
<td>MDCK</td>
<td>No</td>
<td>Mutagenesis of all 3 sites</td>
<td>No change</td>
<td>12</td>
</tr>
</tbody>
</table>

N-glycosylation was prevented by mutation of the consensus N-glycosylation site(s) of the individual protein, or by treatment of cells with tunicamycin, a general inhibitor of N-glycosylation. OK, opposum kidney; MDCK, Madin-Darby canine kidney. *Presize intracellular compartments were not identified.
Furthermore, several proteins such as transmembrane neurotrophin receptor p75 (93), the sodium-dependent purine-selective nucleoside transporter SPNT (50), the sodium-sulfate cotransporter Na,Si-1 (74), and M2 muscarinic acetylcholine receptor (12) are N-glycosylated, but normal N-glycosylation is not necessary for their apical localization. Presumably, these proteins contain other intrinsic sorting signals that are sufficient to ensure their apical distribution. It has been shown that the O-glycosylated stem region of p75 acts as the apical sorting signal (93). The M2 muscarinic acetylcholine receptor contains apical sorting information in its third intracellular loop (12). However, the apical sorting signals for SPNT and Na,Si-1 remain to be identified. It is unclear why N-glycans are important for the apical delivery of some N-glycosylated proteins, but not for others. This remains fertile ground for further studies.

**N-glycans are required for both apical membrane delivery and retention of glycoproteins in the apical membrane.** Many glycoproteins require intact N-glycans to be located on the apical membrane. One possible explanation is that N-glycans act as apical sorting signals and thus promote apical membrane delivery of the proteins. However, the quantity of protein on the apical membrane depends on the rate of both its delivery to this location and endocytosis of the protein.

As noted above, the mutations of N1, N3, N5, or N7 significantly decreased the relative apical content of the H-K ATPase β-subunit (90). Mutations of N1, N3, or N5 also increased the rate of endocytosis of the β-subunit. The rate of endocytosis of the N7 mutant could not be measured due to the very low apical content of the mutant. Mutations of N2, N4, or N6 did not change either the relative apical content or endocytosis. None of the mutations affected the rate of degradation (90). The decrease in the relative apical content of the H-K ATPase β-subunit N1, N3, and N5 mutants could be due to impaired apical membrane delivery and/or enhanced endocytosis. The apical content of all three mutants was decreased to a greater extent than could be expected from an increase in the rate of endocytosis. For example, in the N1 mutant, the apical content was decreased sixfold, but the rate of endocytosis was increased only threefold. This indicates that the apical membrane delivery from the TGN/endosomes was also affected by the mutation.

Therefore, the presence of specific N-glycans is required for both apical delivery and membrane retention of the H-K ATPase β-subunit. The rate of delivery of a protein from the TGN/endosomes to the apical membrane depends on the efficiency of protein sorting into apical transport containers and the rate of delivery of these containers to the apical membrane.

### Table 4. Effect of addition of N-glycans on polarized distribution of proteins in the plasma membrane of epithelial cells

<table>
<thead>
<tr>
<th>Protein</th>
<th>Number of N-Glycans Added</th>
<th>Cell Type</th>
<th>Protein Location Before Addition</th>
<th>Ratio of Apical to Basolateral Abundance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na-K-ATPase β-subunit</td>
<td>1-5*</td>
<td>HGT-1</td>
<td>Basolateral membrane</td>
<td>Increased</td>
<td>91</td>
</tr>
<tr>
<td>Truncated occludin</td>
<td>2*</td>
<td>MDCK</td>
<td>Golgi</td>
<td>Increased</td>
<td>31</td>
</tr>
<tr>
<td>Chimeric ERGIC-53</td>
<td>2*</td>
<td>MDCK</td>
<td>Golgi</td>
<td>Increased</td>
<td>31</td>
</tr>
<tr>
<td>Chimeric non-raft-associated transmembrane protein</td>
<td>2*</td>
<td>MDCK</td>
<td>Mostly intracellular; basolateral membrane</td>
<td>Increased</td>
<td>6</td>
</tr>
<tr>
<td>Tail-anchored chimeric protein</td>
<td>2†</td>
<td>MDCK</td>
<td>Basolateral membrane</td>
<td>Increased</td>
<td>10</td>
</tr>
</tbody>
</table>

*N-glycosylation sites in the protein extracellular domains were engineered by mutagenesis. †Twenty-one-amino acid sequence of bovine opsin containing 2 N-glycosylation sites was added to the COOH terminus of the extracellular domain of a tail-anchored protein.

Fig. 2. H-K-ATPase β-subunits expressed on the apical surface of LLC-PK1 cells are complex-type glycosylated, while the intracellular nonmature fraction contains high-mannose-type subunits. The YFP-linked β-subunit of the H-K-ATPase (YFP-β) stably expressed in LLC-PK1 cells is detected in total cell lysates in 2 forms: EndoH resistant and EndoH sensitive. Only the EndoH-resistant complex-type form reaches the apical surface, as detected by apical surface biotinylation. The intracellular form of YFP-β is converted by EndoH to the deglycosylated protein that has the same gel mobility as YFP-β in cell lysates prepared after exposure of the cells to tunicamycin, the inhibitor of N-glycosylation. The gel mobility of the EndoH-sensitive form of YFP-β is the same as that of YFP-β from the cells exposed to dMM (preserves the high-mannose structure of glycoproteins) and greater than that of YFP-β from the cells exposed to swainsone (preserves the hybrid structure of glycoproteins). These results indicate that the intracellular fraction of YFP-β/H9252 of YFP-β prepared after exposure of the cells to tunicamycin, the inhibitor of N-glycosylation, the apical membrane depends on the rate of both its delivery to the apical membrane and endocytosis of the protein.

**AJP-Renal Physiol** • **Vol. 296** • **March 2009** • www.ajprenal.org
The mutation of N-glycosylation sites in a cargo protein is unlikely to affect export and transport of the containers. Thus the decreased rate of delivery of the glycosylation-deficient mutants to the apical membrane is consistent with the hypothesis that N-glycans facilitate apical sorting. However, it is not clear whether N-glycans per se serve as apical sorting signals or whether they facilitate recognition of other apical sorting signals present in a glycoprotein by providing an optimal conformation of the protein. Also, it is possible that N-glycans facilitate association of glycoproteins with other proteins that contain apical sorting information, thus promoting cosorting of glycoproteins into vesicles destined for the apical membrane.

It is remarkable that the same N-glycans (N1, N3, and N5) of the H-K-ATPase β-subunit that are important for ER-to-Golgi trafficking are also important for apical delivery and membrane retention of the protein (90). These results suggest that, similar to ER-to-Golgi trafficking, both apical delivery and apical membrane retention of the H-K-ATPase β-subunit are lectin dependent and only specific N-glycans are accessible to these lectins. Reduced retention of the mutants in the apical membrane might result from disruption of the interaction of specific N-glycans of the H-K-ATPase β-subunit with an unidentified integral or extracellular lectin at the apical membrane, which acts to anchor the protein in the membrane. As a consequence, the rate of constitutive endocytosis of the mutant subunit would increase. Interactions of lectins with N-glycans attached to other membrane proteins have been shown to be important for their retention in the plasma membrane in several different cell types (11, 47, 64, 65, 68).

The majority of mature glycoproteins are of the complex type. The complex-glycosylated proteins contain the tris- and tetrantsiosyl core (Fig. 1, green rectangle) and terminal chains that are added during rebuilding of high-mannose N-glycans in the Golgi. To examine whether the core region, the terminal chains, or both are necessary for delivery and membrane retention of apical proteins, various inhibitors of N-glycan processing have been employed. Treatment of cells with these inhibitors prevents trimming and further terminal glycosylation of high-mannose N-glycans. This results in the absence of either all (dMM) or most (swainsonine) of the terminal sugars normally present in the complex N-glycans (Fig. 1). Similar to dMM, kifunesine, the inhibitor of the ER α-mannosidase 1, abolishes addition of terminal chains to the high-mannose glycans (not shown).

Treatment of MDCK cells expressing endolyn with dMM or kifunesine decreased the relative apical abundance, biosynthetic apical delivery, and postendocytic apical delivery of the protein to the same extent as tunicamycin (72, 73). These results suggest that the terminal chains rather than the residues of the core region of N-glycans are important for apical sorting of endolyn. However, endolyn contains not only N-glycans, but also O-glycans. It has been shown that disruption of N-glycan processing by dMM dramatically inhibited O-glycosylation of several proteins that contain both N- and O-glycosylation sites (60). Therefore, the possibility that dMM or kifunesine affects apical distribution of endolyn due to the impairment of its O-glycosylation cannot be excluded. In accord with the latter possibility, benzyl-N-acetyl-α-d-galactosaminide that inhibits elongation of both O- and N-glycans also impaired apical polarity of endolyn (72).

In contrast, the apical localization of gp114 (recently identified as a dog homolog of CEACAM proteins) (27) in MDCK cells was not affected by dMM (48), indicating that terminal chains are not important for apical distribution of this protein. Consistently, exposure of LLC-PK1 cells expressing the H-K-ATPase β-subunit to dMM or swainsonine led to only a slight decrease in the relative apical content of the H-K-ATPase β-subunit, while mutation of one of the seven N-glycosylation sites, either N1, N3, N5, or N7, resulted in a severalfold greater reduction in relative apical abundance (90). On the other hand, the rate of endocytosis of the β-subunit was significantly higher in cells exposed to swainsonine than with any of the single site-deficient mutants (90). These data suggest that the core region sugars play the major role in apical delivery of the β-subunit, whereas the terminal monosaccharide residues are important for stabilization and retention of the β-subunit in the apical membrane.

**Putative Apical Sorting Machinery that Recognizes N-Glycans**

**Search for apical sorting receptors.** The mechanism by which the presence of N-glycans can facilitate apical sorting of glycoproteins is poorly understood. The μ1B-subunit of the AP-1B adaptor protein complex binds specifically to basolateral sorting motifs present in the cytoplasmic domains of basolateral proteins, promoting their placement into the basolateral transport containers and subsequent delivery to the basolateral membrane (25, 26, 28). It seems logical that a similar coupling between a sorting receptor and N-glycans would be used by the cell for delivery of glycoproteins to the apical membrane. Since N-glycans are composed of carbohydrates moieties, a priori the apical sorting receptor must be a lectin, i.e., a carbohydrate-binding protein.

One potential apical sorting receptor, the mannos-binding lectin VIP36 (43, 44, 79), is predominantly localized in ERGIC and plasma membrane (34, 82). VIP36 plays a role in the export of glycoproteins from the ER (36, 83) (Table 2). VIP36 was also found to promote apical sorting of several glycoproteins. The apical membrane content of this lectin in MDCK cells was found to be twice as high as the basolateral content, and the plasma membrane glycoproteins recognized by VIP36 were also twofold enriched in the apical membrane compared with the basolateral membrane (34). Overexpression of VIP36 in MDCK cells increased the relative apical content of both VIP36 and VIP36-recognized glycoproteins. In contrast, the overproduction of a mutant version of VIP36, which has no lectin activity, had no effect. Furthermore, VIP36 has been shown to bind to clusterin and α-amylase and to increase the relative apical abundance of these proteins (34, 35). Consistent with a specificity of VIP36 for high-mannose-type glycan (43, 44, 79), both clusterin and α-amylase carry not only complex-type N-glycans but also the high-mannose-type N-glycans (34, 35). However, since the majority of mature glycoproteins contain predominantly complex-type N-glycans, VIP36 is unlikely a universal apical sorting receptor.

A galactose-binding lectin, galectin-3, is another potential apical sorting receptor. This lectin was detected in raft-independent apical carrier vesicles in MDCK cells (15). Galectin-3 has been found to directly interact with three apical glyco-
teins, lactase-phlorizin hydrolase, transmembrane neurotrophin receptor p75, and gp114 (15, 17). This interaction is competitively inhibited by lactose, suggesting that galectin-3 binds to the glycans attached to these proteins. Lactase-phlorizin hydrolase and transmembrane neurotrophin receptor p75 contain both N- and O-glycans, whereas gp114 has only N-glycans Carbohydrate-dependent binding of galectin-3 to p75 requires the presence of the O-glycosylated stalk domain that does not contain putative N-glycosylation sites (15, 17). These data suggest that galectin-3 can bind to both N- and O-glycans.

Depletion of galectin-3 from MDCK cells prevents formation of the high-molecular-weight clusters containing lactase-phlorizin hydrolase, transmembrane neurotrophin receptor p75 or gp114 and results in missorting of all three apical membrane proteins to the basolateral membrane (15, 17). These findings suggest that galectin-3 cross-links glycoproteins to form large galectin-3-containing clusters in the TGN membranes. These clusters presumably bud from the TGN to form apical transport containers (15, 17).

Galectin-3 binds to galactose, a terminal residue that might be exposed at the carbohydrate chain termini of the mature complex N-glycans (69). Consistently, the efficiency of coimmunoprecipitation of gp114 with galectin-3 and formation of the high-molecular-weight clusters containing gp114 and galectin-3, as well as apical distribution of gp114 are severely impaired in galactosylation-deficient MDCKII-RCAr cells (15, 17, 48). However, the apical localization of gp114 in wild-type MDCK cells is not affected by the mannosidase I inhibitor dMM, which prevents formation of hybrid and complex N-glysans and thus abolishes the insertion of the galactose residues into N-glycans (48). The explanation for these contradictory experimental observations is unknown.

Relationship between N-glycans and lipid rafts. Significant evidence has accumulated to suggest involvement of lipid rafts in sorting and delivery of proteins to the apical membrane. Lipid rafts are rigid membrane microdomains enriched in glycolipids, sphingolipids, and cholesterol embedded in the fluid membrane (81). In 1997, Simons and Ikonen (84) postulated that newly synthesized apical proteins accumulate in these microdomains in the TGN, while basolateral proteins are excluded from these lipid rafts. Small lipid rafts are then clustered, forming bigger rigid membrane domains that then bud from the TGN as apical transport containers (41, 81).

Certain apical integral glycoproteins, including influenza viral proteins hemaglutinin and neuraminidase, associate with lipid rafts via their transmembrane domains (4, 80). Although transmembrane domains of these proteins are important for both raft association and apical sorting, the signals for raft association and apical sorting are not identical (4, 49, 80). Raft association appears necessary, but not sufficient, for apical sorting of hemaglutinin (4, 49, 80). In contrast, raft association is not absolutely required for apical sorting of neuraminidase (4, 80).

GPI-anchored proteins associate with lipid rafts due to the favorable packing of the GPI anchor into ordered domains of the membrane (85). Stable association with rafts is required for apical sorting of several chimeric GPI-anchored proteins (18, 66, 75, 81, 85). However, raft association is neither necessary nor sufficient for apical sorting of a natural GPI-anchored glycoprotein, membrane dipeptidase (67, 88). Removal of a GPI anchor excluded the protein from lipid rafts but did not impair its apical distribution. On the other hand, removal of N-glycans from the protein resulted in basolateral sorting but did not affect the protein association with rafts (67, 88).

Oligomerization of apical glycoproteins appears to increase their affinity for rafts. For example, clustering of gp114 molecules in the plasma membrane induced by interaction with the gp114-specific antibodies increases association of gp114 with lipid rafts, as judged by increased detergent resistance of the glycoprotein (92). It has been postulated that intracellular multivalent lectins could cluster apical glycoproteins in rafts in vivo and thus facilitate their apical delivery (41, 81, 92).

However, direct evidence for the importance of N-glycans in clustering of apical N-glycosylated proteins in lipid rafts has not been presented. Furthermore, several pieces of evidence suggest that the presence of N-glycans does not facilitate raft association. First, many N-glycosylated apical proteins are not associated with lipid rafts (Table 3). Second, removal of N-glycans did not affect raft association of the membrane dipeptidase, while removal of a GPI anchor excluded this protein from lipid rafts (67, 88). Finally, apical pyrophosphatase/phosphodiesterase NPP3, but not its basolateral homolog, NPP1, associates with rafts, even though both NPP3 and NPP1 contain N-glycans. Specific positively charged amino acid residues in the cytoplasmic tail rather than N-glycans have been shown to be important for association of NPP3 with lipid rafts (19, 53).

Moreover, lectins that cluster N-glycosylated proteins in lipid rafts have not been found. Lectins of the galectin family were considered as candidates. They usually are multivalent and, therefore, binding of one lectin molecule to N-glycans that belong to different glycoprotein molecules can organize glycoproteins in clusters, bundles, arrays, and lattices (8). Indeed, galectin-3 causes clustering of apical glycoproteins, lactase-phlorizin hydrolase, transmembrane neurotrophin receptor p75, or gp114 (15, 17). However, none of these three proteins are present in rafts. Also, galectin-3 does not bind to sucrase-isomaltase, the apical glycoprotein that associates with lipid rafts (15). These results indicate that galectin-3 does not play a role in clustering of apical glycoproteins in lipid rafts.

Another lectin of the same family, galectin-4, is a major component of lipid rafts (14, 16). Depletion of galectin-4 impairs formation of rafts and affects apical sorting of several proteins in HT-29 cells. However, galectin-4 binds with high affinity to glycolipids but not glycoproteins (16).

Recent studies have shown that association of apical proteins with rafts occurs not only in the TGN (9) or late compartments of the Golgi apparatus (86) but also in the cis-Golgi or even in the ER (3, 78). It is possible that the mannose-binding lectin VIP36 facilitates apical sorting of glycoproteins by stabilizing them in lipid rafts in the early steps of the glycoprotein processing in the ER or cis-Golgi, when the mannose residues are exposed at the termini of the carbohydrate chains. This hypothesis is in agreement with the fact that VIP36 was originally isolated from rafts (23), as well as with the data showing that overexpression of VIP36 increases apical distribution of two raft-associated N-glycosylated proteins, clusterin and α-amylase (34, 35).
Conclusions and Future Directions

N-glycans are attached to the majority of membrane proteins. Besides the direct effect of N-glycans on the conformation and intrinsic properties of proteins, N-glycans play a role in intracellular trafficking that arises from specific interactions of N-glycans with intracellular lectins. The best studied of these are the interactions of N-glycans with the ER-resident lectins that are crucial to folding and quality control of newly synthesized glycoproteins and their export from the ER to Golgi. These glycan-lectin interactions ensure that only properly folded proteins exit the ER (Table 2).

Growing experimental evidence also suggests that N-glycans attached to a number of apical proteins are required for their apical distribution (Tables 3 and 4). Recent studies indicate that N-glycans are important for delivery of certain glycoproteins to the apical membrane (72, 73, 90) and for their retention in the apical membrane (90). However, apical localization of a number of membrane glycoproteins is not affected by changes in N-glycosylation. Moreover, several proteins that are specifically sorted to the basolateral membrane are, in fact, N-glycosylated.

Identification of specific lectins that recognize N-glycans and facilitate apical distribution of glycoproteins is a challenging task. So far, only two lectins have been identified as potential apical sorting receptors: the mannose-binding lectin VIP36 and the galactose-binding lectin galectin-3. It is remarkable that VIP36 has been found to facilitate apical distribution of two raft-associated proteins (34, 35), whereas galectin-3 has been shown to be critical for apical location of three non-raft proteins but not a raft-associated protein (15). Possibly, VIP36 and galectin-3 facilitate apical distribution of glycoproteins by two different mechanisms, raft dependent and raft independent. However, this hypothesis needs to be tested for other apical proteins. Also, given the highly diverse structures of complex N-glycans, it would be expected that other lectins facilitating apical sorting and apical membrane retention of glycoproteins will be identified.

In summary, N-glycans attached to many apical proteins are critical to their maturation, sorting, and trafficking to and retention in the apical membrane. As a consequence, defects in N-glycosylation can lead to cellular abnormalities in different tissues. Further investigations may reveal previously unsuspected roles of N-glycans in trafficking of glycoproteins which will provide insight into normal epithelial development and the pathogenesis of various diseases.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-077149, DK-58333, and DK-053642.

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