N-GLYCAN-DEPENDENT APICAL TRAFFICKING OF THE SIALOMUCIN ENDOLYN

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Crucial to the function of polarized epithelial cells is the generation and maintenance of distinct protein populations at the apical and basolateral domains. Cellular mechanisms sustain this polarity by properly sorting apical and basolateral proteins in both the biosynthetic and postendocytic pathways *via* sorting signals inherent within the protein. N-glycosylation has been specifically implicated as one of several sorting signals responsible for delivery of proteins to the apical surface along the biosynthetic pathway. Whether this sorting signal is also used along the postendocytic pathway is currently not known. To examine N-glycan-dependent trafficking along the biosynthetic pathway and explore a possible role for N-glycans in sorting along the postendocytic pathway, this study focuses on the sialomucin endolyn. Characterization of the biosynthetic sorting signal revealed that two glycans at amino acid positions 68 and 74 were necessary and sufficient for apical delivery of endolyn. Terminal processing of N-glycans is also necessary for efficient apical delivery as incubation with kifunensine, a drug that inhibits terminal processing of N-glycans, resulted in the nonpolarized delivery of endolyn. A similar N-glycan-dependent sorting signal was also found to be important for apical recycling of endolyn along the postendocytic pathway. The use of the same sorting signal suggested that endolyn may be sorted in a compartment that is common to both the biosynthetic and postendocytic pathway. However, further studies revealed that endolyn is sorted in distinct compartments along these two pathways. These data enhance our understanding of how and where N-glycans mediate apical sorting to maintain the integrity of polarized sorting in epithelial cells.

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PREFACE

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1.0 INTRODUCTION

1.1 OVERVIEW

Critical to the proper function of polarized epithelial cells is the division of the plasma membrane into domains, the apical and basolateral, which have distinct protein and lipid compositions. The generation and maintenance of this polarity is largely sustained by cellular sorting mechanisms along the biosynthetic and postendocytic pathways that recognize and sort proteins based on inherent sorting signals. These sorting signals distinguish proteins for delivery to either the apical or basolateral surface. Sorting along the biosynthetic pathway is believed to occur in the most distal compartment of the Golgi complex (the *trans*-Golgi network or TGN) and in post-Golgi recycling endosomes (118). Basolateral biosynthetic sorting signals generally comprise linear peptide sequences in the cytoplasmic portion of the protein and mediate sorting through their interaction with adaptor protein complexes (12, 33, 102, 126). In contrast, apical sorting has been shown to depend on a variety of signals; including cytoplasmically-disposed amino acid motifs; protein association with glycoplipid enriched lipid microdomains (i.e. lipid rafts) through lipid anchors or transmembrane residues; and glycosylation, including both O- and N-glycans (101, 118). Along the postendocytic pathway, apical and basolateral proteins are sorted within common recycling endosomes (37, 148). Although, basolateral proteins use similar sorting signals along these two pathways (8, 93, 106), little research has focused on the sorting of apical proteins along the postendocytic pathway.

The purpose of this dissertation was to further examine the role of N-glycans in apical trafficking along both the biosynthetic and postendocytic pathway using the sialomucin endolyn as a model protein. Apical biosynthetic delivery of endolyn in Madin-Darby canine kidney cells requires N-glycans within the luminal domain of the protein (61). After delivery to the surface, endolyn is recycled back to the apical surface, but it is not known whether N-glycans are necessary for this process (61). My major goals were to characterize the biosynthetic N-glycan-dependent apical sorting signal of endolyn, to determine whether N-glycans were important for apical recycling of endolyn, and to determine where along the biosynthetic pathway endolyn is sorted. The following introduction reviews the sorting of apical and basolateral proteins along both the biosynthetic and postendocytic pathways and presents the sialomucin endolyn as a model protein for this study.

1.2 POLARIZED EPITHELIAL CELLS

Lining all body surfaces and most internal organs is a layer of tightly apposed polarized epithelial cells. This epithelial layer serves as barrier against pathogens and mediates the unidirectional flow of ions, metabolites, and fluids with and between internal and external environments (128). Such specific interaction with these two very different environments could not be accomplished without the asymmetrical division of the plasma membrane into two domains of distinct proteins and lipid compositions, the apical and the basolateral. The apical domain is found at the apex of the cell and is exposed to the external environment or the lumen of an organ. A high population of glycosphingolipids comprises the apical surface, likely aiding

in protection from the harsher external environment (128). The apical surface of absorptive polarized epithelial cells is commonly populated with microvilli, membranous extensions with actin filaments at their core, which increase the surface area for absorption (157). On the other hand, the basolateral domain comprises the lateral membranes that are in contact with neighboring cells and the basal membrane that is contact with the underlying connective tissue and blood supply. Many of the proteins found in this domain are important for nutrient uptake, cell-cell attachment, and growth control (128). This structural arrangement is found in numerous epithelial cells, including those of the intestine and kidney. Hepatocytes are also polarized, but their structure is different from other polarized epithelial cells (Figure 1.1) (157). In these cells the apical surface forms a continuous network called the bile caniculus between cells that drains bile secreted from the apical surface (157). In all polarized epithelial cells, the apical and basolateral surfaces are separated from one another by tight junctions. These multiprotein complexes form a contiguous structure around the cell that presents a physical barrier to the diffusion of apical and basolateral proteins (125).

The establishment of polarity depends on the segregation of apical and basolateral cargo and specific developmental cues, including cell-cell contact. Vegas et al. showed that in the absence of cell-cell contact, apical components were sequestered in vacuolar apical compartments (VACs) and only upon cell-cell contact does VAC exocytosis occur at sites of cell-cell contact (144). Over a time period, microtubules rearrange into an array displacing the apical surface to the apex of the cell (118). The proteins believed to regulate this process have been identified in pivotal studies with *C. elegans* and *D. melanogaster* and include three complexes;PAR3/aPKC/PAR6, Crumbs3/PALS1/PATJ, and Scrib/mDIg/mLgl (125). Several of

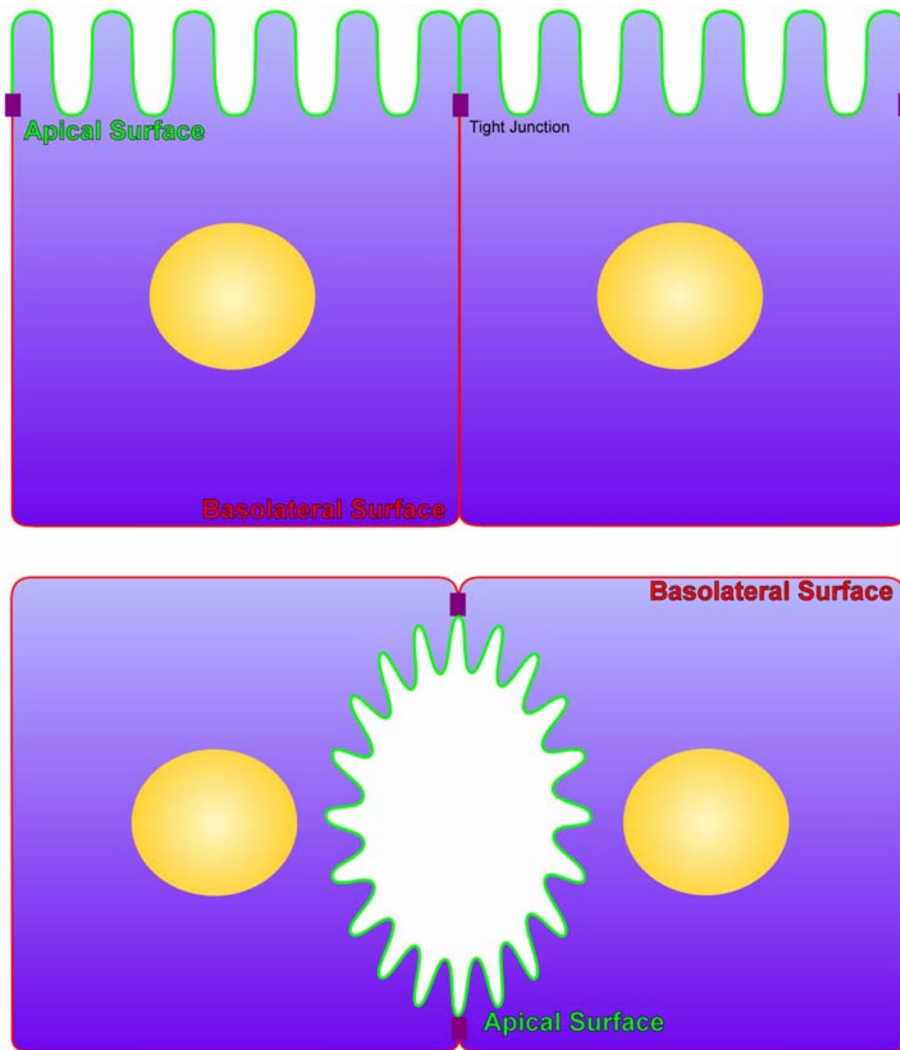


Figure 1.1 Structure of polarized epithelial cells vs. hepatocytes

The apical and basolateral surface in polarized epithelial cells (upper panel) and hepatocytes (lower panel) are outline in green and red, respectively. In polarized epithelial cells, the apical surface is not freely exposed; however, in hepatocytes, the apical surfaces of two adjacent cells form a passage for bile called the bile caniculus.

these proteins are considered to be scaffolding proteins of tight junctions; thus it is believed that the initiation of polarity and the formation of tight junctions are intimately connected (125).

Once polarity is established, tight junctions play a part in maintaining polarity by presenting a physical barrier between the two domains. Polarity, however, is predominantly maintained by the ability of cellular sorting mechanisms to recognize sorting signals inherent within proteins and sort them to either the apical or basolateral domain along both the biosynthetic and postendocytic pathways. Defects in the sorting signals of proteins can alter the trafficking of a protein and result in disease. For instance, hypercholesterolemia can occur in a patient due to a point mutation within the basolateral sorting signal of the low density lipoprotein receptor (LDLR) (131). Mutant LDLR localizes to the apical surface instead of the basolateral and the patient is unable to remove low density lipoproteins from the blood. Thus, it is important to understand how proteins are properly sorted to understand normal physiology as well as pathological states.

1.2.1 MDCK cells: a polarized epithelial model system

Much of our understanding of how apical and basolateral proteins are differentially sorted and transported has come from studies of Madin-Darby canine kidney (MDCK) cells. Growth on permeable filter supports allows these renal epithelial cells to form a polarized monolayer similar to an *in vivo* epithelium and enables individual access to the apical and basolateral domains. The sorting capabilities of polarized cells were first revealed when it was shown that enveloped viruses such as influenza and vesicular stomatitis bud specifically from the apical and basolateral surface of MDCK cells, respectively (13). Importantly, this asymmetric budding of enveloped viruses was found to be dependent on the polarized distribution of envelope proteins, suggesting

that these viral proteins contained sorting signals similar to cellular proteins for trafficking to either the apical or basolateral domain (116). MDCK cells have been utilized in numerous studies since their introduction and are used in the experiments presented here. However, even nonpolarized MDCK cells and other nonpolarized cells, such as fibroblasts, appear to have the capacity to sort newly-synthesized apical and basolateral cargo into distinct transport carriers (71, 97, 152, 154). This suggests that protein sorting machinery is universally expressed in all cell types.

1.3 PROTEIN SORTING ALONG THE BIOSYNTHETIC PATHWAY

Newly-synthesized proteins can be delivered to the apical surface by either a vectorial or transcytotic pathway. In the transcytotic pathway, proteins are first delivered to the basolateral surface then internalized and transcytosed to the apical surface. This pathway is used primarily in hepatocytes and to a lesser extent in intestinal and renal cells (157). MDCK cells predominantly use a vectorial pathway to deliver newly-synthesized proteins to the apical surface (157). It has long been thought that proteins along this pathway are sorted into distinct carriers destined for the apical surface from the basolateral surface. Mounting evidence, however, suggests that sorting may not occur exclusively in the TGN. There is evidence to suggest that apical and basolateral cargoes are segregated from one another as early as the ER (2, 137, 145). Other studies present evidence that sorting may occur in post-Golgi recycling endosomal compartments (6, 107). The sorting signals that direct polarized targeting and the three possible sites for sorting along the biosynthetic pathway are discussed below.

1.3.1 Basolateral Sorting Signals

Early work on the polymeric immunoglobulin receptor (pIgR) revealed that the cytoplasmic portion of the protein was important for basolateral sorting (95). A role in basolateral delivery was confirmed when transfer of a specific sequence of amino acids from pIgR to a normally apical protein resulted in its redistribution to the basolateral surface (21). Similar studies were also performed with LDLR, implicating that specific sequences in the cytoplasmic tail of the receptor were necessary and sufficient for delivery to the basolateral surface (60). Subsequent studies revealed that the two critical domains for basolateral delivery of LDLR were dependent on specific tyrosine residues (91). Later, Hunziker *et al.* revealed that two adjacent leucine residues rather than a critical tyrosine residue was important for sorting the IgG Fc receptor FcRII-B2 to the basolateral surface (58). It is now accepted that basolateral sorting signals rely on specific tyrosine tetrapeptide motifs of the form NPXY and YXXΦ or dileucine motifs ([DE]XXXL[L/I]) in the cytoplasmic portions of proteins, although exceptions do exist. The similarity of these sorting motifs to those used in endocytosis and lysosomal sorting suggested that the recognition of basolateral sorting signals was dependent on adaptor protein (AP) complexes (Figure 1.2). The AP complex family consists of four heterotetrameric complexes (AP-1, AP-2, AP-3, AP-4) that are composed of either a γ, α, δ, ε large subunit, a β large subunit, a μ medium subunit, and a small σ subunit (32). While AP-2 is localized exclusively to the plasma membrane and functions in endocytosis, the remaining complexes associate with both the TGN and endosomal compartments and have been implicated in basolateral delivery as well as delivery from the TGN to endosomal compartments (5, 102, 108, 126).

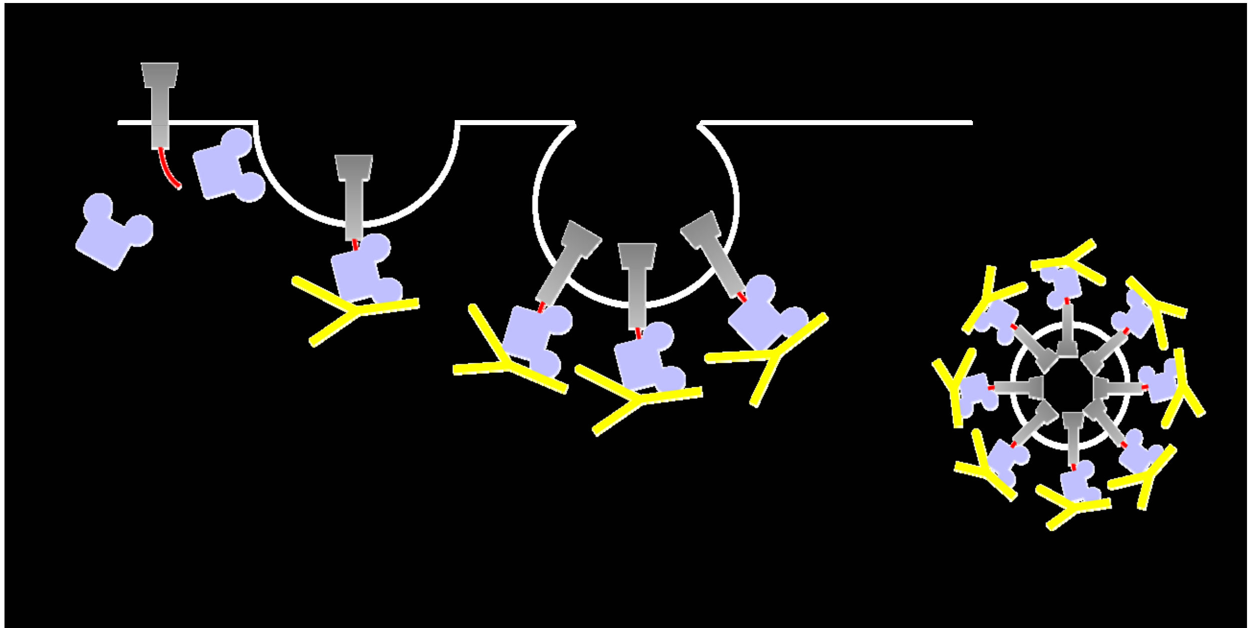


Figure 1.2 Sorting to the basolateral surface along the biosynthetic pathway

Tyrosine-based tetrapeptide motifs such as YXXΦ and dileucine motifs of the form [DE]XXXL[L/I] are found in the in the cytoplasmic domains of basolateral proteins. It is believed that these motifs associate with adaptor (AP) complexes (shown in blue) which interact with a host of cellular proteins, including clathrin (shown in yellow), that are important for the formation of vesicles that are destined for the basolateral surface.

1.3.2 Apical Sorting Signals

Delivery to the apical surface is based on a more diverse group of sorting signals than found for basolateral sorting. Signals can be found in the cytoplasmic, transmembrane, or lumenal domains and rely on associations with lipid rafts, sequence motifs, or glycosylation including O-glycans as well as N-glycans.

1.3.2.1 Associations with Lipid Rafts

It was noted very early that the apical surface is largely populated with glycosphingolipids and that the aggregation of these lipids in the TGN could facilitate sorting of proteins to the apical surface (141). Multiple groups first demonstrated that transference of the GPI-attachment sequence of several proteins could confer an apical distribution to basolateral and neutral proteins, suggesting that the GPI-anchor may in fact be a sorting signal (17, 83). In accordance, Lisanti *et al.* showed that glycosylphosphatidylinositol (GPI)-anchored proteins are predominantly located at the apical surface (84). However it was Brown *et al*. who made the connection between lipid and protein when it was found that GPI-anchored proteins could be isolated in cold Triton X-100 insoluble microdomains enriched in sphingolipids (18). Insolubility in cold Triton X-100 has since become a hallmark of an association with lipid rafts. Additionally the high lipid content of rafts allows their flotation to a low density during gradient centrifugation (18, 127). Further examination of lipid rafts revealed that cholesterol is also an integral component (123). Using fluorescence resonance energy transfer techniques, GPI-anchored proteins have been shown to organize into discrete domains on the surface that disappear in the absence of cholesterol (143). According to the raft hypothesis, sphingolipids and

cholesterol are clustered together in microdomains or rafts that can move through the bilayer and incorporate GPI-anchored proteins due to the long saturated acyl chain of the GPI-anchor (127).

It also appears that transmembrane residues can mediate associations with lipid rafts for delivery to the apical surface. The best studied example is influenza hemaggutinin (HA), a viral envelope protein. Insolubility of HA in cold Triton X-100 is conferred by a sequence of 10 amino acids in the transmembrane domain of the protein (129, 134). Additionally, HA delivery and its insolubility in ice-cold Triton X-100 can be disrupted by lowering cellular cholesterol levels (70). Another influenza envelope protein, neuraminidase, has also been shown to associate with lipid rafts through its transmembrane domain (76).

There is, however, some resistance to the full acceptance of the protein association with lipid rafts as an apical sorting mechanism (118). GPI anchored proteins lacking their GPI attachment signal are secreted predominantly from the apical surface (89, 111). In Fischer rat thyroid cells, GPI-anchored proteins can be found at the basolateral surface (158). Additionally, transference of a GPI attachment signal to rat growth hormone did not result in apical delivery unless an N-glycosylation site was also present within the protein's lumenal domain (9). A possible explanation for these results was recently published by Paladino *et al.* (109). This group suggests that the specific delivery of raft-associated proteins to the apical and basolateral surface is mediated by protein oligomerization. The authors show that only GPI-anchored proteins that are destined for the apical surface can form high molecular weight complexes upon raft association. Disruption of oligomerization of these proteins resulted in a basolateral distribution. The authors speculate that oligomerization either stabilizes the raft architecture or allows for the coalescence of smaller rafts into larger rafts that could increase the curvature of a budding vesicle and thus designate it for delivery to the apical surface (109).

1.3.2.2 Cytoplasmic Tail Determinants

Recent studies indicate that apical sorting can also be mediated within the cytoplasmic domain of a protein. The first apical protein shown to have an apical sorting signal in its cytoplasmic tail was rhodopsin. Truncation of rhodopsin's cytoplasmic tail resulted in its nonpolarized distribution, and addition of rhodopsin's cytoplasmic domain to a nonsorted protein resulted in an apical delivery of the chimera in MDCK cells (23). Studies with the cytoplasmic domain of a megalin minireceptor revealed that the tail of this protein also contains a cytoplasmic apical sorting signal (133). Guanylyl cyclase C is also trafficked to the apical surface of MDCK cells due to a stretch of 11 amino acids in its cytoplasmic domain (51). However, no consensus amino acid sequence or motif that directs apical targeting of proteins has yet been identified.

1.3.2.3 Glycosylation

Glycosylation is a ubiquitous post-translational modification of many proteins that has been shown to play an important role in protein folding, stability, and protein interactions. However, both in combination and individually, N- and O-glycans have been shown to be important for the delivery of a subset of proteins to the apical surface. In the following sections, the complex structure of both N- and O-glycans and the implications for the role in sorting are reviewed. Additionally, two proposed mechanisms by which glycans mediate sorting are discussed.

1.3.2.3.a O-glycosylation

Mucin-type O-linked glycosylation begins with the addition of N-acetylgalactosamine (GalNAc) to a loosely defined consensus site that involves a serine or threonine residue. N-acetylgalactosamine (GalNAc) and galactose are then added to form one of several core structures which can be further processed by N-acetyllactosamine, sialic acid, fucose, galactose,

GlcNAc, GalNAc, and sulfate to form hundreds of possible linear and branched O-glycan structures (Figure 1.3).

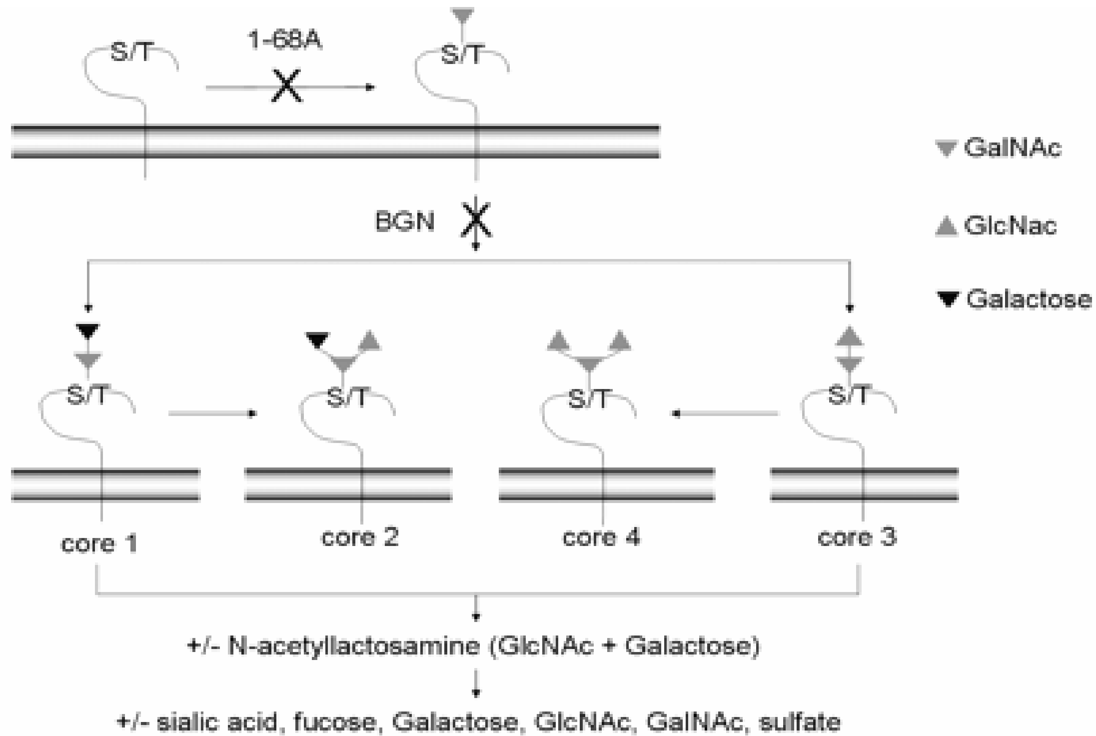


Figure 1.3 Structure of O-glycans and effects of pharmacological perturbants

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. There are two known inhibitors of O-glycan synthesis. 1-68A inhibits the transferase responsible for the initial addition of GalNAc to Ser/Thr. Benzyl-N-acetyl-α-galactosaminide (BGN) acts as an acceptor substrate for both galactosyl- and sialyl transferases, and thus, competes with endogenous glycoprotein substrates to inhibit O-glycan elongation.

A role for O-linked glycans in protein sorting has been implicated in several mutagenesis studies. Yeaman *et al.* 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 MDCK cells (153). Interestingly, this region was also required for apical secretion of a truncated, secreted mutant of p75 (153). Similarly, a combination of the transmembrane domain and adjacent O-glycosylated stalk of the apical membrane hydrolase sucrase-isomaltase (SI) is sufficient to direct apical delivery of the protein (67). Moreover, this region of SI confers apical sorting when attached to rat growth hormone, a normally nonpolarized protein (130).

Other studies have used a pharmacological approach to examine the role of O-glycans in polarized biosynthetic sorting; however, a serious limitation is the lack of a selective inhibitor of O-glycosylation. Recently a compound, 1-68A, was identified that inhibits ppGaNTases but appears to have serious effects on cell function because even short term treatment with this drug induces apoptosis (43). A more commonly used drug to perturb O-glycan structure is benzyl-N-acetyl-α-galactosaminide (BGN) and the related compound phenyl-N-acetyl-α-galactosaminide. These membrane permeant drugs are recognized by galactosyltransferases and sialyltransferases as efficient acceptors and thus competitively inhibit the elongation of endogenous GalNAc residues on O-glycans (54). However a recent study suggests that N-glycan processing is also affected, making it difficult to interpret studies using these drugs (55).

1.3.2.3.b N-glycosylation

Upon cotranslational translocation into the ER, a core glycan structure of $Glc_3Man_9GlcNAc_2$ (where Glc is glucose and Man is mannose) is transferred *en bloc* from a dolichol lipid anchor to a specific consensus site on proteins (Asn-X-Ser/Thr, where X is any amino acid except proline) (Figure 1.4) (74). After transfer to the protein, the three Glc residues and one Man are removed in the ER and additional mannose residues are sometimes removed in the early Golgi. 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 residues are termed high mannose. Those structures with at least five mannose residues and N-acetyllactosamine on only one nonreducing mannose of the core are termed hybrid. Complex N-glycans are formed when N-acetyllactosamine is added to the two nonreducing mannose residues in the core structure. 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 poly N-acetyllactosamine contributes to the heterogeneity of N-glycans.

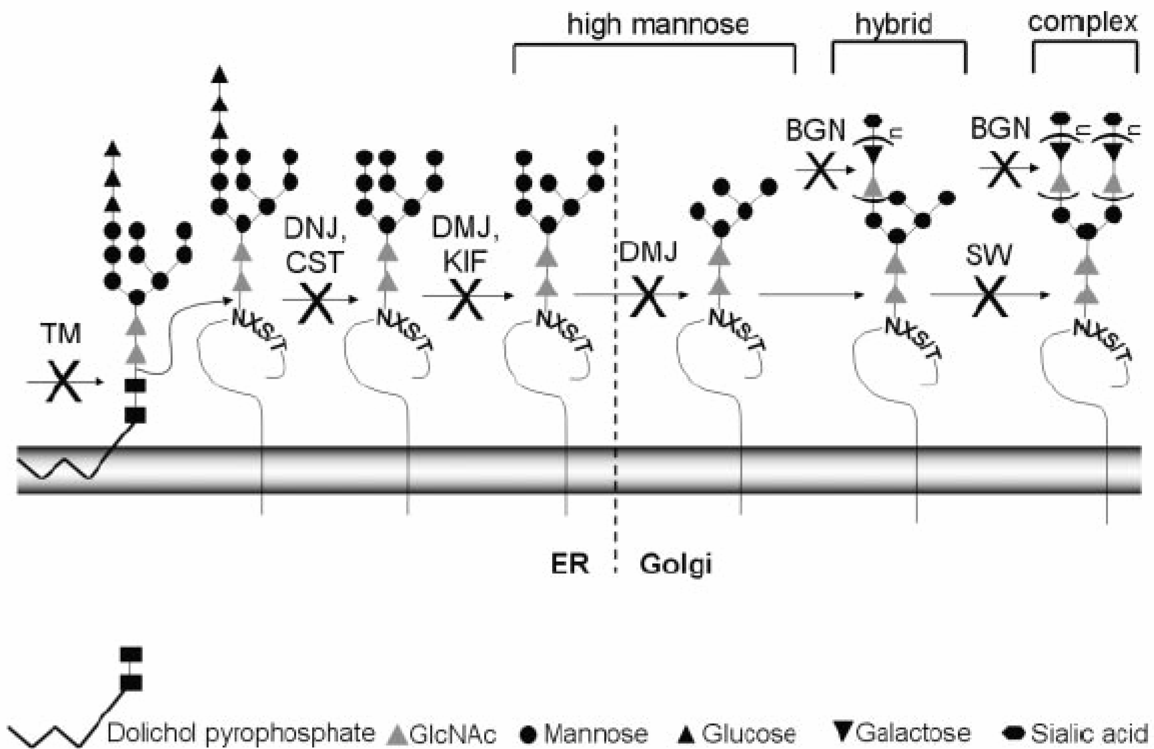


Figure 1.4 Structure of N-glycans and effects of pharmacological perturbants

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 drugs (Table 1.1) and mutant cell lines that perturb the N-glycan processing pathway and mutagenesis techniques have been useful in implicating a role for N-glycans in apical sorting. N-glycans were strongly implicated in apical sorting when Scheiffele *et al.* demonstrated that rat growth hormone, a nonglycosylated neutral protein, could be converted to an apical protein by the addition of two N-glycosylation consensus sites (122). The first endogenous protein shown to be trafficked to the apical surface in an N-glycan-dependent manner was gp80/clusterin (138). Apical secretion of this protein was disrupted when cells were treated with tunicamycin, a potent inhibitor of N-glycan core synthesis (138). Further characterization of the N-glycan-dependent sorting signal using glycosidase inhibitors and the mutant cell line MDCKII-RCA^r demonstrated that core glycan structures, rather than the terminal or penultimate saccharides were critical for proper apical sorting (112, 146). Delivery of the sialomucin endolyn to the apical surface was also shown to be disrupted upon treatment with tunicamycin and was the first transmembrane protein shown to rely on an N-glycan-dependent apical sorting signal (61). It has also been shown that the terminal processing of N-glycans is necessary for the apical sorting of gp114 (79). With a few proteins, specific N-glycans within the protein were found to be important for apical sorting. For instance, it was shown that only one of three N-glycans was important for apical secretion of erythropoietin (73). In another case, mutagenesis of three out of the four N-glycan consensus sequences from the neuronal glycine transporter (GLYT2) resulted in the nonpolarized distribution of this protein in MDCK cells (88). Thus there appears to be specificities in the structure and the number of N-glycans necessary for the N-glycan-dependent delivery of apical proteins. Requirements for the N-

glycan-dependent delivery of endolyn were examined in this study and are presented in Chapter 2.

Table 1.1 N-glycosylation perturbants

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 a glucosylated high mannose N-glycans
Castanospermine	CST	Like DNJ, this drug inhibits ER glucosidases.	Proteins display a 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 mannosidase II	Prevents the conversion of hybrid N-glycans to complex.
Benzyl-N-acetyl-α-galactosaminide	BGN	This drug acts 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.

1.3.2.3.c Proposed Mechanism for Glycan-mediated Protein Sorting

As described above, there is a wealth of information to suggest that both N- and O-glycans play a role in apical targeting. Thus, an important question is then how do these ubiquitous translational modifications specify polarized sorting? No discernable differences have been found between N-glycan structures isolated from apical vs. basolateral membrane proteins, suggesting that there is not a unique saccharide or glycan modification on apical proteins that confers apical targeting information. Thus in the absence of a unifying hypothesis, two models have been proposed to describe the mechanisms by which glycans may enable segregation of proteins into apically-destined carriers (117). One mechanism proposes that a family of receptors exists that sorts proteins into apically-destined vesicles (117). The receptors could recognize the glycans directly or recognize a proteinaceous conformation that is maintained by the glycans. As yet, however, no such receptor has been definitively defined. An early candidate was VIP36, a lectin originally isolated from lipid rafts; however, contradicting results have been observed for the localization of this protein and its role in apical sorting (31, 35, 44). The MAL proteolipid has been implicated in the apical delivery of a variety of apical proteins, not just glycosylated proteins, suggesting that MAL may be necessary for apical transport but not apical sorting (87). Most recently, a member of the galectin family, galectin-3, was implicated as an apical sorting receptor. Several apical proteins, including p75, gp114, and lactose phlorizin hydrolase, were shown to interact with galactin-3 in a carbohydrate-dependent manner. Additionally, siRNA-mediated knockdown of galectin-3 disrupted the expression of these proteins at the apical surface (26, 27). The trafficking of galectin-3, however, makes the role of this protein in apical sorting controversial. Galectins are cytosolic proteins that cannot bind to glycans of proteins within the lumen of the TGN or endocytic compartments. However, these proteins are secreted by a non-classical pathway and then internalized by binding glycoconjugates. It is not known whether

galectins can travel back to the TGN, but it is possible that galectin-3 may affect apical sorting within postendocytic compartments. Thus to define the role of galectin-3 in apical sorting, it is important to determine whether N-glycans are recognized in the TGN or postendocytic compartments along the biosynthetic pathway.

In the second model, glycans facilitate apical sorting by stabilizing a proteinaceous conformation required for sorting in the TGN, perhaps by promoting oligomerization or associations with lipid rafts. In support of this model, addition of N-glycosylation consensus sequences to mutant proteins that were retained in the Golgi complex was found to result in their efficient transport to the apical surface (42, 117). The diversity in requirements (e.g., N- vs. O-glycans, core vs. terminal N-glycan sugars) for glycan-dependent sorting might also argue in favor of a role for glycans in modulating protein conformation; however such a conformation could also be recognized by a receptor in accordance with the first model. At this point definitive evidence that discriminates between these two mechanisms is absent.

1.3.3 Role of Pre-TGN Sorting

Several recent reports using very different approaches have suggested the intriguing possibility that sorting of apical and basolateral cargo may occur in biosynthetic compartments prior to the TGN. Tveit *et al.* recently reported differential processing of apical and basolateral populations of the proteoglycan serglycin in early Golgi compartments in addition to the TGN (137). Proteoglycans are modified by the addition of glycosaminoglycan chains (GAGs) which are composed of a core tetrasaccharide linker which can be sulfated early in the Golgi followed by repeating units of glucoronic acid and either N-acetylglucosamine (heparin sulfate chains) or N-acetyl-galactosamine (chondroitan sulfate chains), which are subsequently sulfated in the TGN

(41). Interestingly, serglycin isolated from the basolateral media contained shorter chondroitan sulfate chains but was more highly sulfated than the apical pool of this proteoglycan (137, 145). GAG chains on basolaterally-delivered serglycin also exhibited primarily 6-O-sulfation versus 4-O-sulfation on the apically-delivered pool (137). Importantly, sulfation of the core tetrasaccharide linker region, which occurs early in the Golgi, was similarly higher in basolateral serglycin (137). These results suggest that apically- and basolaterally-delivered populations of serglycin appear to have differential access to GAG processing machinery prior to transiting the TGN.

Segregation of apical and basolateral cargo in the early Golgi has also been shown by Alfalah *et al.* by demonstrating that fully and incompletely processed forms of apical cargo can be separated from basolateral cargo based on their insolubility in the nonionic detergent Tween 20 (2). In their experiments, several apically-destined proteins were found to be insoluble in Tween 20, whereas basolateral proteins remained soluble (2). The incorporation of newly synthesized apical proteins into Tween 20-insoluble complexes was observed even when cells were incubated at 15°C, which prevents newly-synthesized proteins from leaving the endoplasmic reticulum intermediate compartment (2). Together, these studies suggest that newly-synthesized proteins may access different sorting platforms or microdomains shortly after biosynthesis. However if proteins are segregated early in the biosynthetic pathway, what would be the purpose of having multiple types of apical sorting signals? Additionally, there would still need to be machinery present in either the TGN or recycling endosomes to sort the proteins into apically-destined vesicles.

1.3.4 Protein Sorting in the TGN

Early studies with viral proteins revealed that apical and basolateral proteins traverse the Golgi together and are segregated in a distal compartment of this compartment. Research examining the role of the TGN in sorting has been aided by the ability to stage mature, newly-synthesized proteins in the TGN by a 20°C temperature block (90). Export from the TGN can then be visualized by live cell imaging or isolated biochemically upon warming the cells to 37°C. Using these techniques, two groups were able to show that VSV-G buds from the TGN in tubular structures that are delivered to the plasma membrane in a microtubule-dependent fashion (50, 135). Extending these studies, Keller *et al.* showed the segregation of apical and basolateral cargo at the level of the TGN and the inclusion of the proteins into distinct carriers (71). Biochemically, TGN-derived apical and basolateral carriers have been isolated and shown to have distinct protein and centrifugation profiles (147). Moreover differential requirements have been observed for the release of TGN-staged apical and basolateral proteins *in vitro* (11, 77, 152). Release of basolateral carriers has been shown to be dependent on protein kinase D and CtBP3/BARS, which appears to introduce curvature to the TGN membrane (11, 152). On the other hand, annexin XIIIb has been demonstrated to specifically stimulate apical delivery (77). Together these studies suggest that there is a level of segregation that occurs at the level of the TGN.

A relatively recent complication of the model for apical and basolateral protein segregation in the TGN comes from growing evidence consistent with the existence of different pathways for the TGN export of apical proteins with distinct sorting signals. Jacob *et al*. followed the TGN export of two fluorescently-tagged apical proteins that utilize distinct targeting mechanisms: the lipid-raft associated protein sucrase-isomaltase and the non-raft-

associated protein lactase-phlorizin hydrolase (69). These two apical proteins initially exited the TGN together in large vesicular compartments that subsequently gave rise to smaller vesicles that preferentially contained either of the two cargoes (69). Subsequent studies revealed that post-TGN trafficking of sucrase-isomaltase but not lactase-phlorizin hydrolase was found to be actin-dependent, although both required microtubules for efficient surface delivery (68). Vesicles containing sucrase-isomaltase were subsequently immunoisolated and proteomic analysis identified the motor protein myosin I and its regulator, α-kinase 1 on these vesicles, consistent with a role for actin in transport of raft-associated cargo (46). Another mechanism for actin involvement in biosynthetic trafficking may involve the generation of actin comets that function to propel transport carriers through the cytoplasm (30). Indeed, vesicles containing raft-associated but not raft-independent apical markers have been observed in association with actin comets in MDCK cells (40, 119). Thus, it appears that multiple sorting pathways to the apical surface may originate from the TGN.

1.3.5 Post-TGN Sorting

While there is certainly evidence that sorting occurs in the TGN, there is growing evidence that proteins may be sorted within endosomal compartments. Several studies over the past several years have shown that basolateral proteins transit endosomal compartments before delivery to the surface along the biosynthetic pathway. First, the transferrin receptor, was shown to localize to horseradish peroxidase (HRP)-labeled endosomes by electron microscopy before delivery to the surface (36). Similarly, H1, the major subunit of the asialoglycoprotein receptor, was isolated from endosomes containing cation-independent mannose-6-phospate receptor before delivery to the surface in nonpolarized cells (81). In polarized cells, Orzech *et al.* found that initial

biosynthetic delivery of the polyimmunoglobulin receptor (pIgR) to the basolateral surface involved transit through an endosomal compartment (107). In these studies, pIgR was radiolabeled and chased in the presence of wheat germ agglutinin conjugated to HRP (WGA-HRP) to selectively label endosomal recycling compartments such as the common recycling endosome and apical recycling endosome. After a time period insufficient for surface delivery, cells were treated with diaminobenzidine (DAB) and hydrogen peroxide. These two reagents then react with the HRP in the endosomal compartments to form an insoluble precipitate that can be removed by centrifugation. A loss of radioactive signal was seen for basolateral delivery of pIgR in the treated samples in comparison to non-treated controls, suggesting that pIgR was trafficking to endosomes before delivery to the basolateral surface. More recently, Ang *et al.* extended this approach using a combination of live-cell and biochemical techniques to investigate the significance and extent of endosomal transit of the basolateral marker VSV-G in nonpolarized MDCK cells that stably express the human transferrin receptor (6). A yellow fluorescent protein-tagged variant of VSV-G (VSV-G-YFP) was staged in the TGN while fluorescently labeled transferrin was taken up to label recycling endosomes. At this point the two proteins were segregated from one another, but upon warming for 10 minutes, colocalization was observed. This colocalization was confirmed by electron microscopy and coimmunoisolation of VSV-G and transferrin. To determine whether trafficking through endosomes is required for basolateral delivery of VSV-G, Ang *et al.* internalized transferrin conjugated to HRP into recycling endosomes while staging VSV-G-YFP and then treated cells with DAB and hydrogen peroxide to inactivate the recycling endosome. Cells were then warmed to allow delivery to the surface. Immunofluorescence studies showed a visible decrease in the amount of VSV-G-YFP at the surface in comparison to controls and surface delivery was shown to be inhibited by 80%

using flow cytometry. Live cell techniques were also used to show that vesicles containing E-cadherin fuse with recycling endosomes before delivery to the surface in nonpolarized cells (85).

While the studies discussed above strongly suggest that basolateral proteins are sorted to endosomal compartments, there is little compelling evidence for the involvement of endosomes in sorting apical proteins. Studies have focused on the trafficking of apical mutants of basolateral proteins that still possess sequences which may interact with basolateral machinery. For example, Orzech *et al.* concluded that an apically-directed mutant of pIgR that lacks a casein kinase phosphorylation site traffics indirectly to the cell surface; however this mutant contains an intact AP-1 interaction motif (107). Similarly, Ang *et al.* co-isolated labeled transferrin in compartments that were positive for an apically-delivered mutant of VSV-G; however the AP-interacting sequence in this poorly-defined VSV-G variant also remains intact (6). The role of recycling endosomes in apical biosynthetic trafficking was further characterized in this study using two authentic apical proteins, endolyn and influenza hemagglutinin, and is presented in Chapter 4.

1.4 SORTING ALONG THE POSTENDOCYTIC PATHWAY

With nearly 40% of the membrane of MDCK cells is turned over every hour, maintenance of polarity is dependent on polarized sorting along the postendocytic pathway in addition to sorting along the biosynthetic pathway (19). Upon internalization, proteins are first sorted into apical or basolateral early endosomes (94). These structures are defined by the localization of rab4 and EEA1 (94, 132). It is in these structures that ligands disassociate from their receptor due to the acidic pH of this compartment (94). The ligands are sorted to late endosome and lysosomes,

while the membrane receptor is trafficked back to the surface or recycling endosomes (94). Studies show that apical and basolateral cargo access a common recycling endosome and that cargo is sorted back to the appropriate surface. Odorizzi *et al.* revealed that transferrin receptor (TfR) internalized simultaneously from the apical and basolateral surface meets within an intracellular endosomal compartment and that the majority of the TfR was trafficked to the basolateral surface (105). Futter *et al.* showed that polyimmunoglobulin receptor (pIgR) internalized from the apical surface also meets with TfR in a common endosomal compartment (37). Furthermore the authors showed that tubules emanating from these structures form buds from clathrin coated domains, similar to that described for polarized sorting at the TGN. This structure is distinct from the apical recycling endosome to which rab11 localizes in the apical pole of the cell (148). Common recycling endosomes are localized to the medial portion of the cell and are slightly acidic (19, 148). From the common recycling endosome, apical cargo is trafficked to the surface via apical recycling endosomes, while basolateral cargo is directly trafficked to surface (148). The trafficking of apical and basolateral proteins along the postendocytic pathway is illustrated in Figure 1.5.

1.4.1 Basolateral Postendocytic Sorting Signals

In comparison to the biosynthetic pathway, very few studies have focused on the identification of sorting signals used in the postendocytic pathway. However, it appears that either the same or at least similar sorting signals are used in both pathways. Matter *et al.* found that deletion as well as mutation of the distal tyrosine-based signal in LDLR inhibited delivery along both the biosynthetic and postendocytic pathway, suggesting that the same sorting is used (93).

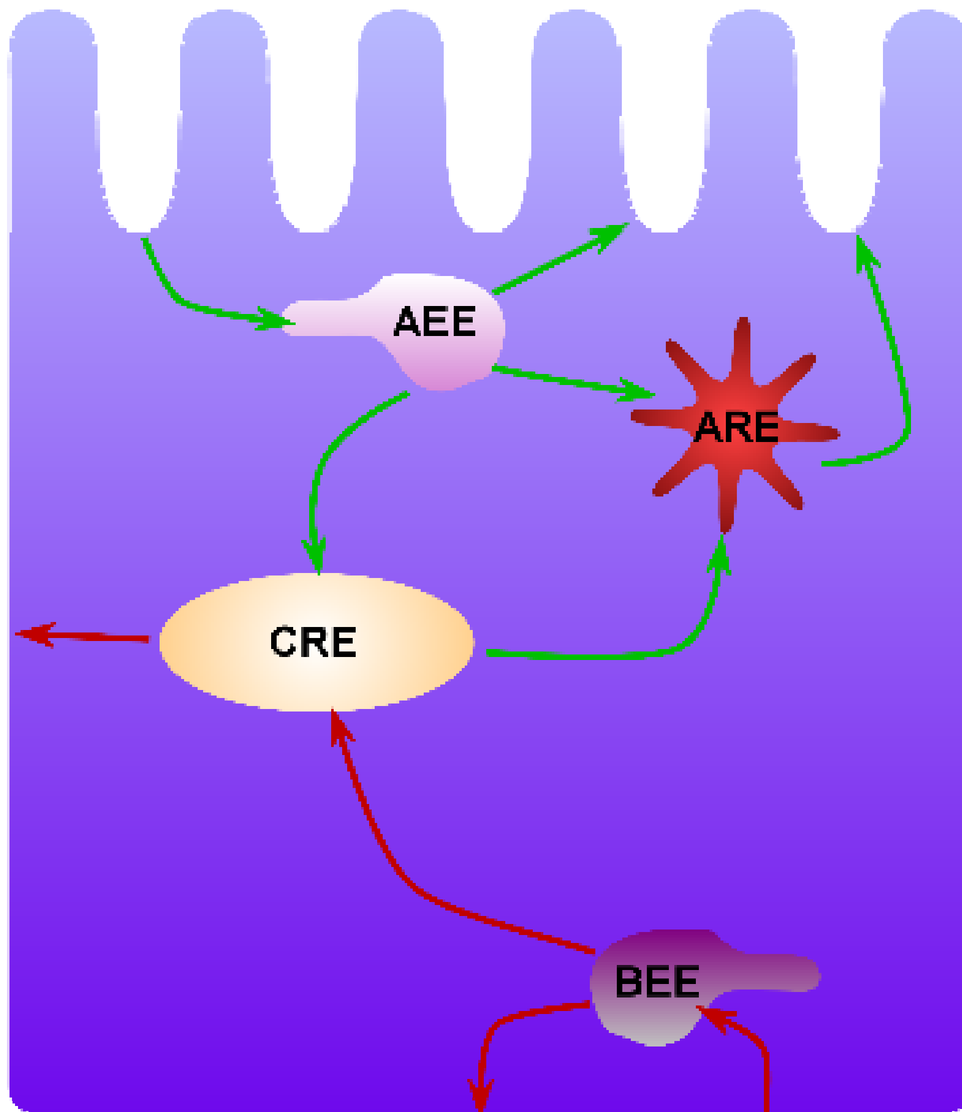


Figure 1.5 Apical and basolateral sorting routes along the postendocytic pathway

In polarized epithelial cells, apical (green) and basolateral (red) proteins internalized from the surface enter into either apical or basolateral early endosomes (AEE and BEE, respectively). From these compartments, proteins are directly or indirectly routed back to their respective surface. Some apical proteins are indirectly trafficked back to the surface via the apical recycling endosome (ARE). Both apical and basolateral proteins can be trafficked to the common recycling endosome (CRE); thus, to maintain polarity, there must be cellular sorting machinery within this compartment. From the CRE, basolateral proteins are immediately trafficked back to the surface while apical proteins are routed through the ARE.

Aroeti and Mostov showed that mutations in the cytoplasmic domain of pIgR could disrupt biosynthetic basolateral delivery and also cause a reduction in recycling after internalization from the basolateral surface (8). In the case of TfR, the same region was implicated in biosynthetic and postendocytic basolateral delivery; however specific point mutations within this region could selectively inhibit basolateral sorting along the two pathways (106). Given the similarity of these sorting signals to those used in the biosynthetic pathway and the localization of AP-complexes to endosomes, it is believed that these or similar complexes function in postendocytic delivery (94).

1.4.2 Apical Postendocytic Sorting Signals

While those sorting signals used for biosynthetic basolateral delivery of proteins also appear to function in the postendocytic pathway, it is not known whether the same is true for apical sorting. This study presents the first examination of whether N-glycans are involved in apical recycling of proteins using endolyn as a model.

1.5 THE SIALOMUCIN ENDOLYN

This dissertation focuses on role of N-glycans in apical delivery along both the biosynthetic and postendocytic pathway. To this end, I used a model protein (endolyn) that is efficiently delivered to the apical surface along both routes. Although the function of this protein is not known, this

mucin-like protein is believed to cycle between the cell surface and lysosomes, suggesting that it has a role at the plasma membrane.

Endolyn is a 78 kD lysosomal type I integral membrane protein consisting of 173 amino acids (62) (Figure 1.6). The lumenal domain is comprised of two mucin-like domains that are connected by a proposed disulfide bond hinge region (62). Within the lumenal domain there are thirty-five possible sites (Ser/Thr) for O-glycosylation and eight consensus sites for N-glycosylation. The short cytoplasmic domain of endolyn is short (13 aa) and the last four residues comprise a tyrosine based tetrapeptide YXXΦ motif (YHTL) that is necessary for delivery to lysosomes (62). Thus, endolyn is found primarily in lysosomes, but a smaller fraction can also be found at the plasma membrane. Studies with the human ortholog of endolyn, CD164, suggest that endolyn does have an important role at the surface. In hematopoietic cells, expression of endolyn at the surface mediates adherence to stromal cells, inhibiting their differentiation (156). It is believed that the downregulation of endolyn from the surface to a more endosomal localization may regulate differentiation of these cells (22).

It is not unusual for a lysosomal protein to be expressed at the surface, as delivery from the biosynthetic pathway is usually directed by two routes: a "direct" pathway from the TGN to late endosomes and then lysosomes, and an "indirect" pathway in which the protein is first delivered to the plasma membrane, internalized, and trafficked along the endocytic pathway to lysosomes (52). Both pathways depend on AP-3, which has been localized to the TGN as well as endosomes (63). Delivery along the direct pathway requires a glycine residue preceding the tyrosine-based lysosomal sorting signal (63). Since, endolyn does not possess a glycine prior to its lysosomal sorting signal, it is delivered to lysosomes primarily via the indirect pathway (62,

63). Indirect trafficking of endolyn was confirmed when inhibition of endocytosis in nonpolarized 3T3 cells resulted in decreased lysosomal delivery (63).

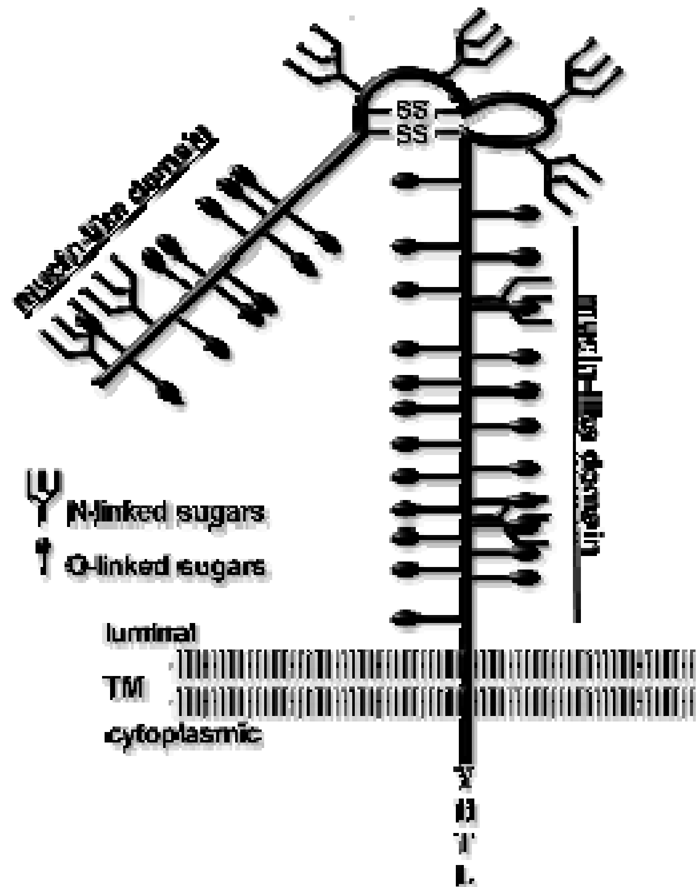


Figure 1.6 Structure of endolyn

Endolyn is a type I transmembrane lysosomal protein. The lumenal portion includes two highly glycosylated mucin-like domains connected by a putative disulfide-bonded loop. The last four residues within the cytoplasmic tail comprise a tyrosine-based tetrapeptide motif of the form YXXΦ.

Lysosomal proteins delivered along the indirect pathway in polarized cells usually transit the basolateral surface due to the similarity between lysosomal and basolateral sorting signals

(59). However, a unique trafficking pattern for endolyn was first revealed in WIF-B cells, a hepatocyte cell line. In these cells, all membrane proteins are first delivered to the basolateral surface then internalized and delivered to their correct destination. In contrast to other lysosomal proteins, endolyn was found to transit a subapical compartment before delivery to lysosomes (64). When endolyn was expressed in polarized MDCK cells, antibodies specific for endolyn were internalized from the apical surface while antibodies specific for lamp-2 were internalized from the basolateral surface, suggesting that endolyn is delivered to the apical surface in these cells (61). After internalization from the apical surface, the majority of endolyn is delivered to lysosomes while a significant fraction is recycled to the apical surface (63).

Apical delivery of newly synthesized endolyn in polarized MDCK cells was attributed to endolyn's lumenal domain, and despite the abundance of O-glycosylation, disruption of N-glycosylation with tunicamycin treatment significantly inhibited this delivery (61). Importantly apical delivery of endolyn predominates despite the fact that the lysosomal sorting signal can act as a basolateral sorting signal (61). Thus the N-glycan-dependent sorting signal of endolyn is the only example in which an apical sorting signal naturally dominates over a basolateral sorting signal. Additionally, apical delivery of endolyn is raft-independent as endolyn is soluble in cold Triton X-100 (61). Thus, endolyn is an excellent model protein to study the role of N-glycans in apical trafficking along both the biosynthetic and postendocytic pathway.

1.6 GOALS OF THIS DISSERTATION

Polarized epithelial cells provide a barrier against bacterial and viral infection and regulate the transport of metabolites, ions, and solutes between two environments. To facilitate these

functions, polarized epithelial cells uniquely organize their plasma membrane into discrete apical and basolateral domains. The apical surface faces the external or luminal milieu while the basolateral domain is in contact with neighboring cells and the underlying extracellular matrix and blood supply. The distinct composition of these two domains is maintained by cellular sorting machinery that recognizes sorting signals inherent within proteins along both the biosynthetic and postendocytic pathways. Sorting of proteins to the basolateral surface along these two pathways relies on specific amino acid motifs within cytoplasmic domains. Little research has focused on apical sorting along the postendocytic pathway; however multiple sorting signals have been proposed for sorting proteins to the apical surface along the biosynthetic pathway. Examples include cytoplasmically-disposed amino acid motifs and protein associations with glycolipid-enriched lipid microdomains (i.e., lipid rafts) through lipid anchors or transmembrane residues. Additionally, both N- and O-glycans have been implicated in the sorting of a subset of proteins. Currently it is not known how glycans mediate apical sorting but it has been proposed that glycans may participate in apical sorting by stabilizing a transport permissive conformation that if preferentially delivered to the apical surface. Alternatively, it has also been proposed that glycans mediate sorting through a receptor-mediated mechanism.

To explore the role of N-glycans in apical sorting, I have studied the apical trafficking of the sialomucin endolyn. Newly-synthesized endolyn is delivered to the apical surface of MDCK cells; and the first goal of this study was to characterize the requirements necessary for the N-glycan-dependent apical sorting of endolyn along the biosynthetic pathway. The second goal of this study was to determine whether N-glycans were important for apical recycling of endolyn, since it has not been whether N-glycans can function as an apical sorting signal along the postendocytic pathway. Additionally, given the growing evidence that sorting may also occur in

postendocytic compartments in addition to the TGN, the third goal of this study was to determine whether N-glycans mediate the sorting of endolyn from postendocytic compartments along the biosynthetic pathway.

2.0 SPECIFIC N-GLYCANS DIRECT APICAL DELIVERY OF TRANSMEMBRANE, BUT NOT SOLUBLE OR GPI-ANCHORED FORMS OF ENDOLYN IN MDCK CELLS

2.1 ABSTRACT

The sialomucin endolyn is a transmembrane protein with a unique trafficking pattern in polarized Madin-Darby canine kidney (MDCK) cells (61). Despite the presence of a cytoplasmic tyrosine motif that, in isolation, is sufficient to mediate basolateral sorting of a reporter protein, endolyn predominantly traverses the apical surface *en route* to lysosomes. Apical delivery of endolyn is disrupted in tunicamycin-treated cells implicating a role for N-glycosylation in apical sorting. Site-directed mutagenesis of endolyn's eight N-glycosylation sites was used to identify two N-glycans that appear to be the major determinants for efficient apical sorting of the protein. In addition, apical delivery of endolyn was disrupted when terminal processing of N-glycans was blocked using glycosidase inhibitors. Missorting of endolyn occurred independently of the presence or absence of the basolateral sorting signal, as apical delivery was also inhibited by tunicamycin when the cytoplasmic tyrosine motif was mutated. However, we found that apical secretion of a soluble mutant of endolyn was N-glycan independent, as was delivery of glycosylphosphatidylinositol (GPI)-anchored endolyn. Thus, specific N-glycans are only essential for the apical sorting of transmembrane endolyn, suggesting fundamental differences in the mechanisms by which soluble, GPI-anchored, and transmembrane proteins are sorted.

2.2 INTRODUCTION

Proper functioning of polarized epithelial cells necessitates the maintenance of differentiated apical and basolateral plasma membranes, which requires appropriate sorting of newly synthesized proteins to these distinct domains. In the biosynthetic pathway, newly synthesized apical and basolateral proteins are sorted upon reaching the *trans*-Golgi network (TGN). Basolateral sorting signals, including some tyrosine-containing tetrapeptide motifs and di-leucine motifs, are generally localized to the cytoplasmically-exposed portions of these proteins (101). These signals are thought to bind directly to adaptor protein complexes, which mediate sorting of the respective transmembrane proteins into basolaterally directed transport vesicles, analogous to the sorting of plasma membrane proteins into endocytic vesicles by AP-2 (12, 33, 126). By contrast, apical sorting signals are less well defined and reside frequently within the membrane- or lumenally-oriented regions of these molecules (101). Membrane-embedded signals include GPI-anchors attached to the carboxyl terminus of some proteins and amino acid sequences within the transmembrane domains of other apical proteins. It has been postulated that association with glycolipid-enriched lipid microdomains may play a role in the apical delivery of these proteins (66, 110). In addition, both N- and O-linked glycosylation have been demonstrated to be necessary for the correct apical delivery of several proteins (100, 121, 122, 146, 153).

We have been examining the trafficking of the sialomucin endolyn, a type I transmembrane protein that contains both lysosomal and apical targeting information (61, 64). The lumenal domain of endolyn consists of two highly O-glycosylated mucin subdomains connected by a non-mucin region with a suggested compact structure defined by two disulfide bridges (62). Each of these regions possesses two to four N-glycosylation sites. Endolyn is often

most highly concentrated in lysosomes at steady state, but is found to varying degrees in other endocytic compartments and at the plasma membrane depending on the cell type and state of differentiation (22, 24, 65). Lysosomal delivery of endolyn requires a carboxyterminal YXXΦ motif (62). While the function of endolyn is poorly understood, studies of the human and mouse proteins (also called CD164) in hematopoietic cells and myoblasts, respectively, indicate a role in cell adhesion and cell differentiation highlighting the importance of the plasma membrane pool of the protein (80, 156).

We previously demonstrated that a sizable fraction of newly synthesized endolyn, unlike other lysosomal membrane proteins that contain similar tyrosine sorting motifs, traverses the apical plasma membrane *en route* to lysosomes in polarized MDCK cells (61). This unusual route is not due to lack of basolateral sorting information in the cytoplasmic tail of the protein, as analysis of chimeras demonstrated that endolyn's tyrosine motif can serve as an efficient basolateral targeting signal. Rather, the lumenal domain of endolyn confers dominant apical targeting information that overrides the lysosomal and basolateral sorting information in the cytoplasmic tail in the biosynthetic pathway. Apical sorting of endolyn occurs by a mechanism that does not involve glycolipid rafts, but, intriguingly, requires N-glycosylation of the protein. Endolyn thus represents the first example of a transmembrane protein in which N-glycosylation appears to generate an apical sorting signal that is dominant over basolateral tyrosine motif-dependent sorting information.

Here we have examined in detail the structural requirements for glycan-dependent apical sorting of endolyn. Whereas all of endolyn's eight N-glycosylation consensus sequences appear to be glycosylated, only a subset of these glycans was found to be both necessary and sufficient for efficient apical sorting of endolyn. In addition, terminal oligosaccharide processing rather

than simply core glycosylation was required for interpretation of the apical sorting information, suggesting that specific receptor-ligand interactions are involved in the process. We also addressed the question whether N-glycans were a general requirement for apical sorting of differentially-tethered forms of the same protein. Interestingly, manufactured soluble and GPI-anchored forms of endolyn were targeted apically independently of N-glycosylation. This indicates that at least two distinct mechanisms exist to sort proteins to the apical surface of MDCK cells. Sorting of transmembrane proteins like endolyn appear to be restricted to one (N-glycan-dependent) mechanism, while secretory and GPI-linked proteins can be sorted in entirely different way(s), possibly in addition to the mechanism used by transmembrane endolyn.

2.3 RESULTS

2.3.1 Only a subset of endolyn's N-glycans is required for efficient apical sorting

Previously, we demonstrated that tunicamycin (TM) treatment dramatically disrupted endolyn polarity in MDCK cells (61). To examine the potential role of individual N-glycans in endolyn sorting, we generated MDCK cell lines stably expressing endolyn mutants in which one or two of the eight N-glycosylation consensus sequences were disrupted by alanine substitution for the respective asparagines(s) (Figure 2.1). To assess the loss of N-glycans, cells were radiolabeled for 3 h prior to solubilization and immunoprecipitation using anti-endolyn mAb. To minimize oligosaccharide heterogeneity and facilitate the electrophoretic analysis, the O-glycosylation inhibitor benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BGN) was included during the radiolabeling period. All of the mutants migrated distinctly from wild-type endolyn on SDS-

PAGE, with the double mutations resulting in a larger shift than the single mutations, suggesting all eight possible glycosylation sites are normally utilized (Figure 2.1B).

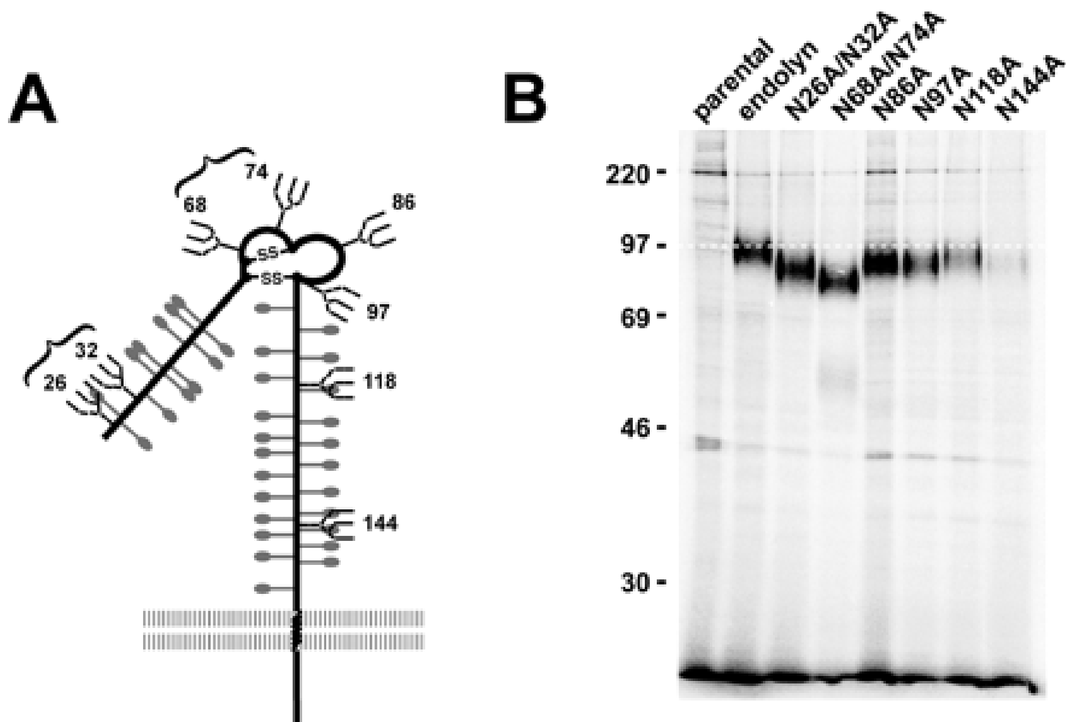


Figure 2.1: Characterization of endolyn N-glycosylation mutants.

A. Schematic of rat endolyn with predicted sites for N- and O-glycosylation indicated according to (61, 64). Numbers indicate the positions of asparagines in N-glycosylation consensus sequences; these sites were mutated individually or in pairs (as indicated by brackets) by substituting alanines for asparagines.

B. Analysis of endolyn glycosylation mutants by SDS-PAGE. MDCK cells expressing the indicated endolyn glycosylation mutants were radiolabeled for 3 h in the presence of benzyl-GalNAc (to reduce oligosaccharide heterogeneity and thus provide sharper resolution of endolyn upon SDS-PAGE), then solubilized, and endolyn immunoprecipitated from lysates using anti-endolyn mAb. The faster migration on SDS-PAGE of all mutants compared with wild type endolyn (white dotted line) suggests that all eight consensus sequences are normally N-glycosylated.

To determine the polarized distribution of these endolyn glycosylation mutants the corresponding cell lines were subjected to domain selective biotinylation after metabolic labeling for 2 h and a 15 min chase (Figure 2.2). Importantly, the fraction of each mutant protein biotinylated at the cell surface (apical and basolateral membranes combined) was similar to wild-type endolyn (with or without tunicamycin treatment), and generally ranged between 10-20% of the total with no statistical difference between any of the cell lines. This suggests that the mutations did not disrupt endolyn folding or cell surface delivery. Only one mutant (N68A/N74A) displayed a cell surface distribution distinct from that of wild-type endolyn (60% vs 73% apical for wild-type endolyn). While the effect of this double mutation did not approach the effect of TM treatment on endolyn polarity (44% apical), the decreased polarity of this mutant was highly reproducible and statistically significant ($P<0.001$). To further dissect the importance of the N-glycans at positions 68 and 74, we generated endolyn constructs in which these sites were individually mutagenized (N68A and N74A, respectively). Whereas the polarity of N68A was slightly reduced relative to wild-type endolyn (64% vs 71% apical; $p<0.02$, $n=21$), the polarity of N74A was indistinguishable from wild-type (71% apical, $n=19$). Thus, within the context of otherwise fully-glycosylated endolyn, the glycan at position 68 appears to be most critical for glycan-dependent apical sorting of the protein.

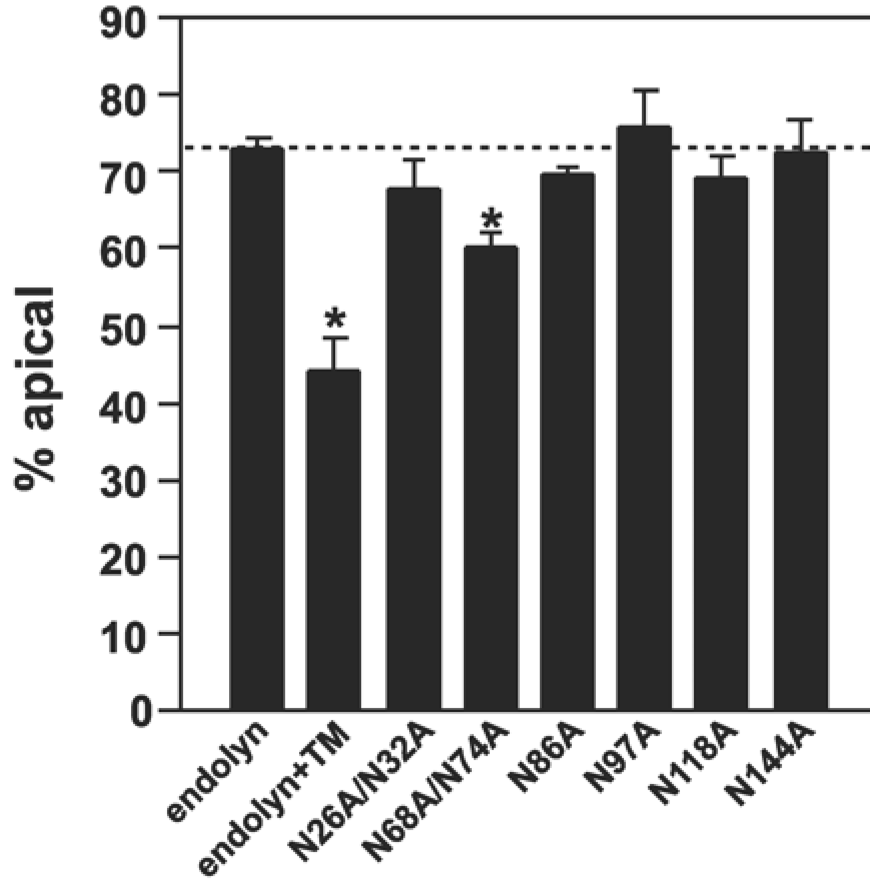
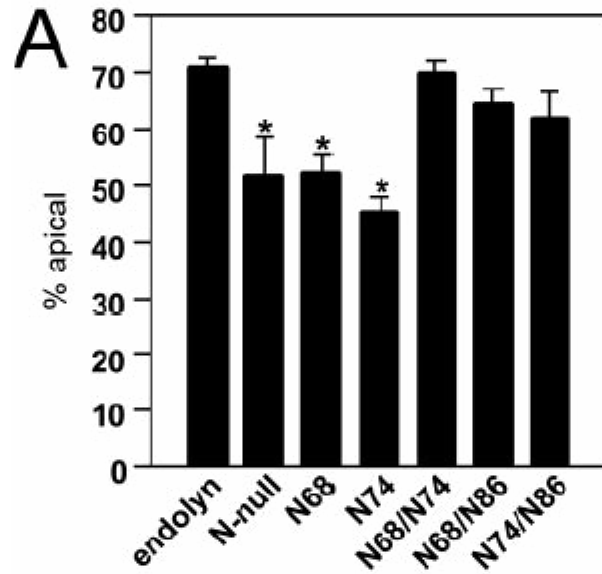


Figure 2.2 Surface polarity of endolyn glycosylation mutants.

Duplicate filters of polarized MDCK cells stably expressing wild-type endolyn or the indicated mutant constructs were radiolabeled for 2 h and chased for 15 min prior to domain-selective biotinylation. Tunicamycin (TM) was included 30 min before and during the radiolabeling period in the indicated samples for cells expressing wild-type endolyn. The apical fraction of biotinylated endolyn in each apical/basolateral pair of filters is shown (mean % apical +/- S.E.M.; endolyn, n=37; endolyn+TM, n=11; N26A/N32A, n=11; N68A/N74A, n=30; N86A, n=11; N97A, n=8; N118A, n=11; N144A, n=4). *$P<0.001$ vs. endolyn by t-test.

To further investigate the role of glycans at positions 68 and 74 in endolyn sorting, we generated an additional panel of endolyn mutants. The parent for these mutants was a construct we call N-null, in which all eight glycosylation sites are mutagenized. Importantly, the distribution of cell surface N-null was non-polarized (Figure 2.3), similar to that of non-N-glycosylated endolyn in TM-treated cells (Figure 2.2). Thus, missorting of endolyn in tunicamycin-treated cells cannot be explained by a global effect on the apical sorting machinery. This is an important control, as a previous study demonstrated that tunicamycin treatment altered the polarity of chromogranin A, a proteoglycan that lacks N-glycans, in MDCK cells (75). Reconstruction of the N-glycosylation consensus sequences at either position 68 or 74 (N68 and N74, respectively) did not restore polarized sorting of endolyn. Strikingly, however, when both N-glycosylation consensus sequences were reintroduced (N68/N74), the resultant construct was sorted with polarity similar to that of wild-type endolyn (70% vs 71%), demonstrating that both glycans contribute to the sorting determinant (Figure 2.3A). Comparison of the N-null mutant with N68/N74 by indirect immunofluorescence after simultaneous binding of anti-endolyn mAb to both surface domains confirms their different distributions: whereas N68/N74 showed preferential apical binding of the antibody like wild-type endolyn, anti-endolyn mAb bound efficiently to both apical and basolateral membranes of cells expressing N-null (Figure 2.3B). We also tested, by surface biotinylation, whether different pairs of glycosylation sites in the non-mucin “knob” domain of endolyn, namely N68/N86 or N74/N86, led to restored apical sorting (Figure 2.3A). Both combinations could partially substitute for N68/N74 with apical polarities of the corresponding mutants of 65% and 62%, respectively.



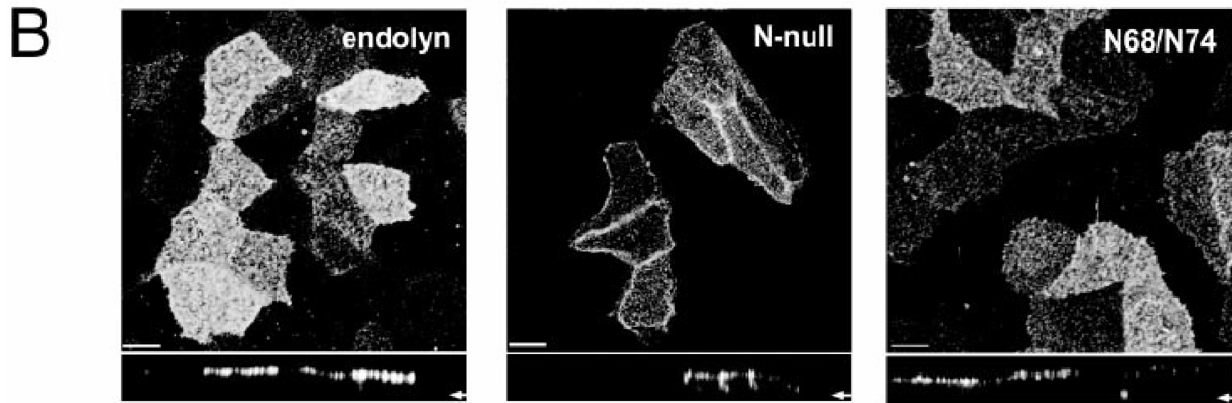


Figure 2.3 Addition of two glycans to endolyn N-null mutant restores apical transport.

Filter-grown MDCK cells stably expressing wild-type endolyn, endolyn lacking all N-glycosylation consensus sequences (N-null), or containing one or two restored sites (N68, N74, and N68/N74) were subjected to domain-selective biotinylation as described in the legend to Fig. 2. The surface polarity of endolyn is plotted (mean % apical +/- S.E.M.; endolyn, n=22; N-null, n=4; N68, n=8; N74, n=8; N68/N74, n=12; N68/N86, n=14; NN74/N86, n=13). *P<0.001 vs endolyn by t-test.

2.3.2 Role of oligosaccharide processing in endolyn sorting

As glycosylated proteins travel through the early biosynthetic pathway, the N-glycan precursors added *en bloc* to asparagine residues are trimmed by glucosidases and mannosidases. Glycan-dependent apical sorting of gp80 (a.k.a. clusterin), the major endogenous protein secreted by MDCK cells has been shown to require core N-glycosylation, but not subsequent processing of the N-glycans (112, 146). Thus we assessed the effects of various inhibitors of glycan processing on apical delivery of endolyn. Deoxynojirimycin (DNJ) inhibits glucose trimming of N-glycan core structures, whereas deoxymannojirimycin (DMJ) and kifunensine (KIF) inhibit mannose trimming and interfere with further processing of the glycan on all branches, i.e., addition of N-acetylglucosamine, galactose and sialic acid. Treatment with these drugs altered the mobility of endolyn on SDS-PAGE (Figure 2.4A), suggesting that N-glycan processing was disrupted in each case, although the effect of DNJ on endolyn mobility was minor and thus more difficult to observe than that of dMJ and KIF.

To determine the effects of these drugs on the apical polarity of endolyn, domain selective biotinylation experiments were performed (Figure 2.4B). While these drugs affect ER export of some proteins, we observed no effect on the overall efficiency of transport of endolyn to the cell surface, consistent with our observations using TM. Strikingly, both DMJ and KIF dramatically altered endolyn polarity; by contrast, DNJ treatment had no effect on endolyn polarity. The effects of DMJ and KIF were comparable to that observed in TM-treated cells or in the N-null mutant, suggesting that mannose trimming of endolyn's N-glycans (allowing subsequent processing of complex glycans) was critical for generating the apical sorting determinant.

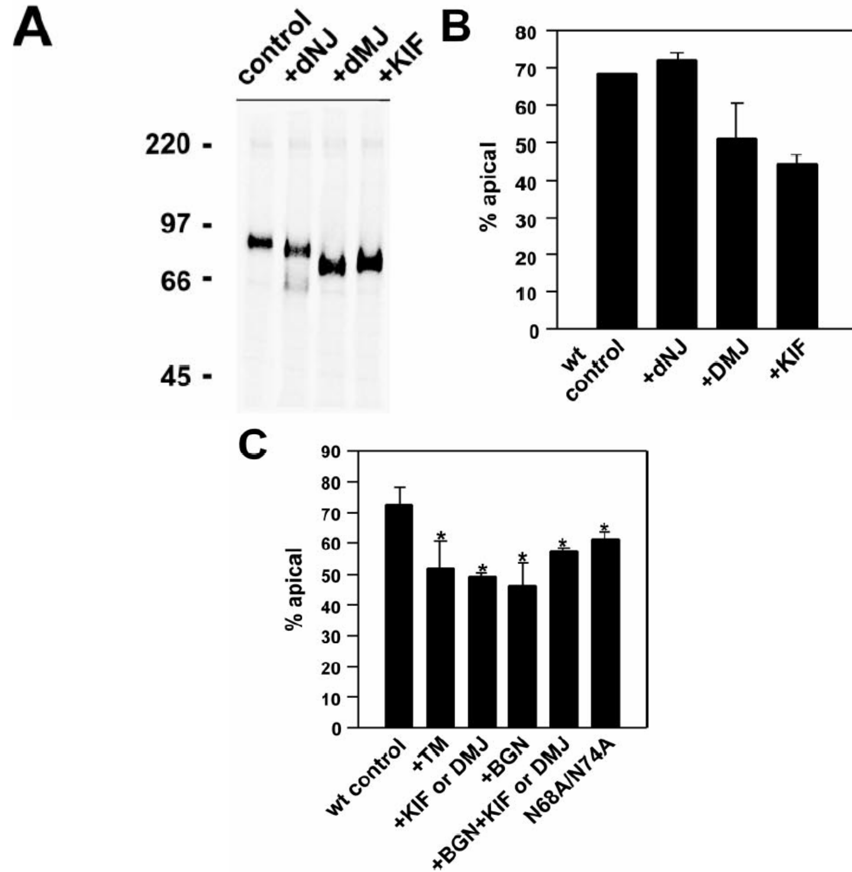


Figure 2.4 Terminal sugars on N-glycans participate in apical sorting of endolyn.

A. Mobility of endolyn from DNJ-, DMJ-, or KIF-treated cells on SDS-PAGE. Endolyn-expressing cells were starved and radiolabeled for 2h in the presence of deoxynojirimycin (DNJ), deoxymannojirimycin (DMJ), or kifunensine (KIF), then chased for 15 min. Endolyn was immunoprecipitated from the cells after solubilization and analyzed on a 10% SDS-PAGE gel.
B. Effect of glycosylation perturbants on surface distribution of endolyn. Endolyn-expressing cells were starved and radiolabeled for 2 h in the presence of the indicated drugs, then chased for 15 min prior to domain-selective biotinylation. The apical fraction of total biotinylated endolyn is plotted (average +/- range of 2 experiments).
C. Effect of glycosylation perturbants on vectorial delivery of endolyn. Cells expressing wild-type endolyn or the N68A/N74A mutant were starved and radiolabeled for 20 min in the presence or absence of the indicated drugs, then chased for 60 min at 37°C with anti-endolyn mAb added to either the apical or basolateral media of a pair of filters. After extensive washing, cells were solubilized and antibody-antigen complexes collected. The remaining endolyn was then immunoprecipitated from the samples, and the fraction of newly synthesized endolyn that reached the apical or basolateral membranes in each pair of filters was quantitated after SDS-PAGE. The fraction of endolyn accessible to apically-added antibody (relative to total endolyn reaching the surface) is plotted [mean +/- S.E.M.; endolyn control, n=9; +TM, n=4; +either KIF or DMJ, n=3; +BGN, n=8; +BGN and either KIF or DMJ, n=2 (average +/- range); N68A/N74A, n=3]. Because very similar effects were observed using KIF and dMJ, statistical analysis was performed on the combined data from cells treated with either of these drugs. *P<0.005 vs. endolyn by t-test.

To confirm that these drugs altered the vectorial delivery of endolyn to the apical plasma membrane, we used an antibody internalization assay designed to assess direct transport of newly synthesized endolyn to either cell surface domain in the biosynthetic pathway (61). Here, cells were radiolabeled over a brief period (20 min) and chased for 60 min in the presence of anti-endolyn mAb in either the apical or basolateral medium. As expected, treatment with TM, DMJ, or KIF disrupted apical sorting of newly synthesized endolyn (Figure 2.4C). We also confirmed that substitution of alanines for asparagines 68 and 74 resulted in significantly lower direct apical transport (62%), consistent with the compromised polarity of the corresponding mutant protein in our biotinylation experiments. In addition, we assessed the effect of BGN in this assay: this drug inhibits O-glycan processing but may also block galactose addition to N-glycans. BGN had a similar effect on endolyn polarity as DMJ, KIF, or TM. Importantly, the effect of BGN on endolyn polarity was not additive to KIF or DMJ, suggesting that BGN's effect was not due to perturbation of O-glycan processing, but N-glycan processing. This result is consistent with the notion that terminal processing, rather than simply inhibition of mannose trimming, is important for formation of endolyn's N-glycan-dependent apical sorting determinant.

2.3.3 Polarized sorting of soluble and GPI-anchored endolyn is N-glycan independent

We previously demonstrated that En_{sol}, a truncated, soluble mutant of endolyn lacking a transmembrane domain, is efficiently secreted into the apical medium of polarized MDCK cells (61). Surprisingly, while En_{sol} mobility on SDS-PAGE was altered by TM treatment of stably expressing cell lines as expected, the polarity of En_{sol} secretion was unaffected by this drug (Figure 2.5). Thus, N-glycosylation-independent apical sorting of En_{sol} might reflect the

presence of additional apical sorting information in the lumenal domain of endolyn. Treatment with BGN also had no effect on the polarity of En_{sol} secretion (data not shown).

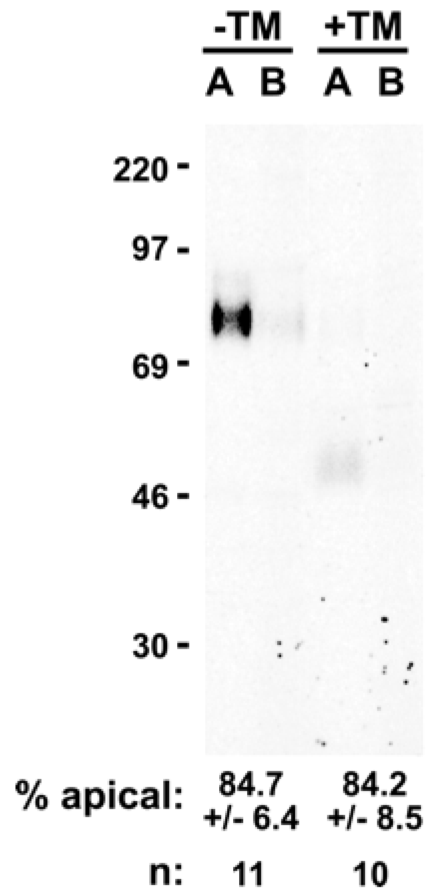


Figure 2.5 Apical secretion of a soluble form of endolyn does not require N-glycans.

Filter-grown En_{sol}-expressing cells were starved and radiolabeled for 3 h in the presence or absence of TM. The apical and basolateral media were collected and secreted En_{sol} immunoprecipitated and analyzed by SDS-PAGE. A representative gel is shown, and the polarity of secreted En_{sol} (mean +/- S.D. determined from 10-11 experiments) is noted below each pair of lanes.

The difference between apical sorting of transmembrane and secreted forms of endolyn in respect to their dependence on glycans could be explained in two ways: The tyrosine-containing motif in the cytoplasmic tail of endolyn, which can function as a basolateral sorting signal, might supercede recognition of redundant apical info and redirect non-N-glycosylated molecules basolaterally. Alternatively, the soluble form of endolyn might be sorted via a mechanism entirely distinct from that of wild-type endolyn. To distinguish between these possibilities, we examined whether polarity of a transmembrane form of endolyn lacking a functional basolateral sorting signal (EEE_{YA}, a mutant in which the critical tyrosine is substituted by alanine; (61)) was compromised by perturbation of N-glycans (Figure 2.6). Interestingly, EEE_{YA} polarity was sensitive to TM treatment, as well as to perturbation of terminal N-glycan processing by KIF treatment (Figure 2.6). The discrepant polarity between fully N-glycosylated and non-N-glycosylated forms of EEE_{YA} was striking under conditions where both forms were simultaneously expressed, i.e., when cells were radiolabeled after relatively short (30 min) pretreatment with TM (Figure 2.6B). These results suggest that the nonpolarized distribution of non-N-glycosylated endolyn and EEE_{YA} does not depend on a functional basolateral sorting signal, and by inference, that En_{sol} is sorted apically via a distinct, glycan-independent mechanism unavailable to transmembrane forms of endolyn.

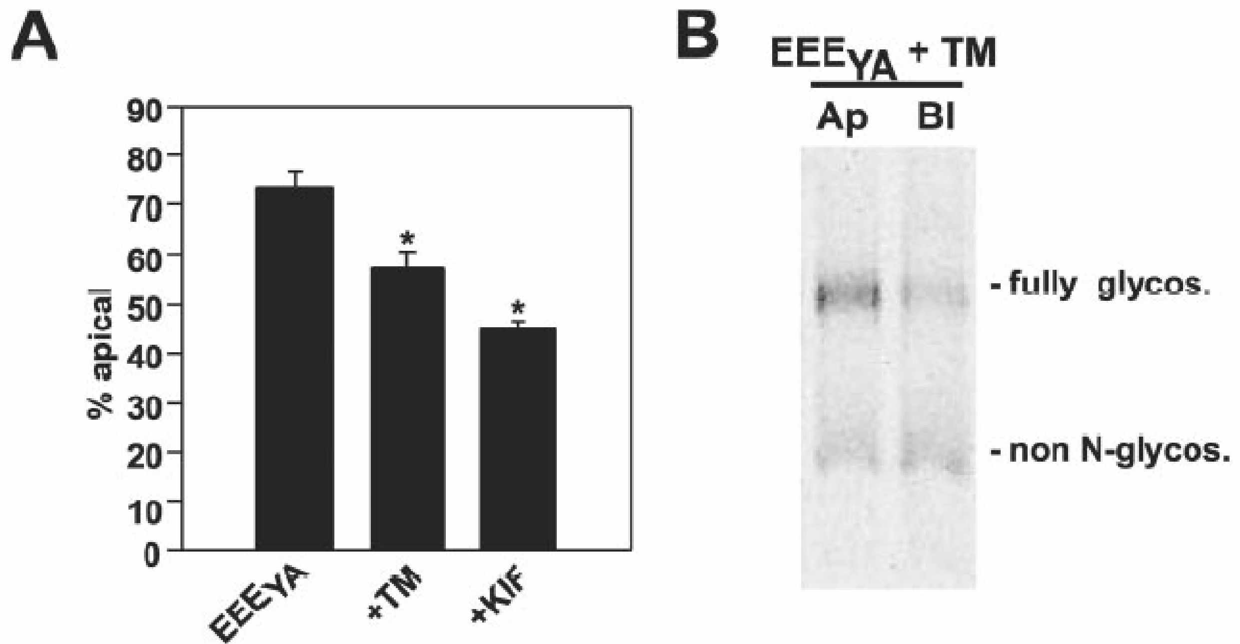


Figure 2.6 Apical delivery of EEE_{YA} is N-glycosylation-dependent

A. EEE_{YA}-expressing cells were starved and radiolabeled for 2h in the presence of TM, BGN, or KIF, then chased for 15 min prior to domain-selective biotinylation and quantitation of cell surface polarity. The apical fraction of total biotinylated EEE_{YA} is plotted (mean +/- S.E.M.; control, n=11; TM, n=6; BGN, n=9; KIF, n=2). *P<0.01 vs. endolyn by t-test.

B. A typical gel showing apically- and basolaterally-biotinylated EEE_{YA} recovered from TM-treated cells, with the migration of fully glycosylated and non-N-glycosylated protein indicated. The polarity of these two EEE_{YA} species in this experiment is also noted.

We next examined whether GPI-anchored endolyn requires N-glycosylation for apical sorting. A mutant expressing GPI-endolyn was constructed by fusing the C-terminal signal sequence of 5'-nucleotidase providing a GPI-anchor attachment site to the luminal domain of endolyn (see Materials and Methods). Indirect immunofluorescence of surface-labeled polarized MDCK cells stably expressing GPI-endolyn demonstrated a predominant apical distribution of the cell surface protein (Figure 2.7A). Domain-selective biotinylation of radiolabeled GPI-endolyn (Figure 2.7C) and antibody internalization experiments after brief metabolic labeling (not shown) confirmed that the protein was delivered primarily to the apical cell surface. Unlike wild-type endolyn (61) and EEE_{YA}, which are completely extracted from cells by Triton X-100,

GPI-endolyn was partly insoluble in cold Triton X-100, similar to virally-introduced influenza hemagglutinin (HA; included as an positive control as this protein is known to have this characteristic; Figure 2.7C). This resistance to extraction by cold Triton X-100 is a hallmark of GPI-anchored proteins that reflects their association with glycolipid-enriched microdomains (18). To determine the role of N-glycans in GPI-endolyn sorting, stably expressing polarized MDCK cells were pretreated with glycosylation perturbants, and the distribution of radiolabeled GPI-endolyn analyzed using domain-selective biotinylation (Figures 2.7C and 2.7D). None of the perturbants tested, including TM (Figures 2.7C and 2.7D), KIF (Figure 2.7C), or BGN (not shown) disrupted the polarity of GPI-endolyn delivery. Thus, similar to the soluble form, apical sorting of GPI-linked endolyn appears to occur independently of the presence of N-glycans.

Finally, we tested whether the mode of anhoring, i.e., a transmebrane anchor in general, or the presence of endolyn-specific transmembrane or cytoplasmic sequences, were additional requirements fro glycan-dependent apical sorting. To address this, we examined the targeting and sensitivity to tunicamycin treatment of two chimeric protein in stably expressing polarized MDCK cells. In one of these chimeras, ECEYA, the transmembrane domain of EEEYA was replaced with that of CD8, an O-glycosylated plasma membrane protein. In the other chimera, EECΔ, the cytoplasmic domain of endolyn was replaced with a neutral cytoplasmic tail consisting of the first 10 amino acids of CD8 tail. Both of these protein were targeted apically with a polarity similar to that of wild-type endolyn (70.3% apical for EECΔ; 73.7% for ECEYA; n=6) and treatment with TM disrupted their apical delivery (54.7 and 48.8% apical, respectively; n=5; $p < 0.05$ versus untreated controls). Treatment with KIF also disrupted the polarized sorting of these mutants (our unpublished data). Thus, N-glycans in endolyn’s luminal domain are required apical sorting determinants uniquely in the context of a transmembrane protein, but the

underlying sorting mechanism does not depend on specific sequences in the transmembrane or cytoplasmic domain of endolyn.

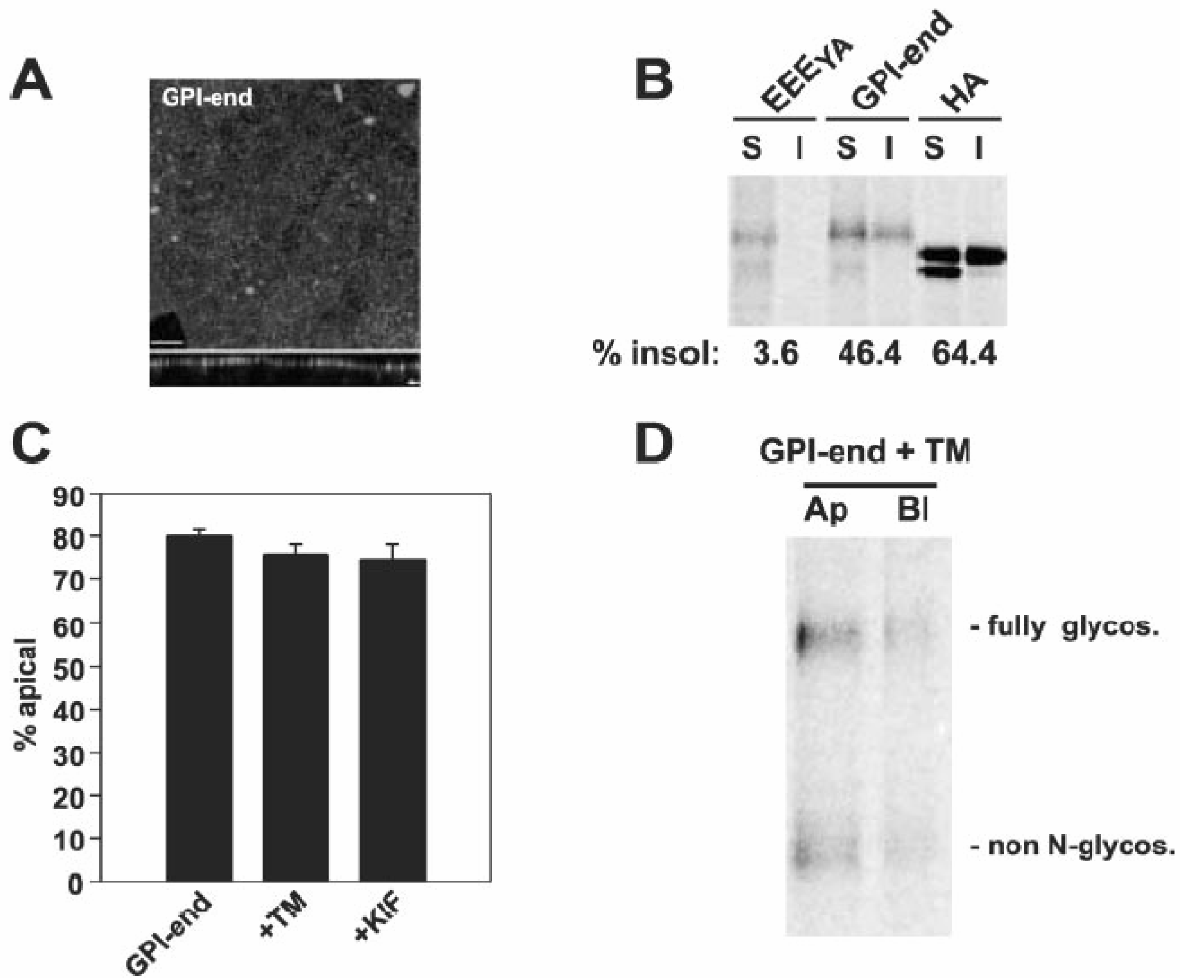


Figure 2.7 Apical delivery of GPI-anchored endolyn does not require N-glycosylation

A. Surface distribution of GPI-endolyn. Filter-grown GPI-endolyn expressing cells were surface-labeled on ice with anti-endolyn mAb, fixed and processed for indirect immunofluorescence, and analyzed by confocal microscopy. An XY section taken at the level of the apical surface (upper panel) and an XZ section through the monolayer (lower panel) are shown. The position of the filter in the lower panel is marked with an arrowhead. Scale bar: X μm.

B. Detergent solubility of GPI-endolyn. MDCK cells expressing influenza HA, EEE_{YA}, or GPI-endolyn were radiolabeled for 1 h, then chased for 1 h prior to solubilization on ice and isolation of detergent-soluble (S) and -insoluble (I) fractions. Immunoprecipitated fractions were analyzed by SDS-PAGE. The fraction of total protein that was insoluble in cold detergent is given beneath each pair of lanes.

C. Effect of glycosylation perturbants on surface distribution of GPI-endolyn. GPI-endolyn-expressing cells were starved and radiolabeled for 2 h in the presence of TM or KIF, then chased for 15 min prior to domain-selective biotinylation. The apical fraction of biotinylated GPI-endolyn is plotted (mean +/- S.E.M.; control, n=13; TM, n=6; KIF, n=6).

D. Representative gel showing apically- and basolaterally-biotinylated GPI-endolyn recovered from TM-treated cells. The polarity of fully-glycosylated and non-N-glycosylated GPI-endolyn that was calculated in this particular experiment is also noted.

2.4 DISCUSSION

We previously found that the sialomucin endolyn is uniquely trafficked to lysosomes via the apical surface of polarized MDCK cells. A dominant N-glycan dependent apical sorting signal was localized to the lumenal domain of endolyn. Here we have further characterized the N-glycosylation requirement for apical sorting of endolyn, revealing specificity both in the position of glycans involved and in glycan structure. The amino acid sequence of endolyn contains eight potential sites for N-glycosylation, all of which appear to be glycosylated *in vivo*. Of these eight, we have identified two specific sites located within a putative disulfide-bonded loop domain that are necessary and sufficient for efficient apical delivery. Proper sorting of newly synthesized endolyn requires terminal processing of endolyn's N-glycans rather than simply the presence of core N-glycans, suggesting that the terminal sugars of these glycans are an integral part of the apical targeting signal. Surprisingly, however, apical sorting of both soluble and GPI-anchored forms of endolyn was found to be N-glycan-independent. These results suggest that closely related apically-destined proteins can be efficiently sorted via distinct, non-redundant mechanisms.

2.4.1 Specific N-glycosylation sites on endolyn are required for apical sorting of endolyn along the biosynthetic pathway

To determine the role of particular N-glycans in apical sorting of endolyn, individual sites or subsets of N-glycosylation consensus sequences were mutagenized and the polarity of expressed mutant proteins was analyzed using domain selective biotinylation. Only one mutant, N68A/N74A (~60% apical), was significantly less polarized than wild-type endolyn (~72%

apical), and another mutant with the single substitution N68A exhibited intermediate polarity (~64% apical). A similar effect on polarity was observed in antibody uptake experiments in cells that were metabolically labeled over shorter periods, demonstrating that the missing N-glycans disrupt direct delivery of newly synthesized endolyn to the apical membrane as opposed to subsequent trafficking of the protein. A mutant that contained intact glycosylation sites *only* at positions 68 and 74, while all others were mutated by alanine substitution, was correctly sorted to the apical domain. This suggests that N-glycans in these positions are sufficient to mediate apical targeting and thus are probably the major sorting determinants in the fully glycosylated protein *in vivo*. Neither N68 nor N74 alone was sufficient to confer apical sorting, indicating that both N-glycans are necessary. However, the effect of the N68A/N74A double-mutation on polarity was less dramatic than that observed for endolyn in tunicamycin-treated cells or for the N-Null mutant in which all asparagines of putative glycosylation sites were substituted by alanines. This partial effect may reflect the ability of the remaining N-glycans to compensate for the loss of N-glycans at positions 68 and 74. In fact, in the context of the N-null background, reconstruction of pairs of N-glycosylation consensus sequences at positions 68 and 86 or 74 and 86 resulted in preferential apical sorting of the corresponding proteins, although the fidelity of sorting appeared to be somewhat lower compared with the N68/N74 mutant.

While the total recovery of non- or singly-N-glycosylated forms of endolyn in our immunoprecipitates was typically less than wild-type (which could be explained either by increased susceptibility to degradation or by poorer recognition by our monoclonal antibodies), the relative fraction of total endolyn recovered at the plasma membrane was not significantly different in any of our mutants. This finding suggests that endolyn mutants lacking N-glycans were not retained in the TGN, but rather that loss of the apical sorting determinant resulted in

nonpolarized transport to the cell surface. This phenotype is in contrast to the results of Gut et al., who demonstrated that inhibition of N-glycosylation blocked the exit of several apical proteins from the TGN, and did not result in their missorting to the basolateral membrane (42). We interpret our results to indicate that apical targeting of endolyn requires N-glycans not as a general structural support to allow a "transport permissive" conformation of the protein, but as a sorting determinant that enables specific interactions with the sorting machinery.

2.4.2 Terminal processing of N-glycans is important for apical delivery of endolyn

When cells stably expressing endolyn were treated with kifunensine and DMJ, drugs that block mannose trimming and further processing of N-glycans, nonpolarized cell surface delivery of endolyn was observed. However, DNJ, a drug that blocks glucose trimming, had no affect on apical delivery. N-glycan processing to form complex oligosaccharides can occur in the absence of endogenous glucosidase activity or in DNJ-treated cells (34, 92). This suggests that the terminal glycans on endolyn expressed under these conditions can form the necessary sorting determinant, while this is not possible when terminal processing is completely abrogated.

Our results using glycosylation inhibitors are in contrast to those published for the primary secreted protein in MDCK cells, gp80 (a.k.a. clusterin), whose proper apical delivery requires the addition of core oligosaccharides but not further processing of N-glycans. Apical delivery of gp80 was unaffected by DMJ treatment or in ricin- and concanavalin A-resistant MDCK cell lines, which like DMJ or KIF-treated cells, are unable to add galactose or sialic acid residues to N-glycans (112, 146). This discrepancy points towards differences in the N-glycan dependent sorting of individual proteins or secreted and transmembrane proteins in general.

We also observed that treatment with BGN, a drug that scavenges UDP-galactose and thus prevents its addition to oligosaccharide chains, significantly disrupted polarized delivery of newly synthesized endolyn. The effects of BGN treatment on the surface distribution of sucrase-isomaltase were previously interpreted to reflect a requirement for O-glycan processing in the apical sorting of this protein (1). However, this drug has also been shown to block terminal processing of N-glycans in some cell lines (39). Treatment with both BGN and DMJ or KIF disrupted endolyn sorting to the same extent as either drug alone, consistent with a common inhibitory mechanism. We conclude that the effect of BGN on endolyn polarity is most likely due to its effects on N-glycosylation rather than to an additional role for O-glycosylation in endolyn sorting; however, the latter cannot be ruled out completely.

Our earlier studies demonstrated that acute (2h) treatment with BGN did not affect the polarized distribution of endolyn in stably-expressing MDCK cell lines as measured using domain-selective binding of radioiodinated anti-endolyn antibody (61). The discrepancy between these results is likely due to the mixing of preexisting recycling endolyn pools with newly synthesized molecules at the apical plasma membrane. Indeed, we have observed that the efficiency of biotinylation of radiolabeled endolyn does not decrease significantly over a 21 h chase, suggesting that endolyn recycles to the cell surface for long periods after synthesis (J.R. Bruns and O.A. Weisz, unpublished). Thus, it is likely that the short-term drug treatment conditions under which the effect of BGN on polarity was previously examined were insufficient to alter the glycosylation profile of the majority of cell-surface endolyn.

2.4.3 Differential sorting requirements for soluble and GPI-anchored endolyn

N-glycans were initially implicated as sorting signals for gp80 and glycosylated human growth hormone, both of which are soluble proteins (112, 122). Thus we were surprised to find that apical delivery of a soluble form of endolyn, En_{sol} was completely insensitive to treatment with TM. The N-glycan-independent delivery of En_{sol} could be due to the unmasking of a recessive apical sorting signal upon removal of endolyn's cytoplasmic tail, which contains a tyrosine-dependent basolateral sorting motif (61). In this scenario, the nonpolarized sorting we observed for TM-treated endolyn or the N-null mutant would reflect the competition between cytoplasmic basolateral sorting information and residual lumenal apical targeting information in these proteins. However, the polarity of EEE_{YA}, a full-length construct in which this critical tyrosine residue was mutagenized to alanine, was disrupted by cell treatment with TM or KIF to an extent equal to that observed for wild-type endolyn. This suggests that the apical sorting determinant responsible for glycan-independent secretion of En_{sol} is absent or inaccessible in EEE_{YA} and wild-type endolyn. Indeed, other studies suggest that soluble and transmembrane proteins can be sorted via distinct mechanisms; for example, trafficking of soluble and membrane proteins is differentially sensitive to perturbation of microtubule structure and pH (10, 20).

We also found that apical delivery of a GPI-anchored form of endolyn is N-glycan independent. The ability of GPI-anchors to function as apical sorting signals is currently controversial (9, 82, 83, 110). Early studies demonstrated that addition of a GPI-attachment signal to herpes simplex glycoprotein D or to human growth hormone resulted in apical delivery (83). It has been hypothesized that the GPI-lipid anchor facilitates the inclusion of proteins into detergent-insoluble glycosphingolipid-enriched membrane domains that are selectively trafficked to the apical surface. However, a recent study, which showed that GPI-anchored proteins lacking

N-glycans are not always apically targeted, has challenged the role of GPI anchors as autonomous apical targeting signals (9). In the case of endolyn, the addition of a GPI-anchor undoubtedly facilitates apical sorting in the absence of N-glycans; however, it is possible that other additional apical targeting information is also needed. This determinant could be identical to the information that directs soluble endolyn to the apical surface. At this stage the nature of such "recessive" apical information is unclear; it could consist of residual O-glycans, a weak peptide motif, and/or depend on a receptor-independent mechanism, such as aggregation of cargo mediated by the lumenal domain of endolyn.

2.4.4 Mechanisms for glycan-dependent sorting

The mechanism by which N-glycans mediate apical sorting is not known; however, two general models have been proposed (117). One possibility is that N-glycans are required to stabilize transport permissive conformations of proteins. Our results are more compatible with the second proposed mechanism: that N-glycans are required for protein binding to a cargo receptor in the TGN that concentrates the protein into apically-destined vesicles.

There is in fact precedence for lectins that function as cargo receptors in the biosynthetic pathway (44, 45). One such lectin, VIP36, has been suggested to act as receptor for soluble and membrane proteins with high-mannose-type N-glycans, including gp80 (44) and is therefore an unlikely candidate to bind endolyn or other proteins that rely on complex N-glycans for sorting. Thus, rather than a single receptor recognizing all proteins that require N-glycans for apical sorting, there may be several such binding proteins.

Our observation that two of endolyn's eight N-glycans are critical for efficient apical sorting suggests that there are specific structural requirements for recognition of the apical

sorting determinant. Similarly, it was previously demonstrated that only one of the three N-glycans on erythropoietin, a secreted apical protein whose distribution is perturbed by tunicamycin, is critical for its apical targeting (73). The key N-glycans identified for endolyn and erythropoietin sorting may be critical because they possess oligosaccharide structures that are selectively recognized by a lectin receptor (either due to their unique structure since they might be more accessible to selective modification by oligosaccharide processing enzymes, or simply because they are the ones accessible to the receptor.) Alternatively, these glycans may stabilize a proteinaceous confirmation that binds to the sorting receptor. The N-glycans at positions 68 and 74 of endolyn are located on a predicted disulfide-bonded loop that carries no or few O-glycans. This part of the protein is likely to have a well-defined secondary structure, different from the mucin-like regions that constitute much of the remaining lumenal domain. Moreover, these N-glycans and several other amino acids in this region are well conserved among species, and could therefore serve as a distinct structural sorting determinant. It is important to keep in mind that complex N-glycans are bulky carbohydrate clusters that can reach more than 3 nm from the protein surface and that their outer parts can therefore act as independent domains of glycoproteins (47). Thus, terminal sugars could be part of an epitope recognized by a lectin receptor, or they alone could create the crucial epitope.

2.4.5 Glycans and endolyn function

Glycans have previously been implicated in the function of endolyn/CD164. A monoclonal antibody recognizing an N- and O-glycan-dependent epitope on the N-terminal mucin domain of human CD164 has been shown to reduce the adhesion of hematopoietic progenitor cells to bone marrow stroma and to negatively regulate cell proliferation *in vitro* (28, 156). This observation

agrees with the paradigm that specific sugar modifications are essential for the interaction of sialomucins with their extracellular binding partners (142).

Assuming that similar principles operate in human and rat, the N-glycans involved in apical targeting and those possibly involved in extracellular protein-protein interactions do not appear to be the same. The amino-terminal epitopes of human CD164 are differentially expressed in various tissues (150). We do not yet know whether the N-glycans (or their specific modifications) important for apical sorting in polarized MDCK cells are ubiquitously expressed. Because the size and structure of N- and O-glycans is developmentally regulated (136, 155), it is possible that the terminal carbohydrates on these N-glycans are not always processed equivalently. The requirement for terminal glycan processing for apical delivery of endolyn could therefore reflect a built-in mechanism to modulate endolyn trafficking. Thus, glycan processing may be one of the modifications by which the trafficking of endolyn/CD164 is regulated to adjust its distribution between cell surface and endosomal/lysosomal compartments according to the functional requirements for this protein at different stages during tissue development.

3.0 N-GLYCAN MEDIATE APICAL RECYCLING OF THE SIALOMUCIN ENDOLYN IN POLARIZED MDCK CELLS

3.1 ABSTRACT

Apical and basolateral proteins are maintained within distinct membrane subdomains in polarized epithelial cells by biosynthetic and postendocytic sorting processes. Sorting of basolateral proteins in these processes has been well studied; however, the sorting signals and mechanisms that direct proteins to the apical surface are less well understood. We previously demonstrated that an N-glycan dependent sorting signal directs the sialomucin endolyn to the apical surface in polarized Madin-Darby canine kidney cells. Terminal processing of a subset of endolyn's N-glycans is key for polarized biosynthetic delivery to the apical membrane. Endolyn is subsequently internalized, and via a cytoplasmic tyrosine-based sorting motif is targeted to lysosomes from where it constitutively cycles to the cell surface. Here we examine the polarized sorting of endolyn along the postendocytic pathway in polarized cells. Our results suggest that similar N-glycan sorting determinants are required for apical delivery of endolyn along both the biosynthetic and postendocytic pathways.

3.2 INTRODUCTION

Crucial to the function of polarized epithelial cells is the ability to sort proteins along both the biosynthetic and endocytic pathways in order to generate and preserve distinct populations of membrane proteins at their apical and basolateral membrane domains. Intense research has been focused on identifying sorting signals within proteins as well as the cellular sorting machinery responsible for targeting proteins to the correct domain. This has led to a greater understanding of how proteins are sorted along the biosynthetic pathway. Basolateral sorting is generally dependent on cytoplasmic peptide sorting sequences, some of which conform to tyrosine-based tetrapeptide or dileucine motifs (101). Understanding apical sorting, on the other hand, has been more elusive. Proposed apical sorting signals include glycosylphosphatidylinositol (GPI) lipid anchors, specific amino acid sequences within the transmembrane or cytoplasmic domains of proteins, and both N- and O-glycans (101, 118, 124).

Polarized sorting of newly synthesized proteins into distinct transport carriers has been demonstrated to occur in the *trans*-Golgi network (TGN) in Madin-Darby canine kidney cells (MDCK). More recently it has been shown that biosynthetic sorting of some proteins may occur within endosomal compartments as well (6, 36, 107). Tyrosine-based tetrapeptide and dileucine motifs can interact with adaptor protein complexes in these compartments to direct proteins to endosomes, lysosomes, or the basolateral cell surface (12). The association with glycolipid enriched microdomains in the Golgi complex may facilitate the concentration of GPI-anchors and proteins whose apical sorting signals reside in their transmembrane domains into apically-destined transport carriers (109). A mechanism for the sorting of apical proteins via glycan-dependent sorting signals has yet to be identified.

In addition to polarized biosynthetic delivery, the maintenance of cellular polarity requires efficient sorting of proteins along the postendocytic pathway. After internalization from the apical or basolateral cell surface, proteins can be recycled back to the appropriate cell surface domain, transcytosed to the opposing membrane domain, or be targeted to late endosomes and lysosomes. A significant fraction of apically- and basolaterally-internalized cargoes is known to intermix in common endosomes prior to recycling; thus there must exist cellular sorting mechanisms in this compartment that enable efficient polarized segregation of distinct proteins (3, 148).

An obvious question of interest is whether polarized sorting in the postendocytic pathway requires the same signals used to direct proteins along the biosynthetic pathway. In the case of basolateral proteins, studies have demonstrated that the postendocytic sorting signals of the polymeric immunoglobulin receptor and the LDL receptor are similar to those that direct their polarized delivery along the biosynthetic pathway (8, 93). In contrast, basolateral biosynthetic and postendocytic sorting signals in the transferrin receptor are contained within the same cytoplasmic region of the protein but are not identical (106). Whether similar or distinct apical sorting determinants operate along the biosynthetic and postendocytic pathways has been unexplored.

Our previous studies have focused on dissecting the trafficking signals that govern polarized biosynthetic trafficking of endolyn, a sialomucin that localizes to lysosomes and apical membranes in polarized MDCK cells (61). Lysosomal targeting of endolyn is conferred by a tyrosine-based tetrapeptide motif in the cytoplasmic tail of the protein (61, 62); however, along the biosynthetic pathway, the basolateral/lysosomal sorting information in this signal is largely overridden by N-glycan dependent apical sorting information in the lumenal domain of the

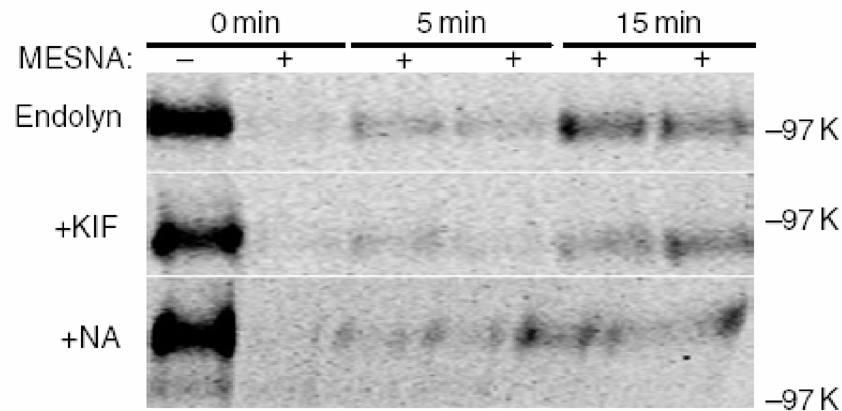
protein (61, 62). Thus, unlike other lysosomal membrane proteins, which transit the basolateral surface *en route* to lysosomes, a sizable fraction of newly synthesized endolyn is delivered apically prior to internalization and transport to lysosomes (61, 98). Based on antibody uptake studies, we estimate that at least 50% of newly synthesized endolyn traverses this indirect pathway, whereas the remainder is transported along the direct route to lysosomes in MDCK cells (61). Biosynthetic apical sorting of endolyn requires specificity in both the structure and position of N-glycans (115). Polarized delivery also appears to be raft-independent as endolyn is soluble in cold TX-100 although it remains possible that a distinct type of non-classical raft is involved in endolyn transport (61). Additionally, our studies revealed that a significant fraction of endolyn recycles to the apical surface after internalization from the surface. Moreover, endolyn appears to constitutively recycle between lysosomes and the plasma membrane (63). Here we have further explored the post-endocytic trafficking route of endolyn in polarized cells. Our data suggest that endolyn is efficiently recycled from endosomes and lysosomal compartments to the apical surface and that polarized postendocytic sorting of this protein relies on the same N-glycan-dependent sorting information that is important for its polarized biosynthetic delivery. Endolyn is thus the first apical protein shown to use the same sorting signals in both the biosynthetic and postendocytic pathways.

3.3 RESULTS

3.3.1 Internalization of endolyn is not affected by perturbation of terminal glycosylation

Our previous studies demonstrated that mutants of endolyn lacking N-glycans at positions 68 and 74, or endolyn synthesized in the presence of the mannosidase inhibitors kifunensine (KIF) or

deoxymannojirimycin were delivered in a nonpolarized manner to the plasma membrane of polarized MDCK cells (115). In order to determine whether these structural features are also important for sorting of endolyn along the endocytic pathway, we first characterized the entry kinetics of endolyn from the apical plasma membrane because glycosylation status has been demonstrated to affect the internalization rate of some proteins (4, 139). Internalization of endolyn from clathrin-coated pits is mediated by a tyrosine-based tetrapeptide motif in its cytoplasmic tail that also functions as the lysosomal targeting signal for this protein (61-63). Using a standard biotinylation-based endocytosis assay to quantitate the kinetics of endolyn internalization from the apical surface, we determined that the initial rate of wild-type endolyn internalization was roughly 1%/min (Figure 3.1). A similar rate was observed when using an antibody uptake assay (data not shown). This is comparable to the rate measured for apical internalization of a mutant influenza hemagglutinin protein that contains a tyrosine-based internalization motif (99). We next measured the internalization kinetics of endolyn synthesized in the presence of KIF, as well as endolyn whose N- and O-glycans were desialylated by cell surface treatment with neuraminidase. Treatment with KIF or neuraminidase altered the mobility of endolyn on SDS-PAGE as predicted; however, the internalization rate of endolyn over 15 min was similar to control conditions in either case (Figure 3.1). Thus, the rate of internalization of endolyn appears to be largely independent of its terminal glycosylation status.



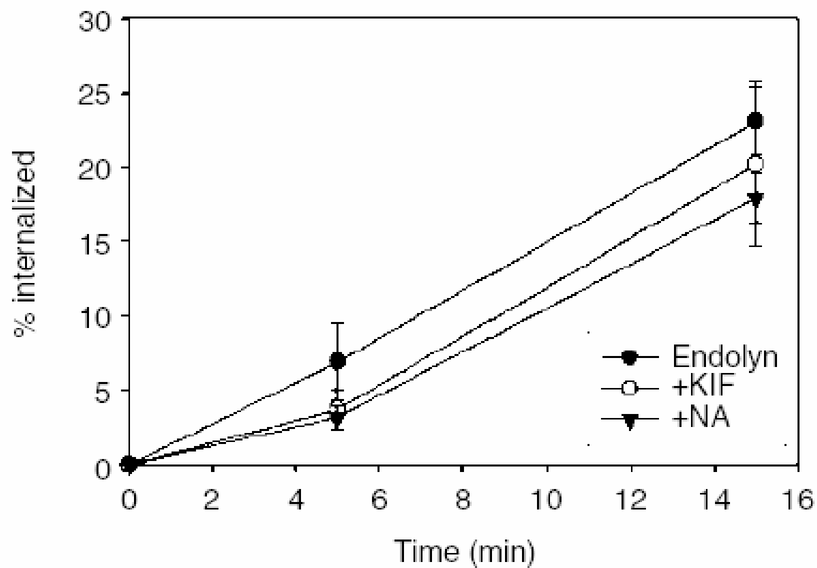


Figure 3.1 Endolyn is efficiently internalized.

Polarized endolyn-expressing MDCK cells were radiolabeled for 2 h in the presence or absence of kifunensine (KIF) and chased for 15 min prior to biotinylating the apical surface with sulfo-NHS-SS-biotin. Where indicated, filters were then incubated with apically added neuraminidase (NA) on ice. The cells were rapidly warmed to 37°C for 0, 5, or 15 min, and biotin was stripped from the surface using MESNA. One of the 0 min samples was left unstripped to extrapolate the total amount of endolyn initially present at the apical surface. The cells were solubilized and the biotinylated fraction of endolyn was recovered and analyzed as described in Methods. Quantitation of the data is shown below.

3.3.2 Endolyn recycles from common recycling endosomes and late endocytic compartments

Once internalized into apical early endosomes, endolyn could be recycled back to the cell surface or be targeted deeper into the endocytic pathway towards lysosomes. Our previous studies following the fate of internalized radioiodinated anti-endolyn antibodies suggest that both events occur to a significant extent (61). The majority of the fraction that is not recycled from early compartments is efficiently targeted to lysosomes; however, because the internalized antibodies are degraded in this compartment, potential recycling of endolyn from late endosomes and lysosomes cannot be detected using this assay. Nevertheless, there is good evidence that endolyn constitutively recycles from these deeper endocytic compartments. Because they are accessed by both apically- and basolaterally- internalized proteins, the return of endolyn from late endosomes and lysosomes to the apical membrane would require active polarized sorting.

To test whether apically-internalized endolyn enters common endosomes, iron-loaded canine transferrin and prebound anti-endolyn-antibody were co-internalized from the basolateral and apical surfaces, respectively, for 45 min at 37°C prior to fixation and subsequent processing for indirect immunofluorescence. Modest colocalization of these two cargo molecules was observed in confocal sections taken through the medial portion of the cells (Figure 3.2), consistent with the reported distribution of common endosomes (148). No colocalization was seen at or above the level of the tight junctions (apical) or just above the filter support (basal). Thus, at least a fraction of apically-internalized endolyn gains access to common endosomes from which it must be actively sorted prior to return to the apical cell surface.

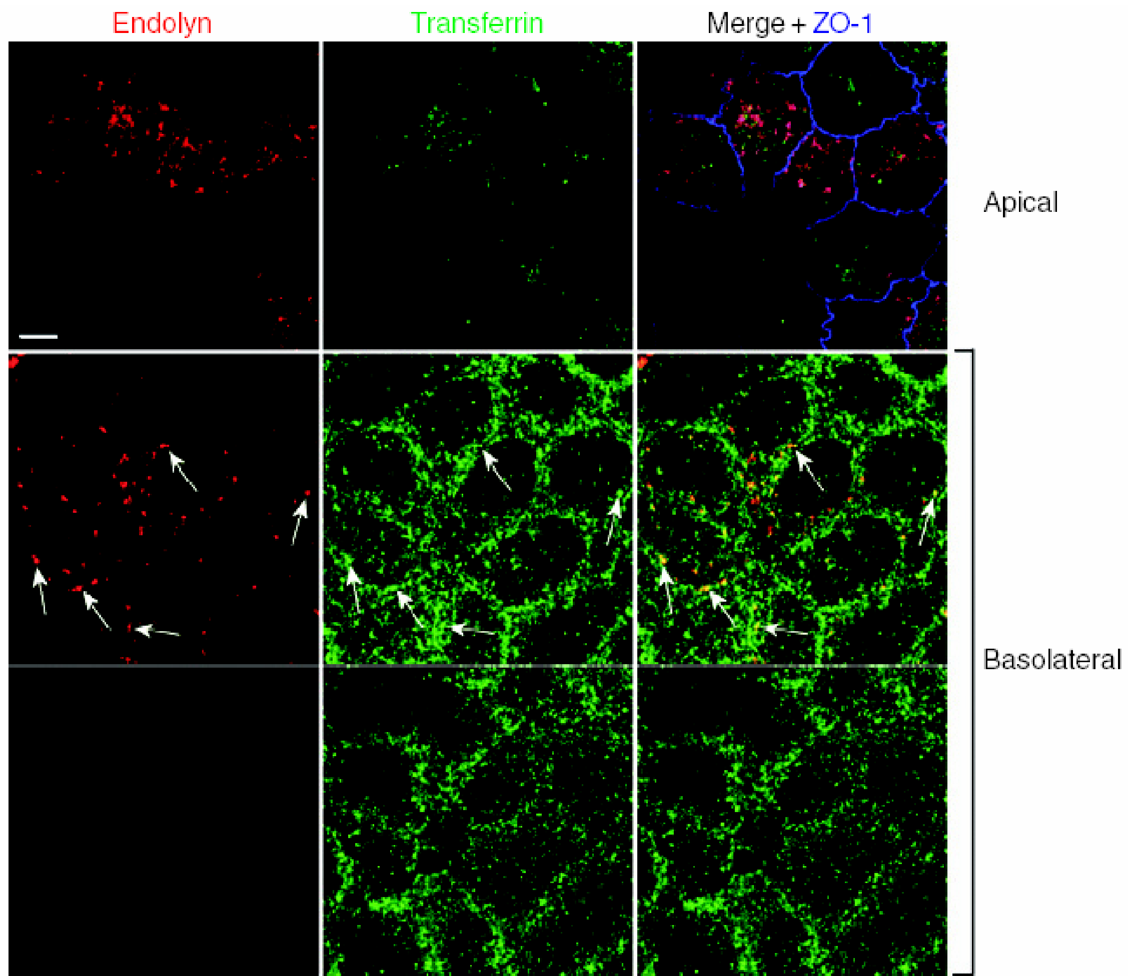


Figure 3.2 Apically-internalized endolyn colocalizes with basolaterally-internalized transferrin in endosomes.

Anti-endolyn antibody was prebound to the apical surface of polarized endolyn-expressing MDCK cells for 1 h on ice. After washing, cells were warmed to 37°C in the presence of basolaterally-added iron-loaded transferrin for 45 min. Cells were then fixed, permeabilized, processed for indirect immunofluorescence to detect endolyn, transferrin, and the tight junction marker ZO-1, and examined by confocal microscopy. Single optical sections taken at the tight junction (apical), immediately below the tight junction, and near the basal surface are shown. The individual staining patterns for endolyn (red) and transferrin (green) are shown, along with merged images that include the ZO-1 staining pattern. Colocalization (arrows) between endolyn and transferrin was observed only in medial sections. Scale bar: 4 μm.

In addition to recycling from early and common endosomes, there is evidence that endolyn constitutively recycles from lysosomes. Ihrke et al. (2004) demonstrated that inhibition of AP-2-mediated endocytosis upon overexpression of dominant-negative AP-180 in 3T3 cells resulted in the redistribution of endolyn from lysosomes to the cell surface (63). To examine this possibility more quantitatively, we used domain selective biotinylation to quantitate the fraction of newly-synthesized radiolabeled endolyn that was present at the plasma membrane (apical and basolateral) of MDCK cells over a long chase period (Figure 3.3). Endolyn-expressing cells were radiolabeled for 2 h, then chased and biotinylated 0, 6, or 21 h later. These experiments were performed using filter pairs of endolyn that were biotinylated apically or basolaterally; the biotinylated fraction was calculated as the total % biotinylated in each filter pair. Initially after the radiolabeling period, roughly 20% of endolyn was present at the cell surface. After 6 h of chase, this fraction decreased to ~8%, consistent with the internalization and lysosomal delivery of newly-synthesized endolyn. This appears to represent the steady state distribution of endolyn between cell surface and intracellular pools in these cells, as the same percentage of pre-synthesized endolyn was detected at the cell surface after 21 h of chase. Given the internalization rate of endolyn that we measured and the relatively long half life of the protein (~21 h), our data suggest that endolyn recycles from both early and late endocytic compartments and requires the active segregation from basolateral cargo.

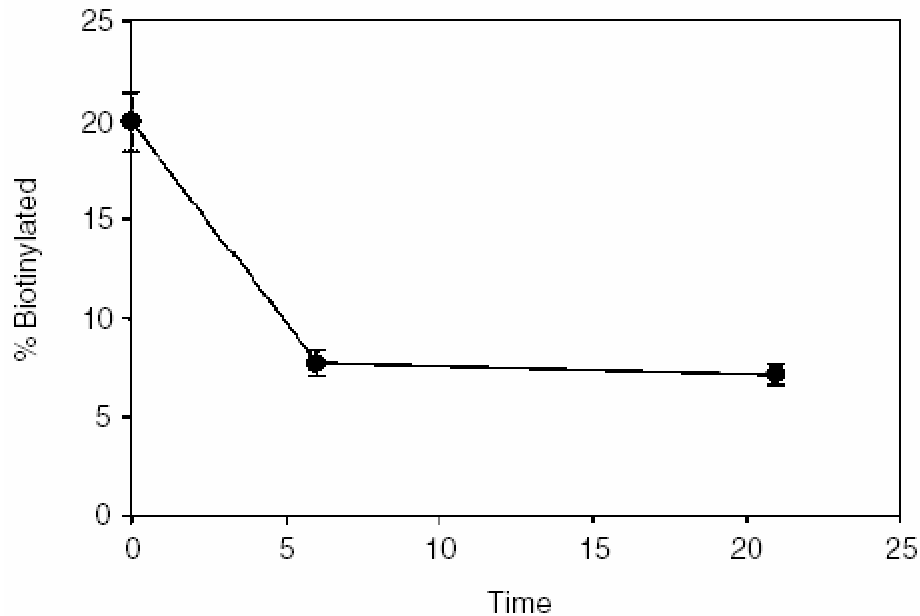


Figure 3.3 Newly-synthesized endolyn recycles to the cell surface over a long chase period.

Polarized MDCK cells stably expressing endolyn were radiolabeled for 2 h then chased for 0, 6, or 21 h. At each time point, cells were biotinylated. The total percent of presynthesized endolyn biotinylated recovered at the plasma membrane (apical + basolateral) at each time point is plotted (mean =/- S.E.M.; n=17-22).

3.3.3 Endolyn does not recycle via the TGN

In a few cases, apically internalized proteins have been shown to recycle via the TGN (15). While the short cytoplasmic tail (13 aa) of endolyn is unlikely to contain a TGN targeting signal, it was important to rule out the possibility that polarized postendocytic sorting of this protein occurs in this compartment. To determine whether internalized endolyn returns to the TGN, we used both immunofluorescence and biochemical approaches (Figure 3.4). In our immunofluorescence approach, the apical surfaces of polarized MDCK cells stably expressing endolyn were preincubated with anti-endolyn antibody for 1 h on ice and the cells were then warmed to 37°C for 45 min. After fixation and permeabilization, cells were processed for double

label indirect immunofluorescence to detect endolyn and either the TGN marker furin (Figure 3.4A) or the Golgi complex marker giantin (not shown). No colocalization was observed with either marker, suggesting that endolyn does not traffic back to the TGN. Moreover, no colocalization was observed when the internalization step was performed for 4 h at 20°C to retain any retrieved proteins in the TGN (not shown).

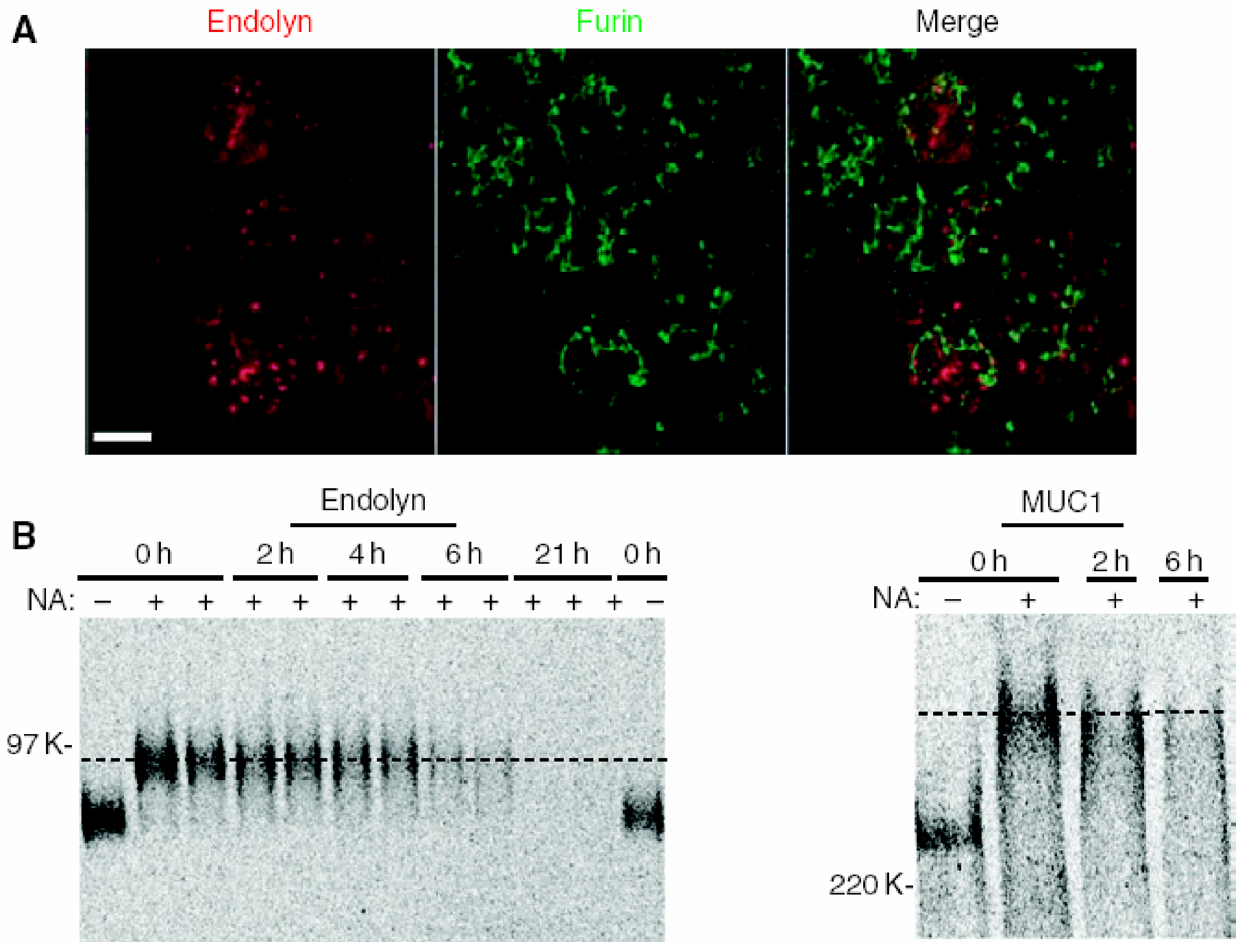


Figure 3.4 Internalized endolyn does not transit the TGN

A. Anti-endolyn antibody was prebound to the apical surface of MDCK cells for 1 h on ice, and the cells were rapidly warmed to 37°C for 45 min. After fixation, cells were processed for indirect immunofluorescence to visualize endolyn (red) and the TGN marker furin (green) and examined by confocal microscopy. A supranuclear section containing the Golgi complex is shown. Scale bar: 8 μm.

B. Polarized endolyn expressing MDCK cells were radiolabeled for 2 h and then chased for 15 min. Both the apical and basolateral surfaces were biotinylated on ice, and the indicated samples were incubated on ice with neuraminidase (NA) to desialylate cell surface proteins. The cells were returned to culture for 0 to 21 h, then solubilized and the biotinylated pool of endolyn recovered and analyzed by SDS-PAGE. The decrease in mobility of desialylated endolyn relative to wild-type is characteristic of mucin like proteins. Similar results were observed in two experiments**.**

In the biochemical approach, we determined whether endolyn desialylated at the cell surface could be resialylated upon return to culture for up to 21 h. Sialyltransferases reside in the distal compartments of the Golgi complex, and this approach has previously been used to document recycling of proteins via the TGN in other cell types (15, 104). Both the apical and basolateral surfaces of polarized MDCK cells were biotinylated and then subsequently treated with neuraminidase on ice. The cells were returned to culture at 37°C for 0 to 21 h, solubilized, and then immunoprecipitated with anti-endolyn antibody. The biotinylated fraction was isolated using streptavidin-agarose and examined by SDS-PAGE. Similar to other mucin-like proteins, efficient desialylation resulted in significantly slower mobility upon SDS-PAGE due to a decrease in the overall negative charge of endolyn (Figure 3.4B, compare 0 h +/-NA lanes). The stability of desialylated endolyn over the 21 h time course was significantly lower than that of wild type endolyn (not shown), presumably reflecting enhanced degradation of the protein upon delivery to lysosomes; however, we could follow the radiolabeled, biotinylated protein for at least 6 h. No shift in electrophoretic mobility of desialylated endolyn was observed during this period, confirming that recycling endolyn is unlikely to access sialyltransferase-containing compartments.

3.3.4 Polarized recycling of endolyn is N-glycan-dependent

We next sought to determine whether postendocytic delivery to the cell surface requires the same N-glycan-dependent apical sorting information we have shown to be important for biosynthetic sorting. To determine whether terminal processing is important for postendocytic sorting of endolyn, we compared the surface polarity of newly synthesized endolyn synthesized in the absence or presence of KIF over a long time course during which multiple rounds of recycling

would occur. Polarized MDCK cells stably expressing endolyn were radiolabeled with [^{35}S]-cysteine for 2 h, then chased for 0, 6, or 21 h. KIF was included in the indicated samples during the starve and radiolabeling steps, but omitted from the chase. At each time point cells were subjected to domain-selective biotinylation, then solubilized and the polarity of the pre-synthesized endolyn was determined as described in Methods. Figure 3.5A depicts the possible outcomes of these experiments: if N-glycans are *not* important for postendocytic sorting, we predict that newly-synthesized proteins that are initially delivered in a nonpolarized manner would gradually regain a polarized distribution upon multiple rounds of recycling. In contrast, if the same N-glycan-dependent signal is important for biosynthetic and postendocytic sorting, then those proteins that are initially delivered in a nonpolarized manner would maintain a nonpolarized distribution after numerous rounds of recycling.

Our results conform to the second scenario. Endolyn maintained a polarized distribution (~70% apical) throughout the time course, confirming that the wild-type protein is preferentially returned to the apical surface after internalization. When endolyn was synthesized in the presence of KIF, it was delivered in a nonpolarized manner, similar to our previous observation (Figure 3.5B, 52% apical) and, importantly, we observed no change in its distribution over the 21 h chase period. The consistent difference that we observed in the distribution of wild-type and incorrectly glycosylated endolyn is not a reflection of altered trafficking rates or reduced stability of KIF-treated endolyn, as both proteins have long half-lives (>18 h) and are internalized and recycled with similar rates. Thus, the continued nonpolarized distribution of KIF-treated endolyn after numerous rounds of internalization and recycling suggests that the absence of terminally-processed N-glycans prevents polarized sorting of this protein along the postendocytic pathway.

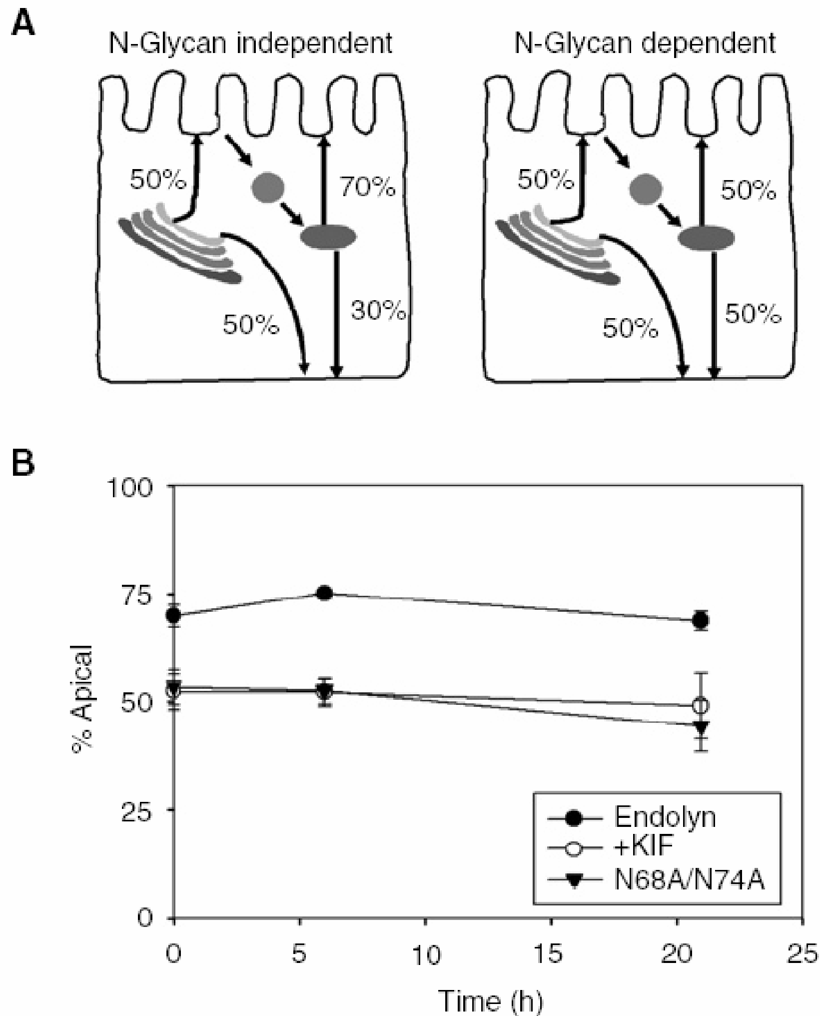


Figure 3.5 Polarized postendocytic sorting of endolyn is N-glycan-dependent.

A. A schematic illustrating the predictions for the long time course polarity experiments. KIF-treated endolyn is initially delivered to the plasma membrane of polarized cells in a non-polarized fashion. Delivery to the cell surface may occur directly from the TGN as depicted or via an endosomal intermediate (not shown). If an N-glycan-*independent* signal is required for polarized postendocytic sorting (left hand panel), then endolyn constructs that are initially delivered to the surface in a non-polarized manner will gradually redistribute apically. In contrast, if N-glycans are required for polarized postendocytic sorting (right hand panel), then proteins initially delivered in a non-polarized manner will retain their nonpolarized distribution over multiple rounds of recycling.
B. The polarity of endolyn and glycosylation mutants is maintained over long chase periods. Polarized MDCK cells expressing endolyn, the N68A/N74A mutant (which lacks N-glycans at positions 68 and 74), or endolyn synthesized in the presence of KIF were radiolabeled for 2 h, and then chased for 0, 6, or 21 h prior to domain-selective biotinylation. The percentage of total biotinylated endolyn present at the apical surface at each time point is plotted (mean +/- S.E.M.; n=17-22). There is no statistical difference in the surface distribution of wild-type, KIF-treated, or mutant endolyn after 21 h relative to their initial delivery.

We also examined the behavior of mutant endolyn missing the two N-linked glycans at positions 68 and 74 (N68A/N74A), which shows reduced biosynthetic apical polarity (115). Although this mutant is cleaved intramolecularly over time and is therefore less stable than wild-type endolyn ($t_{1/2}$ <6; data not shown), we were able to monitor the cell surface polarity of newly-synthesized intact N68A/N74A over a 21 h chase period (Figure 3.5B). The initial polarity of N68A/N74A was 53% apical, similar to that of KIF-treated endolyn, and the distribution of this mutant remained non-polarized during the time course. Taken together, these data suggest that similar N-glycosylation requirements govern the efficiency of endolyn sorting along the biosynthetic and postendocytic pathways.

3.3.5 Post-endocytic apical sorting of endolyn relies on terminal sialylation

If glycosylation directs polarized postendocytic sorting of endolyn, then acute perturbation of endolyn glycans at the apical cell surface might result in redistribution of the protein. Because we previously implicated terminal processing of N-glycans in the proper biosynthetic sorting of endolyn, we asked whether cell surface treatment with neuraminidase would interfere with postendocytic apical sorting of the protein. Polarized MDCK cells stably expressing endolyn were radiolabeled for 2 h, chased for 10-15 min, and the apical sides of the filters were treated with or without neuraminidase. The cells were then returned to culture for 0 or 2 h and biotinylated to determine the polarity of wild-type and desialylated endolyn (Figure 3.6). Mock-treated endolyn displayed a primarily apical distribution at both 0 and 2 h. In the samples treated with NA, efficient desialylation of endolyn was observed in the apically biotinylated samples at the 0 h time point (Figure 3.6, +NA, 0 h). Importantly, upon return to culture, a significant fraction of the desialylated endolyn was rapidly transcytosed to the basolateral surface,

consistently resulting in a nonpolarized distribution of this pool of protein. In the neuraminidase-treated samples, we also noted the appearance of sialylated endolyn at the apical surface after 2 h; this likely reflects preferential apical delivery of correctly glycosylated endolyn from both intracellular compartments and the basolateral cell surface. Thus, terminal sialylation of N-glycans may contribute to the apical sorting determinant that governs polarized sorting of endolyn along the postendocytic pathway.

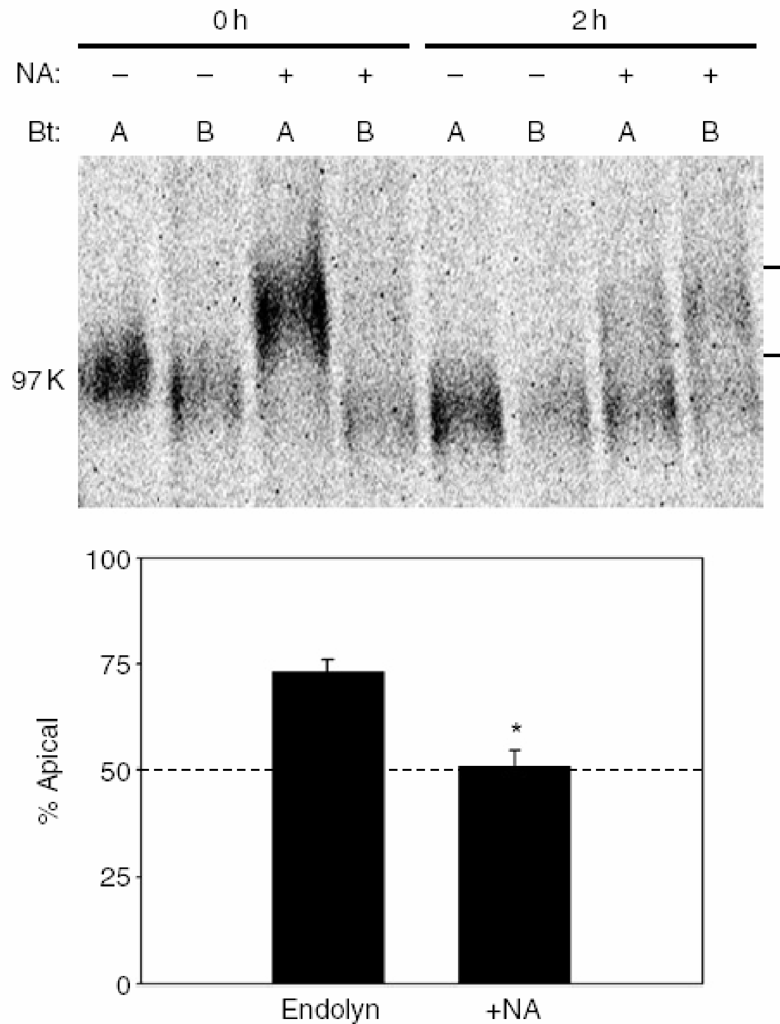


Figure 3.6 The polarity of endolyn is rapidly altered upon cell surface neuraminidase treatment

MDCK cells stably expressing endolyn were radiolabeled, and the apical surfaces were mock-treated or treated with neuraminidase (NA) on ice for 1 h. Subsequently cells were returned to culture at 37°C for 0 or 2h, and the apical (A) or basolateral (B) surface was biotinylated. Samples were analyzed by SDS-PAGE and a representative gel is shown. The mobility of desialylated endolyn is denoted by the bracket. The apically-desialylated pool of endolyn is shifted to a nonpolarized distribution by 2 h. The graph below shows the polarity of mock-treated and apically-desialylated endolyn after 2 h in culture (mean +/- S.E.; n=10-11). *p <0.001 vs. mock-treated by Student's t test. The dotted line at 50% denotes the distribution of a nonpolarized protein.

3.4 DISCUSSION

Our data demonstrate that endolyn recycles constitutively to the apical surface of polarized MDCK cells via compartments in which it is actively segregated from basolateral and lysosomal cargo. Moreover, although internalized endolyn does not recycle through the TGN, the polarized sorting of endolyn along the postendocytic route depends on an N-glycan-dependent signal with characteristics similar to the determinants that are important for polarized biosynthetic delivery of newly-synthesized endolyn. Two specific N-glycans of endolyn are essential for efficient apical delivery in both pathways, and in particular, terminal sugar modifications appear to be key determinants for apical sorting. Our study is the first to demonstrate a role for N-glycans in polarized apical recycling.

It is generally thought that polarized biosynthetic and postendocytic sorting occur in distinct cellular compartments (124). Several groups have demonstrated, using live cell imaging, the segregation of newly synthesized "apical" and "basolateral" proteins into distinct transport carriers emanating from the TGN in both polarized and nonpolarized cells (71, 120). Moreover, differential requirements for the release of TGN-staged apical and basolateral proteins have been observed *in vitro* (29, 77, 96, 152) and distinct TGN-derived transport carriers enriched in apical vs. basolateral markers have been immunoisolated (147). However, recent evidence suggests that a significant fraction of some newly-synthesized basolateral proteins also traverse endosomal compartments *en route* to the cell surface (6, 36, 107, 113). In addition, mutants of basolateral proteins that are mistargeted apically have also been detected in recycling endosomes prior to surface delivery, although direct delivery of proteins from the TGN to the apical surface has also been observed (6, 72, 107). Whether the indirect route via an endosomal intermediate represents a significant pathway for the delivery of endogenous apical proteins has not yet been

demonstrated. Thus, it is possible that N-glycan dependent sorting of endolyn along the biosynthetic and postendocytic routes occurs at the same intracellular site. Regardless, our data demonstrate for the first time that N-glycans can direct polarized apical sorting within the postendocytic pathway.

After internalization, proteins in polarized cells can be returned to the same or opposing cell surface (recycling and transcytosis, respectively), or be targeted deeper into the endocytic pathway for delivery to lysosomes. Polarized recycling requires active sorting of internalized cargo and is essential for maintaining the appropriate cell surface distribution of membrane proteins. Endocytic sorting of basolateral transmembrane proteins appears to be mediated by cytoplasmic sorting motifs binding to adaptor protein complex(es) (37, 38). However, little is known about the sorting mechanisms for apical proteins in the postendocytic pathway. A study in polarized hepatic cells (WIF-B) showed that during basolateral-to-apical transcytosis, exit of several apical membrane proteins from early endosomes depended on the presence of cholesterol and glycosphingolipids, although only some of these proteins were detergent-insoluble in cold Triton X-100 (103). Moreover, recent evidence suggests that some GPI-linked proteins take a transcytotic route to the apical domain in MDCK cells, rather than a direct route as previously thought (113). These results suggest that incorporation into lipid rafts may serve as an apical sorting mechanism both at the TGN and in endosomes; however, this mechanism may not apply to all apical proteins and cell-type specific differences may also exist.

In this study, we have examined the signals that govern apical postendocytic trafficking of the sialomucin endolyn. After internalization, a large fraction of endolyn is trafficked to lysosomes, while a smaller but still significant proportion is recycled back to the apical surface (61). The recycling of this latter fraction can occur from common endosomes, as we have shown

that a population of endolyn enters into these compartments and intermixes with basolateral cargo or from peripheral early endosomes/apical recycling endosomes. In addition, our ability to detect newly-synthesized endolyn at the apical surface over a long chase period suggests continuous recycling of endolyn from later endocytic compartments including lysosomes. Additional evidence for the constitutive cycling of endolyn between the cell surface and lysosomes in nonpolarized cells has been previously documented (63). Trafficking of endolyn from lysosomes to the apical membrane could be direct or via an endosomal intermediate; but in either case must involve active sorting of the protein from basolaterally-destined cargo.

In our previous studies, we examined the fate of apically- and basolaterally-internalized radioiodinated anti-endolyn antibodies (61). This method allows more sensitive quantitation of internalization and recycling rates compared with the biochemical methods used in this study. Unfortunately, the long half-life of endolyn does not allow us to replace the entire cellular population of wild-type endolyn with KIF-treated protein and thus precludes the use of this technique to compare the trafficking of these two proteins. However, it is important to note that the rates of internalization and recycling that we measured for endolyn using biotinylation-based methods are consistent with our determinations using an antibody approach (data not shown). We did attempt to use radioiodinated anti-endolyn antibodies to measure differences in the trafficking rates between cells stably expressing wild-type endolyn and the missorted glycosylation mutant N68A/N74A. However, straightforward interpretation of these experiments was confounded by the considerably shorter half life of N68A/N74A compared with wild-type endolyn (<6 h vs ~21 h), which resulted in significantly less recycling of antibody internalized from either cell surface domain. The decreased stability of N68A/N74A likely reflects susceptibility to cleavage within a putative disulfide–bound loop that houses the two missing

glycans. Moreover, it should be noted that all of the available anti-endolyn antibodies recognize reduction-sensitive epitopes that appear to be localized within the loop domain. It is therefore possible that antibody binding perturbs recognition by the sorting machinery of glycan-dependent trafficking signals in this region of wild-type endolyn.

In our extended time course experiments we demonstrated that KIF-treated endolyn and the N68A/N74A mutant maintain a nonpolarized distribution, suggesting that the same N-glycan-dependent sorting signal that is utilized in the biosynthetic pathway also directs polarized postendocytic sorting. To examine whether glycan-dependent sorting occurs in early endocytic compartments, we compared the recycling of wild-type or KIF-treated endolyn. We found that ~40% of pre-internalized endolyn recycled to the apical surface within 15 min, but could detect no discernable difference in the rate or fidelity between the wild-type or KIF-treated protein. This suggests that early recycling occurs predominantly from peripheral apical early/recycling endosomes that do not receive basolateral cargo and from which recycling to the apical cell surface may occur by "default". In this model, polarized sorting of endolyn occurs from compartments deeper within the cell, such as common recycling endosomes and lysosomes. Because a large fraction of endolyn is efficiently sorted to and recycles from these late endocytic compartments, these organelles must either have sorting capability or correspond with other endosomes where sorting from basolateral cargo occurs. It is currently not known whether recycling and polarized sorting can take place in late endosomes/lysosomes. In any case, our results indicate that return to the apical surface from these "deeper" endocytic compartments is dependent on intact N-glycan processing of endolyn.

Our experiments further suggest that terminal sialic acid residues play an important role in sorting, as acute cell surface neuraminidase treatment rapidly redistributed a population of

apical endolyn to the basolateral cell surface. A previous study suggested a role for terminal glycan processing of the apical protein gp114 based on its mislocalization in galactosylation-deficient ricin resistant cells; however, in contrast to our observations with endolyn, the effect could not be reproduced by deoxymannojirimycin treatment of wild-type cells (79). Similarly, a role for α2,3-linked sialic acid in the biosynthetic apical delivery of mucins has previously been suggested based on the effects of pharmacological disruption of glycan synthesis using BGN (55). An elegant recent study from the same group suggests that the effects of this drug on apical sorting may be due to changes in the composition and stability of lipid rafts rather than to altered mucin glycosylation (27), and it is predicted that similar effects on raft composition would be found in ricin-resistant cells. Such a mechanism is unlikely to be the cause for the non-polarized redistribution of endolyn that we observed when the protein was synthesized in the presence of KIF or deoxymannojirimycin, or upon cell surface treatment with neuraminidase. Because neuraminidase desialylates both N- and O-linked glycans, it remains to be shown whether sialic acids on endolyn's O-glycans may facilitate apical sorting. Nonetheless, since terminal processing of N-glycans is a requirement for polarized sorting of endolyn in the biosynthetic and post-endocytic pathway, our data are consistent with the idea that sialylation of N-glycans is important for efficient recognition of the apical sorting determinant.

The mechanism by which glycosylation-dependent sorting signals are recognized is not known. Current models propose that glycans may stabilize a proteinaceous conformation that confers a transport-permissive structure to a protein, or alternatively, that glycans are directly recognized by a lectin-like receptor (117). While our data do not distinguish between these models, our observations suggest that common mechanisms are utilized along both the

biosynthetic and post-endocytic pathways. Future studies will be required to unravel how these N-glycan dependent sorting signals are decoded.

4.0 ENDOLYN IS SORTED IN DISTINCT COMPARTMENTS WITHIN THE BIOSYNTHETIC AND POSTENDOCYTIC PATHWAYS

4.1 ABSTRACT

While the *trans*-Golgi network has long been thought to be the site of polarized sorting along the biosynthetic pathway, recent studies suggest that endosomal recycling compartments may also function as an additional site for polarized sorting. However, in these studies, apical trafficking was studied using mutant basolateral proteins, making it difficult to conclude that true apical proteins are sorted in these compartments. The sialomucin endolyn was recently shown to require a similar N-glycan-dependent apical sorting signal along both the biosynthetic and postendocytic pathways. This suggests that sorting of this protein may occur in a common compartment along both pathways, such as recycling endosomes. In this study, I examined the biosynthetic trafficking of endolyn in comparison to the raft-associated apical protein, influenza hemagglutinin (HA). Neither protein was found to transit common recycling endosomes along the biosynthetic pathway. However, HA was found to traverse an apical endocytic compartment while endolyn was not. This study suggests that endolyn, despite the use of similar N-glycan-dependent apical targeting signals for biosynthetic and postendocytic delivery, is sorted in distinct compartments along these pathways.

4.2 INTRODUCTION

The previous chapters revealed that trafficking of endolyn along both the biosynthetic and postendocytic pathways is dependent on the terminal processing of a subset of N-glycans (114, 115). This finding raises the possibility that endolyn may be sorted in a compartment common to both pathways. Both the TGN and endocytic compartments have been implicated to have roles in both the biosynthetic and postendocytic pathways (6, 15, 107). My biochemical and immunofluorescence data presented in the last chapter suggest that endolyn does not traverse the TGN after internalization from the surface. Therefore, I tested the possible role of endocytic compartments in the trafficking of endolyn along the biosynthetic pathway.

To date, several basolateral proteins have been shown to traffic to the surface *via* endocytic compartments in nonpolarized cells. The first proteins to be implicated were the transferrin receptor and the H1 subunit of the asialoglycoprotein receptor (36, 81). Recently, a temperature-sensitive mutant of the vesicular stomatitis virus glycoprotein G (VSV-G) conjugated to yellow fluorescent protein (VSV-G-YFP) was shown by Ang *et al.* to traffic to transferrin-positive recycling endosomes before delivery to the surface using live cell techniques (6). To determine whether this indirect trafficking was necessary for delivery, Ang *et al.* assessed the surface delivery of VSV-G-YFP after inactivation of transferrin-postive recycling endosomes (6). For these studies transferrin-conjugated to horseradish peroxidase (HRP-Tf) was internalized into recycling endosomes then treated with diaminobenzidine (DAB) and hydrogen peroxide, both of which are membrane permeable. In the presence of hydrogen peroxide, DAB reacts with the HRP located in the recycling endosome to form an insoluble precipitate. Inactivation of recycling endosomes using this method inhibited subsequent delivery of VSV-G-YFP to the surface. In nonpolarized cells, transferrin-positive recycling endosomes are also positive for

rab11 and using live cell techniques Lock et al. showed that E-cadherin enters rab11-positive structures before delivery to the surface (85). Additionally, expression of rab11 mutants resulted in a decrease in the amount of E-cadherin found at the surface (85).

Though it has been shown that the endocytic pathway in polarized cells is organized differently, endocytic recycling compartments have also been implicated in biosynthetic trafficking in these cells. In polarized cells, the transferrin receptor (TfR) is localized to common recycling endosomes that contain both apical and basolateral cargo but lack rab11 (149). Rab11 is instead found in apical recycling endosomes (149). Orzech *et al.* implicated these two recycling compartments when ablation of these compartments affected the initial biosynthetic delivery of the pIgR to the basolateral surface (107). Thus it appears that basolateral proteins are trafficked to the surface *via* endocytic intermediates in both nonpolarized and polarized cells.

Whether apical proteins also traverse endocytic compartments is currently not clear. In several of the studies mentioned above apical mutants of the basolateral proteins were studied. However, several of these 'apical' proteins still possessed sequences that can be recognized by basolateral sorting machinery. An apical mutant of VSV-G-YFP was found to traverse recycling endosomes, however the AP-interacting sequence in this mutant is intact (6). Similarly, an apical mutant of pIgR that retains an AP-interacting sequence was also shown to traverse an endocytic compartment before delivery to the basolateral surface in polarized cells (107). Studies have also examined the trafficking of apical GPI-anchored proteins but contrasting results have been observed. Futter et al. showed that the GPI-anchored protein alkaline phosphatase was not found to traverse endocytic compartments before delivery to the cell surface of nonpolarized cells (36). However a recent study using live cell techniques in polarized cells shows that a GPI-anchored construct of YFP is found in a subapical compartment before delivery to the apical surface (53).

Taken together, these studies make it difficult to conclude that apical proteins are sorted in endocytic compartments.

The role of endocytic compartments in the sorting of apical proteins is further complicated by the fact that there are multiple pathways to the apical surface. For instance, raft-associated proteins have been shown to traffic to the apical surface in a manner distinct from non-raft-associated proteins (69). Jacob et al. showed that raft-associated- and non-raft-associated-proteins exit the TGN in the same vesicular carrier but are subsequently found in separate carriers (69). Delivery of the raft-associated proteins was also shown to be affected by the actin depolymerization induced by incubation with cytochalasin D (68). Consistent with a role for actin, the motor protein myosin I and its regulator, α-kinase 1 were found to specifically associate with immunoisolated vesicles containing the raft-associated cargo (46). Data from our own lab shows that delivery of influenza hemagglutinin (HA) is specifically enhanced in comparison to non-raft-associated proteins when phosphatidylinositol-5-kinase is overexpressed (40). Thus, in this study, the trafficking of endolyn along the biosynthetic pathway was compared to HA.

4.3 RESULTS

4.3.1 Apical proteins do not traverse common recycling endosomes along the biosynthetic pathway

Similarly to Ang *et al.*, I wanted to inactivate specific endocytic compartments and determine whether this has any affect on the surface delivery of endolyn and HA along the biosynthetic pathway (6). To validate this approach, I first confirmed that the inactivation of transferrin-

positive compartments inhibits the delivery of VSV-G-YFP to the surface of nonpolarized cells. MDCK cells that stably express human transferrin receptor (TfR; PTR9 cells) were grown on coverslips and infected with replication deficient adenovirus encoding VSV-G-YFP and incubated at 40°C overnight. The next day recycling endosomes were loaded with or without Tf conjugated to HRP (HRP-Tf) how. Cells were then incubated with or without diaminobenzidine (DAB) and hydrogen peroxide for 1 hr on ice. After a 1 hr chase at the permissive temperature of 31.5°C in the presence of cycloheximide, the cells were fixed and imaged (Figure 4.1). In concordance with the observations of Ang et al., delivery of VSV-G-YFP to the surface was only inhibited when recycling endosomes were successfully inactivated by incubation with both DAB and hydrogen peroxide (6).

The next step was to determine whether the same results could be obtained in polarized cells using a biochemical approach. In this experiment, filter-grown PTR9 cells were infected and incubated overnight at 40°C. The next day cells were radiolabeled in the presence or absence of HRP-Tf and then cells were incubated with or without DAB and hydrogen peroxide for 1 hr on ice. Cells were then chased at 31.5°C for 0 or 90 min, after which the basolateral surface was biotinylated. The total fraction of VSV-G-YFP found at the surface after 0 or 90 min of chase is plotted (Figure 4.2). A considerable decrease in the amount of VSV-G-YFP at the surface was only seen when transferrin-positive common recycling endosomes (CREs) were inactivated, suggesting that VSV-G-YFP traverses this compartment along the biosynthetic pathway in polarized cells.

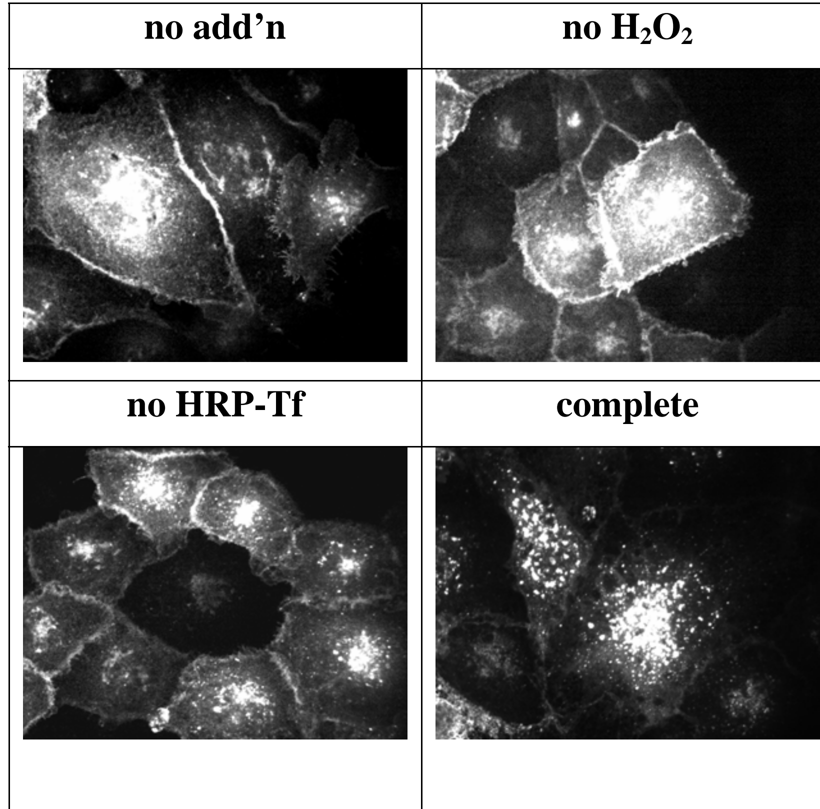


Figure 4.1 Inactivation of Tf-containing endosomes inhibits surface delivery of VSV-G-YFP

PTR9 cells grown on coverslips were infected to express tsO45VSV-G-YFP and incubated overnight at 40°C. The next day cells were starved in serum-free media for 30 min before incubation with HRP-Tf for 45 min at 40°C. HRP-Tf remaining on the surface was removed by incubating twice with 0.15 M NaCl and 20 mM citric acid pH 5.0 for 5 min. On ice, cells were washed with PBS and incubated with DAB and H_2O_2 for 1 h. Three different sets of controls were done in this experiment. In one control set cells were not incubated with HRP-Tf, DAB, or H_2O_2 (no add’n). In a second control set, cells were incubated with only HRP-Tf and DAB (no H_2O_2). The third control set was incubated with only DAB and H_2O_2 (no HRP-Tf). Subsequently cells were chased at 31.5°C in the presence of cycloheximide for 1h. After washing with PBS, cells were fixed and imaged.

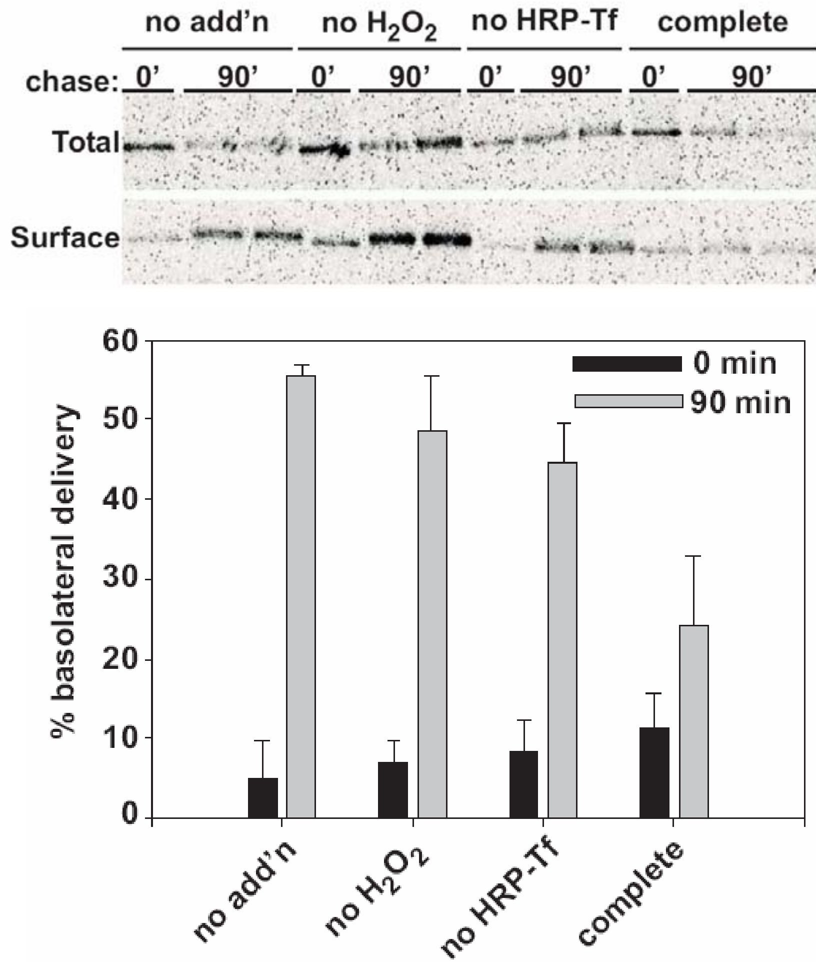


Figure 4.2 Inactivation of common recycling endosomes in polarized cells disrupts delivery of VSV-G-YFP to the basolateral surface

Polarized PTR9 cells were infected to express VSV-G-YFP and incubated overnight at 40°C. The next day. cells were starved in serum-free media for 30 min, then incubated in media devoid of cysteine and methionine for 30 min and radiolabeled for 15 min at 40°C. During this 45 min, cells were also incubated with HRP-Tf on the basolateral surface. HRP-Tf remaining at the surface was removed and cells were treated with DAB and H_2O_2 for 1 h on ice. Controls were done as described in Figure 4.1. After washing, cells were chased for 0 or 90 min at 31.5°C. Delivery to the basolateral surface was quantitated using domain selective biotinylation. Cells were solubilized and VSV-G-YFP was immunoprecipitated. Four-fifths of the sample after elution was incubated with streptavidin-agarose beads to recover the biotinylated (surface) protein. The remaining fraction was retained to calculate the total amount of endolyn. Samples were analyzed by SDS-PAGE and the gels from a representative experiment are shown (upper panel). The mean +/- SD of 2 experiments is plotted (lower panel).

Having validated the inactivation of the CRE in polarized cells and the use of biochemical techniques to measure surface delivery, I next determined the effect of inactivating the CRE on apical trafficking of endolyn and HA. These experiments were performed as described above using adenoviruses that expressed either HA or endolyn. Surface delivery of HA was assessed using cell surface trypsinization while delivery of endolyn was quantitated after surface biotinylation. Inactivation of CRE had no effect on the amount of newly synthesized endolyn reaching the apical surface (Figure 4.3), suggesting that this protein does not enter this compartment along the biosynthetic pathway. I routinely observed that a population of immature protein was found only at the apical surface of cells that received hydrogen peroxide, suggesting that terminal glycosylation processes can be affected by this reagent (compare 90 min lanes for no HRP-Tf and complete to 0 min lane); however, this does not appear to affect the efficiency of surface delivery, as similar amounts of endolyn were found in these cells compared with non-peroxide treated cells. Similarly it appears that HA does not traverse the CRE as inactivation of this compartment had no effect on the delivery of HA to the apical surface (Figure 4.4). In experiments with either protein, a slight increase in the average amount of protein at the apical surface after inactivation of recycling endosomes was observed. It was possible that the increase in apical delivery was due to an overall change in the polarity after inactivation of the CRE; however there was no effect on the polarity of HA (data not shown). Alternatively it is possible that inactivation or recycling endosomes could inhibit the internalization of proteins from the surface and maintain this pool that normally recycles at the apical surface. However, HA has not been shown to be efficiently recycled like endolyn.

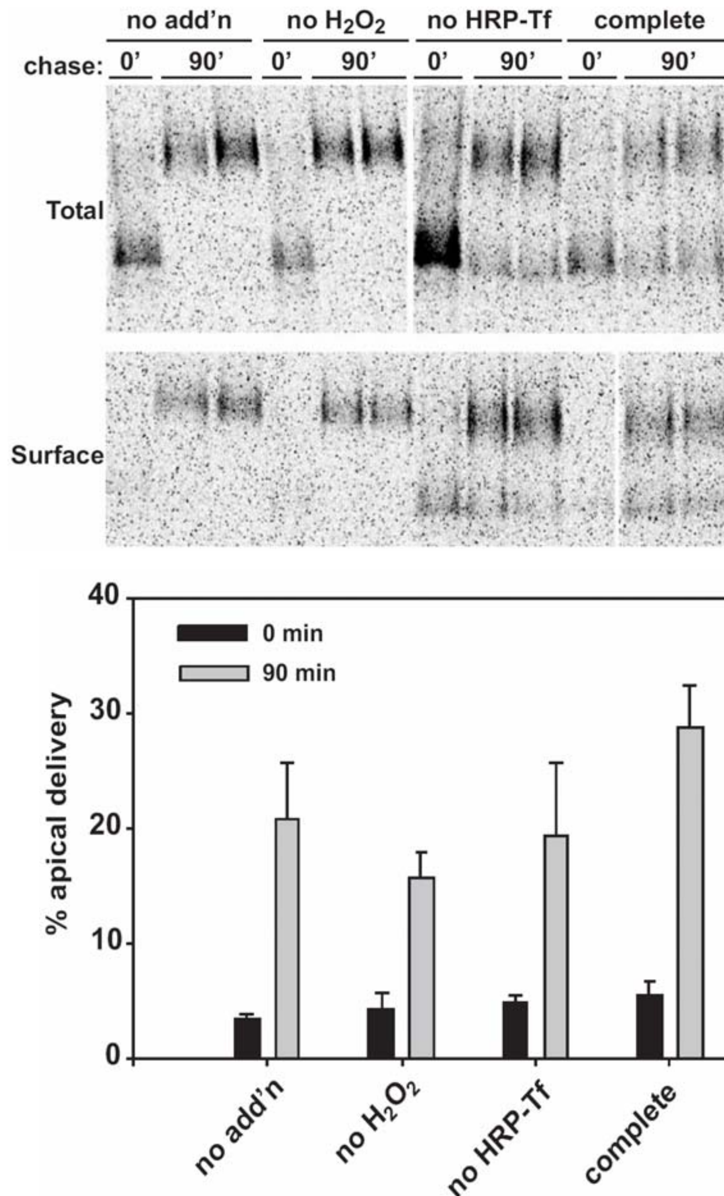


Figure 4.3 Biosynthetifc delivery of endolyn to the apical surface is not disrupted by inactivation of common recycling endosomes

Filter-grown PTR9 cells were infected to express endolyn and incubated overnight at 37°C. Cells were radiolabeled and incubated with HRP-Tf at 37°C. Cells were processed as described for VSV-G-YFP except cells were chased for 0 or 90 min at 37°C. Apical delivery at each time point was quantitated using domain selective biotinylation techniques. Samples were analyzed by SDS-PAGE and the gels from a representative experiment are shown (upper panel). The mean +/- SD of 3 experiments is plotted (lower panel).

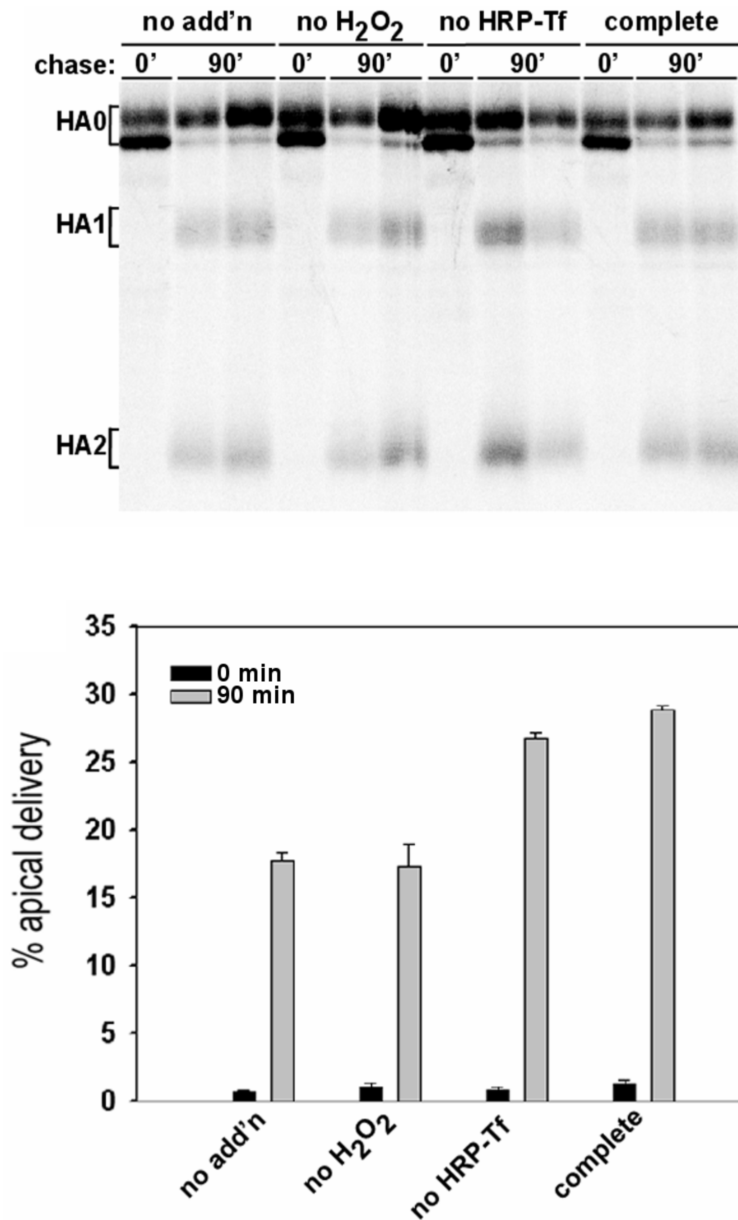


Figure 4.4 Apical biosynthetic delivery of HA is not disrupted by inactivation of common recycling endosomes

Polarized PTR9 were infected to express HA and processed as described in Figure 4.3, except that cell surface trypsinization was used to quantitate apical surface delivery. Samples were analyzed by SDS-PAGE and the gel from a representative experiment is shown (upper panel). The mean +/- SD of 4 experiments for each condition is plotted (lower panel).

4.3.2 Apical endocytic compartments are differentially used along the biosynthetic pathway

Since Orzech et al. implicated a possible role for the apical recycling endosome (ARE) along the biosynthetic pathway, I wanted to determine whether endolyn or HA traverses this compartment (107). To inactivate endosomal recycling compartments such as the ARE and the CRE, Orzech et al. used wheat germ agglutinin (WGA) conjugated to HRP (HRP-WGA) (107). WGA binds to glycans on membrane proteins and is efficiently internalized into early endosomes; subsequently, it partitions with membrane proteins along the recycling pathway. Localization of WGA-HRP to recycling endosomes was confirmed by Orzech et al. as intracellular staining of WGA-HRP-FITC internalized apically for 15 min at 37°C in MDCK cells displayed a diffuse, tubular pattern consistent with recycling compartments (107). In my studies, HRP-WGA was internalized for 1 hr at 18.5^{o}C to enhance localization with the ARE. However, in my experiments, confocal images of internalized WGA-FITC under these and other internalization conditions showed significant colocalization with EEA1, a marker of early endosomes, but little colocalization with markers for the ARE or CRE (Figure 4.5). Nonetheless, inactivation of compartments containing WGA-HRP inhibited the apical delivery of HA but not of endolyn, suggesting that these two proteins are delivered to the apical surface by distinct pathways (Figures 4.6 and 4.7).

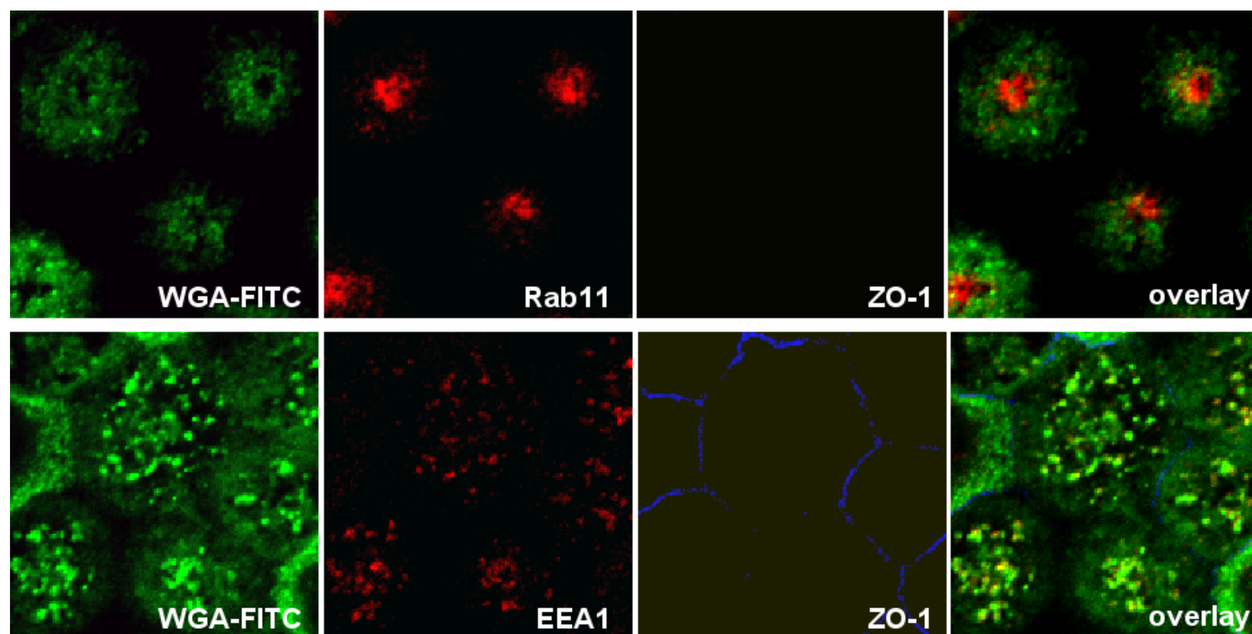


Figure 4.5 Characterization of HRP-WGA internalization conditions

WGA-FITC was internalized from the apical surface of polarized PTR9 cells for 1 h at 18.5°C. Remaining WGA-FITC at the surface was stripped with two 10 min incubations with 100 mM N-acetyl-D-glucosamine. After washing, cells were fixed, permeabilized, and processed for indirect immunofluorescence to detect ZO-1 (tight junctions) and either Rab11 (upper panel) or EEA1 (lower panel), representing markers of recycling and early endosomes, respectively. Overlayed images are shown in the right-hand panels. Image provided by Asli Oztan (Apodaca Lab, University of Pittsburgh).

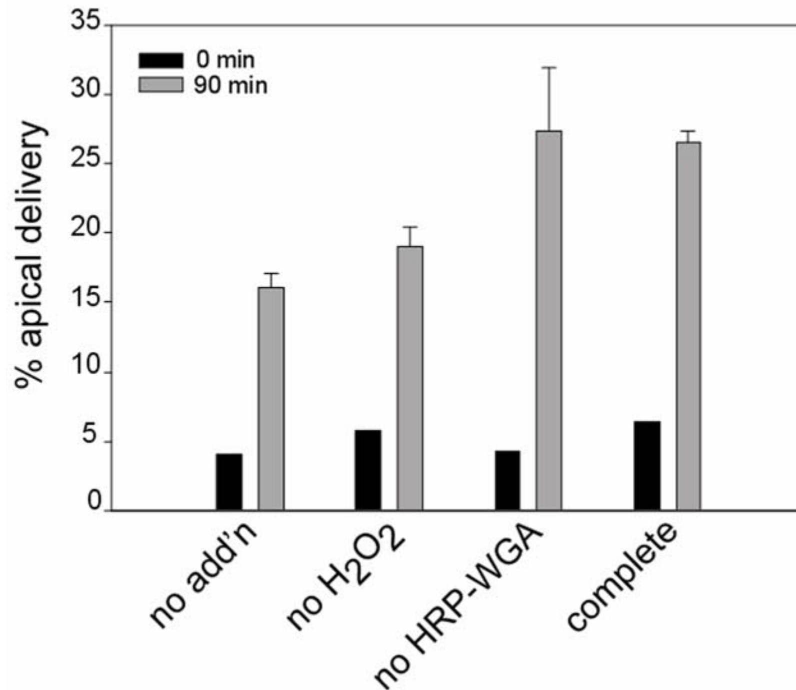


Figure 4.6 Inactivation of HRP-WGA-containing compartments does not disrupt apical delivery of endolyn.

Filter-grown PTR9 cells were infected to express endolyn and incubated overnight at 37°C. The next day, cells were starved and radiolabeled for 15 min. Cells were apically incubated with HRP-WGA for 1 h at 18.5°C. Remaining HRP-WGA was removed from the surface with 100mM N-acetyl-D-glucosamine and washed several times before the inactivation procedure as described in Figure 4.2. After inactivation, cells were chased for 0 or 90 min at 37°C. Apical delivery was quantitated using domain selective biotinylation techniques. The mean +/- range of a representative experiment is plotted**.** Similar results were obtained in 3 experiments.

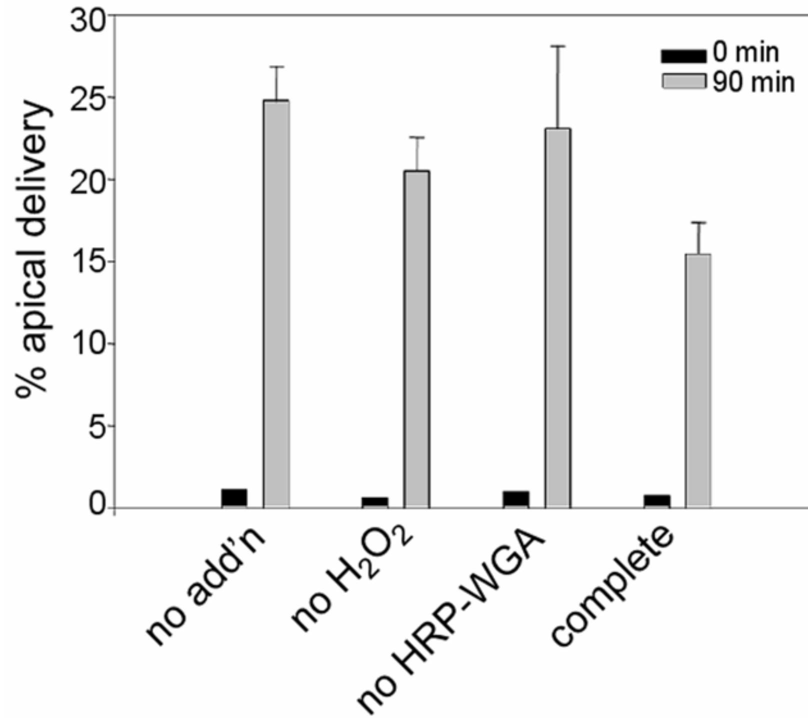


Figure 4.7 Biosynthetic apical delivery of HA is disrupted upon inactivation of HRP-WGA-containing endosomal compartments

Polarized PTR9 cells infected to express HA were processed as described in Figure 4.6, except that cell surface trypsinization was used to quantitate delivery to the apical surface. The mean +/- range of a representative experiment is plotted**.** Similar results were obtained in 5 experiments.

4.4 DISCUSSION

To determine whether endolyn or HA traverse endocytic recycling compartments, I adapted a technique described by Ang et al. to inactivate common recycling endosomes in polarized cells (6). This approach was validated by showing that inactivation inhibits the delivery of VSV-G-YFP to the basolateral surface in polarized cells. Inactivation of common recycling

endosomes, however, did not disrupt the N-glycan-dependent apical delivery of endolyn or of the raft-associated protein HA.

To determine whether endolyn or HA traffic via the apical recycling endosome as suggested by Orzech et al., endocytic compartments containing HRP-WGA were inactivated (107). While there was no effect on endolyn, delivery of HA to the apical surface was inhibited under these conditions. However, it cannot be determined whether the affect on HA was due to a specific inactivation of apical recycling endosomes. My confocal images of the internalization of WGA-FITC suggest that inactivation of WGA-HRP-containing compartments would be largely limited to apical early endosomes. However, it is possible that little colocalization was seen between WGA-FITC and rab11 because these markers localize to different subdomains within the ARE. To discriminate between these possibilities, fluid phase HRP, which should only label early endosomes, was internalized from the apical surface for 15 min at 37°C to specifically inactivate apical early endosomes. Results showed no effect on the apical delivery of HA (data not shown). Thus further studies are necessary to clearly define a role for the ARE in the biosynthetic trafficking of HA. For example, IgA conjugated to HRP could be used to selectively inactivate the ARE as this protein has been shown to transcytose from the basolateral surface to the apical surface via apical recycling endosomes. Additionally, disruption of the expression or function of proteins such as rab11 and myosinVb, which play important roles in the trafficking of proteins from the ARE could implicate the involvement of this compartment in the trafficking of HA (78, 85).

Taken together these data suggest that there are multiple pathways to the cell surface along the biosynthetic pathway. It is still unclear whether endocytic compartments can function in polarized sorting since VSV-G-YFP and HA were found to transit distinct endocytic

compartments along the biosynthetic pathway. If these compartments do not function in polarized sorting along the biosynthetic pathway, it is possible that traversing endocytic intermediates is important for regulating the amount of protein at the surface. A limitation of my experiments is that it is not possible to quantitate the extent to which proteins utilize endocytic intermediates. This is because the inactivation of endocytic compartments did not completely inhibit delivery of VSV-G-YFP or HA. It is possible that a given protein uses multiple pathways to traffic to the cell surface and that the inhibition I observed accurately represents the fraction that traverses endosomes. It is also possible that trafficking via endocytic intermediates is a major pathway in cells but that cells can develop alternate pathways when this route is inaccessible. For instance, it has been shown that when clathrin-mediated endocytosis is inhibited, proteins that normally use this pathway will enter via clathrin-independent mechanisms (25). Alternatively, it is possible that I was unable to completely inactivate the entire population of a given endocytic compartments and thus incapable of completely perturbing this trafficking pathway in cells.

In contrast to VSV-G-YFP and HA, my studies suggest that endolyn is trafficked directly to the apical surface along the biosynthetic pathway. Thus, endolyn, despite the use of a similar N-glycan-dependent sorting signal along the biosynthetic and postendocytic pathway, is sorted in distinct compartments along these two pathways.

5.0 CONLUSIONS AND FUTURE DIRECTIONS

The segregation of the plasma membrane into apical and basolateral domains in polarized epithelial cells allows these cells to simultaneously interact with the external and internal environments, respectively. Maintenance of these distinct domains has been attributed to the ability of the cell to differentiate and sort apical from basolateral cargo based on inherent signals within proteins. While the basolateral sorting signals identified so far rely exclusively on sequence motifs in the cytoplasmic domains of proteins; apical sorting signals rely on a variety of signals including glycosylation, cytoplasmic sequences, and an association with lipid rafts. Understanding the role of glycosylation in the trafficking of apical proteins has been challenging given the ubiquitous and complex nature of these moieties and their involvement in sorting only a subset of proteins. In this dissertation, I have examined the role of N-glycans in apical trafficking using the sialomucin endolyn as a model protein. I found that polarized apical biosynthetic delivery is dependent on the terminal processing of two N-glycans found in the luminal domain of the protein. Moreover, my studies show that similar N-glycan dependent signals are necessary for polarized apical sorting of endolyn, indicating for the first time that the glycosylation status can influence the fidelity of postendocytic cargo sorting. This observation suggested that newly synthesized and recycling endolyn may be sorted in a common compartment. However, the experiments in chapter 4 demonstrate that despite the use of similar apical targeting signals, endolyn is sorted in distinct compartments in these pathways. These

studies provide a comprehensive model for the itinerary for the sorting and delivery of endolyn, which can be used to illuminate the functional significance of endolyn expression at steady state. Future studies will be geared towards defining an N-glycan-dependent apical sorting mechanism and the proteins involved.

The intracellular trafficking of endolyn in polarized epithelial cells is not dependent on one signal but rather two signals; an N-glycan-dependent apical sorting signal in its lumenal domain and the lysosomal sorting YXXΦ motif (YHTL) in its cytoplasmic domain (61, 62). It is the differential recognition of these two sorting signals that results in the indirect trafficking of endolyn to lysosomes (61, 63). Along the biosynthetic pathway the majority of endolyn is trafficked from the TGN to the apical surface via the N-glycan-dependent apical sorting signal (61) (Chapters 2&4). A small fraction is also trafficked indirectly via the basolateral surface, because the lysosomal sorting signal can also act as a basolateral sorting signal (61). After delivery to the surface, endolyn is internalized into the postendocytic pathway and the majority of endolyn is now trafficked to lysosomes (61). However a significant fraction of endolyn recycles back to the apical surface via the N-glycan-dependent apical sorting signal (Chapter 3).

One question that arises from this trafficking model is what accounts for the apparent differential recognition of endolyn's lysosomal sorting signal in the TGN vs. endosomes. Newly synthesized endolyn is largely diverted to the cell surface, whereas internalized endolyn is efficiently shunted to lysosomes. Lysosomal trafficking of endolyn is dependent on AP-3 (63). This adaptor complex has been localized to the TGN and endosomes; However, AP-3 only recognizes endolyn's lysosomal sorting signal in endosomes (63). Ihrke et al. propose that a post-translational modification, such as phosphorylation, of endolyn's cytoplasmic tail may regulate the affinity of endolyn for AP-3 in distinct compartments (63). Indeed, such a mechanism has

been implicated for lysosomal delivery of Aquaporin 4 (86). The serine preceding the tyrosine based motif in the cytoplasmic domain of this protein was shown to be phosporylated. Additionally replacement of the serine residue with aspartic acid, mimicking phosphorylation, was shown to increase binding of the tyrosine motif to AP-3 in a two-hybrid assay and increase lysosomal delivery in MDCK cells (86). Endolyn has a cytoplasmic serine residue five amino acids prior to its lysosomal sorting signal, thus it is possible that phosphorylation of this residue at the membrane or at endosomes increases the interaction between endolyn and AP-3. Alternatively, it is possible that different accessory machinery can associate with endosomes in comparison to the TGN to aid in AP-3 binding at this site.

The apical delivery of endolyn along both the biosynthetic and postendocytic pathways suggests that endolyn has a functional purpose at this site. Indeed, mRNA levels of endolyn are highest in liver, kidney, spleen, and lung tissue; all of which contain polarized epithelial cells (62). Furthermore, studies with the human ortholog of endolyn, CD164, have revealed an important role for this protein at the cell surface in differentiation during both myogenesis and hemopoiesis (80, 156). Endolyn contains two mucin-like domains containing sialylated glycans and thus may provide a protective role similar to mucins or act as a receptor at the apical surface (62) (Chapter 3). The highly glycosylated filamentous structure of mucins allows these proteins to protect the surface from the harsh external environment (140). However, bacteria and viruses have adapted to use terminal sugars, such as sialic acid, for adhesion (140). To combat invading pathogens, binding to endolyn may trigger phosphorylation of its cytoplasmic tail, resulting in delivery to lysosomes. The pathogen would then be destroyed in the lysosome and endolyn could be dephosphorylated and recycled back to the apical surface. It is also possible that endolyn acts as a specific receptor, as the disulfide loop domain that connects the two mucin-like domains has

consensus sequences that have only been found in growth factor and cytokine receptors. Binding of putative ligands to endolyn may induce phosphorylation within its cytoplasmic domain resulting in a signaling pathway and/or its degradation. Thus, it would be interesting to determine whether site-directed mutagenesis of the serine preceding endolyn's lysosomal sorting motif has any effect on its trafficking.

A final question that arises from the trafficking of endolyn is how do N-glycans mediate sorting to the apical surface? Two mechanisms have been envisioned for the sorting of proteins that contain N-glycan-dependent apical sorting signals. One model proposes that there is a family of receptors that sort apical proteins, either through a direct interaction with N-glycans or recognition of a proteinaceous conformation that is stabilized by N-glycans (117). The second mechanism proposes that N-glycans stabilize a tertiary conformation that promotes oligomerization or associations with lipid rafts for export from the TGN (117). Experiments performed to discriminate between these mechanisms by identifying specific N-glycan requirements have been unsuccessful in elucidating a specific mechanism; however, these studies, including my own, suggest that specific N-glycans and/or structures are necessary (73, 79, 88, 112, 146). Thus, further specific characterizations of N-glycan-dependent sorting signals may be influential. For instance, the two key N-glycans necessary for apical delivery of endolyn are located on a proposed disulfide loop domain within the protein. It is possible that the two key N-glycans are important because they stabilize this structure, which is then recognized by a receptor. To test this, delivery of endolyn in the absence of this structure could be examined. Site-directed mutagenesis techniques could be used to disrupt the critical cysteine residues within the disulfide loop region of the endolyn construct in which only the two key N-glycans are present (null+N68+N74). Appropriate delivery to the surface would be assessed by domain

selective biotinylation techniques. If endolyn is delivered to the apical surface without the presence of the disulfide loop, this would suggest that the loop domain is not important and would support a mechanism in which glycans are directly recognized. Another possibility is that the disulfide loop domain allows the two key N-glycans to be processed differently than the other eight N-glycans.

Alternatively experiments geared to test the possible mechanisms may be more influential in identifying a mechanism. Preliminary experiments have been initiated within the lab to identify the mechanism by which endolyn is specifically trafficked to the apical surface. To determine whether oligomerization rather than raft-association is important for endolyn delivery, I examined whether there is a difference in the oligomerization status of sorted and nonsorted endolyn constructs. Using sucrose gradient techniques, no differences were observed. Experiments were also performed to determine whether delivery of endolyn to the surface is saturable to determine whether endolyn uses a receptor-mediated mechanism for its N-glycan-dependent apical delivery. Preliminary studies examining the polarity of overexpressed endolyn were consistent with this possibility; however, these results could not be replicated in subsequent attempts. As an alternative approach, I attempted an *in vivo* coimmunoprecipitation approach to identify potential receptor(s) that selectively associate with properly sorted variants of endolyn. Preliminary studies using this approach, however, were unsuccessful. A possible explanation for this is that the antibody used for immunoprecipitation may have disrupted an interaction with the receptor, since it recognizes an epitope within the disulfide loop domain. Thus, future studies using a different antibody that recognizes a distinct site on endolyn or an epitope-tagged version of endolyn may be informative.

Another approach to identifying a mechanism may be to examine whether the apical delivery of endolyn is affected by any of the lectin-like proteins that have been found to associate with apical cargo such as VIP36, MAL, and galectins (26, 27, 44, 87). Recently, intriguing roles for members of the galectin family have emerged in apical sorting of both raft- and non-raft-associated proteins. Unlike conventional secreted proteins, galectins are synthesized in the cytosol and exit cells via a poorly-defined non-classical pathway; however upon secretion they can avidly bind cell surface glycoconjugates and be internalized (57). Internalized galectins can reach endosomal compartments; however, it is not yet clear if they can travel as far back as the TGN (57). Thus their involvement in sorting may only occur in the postendocytic pathway, consistent with the possibility that distinct sorting sites are used in the biosynthetic versus postendocytic pathway. Fourteen different galectins have been identified in mammals and each contains at least one carbohydrate recognition domain that designates its binding specificity (56). Galectin-4 contains two CRDs and appears to preferentially bind glycosphingolipids (27). Upon knockdown of galectin 4 in HT-29 cells, apical protein association with lipid rafts was reduced and the proteins accumulated intracellularly (27). A model has been proposed in which galectin-4 organizes ‘super rafts’ that act as sorting platforms for apical cargo (14).

Another member of the galectin family, galectin-3, is expressed in many cell-types including MDCK cells and was recently shown to preferentially associate with vesicles containing the non-raft-associated protein lactose-phlorizin hydrolase (LPH) in comparison to the raft-associated sucrase-isomaltase (26). Additionally, siRNA-mediated knockdown of galectin-3 disrupted the apical delivery of LPH as well as of two other non-raft-associated glycoproteins, p75 neurotrophin receptor and gp114 (26). Association of galectin-3 and the non-raft associated cargi was shown to be carbohydrate-dependent but a specific role for N-glycans

was not explored (26). To examine a role for galectin-3 in the N-glycan-dependent trafficking of endolyn, the effects of siRNA mediated galactin-3 knockdown on the apical delivery of endolyn along the biosynthetic and postendocytic pathway could be assessed using domain selective biotinylation techniques. If galectin-3 does not affect apical delivery of endolyn it is still possible that another galectin family member may be involved.

In conclusion, maintenance of the apical surface is important for polarize epithelial cell function and a subset of proteins rely on N-glycans for apical delivery. My studies demonstrate that specific N-glycans and structures are recognized in distinct compartments along both the biosynthetic and postendocytic pathways. How these specific requirements translate into a mechanism is not currently understood; however it is anticipated that the future studies described in this dissertation will be influential in this endeavor.

6.0 MATERIALS AND METHODS

6.1 CDNA CONSTRUCTS

The cDNA of wild type rat endolyn and construction of the EEE_{YA} and En_{sol} mutants have been described previously (61, 64). Individual and double glycosylation mutants were generated by site-directed mutagenesis using primers to complementary DNA sequences in the region of interest, both containing the desired mutation. Generation of the N-null construct was done by sequentially introducing the N68A/N74A, N86A, N26A/N32A, N97A, and N144A mutations. Constructs with one or two intact glycosylation sites were generated by reintroducing the required codons into the N-null construct. To make the GPI-linked version of endolyn, we first generated an intermediate construct in pBSII SK^- coding for the lumenal domain of endolyn and ending with a Pst I site introduced by PCR. The fragment of rat 5'-nucleotidase cDNA coding for the C-terminal signal sequence necessary for GPI-anchor attachment to the protein was retrieved from the full-length cDNA by digest with Pst I/BamH I and then cloned into the Pst I site of the intermediate. The resulting expressed amino acid sequence at the domain border was KSTFSAAS, with the first four amino acids from endolyn and the last four from 5'nucleotidase. All mutations were initially introduced into cDNAs inserted in pBSII SK-, which were then subcloned behind the butyrate-inducible cytomegalovirus promoter of the pCB6 vector (16) and verified by DNA sequencing.

6.2 CELL LINES

MDCK II stable cell lines expressing endolyn, EEE_{YA}, En_{sol}, and EECΔ were previously generated (61). Generation of MDCK II stable transfectants expressing endolyn N-glycsoylation mutants and the endolyn/CD8α chimera ECE_{YA} was performed the calcium phosphate method as described previously (61, 151). For most constructs, the polarity of at least two individual clones of drug-resistant cells was assessed. Cells were cultured in modified Eagle's medium (Sigma) supplemented with 10% FBS and 400 μg/ml G418. PTR9 MDCK cells stably expressing the human transferring receptor, rabbit pIgR, and the tetracycline transactivator were a gift from Dr. Gerard Apodaca (University of Pittsburgh). These cells were cultured in modified Eagle's medium supplemented with 10% FBS and 0.05% hygromycin B (Sigma).

6.3 ANTIBODIES

Rabbit antiserum no. 6431 and mouse monoclonal antibodies (mAbs) 501 and 502 to rat endolyn have been described previously; the latter were used interchangeably with similar results. Antibodies for VSV-G-YFP and HA were obtained from supernatants of cultured hybridomas (8G5 from Dr. Douglas Lyles, Wake Forest University and Fc125 from Dr. Thomas Braciale, University of Virginia).

6.4 DRUG TREATMENTS

The following glycosylation perturbants were obtained from Sigma Aldrich and used at the designated concentrations: tunicamycin (TM, 3 μg/ml), deoxymannojirimycin (DMJ, 1 mM), deoxynojirimycin (DNJ, 1 mM), and benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BGN, 4 mM). The α-mannosidase inhibitor KIF, which prevents terminal processing of N-glycans, was purchased from BIOMOL (Plymouth Meeting, PA) and used at a concentration of 21.5 μM. Drugs were added 0.5-2 h prior to the radiolabeling of cells, and were included in the starvation and pulse steps but omitted thereafter. Short pretreatment with tunicamycin (30 min) resulted in synthesis of both fully N-glycosylated and non-N-glycosylated forms of proteins, allowing direct comparison of their polarized distribution in the same gel lanes (Figs. 5, 6, and 7). Upon longer pretreatment (2 h), only the non-N-glycosylated forms were observed. The polarized distribution of non-N-glycosylated forms was the same regardless of the pretreatment protocol used. Cell surface neuraminidase treatment was performed for 2 h at 4°C using 20 mU/ml α2-3,6,8-Neuraminidase isolated from *Vibrio cholerae* (Calbiochem, La Jolla, CA).

6.5 REPLICATION-DEFECTIVE RECOMBINANT ADENOVIRUSES AND INFECTION

Replication-defective recombinant adenoviruses expressing tetracycline-repressible HA (Japan serotype) and endolyn were generated and purified as previously described (48). Replication-defective recombinant adenovirus expressing tsO45 VSV-G-YFP was a gift from Dr. Patrick Keller (European Molecular Biology Laboratory). For infection, PTR9 cells stably expressing

the tetracycline transactivator grown on coverslips or transwells were first incubated in calcium-free PBS containing 1mM $MgCl_2$ (PBS-M) for 5 min. The PBS-M was then replaced with PBS-M containing enough adenovirus sufficient for a multiplicity of infection of 50. Cells were then returned to a 37°C incubator for 1 h. Cells were then washed in PBS-M and incubated in complete media overnight at 37°C for cells expressing endolyn and HA and 40°C for cells expressing VSV-G-YFP.

6.6 DETERMINATION OF POLARITY

Domain selective biotinylation was performed as previously described (61, 115). Briefly, polarized cells stably expressing wild-type or mutant endolyn were starved in cysteine- and methionine-free media for 30 min and radiolabeled for 2 h with [^{35}S]-cysteine. In some experiments, the apical or basolateral surface was then incubated with neuraminidase as described above. Samples were then chased for the indicated periods and either the apical or basolateral surface of a pair of filters was biotinylated on ice. Cells were solubilized and immunoprecipitated with anti-endolyn antibody. Mouse monoclonal antibodies (mAbs) 501 and 502 to rat endolyn have been describedand were used interchangeably with similar results. As described previously, four-fifths of the sample after elution was incubated with streptavidin-agarose beads to recover the biotinylated (surface) fraction of protein. The remaining fraction was retained to calculate the total amount of endolyn. After electrophoresis of the surface and total protein samples, the fraction of cell surface endolyn recovered from each apically or basolaterally biotinylated filter was determined by normalizing to the total endolyn recovered from that filter. The ratio of apical surface endolyn to total surface endolyn (apical plus

basolateral) in each filter pair was then calculated. Data were analyzed using Student's t- test and outlying numbers were discarded using Chauvenet's criterion.

6.7 DETERMINATION OF DETERGENT INSOLUBILITY

The insolubility of EEE_{YA}, GPI-endolyn, and HA in ice-cold Triton X-100 was performed as previously described (61). MDCK cells expressing EEE_{YA}, GPI-endolyn, or virally expressing HA were radiolabeled for 1 h and chased for 1 h. Cells were then solubilized for 20 min in ice-cold TNE (250mM Tris-HCl, 150 mM NaCl, 5 mM EDTA pH 7.5) containing 1% Triton X-100. After centrifugation at 4°C for 20 min at 16,000 g the TX-100 soluble and insoluble fractions were immunoprecipitated and analyzed by SDS-PAGE. Quantitation was done using a phosphoimager.

6.8 IMMUNOFLUORESCENCE MICROSCOPY

Chapter 2: Both surfaces of filter-grown cells expressing endolyn, N-null, and N68.N74 were exposed to anti-endolyn mAb (25 μg/ml) for 1 h on ice. Filters were washed extensively before fixation and incubation with Cy3-conjugated goat-anti-mouse antibody (Jackson Immunoresearch Laboratories, West Grove, PA). Confocal images were collected using a Leica TCS SP system equipped with a 63X Leitz Plan-Apo objective (NA 1.4) at a resolution of 1024x1024 pixels and a zoom of 2.0-3.0. Adobe Photoshop software (Adobe Systems, Mountain View, CA) was used for image processing.

Chapter 3: The apical surface of polarized endolyn-expressing cells was incubated with anti-endolyn antibody (25 μg/ml) on ice for 1 h. For studies with transferrin, cells were starved in serum-free medium for 45 min prior to incubation with apical anti-endolyn antibody on ice for 1 h. After washing, the filters were then transferred to 37°C for 45 min to allow internalization of antibody from the apical surface and transferrin from the basolateral surface. The cell surfaces were then acid-stripped for 60 min at 4°C. Cells were prepared for confocal microscopy using a pH-shift fixation method as previously described (7). After fixation, excess formaldehyde was quenched in PBS containing 20 mM glycine pH 8.0. The cells were washed and then permeabilized with 0.1% Triton X-100 in PBS containing 1% BSA (PBSA). This was followed by incubation in PBS containing 5% normal goat serum (Sigma). Cells were immunostained with appropriate primary antibodies diluted in PBSA for 1 h. Mouse anti-endolyn (25 μg/ml) and rabbit anti-furin (1:400 dilution, Affinity Bioreagents, Golden, CO) or mouse anti-endolyn, rabbit anti-canine transferin (gift from Gerard Apodaca), and rat anti-ZO-1 (1:20 dilution, a gift from Gerard Apodaca). After washing, samples were incubated with fluorescent-labeled secondary antibodies diluted in blocking buffer. Alexa 488 goat anti-mouse and Alexa-647 goat anti-rabbit secondary antibodies were used (Molecular Probes, Eugene, OR). For the transferring study, Cy3 goat anti-rat secondary antibodies were also used (Jackson Immunoresearch). Filters were washed and mounted onto glass coverslips with Aqua-polymount (Polysciences, Inc., Warrington, PA). Imaging was performed on a TCS-SL confocal microscope (Leica, Dearfield, IL) equipped with argon, green helium-neon, and red-helium-neon lasers. Acquisition of images was performed with 100x plan-apochromat oil objective (NA 1.4) and the appropriate filter combination. The images were saved as TIFFs and imported into Adobe Photoshop (Adobe, San Jose, CA) where contrast was corrected.

Chapter 4: WGA-FITC was internalized from the apical surface of filter-grown PTR9 cells for 1 h at 18.5^{o}C. WGA-FITC remaining at the cell surface was removed by treating the surface with 100 mM N-acetyl-D-glucosamine for 2X10 min. Cells were then washed 2X in PBS and fixed in with 4% paraformaldehyde in 100 mM NaCacodylate for 10 min at room temperature. Cells were washed 3X in PBS and any remaining formaldehyde was quenched by incubating in PBS containing 20 mM glycine (pH 8.0) for 5 min. After 3 washes in PBS cells were blocked in 10% normal goat serum containing fish skin gelatin and saponin for 10 min. Cells were incubated with the appropriate primary antibodies diluted in the blocking solution (rat anti-ZO-1 diluted 1:10 and rabbit anti-EEA1 was diluted 1:500 or rabbit anti-rab11 diluted 1:500). After washing 3-5X with blocking solution, cells were incubated with secondary antibodies diluted in blocking solution (Cy5 conjugated goat anti-rabbit diluted 1:100 and Cy3-conjugated goat anti-rat diluted 1:1000 (Jackson Immunoresearch). This incubation was followed by three washes with blocking solution and 3 washes with PBS. Cells were then post-fixed in 4% paraformaldehyde in 100 mM NaCacodylate for 10 min. Before mounting the cells were washed 3X with PBS, 3X with PBS containing 20 mM glycine pH 8.0, and 3X with PBS. Imaging was performed on a TCS-SL confocal microscope (Leica, Dearfield, IL) equipped with argon, green helium-neon, and red-helium-neon lasers. Acquisition of images was performed with 100x plan-apochromat oil objective (NA 1.4) and the appropriate filter combination. Images were analyzed with Volocity software Improvision.

6.9 INTERNALIZATION ASSAY

The internalization rate of endolyn was determined using the procedure described by Altschuler et al (4). Briefly, endolyn-expressing MDCK cells plated on Costar 6-well transwells were radiolabeled with [^{35}S]-cysteine for 2 h and chased for 10-15 min. The apical surface was biotinylated on ice twice for 10 min with sulfo-NHS-SS-Biotin (0.5 mg/ml; Pierce) in TEA-buffered saline (pH 7.6). The reaction was quenched by washing the cells with culture medium containing fetal bovine serum. After biotinylation, cells were incubated at 37°C for 0, 5, or 15 min in HEPES-buffered MEM. After each time point, cells were washed with ice cold PBS and the apical surface incubated with Sodium 2-mercaptoethansulfonate (MESNA) to strip remaining biotinylated proteins. Iodoacetic acid (120mM) was then added to quench any remaining MESNA. A duplicate 0 min time point was left untreated with MESNA in order to determine the total amount of endolyn at the apical cell surface (100%). The cells were then solubilized, immunoprecipitated, and then incubated with immobilized streptavidin (Pierce). Samples were analyzed by SDS-PAGE to determine the percent of endolyn internalized relative to the total amount initially present at the apical surface.

6.10 INACTIVATION OF TF- AND WGA-CONTAINING ENDOSOMAL COMPARTMENTS AND ASSESSMENT OF SURFACE DELIVERY

This technique was performed as described by Ang *et al.* (6). To label Tf-containing endosomal compartments, nonpolarized PTR9 cells virally expressing VSV-G-YFP and polarized PTR9 cells virally expressing VSV-G-YFP, endolyn, or HA were first incubated in serum-free media

for 30 min. Cells were then incubated with 0.01 mg/ml HRP-Tf for 45 min. For biochemical studies, HRP-Tf was internalized while cells were starved in media devoid of cysteine and methionine for 30 min and radiolabeld with 1 mCi/ml Trans-^{35}S-label for 15 min. For cells expressing VSV-G-YFP these step were performed at 40°C rather than 37°C. HRP-Tf remaining at the cell surface was removed after 2 X 10 min incubations with 0.15M NaCl and 20mM citric acid pH 5.0. To label WGA-containing endosomal compartments, cells were radiolabeled for 15 min and then the apical surface of cells was incubated with 25 μg/ml HRP-WGA (EY Laboratories, San Mateo, CA) for 1 h at 18.5^{o}C. HRP-WGA remaining at the surface was removed by 2 X 10 min incubations with 100 mM N-acetyl-D-glucosamine (Sigma).

For HRP inactivation, cells were first washed for 3 X 5 min in PBS and then incubated with PBS containing 0.1 mg/ml DAB (Sigma) and 0.025% H_2O_2. Cells were incubated in the dark on ice for 1 h. The reaction was quenched in PBS containing 1% BSA (Sigma) for 5 min and then washed 3X with PBS. As controls, HRP-Tf or HRP-WGA was not internalized and samples were not incubated with DAB and H_2O_2 (no add'n), while in other samples only the H_2O_2 or HRP-Tf/HRP-WGA was omitted (no H_2O_2 or no HRP-Tf/no HRP-WGA respectively). For immunofluorescence studies, nonpolarized PTR9 cells expressing VSV-G-YFP were chased in the presence of cycloheximide at 32°C for 60 min. Cells were then fixed and imaged on a microscope. For biochemical studies, PTR9 cells expressing VSV-G-YFP were chased for at 32°C and PTR9 cells expressing endolyn or HA were chased at 37°C for 0 or 90 min. At each time point delivery to the surface was quantitated. For cells expressing VSV-G-YFP or endolyn, biotinylation of the basolateral or apical surface was performed as described above. To quantitate apical delivery of HA to the surface, cell surface trypsinization techniques were used as described by Henkel *et al.* (49). Briefly, the apical surface incubated with 100 μg/ml TPCK-

treated trypsin for 30 min on ice. The apical surface was then incubated with 200 µg/ml soybean trypsin inhibitor. Cells were then rinsed with PBS and solubilized. HA was immunoprecipitated and analyzed by SDS-PAGE under reducing conditions. The trypsin treatment cleaves HA at the surface into subunits that remain associated via disulfide bonds during immunoprecipitation but are disrupted under reducing conditions. The percentage of cleaved HA1 and HA2 subunit in comparison to the total amount of HA within the cell was quantitated using a phosphoimager.

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