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N-Glycans Mediate Apical Recycling of the Sialomucin Endolyn in Polarized MDCK Cells

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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 the postendocytic pathways.

Key words: polarized trafficking, endocytosis, sialic acid

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Crucial to the function of polarized epithelial cells is the ability to sort proteins along both the biosynthetic and the post endocytic pathways 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- and dileucine-based motifs (1). 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 (1–3).

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 (4–6). Tyrosine- and dileucine-based motifs can interact with adaptor protein complexes in these compartments to direct proteins to endosomes, lysosomes or the basolateral cell surface (7). 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 (8). The sorting of some apical proteins is glycan-dependent; however, the underlying mechanisms that direct apical sorting have 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 (9,10).

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 low-density lipoprotein receptor are similar to those that direct their polarized delivery along the biosynthetic pathway (11,12). 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 (13). Whether similar or distinct apical sorting determinants operate along the biosynthetic and the postendocytic pathways has been unexplored.

Our previous studies have focussed 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 (14). Lysosomal targeting of endolyn is conferred by a tyrosine-based tetrapeptide motif in the cytoplasmic tail of the protein (14,15); 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 (14,15). Biosynthetic apical sorting of endolyn requires specificity in both the structure and the position of N-glycans (16) and appears to be lipid raft-independent as endolyn is soluble in cold TX-100 (14). Because a significant fraction of endolyn recycles to the apical surface after internalization from the surface, we have used this protein as a model to examine the requirements for N-glycans in postendocytic sorting. 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 the postendocytic pathways.

Results

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 non-polarized manner to the plasma membrane of polarized MDCK cells (16). 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 (17,18). 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 (14,15,19). Using a standard biotinylation-based endocytosis assay to quantitate the kinetics of endolyn internalization, we determined that the initial rate of wild-type endolyn internalization from the apical surface was roughly 1% per minute (Figure 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 (20). 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

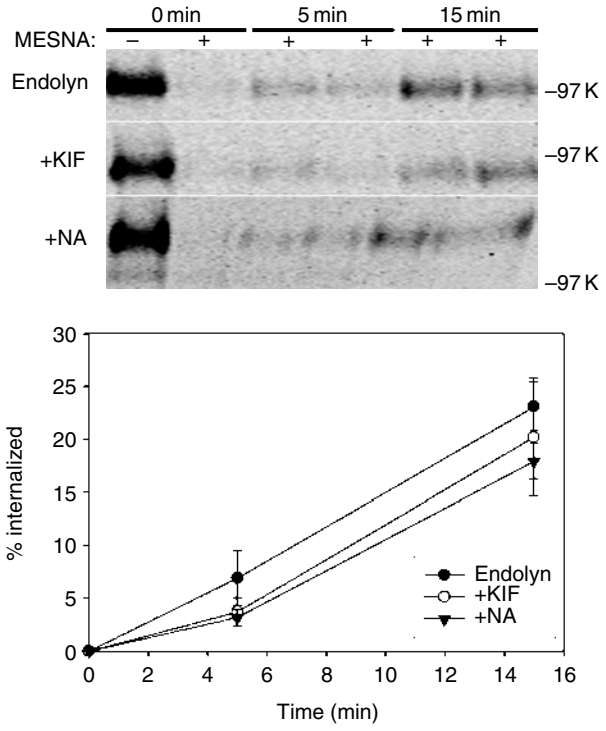

Figure 1: Endolyn is efficiently internalized. Polarized endolyn-expressing MDCK cells were radiolabeled for 2 h in the presence or absence of KIF and chased for 15 min prior to biotinylating the apical surface with sulfo-NHS-SS-biotin. Where indicated, filters were then incubated with apically added 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 *Materials and Methods*. Quantitation of the data are shown below.

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 1). Thus, the rate of internalization of endolyn appears to be largely independent of its terminal glycosylation status.

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 (14). 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 cointernalized from the basolateral and apical surfaces, respectively, for 45 min at 37 °C prior to fixation and subsequent processing for indirect immunofluorescence. Modest co-localization of these two cargo molecules was observed in confocal sections taken through the medial portion of the cells (Figure 2), consistent with the reported distribution of common endosomes (10). No co-localization 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.

In addition to recycling from early and common endosomes, there is evidence that endolyn constitutively recycles from lysosomes. Ihrke *et al.* (2004) (19) 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. To examine recycling from late compartments 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). 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 percentage 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 approximately 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 presynthesized 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 (approximately 21 h), our data suggest that endolyn recycles from both early and late endocytic compartments and requires the active segregation from basolateral cargo.

Endolyn does not recycle via the TGN

In a few cases, apically internalized proteins have been shown to recycle via the TGN (21). 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 4). In our

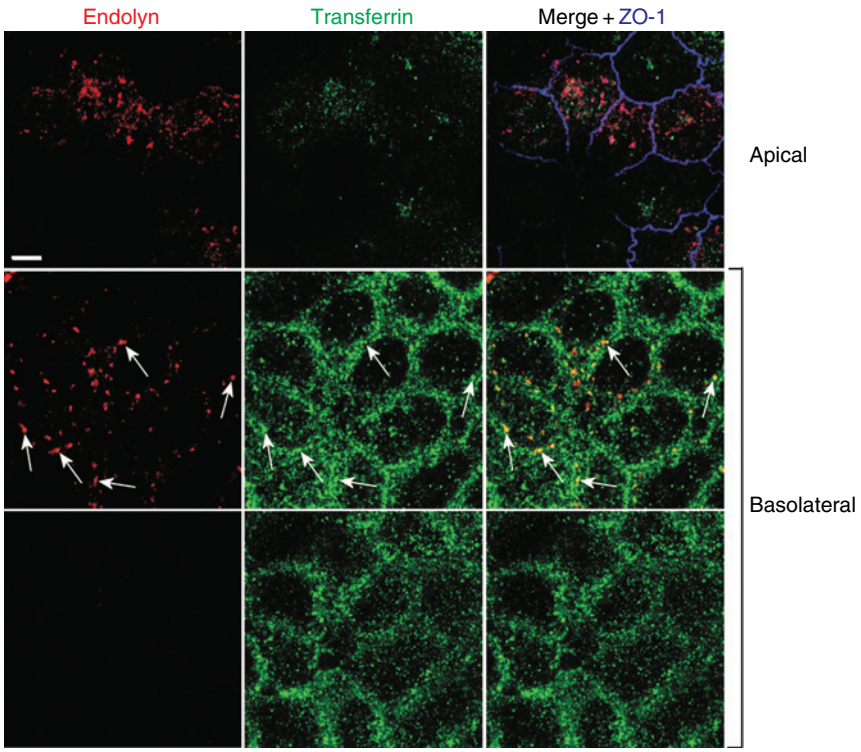

Figure 2: Apically internalized endolyn co-localizes with basolaterally internalized transferrin in endosomes. Anti-endolyn antibody was prebound to the apical surface of polarized endolyn-expressing MDCK cells for 1 h on ice. After washing, cells were warmed to 37 °C in the presence of basolaterally 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. Co-localization (arrows) between endolyn and transferrin was observed only in medial sections. Scale bar: 4 μm.

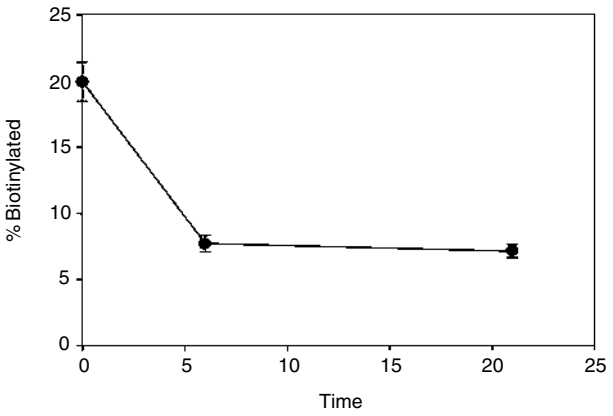

Figure 3: Newly synthesized endolyn recycles to the cell surface over a long chase period. Polarized MDCK cells stably expressing endolyn were radiolabeled for 2 h then chased for 0, 6 or 21 h. At each time-point, cells were biotinylated. The total percent of presynthesized endolyn that was biotinylated at the plasma membrane (apical + basolateral) at each time-point is plotted (mean = +/–SEM; $n = 17–22$).

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 4A) or the Golgi complex marker giantin (not shown). No co-localization was observed with either marker, suggesting that endolyn does not traffic back to the TGN. Moreover, no co-localization was observed when the internalization step was performed for 4 h at 20 °C to retain any retrieved proteins in the TGN (not shown).

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 (21,22). Both the apical and the 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 0–21 h, solubilized, and then immunoprecipitated with anti-endolyn antibody. The biotinylated fraction was isolated using streptavidin-agarose and examined by SDS-PAGE. Similar

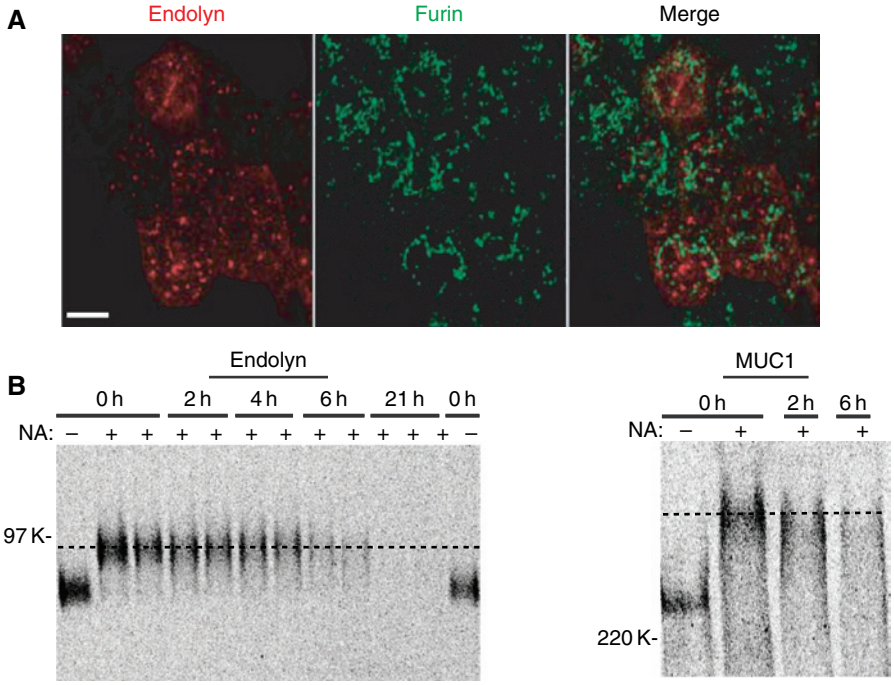

Figure 4: Internalized endolyn does not transit the TGN. (A) Anti-endolyn antibody was prebound to the apical surface of MDCK cells for 1 h on ice, and the cells were rapidly warmed to 37 °C for 45 min. After fixation, cells were processed for indirect immunofluorescence to visualize endolyn (red) and 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 MDCK cells stably expressing endolyn or MUC1 were radiolabeled for 2 h or overnight, respectively. Both the apical and basolateral surfaces were biotinylated on ice, and the indicated samples were incubated on ice with NA to desialylate cell-surface proteins. The cells were returned to culture for 0–21 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. A dotted line is drawn through the center of the 0 h time-point to emphasize the presence or absence of a shift in electrophoretic mobility. Similar results were observed in three experiments for each construct.

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 4B, compare 0 h ± neuraminidase (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. This result was also confirmed by isoelectric focusing (not shown). To verify that our approach could indeed detect the recycling of a protein through the TGN, we examined the trafficking of the transmembrane mucin MUC1. Litvinov and Hilkens (23) previously showed that a premature desialylated form of MUC1 is delivered to the surface of non-polarized cells and then subsequently sialylated after multiple rounds of internalization and recycling via the TGN. Consistent with this, we reproducibly observed a shift in the electrophoretic mobility of desialylated cell-surface MUC1 in stably transfected MDCK cells after returning to culture for 6 h (Figure 4B).

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. Kifunensine 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 presynthesized endolyn was determined as described in *Materials and Methods*. Figure 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 non-polarized 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 non-polarized manner would maintain a non-polarized distribution after numerous rounds of recycling.

Our results conform to the second scenario. Endolyn maintained a polarized distribution (approximately 70% apical) throughout the time–course, confirming that the wild-type protein is preferentially returned to the apical surface after internalization. When endolyn was

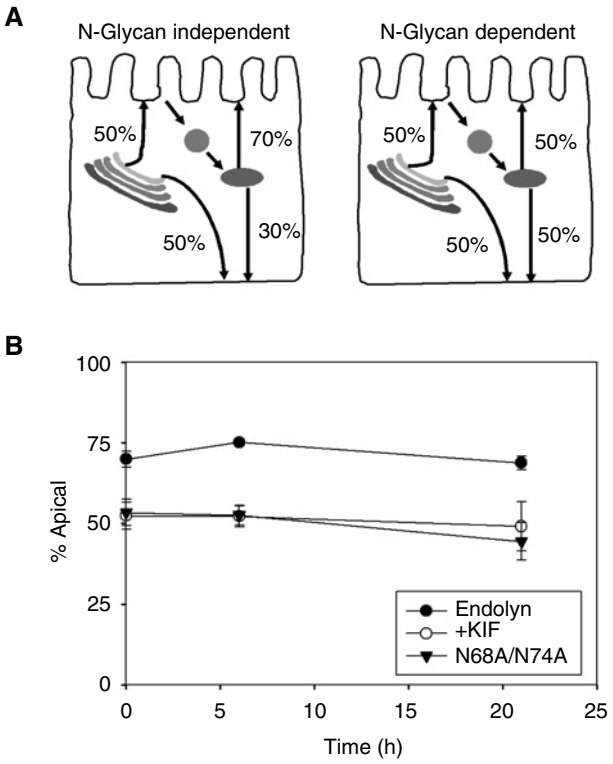

Figure 5: Polarized postendocytic sorting of endolyn is N-glycan-dependent. (A) A schematic illustrating the predictions for the long time–course polarity experiments. Kifunensine-treated endolyn and N68A/N74A are initially delivered to the plasma membrane of polarized cells in a non-polarized fashion. 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 non-polarized 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 ± SEM; $n = 17$–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.

synthesized in the presence of KIF, it was delivered in a non-polarized manner, similar to our previous observation (Figure 5B, 52% apical) and, importantly, we observed no change in its distribution over the 21-h chase period. The consistent difference 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 non-polarized 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.

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 (16). 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 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 the postendocytic pathways.

Postendocytic 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 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 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 non-polarized 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 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.

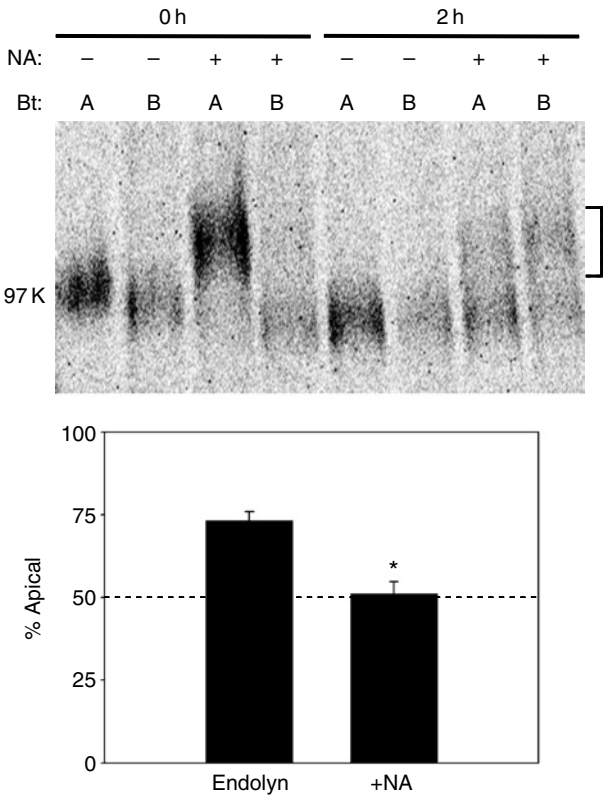

Figure 6: The polarity of endolyn is rapidly altered upon cell-surface neuraminidase treatment. Madin-Darby canine kidney cells stably expressing endolyn were radiolabeled, and the apical surfaces were mock-treated or treated with NA on ice for 1 h. Subsequently cells were returned to culture at 37 °C for 0 or 2 h, 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 non-polarized distribution by 2 h. The graph below shows the polarity of mock-treated and apically desialylated endolyn after 2 h in culture (mean ± SE; $n = 10$–11). *$p < 0.001$ versus mock-treated by Student's *t*-test. The dotted line at 50% denotes the distribution of a non-polarized protein.

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 has long been thought that polarized biosynthetic and postendocytic sortings occur in distinct cellular compartments in MDCK cells (2). However, recent evidence suggests that a significant fraction of some newly synthesized basolateral proteins also traverses endosomal compartments *en route* to the cell surface (4–6,24).

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) (25,26). 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 (27). 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 (24). 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 transported to lysosomes, while a smaller but still significant proportion is recycled back to the apical surface (14). 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 the lysosomes in non-polarized cells has been previously documented (19). 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 (14). 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 versus approximately 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 non-polarized 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 approximately 40% of preinternalized 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 the 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 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 that glycan the apical protein gp114 is mislocalized in galactosylation-deficient ricin resistant cells due to defective terminal glycosylation; however, in contrast to our observations with endolyn, gp114 mislocalization was not observed upon deoxymannojirimycin treatment of wild-type cells (28). Similarly, a role for α-2,3-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 Benzyl-N-acetyl-α-galactosaminide (BGN) (29). 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 (30), 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, because terminal processing of N-glycans is a requirement for polarized sorting of endolyn in the biosynthetic and postendocytic 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 (31). While our data do not distinguish between these models, our observations suggest that common mechanisms are utilized along both the biosynthetic and the postendocytic pathways. Future studies will be required to unravel how these N-glycan-dependent sorting signals are decoded.

Materials and Methods

Cell lines, antibodies and drug treatments

The generation of MDCK II cells stably expressing wild-type endolyn and endolyn glycosylation mutants was described previously (14,16). The α-mannosidase inhibitor KIF, which prevents terminal processing of N-glycans, was purchased from BIOMOL and used at a concentration of 21.5 μM. Cells were pretreated with KIF for 1 h at 37 °C prior to initiating the pulse-chase protocol and was included during the starve (30 min) and pulse (2 h) steps. Cell-surface neuraminidase treatment was performed for 2 h at 4 °C using 20 mU/mL α-2-3,6,8-Neuraminidase isolated from *Vibrio cholerae* (Calbiochem, La Jolla, CA, USA). Anti-endolyn (mAb 501) was used at 25 μg/mL; rabbit anti-canine transferrin antibody was a gift from Gerard Apodaca; rabbit anti-furin was used at 1:400 dilution (Affinity Bioreagents, Golden, CO, USA), and rat anti-ZO-1 (1:20) was a gift from Gerard Apodaca. Affinity purified, minimal cross reacting Alexa-488 and Alexa-647 conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) were used at 1:500 dilution.

Internalization assay

The internalization rate of endolyn was determined using the procedure described by Altschuler *et al.* (17). 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, Rockford, IL, USA) in triethanolamine (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 (120 mM) was then added to quench any remaining MESNA. A duplicate 0-min time-point was left untreated with MESNA 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.

Immunofluorescence microscopy

The apical surface of polarized endolyn-expressing MDCK cells was incubated with anti-endolyn antibody (25 μg/mL) on ice for 1 h. For the studies with transferrin, cells were starved in serum-free medium for 45 min prior to incubation with apical anti-endolyn antibody on ice. 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 (32). 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, Saint Louis, MO, USA). Cells were immunostained with appropriate primary antibodies diluted in PBSA for 1 h, washed and incubated with fluorescent-labeled secondary antibodies diluted in blocking buffer. Filters were washed and mounted onto glass coverslips with Aqua-polymount (Polysciences, Inc., Warrington, PA, USA). Imaging was performed on a TCS-SL confocal microscope (Leica, Dearfield, IL, USA) equipped with argon, green helium-neon and red-helium-neon lasers. Acquisition of images was performed with ×100 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, USA) where contrast was corrected.

Determination of polarity

Domain selective biotinylation was performed as previously described (14,16). 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. 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.

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References

1. Nelson WJ, Yeaman C. Protein trafficking in the exocytic pathway of polarized epithelial cells. Trends Cell Biol 2001;11:483–486.
2. Schuck S, Simons K. Polarized sorting in epithelial cells: raft clustering and the biogenesis of the apical membrane. J Cell Sci 2004;117: 5955–5964.
3. Rodriguez-Boulan E, Kreitze G, Musch A. Organization of vesicular trafficking in epithelia. Nat Rev Mol Cell Biol 2005;6:233–247.
4. Ang AL, Taguchi T, Francis S, Folsch H, Murrells LJ, Pypaert M, Warren G, Mellman I. Recycling endosomes can serve as intermediates during transport from the Golgi to the plasma membrane of MDCK cells. J Cell Biol 2004;167:531–543.
5. Futter CE, Connolly CN, Cutler DF, Hopkins CR. Newly synthesized transferrin receptors can be detected in the endosome before they appear on the cell surface. J Biol Chem 1995;270:10999–11003.
6. Orzech E, Cohen S, Weiss A, Aroeti B. Interactions between the exocytic and endocytic pathways in polarized Madin-Darby canine kidney cells. J Biol Chem 2000;275:15207–15219.
7. Bonifacino JS, Traub LM. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem 2003;72:395–447.
8. Paladino S, Sarnataro D, Pillich R, Tivodar S, Nitsch L, Zurzolo C. Protein oligomerization modulates raft partitioning and apical sorting of GPI-anchored proteins. J Cell Biol 2004;167:699–709.
9. Altschuler Y, Hodson C, Milgram SL. The apical compartment: trafficking pathways, regulators and scaffolding proteins. Curr Opin Cell Biol 2003;15:423–429.
10. Wang E, Brown PS, Aroeti B, Chapin SJ, Mostov KE, Dunn KW. Apical and basolateral endocytic pathways of MDCK cells meet in acidic common endosomes distinct from a nearly-neutral apical recycling endosome. Traffic 2000;1:480–493.
11. Matter K, Whitney JA, Yamamoto EM, Mellman I. Common signals control low density lipoprotein receptor sorting in endosomes and the Golgi complex of MDCK cells. Cell 1993;74:1053–1064.
12. Aroeti B, Mostov KE. Polarized sorting of the polymeric immunoglobulin receptor in the exocytotic and endocytotic pathways is controlled by the same amino acids. EMBO J 1994;13:2297–2304.
13. Odorizzi G, Trowbridge IS. Structural requirements for basolateral sorting of the human transferrin receptor in the biosynthetic and endocytic pathways of Madin-Darby canine kidney cells. J Cell Biol 1997;137:1255–1264.
14. Ihrke G, Bruns JR, Luzio JP, Weisz OA. Competing sorting signals guide endolyn along a novel route to lysosomes in MDCK cells. EMBO J 2001;20:6256–6264.
15. Ihrke G, Gray SR, Luzio JP. Endolyn is a mucin-like type I membrane protein targeted to lysosomes by its cytoplasmic tail. Biochem J 2000;345:287–296.
16. Potter BA, Ihrke G, Bruns JR, Weixel KM, Weisz OA. Specific N-glycans direct apical delivery of transmembrane, but not soluble or glycosylphosphatidylinositol-anchored forms of endolyn in Madin-Darby canine kidney cells. Mol Biol Cell 2004;15:1407–1416.
17. Altschuler Y, Kinlough CL, Poland PA, Bruns JB, Apodaca G, Weisz OA, Hughey RP. Clathrin-mediated endocytosis of MUC1 is modulated by its glycosylation state. Mol Biol Cell 2000;11:819–831.
18. Vagin O, Turdikulova S, Sachs G. The H,K-ATPase beta subunit as a model to study the role of N-glycosylation in membrane trafficking and apical sorting. J Biol Chem 2004;279:39026–39034.
19. Ihrke G, Kyttala A, Russell MR, Rous BA, Luzio JP. Differential use of two AP-3-mediated pathways by lysosomal membrane proteins. Traffic 2004;5:946–962.
20. Naim HY, Dodds DT, Brewer CB, Roth MG. Apical and basolateral coated pits of MDCK cells differ in their rates of maturation into coated vesicles, but not in the ability to distinguish between mutant hemagglutinin proteins with different internalization signals. J Cell Biol 1995;129:1241–1250.
21. Brandli AW, Simons K. A restricted set of apical proteins recycle through the trans-Golgi network in MDCK cells. EMBO J 1989;8:3207–3213.
22. Obermuller S, Kiecke C, von Figura K, Honing S. The tyrosine motifs of Lamp 1 and LAP determine their direct and indirect targetting to lysosomes. J Cell Sci 2002;115:185–194.
23. Litvinov SV, Hilkens J. The epithelial sialomucin, episialin, is sialylated during recycling. J Biol Chem 1993;268:21364–21371.
24. Polishchuk R, Di Pentima A, Lippincott-Schwartz J. Delivery of raft-associated, GPI-anchored proteins to the apical surface of polarized MDCK cells by a transcytotic pathway. Nat Cell Biol 2004;6:297–307.
25. Futter CE, Gibson A, Allchin EH, Maxwell S, Ruddock LJ, Odorizzi G, Domingo D, Trowbridge IS, Hopkins CR. In polarized MDCK cells basolateral vesicles arise from clathrin-gamma-adaptin-coated domains on endosomal tubules. J Cell Biol 1998;141:611–623.
26. Gan Y, McGraw TE, Rodriguez-Boulan E. The epithelial-specific adaptor AP1B mediates post-endocytic recycling to the basolateral membrane. Nat Cell Biol 2002;4:605–609.
27. Nyasae LK, Hubbard AL, Tuma PL. Transcytotic efflux from early endosomes is dependent on cholesterol and glycosphingolipids in polarized hepatic cells. Mol Biol Cell 2003;14:2689–2705.
28. Le Bivic A, Garcia M, Rodriguez-Boulan E. Ricin-resistant Madin-Darby canine kidney cells missort a major endogenous apical sialoglycoprotein. J Biol Chem 1993;268:6909–6916.
29. Huet G, Gouyer V, Delacour D, Richet C, Zanetta JP, Delannoy P, Degand P. Involvement of glycosylation in the intracellular trafficking of glycoproteins in polarized epithelial cells. Biochimie 2003;85:323–330.
30. Delacour D, Gouyer V, Zanetta JP, Drobecq H, Leteurtre E, Grard G, Moreau-Hannedouche O, Maes E, Pons A, Andre S, Le Bivic A, Gabius HJ, Manninen A, Simons K, Huet G. Galectin-4 and sulfatides in apical membrane trafficking in enterocyte-like cells. J Cell Biol 2005;169:491–501.
31. Rodriguez-Boulan E, Gonzalez A. Glycans in post-Golgi apical targeting: sorting signals or structural props? Trends Cell Biol 1999;9: 291–294.
32. Apodaca G, Katz LA, Mostov KE. Receptor-mediated transcytosis of IgA in MDCK cells is via apical recycling endosomes. J Cell Biol 1994;125:67–86.