The EMBO Journal vol.8 no.11 pp.3207 - 3213, 1989

A restricted set of apical proteins recycle through the *trans*-Golgi network in MDCK cells

A.W.Brändli and K.Simons

European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg, FRG

Communicated by K.Simons

Sorting of newly synthesized proteins destined for the apical plasma membrane takes place in the *trans*-Golgi network (TGN) in MDCK cells. This process is most likely receptor mediated and requires components that recycle between both compartments. We have developed an assay to detect apical proteins that recycle through the sialyltransferase-containing TGN. Cell surface glycoproteins were exogalactosylated apically using a mutant cell line derived from MDCK, MDCKII-RCA^r. The mutant exhibits impaired galactosylation of glycoconjugates and thereby allows maximal incorporation of exogenously added galactose in the presence of galactosyltransferase. Upon reculture at 37°C, a time-dependent increase of sialylated apical surface glycoproteins was observed by lectin binding as well as by the sialic acid-specific $NaIO_4/NaB[^3H]_4$ labeling technique. This indicates that some galactosylated surface molecules had returned to the TGN. Recycling through the TGN was blocked, if exogalactosylated cells were incubated at 20°C. Two-dimensional gel electrophoresis identified three apical proteins which recycle through the TGN, suggesting that this pathway is selective for a subset of the apical surface proteins.

Key words: endocytosis/MDCK cells/plasma membrane/sialylation/*trans*-Golgi network

Introduction

Plasma membrane polarity in simple epithelial cells is maintained by intracellular sorting of apical and basolateral proteins in the *trans*-Golgi network (TGN) (Griffiths and Simons, 1986; Bennett *et al.*, 1988; Hughson *et al.*, 1988; Rodriguez-Boulan and Salas, 1989). The molecular mechanism responsible for this process is not known. The sorting event in the TGN requires recognition of newly synthesized apical and basolateral proteins. In the simplest case, specific recognition is necessary for only one of the two pathways of polarized transport (Pfeffer and Rothman, 1987). Since the apical plasma membrane facing the external environment is unique to epithelia, it has been postulated that sorting in the apical direction would be the signal-mediated pathway and require specific receptors, whereas the basolateral pathway is equivalent to the exocytic pathway in unpolarized cells and could operate by default (Wieland *et al.*, 1987; Simons and van Meer, 1988; Wandinger-Ness and Simons, 1989). The putative apical sorting receptors would recognize newly synthesized apical proteins in the TGN and segregate with them into specific transport vesicles which subsequently fuse with the apical membrane. After apical delivery, the sorting receptors would be endocytosed and returned to the TGN to initiate a new round of sorting. Evidence for the recycling of components of the sorting machinery is provided by the observation that sorting proceeds for hours after inhibition of host protein synthesis in virus-infected epithelial cells (Rindler *et al.*, 1984).

A number of studies have reported the return of plasma membrane proteins of the Golgi complex (Herzog, 1981; Farquhar, 1985; van Deurs *et al.*, 1988). However, recycling through the TGN, defined as transport of internalized surface molecules to the TGN and subsequent return to the plasma membrane, has only been convincingly demonstrated for transferrin and its receptor (Regoeczi *et al.*, 1982; Snider and Rogers, 1985; Reichner *et al.*, 1988) and for the two mannose 6-phosphate receptors (Duncan and Kornfeld, 1988; Reichner *et al.*, 1988; Jin *et al.*, 1989). In fact, most plasma membrane glycoproteins do not recycle through the TGN (Reichner *et al.*, 1988). Duncan and Kornfeld (1988) have shown that mannose 6-phosphate receptors are not transported to the more proximal, *trans*-cisternae of the Golgi. Further, there is no evidence for significant recycling of surface glycoproteins through *cis*- or *medial*-Golgi elements (Duncan and Kornfeld, 1988; Neefjes *et al.*, 1988), suggesting that recycling cell surface glycoproteins do not travel through more proximal Golgi compartments before entering the TGN. Given the evidence for recycling of cell surface proteins through the TGN, it was of interest to determine whether a selected set of apical surface proteins can be identified that shuttle between the apical surface and the TGN.

We have designed experiments to detect and to identify such molecules. Our strategy relies on previous work (Duncan and Kornfeld, 1988) and involves making covalent alterations to cell surface components so that they become substrates for enzymes of known intracellular location. Transport of proteins to the enzyme-containing internal compartment results in a covalent modification of the molecule, which is detected upon its return to the cell surface. Cell surface glycoproteins are modified with galactose residues by exogalactosylation to generate substrates for sialyltransferase. The transfer of sialic acid by sialyltransferase, an enzyme localized in the TGN (Roth *et al.*, 1985; Berger *et al.*, 1987), is used to monitor the recycling of cell surface glycoproteins to the TGN. We have detected a subset of exogalactosylated proteins that acquire sialic acids upon reculture indicating the existence of a selective recycling pathway through the TGN.

Results

Experimental scheme to detect cell surface molecules recycling through the TGN

The following experimental protocol shown in Figure 1 was used to detect apical cell surface molecules that return to

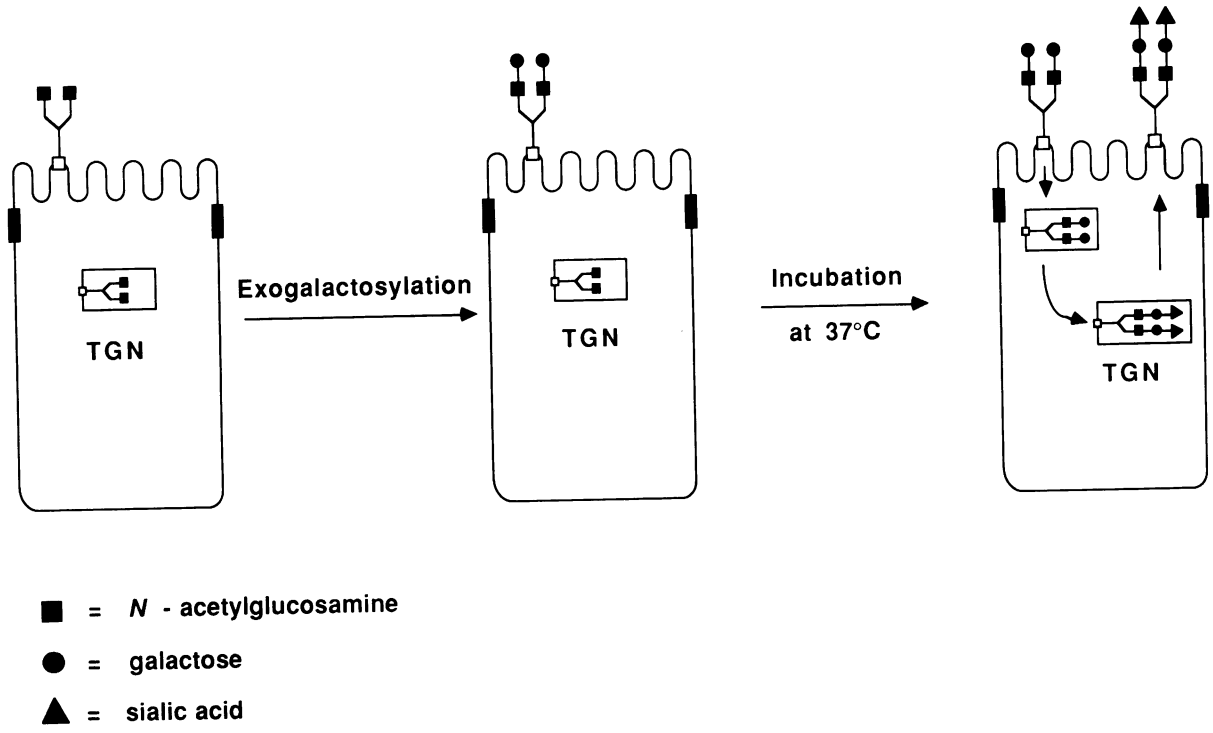

Fig. 1. Experimental protocol used to detect cell surface glycoproteins recycling through the *trans*-Golgi network by sialylation.

the TGN in MDCK cells. Cell surface glycoproteins were galactosylated by incubation with UDP-galactose and galactosyltransferase. Exogalactosylation was restricted to the apical plasma membrane domain by using MDCK cells grown on filters. Incubating the cells at 37°C allowed internalization of galactosylated proteins. Acquisition of sialic acid in the TGN was monitored after return to the apical cell surface using either the sialic acid-specific lectin *Limax flavus* agglutinin (LFA) or the $NaIO_4/NaB[^3H]_4$ labeling technique.

A mutant cell line of MDCK, MDCKII-RCA^r, was used to permit maximal incorporation of galactose during exogalactosylation. MDCKII-RCA^r cells, isolated by Meiss *et al.* (1982), polarize in culture and develop transepithelial resistance. However, they are impaired in the synthesis of galactose-containing glycosphingolipids and glycoproteins due to the inability of the Golgi apparatus to translocate UDP-galactose from the cytosol (Brändli *et al.*, 1988). Therefore, glycoproteins bearing *N*-linked glycans lack galactose as well as sialic acid residues, and terminate with *N*-acetyl-glucosamine residues (GlcNAc).

Labeling of sialoglycoproteins by the $NaIO_4/NaB[^3H]_4$ technique

Sialic acid residues can be selectively oxidized by low concentrations of periodate to produce an aldehyde which can then be tritium-labelled by reduction with $NaB[^3H]_4$ (Van Lenten and Ashwell, 1971). Under the conditions used, periodate is membrane impermeable and therefore, only cell surface-exposed sialic acid residues are oxidized (Gahmberg and Andersson, 1977). MDCK cells were treated either apically or basolaterally by the $NaIO_4/NaB[^3H]_4$ technique and the labeled sialoglycoproteins were analyzed by SDS–PAGE. The apical and basolateral patterns of labeled sialoglycoproteins were strikingly different (Figure 2). Thus,

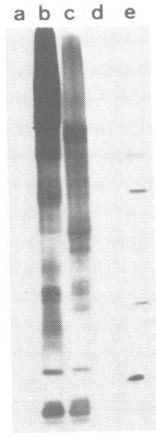

Fig. 2. Plasma membrane domain-restricted labeling of sialoglycoproteins of MDCKII cells by the $NaIO_4/NaB[^3H]_4$ technique. MDCKII cells were grown on polycarbonate filters for 3 days, subjected to apical (**lanes a** and **b**) or basolateral (**lanes c** and **d**) $NaIO_4/NaB[^3H]_4$ labeling as described in Materials and methods. Oxidation by $NaIO_4$ was omitted in **lanes a** and **d**. The mol. wts of the proteins in **lane e** are as follows: 92 500, 69 000, 46 000, 30 000 and 14 000. Labeled sialoglycoproteins were precipitated with trichloroacetic acid–phosphotungstic acid and analyzed by SDS–PAGE using a 5–15% acrylamide gradient gel.

tritiation of sialoglycoconjugates was restricted to a specific plasma membrane domain and the assay conditions did not lead to opening of the tight junctions. The radioactivity, recovered as acid-insoluble material, comprised <4% of the total when the oxidation step was omitted. A 70% reduction of apical surface sialic acid residues in the mutant cells as compared to wild-type cells was detected by this method (Figure 3). Similar results were obtained using labeled sialic acid-specific LFA in binding assays (Brändli *et al.*, 1988).

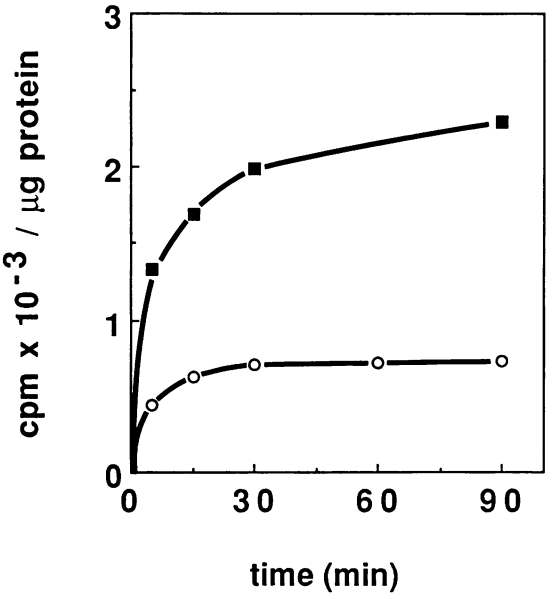

Fig. 3. Labeling of apical sialoglycoproteins of wild-type and mutant MDCKII cells by the $NaIO_4/Na[^3H]_4$technique. Filter-grown cells were exposed apically to 1 mM $NaIO_4$ for various times. Oxidized sialic acid residues were reduced by $NaB[^3H]_4$, lysates were prepared, labeled sialoglycoproteins were precipitated with trichloroacetic acid–phosphotungstic acid and the incorporated radioactivity was determined. Oxidation by $NaIO_4$ was omitted for control cells and the obtained values were substracted from the data points shown. Each point represents the average of duplicate samples. ■, MDCKII cells; ○, MDCKII-RCA^r cells.

Apically exogalactosylated glycoconjugates are internally sialylated and reappear on the apical plasma membrane

Filter-grown MDCKII-RCA^r cells were exogalactosylated apically for 1 h at 37°C to follow apical glycoconjugates which recycle through the TGN. Galactosylated cells were washed and further incubated for various times at 37°C. Recycling of apical surface glycoconjugates through the TGN was monitored by following the appearance of sialic acid-containing glycoconjugates on the apical plasma membrane using the europium-labeled LFA (Figure 4A) or by the $NaIO_4/NaB[^3H]_4$ technique (Figure 4B). The amount of sialic acid observed 8 h after exogalactosylation increased 1.3-fold (by LFA binding) to 1.6-fold (by $NaIO_4/NaB[^3H]_4$ labeling) compared to control cells. The minor discrepancy between the two values obtained using two independent methods might be due to a fraction of sialic acid residues, which are accessible to small molecules, like $NaIO_4$ and $NaB[^3H]_4$, but not to LFA because of steric hindrance. The increase was small and the cell surface exposed sialic acid residues did not reach wild-type levels. However, these results suggest that recycling of proteins between the apical plasma membrane and the TGN occurs and can be detected biochemically.

Recycling of apical surface proteins through the TGN is inhibited by incubation at 20°C

Cells incubated at temperatures below 20°C are not blocked in exocytic protein transport from the TGN to the plasma membrane (Matlin and Simons, 1983), but also in endocytic protein traffic from early to late endosomes and lysosomes

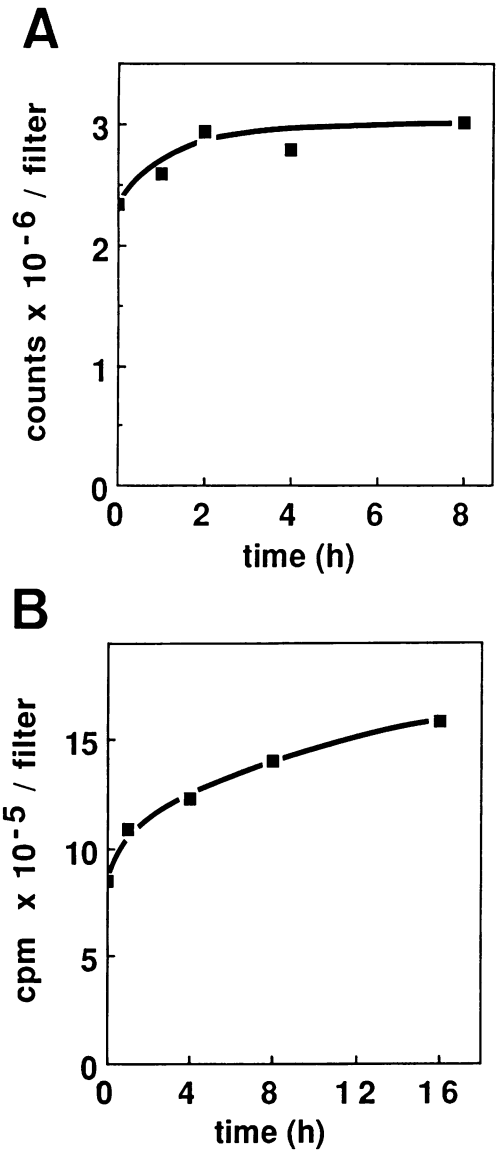

Fig. 4. Apical cell surface glycoproteins acquire sialic acids following exogalactosylation. MDCKII-RCA^r cells were exogalactosylated apically, washed and recultured for various times at 37°C. Apical cell surface sialoglycoproteins were detected by lectin binding assays using Eu^{3+}-labeled *Limax flavus* agglutinin (**panel A**) or by determining the amount of acid-insoluble radioactivity after $NaIO_4/NaB[^3H]_4$ labeling (**panel B**). The values at $t = 0$ h were obtained with control cells in the absence of exogalactosylation. Each point represents the average of duplicate samples. For experimental details see Materials and methods.

(Dunn *et al.*, 1980; Marsh *et al.*, 1983, 1986; Griffiths *et al.*, 1988).

MDCKII-RCA^r cells were exogalactosylated for 1 h at 20°C, and subsequently incubated either at 20 or at 37°C (Figure 5). After 2 h at 20°C no sialylation of apical surface glycoproteins was observed and after 4 h a slight increase (1.1-fold) over the values for control cells was seen. Cells incubated at 37°C showed a 1.4-fold increase in apical sialic acid residues after 4 h compared to control cells. This result is consistent with previous findings that temperatures below 20°C block the transport of the transferrin receptor (Snider and Rogers, 1985) and of the mannose 6-phosphate receptors (Duncan and Kornfeld, 1988; Jin *et al.*, 1989) from the plasma membrane to the TGN.

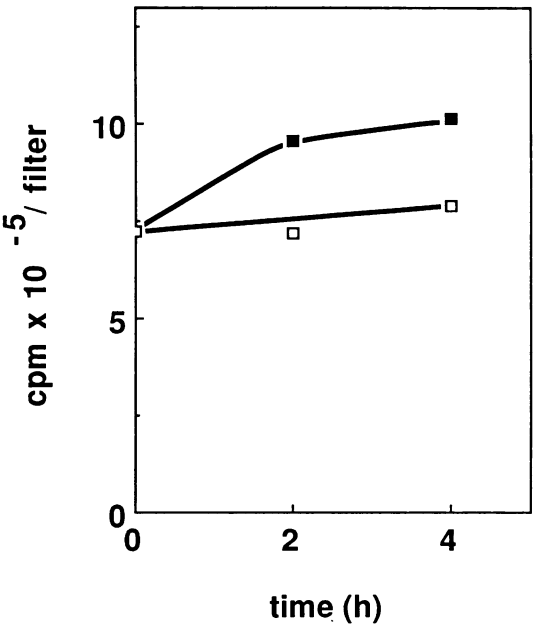

Fig. 5. Incubation at 20°C blocks sialylation of exogalactosylated apical cell surface proteins. MDCKII-RCA^r cells were exogalactosylated apically at 20°C, washed and recultured for various times at 20°C (□) and 37°C (■). Sialylation of apical cell surface glycoproteins was detected by determining the amount of acid-insoluble radioactivity after $NaIO_4/NaB[^3H]_4$ labeling. Each point represents the average of duplicate samples.

Table I. Sialyltransferase activity in the absence and presence of detergent

Acceptor	Temperature (0°C)	Sialyltransferase activity (nmol/mg protein/min)	
		(−) TX-100	(+) TX-100
Asialofetuin	0	0.14 ± 0.12	4.14 ± 0.42
−	0	ND	0.81 ± 0.28
Asialofetuin	37	0.12 ± 0.10	20.86 ± 1.51
−	37	ND	0.83 ± 0.23

TX-100, Triton X-100; ND, not determined.
Cell surface sialyltransferase activity was measured as transfer of *N*-acetyl[^{3}H]neuraminic acid to a soluble acceptor, asialofetuin. Filter-grown MDCKII-RCA^r cells were incubated apically with asialofetuin and CMP-*N*-acetyl[^{3}H]neuraminic acid for 30 min at the indicated temperatures. Supernatants were recovered and pooled with two washes. Asialofetuin was precipitated by trichloroacetic acid−phosphotungstic acid and acid-insoluble radioactivity was measured. Total sialyltransferase activity was determined as follows. Filter-grown cells were scraped, lysed with 1% Triton X-100, incubated with asialofetuin and CMP-*N*-acetyl[^{3}H]neuraminic acid for 30 min at the indicated temperatures, and processed as mentioned above. Asialofetuin was omitted in control assays. Each assay was carried out with duplicate samples. Mean values are indicated. For experimental details see Materials and methods.

Absence of cell surface sialyltransferase activity

Both biochemical and immunochemical approaches have provided evidence for the existence of glycosyltransferases, and particularly galactosyltransferase, on the cell surface of some cell types, where they might participate in fertilization, cell adhesion and cell migration (Pierce *et al.*, 1980; Hathaway and Shur, 1988). We tested whether sialyltransferase activity was present on the apical plasma membrane of MDCKII-RCA^r cells. Table I shows that significant amounts of sialyltransferase activity could only be detected in the presence of detergent. At 37°C, sialyltransferase activity measured in the presence of detergent increased at least 170-fold. This suggested that <0.6 (± 0.5)% of the total activity was external. The very small amount of sialyltransferase activity measured in the absence of detergent was variable and did not show temperature sensitivity. It is most likely due to radiolabeled CMP-sialic acid trapped within the pellet of acid-insoluble material and is negligible. Therefore, sialyltransferase activity is essentially restricted to internal compartments in MDCKII-RCA^r cells.

A distinct set of apical surface proteins recycle through the TGN

In the above experiments only a small increase of sialylation of apical surface proteins occurred after exogalactosylation and subsequent incubation of MDCKII-RCA^r cells. This could either be due to the fact that a fraction of all apical proteins recycle back to the TGN or, alternatively, that a limited subset of apical proteins are recycling. In order to address this question, MDCKII-RCA^r cells were exogalactosylated apically and incubated at 37°C. Sialoglycoproteins were tritiated by the $NaIO_4/NaB[^3H]_4$ technique and analyzed by two-dimensional gel electrophoresis. Ungalactosylated control cells (Figure 6A) displayed a rather simple pattern of sialoglycoproteins with <10 major labeled species. These proteins were also present in cell lysates from exogalactosylated cells (Figure 6B and C). However, at least three additional labeled proteins were detected already 2 h after exogalactosylation. Two of these proteins were very acidic and had a mol. wt of 75 kd and 45−55 kd respectively. A third, less acidic 75 kd protein was observed. This protein displayed extensive charge heterogeneity and spreads over a large pl range. Densitometric analysis of the fluorograms revealed a 5- to 15-fold increase in the labeling of the recycling proteins after 4 h of reculture. At this time-point, they were among the major labeled polypeptides. Incubation for longer time points did not reveal further proteins (data not shown).

These results suggest that the acquisition of sialic acid by apical cell surface proteins after exogalactosylation is restricted to a limited number of apical proteins which move between the TGN and the cell surface.

Discussion

We have developed a novel assay to study endocytosis and recycling of surface proteins through the TGN in epithelial cells. Galactosylation of surface glycoproteins with exogenously added UDP-galactose and galactosyltransferase produces *N*-linked oligosaccharides which are substrates for sialyltransferase. Sialylation of the galactosylated protein was used as an indicator for passage through the TGN. Reappearance of sialylated proteins on the cell surface was detected by binding assays with a sialic acid-specific lectin, *Limax flavus* agglutinin or by the $NaIO_4/NaB[^3H]_4$ labeling technique. The second method introduced a radioactive label into surface sialoglycoproteins. This feature makes the assay unique compared to previously reported assays for recycling through the TGN (Snider and Rogers, 1985; Duncan and Kornfeld, 1988), since it allows the identification of individual proteins that have recycled through the TGN by comparative two-dimensional gel analysis. In addition, only

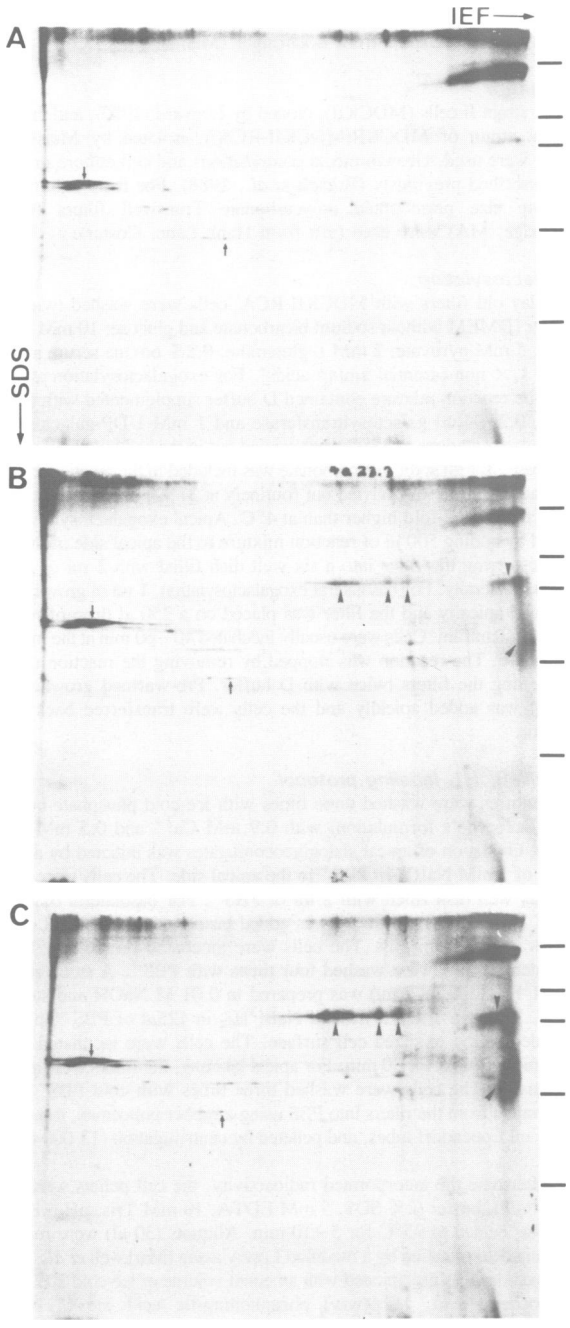

Fig. 6. A distinct set of apical proteins recycle through the *trans*-Golgi network. Filter-grown MDCKII-RCA^r cells were exogalactosylated apically at 20°C, washed and recultured for 2 h (**panel B**) or 4 h (**panel C**) at 37°C. A filter with control cells was not exogalactosylated (**panel A**). Apical sialoglycoproteins were labeled by the $NaIO_4/NaB[^3H]_4$ technique. Half of the cell lysate from one filter was resolved by two-dimensional IEF/SDS–PAGE. The migration positions of the indicated mol. wt markers are as follows: 200 000, 92 500, 69 000, 46 000 and 30 000. Arrows denote examples of sialoglycoproteins found also in lysates of control cells. Arrowheads indicate sialoglycoproteins specific for exogalactosylated and recultured cells.

molecules that have completed a full cycle and have reappeared on the correct plasma membrane domain are labeled, due to the membrane-impermeability of the reagents (Figure 2; Gahmberg and Anderson, 1977).

Movement of surface glycoproteins through the TGN was followed in the polarized epithelial cell line MDCKII-RCA^r. MDCKII-RCA^r cells have a strongly reduced amount of surface sialic acid residues associated with glycoproteins and are essentially devoid of sialylated glycosphingolipids (Figure 3; Brändli *et al.*, 1988). This allows a very sensitive monitoring of changes in the levels of sialic acid residues with minimal background. The observed residual sialylation of glycoconjugates is most likely due to sialic acids linked to sugars other than galactose, such as *N*-acetylgalactosamine (GalNAc) in *O*-linked glycans (Brändli *et al.*, 1988). Neuramindase treatment of control MDCKII-RCA^r cells prior to $NaIO_4/NaB[^3H]_4$ labeling reduced the incorporation of ^3H into acid-insoluble material by >60% (A.Brändli, unpublished observation). The cellular machinery needed for sialylation of glycoconjugates, such as nucleotide sugar translocators for CMP-sialic acid and sialyltransferases, are operational in MDCKII-RCA^r cells (Brändli *et al.*, 1988).

Exposure of apical membrane glycoproteins to sialyltransferase was used to study recycling through the TGN. Roth *et al.* (1985) have localized sialyltransferase to the TGN in hepatocytes. Berger *et al.* (1987) and Duncan and Kornfeld (1988) have reported evidence for different subcellular locations for galactosyl- and sialyltransferase in cultured cells. Altogether, this work suggests that galactosyltransferase resides in the *trans*-cisternae of the Golgi complex, while sialyltransferase is restricted to the TGN. However, this may not be the case in all cell types (Roth, 1987). Sialyltransferase has not yet been immunolocalized in MDCK cells. Several lines of indirect evidence support a localization in the TGN. First, newly synthesized glycoproteins of vesicular stomatitis virus accumulating in the TGN at 20°C became completely sialylated (Fuller *et al.*, 1985). Second, in cell fractionation experiments, the specific activity of sialyltransferase was highest in Golgi-derived membranes (A.Brändli, unpublished observation). Third, sialyltransferase activity was not detected on the apical cell surface (Table I). Finally, the absence of sialic acid addition to apical proteins at 20°C (Figure 5) suggests that sialylation takes place in a post-endosomal compartment.

Using MDCKII-RCA^r cells, we have demonstrated that apically exogalactosylated cells acquire surface sialic acids in a time- and temperature-dependent manner, as expected for apical proteins recylcing through the TGN. Since exogalactosylation was usually carried out at 37°C, it is possible that some of the galactosyltransferase and UDP-galactose entered endosomal compartments and acted on molecules that recycle between endosomes and the TGN. However, if this subset of sialylated molecules were to appear on the cell surface it would not be detected by this assay. The increase in cell surface sialic acids is only minor (1.3- to 1.7-fold over background) and never reaches the levels observed in wild-type MDCKII cells. The low amounts of sialylation of apical cell surface glycoproteins could indicate that either all apical glycoproteins cycle through the TGN at a very slow rate or that a small subset of molecules cycles while the remainder of the molecules never return to the TGN. The second possibility is favored by the observation that only a limited set of sialylated apical molecules were detected by two-dimensional gel electrophoresis, even if the cells were incubated for as long as 16 h after exogalactosylation. The kinetics of sialylation of these apical cell surface molecules is reminiscent to those reported for the sialylation of the transferrin receptor and the mannose

6-phosphate receptors with half-times of 2–4 h (Snider and Rogers, 1985; Duncan and Kornfeld, 1988; Jin *et al.*, 1989). It should be noted that the assay we are using also has limitations. First, the assay is restricted to glycoproteins carrying *N*-linked glycans; second, recycling of glycoproteins bearing sialylated *O*-linked glycans in addition to *N*-linked glycans might be missed, because they appear as ^{3}H-labeled sialoglycoproteins already on two-dimensional gels of mock-treated cells. Finally, the analysis by the two-dimensional electrophoresis system used in this study might not resolve all sialoglycoproteins. However, we can conclude that there is no evidence for extensive recycling of proteins from the apical plasma membrane domain through the TGN in MDCK cells. Preliminary results suggest that this conclusion also applies to the basolateral plasma membrane of MDCK cells. Using specific antibodies directed against the cation-independent mannose 6-phosphate receptor in conjugation with the sialylation assay, it was possible to demonstrate that this protein which is basolaterally localized in MDCK cells recycles between the basolateral cell surface and the TGN (K.Prydz, A.W.Brändli and K.Simons; in preparation). Altogether these results obtained with MDCK cells are in line with previous reports that have shown that in unpolarized cells, like CHO cells, cell surface glycoproteins as a whole do not routinely recycle through the Golgi complex (Duncan and Kornfeld, 1988; Neefjes *et al.*, 1988; Reichner *et al.*, 1988; van Deurs *et al.*, 1988). Reichner *et al.* (1988) found only a small amount (<10%) of endogenous resialylation in the absence of protein synthesis in EL-4 and K562 cells. These findings suggest that there is a mechanism for selective endocytosis, targeting and recycling of a restricted set of surface molecules through the TGN.

This is the first report of apical proteins in epithelial cells that are able to recycle along such a pathway. The function of these proteins, however, is unknown. Since transport to the TGN appears to be selective, it is unlikely that the recycling represents a general mechanism that allows the continuous repair of asialoglycoproteins by sialylation. Two recent reports support the existence of a route for membrane traffic from the apical plasma membrane to the TGN. Ricin molecules bound to the cell surface are endocytosed and a small fraction is transported to the Golgi complex (van Deurs *et al.*, 1988). Translocation of internalized ricin toxin into the cytosol occurs most likely in the Golgi complex (Youle and Colombatti, 1987). *In vivo*, ingested ricin molecules are taken up by epithelial cells lining the gastro-intestinal tract via the apical membrane and might, therefore, take advantage of endogenous apical molecules recycling through the TGN to gain access to this compartment. Whether one of the recycling apical proteins, has the physiological function to sort apical proteins into carrier vesicles in the TGN remains to be proven in the future.

Materials and methods

Materials

Media and reagents for cell culture were purchased from Gibco BRL and from Seromed (West Berlin, FRG). CMP-*N*-acetyl[9-^{3}H]neuraminic acid (30.7 Ci/mmol), Entensify and Protosol tissue solubilizer were obtained from Du Pont–New England Nuclear. UDP-D-[6-^{3}H]galactose (20 Ci/mmol) and NaB[^{3}H]$_4$ (66.9 Ci/mmol) were from Amersham. Asialofetuin, type I; ovalbumin, grade V; CMP-*N*-acetylneuraminic acid, sodium salt; *N*-acetylneuraminic acid, Type VI; galactosyltransferase from bovine milk; Dulbecco's modified Eagle's medium (DMEM) without glucose and sodium bicarbonate; and sodium borohydride were from Sigma. UDP-galactose was purchased from Boehringer (Mannheim, FRG).

Cell culture

MDCK strain II cells (MDCKII), cloned by Louvard (1980), and the ricin-resistant strain of MDCKII(MDCKII-RCA^r), isolated by Meiss *et al.* (1982), were used. Growth media compositions and cell culture protocols were described previously (Brändli *et al.*, 1988). For filter cultures, 0.4 μm pore size premounted polycarbonate Transwell filters (Costar, Cambridge, MA) were used (gift from Hank Lane, Costar).

Exogalactosylation

Three day old filters with MDCKII-RCA^r cells were washed twice with D buffer [DMEM without sodium bicarbonate and glucose; 10 mM Hepes, pH 7.2; 5 mM pyruvate; 2 mM L-glutamine; 0.2% bovine serum albumin (BSA); 1 × non-essential amino acids]. For exogalactosylation at 4 and 20°C, the reaction mixture contained D buffer supplemented with 10 mM $MnCl_2$, 0.25 U/ml galactosyltransferase and 1 mM UDP-galactose.

Exogalactosylations at 37°C were carried out in the cell culture incubator. In this case, 3.7 g/l sodium bicarbonate was included in the reaction mixture. Exogalactosylation was carried out routinely at 37°C, because the transfer of galactose was 2-fold higher than at 4°C. Apical exogalactosylation was initiated by adding 500 μl of reaction mixture to the apical side of the filter and transferring the filter into a six-well dish filled with 2 ml of growth media basolaterally. For basolateral exogalactosylation, 1 ml of growth media was added apically and the filter was placed on a 250 μl drop of reaction mixture on Parafilm. Cells were usually incubated 40–60 min at the indicated temperature. The reaction was stopped by removing the reaction mixture and washing the filters twice with D buffer. Pre-warmed growth media (1.5 ml) was added apically and the cells were transferred back to the incubator.

$NaIO_4$/NaB[^{3}H]$_4$ labeling protocol

Filter cultures were washed three times with ice-cold phosphate-buffered saline (Dulbecco's formulation) with 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+} (PBS^+). Oxidation of apical sialoglycoconjugates was initiated by addition of 1 ml of 1 mM $NaIO_4$ in PBS^+ to the apical side. The cells were placed into a six-well dish filled with 2 ml of PBS^+. For basolateral oxidation, 2 ml of 1 mM $NaIO_4$ in PBS^+ was added basolaterally and 1 ml of cold PBS^+ was added apically. The cells were incubated on ice for 30 min. Subsequently, they were washed four times with PBS^+. A stock solution of NaB[^{3}H]$_4$ (200 mCi/ml) was prepared in 0.01 M NaOH and stored at −70°C. Between 1 and 5 mCi of NaB[^{3}H]$_4$ in 125μl of PBS^+, pH 8.0, was added to the oxidized cell surface. The cells were incubated on ice in a humid chamber for 30 min. For apical labeling, the filters were agitated occasionally. The cells were washed three times with cold PBS^+. Cells were scraped from the filters into PBS using a rubber policeman, transferred into 1.5 ml Eppendorf tubes, and pelleted by centrifugation (13 000 r.p.m., 5 min).

To determine the incorporated radioactivity, the cell pellets were lysed with 500 μl L buffer (2% SDS, 5 mM EDTA, 10 mM Tris, pH 6.8). The lysate was heated to 95°C for 5–10 min. Aliquots (30 μl) were removed for protein determination by a modified Lowry assay (Markwell *et al.*, 1978). The remainder was precipitated with an equal volume of ice-cold 20% (w/v) trichloroacetic acid, 1% (w/v) phosphotungstic acid in 0.5 N HCl (TCA–PTA) for 30 min at 4°C. The precipitate was pelleted by centrifugation in an Eppendorf centrifuge (13 000 r.p.m., 10 min), washed twice with 500 μl of TCA–PTA, and dissolved in 500 μl Protosol. Ready Safe scintillation cocktail (4.5 ml; Beckman) was added and the samples were counted in a 1218 Rackbeta liquid scintillation counter (Wallac OY, Turku, Finland). For one-dimensional gel electrophoresis, TCA–PTA precipitates were solubilized in SDS–PAGE sample buffer [50 mM Tris–HCl, pH 6.8, 2.5 mM EDTA, 2% SDS, 5% (v/v) glycerol, 0.01% bromophenol blue and 2.5% (v/v) 2-mercaptoethanol]. Samples were resolved on 5–15% acrylamide gradient gels. After electrophoresis the gels were fixed in 45% methanol and 7% acetic acid and treated for fluorography using Entensify. For two-dimensional IEF–SDS polyacrylamide gel electrophoresis, cell pellets were lysed and processed as described by Bravo (1984). Fifteen per cent acrylamide gels were used for the second dimension.

Eu^{3+}-LFA binding assay

Limax flavus agglutinin (LFA) was purchased from Calbiochem and labeled with europium (Eu^{3+}) by I.Hemmilä (Wallac OY, Turku, Finland) essentially as described (Hemmilä *et al.*, 1984).

MDCKII-RCA^r cells were grown on filters, exogalactosylated and recultured as described above. The binding assay (Brändli *et al.*, 1988) was modified as follows. Cells were washed apically twice with PBS without

Ca^{2+} and Mg^{2+}, and three times with PBS containing 5 mg/ml BSA (PBS–BSA). Eu^{3+}-labeled LFA (92 μg/ml; 6.2 Eu^{3+}/molecule) was diluted to 1 μg/ml with PBS–BSA and 500 μl was added apically per filter. If 100 mM sialic acid was included in the incubation, the binding of LFA was reduced by 99.5%. After 60 min at 4°C, the lectin solution was removed, and the cells were washed successively three times with PBS–BSA and PBS. Cells were scraped from the filters as described. The cell pellets were lysed with 500 μl of Delfia fluorescence enhancement solution (Wallac OY) (Hemmilä *et al.*, 1984) and incubated at 4°C overnight on a rotating shaker. The cell lysate was cleared by centrifugation (13 000 r.p.m., 10 min). Duplicate 200 μl aliquots of the supernatant were measured by a time-resolved fluorometer, 1230 Arcus (Wallac OY).

Cell surface sialyltransferase assay

Filter-grown MDCKII-RCA^r cells were washed three times with PBS^+. The reaction mixture contained in a total volume of 250 μl of DMEM (without glucose and sodium bicarbonate): 20 mM Hepes, pH 7.2, 1.25 mg asialofetuin, 50 μM CMP-*N*-acetylneuraminic acid and 0.5 μCi CMP-*N*-acetyl[9-^{3}H[neuraminic acid (30.7 Ci/mmol). The assay was initiated by adding the reaction mixture apically to the cells. The filters were placed into six-well plates with 2 ml growth media, containing 0.35 g/l sodium bicarbonate basolaterally. The cells were incubated for 30 min at 4°C on ice or at 37°C. The supernatants were collected and the cells were washed twice with 125 μl of PBS^+. The washes were pooled with the supernatants. Precipitation was initiated by the addition of 500 μl of TCA–PTA for 30 min at 4°C. The precipitates were pelleted by centrifugation and processed for liquid scintillation counting. To determine the amount of CMP-*N*-acetyl[9-^{3}H]neuraminic acid trapped in the precipitate of asialofetuin, samples of the reaction mixture were incubated in the absence of cells.

For determining the total sialyltransferase activity, cells were scraped from the filters as described above. The assay was initiated by adding 250 μl of the reaction mixture containing, in addition, 1% (w/v) Triton X-100 to the cell pellets. The cells were dissolved using a vortex and incubated for 30 min at 4 or 37°C. The reaction was stopped by adding 250 μl of PBS^+ and 500 μl of TCA–PTA. The precipitates were processed for liquid scintillation counting.

Acknowledgements

We wish to express our thanks to Hank Lane (Costar, Cambridge, MA) for providing polycarbonate filters, Illka Hemmilä (Wallac Oy, Turku, Finland) for preparing Eu^{3+}-labeled reagents, and Hilkka Virta for help in tissue culture. In addition, we thank Bob Bacallao, Mark Bennett, Morgane Bomsel, Carlos Dotti, Kristian Prydz and Angela Wandinger-Ness for critical reading of the manuscript. A.W.B. was supported by an EMBL predoctoral fellowship.

References

Bennett,M.K., Wandinger-Ness,A. and Simons,K. (1988) *EMBO J.*, **7**, 4075–4085.
Berger,E.G., Turnher,M. and Müller,U. (1987) *Exp. Cell Res.*, **173**, 267–273.
Brändli,A.W., Hansson,G.C., Rodriguez-Boulan,E. and Simons,K. (1988) *J. Biol. Chem.*, **263**, 16283–16290.
Bravo,R. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4848–4850.
Duncan,J.R. and Kornfeld,S. (1988) *J. Cell Biol.*, **106**, 617–628.
Dunn,W.A., Hubbard,A.L. and Aronson,N.N.,Jr (1980) *J. Biol. Chem.*, **255**, 5971–5978.
Farquhar,M.G. (1985) *Annu. Rev. Cell Biol.*, **1**, 447–488.
Fuller,S.D., Bravo,R. and Simons,K. (1985) *EMBO J.*, **4**, 297–307.
Gahmberg,C.G. and Andersson,L.C. (1977) *J. Biol. Chem.*, **252**, 5888–5894.
Griffiths,G. and Simons,K. (1986) *Science*, **234**, 438–443.
Griffiths,G., Hoflack,B., Simons,K., Mellman,I. and Kornfeld,S. (1988) *Cell*, **52**, 329–341.
Hathaway,H.J. and Shur,B.D. (1988) *BioEssays*, **9**, 153–158.
Hemmilä,I., Dakubu,S., Mukkala,V.-M., Siitari,H. and Lövgren,T. (1984) *Anal. Biochem.*, **137**, 335–343.
Herzog,V. (1981) *Trends Biochem. Sci.*, **6**, 319–322.
Hughson,E., Wandinger-Ness,A., Gausepohl,H., Griffiths,G. and Simons,K. (1988) In Schwartz,M. (ed.), *Molecular Biology and Infectious Disease*. Centenary Symposium of the Pasteur Institute, Elsevier, Amsterdam, pp. 75–90.
Jin,M., Sahagian,G.G. and Snider,M. (1989) *J. Biol. Chem.*, **264**, 7675–7680.
Louvard,D. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 4132–4136.
Markwell,M.A.K., Haas,S.M., Bieber,L.L. and Tolbert,N.E. (1978) *Anal. Biochem.*, **87**, 206–210.
Marsh,M.E., Bolzau,E. and Helenius,A. (1983) *Cell*, **32**, 931–940.
Marsh,M.E., Griffiths,G., Dean,G.E., Mellman,I. and Helenius,A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2899–2903.
Matlin,K.S. and Simons,K. (1983) *Cell*, **34**, 233–243.
Meiss,H.K., Green,R.F. and Rodriguez-Boulan,E.J. (1982) *Mol. Cell. Biol.*, **2**, 1287–1294.
Neefjes,J.J., Verkerk,J.M.H., Broxterman,H.J.G., van der Marel,G.A., van Boom,J.H. and Ploegh,H.L. (1988) *J. Cell Biol.*, **107**, 79–87.
Pierce,M., Turley,E.A. and Roth,S. (1980) *Int. Rev. Cytol.*, **65**, 1–47.
Pfeffer,S.R. and Rothman,J.E. (1987) *Annu. Rev. Biochem.*, **56**, 829–852.
Regoeczi,E., Chendemi,P.A., Debanne,M.T. and Charlwood,P.A. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 223–229.
Reichner,J.S., Whiteheart,S.W. and Hart,G.W. (1988) *J. Biol. Chem.*, **263**, 16316–16326.
Rindler,M.J., Ivanov,I.E., Plesken,H., Rodriguez-Boulan,E. and Sabatini,D.D. (1984) *J. Cell Biol.*, **98**, 1304–1319.
Rodriguez-Bouland,E. and Salas,P.J.I. (1989) *Annu. Rev. Physiol.*, **51**, 741–754.
Roth,J. (1987) *Biochim. Biophys. Acta*, **906**, 405–436.
Roth,J., Taatjes,D.J., Lucocq,J.M., Weinstein,J. and Paulson,J.C. (1985) *Cell*, **43**, 287–295.
Simons,K. and van Meer,G. (1988) *Biochemistry*, **27**, 6197–6202.
Snider,M.D. and Rogers,O.C. (1985) *J. Cell Biol.*, **100**, 826–834.
van Deurs,B., Sandvig,K., Petersen,O.W., Olsnes,S., Simons,K., and Griffiths,G. (1988) *J. Cell Biol.*, **106**, 253–267.
van Lenten,L. and Ashwell,G. (1971) *J. Biol. Chem.*, **246**, 1889–1894.
Wandinger-Ness,A. and Simons,K. (1989) In Hannover,J. and Steer,C. (eds), *Intracellular Trafficking of Proteins*. Cambridge University Press, Cambridge, in press.
Wieland,F.T., Gleason,M.L., Serafini,T.A. and Rothman,J.E. (1987) *Cell*, **50**, 289–300.
Youle,R.J. and Colombatti,M. (1987) *J. Biol. Chem.*, **262**, 4676–4682.

Received on May 24, 1989; revised on August 2, 1989