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Microtubule perturbation retards both the direct and the indirect apical pathway but does not affect sorting of plasma membrane proteins in intestinal epithelial cells (Caco-2)

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Endogenous plasma membrane proteins are sorted from two sites in the human intestinal epithelial cell line Caco-2. Apical proteins are transported from the Golgi apparatus to the apical domain along a direct pathway and an indirect pathway via the basolateral membrane. In contrast, basolateral proteins never appear in the apical plasma membrane. Here we report on the effect of the microtubule-active drug nocodazole on the post-synthetic transport and sorting of plasma membrane proteins. Pulse-chase radiolabeling was combined with domain-specific cell surface assays to monitor the appearance of three apical and one basolateral protein in plasma membrane domains. Nocodazole was found to drastically retard both the direct transport of apical proteins from the Golgi apparatus and the indirect transport (transcytosis) from the basolateral membrane to the apical cell surface. In contrast, neither the transport rates of the basolateral membrane nor the sorting itself were significantly affected by the nocodazole treatment. We conclude that an intact microtubular network facilitates, but is not necessarily required for, the transport of apical membrane proteins along the two post-Golgi pathways to the brush border.

Key words: epithelia/microtubules/plasma membrane proteins/sorting/transcytosis

Introduction

Many epithelial cells display a morphological and functional surface polarity. The apical (also designated 'luminal', 'microvillar', or 'brush border') membrane faces the exterior and the basolateral membrane faces the internal milieu. The two cell surface domains are separated by tight junctions and exhibit distinct protein and lipid compositions. Proper sorting of these membrane components to their corresponding plasma membrane domains is essential for the maintenance of the various epithelial functions (for review see Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989).

In Madin-Darby canine kidney (MDCK) cells the sorting of apical and basolateral membrane glycoproteins has been shown to occur intracellularly (Matlin and Simons, 1984; Misek *et al.*, 1984; Rindler *et al.*, 1985; Caplan *et al.*, 1986; Lisanti *et al.*, 1989) most likely in the *trans*-Golgi network (Griffiths and Simons, 1986). In contrast, in hepatocytes apical proteins are routed to the basolateral membrane prior to their insertion into the apical domain (Bartles *et al.*, 1987). In the intestinal epithelial cell line Caco-2 apical proteins can be transported from the Golgi apparatus to the brush border membrane both along a direct pathway bypassing the basolateral membrane and an indirect route via the basolateral membrane (Matter *et al.*, 1990a). Thus, sorting of endogenous plasma membrane glycoproteins occurs from two sites: the Golgi apparatus and the basolateral membrane. The sorting process in the Golgi apparatus exhibits different efficiencies for different apical proteins. In contrast, in all investigated epithelial cell types endogenous basolateral membrane glycoproteins are routed to the basolateral membrane only (Caplan *et al.*, 1986; Bartles *et al.*, 1987; Matter *et al.*, 1990a).

Microtubules have been demonstrated to play an important role in the axoplasmic transport of organelles and vesicles (for reviews see Vale, 1987; Vale and Goldstein, 1990; Stebbings, 1990) as well as for the maintenance of the morphology of the Golgi apparatus (Rogalski and Singer, 1984; Thyberg and Moskalewski, 1985; Turner and Tartakoff, 1989). Therefore, microtubules may be important determinants for protein transport particularly for long distance pathways (Kelly, 1990). The effect of microtubule depolymerizing drugs on sorting and transport of epithelial plasma membrane proteins has been studied in different systems. For MDCK cells the published results are controversial. Rindler *et al.* (1987) reported a reduced apical appearance as well as a mis-sorting to the basolateral membrane of an apical membrane glycoprotein in nocodazole-treated cells, whereas Salas *et al.* (1986) were unable to detect any effect of microtubule-active drugs in the same model system. *In vivo* and organ culture studies have pointed to an involvement of microtubules in the transport of apical intestinal proteins (Herbst *et al.*, 1970; Quaroni *et al.*, 1979; Danielsen *et al.*, 1983; Bennett *et al.*, 1984; Hugon *et al.*, 1987; Achler *et al.*, 1989). Likewise, in Caco-2 cells nocodazole was found to disturb the transport of an apical membrane glycoprotein (Eilers *et al.*, 1989). However, the results obtained with intestinal cells were difficult to interpret since the transport pathways were not known in detail.

In this study, we have reinvestigated the effect of nocodazole on the cell surface appearance and sorting of plasma membrane glycoproteins in Caco-2 cells by applying three previously described cell surface assays (Matter *et al.*, 1990a). We present evidence that microtubules facilitate the transport of three apical membrane proteins to the brush border membrane both from the Golgi apparatus and from the basolateral membrane. An intact microtubular network is, however, not necessary for the proper sorting of apical and basolateral proteins.

Results

The microtubule depolymerizing drug nocodazole (De Brabander *et al.*, 1976) was used to destroy the microtubular

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network. Eilers *et al.* (1989) demonstrated by immunofluorescence that the microtubules of filter-grown Caco-2 cells can be depolymerized by 10 μg/ml nocodazole. Since long chase times (i.e. overnight) are required to monitor the cell surface appearance of some brush border hydrolases in Caco-2 cells (Matter *et al.*, 1990a) we tested the effect of long and short incubations of nocodazole on the degree of depolymerization of α-tubulin by measuring the extractibility of α-tubulin under microtubule-stabilizing conditions (Solomon, 1986). Filter-grown Caco-2 cells were incubated with 10 μg/ml nocodazole for either 3 h or overnight, or were mock-treated with the solvent DMSO. After extracting the cells (for details see Materials and methods) first under stabilizing conditions (free α-tubulin: extract I) and then under destabilizing conditions (polymeric α-tubulin: extract II) the amount of α-tubulin in each extract was determined by immunoblotting. Figure 1 shows that the effect of nocodazole is similar after incubations of 3 h or overnight. The quantification of these immunoblots (Table I) indicates that the effect of nocodazole is slightly more pronounced after the overnight incubation (99% free α-tubulin) than after a preincubation of 3 h (97% free α-tubulin). Polymeric tubulin in the same low range was also detected when the cells were preincubated on ice with nocodazole (not shown). Immunoblot analysis with an antibody specific for detyrosinated α-tubulin (Kreis, 1987) was inconclusive due to too weak signals. By immunofluorescence no differences were visible between the different methods of microtubule depolymerization (different times of nocodazole incubation +/− cold treatment).

In order to apply the cell surface assays it was necessary that the Caco-2 monolayers remained tight even after long incubations with nocodazole. As a parameter for the tightness of Caco-2 monolayers we measured the electrical resistance which is about 250 Ωcm^2 for passages 65−80 (Matter *et al.*, 1990a). The electrical resistance of Caco-2 monolayers was found not to be altered by nocodazole or DMSO.

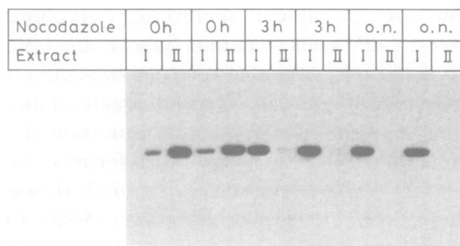

Fig. 1. Depolymerization of microtubules. Caco-2 cells were incubated with 10 μg/ml nocodazole for 3 h or overnight (o.n.) whereas control cells were incubated for 3 h or overnight with DMSO only (0 h). Thereafter, the cells were extracted (see Materials and methods) using first microtubule stabilizing conditions (Extract I) followed by microtubule destabilizing conditions (Extract II). The amount of α-tubulin in these extracts was determined by immunoblotting followed by autoradiography. Shown are duplicate experiments for each condition.

Table I. Depolymerization of microtubules by nocodazole

Nocodazole	0 h	3 h	o.n.
Extract I	28 ± 2	97 ± 1	99 ± 1
Extract II	72 ± 3	3 ± 1	1 ± 1

Caco-2 cells were preincubated with nocodazole or DMSO and the amount of free (Extract I) and polymeric (Extract II) α-tubulin was determined by immunoblotting (see Figure 1) and densitometric scanning of autoradiographs. Given are mean values ± 1 standard deviation of 4 independent experiments. (o.n., overnight).

Effect of nocodazole on the appearance of plasma membrane proteins in cell surface domains

In order to monitor the influence of nocodazole on the appearance of the three apical membrane glycoproteins dipeptidylpeptidase IV (DPPIV), sucrase isomaltase (SI), aminopeptidase N (ApN) and one basolateral membrane glycoprotein (BIMg: defined by a monoclonal antibody, see Eilers *et al.*, 1989) in the cell surface domains of Caco-2 cells we combined pulse-chase labeling with three polar cell surface assays (Matter *et al.*, 1990a). The appearance of each of these plasma membrane proteins was studied by two of these assays which are based on the accessibility of the basolateral membrane of intact Caco-2 cells grown on nitrocellulose filters to either antibodies, proteases, or to the biotinylation agent.

The transport to the cell surface of the basolateral protein BIMg was studied by the cell surface immunoprecipitation assay (Figure 2; Matter *et al.*, 1990a). In nocodazole-treated cells as in control cells BIMg could only be detected in the basolateral membrane suggesting that the sorting of BIMg is not affected by nocodazole. This conclusion was already drawn by Eilers *et al.* (1989) since the cell surface distribution of BIMg was not affected by the drug after a chase of 2 h (monitored by the protease assay). To compare the rates of transport (time of half maximal appearance) the experiments were quantified by densitometric scanning of fluorographs and the values were corrected for the total incorporation of [^{35}S]methionine into BIMg (not shown). This correction is necessary because the incorporation of the radioactive amino acid varies from one filter culture to another. For this reason the intensities of the bands in Figure 2 cannot be compared directly. These calculations revealed that the rates of transport were not significantly affected by nocodazole (not shown). We conclude that neither the sorting nor the rate of transport of the basolateral protein BIMg is affected by nocodazole.

Previously, the apical protein SI was found to be transported to the brush border membrane mainly by the direct pathway implying that it was sorted with high efficiency in the Golgi apparatus (Matter *et al.*, 1990a). The

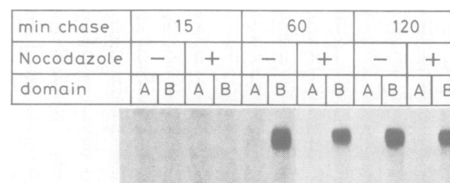

Fig. 2. Nocodazole does not affect appearance of the basolateral membrane glycoprotein BIMg in plasma membrane domains. Caco-2 cells preincubated for 3 h with 10 μg/ml nocodazole (+) or with solvent DMSO (−) were pulse labeled and chased for the indicated intervals of time. After incubating the cells at 4°C with a BIMg specific MAb either in the apical (A) or basolateral (B) medium the cells were extracted with Triton X-100 and the extracts were divided into two samples. One aliquot was used for precipitating the BIMg−IgG immunocomplexes and the other one was used for the determination of totally labeled BIMg by the use of a MAb recognizing an epitope other than the MAb used for the surface binding (not shown). The precipitates were analyzed by SDS−PAGE and fluorography.

effect of nocodazole on the sorting and transport of SI was studied by combining the metabolic labeling with the cell surface immunoprecipitation assay. The fluorograph in Figure 3A shows that only very small amounts of SI were detectable in the basolateral membrane independent of the presence or absence of the microtubule depolymerizing drug. Together with the polar expression after the overnight chase (see also the quantification in Figure 3B) this result suggests that the sorting of SI was not disturbed by nocodazole. The transport to the apical cell surface was, however, drastically retarded in the presence of the drug. Since the rate of transport to and through the Golgi apparatus (as deduced from the rate of complex glycosylation) was not affected (not shown) the retardation in the presence of nocodazole is due to the transport from the Golgi apparatus to the apical membrane. It can be concluded, therefore, that an efficient transport along the direct pathway from the Golgi apparatus to the apical plasma membrane depends on an intact microtubular network. Interestingly, after an overnight chase the levels of SI in the brush border membrane reached normal values in the absence of microtubules indicating that there is no absolute requirement for an intact microtubular network.

The two apical proteins DPPIV and ApN are transported from the Golgi apparatus to the apical cell surface along two pathways: a direct Golgi-to-brush border route and an indirect pathway via the basolateral membrane (Matter *et al.*, 1990a). Figures 4 (DPPIV) and 5 (ApN) (A: fluorographs; B: quantification by densitometric scanning) show the effect of nocodazole on the cell surface appearance of these two peptidases as monitored by the cell surface biotinylation assay. The rates of transport to the apical plasma membrane were similarly affected as in the case of SI (Figure 3). Interestingly, the rates of transport to the basolateral membrane were unchanged as for the basolateral protein BIMg (Figure 2). This may suggest that DPPIV and ApN are transported to the basolateral membrane along the same pathway as BIMg. Once in the basolateral membrane DPPIV

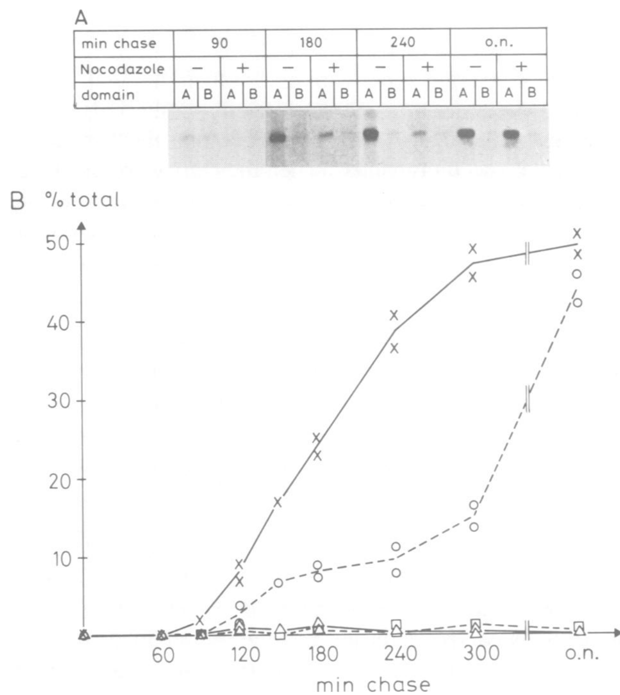

Fig. 3. Nocodazole retards apical appearance of SI. (**Panel A**) After a preincubation of 3 h with (+) or without (−) nocodazole Caco-2 cells were pulse labeled and chased as indicated. The cell surface appearance of SI was monitored by incubating the monolayers with MAb HBB2/614 in either the apical (A) or basolateral (B) medium at 4°C. Triton X-100 extracts were divided into two aliquots. One sample was incubated with protein A-Sepharose (isolation of surface bound antigens, panel A) and from the other SI was immunoprecipitated with MAbs specific for an epitope other than that recognized by antibody HBB2/614 (not shown) in order to determine the total amount of radioactive SI. The precipitates were analyzed by SDS−PAGE and fluorography and the latter were quantified by densitometric scanning. (**Panel B**) Quantification; cell surface immunoprecipitated SI is given as the percent of totally immunoprecipitable radioactive SI derived from the same cell extract. (———) control cells: (X) apical domain, (△) basolateral domain; (---------) nocodazole treated cells: (○) apical domain, (□) basolateral domain; o.n., overnight.

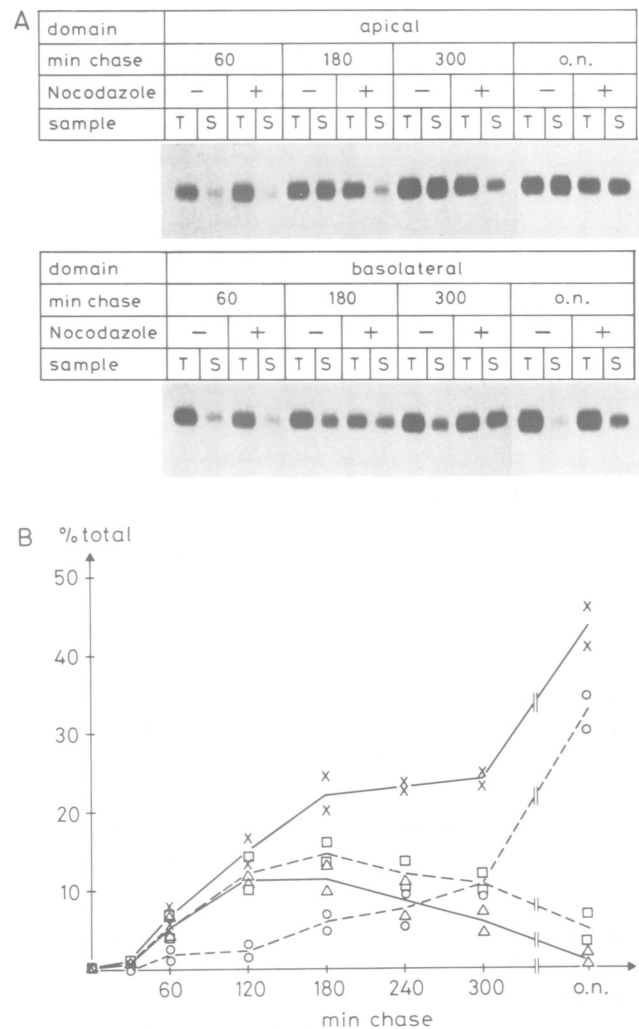

Fig. 4. Effect of nocodazole on cell surface appearance of DPPIV. After pulse−chase labeling with (+) or without (−) nocodazole either the apical or basolateral membrane of Caco-2 cells was biotinylated with NHS-SS-Biotin. The Triton X-100 extracts were divided into two samples and from both DPPIV was immunopurified. One immunoprecipitation was loaded directly on SDS−PAGE gels (T, used for quantification in panel B) and from the other one the immunoprecipitated DPPIV was eluted and further purified with avidin−agarose (S). Shown are fluorographs of 10% gels (**panel A**). Fluorographs were quantified by densitometric scanning (**panel B**). The amount of precipitated biotinylated antigen is expressed as the percent of totally immunoprecipitable DPPIV from the same cell extract. (———) control cells; (X) apical domain, (△) basolateral domain; (---------) nocodazole treated cells; (○) apical domain, (□) basolateral domain; o.n., overnight.

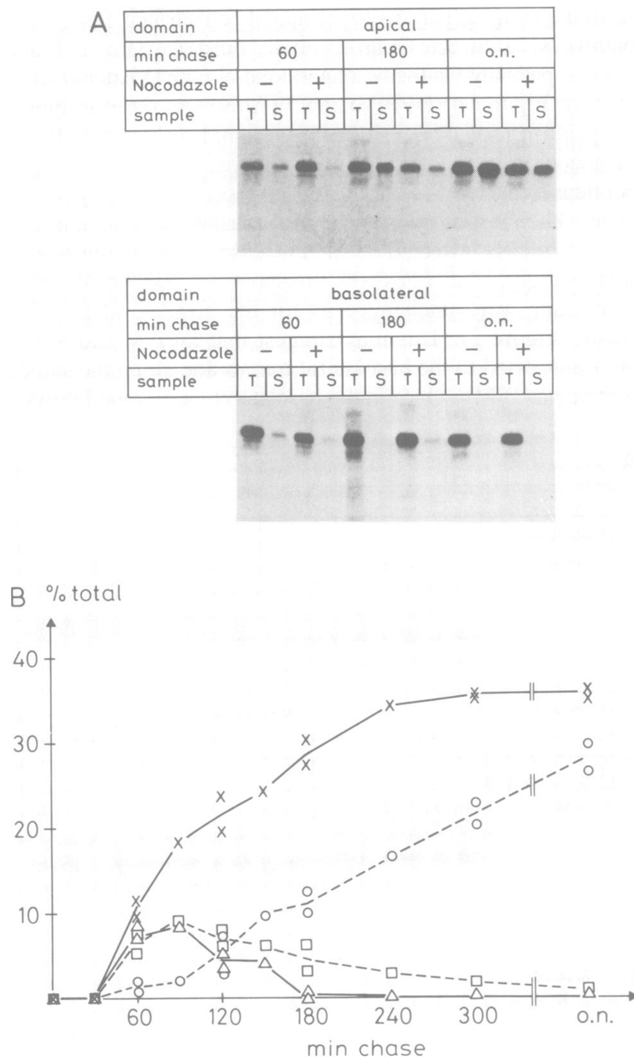

Fig. 5. Effect of nocodazole on cell surface appearance of ApN. After a preincubation of 3 h with (+) or without (−) nocodazole Caco-2 cells were pulse–chase labeled and the appearance of ApN was analyzed by the biotinylation assay as described for DPPIV in Figure 4. The fluorographs were quantified and the amount of biotinylated antigen was expressed as the percent of totally immunoprecipitable ApN. **Panel A**: T, total immunoprecipitable ApN; S, biotinylated ApN. **Panel B**: (———) control cells: (X) apical domain, (△) basolateral domain; (---------) nocodazole treated cells: (○) apical domain, (□) basolateral domain; o.n., overnight.

and ApN are internalized and transcytosed to the apical membrane (Matter *et al.*, 1990a). Both peptidases exhibited a longer half-life in the basolateral membrane in the presence of the drug (Figures 4 and 5). Whether this is due to retarded endocytosis from the basolateral membrane or to a slowed-down delivery to the basolateral membrane needs measuring of the rate of internalization (see below). That in the presence of nocodazole the proteins appear with the same kinetics in the basolateral membrane as in control cells but do not reach a significantly higher maximal level already favors the notion that sorting in the Golgi apparatus is not affected by nocodazole. This conclusion is further supported by the finding that sorting of SI is not affected by microtubule depolymerization (Figure 3). The slower disappearance from the basolateral membrane explains the results of Eilers *et al.* (1989) where ApN was found at an enhanced level in the basolateral membrane and at a reduced level in the apical membrane of nocodazole-treated cells after a [^{35}S]methionine chase of 2 h. In the present study the nocodazole-induced increase of ApN in the basolateral membrane at 2 h was less pronounced than in our previous paper (Eilers *et al.*, 1989). This is most likely due to the following two facts. First, in the present study a lower passage of Caco-2 cells was used in which the transport kinetics of ApN are somewhat slower than in Eilers *et al.* (1989) (K.Matter and H.-P.Hauri, unpublished). Second, the biotinylation assay, since performed at 0°C, exclusively measures cell surfaces ApN, while the previously used protease assay, since performed at 37°C, may also measure some internalized ApN.

Surprisingly, the levels of ApN and DPPIV in the brush border membrane rise enormously during an overnight chase in the presence of nocodazole but they never reach normal values. Extending the times of chase up to 30 h did not lead to a further increase of these proteins in the brush border membrane. Nevertheless, basolateral values are very low after overnight incubations indicating that the polar cell surface expression of these two peptidases does not critically depend on an intact microtubular network as for SI.

Nocodazole retards transcytosis of DPPIV and ApN

For a more precise interpretation of the transport kinetics of ApN and DPPIV the effect of nocodazole on the corrective transcytosis of newly synthesized peptidases was studied. This was achieved by the previously described combination of the biotinylation assay with one of the other cell surface assays (Matter *et al.*, 1990a). Briefly, after pulse-chase labeling of the cells with [^{35}S]methionine the basolateral membrane was biotinylated. Then the cells were incubated again at 37°C and finally subjected to the cell surface immunoprecipitation procedure (DPPIV) or the protease assay (ApN) in order to visualize the basolateral disappearance and the apical appearance of the biotinylated peptidases.

Figure 6 demonstrates that the basolateral disappearance of DPPIV is less retarded by nocodazole than the apical appearance of basolaterally biotinylated DPPIV. Similar results were obtained for ApN (Figure 7). The reduced rates of internalization from the basolateral membrane explain why the newly synthesized peptidases exhibit a longer half-life in the basolateral membrane (Figures 4 and 5) and, therefore, do not contradict our conclusion that sorting of DPPIV and ApN in the Golgi apparatus is unaffected by nocodazole. The kinetic difference between basolateral internalization and apical reappearance indicates that the transcellular transport itself is the most sensitive step of basolateral-to-apical transcytosis.

Since after the overnight incubation the transport of basolaterally biotinylated peptidases to the apical membrane was nearly complete in nocodazole-treated cells one can draw the same conclusions for the effect of nocodazole on the indirect pathway as for its influence on the direct pathway. First, basolateral-to-apical transcytosis does not necessarily require an intact microtubular network but it is drastically retarded in the presence of nocodazole and second, sorting to the apical membrane after internalization from the basolateral membrane is not affected by nocodazole.

Apical endocytosis and recycling are not affected by nocodazole

DPPIV and to a lesser extent SI are endocytosed from the brush border membrane of Caco-2 cells and efficiently

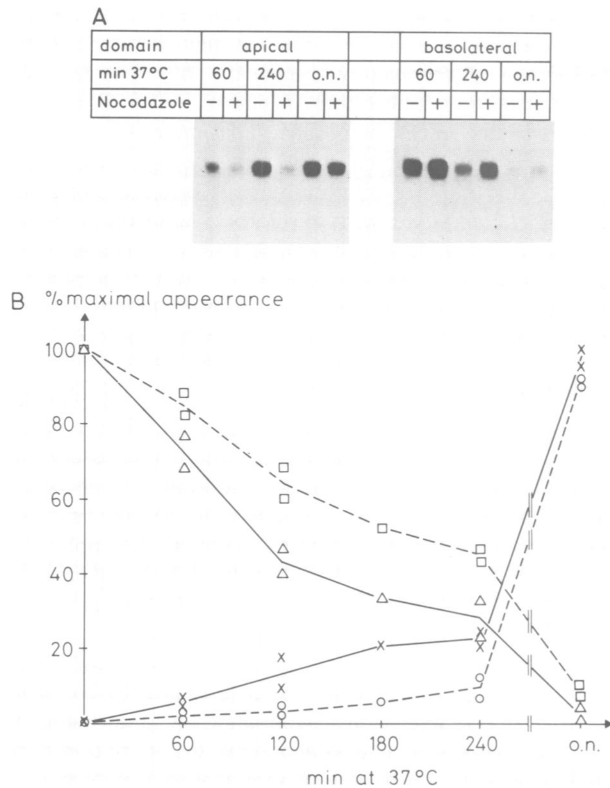

Fig. 6. Nocodazole retards transcytosis of DPPIV. **Panel A**: after preincubating with (+) or without (−) nocodazole Caco-2 cells were pulse labeled for 30 min and chased for 2 h. Thereafter the basolateral membrane was biotinylated at 4°C, and the modified cells were incubated at 37°C for the indicated intervals of time in the presence or absence of the drug. After cooling the cells of 4°C surface DPPIV was immunoprecipitated, and after elution from the immunobeads further purified with avidin−agarose beads. The resulting biotinylated DPPIV derived from either the apical or basolateral plasma membrane domain was analyzed by SDS−PAGE and fluorography (panel A). **Panel B**: the amount of basolaterally biotinylated immunoprecipitated DPPIV derived from either of the membrane domains was divided by the amount of biotinylated DPPIV obtained from the same detergent extracts, and the resulting ratio was expressed as the percent of the maximally obtained value (panel B). (———) control cells: (X) apical domain. (△) basolateral domain; (---------) nocodazole treated cells: (○) apical domain, (□) basolateral domain; o.n., overnight.

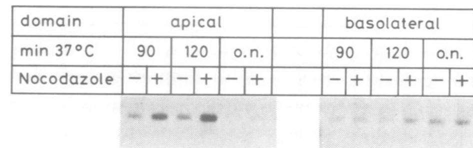

Fig. 7. Transport of newly synthesized ApN from the basolateral to the apical membrane is retarded by nocodazole. After preincubating with (+) or without (−) nocodazole Caco-2 cells were pulse labeled, chased for 75 min, cooled to 4°C, and the basolateral membrane was biotinylated. Thereafter the cells were incubated again at 37°C in the presence or absence of nocodazole. After the indicated intervals of time cells were digested from either side of the monolayer, and extracted with Triton X-100 followed by purification of biotinylated ApN (fluorographs of 10% gels).

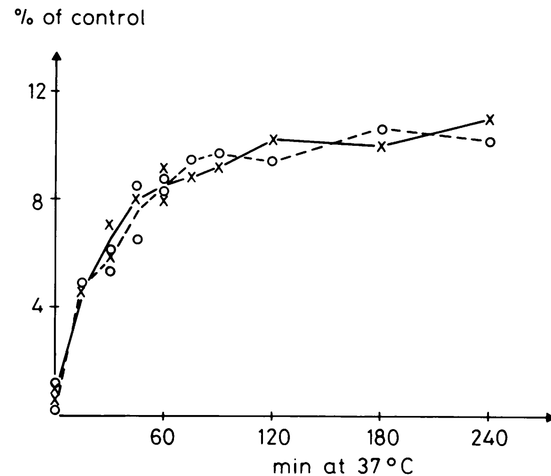

Fig. 8. Nocodazole does not influence endocytosis and recycling of apical DPPIV. Caco-2 cells were preincubated for 3 h with nocodazole or DMSO only. Thereafter the cells were labeled at 4°C with [^{125}I]Fab fragments specific for DPPIV, and incubated at 37°C for different intervals of time in the presence or absence of nocodazole. After cooling to 4°C Fab fragments bound to surface DPPIV were eluted by low pH, the remaining cell-associated radioactivity was measured and expressed as the percent of the radioactivity bound to identically incubated cells which were not treated with the low pH buffer. (X———X) control cells; (○---------○) nocodazole treated cells.

re-externalized to the apical plasma membrane (Matter *et al.*, 1990b). Therefore, the finding that the peptidases never reached the normal apical level in the presence of nocodazole (Figures 4 and 5) could be due to an interference of nocodazole with the equilibrium of apical endocytosis and recycling. To test this possibility we measured this equilibrium in nocodazole-treated cells using a previously described assay (Matter *et al.*, 1990b) as follows. Caco-2 cells were labeled with [^{125}I]Fab fragments specific for DPPIV and the antibody fragments remaining (or reappearing) at the cell surface after an incubation at 37°C for different intervals of time were eluted by low pH. Figure 8 shows that the equilibrium between endocytosed and re-externalized Fab fragments is not affected by microtubule perturbation. The original rates of internalization as well as the equilibrium levels of internal DPPIV are identical with or without nocodazole. Since the reduced maximal apical level of DPPIV in the presence of nocodazole was found after an overnight chase this reduction may be a long-term effect. A preincubation with nocodazole for 20 h, however, did not change the result of the endocytosis assay (not shown). Thus, the process of apical endocytosis and re-externalization of DPPIV was not influenced by nocodazole. In the course of this study we noticed that in Caco-2 cells of passages around 70 the equilibrium of apically internalized DPPIV was at 10%, hence somewhat lower than in passages around 140 (Matter *et al.*, 1990b).

Discussion

This study focuses on the effect of microtubular perturbation on the cell surface appearance of three apical and one basolateral plasma membrane glycoproteins in the intestinal epithelial cell line Caco-2. The experiments indicate that neither the transport to the basolateral membrane nor the sorting of both classes of plasma membrane proteins is influenced by nocodazole. Transport along both pathways to the apical membrane, however, is drastically retarded in the absence of an intact microtubular network.

In intestinal tissue transport of glycoproteins to the apical cell surface domain of enterocytes has been shown to be influenced by microtubular perturbation either by using electron microscopical (Blok *et al.*, 1981; Pavelka and

Ellinger, 1981; Ellinger *et al.*, 1983; Bennett *et al.*, 1984; Hugon *et al.*, 1987; Achler *et al.*, 1989) or biochemical approaches (Quaroni *et al.*, 1979; Danielsen *et al.*, 1983). A previous study from our laboratory has pointed to a similar effect of nocodazole in the intestinal epithelial cell line Caco-2 (Eilers *et al.*, 1989). It was found that nocodazole led to an increased appearance of aminopeptidase N in the basolateral membrane. Since the routes to the cell surface were unknown at the time and a detailed kinetic analysis was not performed it was unclear if the results indicated inhibition of a transport pathway via the basolateral membrane or mis-sorting. The present experiments clearly demonstrate that the previously described effect of nocodazole is not due to mis-sorting in the Golgi apparatus but to a retardation of the indirect pathway. More precisely, the internalization of apical proteins in the basolateral membrane is slowed down. Even more pronounced is the inhibition of the indirect pathway at an intracellular site. The drastically retarded apical appearance, however, it caused by a combined effect of nocodazole on the direct route and on transcytosis.

Interestingly, transport of secretory and plasma membrane proteins are affected in a similar way by nocodazole. Eilers *et al.* (1989) have shown that the apical secretion of cathepsin D and acid α-glucosidase after a chase of 2 h was drastically reduced by nocodazole. In contrast, the amount of basolaterally secreted cathepsin D and α-glucosidase remained unaffected and was not increased. Thus, neither apical membrane proteins nor apically secreted proteins are mis-sorted in Caco-2 cells treated with nocodazole but their transport to the apical cell surface is inhibited by microtubule perturbation.

For MDCK cells data on the role of microtubules for protein sorting are conflicting. Whereas Rindler *et al.* (1987) detected a mis-sorting of the apical glycoprotein influenza virus hemagglutinin in nocodazole treated MDCK cells, Salas *et al.* (1986) did not find any effect of microtubule depolymerization. Furthermore, two secretory proteins were found to be mis-sorted after microtubule disruption (Parczyk *et al.*, 1989). Lipid sorting in MDCK cells exhibited yet another nocodazole effect. Transport to the apical membrane was reduced but no mis-sorting was detectable upon nocodazole treatment (H.G.van Meer, personal communication). Currently, it is difficult to decide if these differences between MDCK and Caco-2 cells are due to tissue differences similar to the variations in the sorting pathways of apical membrane proteins in these two cell lines. Transport of basolateral membrane proteins, on the other hand, was affected neither in MDCK (Salas *et al.*, 1986; Rindler *et al.*, 1987) nor in Caco-2 cells by microtubule depolymerization. Nevertheless, in a study on the architecture of microtubules in MDCK cells Bacallao *et al.* (1989) hypothesized that microtubules may be involved in the transport to the basolateral membrane as well and van der Sluijs *et al.* (1990) observed attachment of basolateral transport vesicles to microtubules *in vitro*. The question arises therefore if the basolateral transport depends on nocodazole-resistant microtubules. In the present study protein transport to the basolateral membrane was not even inhibited by a combination of nocodazole with cold treatment (data not shown), a procedure which also depolymerizes the more stable microtubules (Bacallao *et al.*, 1989). Nevertheless, we cannot formally exclude the possibility that the basolateral vesicle transport occurs along extremely resistant microtubules.

An interesting aspect of the nocodazole effect on Caco-2 cells is that the transport to the apical membrane was only retarded but not completely abolished. This indicates that microtubules are not absolutely necessary but facilitate the transport to the apical membrane. That microtubules may serve as tracks to the apical membrane is supported by two findings. Microtubules are preferentially oriented along the long axis of enterocytes *in vivo* (Gorbski and Borisi, 1985; Hugon *et al.*, 1987), and they penetrate the terminal web and end in close vicinity of the brush border membrane (Sandoz *et al.*, 1985; Hagen *et al.*, 1987). This is in line with the notion that translocation of apical transport vesicles to the apical plasma membrane may be speeded up by microtubules possibly via microtubule motors (Schnapp *et al.*, 1985; Vale *et al.*, 1985; Paschal *et al.*, 1987; Vale, 1987; Scholey, 1990), but that the information necessary for the fusion with the correct target membrane is a property of the vesicles themselves. It is important to note that the major inhibition of the transcytotic pathway was found to be the transport of basolaterally internalized ApN and DPPIV to the apical membrane. Thus, it is the actual transport step again which is facilitated by an intact microtubular network. The same is true for the polyimmunoglobulin receptor in colchicine-treated HT29 cells (Nagura *et al.*, 1979). It is difficult to completely rule out that a few microtubules survive the nocodazole treatment and that the apical transport would in fact have an absolute requirement for microtubules. We believe this to be unlikely since a combination of nocodazole and cold treatment did not modify the nocodazole results.

That both pathways to the apical membrane are sensitive to nocodazole may suggest that apical proteins internalized from the basolateral membrane return to the *trans*-Golgi network prior to be routed to the apical membrane along the direct pathway. This appears unlikely since in MDCK cells the Golgi apparatus has been shown not to participate in transcytosis, at least in an apical to basolateral direction (Pesonen *et al.*, 1984). Furthermore, by using sulfation of tyrosine residues as a marker for passage through the trans-Golgi network we obtained no evidence that basolaterally as well as or apically endocytosed DPPIV passes through the Golgi complex prior delivery to the apical cell membrane (K.Matter and H.-P.Hauri, unpublished data).

During the overnight chase the levels of apical proteins in the brush border membrane increased in the presence of nocodazole but did not quite reach normal values particularly for the peptidases. This may be due to an effect of nocodazole on apical recycling of brush border enzymes (Matter *et al.*, 1990b). Internalization and recycling of DPPIV, however, were identical in the presence or absence of nocodazole. Another possibility is that nocodazole leads to an enhanced delivery of apical proteins to lysosomes via a previously described pathway which bypasses the apical membrane (Matter *et al.*, 1990b). Yet another possibility is that a fraction of the peptidases is arrested somewhere inside the cells. Two groups have reported the formation of basolateral microvilli and microvilli-covered vacuoles in enterocytes upon treating rats with colchicine (Pavelka and Gangl, 1983; Achler *et al.*, 1989). Achler *et al.* (1989) found that these unusual microvillar structures indeed carry certain brush border enzymes and that basolateral microvilli are endocytosed and fuse with the vacuoles. Finally, these vacuoles fuse with the brush border membrane. A similar mechanism would fit with our data since the two apical proteins which

are transported to the basolateral membrane in significant amounts (i.e. DPPIV and ApN) are actually those which exhibit the most extensive reduction of the maximal apical level. However, when Caco-2 cells were treated with nocodazole neither basolateral nor intracellular microvillar structures could be detected (U.Eilers, J.Klumperman and H.-P.Hauri, unpublished data). Futhermore, the lower levels of ApN and DPPIV in the brush border membrane were stable and did not further increase during prolonged chase times. It is not clear why Caco-2 cells and enterocytes *in vivo* display such morphological differences after microtubule perturbation. A possible reason may be a lower potential to form microvilli: Caco-2 cells display a less developed brush border than enterocytes *in vivo*. Alternatively, the difference may be due to the drugs (nocodazole versus colchicine).

In conclusion, in filter-grown Caco-2 cells the transport of newly synthesized proteins along both pathways to the apical membrane, i.e. the direct route as well as the indirect transcytotic pathway is facilitated by an intact microtubular network. In contrast, neither sorting itself nor the rates of transport to the basolateral membrane of basolateral as well as apical proteins are influenced by nocodazole. A future challenge will be the identification of the motors which move transport vesicles along microtubules.

Materials and methods

Cell culture and labeling with [^{35}S]methionine

Caco-2 cells were cultured as previously described (Hauri *et al.*, 1985; Stieger *et al.*, 1988). For cell surface assays the cells were grown in Millicell chambers (Millipore Corp., Bedford, MA) and labeled with [^{35}S]methionine as described (Matter *et al.*, 1990a). In all experiments Caco-2 passages between 65 and 80 were used. Nocodazole incubations were carried out as described by Eilers *et al.* (1989) using 10 μg/ml nocodazole (diluted from a fresh 3 mg/ml stock solution in DMSO). Controls were incubated with the same dilution of DMSO. For pulse–chase experiments the cells were preincubated for 3 h in nocodazole-containing medium.

Cell surface assays, immunological techniques, and SDS–PAGE

Cell surface immunoprecipitation. (Matter *et al.*, 1990a) An MAb (monoclonal antibody) specific for a given plasma membrane protein was added to either the apical or basolateral medium at 4°C. After rinsing the cells were solubilized in the presence of a Fab fragment derived from the same MAb and IgG–antigen complexes were precipitated with protein A–Sepharose beads. Total values of incorporated [^{35}S]methionine were determined by precipitating the same antigen with an antibody specific for another epitope.

Cell surface biotinylation. (Matter *et al.*, 1990a) This operation was performed with NHS-SS-Biotin (Pierce, The Netherlands). After biotinylation of either the apical or basolateral plasma membrane domain the cells were solubilized and the antigens were immunoprecipitated with MAbs. The antigens were eluted from the immunobeads by low pH treatment and the biotinylated proteins were further purified with avidin–agarose beads.

Protease assay. This operation was performed with papain and elastase as described previously (Eilers *et al.*, 1989). For normalizing unequal [^{35}S]methionine incorporations MAb G1/93 against an intracellular antigen (Schweizer *et al.*, 1988) was used.

The following MAbs against plasma membrane glycoproteins were used. DPPIV: HBB3/775 (Hauri *et al.*, 1985) and HBB3/456 (Matter *et al.*, 1990b); SI: HBB3/705, HBB2/614, HBB2/219, and HBB1/691 (Hauri *et al.*, 1985); ApN: HBB3/153 (Hauri *et al.*, 1985) and HBB3/344 (Eilers *et al.*, 1989); BIMg: G1/136, G1/75 (Eilers *et al.*, 1989), and G1/110 (Matter *et al.*, 1990a). Fab fragments were prepared and purified as described previously (Matter *et al.*, 1990b). Endocytosis of apical DPPIV was measured by labeling cells with [^{125}I]Fab fragments (derived from MAb HBB3/775) followed by acid stripping after incubating the cells at 37°C (Matter *et al.*, 1990b). Immunoprecipitates were analyzed by SDS–PAGE and fluorography (Hauri *et al.*, 1985) and fluorographs were quantified with a Camag LTC Scanner II (Stieger *et al.*, 1988).

Tubulin extraction

Tubulin was extracted essentially as described by Solomon (1986) using filter-grown Caco-2 cells. The chambers were disassembled and the filters carrying the cells were washed at 37°C twice with PBS and once with PM2G (100 mM PIPES, 2 mM EDTA, 5 mM $MgCl_2$, 2 M glycerol, 40 μg/ml phenylmethylsulfonyl fluoride, pH 6.9). Filters were then transferred into 1 ml of prewarmed (37°C) PM2G containing 0.2% Nonidet P-40 and incubated at 37°C. After 20 min the extract was transferred to a plastic tube and 1 ml of the same buffer was added to the cells. After another 15 min at 37°C this second extract was pooled with the first extract (Extract I). Up to this step all used buffers contained either 10 μg/ml nocodazole derived from a 3 mg/ml stock solution in DMSO (if nocodazole-treated cells were extracted) or a corresponding amount of DMSO (extraction of control cells). The cells were washed once with PM2G (37°C) and transferred to a precooled tube with 1 ml of PM2G containing 5 mM $CaCl_2$ and 0.2% Nonidet P-40. After 20 min on ice the extract was transferred to a new tube and the incubation was repeated with 1 ml of the same buffer. After 15 min this last extract was pooled with the other Ca^{2+}-extract (Extract II). Equal volumes of extracts I and II were processed for SDS–PAGE (10% gels). Immunoblotting was performed as described (Hauri and Bucher, 1986) using antibody αT13 which is specific for tyrosinated α-tubulin (Kreis, 1987). If the material remaining on the nitrocellulose filters after these extractions was analyzed no tubulin was detectable.

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