Nocodazole, a Microtubule-active Drug, Interferes with Apical Protein Delivery in Cultured Intestinal Epithelial Cells (Caco-2)

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Abstract. The polarized delivery of membrane proteins to the cell surface and the initial secretion of lysosomal proteins into the culture medium were studied in the polarized human intestinal adenocarcinoma cell line Caco-2 in the presence or absence of the microtubule-active drug nocodazole. The appearance of newly synthesized proteins at the plasma membrane was measured by their sensitivity to proteases added either to the apical or the basolateral surface of cells grown on nitrocellulose filters. Nocodazole was found to reduce the delivery to the cell surface of an apical membrane protein, aminopeptidase N, and to lead to its partial missorting to the basolateral surface, whereas the drug had no influence on the delivery of a basolateral 120-kD membrane protein defined by a monoclonal antibody. Furthermore, nocodazole selectively blocked the apical secretion of two lysosomal proteins, cathepsin D and acid α-glucosidase, whereas the drug had no influence on their basolateral secretion. These results suggest that in Caco-2 cells an intact microtubular network is important for the transport of newly synthesized proteins to the apical cell surface.

MANY epithelial cells display two clearly distinguishable plasma membrane domains: an apical (also designated "luminal" or "brush border") membrane facing the exterior and a basolateral membrane. These two domains are separated by tight junctions and show a strikingly different ultrastructure and protein composition (for review see Simons and Fuller, 1985). The mechanisms by which epithelial cells generate and maintain cell surface polarity are unknown. Several observations have demonstrated that epithelial cells have the capacity to sort newly synthesized proteins destined for either of the two cell surface domains already intracellularly (Matlin and Simons, 1984; Misek et al., 1984; Rindler et al., 1985), presumably in the *trans*-Golgi network (Griffiths and Simons, 1986). On the other hand, sorting pathways have been postulated by which apical proteins are transported to the basolateral surface before their insertion in the apical domain (Quaroni et al., 1979; Bartles et al., 1987; Massey et al., 1987, see Bartles and Hubbard, 1988 for review). Cytoskeletal elements, in particular microtubules, may play an important role in this sorting process. Studies in whole animals have pointed to an involvement of microtubules in the transport of proteins to the apical surface (Herbst et al., 1970; Quaroni et al., 1979; Danielsen et al., 1983; Bennett et al., 1984) whereas results obtained with cultured epithelial cell lines have been controversial (Salas et al., 1986; Rindler et al., 1987).

In this study, we examined the polarized delivery of endogenous membrane proteins and secreted lysosomal proteins of the human intestinal cell line Caco-2. Using nocodazole as a microtubule-active agent we present evidence that an intact microtubular network is important for efficient protein transport to the apical cell surface.

Materials and Methods

Monoclonal Antibody Technique

A fraction enriched in Golgi membranes of Caco-2 cells (essentially according to Stieger et al., 1988) was used as an antigen to produce monoclonal antibodies in mice. Balb/c mice were intracutaneously injected with 425 μg membrane protein in 200 μl PBS emulsified with 300 μl complete Freund's adjuvant. A booster injection with 610 μg antigen emulsified with incomplete Freund's adjuvant was administered intraperitoneally 7 wk later. 11 wk after the first immunization a final booster was given with 685 μg protein in PBS, and 3 d later cell fusion was carried out according to established techniques (Fazekas de St. Groth and Scheidegger, 1980; Galfré et al., 1977; Hauri et al., 1985) using PAI myeloma cells (Stocker et al., 1982). Initial screening of culture supernatants was performed by the dot-immunobinding assay described by Hawkes et al. (1982). Positive cultures were expanded and the cells were frozen in liquid N_2. The culture media were concentrated 10 times by ammonium sulfate precipitation (to 50% saturation) and subsequent dialysis against PBS pH 7.4 containing 0.1% sodium azide. Immunofluorescence (see below) was used for secondary screening. Hybridoma lines secreting basolateral-specific antibodies were subcloned and two monoclonal antibodies G1/136 and G1/75 were obtained. Both antibodies were of the IgG_1 subclass as assessed by Ouchterlony double diffusion assays and subclass-specific secondary antibodies and were specific for the same 120-kD protein (see Results).

Other monoclonal antibodies used in this study are: G1/93, against a 53-kD protein localized in putative transport vesicles at the *cis* side of the Golgi apparatus (Schweizer et al., 1988) and HBB3/344, against human aminopeptidase *N*. The latter antibody, an IgG2a, was prepared according to Hauri et al. (1985) and was found to immunoprecipitate aminopeptidase *N* more efficiently than the previously described antibody HBB3/153. Monoclonal

antibodies were either used as 10× concentrated culture supernatants or in ascites form with identical results. For the precipitation of α-glucosidase the monoclonal antibody 118 G3 was used which was raised against acid α-glucosidase purified from human placenta (Hilkens et al., 1981). Cathepsin D was immunoprecipitated with a polyclonal rabbit antiserum (kindly provided by Dr. J. M. Tager, University of Amsterdam, The Netherlands). Tubulin was localized with a monoclonal antibody against Tyr tubulin kindly provided by Dr. T. Kreis (EMBL, Heidelberg, FRG; Kreis, 1987).

Immunofluorescence

Immunofluorescence was performed by an indirect technique essentially according to Fuller et al. (1984). In brief, filter-grown Caco-2 cells were fixed for 30 min with 3% formaldehyde at room temperature, washed with PBS, and quenched with 50 mM ammonium chloride in PBS. The cells were then permeabilized with 0.2% (wt/vol) Triton X-100 in PBS containing 0.2% (wt/vol) gelatin for 4 min and stained with monoclonal antibodies against tubulin (ascites fluid, 1:1,000 diluted) or the 120-kD protein (ascites fluid, 1:300 diluted) for 25 min at 37°C. The secondary antibody was a rhodamine-conjugated goat anti-mouse IgG (Cappel Laboratories, Malverne, PA) used at a dilution of 1:50. Photographs were taken with a Leitz Ortholux II fluorescence microscope.

Immunoelectron Microscopy

5–8 d after confluency of the cells, the filters were taken out of the chambers and rinsed three times with PBS. Subsequently, the cells were fixed in a mixture of 0.1% glutaraldehyde and 1% freshly prepared formaldehyde in 0.15 M sodium bicarbonate buffer (pH 7.4) for 1 h at room temperature. The use of sodium bicarbonate buffer instead of phosphate buffer resulted in a better preservation of the ultrastructure of the cells. Especially the fixation of glycogen was improved (Artvinli, 1975). After fixation, the cells were washed again and gently scraped off the filter with a rubber policeman. The cells were pelleted in 10% gelatin and subsequently postfixed and stored for at least 24 h at 4°C in 1% formaldehyde in 0.1 M phosphate buffer (pH 7.4). Ultrathin cryosections were cut with a Reichert-Jung ultracut E with cryoattachment FC 4D. The sections were incubated with the specific antibody. In the case of a monoclonal antibody a second incubation step with rabbit anti-mouse was added. Antibody binding was visualized with protein A-colloidal gold (Slot and Geuze, 1985). The sections were examined in a Philips EM 410 or EM 201 electron microscope.

Pulse-Chase Experiments

For labeling of newly synthesized proteins with [^{35}S]methionine, Caco-2 cells were grown in Mini Marbrook chambers on nitrocellulose filters (Millipore Continental Water Systems, Bedford, MA). All experiments were performed 5–8 d after the cells had reached confluency. The cells were preincubated upside down for 10 min at 37°C in PBS containing 20% dialyzed FCS. After a 15-min pulse at 37°C with 100 μCi [^{35}S]methionine per filter (added in 300 μl PBS + 20% FCS to the basolateral side) the cells were washed with PBS and chased for the indicated times in normal culture medium (Dulbecco's MEM, 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml fungizone, 1 ml/100 ml nonessential amino acids [100×]; Gibco Laboratories, Grand Island, NY) plus 10 mM unlabeled methionine.

Nocodazole Incubation

A 3 mg/ml stock solution of nocodazole (Aldrich Chemical Co., Milwaukee, WI) was prepared in DMSO. For all experiments the cells were preincubated with nocodazole in complete medium for 3 h at 37°C at a final concentration of 10 μg/ml. The controls were incubated with the corresponding amount of DMSO alone. The subsequently used pulse and chase media also contained nocodazole and/or DMSO in the same concentrations.

Protease Assay and Immunoprecipitation

The appearance of newly synthesized membrane proteins at the cell surface was studied by protease digestion. After different times of chase the cells were washed twice with PBS at 37°C. For digestion 10 mg/ml papain (E. Merck, Darmstadt, FRG) and 0.5 mg/ml elastase (Serva Fine Biochemicals Inc., Garden City Park, NY) in 10 mM cysteine hydrochloride in PBS pH 6.8 were added either from the apical or the basolateral side to the cell monolayer (i.e., inside or outside of the Mini Marbrook chamber). As a control, cells were incubated in PBS without proteases. After exactly 5 min at 37°C the digestion was stopped with ice-cold 20 mM iodoacetic acid and 40 μg/ml PMSF in PBS. The filters were washed three times for 10 min in the stopping buffer. After the first washing step the filters were removed from the Mini Marbrook chambers. Finally, the cells were scraped from the filter in 1 ml 100 mM sodium phosphate, pH 8.0, containing 0.1% sodium azide, 1% Triton X-100, 10 mM iodoacetic acid, and 40 μg/ml PMSF. After homogenization with a syringe connected to a 25-gauge needle followed by a 1-h solubilization on ice, the cells were centrifuged at 100,000 *g* for 1 h at 4°C. Identical radioactivity aliquots (based on TCA precipitation) were immunoprecipitated (2 h, 4°C) with protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) to which the monoclonal antibodies had been adsorbed at pH 8.0 (Hauri et al., 1985). After four wash steps with 100 mM sodium phosphate, pH 8.0, 0.2% BSA, 1% Triton X-100, 0.1% sodium azide, and 40 μg/ml PMSF, and two additional wash steps with 100 mM sodium phosphate without Triton X-100 and BSA and one step with 10 mM sodium phosphate, the immunoprecipitates were subjected to SDS-PAGE. 10% SDS polyacrylamide slab gels were used and labeled proteins were visualized by fluorography using EN^{3}HANCE (New England Nuclear, Boston, MA).

Immunoblotting

Caco-2 cells were immunoprecipitated as described above with either of the two antibodies against the 120-kD basolateral antigen (G1/75 and G1/136). The method of Towbin et al. (1979) was used to transfer the immunoprecipitated proteins from SDS gels to nitrocellulose sheets. 3-mm nitrocellulose strips were either incubated with the same antibody as used for the immunoprecipitation or with the other antibody to assess whether the two probes bound to the same protein. The immunoreaction was visualized by ^{125}I-protein A using 1% powdered milk in PBS as a blocking solution (Hauri and Bucher, 1986).

Calculation of Results

Preflashed films were used for fluorography and the fluorograms were scanned with a Camag TLC scanner II connected to an SP 4290 integrator. Results for aminopeptidase *N* or the basolateral 120-kD antigen were corrected for nonspecific proteolysis and unequal incorporation by normalizing the values according to the amount of coprecipitated intracellular 53-kD protein present in each gel lane. The data were expressed as means ± 1 SD. We observed that the efficiency of digestion in the surface protease assay was more variable between than within experimental series. For this reason the data were statistically evaluated by a paired *t* test in which nocodazole-treated cultures were directly compared with the corresponding controls of the same series.

Results

Identification of a Basolateral Membrane Protein by Monoclonal Antibodies

In a previous study we have described several antibodies that specifically recognize enzyme glycoproteins of the brush border membrane (Hauri et al., 1985). A hybridoma of one of the fusions described in that study was subcloned to yield antibody HBB 3/344. This mAb was characterized and found to be specific for aminopeptidase *N*. It precipitated aminopeptidase *N* more efficiently than the previously described mAb HBB 3/153. To obtain antibodies against basolateral membrane proteins, hybridoma supernatants, originally obtained from a mouse that was immunized with a fraction enriched in Golgi membranes of Caco-2 cells (Stieger et al., 1988), were screened for the presence of basolateral specific antibodies by immunofluorescence microscopy. The positive hybridoma were subcloned and two clones were isolated which were designated G1/136 and G1/75.

A typical basolateral pattern of immunofluorescence is shown in Fig. 1 in which Triton X-100–permeabilized Caco-2 cells grown on Millipore filters were labeled with antibody G1/136. Identical patterns were obtained with G1/75. Anti-

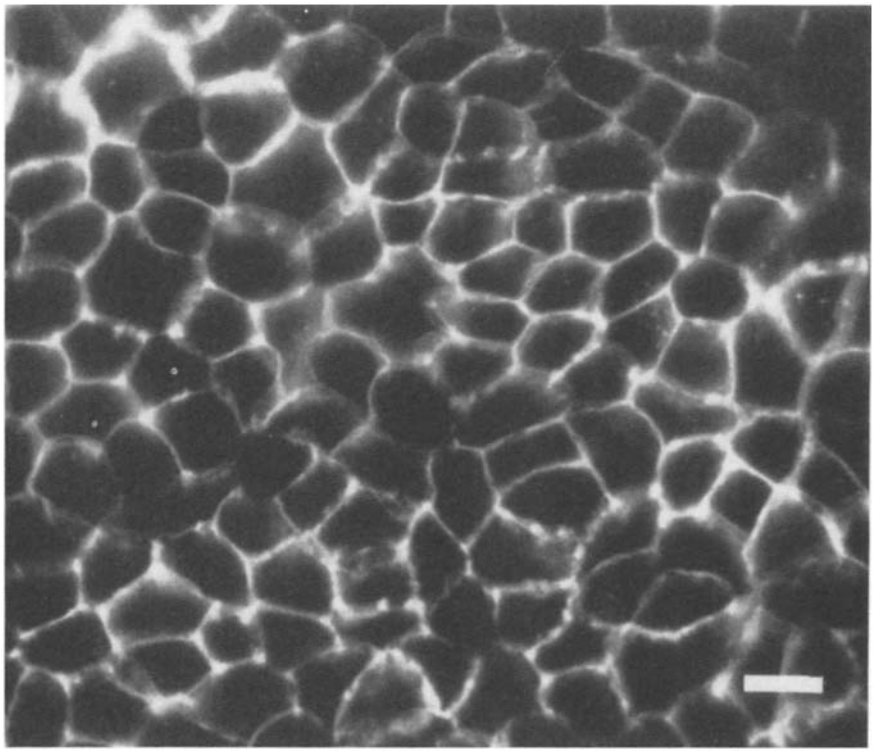

Figure 1. Immunofluorescence staining of Caco-2 cells with monoclonal antibody G1/136. The cells were grown to confluency on Millipore filters in a Mini Marbrook chamber, fixed with 2% formaldehyde, permeabilized with 0.2% Triton X-100, and incubated with the monoclonal antibody (ascites fluid 1:300) followed by rhodamine-labeled goat anti-mouse IgG. Note the typical polygonal staining pattern which is characteristic for a basolateral antigen. Bar, 10 μm.

body G1/75, but not G1/136, was useful for immunolocalization studies at the ultrastructural level (Fig. 2). G1/75 labeled the basolateral membrane while the brush border membrane and the cell junctions were devoid of label. G1/75 and G1/136 were found to bind to the same antigen as assessed by immunoprecipitation in conjunction with immunoblotting (Fig. 3). The antibodies precipitated a 120-kD glycoprotein from [^{35}S]methionine-labeled Caco-2 cells which is synthesized as a 100-kD endoglycosidase H-sensitive precursor protein and is membrane associated in its mature form as probed by sodium carbonate extraction at pH 11 (Fujiki et al., 1982).

Nocodazole Selectively Affects the Transport of an Apical Membrane Protein

To analyze the effects of nocodazole on the intracellular protein transport, we made use of a modification of the protease assay developed by Matlin and Simons (1984). The cells were grown on nitrocellulose filters. 5–8 d after confluency they were labeled with [^{35}S]methionine. Thereafter proteases (for details see Materials and Methods) were added either from the apical or the basolateral side to the intact monolayer. In this assay, surface-exposed proteins are digested whereas intracellular proteins are not accessible to the proteases. The digestion was stopped after 5 min and the protease-resistant antigen fraction was determined by quantitative immunoprecipitation.

Two membrane proteins were analyzed in detail: aminopeptidase *N*, a 158-kD glycoprotein of the brush border (Hauri et al., 1985), and the basolateral 120-kD antigen defined by antibody G1/136. Among a multitude of proteases tested, papain was the only one to which aminopeptidase *N* was sensitive at relatively high concentrations. Protease resistance is a well-known property of intestinal brush border enzymes and is of obvious physiological importance in vivo. The 120-kD protein was most efficiently digested with elastase while papain had no effect. Based on these results a mixture of papain and elastase (see Materials and Methods) was used for all the protease experiments irrespective of whether the digestion was carried out from the apical or basolateral side. Fig. 4 shows a typical result obtained with the surface protease assay. The apical marker aminopeptidase *N* can be digested only from the apical side whereas the basolateral antigen can only be digested when the protease is added from the basolateral side. As a indicator for nonspecific proteolysis we coprecipitated in all experiments an intracellular vesicle-associated 53-kD protein with the monoclonal antibody G1/93 (Schweizer et al., 1988). This antigen is protease-sensitive in detergent-permeabilized but not intact cells (Fig. 5). It is important to note that the intracellular 53-kD protein remained undigested (Fig. 4). We conclude that the assay can indeed be used to study the arrival of proteins at their respective membrane surface.

Fig. 6 shows a time course for the transport of aminopeptidase *N* to the apical cell surface and of the 120-kD protein to the basolateral membrane. Cells were labeled for 15 min with [^{35}S]methionine and chased for the indicated times before protease digestion from the apical and basolateral side, respectively. Based on densitometric scanning of fluorograms the maximal protease sensitivity was observed after 120 min for both proteins indicating that the majority of newly synthesized aminopeptidase *N* and 120-kD protein had arrived at the cell surface at this time.

In the following experiments with nocodazole, a chase point of 2 h was chosen based on the above transport kinetics.

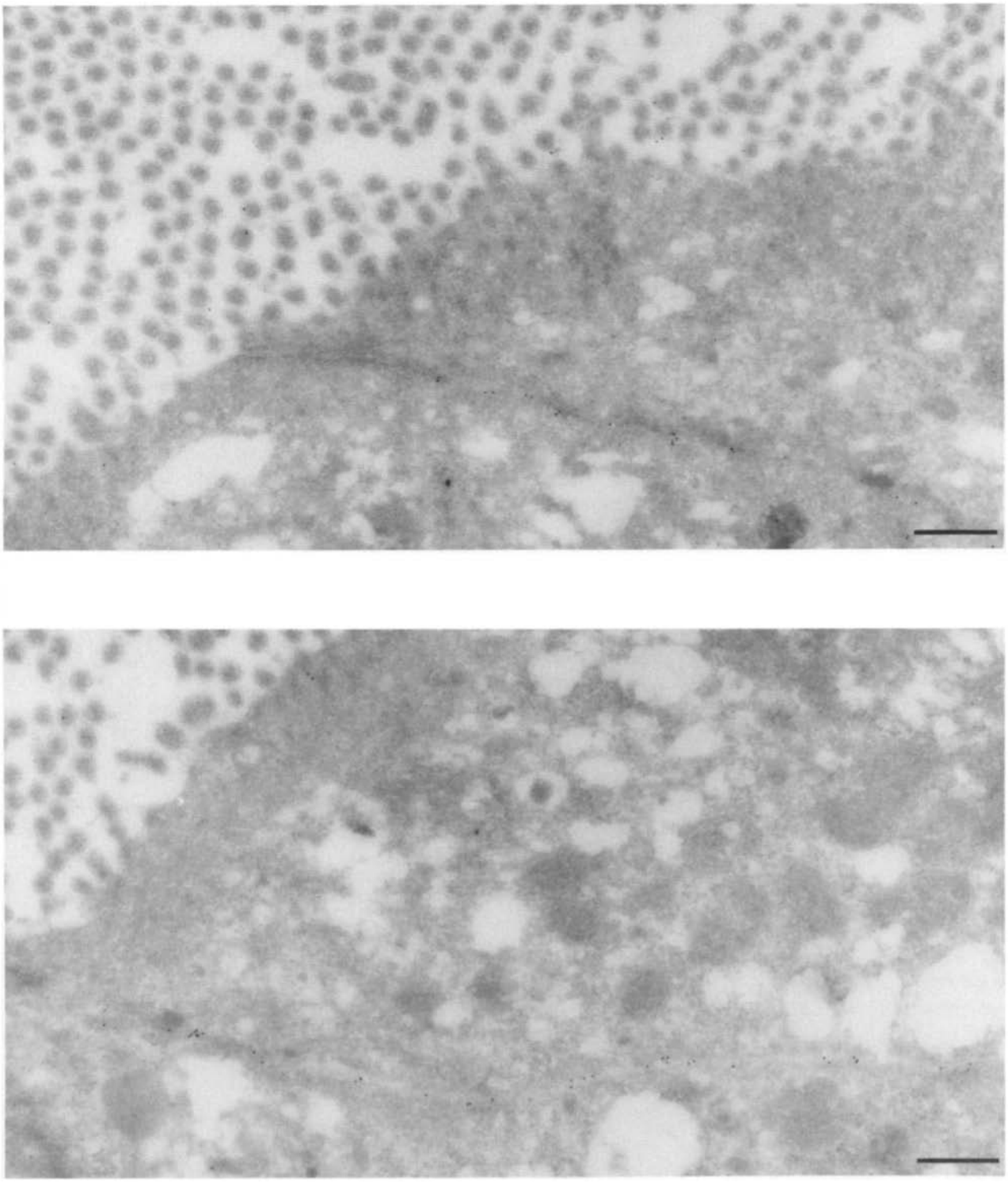

Figure 2. Immunoelectron microscopical localization of a basolateral antigen recognized by monoclonal antibody G1/75 with the protein A-colloidal gold technique. Confluent filter-grown Caco-2 cells were fixed in situ with 1% formaldehyde/0.1% glutaraldehyde, scraped from the filter and processed for ultrathin cryosectioning. Cryosections were sequentially incubated with the monoclonal antibody (ascites fluid 1:100), rabbit anti-mouse IgG and protein A-colloidal gold (5 nm). Note: Gold particles are exclusively confined to the basolateral membrane. The junctional area is not labeled.

The cells were treated with 10 μg/ml nocodazole at 37°C for 3 h before metabolic labeling. This concentration was found to disrupt the microtubular network of Caco-2 cells grown on Millipore filters, as visualized by immunofluorescence using an antitubulin monoclonal antibody (Fig. 7). However, we noticed that the microtubules were not completely depolymerized after the nocodazole treatment in contrast to Caco-2 cells grown on microscope slides which had under-

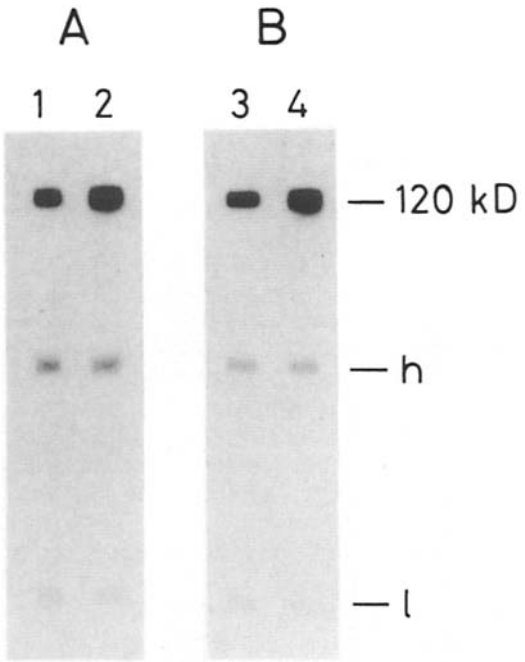

Figure 3. Monoclonal antibodies Gl/136 and Gl/75 recognize the same 120-kD antigen. The antigen was immunoprecipitated with Gl/136 (lanes *1* and *2*) or Gl/75 (lanes *3* and *4*) and the immunoprecipitates were subjected to SDS-PAGE followed by Western blotting. The nitrocellulose blots were either incubated with Gl/136 (lanes *1* and *3*) or Gl/75 (lanes *2* and *4*) and the antibody binding was visualized with ^{125}I-protein A and autoradiography. *h* and *l*, heavy and light chains of mAbs, respectively.

gone the same treatment. The reason for the difference in nocodazole sensitivity is unknown. Nocodazole treatment did not interfere with the tightness of the monolayer in respect to protease accessibility as can be seen in Fig. 8. Indeed, the basolateral antigen could not be digested from the apical side in the nocodazole-treated cells. The rate of incorporation of [^{35}S]methionine was not significantly affected by nocodazole. This suggests that nocodazole did not lead to nonspecific cell damage under the experimental conditions.

Fig. 8 shows representative results for the delivery of aminopeptidase *N* and the 120-kD antigen to the two different cell surface domains in the presence or absence of nocodazole. The chase time chosen in this assay was 2 h. Nocodazole did not have any influence on the transport of the 120-kD protein to the basolateral surface and no missorting to the apical cell surface occurred. However, nocodazole altered the polarized expression of aminopeptidase *N*: in comparison to the untreated cells less protein was digestible from the apical membrane and more protein was digestible from the basolateral side (see quantification, Fig. 9). These results suggest that nocodazole interfered with the apical delivery of aminopeptidase *N* causing a reduced transport to the brush border as well as partial missorting to the basolateral surface. To quantitate the data, the fluorograms from different experiments were scanned and the results were statistically evaluated. Fig. 9 shows the results for aminopeptidase *N* and the 120-kD protein. The histogram indicates the relative amounts of protease-resistant antigen obtained under the different conditions. The total amount of immunoprecipitable aminopeptidase *N* (Fig. 9 *A*) without protease digestion and in the absence of nocodazole was set to 100% (*t*). In the nocodazole-treated cells the protease-resistant aminopeptidase *N* increased from 39% in the control to 75% (*a*) when the digestion was performed from the apical side. Thus, less aminopeptidase *N* was transferred to the apical surface. On the other hand the fraction of protease-sensitive aminopeptidase *N* at the basolateral plasma membrane increased after nocodazole treatment from 0 to 23% (compare *b* bars). These data suggest nonpolarized cell surface delivery of aminopeptidase *N* in the presence of nocodazole. Assuming that aminopeptidase *N* can equally well be digested from the apical and basolateral sides it can be calculated that 13% less enzyme is transported to the cell surface after nocodazole treatment as compared to nontreated controls. This 13% may remain intracellularly. Nocodazole had no influence on the basolateral targeting of the 120-kD protein and no missorting to the apical cell surface occurred (Fig. 9 *B*).

Nocodazole Specifically Interferes with the Initial Secretion of Lysosomal Enzymes into the Apical Medium

The role of microtubules was also studied for the initial secretion of the two lysosomal enzymes cathepsin D and acid α-glucosidase. Under normal culture conditions a fraction of

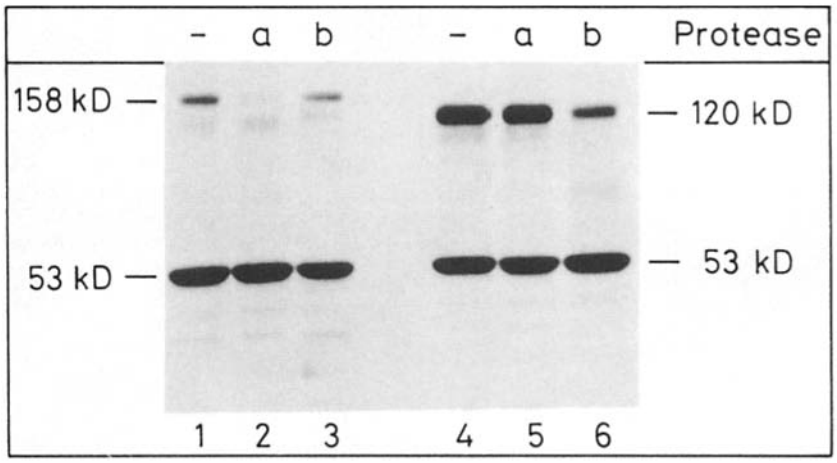

Figure 4. Selective surface digestion of aminopeptidase *N* (158 kD) and the 120-kD protein with papain and elastase from the apical (*a*) or basolateral (*b*) side of the monolayer (fluorogram). Filter-grown Caco-2 cells were metabolically labeled with [^{35}S]methionine over night and the surface protease assay was carried out as described under Materials and Methods. Monolayer-associated aminopeptidase *N* was immunoprecipitated and the immunoprecipitates were subjected to SDS-PAGE followed by fluorography. A 53-kD intracellular membrane protein was coprecipitated as a control for monitoring cell integrity. Note that aminopeptidase *N* can only be digested from the apical side while the 120-kD protein can only be digested from the basolateral side.

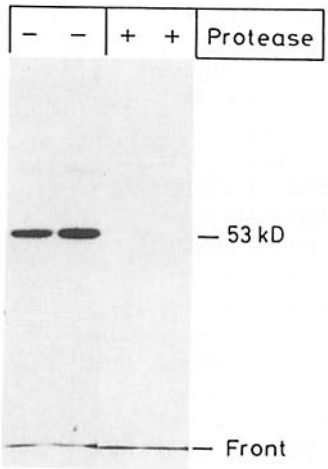

Figure 5. Protease sensitivity of the 53-kD protein in Triton X-100–permeabilized Caco-2 cells. Caco-2 cells were metabolically labeled with [^{35}S]methionine over night, permeabilized with Triton X-100, and treated with (+) or without (–) a mixture of papain and elastase for 5 min as described under Materials and Methods. The samples were immunoprecipitated with mab G1/93 and the immunoprecipitates were subjected to SDS-PAGE. Shown is a fluorogram with duplicate samples. Note that the proteases lead to a complete degradation of the 53-kD protein in the detergent-permeabilized cells.

these proteins was found to be secreted by Caco-2 cells both into the apical and basolateral culture medium as uncleaved pro-forms. Filter-grown Caco-2 cells were pulse labeled as described above in the presence or absence of nocodazole. After a 2-h chase the culture media were collected separately from the apical and from the basolateral chamber. The media were immunoprecipitated with specific antibodies directed against the two lysosomal enzymes α-glucosidase and cathepsin D (Oude Elferink et al., 1985). As illustrated in Fig. 10, nocodazole treatment selectively reduced the secretion of these proteins into the apical but not the basolateral medium. Quantification by densitometric scanning of the fluorograms confirmed this impression (Fig. 11). These results are consistent with those obtained for the plasma membrane proteins described in the previous paragraph with the exception that secretion to the basolateral medium was not increased in the presence of the drug within the 2 h of chase.

Discussion

In this study we have investigated the effect of nocodazole on the surface expression of newly synthesized plasma membrane proteins and on the polarized secretion of lysosomal enzymes in the human adenocarcinoma cell line Caco-2. The findings suggest that an intact microtubular network is essential for efficient sorting of these endogenous proteins to the apical but not to the basolateral cell surface. Considering the fact that we were not able to completely depolymerize the microtubules in filter-grown Caco-2 cells we cannot exclude the possibility that the basolateral pathway depends on a subset of microtubules that are resistant to high doses of nocodazole.

How do our results compare with those previously reported concerning the role of microtubules in epithelial cells? Numerous authors have used electron microscopic autoradiography (Blok et al., 1981; Pavelka and Ellinger, 1981; Ellinger et al., 1983; Bennett et al., 1984; Hugon et al., 1987) or subcellular fractionation (Quaroni et al., 1979; Danielsen et al., 1983) to trace newly synthesized glycoproteins in the presence of antimicrotubular drugs using intestinal tissue in vivo or in organ culture. These studies have consistently shown that a prolonged exposure to microtubule-active agents causes a decrease, but not a complete disappearance, of [^{3}H]fucose label associated with the brush border membrane concomitant with an increased labeling of

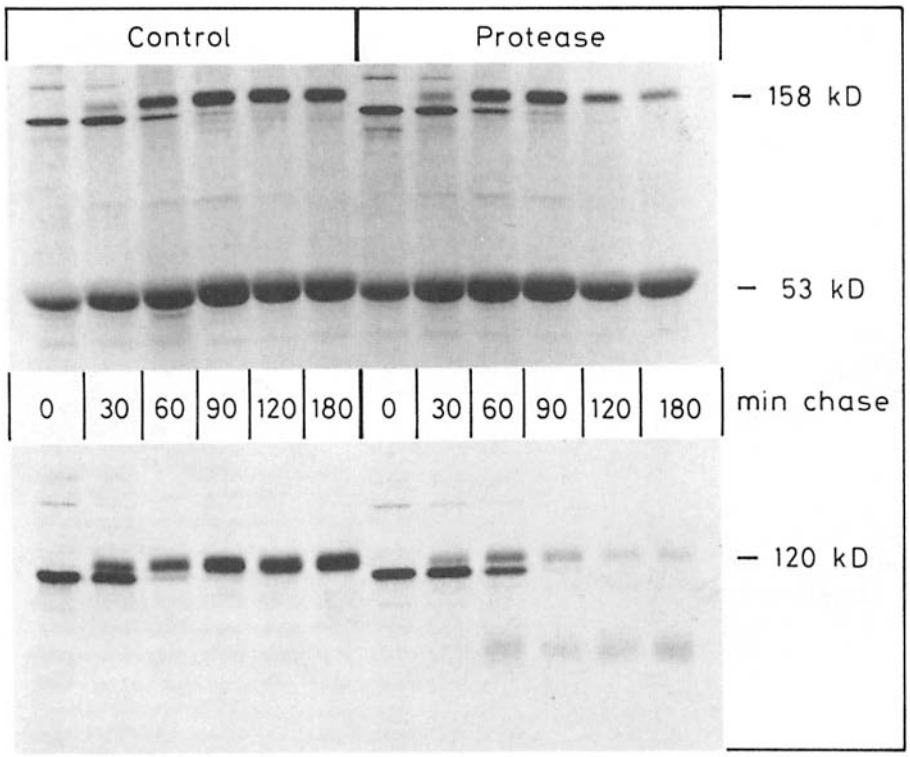

Figure 6. Surface appearance of the newly synthesized aminopeptidase *N* (*upper gel*) and the 120-kD protein (*lower gel*) monitored by the cell surface protease assay (fluorogram). Caco-2 cells were pulsed for 15 min with [^{35}S]methionine and chased for the indicated times. *Control*, no protease added; *protease*, the monolayers were treated with papain and elastase before immunoprecipitation. The positions of mature aminopeptidase (*158 kD*), mature 120-kD protein (*120 kD*), and the intracellular marker (*53 kD*) are indicated.

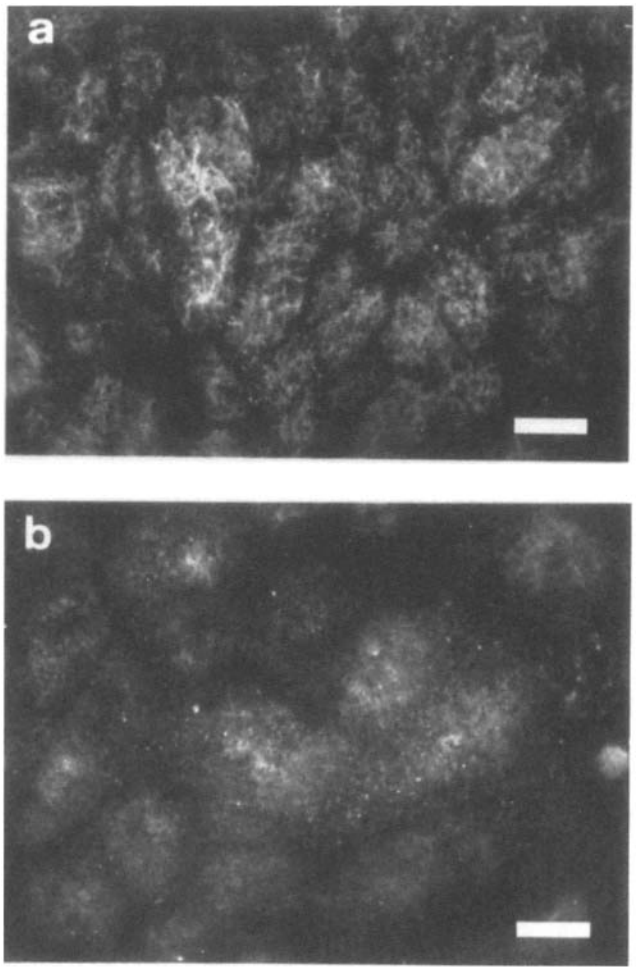

Figure 7. Effects of nocodazole on microtubules in filter-grown Caco-2 cells visualized by immunofluorescence. Filter-grown Caco-2 cells were cultured in the presence (*b*) or absence (*a*) of 10 μg/ml nocodazole for 3 h and the microtubules were labeled with a monoclonal antibody to tubulin in the formaline-fixed and detergent-permeabilized cells. Bar, 14 μm.

the basolateral membrane and in most instances of the Golgi apparatus. Shorter labeling times (90 min), however, did not lead to an increased labeling over the basolateral membrane (Bennett et al., 1984). Moreover, treatment of rats with colchicine for >3 h caused formation of brush border structures along lateral and basal surfaces of enterocytes in vivo (Pavelka et al., 1983). Collectively, these data are in line with the notion that an intact microtubular network is important for the maintenance of intestinal cell polarity. The transport of individual proteins was not investigated in any of these studies. The present data establish that cell surface delivery in Caco-2 cells of an apical enzyme marker protein, aminopeptidase *N*, is reduced by nocodazole and that this treatment leads to a partial missorting of the enzyme to the basolateral membrane. In contrast, the delivery of a basolateral 120-kD protein to its correct membrane domain is not affected by nocodazole and no missorting to the apical cell surface occurred. These results are in line with and extend the in vivo data, which did not allow any conclusion on basolateral glycoproteins.

A similar role of microtubules has been postulated by Rindler et al. (1987) for the polarized expression of viral proteins in the kidney cell line MDCK. These authors reported that colchicine or nocodazole treatment resulted in a nonpolarized delivery of influenza hemagglutinin which is sorted to the apical plasma membrane in untreated cells. On the other hand the sorting of vesicular stomatitis virus (VSV) G protein to the basolateral membrane was not disturbed under these conditions. However, this study is at variance with that of Salas et al. (1986) who reported that neither colchicine nor nocodazole had any effect on the polarized expression of the same two proteins in the same cells. The reason for this discrepancy remains unexplained.

In the present study nocodazole was also found to reduce the apical secretion of two lysosomal enzymes. It was always the enzyme fraction appearing in the apical culture medium which was reduced after nocodazole treatment. This is strong evidence for two independent secretory pathways to the two surface domains in Caco-2 cells, only the apical of which is sensitive to nocodazole. Interestingly, nocodazole did not lead to increased secretion of acid α-glucosidase to the basolateral chamber, even after 4 h (not shown), which might indicate that secretory and membrane proteins are transported in different vesicles. However, an extended study will be required to unequivocally establish this point. The important message in the present work is that nocodazole selectively affects initial apical secretion.

It is conceivable that the loss of a microtubular framework affects the apical transport pathway by two mechanisms. First, it has been shown in numerous reports that antimicrotubular drugs lead to a displacement and partial fragmentation of the Golgi apparatus, and second, intact microtubules might serve as tracks for apical membrane vesicles. The observed orientation of microtubules preferentially along the long axis of enterocytes in vivo (Gorbski and Borisi, 1985; Hugon et al., 1987) and the fact that they penetrate the terminal web and end in close vicinity of the brush border membrane (Sandoz and Lainé, 1985; Hagen et al., 1987) is certainly compatible with a track mechanism. Furthermore, it is important to note that in vivo the distance between the Golgi apparatus and the apical membrane is considerably longer than to the basolateral membrane. Indeed, vesicular transport over long distances, as in axons of neuronal cells, is known to be mediated by microtubules (Vale, 1987).

Two alternative mechanisms have been postulated for the sorting of apical and basolateral membrane proteins in epithelial cells: intracellular sorting (Matlin and Simons, 1984; Misek et al., 1984; Rindler et al., 1985) in the *trans*-Golgi network (Griffiths and Simons, 1986) or sorting at the cell surface in the basolateral membrane (Quaroni et al., 1979; Massey et al., 1987; Bartles et al., 1987). According to the latter hypothesis all the plasma membrane proteins would initially be delivered to the basolateral surface. Proteins destined for the apical domain would then be recruited for a second transport step, i.e., transcytosis, identical to the pathway taken by the polyimmunoglobulin receptor (Ahnen et al., 1985; Mostov and Simister, 1985). It is possible that the sorting mechanism is not identical in all the different epithelial cells. In particular, there is good evidence by means of subcellular fractionation that apical membrane proteins of hepatocytes pass through the basolateral membranes on their way to the apical cell surface. In the present study we have

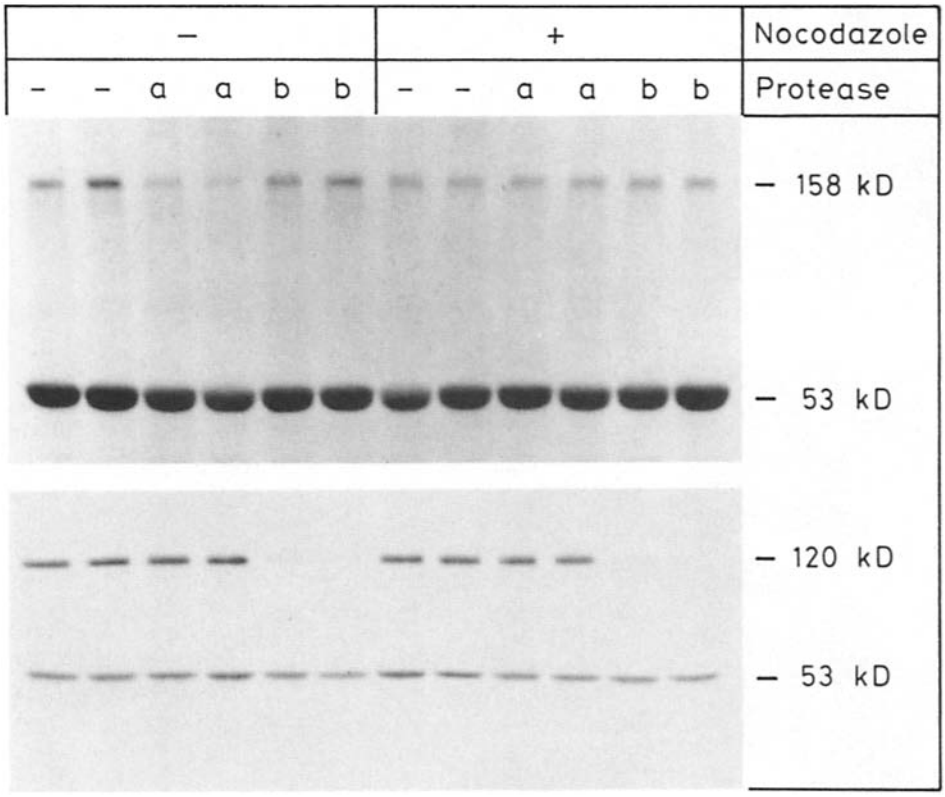

Figure 8. Effect of nocodazole on surface appearance of the aminopeptidase *N* and of the 120-kD protein. Filter-grown cells (duplicate samples) were cultured in the absence or presence of 10 μg/ml nocodazole for 3 h, pulsed for 15 min with [^{35}S]methionine and chased for 2 h in the presence of excess unlabeled methionine. At the end of the chase the monolayers were treated with protease from the apical side (*a*), the basolateral side (*b*), or were incubated with buffer (– indicates control). The apical marker aminopeptidase *N* (*158 kD*) or the basolateral 120-kD protein (*120 kD*) were immunoprecipitated from the cells with monoclonal antibodies and the immunoprecipitates were separated by SDS-PAGE and visualized by fluorography. A 53-kD intracellular membrane protein was coprecipitated as a reference for quantification. The upper and lower fluorograms originate from different gels, each lane of which originates from a single filter culture. The broad appearance of the 53-kD protein in the upper fluorogram is due to comigration of this band with the heavy chain of the mAb against aminopeptidase *N*.

not directly addressed the question of where apical and basolateral membrane proteins are sorted out in Caco-2 cells, since we feel that our protease assay in its present form might not be sensitive enough to uncover a rapid passage of apical proteins through the basolateral membrane. A priori our results appear to be compatible with both possibilities. The appearance of aminopeptidase *N* in the basolateral membrane of nocodazole-treated cells may reflect an initial missorting along a microtubule-independent pathway due to the disruption of the direct microtubule-dependent route. Alter-

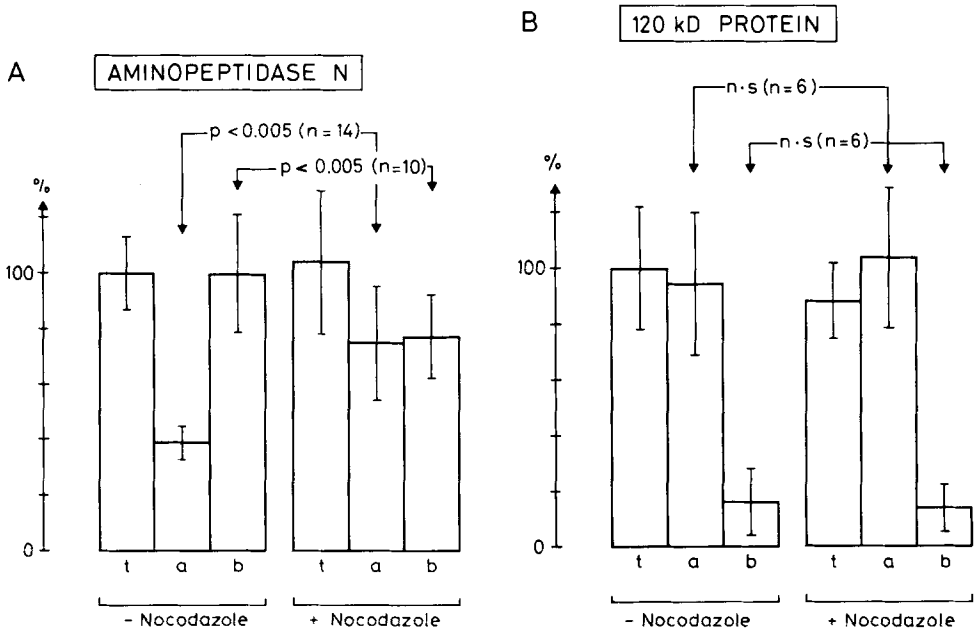

Figure 9. Effect of nocodazole on surface appearance of aminopeptidase *N* and the 120-kD protein (quantification). Pulse–chase experiments were carried out as in Fig. 8. After 2 h chase the monolayers were treated with proteases from the apical side (*a*), the basolateral side (*b*), or were processed without earlier protease treatment (*t*). The fluorograms were scanned and the mean of all the control values (minus nocodazole) was set to 100%. The bars represent cell-associated immunoprecipitable aminopeptidase *N* (*A*) or 120-kD protein (*B*). The values are given as means ± SD of 6–14 different filter cultures of three to seven experimental series. The *p* values were calculated by using the paired *t* test (see Materials and Methods). *ns*, nonsignificant.

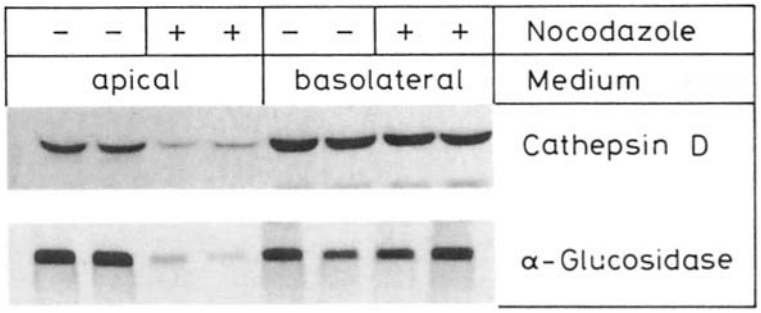

Figure 10. Effect of nocodazole on the initial secretion of the lysosomal enzymes cathepsin D and acid α-glucosidase (fluorogram). Filter-grown confluent Caco-2 cells were cultured with (+) or without (−) nocodazole for 3 h, pulsed for 15 min, and chased for 2 h. The apical and basolateral medium was harvested, adjusted to the same volume, and separately subjected to immunoprecipitation followed by SDS-PAGE and fluorography. Shown are duplicate values. Note that cathepsin D and α-glucosidase are secreted from both cell surface domains but it is always the apical secretion that is affected by nocodazole.

natively this finding may indicate a block of the normal pathway at an intermediate stage in the basolateral membrane. Considering the latter possibility, it is interesting to note that transcytosis but not internalization of the polyimmunoglobulin receptor is blocked by antimicrotubular drugs (Nagura et al., 1979). In fact, internalization of this receptor at the basolateral membrane of an intestinal adenocarcinoma cell line was found to be a rapid event even in colchicin-treated cells (Nagura et al., 1979). Therefore, if aminopeptidase *N* would indeed pass through the basolateral membrane one would expect its accumulation preferentially at an intracellular site rather than at the basolateral surface after nocodazole treatment. The present study does not lend support for such an accumulation since in the drug treated cells the apparent protease-resistant fraction increased by only 13% while the protease-sensitive fraction at the basolateral membrane increased by 23%.

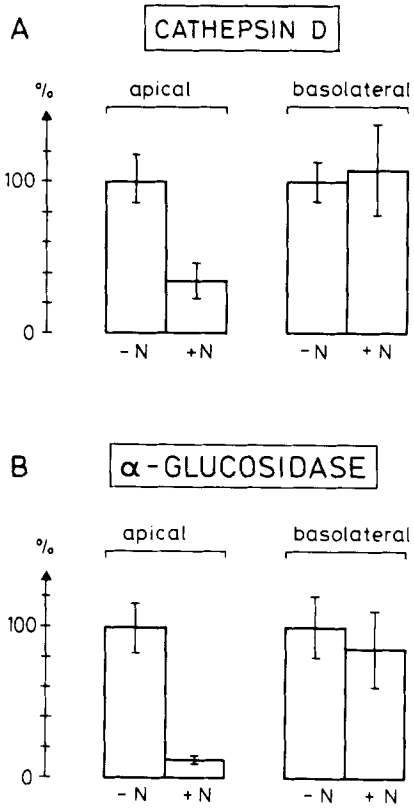

Figure 11. Effect of nocodazole (+*N*) on the initial secretion of cathepsin D and α-glucosidase (quantification). Experiments were performed as described in Fig. 10 and the bands on fluorograms were quantified by densitometry. The amount of secreted lysosomal enzyme to the apical and to the basolateral chamber, respectively, in the absence of nocodazole (−*N*) was set to 100%. The values represent means ± SD of six different filter cultures of three experimental series. For both enzymes nocodazole significantly reduced secretion to the apical chamber but had no effect on the secretion to the basolateral chamber as assessed by a *t* test.

In conclusion, we have shown that in filter-grown Caco-2 cells nocodazole selectively interferes with the apical protein delivery. It remains to be shown whether the transport of basolateral membrane proteins is indeed independent on microtubules or whether this pathway depends on a subset of microtubules that are insensitive to high doses of nocodazole. This experimental system may be useful to further elucidate mechanisms underlying the selective targeting of membrane proteins to the cell surface.

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