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1990-12-01

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Effect of Nocodazole on Vesicular Traffic to the Apical and Basolateral Surfaces of Polarized MDCK Cells

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Abstract. A polarized cell, to maintain distinct basolateral and apical membrane domains, must tightly regulate vesicular traffic terminating at either membrane domain. In this study we have examined the extent to which microtubules regulate such traffic in polarized cells. Using the polymeric immunoglobulin receptor expressed in polarized MDCK cells, we have examined the effects of nocodazole, a microtubule-disrupting agent, on three pathways that deliver proteins to the apical surface and two pathways that deliver proteins to the basolateral surface. The biosynthetic and transcytotic pathways to the apical surface are dramatically altered by nocodazole in that a portion of the protein traffic on each of these two pathways is misdirected to the basolateral surface. The apical recycling pathway is slowed in the presence of nocodazole but targeting is not disrupted. In contrast, the biosynthetic and recycling pathways to the basolateral surface are less affected by nocodazole and therefore appear to be more resistant to microtubule disruption.

T HE cytoskeleton plays a central role in eukaryotic cell biology and physiology as it contributes to such diverse processes as cell motility, muscle contraction, mitosis, and maintenance of cell shape. Many of the motile activities of eukaryotic cells depend on several cytoskeletal-based molecular motors (Vale and Goldstein, 1990). These motors, which couple the hydrolysis of ATP to the generation of force, include actin/microfilament-based myosin-1 and myosin-2, and at least three different microtubule-based motors which provide for the movement of organelles toward the plus or minus ends of microtubules, and the sliding between sets of microtubules (Scholey, 1990). The presence in eukaryotic cells of a polarized network of microtubules, microtubule-based molecular motors and organelles that can preferentially bind these motors, suggests a role for the microtubular system in the sorting and delivery of membranous organelles within cells (Vale, 1987).

The extent to which microtubules and their motors contribute to directed vesicular movement is now emerging (Kelly, 1990). To address this issue, investigators have used membrane proteins as markers for vesicular traffic, and drugs that interfere with microtubule formation to see if drug treatment alters the distribution or movement of the marker proteins. For example, in nonpolarized cells, colcemid-sensitive microtubules were not found to be critical in the biosynthetic delivery of newly synthesized proteins to the cell surface (Stults et al., 1989). Rivas and Moore (1989) found that the regulated but not the constitutive pathway of secretion was dependent on intact microtubules in AtT-20 cells. In addition, several groups have demonstrated that microtubule-disrupting agents partially block transport from endosomes to the lysosome (Wolkoff et al., 1984; Matteoni and Kreis, 1987; Gruenberg et al., 1989).

A polarized cell, in order to maintain distinct basolateral and apical membrane domains, must tightly regulate vesicular traffic terminating at either membrane domain. In general, there are three routes for proteins to reach the apical domain: from the Golgi apparatus (biosynthetic route), recycling from the apical surface (via the apical endosomal compartment), and transcytosis from the basolateral domain. There are three corresponding routes to reach the basolateral surface (see Fig. 1). A few of these pathways have been examined for their dependence on an intact microtubule system. However, the results from these studies have been controversial (Vale, 1987). In rat intestine in vivo, colchicine causes missorting of three apical proteins but does not affect the normal basolateral distribution of Na^+/K^+ ATPase (Achler et al., 1989). Similarly, Eilers and co-workers (1989) found that nocodazole disrupted the apical delivery of aminopeptidase N but did not affect the basolateral in delivery of a 120-kD protein in Caco-2 cells. Thus, in intestinal cells and cell lines, the delivery of newly synthesized proteins to the apical surface may be more sensitive to microtubule disruption than delivery to the basolateral surface. However, this paradigm may not hold for all polarized cell types.

For example, in MDCK cells, some investigators have found that the apical biosynthetic pathway is sensitive to microtubule disruption with others finding this pathway to be resistant to microtubule disruption. Rindler and co-workers (1987) demonstrated that the steady state of distribution of

Apical Pathways **Basolateral Pathways**
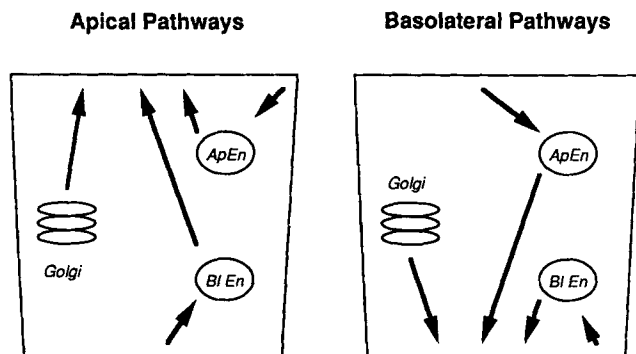
Figure 1. Apical and basolateral pathways in polarized cells. Pathways which deliver vesicular traffic to either surface of a polarized cell include a biosynthetic pathway from the Golgi complex, a transcytotic pathway from one surface to another, and a recycling pathway. The apical endosome (*ApEn*) and the basolateral endosome (*Bl En*) are separate compartments in MDCK cells.

influenza virus hemagglutinin (HA,[1] an apical membrane protein) is altered by microtubule disruption in MDCK cells. They found that a portion of HA is missorted to the basolateral surface, yet a basolateral membrane protein (VSV-G protein) is normally sorted. The secretion of soluble apical proteins was also found to be inhibited by microtubule disruption in MDCK cells (Parczyk et al., 1989). In contrast, the steady-state distribution of an endogenous apical membrane protein of MDCK cells has been found to be unaffected by disruption of microtubules by nocodazole (Ojakian and Schwimmer, 1988). Similarly, Salas and co-workers (1986) found the distribution of influenza virus HA was unaffected by microtubule disruption. Thus, for MDCK cells, it is still unclear to what extent the biosynthetic pathways that deliver newly synthesized proteins to the apical and basolateral surfaces are dependent on intact microtubules.

Transcytotic vesicular traffic (Mostov and Simister, 1985; Bartles and Hubbard, 1988) is unique to the polarized cell. Two such pathways are present in polarized cells: one that transports apical proteins to the basolateral surface, and one that transports basolateral proteins to the apical surface. Several studies have suggested that the basolateral to apical transcytotic pathway of the polymeric immunoglobulin receptor (pIg-R) in rat liver is inhibited by microtubule disruption (Mullock et al., 1980; Perez et al., 1988). Similarly, studies by Nagura and co-workers (1979) suggested that basolateral to apical transcytosis of the pIg-R in HT-29 cells was dependent on intact microtubules.

To help clarify the role of microtubules in vesicular traffic in polarized cells, our current study addresses which of the pathways outlined in Fig. 1 is directed or facilitated by an intact microtubular system. Our approach has been to use the pIg-R expressed in polarized MDCK cells as a model system.

The pIg-R and its ligand traverse a complex pathway through the polarized cell (Takahashi et al., 1982). After synthesis in the rough ER and posttranslational modification

1. *Abbreviations used in this paper:* HA, hemagglutinin; pIg-R, polymeric Ig receptor; SC, secretory component.

in the Golgi complex, the pIg-R is vectorially delivered to the basolateral surface. There it binds ligand (polymeric immunoglobulin [pIg]), is rapidly endocytosed, sorts through the basolateral endosomal compartment and is then delivered to the apical surface where the extracellular portion of the receptor (termed secretory component [SC]) is cleaved and released. We have recently shown that the ligand for the pIg-R can be used as a marker for two additional intracellular pathways (Breitfeld et al., 1989b). First, a portion of basolaterally endocytosed ligand recycles to the basolateral medium. Second, since not all pIg-R is immediately cleaved upon arrival at the apical surface, uncleaved apical pIg-Rs are available to endocytose ligand, which then largely returns to this surface. In addition, we have expressed in MDCK cells a mutant of the pIg-R that lacks the entire cytoplasmic domain of the receptor (termed tail-minus pIg-R) and have shown that it is vectorially delivered to the apical surface from the Golgi (Mostov et al., 1986). Thus, five of the six pathways described above are followed by either the wild-type pIg-R, the tail-minus receptor, or the ligand for the pIg-R. Because we have expressed the pIg-R in a cultured polarized cell line (MDCK cells) (Mostov and Deitcher, 1986), we have been able to develop biochemical assays that follow each of these five pathways (Breitfeld et al., 1989a; Casanova et al., 1990).

Using the pIg-R system to assess the role of microtubules in directing or facilitating each of five pathways which terminate at either the apical or basolateral surface in a single polarized cell line (MDCK cells), we find that intracellular pathways whose destination is the apical surface are affected by microtubule disruption, whereas pathways terminating at the basolateral surface are relatively resistant to microtubule disruption.

Materials and Methods

Cell Culture on Suspended Filters

MDCK cells were cultured on 0.4-μm-pore size, 6.5-mm-diam Transwell chambers (Costar Corp., Cambridge, MA). Cells from a confluent 10-cm tissue culture dish were removed with trypsin, washed with medium, and resuspended in 10 ml of medium. An aliquot of cells (0.2 ml) was added to each chamber. Fresh medium was added daily. Chambers were used for study after 4–6 d of growth. Cells were cultured in Eagles's MEM with 5% FCS, penicillin, and streptomycin. MDCK cells that express the wild-type pIg-R (Mostov and Deitcher, 1986), the tail-minus pIg-R (Mostov et al., 1986), or parent MDCK cells were used for this study.

Drug Treatment

Before each experiment, filter-grown cells were chilled to 4°C and incubated with 0.2% DMSO in the absence or presence of 33 μM nocodazole for 2 h. Cells were then warmed to 37°C in the continued absence or presence of nocodazole for 30 min. The experiment of interest was then performed in the continued absence or presence of nocodazole. Nocodazole (Sigma Chemical Co., St. Louis, MO) was prepared as a stock solution of 5 mg/ml in DMSO.

Metabolic Labeling and Analysis

Metabolic labeling of filter-grown MDCK cells with [^{35}S]cysteine was performed as previously described (Breitfeld et al., 1986b). pIg-R and SC were identified by immunoprecipitation with guinea pig anti-rabbit SC antibody, SDS-PAGE, and fluorography (Breitfeld et al., 1989a).

Detection of Metabolically Labeled pIg-R at the Cell Surface

As previously described (Casanova et al., 1990), MDCK cells were metabolically labeled from the basolateral surface with [^{35}S]cysteine (New England Nuclear, Boston, MA) for 10 min at 37°C. After this pulse period, cells were chased for the indicated time in MEM/0.6% BSA/20 mM Hepes, pH 7.3. To detect basolaterally delivered molecules, the chase was performed with 25 μg/ml trypsin in the basolateral medium and soybean trypsin inhibitor at 200 μg/ml in the apical medium. To detect apically delivered molecules, the chase was performed with 25 μg/ml trypsin in the apical medium and soybean trypsin inhibitor at 200 μg/ml in the basolateral medium. At the end of the chase period, cells were harvested and immunoprecipitated as described above.

Iodination of Anti-SC Fab Fragments

The generation of affinity-purified guinea pig anti-SC Fab fragments has been previously described (Breitfeld et al., 1989a). They were iodinated with the iodine monochloride method (Goldstein et al., 1983) to a specific activity of 5×10^7 cpm/μg. Unincorporated ^{125}I was removed by dialysis.

Assay of Continuous Transcytosis

Filter-grown MDCK cells that express the wild-type pIg-R were incubated at 37°C with ^{125}I-anti-SC Fab fragments in the basolateral chamber (0.4 μg/ml) for 24 h. Apical medium was sampled at various times and precipitated with trichloroacetic acid. After a 15-min spin at 15,000 g at 4°C, radioactivity in the pellets was determined by gamma counting. Parallel experiments were performed with MDCK cells that do not express the pIg-R and these values were subtracted as nonspecific background.

Fate of Endocytosed Ligand for the pIg-R

Filter-grown MDCK cells that express the wild-type pIg-R were allowed to endocytose ^{125}I-anti-SC Fab fragments (40 μg/ml) at 37°C for the time indicated from the basolateral or apical surface. Cells were then washed four times with medium and placed into culture wells containing fresh MEM/0.6% BSA/20 mM Hepes, pH 7.3, all at 37°C. Medium from the apical or basolateral chamber was collected at the appropriate time and made to 15% TCA. Samples precipitated for 1 h at 4°C and the soluble (degraded) and insoluble (intact) material was separated by centrifugation at 15,000 g for 15 min at 4°C and counted in a gamma counter. Filters were counted directly at the end of the experiment. MDCK cells that do not express the pIg-R were analyzed in parallel to control for nonspecific binding and fluid phase uptake of ligand. Values obtained at each time point for these cells were subtracted as nonspecific background.

Tubulin Western Blot

Filter-grown MDCK cells were washed with PBS and then incubated with 0.5 ml extraction buffer (2 M glycerol, 0.1 M Pipes, pH 6.75, 1 mM MgSO₄, 1 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM benzami-

dine, 5 μg/ml pepstatin A, 5 μg/ml O-phenanthroline, 0.5 mM PMSF) with 0.1% TX-100 at 37°C for 15 min in two successive incubations. The resulting supernatant containing unassembled monomeric tubulin was combined with an equal volume of 2× lysis buffer (50 mM Tris, pH 7.4, 0.8 M NaCl, 1% SDS) at room temperature. This was then boiled for 3 min, centrifuged at 15,000 g for 2 min and the resulting supernatant was harvested. β-Mercaptoethanol was added to 0.1%, the sample was boiled for 3 min and then placed on ice.

Polymeric tubulin was recovered by incubating the previously extracted filter-grown MDCK cells with lysis buffer (25 mM Tris, pH 7.4, 0.4 M NaCl, 0.5% SDS) for 5 min at 37°C. This supernatant was then boiled for 3 min and centrifuged at 15,000 g for 2 min. The supernatant was recovered, made to 0.1% β-mercaptoethanol, boiled for 3 min, and placed on ice.

Total cellular tubulin was recovered from a separate filter containing MDCK cells. The cells were washed with PBS, and lysis buffer was added for 5 min at 37°C. The supernatant was harvested, boiled for 3 min and centrifuged at 15,000 g for 2 min. The supernatant was recovered, made to 0.1% β-mercaptoethanol, boiled for 2 min and placed on ice.

Aliquots from total cellular tubulin, polymeric tubulin and monomeric tubulin fractions were subjected to SDS-PAGE and Western blotting (Burnette, 1981) using antitubulin antibody (generously provided by Ken Sawin and Tim Mitchison, UCSF, San Francisco, CA) and the ECL detection system (Amersham Corp., Arlington Heights, IL) according to the manufacturers' instructions. The resulting bands were quantitated by laser densitometry.

The above tubulin analysis was performed on MDCK cells incubated in the absence or presence of nocodazole (see Drug Treatment, above). In the absence of nocodazole, 63% of total cellular tubulin was recovered in the polymeric tubulin fraction with 37% recoverable as monomeric tubulin. In the presence of nocodazole, all of the cellular tubulin was recoverable in monomeric form. There was no detectable polymeric tubulin by Western blot in the presence of nocodazole.

Results

In this study, we have disrupted the microtubular network of polarized MDCK cells with nocodazole (see Materials and Methods) and examined the effect of this treatment on the delivery of the pIg-R or its ligand from various intracellular sites to the apical or basolateral surface.

Delivery from Golgi Complex to Basolateral Surface

Newly synthesized wild-type pIg-R is vectorially delivered from the Golgi complex to the basolateral surface of MDCK cells within 45 min of synthesis (Breitfeld et al., 1990). To examine whether an intact microtubular system facilitates and/or directs this movement, we determined the time course and site of delivery of newly synthesized pIg-R in the absence or presence of nocodazole, which inhibits polymeriza-
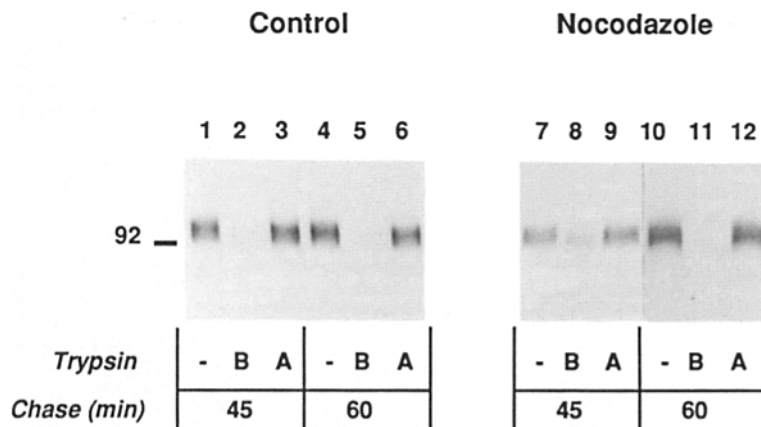
Figure 2. Effect of nocodazole on the biosynthetic delivery of wild-type pIg-R to the basolateral surface. Cells were pre-treated without (*left*) or with nocodazole (*right*), pulsed with [^{35}S]cysteine and then chased for the times indicated in the absence (−) or presence of basolateral (*B*) or apical (*A*) trypsin. The wild-type pIg-R was identified by immunoprecipitation, followed by SDS-PAGE and fluorography. Molecular weight marker at 92 kD is indicated on the left (phosphorylase A). This experiment was repeated twice.
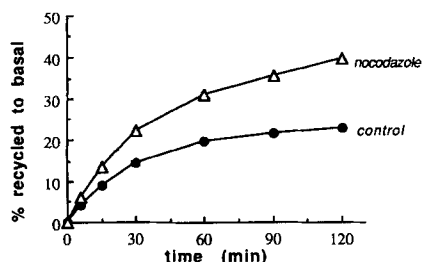
Figure 3. Effect of nocodazole on basolateral recycling of ligand for the wild-type pIg-R. MDCK cells which express the wild-type pIg-R were allowed to endocytose ¹²⁵I-anti-SC Fab fragments for 10 min from the basolateral surface, washed quickly, and then the basolateral medium was sampled for the appearance of ligand at the times indicated. The entire experiment was performed at 37°C, in the absence (*closed circles*) or presence (*open triangles*) of nocodazole. MDCK cells which do not express any form of the pIg-R were analyzed in parallel and these values were subtracted as nonspecific uptake (specific: nonspecific uptake = 5.1:1.0). This experiment was repeated four times.

tion of tubulin monomers (De Brabander et al., 1976). For this and all other experiments, microtubules were disrupted by chilling cells to 4°C for 2 h in the presence of 33 μM nocodazole, followed by a 30-min incubation at 37° C in the continued presence of nocodazole. Similar treatments have been used by other investigators and have been shown to disrupt at least 95% of microtubules in MDCK cells (Salas et al., 1986). In addition, by tubulin Western blot, we determined that no detectable polymeric tubulin is present in polarized MDCK cells after such treatment (see Materials and Methods).

To detect newly synthesized wild-type pIg-R as it arrives at the cell surface, we used a previously described trypsin proteolysis assay (Casanova et al., 1990). Briefly, cells were pretreated in the absence or presence of nocodazole, metabolically labeled with [³⁵S]cysteine for 10 min, and then chased for various periods of time in the absence or presence of apical or basolateral trypsin. The pIg-R was identified by immunoprecipitation and SDS-PAGE. Loss of immunoprecipitable receptor indicates arrival of the pIg-R at the surface exposed to trypsin. As previously reported, we found that by 45 min of chase, 90% of newly synthesized wild-type pIg-R is digested by basolateral trypsin (Fig. 2, lane 2) but is not sensitive to apical trypsin (lane 3). By 60 min, basolateral trypsin digests virtually all newly synthesized pIg-R (lane 5) and apical trypsin has no effect (lane 6). These results indicate that newly synthesized pIg-R is delivered to the basolateral surface of MDCK cells in the absence of nocodazole. In the presence of nocodazole, only 70% of newly synthesized receptor is digested by basolateral trypsin at 45 min (lane 8). Apical trypsin digests no pIg-R (lane 9). By 60 min, in the presence of nocodazole, virtually all newly synthesized pIg-R is digested by basolateral trypsin (lane 11), whereas apical trypsin has no effect (lane 12). Together, these results indicate that nocodazole does not cause missorting of newly synthesized pIg-R but that its rate of delivery to the basolateral surface is slightly delayed.

Delivery from Endosome to Basolateral Surface

Molecules endocytosed from the basolateral surface of MDCK cells pass through a basolateral endosomal compartment be-

fore reaching their final destination (Bomsel et al., 1989). For example, transferrin and its receptor are endocytosed from the basolateral surface, and after sorting through the endosome, are recycled back to the basolateral surface. The basolateral endosome to basolateral surface recycling pathway is a critical mechanism that maintains the basolateral polarity of the transferrin receptor (Fuller and Simons, 1986).

We have previously shown that the anti-SC Fab fragments functionally substitute as ligand for the pIg-R. In addition, if endocytosed from the basolateral surface of MDCK cells, a portion of the ligand recycles to the basolateral surface (Breitfeld et al., 1989b). It is not yet clear whether this represents cycling of receptor–ligand complexes or ligand alone. Nevertheless, basolaterally endocytosed ligand for the pIg-R may be used as a marker for the basolateral recycling pathway. We have now examined the effect of nocodazole on this basolateral ligand recycling pathway. To do so, we have used a previously described ligand uptake assay that allows one to determine the fate of a single cohort of endocytosed ligand (Casanova et al., 1990). Therefore, MDCK cells that express the pIg-R were allowed to basolaterally endocytose anti-SC Fab fragments for 10 min. Cells were washed to remove nonendocytosed Fab fragments and the fate of endocytosed Fab fragments was then determined in the absence or presence of nocodazole. To insure that this assay was indeed following a cohort of endocytosed ligand, at the end of the washing step, for several representative filters, we determined the amount of filter-associated radioactivity that was intracellular or remaining at the basolateral cell surface using a previously described proteolysis "stripping" assay (Breitfeld et al., 1989b). In the absence of nocodazole, after the initial ligand uptake period and washing, 94% of filter-associated ligand was intracellular (protease-resistant) and 6% remained at the cell surface (protease-sensitive). In the presence of nocodazole, the absolute amount of filter-associated ligand was reduced by one-third. However, 89% of filter-associated ligand was intracellular and only 11% was at the cell surface. Thus, after the initial ligand uptake and wash period at 37°C, ~90% or greater of filter-associated ligand is indeed intracellular in the absence or presence of nocodazole.

Using this assay, we found that, as previously demonstrated (Casanova et al., 1990), in MDCK cells that express the pIg-R, 23% of a single cohort of anti-SC Fab fragments

Table I. Effect of Nocodazole on Fate of Basolaterally Endocytosed Ligand

	Transcytosed	Degraded	Recycled	Intracellular
		%		
Control	51	4	23	22
Nocodazole	20	3	40	37

MDCK cells expressing the wild-type pIg-R were allowed to endocytose anti-SC Fab fragments for 10 min from the basolateral surface of MDCK cells. After washing, the apical and basolateral medium was sampled at various times up to 120 min. The percent of endocytosed ligand transcytosed to the apical medium, degraded and released into the medium, recycled into the basolateral medium, or remaining intracellularly after the 120-min incubation is displayed in the table. The time course for the release of endocytosed ligand into the basolateral medium and the apical medium is displayed in Figs. 3 and 7 *B* respectively. The entire experiment was performed in the absence or presence of nocodazole.

2368

endocytosed from the basolateral surface recycle to the basolateral medium by 120 min (Fig. 3 and Table I). During this experiment, 51% of endocytosed ligand was transcytosed to the apical medium (see Fig. 7 B and Table I), 4% was degraded and 22% was left intracellularly at the end of the 120-min incubation (Table I). MDCK cells treated with nocodazole retained their ability to basolaterally recycle endocytosed anti-SC Fab fragments (Fig. 3 and Table I) as the percentage of ligand recycled in the presence of nocodazole was increased almost twofold over cells without nocodazole treatment (40 vs. 23%). In addition, in the presence of nocodazole, only 20% was transcytosed (see Fig. 7 B), 3% was degraded and 37% remained intracellularly at the end of the 120-min incubation (Table I). Therefore, nocodazole enhances recycling of the ligand for the pIg-R, whereas it inhibits transcytosis (see discussion of Fig. 7 B below).

Delivery from Golgi Complex to Apical Surface

When the cytoplasmic domain of the pIg-R is deleted, the mutant receptor (tail-minus receptor) is vectorially delivered from the Golgi complex to the apical surface of MDCK cells (Mostov et al., 1986) where it is cleaved and SC is released into the apical medium. SC release into the apical medium is therefore a convenient measure of the delivery of the tail-minus receptor from the Golgi complex to the apical surface. To assess the role of microtubules in this pathway, in the absence or presence of nocodazole, we metabolically labeled MDCK cells that express the tail-minus receptor and determined the amount of SC released into the apical or basolateral medium as a function of time. Fig. 4 A demonstrates that at 5 h in the absence of nocodazole, 75% of newly synthesized tail-minus receptor has been cleaved and released into the apical medium as SC. by 24 h, all newly synthesized tail-minus receptor has been cleaved and released into the apical medium as SC and there is no detectable tail-minus pIg-R left in the cell. However, in the presence of nocodazole, only 44% of newly synthesized tail-minus receptor is cleaved to SC and released into the apical medium by 24 h (Fig. 4 A). Surprisingly, in the presence of nocodazole, 47% of newly synthesized tail-minus receptor was recovered at 24 h in the basolateral medium as SC (Fig. 4 B) with the remainder (9%) left intracellularly.

One possible explanation for the detection of SC in the basolateral medium in the presence of nocodazole is that nocodazole disrupts the integrity of the monolayer, allowing apically released SC to leak into the basolateral medium. This is unlikely, as nocodazole does not enhance apical to basolateral transport of anti-SC Fab fragments across the parent MDCK cell line (data not shown).

We suggest instead that in the presence of nocodazole, approximately half of the tail-minus receptor is misdirected to the basolateral surface. After reaching the basolateral surface the tail-minus receptor (unlike the wild-type) is unable to be endocytosed and is eventually cleaved by a protease. We have previously observed that a small fraction (<5%) of the wild-type pIg-R is also cleaved to SC at the basolateral surface. The protease responsible for cleaving both the wild-type and tail-minus receptors at the basolateral surface appears to differ in two ways from the protease acting at the apical surface. First, in both cases the basolaterally released material migrates slightly slower on SDS-PAGE compared
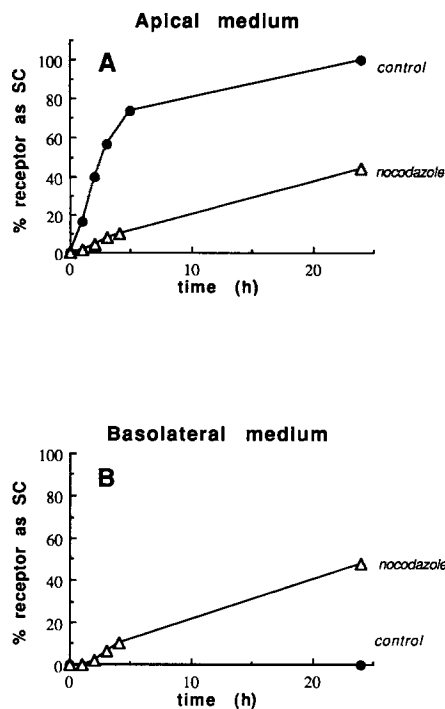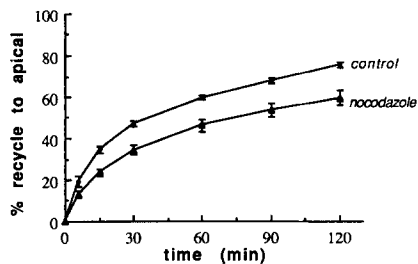

Figure 4. Effect of nocodazole on the rate of delivery of newly synthesized tail-minus pIg-R to the apical or basolateral surface. MDCK cells that express the tail-minus pIg-R were pulsed with [^{35}S]cysteine for 20 min and then chased for 24 h. At the times indicated, the apical or basolateral medium was sampled, SC immunoprecipitated, and then subjected to SDS-PAGE and fluorography. Cells at the end of the 24-h incubation were processed in identical fashion. The gels were scanned by laser densitometry to determine the percentage of receptor released into the apical or basolateral medium as SC. The experiment was performed in duplicate, and in the absence (*closed circles*) or presence (*open triangles*) of nocodazole. This experiment was repeated three times.

with apically released material. Second, only apical cleavage is inhibited by leupeptin (data not shown).

In summary, nocodazole dramatically alters the vectorial delivery of the tail-minus pIg-R as approximately half of newly synthesized mutant receptor is misdirected to the basolateral surface. In addition, the rate of vectorial delivery of the tail-minus pIg-R to the apical surface is dramatically slowed (Fig. 4 A) in the presence of nocodazole.

Delivery from Endosome to Apical Surface

Apically endocytosed proteins pass through an apical endosomal compartment prior to reaching their final destination. A fluid phase marker endocytosed from the apical surface of MDCK cells has been shown to recycle back to the apical medium (45%) or to transcytose to the basolateral medium (45%) (Bomsel et al., 1989). We have previously shown that there is sufficient pIg-R at the apical surface of MDCK cells that express the receptor to allow for the detection of apically endocytosed ligand for the pIg-R. In addition, the fate of apically endocytosed ligand for the pIg-R is substantially different from a fluid phase marker in that the ligand for the pIg-R primarily recycles to the apical medium (Breitfeld et al., 1989b). We have therefore asked what effect nocodazole has on this apical ligand recycling pathway.

Figure 5. Effect of nocodazole on the return of apically endocytosed anti-SC Fab fragments to the apical medium. MDCK cells which express the wild-type pIg-R were allowed to endocytose ^{125}I-anti-SC Fab fragments for 15 min, washed quickly, and then the apical medium was sampled at the times indicated. The entire experiment was performed at 37°C in the absence (closed circles) or presence (open triangles) of nocodazole. MDCK cells which do not express any form of the pIg-R were analyzed in parallel and these values were subtracted as nonspecific uptake (specific: nonspecific uptake = 2.5:1.0). The experiment was performed in quadruplicate and because the observed differences were small, the mean ± SEM is displayed for each value. At 120 min, the difference is statistically significant by t test ($p < 0.005$). This experiment was repeated three times.

Therefore, MDCK cells which express the pIg-R were allowed to endocytose anti-SC Fab fragments from the apical surface for 15 min. The fate of apically endocytosed ligand was determined in the absence or presence of nocodazole. As we have previously shown (Breitfeld et al., 1989b), in the absence of nocodazole, 75% of the endocytosed ligand returns to the apical medium after the 120-min incubation (Fig. 5). Very little is transcytosed to the basolateral medium (3%) or degraded and released (7%). 15% remains cell associated at the end of the 120-min incubation. In the presence of nocodazole, 59% recycles to the apical medium (Fig. 5), 7% is transcytosed to the basolateral medium, and 6% is degraded and released. 28% remains cell associated at the end of the incubation. Although there is only a small reduction in the amount of ligand recycled to the apical medium in the presence of nocodazole (a 21% reduction compared with control), this difference is statistically significant ($p < 0.005$, at 120 min). This suggests that the return to the apical medium of apically endocytosed ligand for the pIg-R is mildly impaired by nocodazole.

Although the pIg-R system does not provide a good measure of the apical to basolateral transcytotic pathway (Simister and Mostov, 1989; Hunziker and Mellman, 1989), it is of note that the small amount of apical to basolateral transcytosis observed for apically endocytosed ligand for the pIg-R is not inhibited by nocodazole and may actually be increased (3 vs. 7%).

Delivery from the Basolateral Surface to Apical Surface (Transcytosis)

Proteins may be delivered to the apical surface from the basolateral surface. This process is termed transcytosis (Mostiv and Simister, 1985) and has now been demonstrated for a number of integral membrane proteins (Bartles and Hubbard, 1988; Matter et al., 1990). We have previously shown that both the pIg-R and its ligand are transcytosed by MDCK cells that express an exogenous pIg-R (Mostov and Deitcher,

1986). Thus, the transcytosis of the pIg-R with its ligand serves as an excellent model system to examine the factors that regulate this basolateral to apical transcytotic pathway. Since SC is released at the apical surface after transcytosis of the pIg-R, transcytosis of the receptor can be assayed by measuring the amount of SC released into the apical medium over time. To examine the dependence of this pathway on microtubules, we metabolically labelled MDCK cells that express the wild-type pIg-R and chased for various periods of time in the absence or presence of nocodazole. We quantitated the amount of SC released into the apical medium as a function of time. Fig. 6 demonstrates that in the absence of nocodazole, SC accumulates in the apical medium such that by 5 h of chase, 60% of metabolically labeled receptor has been cleaved to SC and therefore transcytosed. In the presence of nocodazole, only 13% of metabolically labelled receptor has been transcytosed at 5 h (Fig. 6). This represents at 80% reduction in basolateral to apical transcytosis of the wild-type receptor over the 5-h incubation.

We also examined the effect of nocodazole on the basolateral to apical transcytosis of the ligand for the pIg-R. We examined this in two ways. First, in the absence or presence of nocodazole, ligand was placed in the basolateral chamber and allowed to continuously transcytose to the apical medium for 24 h. The amount of ligand transcytosed to the apical medium was determined at various times. Fig. 7 A demonstrates that by 24 h, cells treated with nocodazole transcytose 42% less ligand than control cells.

We have demonstrated in Fig. 3 that a single cohort of basolaterally endocytosed ligand for the pIg-R is more likely to recycle back to the basolateral medium in the presence of nocodazole. Thus, during the experiment depicted in Fig. 3, we also determined the time course of delivery of ligand to the apical medium. As previously stated (see discussion of Fig. 3), Fig. 7 B demonstrates that in the absence of nocodazole, 51% of internalized ligand is transcytosed to the apical medium within 120 min. In the presence of nocodazole, only 20% is transcytosed into the apical medium. This represents
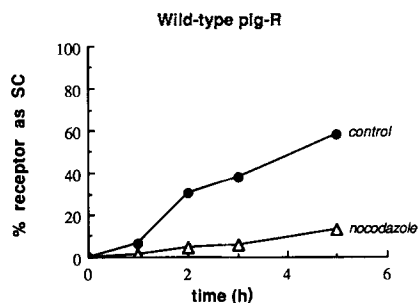
Figure 6. Effect of nocodazole on the basolateral to apical transcytosis of wild-type pIg-R. MDCK cells which express the wild-type pIg-R were pulsed with [^{35}S]cysteine for 20 min and then chased for 5 h. At the times indicated, the apical medium was sampled, SC immunoprecipitated, and then subjected to SDS-PAGE and fluorography. Cells at the end of the 5-h incubation were processed in identical fashion. The gels were scanned by laser densitometry to determine the percentage of receptor released into the apical medium as SC. The experiment was performed in duplicate, and in the absence (closed circles) or presence (open triangles) of nocodazole. This experiment was repeated three times.
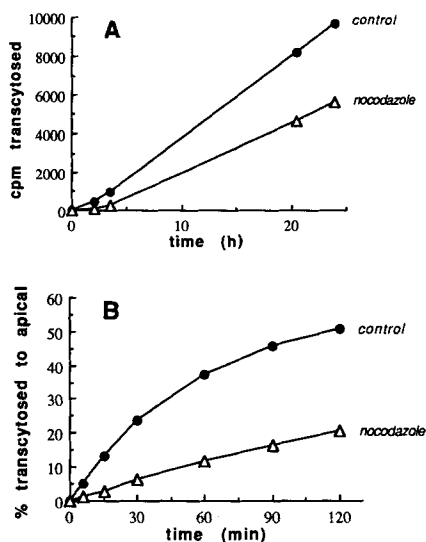
Figure 7. (*A* and *B*) Effect of nocodazole on the basolateral to apical transcytosis of anti-SC Fab fragments. (*A*) MDCK cells which express the wild-type pIg-R were incubated with ^{125}I-anti-SC Fab fragments in the basolateral chamber for 24 h. At the times indicated the apical medium was sampled. After precipitation with TCA, the amount of intact ligand transcytosed was determined by centrifugation and gamma counting of the pellet fraction. The experiment was performed in the absence (*closed circles*) or presence (*open triangles*) of nocodazole. MDCK cells that do not express any form of the pIg-R were analyzed in parallel and these values were subtracted as nonspecific uptake (MDCK parent clone at 24 h: 941 cpm). (*B*) MDCK cells which express the wild-type pIg-R were allowed to endocytose ^{125}I-anti-SC Fab fragments for 10 min from the basolateral surface, washed quickly, and then the apical medium was sampled for the appearance of ligand at the times indicated. The entire experiment was performed at 37°C, in the absence (*closed circles*) or presence (*open triangles*) of nocodazole. MDCK cells that do not express any form of the pIg-R were analyzed in parallel and these values were substracted as nonspecific uptake (specific: nonspecific uptake = 5.0:1.0). This experiment was repeated four times.

a 60% reduction in ligand transcytosis in the presence of nocodazole. Thus, nocodazole dramatically inhibits the basolateral to apical transcytosis of both the pIg-R and its ligand.

Since Fig. 3 and 7 *B* depict separate data from the same experiment, we can precisely relate the portion of endocytosed ligand recycled to that transcytosed, degraded and left intracellularly (see Table I). This analysis suggests that after basolaterally endocytosed ligand enters the endosomal compartment, nocodazole partially blocks ligand transcytosis and that the portion of ligand that fails to be transcytosed compared to control (51% − 20% = 31%) has two fates. Approximately half is left intracellularly (37% − 22% = 15%) and the other half is redirected to the basolateral medium (40% − 23% = 17%). Nocodazole thus not only inhibits basolateral to apical transcytosis but in addition, induces the partial missorting of basolaterally endocytosed ligand for the pIg-R.

Discussion

The regulation of vesicular traffic in eukaryotic cells is a major issue in cell biology. We have focused on this issue in polarized cells using the pIg-R as a model system. Polarized cells are characterized by distinct apical and basolateral surface domains. The polarity of these surface domains is maintained by the fidelity of vesicle delivery from a variety of intracellular locations to the appropriate domain. In the current study, we have examined the role of microtubules in directing vesicular traffic to both the basolateral and apical surfaces of MDCK cells. We have pursued this for several reasons: (*a*) studies on the role of microtubules in the biosynthetic delivery of proteins to the apical surface of MDCK cells have generated conflicting data; (*b*) the role of microtubules in directing vesicular traffic in various cells may differ; and (*c*) most individual studies have examined only one or two vesicular pathways.

Biosynthetic Delivery of Apical Membrane Proteins

Apical membrane proteins are delivered to the apical surface by at least two different pathways. First, they may be vectorially delivered from the Golgi as is the case for the influenza virus HA when expressed in MDCK cells (Matlin and Simons, 1984). Alternatively, they may be first delivered to the basolateral surface, endocytosed, and then transcytosed to the apical surface, as is the case with hepatic apical membrane proteins (Bartles and Hubbard, 1988). Finally, a given apical membrane protein may utilize both the vectorial and transcytotic routes to the apical surface as is the case for several intestinal epithelial proteins (Matter et al., 1990).

For MDCK cells, the role of microtubules in directing the apical membrane protein HA on the vectorial route is controversial as one study found missorting of HA to the basolateral surface when microtubules were disrupted (Rindler et al., 1987) and another found no effect (Salas et al., 1986). Parczyk and co-workers (1989) have found that in MDCK cells, apical secretion of soluble proteins is inhibited by nocodazole supporting the hypothesis that the apical vectorial route of protein delivery is at least facilitated by intact microtubules. In the current study, we provide further support for this hypothesis in MDCK cells by demonstrating that the vectorial apical delivery of the tail-minus pIg-R, an apical membrane protein, is inhibited by nocodazole. Furthermore, we show that this inhibition is not only a kinetic intracellular block. A portion of molecules that fail to reach the apical surface are missorted to the basolateral surface, in agreement with Rindler and co-workers (1987) and Parczyk and co-workers (1989).

In intestinal cells, the delivery of apical membrane proteins has been blocked by microtubule disruption (Achler et al., 1989; Eilers et al., 1989). In these studies, missorting to the basolateral surface or to vesicles in the basolateral cytoplasm was demonstrated. Because the model proteins studied (for example, aminopeptidase N) travel to the apical surface normally by both the vectorial and transcytotic routes, it is not yet clear whether the observed missorting results from a microtubule dependence of the vectorial pathway to the apical surface, the transcytotic pathway, or both. In contrast to this, our current results with the tail-minus pIg-R in MDCK cells indicates that missorting of this apical membrane protein occurs as a result of improper targetting on the vectorial pathway.

Basolateral to Apical Transcytosis

Studies in whole rat liver (Mullock et al., 1980; Perez et al.,

1988) and in cultured HT-29 cells (Nagura et al., 1979) have suggested that pIg transport is a microtubule-dependent process. All of these studies suggested that microtubule disruption caused a block in basolateral to apical transport of pIg with intracellular accumulation of ligand. In the current study, we have examined the transcytotic pathway in a well-characterized polarized epithelial monolayer (MDCK cells) cultured on permeable filter supports. This system has several advantages over the whole liver and HT-29 studies. In the whole liver studies, it is difficult to control for non-specific effects of microtubule disrupting agents on tight junction integrity and polarity. In the HT-29 study, the HT-29 cells were cultured in glucose containing medium. It was subsequently discovered that in these conditions, HT-29 cells are largely in an undifferentiated multilayer. HT-29 cells can be induced to differentiate into a polarized monolayer by growth in glucose-free medium (Huet et al., 1987), but transcytosis of dIgA under these conditions has not been reported. Finally, in both the HT-29 cells cultured on standard tissue culture plastic and the whole liver studies, a quantitative measurement of ligand recycling to the basolateral surface cannot be made. Thus, we have built upon the results of these studies and in agreement with these studies, we observe some additional intracellular accumulation of ligand in the presence of nocodazole (see Table I). In addition, in the presence of nocodazole, we detect enhanced recycling to the basolateral surface of a portion of nontranscytosed ligand. We can assay return of endocytosed ligand to the basolateral medium since filter-grown MDCK cells allow for independent access to both the apical and basolateral medium. The mechanism responsible for the missorting of the tail-minus pIg-R on the apical vectorial pathway and the apparent missorting of endocytosed ligand for the wild-type pIg-R on the basolateral to apical transcytotic pathway may be similar and may represent random delivery of transport vesicles to either surface in the presence of nocodazole.

Apical Recycling

The final inhibitory effect observed with nocodazole in the current study was on the apical recycling of the ligand for the pIg-R. Although the reduction in apical recycling of the ligand for the pIg-R in the presence of nocodazole was only 21%, this effect was reproducible and statistically significant. We did not note any significant mistargeting of apically endocytosed ligand as only a small percentage of apically endocytosed ligand was transcytosed in the apical to basolateral direction (3% control vs. 7% with nocodazole). In summary, those pathways which terminate at the apical surface are altered to various degrees by nocodazole whereas those that terminate at the basolateral surface are much less affected.

Microtubule Orientation in MDCK Cells

This difference in sensitivity may be explained by the orientation of the microtubular network in polarized cells which differs substantially from nonpolarized cells. In nonpolarized cells, microtubules are nucleated and assembled from the centrosome, with their minus ends anchored to the centrosome and their plus ends radiating toward the cell periphery (Bergen et al., 1980). However, polarized MDCK cells grown on filters for at least 5 d display a very different

microtubular array (Bacallao et al., 1989). Microtubule bundles are arranged in a longitudinal fashion along the apical–basal axis of the cell. The minus ends are spread over the apical region of the cell with the plus ends toward the basal region. Thus, apically directed traffic in MDCK cells travels toward the minus ends of microtubules. Since our data suggest that apically directed traffic is sensitive to microtubule disruption, it may be that traffic in the direction of the minus ends (apical) is more facilitated by the microtubular network than is movement directed toward the plus ends (basolateral). Other minus end directed movement in nonpolarized cells is known to be sensitive to microtubule disruption such as vesicular delivery from early to late endosomes and subsequently the lysosome (Gruenberg et al., 1989). Also, the maintenance of the Golgi complex (Turner and Tartakoff, 1989) and lysosome architecture (Matteoni and Kreis, 1987) are thought to be directed by microtubules in the minus direction and are affected by nocodazole. Recently, retrograde transport of proteins to the ER from a Golgi recycling compartment has been shown to be sensitive to microtubule disruption (Lippincott-Schwartz et al., 1990). It is tempting to speculate that this also is a minus end–directed movement. Thus, the sensitivity of minus end directed traffic to microtubule disruption may be a general phenomenon. Whether cytoplasmic dynein is responsible for both the direction of movement and the selection of vesicles for minus directed microtubule movement remains to be determined.

In addition, the degree of inhibition of apical transport which we observed in the presence of nocodazole varied for the three pathways. The most dramatic effects were observed for basolateral to apical transcytosis, with the least dramatic effect being the apical recycling pathway. Perhaps the simplest explanation for these differences is that, at least for apically directed pathways, the degree to which microtubules enhance vesicle movement varies directly with the distance of the pathway (see Fig. 1). Basolateral to apical transcytosis involves movement across the full height of the cell and would therefore be most sensitive to nocodazole. Apical recycling presumably involves a much shorter movement from the apical endosome back to the apical surface, and so relying on diffusion alone may not cause as large an effect. This hypothesis is particularly attractive since axonal vesicular transport, which must be tightly controlled to achieve delivery across large distances, appears heavily dependent on microtubules (Vale et al., 1986).

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