

Functional Specialization of Stable and Dynamic Microtubules in Protein Traffic in WIF-B Cells

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Abstract. We found that the magnesium salt of ilimaquinone, named 201-F, specifically disassembled dynamically unstable microtubules in fibroblasts and various epithelial cell lines. Unlike classical tubulin-interacting drugs such as nocodazole or colchicine which affect all classes of microtubules, 201-F did not depolymerize stable microtubules. In WIF-B-polarized hepatic cells, 201-F disrupted the Golgi complex and inhibited albumin and alpha1-antitrypsin secretion to the same extent as nocodazole. By contrast, 201-F did not impair the transport of membrane proteins to the basolateral surface, which was only affected by the total disassembly of cellular microtubules. Transcytosis of two apical membrane proteins—the alkaline phosphodiesterase B10 and dipeptidyl peptidase IV—was af-

ected to the same extent by 201-F and nocodazole. Taken together, these results indicate that only dynamically unstable microtubules are involved in the transport of secretory proteins to the plasma membrane, and in the transcytosis of membrane proteins to the apical surface. By contrast, stable microtubules, which are not functionally affected by 201-F treatment, are involved in the transport of membrane proteins to the basolateral surface. By specifically disassembling highly dynamic microtubules, 201-F is an invaluable tool with which to study the functional specialization of stable and dynamic microtubules in living cells.

Key words: microtubules • stability • hepatocyte • protein transport • ilimaquinone

EPITHELIAL cells specifically target newly synthesized secretory and membrane proteins to appropriate domains of their plasma membrane. Among the mechanisms involved in such vectorial targeting, microtubules (MTs)¹ play a fundamental role. They facilitate the transport of vesicular carriers across significant distances, participate in their selective delivery to the basolateral or apical membrane, and are also involved in organelle segregation and localization in the cytoplasm (for review see Cole and Lippincott-Schwartz, 1995). In hepatocytes in which the Golgi complex is localized near the apical membrane, MT involvement is even more crucial given that

protein addressing to the apical (biliary) membrane involves an indirect pathway via the basolateral (sinusoidal) membrane (Bartles et al., 1987).

Within the secretory pathway, MTs are known to play important roles in mediating the anterograde and retrograde traffic of vesicular and tubular intermediates between the ER and the Golgi complex (Lippincott-Schwartz et al., 1990). There is also a large body of evidence that MTs are involved in post-Golgi protein trafficking: MTs are required for efficient transport of membrane proteins to the apical surface via a direct (see Lafont et al., 1994) or indirect transcytotic pathway (Breitfield et al., 1990; Hunziker et al., 1990). They are also involved in basolateral targeting of secretory proteins in various cell types including hepatocytes, but their role in the transport of membrane proteins to the basolateral surface remains controversial (De Almeida and Stow, 1991; Boll et al., 1991; Gilbert et al., 1991; Saucan and Palade, 1992; Van der Sluis et al., 1990; Lafont et al., 1994). One explanation to reconcile these conflicting opinions is that distinct classes of MTs, possibly with different turnover rates, would be involved in very specialized transport steps.

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1. *Abbreviations used in this paper:* AAT, α 1-antitrypsin; IQ, ilimaquinone; MAP, microtubule-associated protein; MT, microtubule.

In eukaryotes, cell differentiation is often accompanied by modifications of MT dynamics. Besides the main subset of highly dynamic MTs with half-lives of ~ 10 min, there is a second population of MTs that can be stabilized for several hours (Webster et al., 1987). Although the molecular basis for such stability is still unknown, stable MTs can be distinguished from dynamically unstable ones since they are less sensitive to MT-depolymerizing drugs, and are enriched in posttranslationally modified subunits of α -tubulin (for review see Mac Rae, 1997). These modifications include enzymatic removal of a single Tyr residue at the carboxy terminus, generating Glu-tubulin (Kreis, 1987; Webster et al., 1987), acetylation of the Lys residue in position 40 (LeDizet and Piperno, 1987; Webster and Borisy, 1989), or more complex modifications such as polyglutamylation (Eddé et al., 1990) or polyglycylation (Re-deker et al., 1994).

A significant effort has been made over the past few years to understand the biological roles of stable vs. dynamic MTs. With respect to cell functions, stable MTs appear to be specialized; they are involved in fibroblastic migration (Nagasaki et al., 1994), in localizing vimentin intermediate filaments in fibroblasts (Gurland and Gundersen, 1995), and in axonal growth (Tanaka et al., 1995). However, except for one study suggesting involvement of stable MTs in the traffic of secretory proteins from the ER to the Golgi in HepG2 cells (Mizuno and Singer, 1994), there are virtually no data regarding the functions of stable and dynamic subsets of MTs in vesicular protein trafficking.

Are stable and dynamic MTs involved in distinct steps of protein trafficking? We addressed this question in hepatic cells using the polarized WIF-B hybrid cell line, which has been validated previously as a model to study hepatic protein transport in vitro (Cassio et al., 1991; Ihrke et al., 1993; Shanks et al., 1994). The most straightforward approach would be to depolymerize a subset of MTs in living cells selectively and study the functions of the remaining population. Using drugs that depolymerize MTs by interacting directly with tubulin—such as colchicine or nocodazole—has proven to be unsatisfactory in that dynamically unstable MTs rapidly disassemble, but stable MTs that are more resistant are still affected in a time-dependent manner (Khawaja et al., 1988). On the other hand, inhibitors of protein phosphatases such as okadaic acid (Gurland and Gundersen, 1993) used to disassemble stable MTs selectively were effective in WIF-B cells, but were too toxic to allow functional studies. We found an alternative way to select a subset of MTs in living cells using a drug named 201-F, which disassembled highly dynamic MTs and preserved stable MTs for several hours. 201-F is the magnesium salt of the sesquiterpene-quinone ilimaquinone (IQ), which was isolated from the marine sponge *Smenospongia* sp. (Konracki and Guyot, 1989). IQ treatment of various cell lines causes both MT disassembly and MT-independent breakdown of the Golgi complex into small vesicles (Takizawa et al., 1993; Veit et al., 1993). IQ also causes brefeldin A-like inhibition of vesicular transport without eliciting retrograde transport (Takizawa et al., 1993). Surprisingly, at concentrations up to 50 μ M, IQ had no effect in isolated rat hepatocytes or WIF-B cells, although cells were normally sensitive to brefeldin A (L. Barbot, unpub-

lished data). The specific properties of 201-F on MTs and the guarantee that, even though it would revert to IQ in living cells, we should not observe IQ effects, gave us the opportunity to study the functional specialization of stable and dynamic MTs in various steps of protein traffic in the WIF-B cell line.

Materials and Methods

Antibodies and Chemicals

201-F was prepared by treating IQ with one equivalent of magnesium ethoxide in tetrahydrofuran. The mixture was stirred for 3 h at room temperature and then filtered. The solvent was removed under reduced pressure to yield the magnesium salt, which was used directly. A concentrated (5 mM) stock solution of 201-F in ethanol was kept at 4°C.

Native (clone DM1A), acetylated (clone 6-11B-1), and tyrosinated (clone TUB-1A2) anti- α tubulin were purchased from Sigma Chemical Co. (St Louis, MO). Anti-rabbit IgG TRITC conjugates, colchicine, nocodazole, paclitaxel (Taxol®), saponin, protein A-Sepharose, and protein G-Sepharose were also purchased from Sigma Chemical Co. NHS-LC-biotin and streptavidin-agarose beads were from Pierce (Rockford, IL). Anti-rabbit and anti-mouse IgG FITC conjugates were from Sanofi Diagnostics Pasteur (Marnes-la-Coquette, France). Anti-Glu-tubulin polyclonal antibodies and monoclonal antibodies to polyglutamylated tubulin (clone GT335) were kindly provided by Dr. L. Lafanechère (Commissariat à l'Energie Atomique, Département de Biologie Moléculaire et Structurale, Grenoble, France) and Dr. P. Denoulet (Centre National de la Recherche Scientifique Unité Propre de Recherche 9065, Collège de France, Paris), respectively. Monoclonal antibodies against basolateral B1 and apical B10 hepatocyte membrane proteins were obtained as previously described (Maurice et al., 1985). Rat α 1-antitrypsin (AAT) was prepared according to Carlson and Stenflo (1982). Anti-albumin and antiAAT antibodies were raised in rabbits as previously described (Biou et al., 1984). The anti-rat DPP IV monoclonal antibody was from Serotec Ltd. (Kidlington, Oxford, United Kingdom), and that to rat mannosidase II (clone 53FC3) was from Berkeley Antibody Co., Inc. (Richmond, CA).

Cell Culture and Treatments

WIF-B cells were cultured in F12 Coon's-modified medium (Sigma Chemical Co.) supplemented with 5% FCS (Dutscher, Rungis, France) and HAT mixture (10^{-5} M hypoxanthine, 4×10^{-7} M aminopterin and 1.5×10^{-5} M thymidine; Polylabo, Strasbourg, France). Cells were confluent and normally polarized 8–10 d after plating at a initial density of 7,000 cells/cm². Fao cells were cultured in the same medium as WIF-B cells without HAT mixture. Human pulmonary fibroblasts (kindly provided by Dr. M. Dehoux, Laboratoire de Biochimie A, Hôpital Bichat, Paris, France) and HeLa cells (kindly provided by Dr. A. Servin, INSERM CJF 94-07, Châtenay-Malabry, France) were cultured in Dulbecco's MEM containing nonessential amino acids and supplemented with 5% FCS. MDCK cells were grown under a 10% CO₂ atmosphere in Dulbecco's MEM supplemented with nonessential amino acids and 10% FCS. Drugs were diluted in culture medium before use, starting from stock solutions in ethanol (5 mM 201-F) or in dimethyl sulfoxide (10 mM colchicine, nocodazole or taxol). The working concentration was 10 μ M for colchicine, nocodazole, and taxol. 201-F toxicity on WIF-B cells was measured by trypan blue exclusion after 4 h of treatment with concentrations ranging from 3.1 to 50 μ M. Cell mortality was <10% between 3.1 and 25 μ M, but 60% at 50 μ M. The maximum morphological effect on albumin scattering was observed by treating cells for 1 h with 25 μ M 201-F. This concentration was used in all experiments.

Western Blot Analysis of α -tubulin and Acetylated Tubulin

WIF-B cells grown for 8 d in 12-well plates were subjected to MT-acting drug treatment in F12 culture medium. At the end of the treatments, cells were rinsed twice in MT-stabilizing buffer (80 mM Pipes, 1 mM MgCl₂, 2 mM EGTA, pH 6.9, supplemented with antiprotease mixture: 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, and 20 μ M leupeptin), and were then extracted with MT-stabilizing buffer containing 0.15% Tri-

ton X-100 (37°C for 1 min on a rocking platform) and washed once with Triton-free buffer. We verified that, in such conditions, cellular MTs were actually preserved at the morphological level (not shown). Cells were lysed directly in SDS-PAGE sample buffer while extraction media were adjusted with 4× SDS-PAGE sample buffer. Proteins were blotted onto polyvinylidene difluoride membranes, and α -tubulin or acetylated tubulin were measured using enhanced chemiluminescence on BioMax films (Eastman Kodak Co., Rochester, NY) and densitometry. The linearity of the assay was verified by blotting and revealing tubulin from serial dilutions of cell lysates.

Immunofluorescence

WIF-B cells were cultured on glass coverslips, and after appropriate treatments were rinsed three times with 0.1 M PBS, pH 7.4, at room temperature, and were then fixed and permeabilized with methanol at -20°C for 5 min. To detect small fragments of stable MTs in WIF-B cells treated for various times with MT-depolymerizing drugs, soluble tubulin was eliminated first to reduce the background fluorescence. For this purpose, cells were permeabilized in MT-stabilizing buffer (80 mM Pipes, 1 mM MgCl_2 , 2 mM EGTA, pH 6.9) containing 0.15% Triton X-100 (37°C, 1 min), and then twice with Triton-free buffer and twice with PBS at room temperature. Soluble tubulin extraction was also performed with a slightly different protocol (0.02% Triton X-100 in MT-stabilizing buffer, 4× for 1 min each, 37°C) for high-contrast imaging of Fao, fibroblasts, HeLa, and MDCK cells treated with MT-depolymerizing agents. All cells were then fixed with methanol (-20°C , 5 min) except MDCK cells, which were fixed with 2% paraformaldehyde in MT-stabilizing buffer.

All antibody incubations and washes were performed in PBS. Cells were incubated with primary antibodies for 1 h at 37°C, washed three times and incubated (1 h, 37°C) with FITC-conjugated or a mixture of FITC- and TRITC-conjugated antibodies for single- and double-labeling experiments, respectively. After three washes, the coverslips were mounted in Citifluor™ antifade mounting medium (Citifluor Laboratories, Birmingham, United Kingdom) and examined with a BH-2 microscope (Olympus, Tokyo, Japan) and a CCD camera (491×; COHU, San Diego, CA). Confocal microscopy was performed using a TCS 4D laser scanning microscope (Leica, Heidelberg, Germany).

To assess the stability of MTs that resisted 201-F, a dilution-induced depolymerization assay was performed. WIF-B cells were incubated (5× for 1 min at 37°C) in Hepes-buffered medium (100 mM Hepes, 2 mM MgCl_2 , 1 mM EGTA, pH 6.9) containing 200 $\mu\text{g}/\text{ml}$ saponin, and were then fixed and processed for the immunofluorescence labeling of acetylated tubulin.

To study the transcytosis of B10 and DPP IV, proteins transiently expressed at the basolateral membrane were labeled by adding 1:200 (vol/vol) anti-B10 or anti-DPP IV ascites fluids to the culture medium and incubating cells for 2 h at 37°C. After three washes in PBS, cells were fixed and permeabilized with methanol (-20°C for 5 min). Incubation with FITC-conjugated secondary antibodies and subsequent processing was identical to that described for indirect immunofluorescence.

Electron Microscopy

After 201-F treatment, cells were rinsed twice in PBS and fixed for 30 min at room temperature in 0.1 M sodium phosphate buffer (pH 7.4) containing 3% glutaraldehyde (Sigma Chemical Co.). After scraping, cells were centrifuged and postfixed in 1% osmium tetroxide solution for 1 h at 4°C. Dehydrated cells were embedded in Epon (Electron Microscopy Sciences, Fort Washington, PA), and ultrathin sections were stained with uranyl acetate and lead citrate.

Pulse-Chase Experiments

For secretion studies, WIF-B cells cultured in 60 mm-diameter dishes were depleted of both methionine and cysteine in depletion medium (Eagle's MEM with nonessential amino acids containing 10 mM calcium lactate and 10 mM Hepes, pH 7.4, and lacking L-methionine and L-cysteine). Pulse labeling was performed for 15 min in the same medium supplemented with 40 $\mu\text{Ci}/\text{ml}$ [^{35}S]-L-methionine and L-cysteine mixture (Tran ^{35}S -Label; ICN Pharmaceuticals Inc., Irvine, CA). Cells were washed twice in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.4, containing 10 mM L-methionine and 10 mM L-cysteine), and were then chased for various times in chase medium (depletion medium containing 10 mM L-methionine and 10 mM L-cysteine). When appropriate, drugs were added to the culture, depletion, pulse, and chase media. In all the experiments after the

chase periods, cells were rinsed twice with Tris-buffered saline and lysed in 20 mM Tris buffer containing 20 mM NaCl, 1% (wt/vol) sodium deoxycholate, 1% (vol/vol) Triton X-100, 10 mM L-methionine, and 10 mM L-cysteine, pH 8.0. Chase supernatants and cell lysates were immediately supplemented with antiprotease mixture, and supernatants were adjusted to the characteristics of the immunoprecipitation buffer (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1% (vol/vol) Triton X-100, 5 mM EDTA, 10 mM L-methionine, 10 mM L-cysteine) with 1:10 (vol/vol) of 10× stock solution.

Surface Biotinylation of Proteins

Cell surface proteins were covalently labeled with biotin according to Ihrke (Ihrke et al., 1993). In brief, at the end of a pulse-chase experiment cells were washed twice with ice-cold PBS containing 0.9 mM CaCl_2 , 1 mM MgCl_2 , and 0.16 mM MgSO_4 , pH 7.4, and incubated twice for 20 min at 20°C with NHS-LC-biotin (0.6 mg/ml) in biotinylation buffer (10 mM sodium tetraborate, 137 mM NaCl, 3.8 mM KCl, 0.9 mM CaCl_2 , 1 mM MgCl_2 , pH 9.0). The reaction was stopped by adding serum- and biotin-free culture medium buffered with 20 mM Hepes.

Immunoprecipitation

Sample clarification was performed twice, either overnight at 4°C, or for 2 h at room temperature using nonimmune rabbit or mouse serum (80 μl per sample) and 300 μl of 1:4 suspension of protein A (or protein G)-Sepharose in immunoprecipitation buffer. After removing the Sepharose beads, 5–10 μl of immune sera or antibodies were added to samples in the same conditions. Immune complexes were then precipitated with 100 μl of protein A (or protein G)-Sepharose, washed three times with immunoprecipitation buffer containing 0.1% SDS, and then eluted in SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE fluorography using BioMax films (Eastman Kodak Co.) and quantified by densitometry. In biotinylation experiments, B1 and B10 proteins were first immunoprecipitated and eluted from protein-G Sepharose beads by boiling in 10% aqueous SDS solution, and an aliquot was kept for gel analysis. The eluate was diluted 1:25 in avidin precipitation buffer (20 mM Tris, 150 mM NaCl, 1% [vol/vol] Triton X-100, 0.2% [wt/vol] BSA, 5 mM EDTA, 10 mM L-methionine, and 10 mM L-cysteine, pH 8.0). The biotinylated fraction was then specifically precipitated with 50 μl of streptavidin-agarose bead suspension and eluted in SDS-PAGE sample buffer.

Results

201-F Selectively Disassembles Dynamic MTs

In WIF-B cells treated for 60 min with 25 μM 201-F, the immunofluorescence labeling of α -tubulin showed a markedly disorganized MT network (Fig. 1 *b*). Treated cells exhibited far fewer MTs than did controls (Fig. 1 *a*), and a 4-h treatment did not induce more pronounced breakdown of MTs than a 1-h treatment as shown in Fig. 1 *c*. The morphology of the remaining MTs was evocative of stable MTs, which are very sinuous compared with straight dynamic MTs (Kreis, 1987). Thus, we performed immunofluorescence labeling of various posttranslationally matured forms of α -tubulin usually found in stable MTs. In both control and 201-F-treated WIF-B cells, acetylated tubulin exhibited similar labeling patterns, suggesting that acetylated MTs were not affected by 201-F treatment (Fig. 1, *d–e*). By contrast, antibodies to detyrosinated or polyglutamylated tubulin did not allow labeling of any MT in WIF-B cells, but at least one fluorescent spot per cell was observed, generally localized by the bile canaliculi (Fig. 1, *f–g*). These observations strongly suggest that acetylation is the predominant (if not only) posttranslational maturation of tubulin in WIF-B cells, and that 201-F did not disassemble this class of MTs. As virtually no detyrosinated tubulin could be observed in WIF-Bs, dynamic MTs were

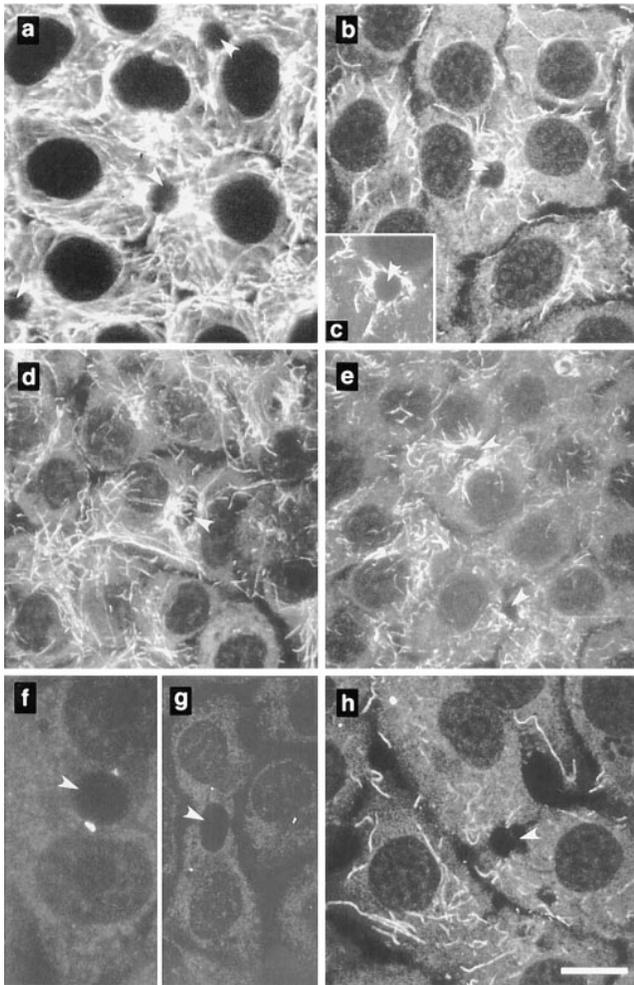


Figure 1. Effect of 201-F on the MT network of WIF-B cells. Cells grown for 8 d on glass coverslips were treated for 60 min without (*a, d, f, g*) or with 25 μM 201-F (*b, e, h*). 201-F treatment was extended to 4 h in *c*. After fixation and permeabilization for 3 min in -20°C methanol, MTs were labeled using antibodies to α -tubulin (*a, b, c*), acetylated tubulin (*d, e*), detyrosinated (*f*), polyglutamylated (*g*), or tyrosinated tubulin (*h*). After incubation with FITC-labeled secondary antibodies, coverslips were examined by confocal microscopy. Bile canaliculi are indicated by arrowheads. Bar, 10 μm .

unlikely to be specifically labeled by anti-Tyr-tubulin antibody: the images were similar to those obtained by labeling α -tubulin or acetylated tubulin in 201-F-treated cells (Fig. 1, *b, e*, and *h*).

To better understand the effects of 201-F on MTs, we compared its effect with that of nocodazole. Biochemical quantifications of α -tubulin and acetylated tubulin that remained polymerized in MTs during the time course of 201-F and nocodazole treatments were performed by a Western blotting technique after soluble tubulin extraction. Nocodazole was chosen as a reference MT-acting drug that affects primarily dynamically unstable MTs, and then gradually causes stable MT depolymerization as the duration of the treatment increases (Khawaja et al., 1988; Gelfand and Bershadsky, 1991). As shown in Fig. 2 *a*, MT

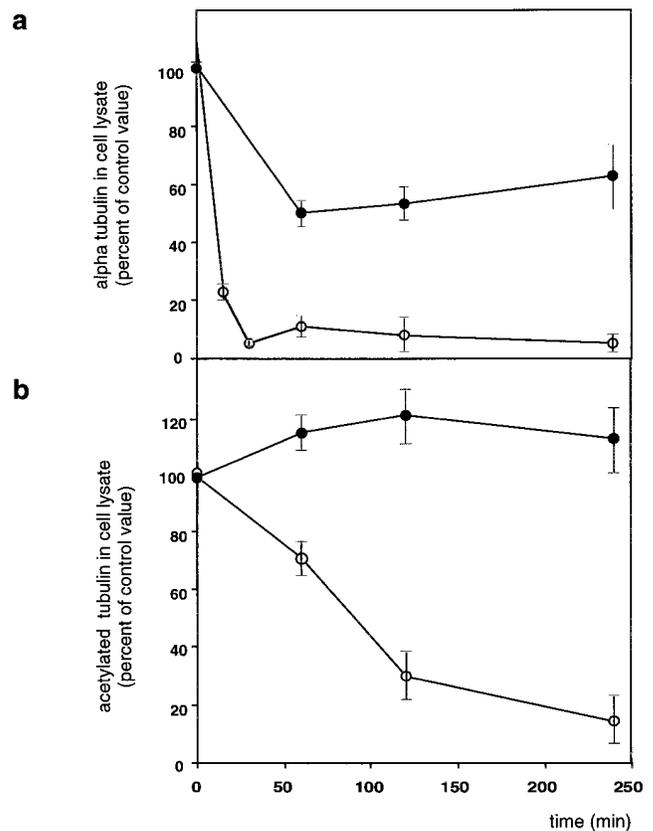


Figure 2. Quantification of α - and acetylated tubulin in MTs during 201-F and nocodazole treatments. WIF-B cells cultured in 12-well plates were incubated for times ranging between 15 min and 4 h with either 25 μM 201-F (*closed symbols*), or 10 μM nocodazole (*open symbols*). At the end of the treatment, soluble tubulin was extracted in MT-stabilizing buffer containing 0.15% Triton X-100 (1 min at 37°C) followed by one rinse in Triton-free buffer. Cells were lysed directly using SDS-PAGE sample buffer. Sample lysates and the corresponding cell extracts were analyzed by Western blotting. α - and acetylated tubulin were quantified using enhanced chemiluminescence assay on BioMax films (Eastman Kodak Co.). Data are expressed relative to the MT contents measured in untreated cells. Each value is the mean \pm SD of three to five independent experiments.

α -tubulin decreased within the first hour of treatment with both drugs. Beyond 1 h of treatment, MT α -tubulin contents reached a steady-state at a much higher level in cells treated with 201-F than that observed with nocodazole, showing that the subset of 201-F-resistant MTs remained constant for at least 4 h (Fig. 2 *a*) as observed morphologically (see Fig. 1 *c*). During nocodazole treatment, the MT contents in acetylated tubulin also decreased, but at a slower rate than that observed for α -tubulin (Fig. 2 *b*). Similar results were found using 10 μM colchicine (not shown). These results indicate that, although the overall population of polymerized tubulin decreased, nocodazole-resistant MTs were enriched in acetylated tubulin. The same holds true during 201-F treatment, but to a much greater extent, as the whole MT subset that resisted 201-F appeared to contain acetylated tubulin (Fig. 2 *b*). A moderate increase in tubulin acetylation even occurred in 201-F-treated cells.

This first set of morphological and biochemical observations indicates that 201-F has a unique effect on MTs that is clearly different from that observed with classical MT-acting drugs such as nocodazole. It seems that 201-F caused the loss of dynamically unstable MTs, but preserved a stable acetylated MT subset for several hours. To verify this hypothesis, we needed to determine if acetylated MTs selected by 201-F treatment were actually stable, and if there were no dynamic MTs left in 201-F-treated cells.

To measure MT stability after 201-F treatment, WIF-B cells were perforated with saponin at 37°C to trigger dilution-induced depolymerization of dynamically unstable MTs. As shown morphologically in Fig. 3, *a* and *b*, acetylated MTs resisted depolymerization both in control and 201-F-treated cells, indicating that 201-F treatment actually preserved the stability of acetylated MTs. This inter-

pretation was confirmed by experiments in which nocodazole was added for 15 or 60 min to the culture medium of 201-F-pretreated cells: immunofluorescent labeling of α -tubulin (Fig. 3, *c-f*) indicated that MTs were not further depolymerized by nocodazole than by a 1-h treatment with 201-F alone. These results were confirmed using biochemical techniques to quantify MT contents after soluble tubulin extraction. In this case also, after nocodazole addition MT α -tubulin levels remained identical to those measured with 201-F alone (Fig. 3 *g*). These experiments also demonstrate that 201-F caused quantitative disassembly of dynamic MTs in WIF-B cells as we could not measure any significant short-term effect of nocodazole on a putative residual fraction of dynamic MTs.

To make sure that the effects of 201-F are not restricted to WIF-B cells, we tested various cell types and evaluated the stability of 201-F-resistant MTs (Fig. 4). The experi-

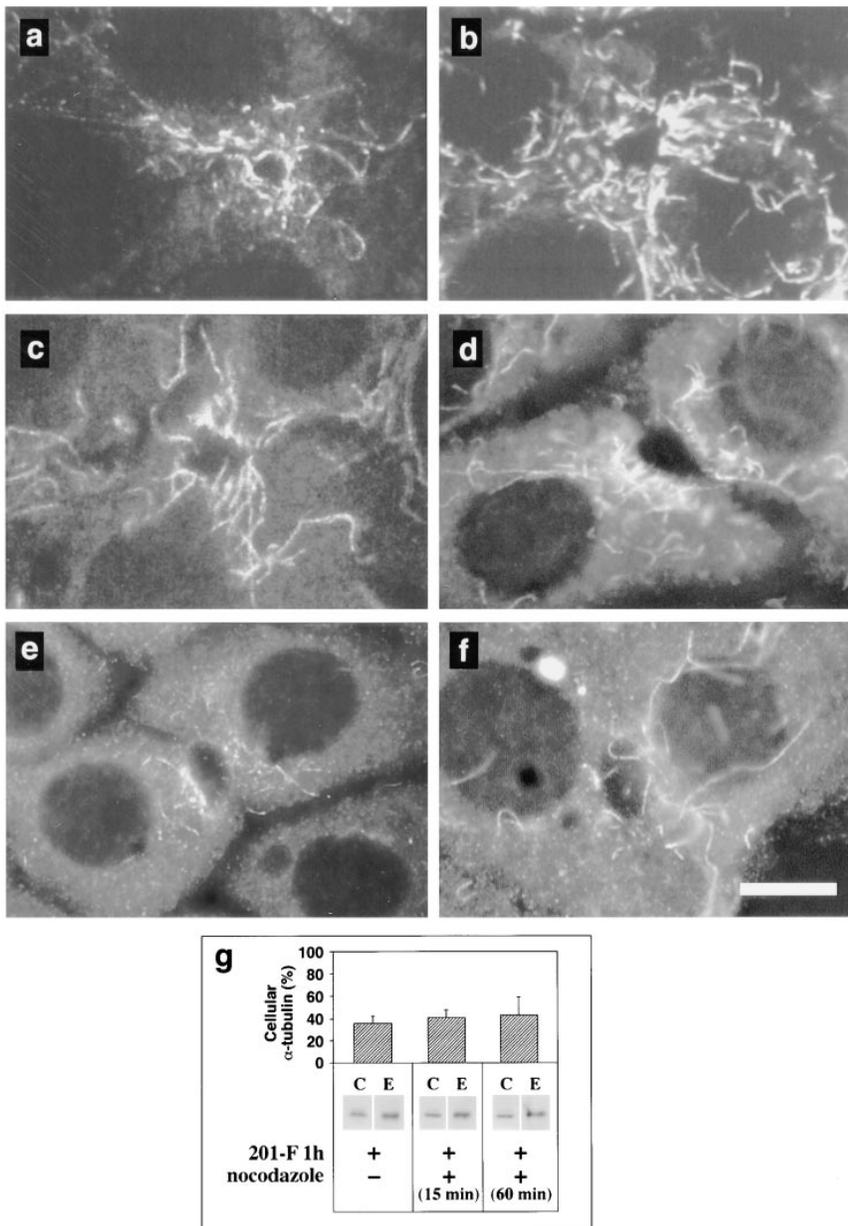


Figure 3. MTs that resist 201-F treatment are stable. In a first series of experiments, control (*a*) and 201-F-treated cells (*b*) were permeabilized using 0.2 mg/ml saponin in HEPES buffer to trigger dilution-induced depolymerization of MTs. MTs were labeled with antibodies to acetylated tubulin, and were observed by means of confocal microscopy. Images are the superimposition of four equidistant optical sections ($\Delta z = 1.5 \mu\text{m}$). Bar, 10 μm . In a second set of experiments (*c-g*), cells were treated with 25 μM 201-F for 1 h. 10 μM nocodazole was then added into the culture medium for 15 (*d*) or 60 min (*f*). 1-h 201-F and 1-h-nocodazole controls are shown in *c* and *e*, respectively. Cells were then fixed and processed for the immunofluorescent labeling of α -tubulin (*c-f*). Soluble α -tubulin was also extracted with MT-stabilizing buffer containing 0.15% Triton X-100. Cellular (C) and extracted (E) α -tubulin was analyzed by Western blot ($n = 3$; *g*).

ments shown were performed using Fao rat hepatoma cells from which WIF-B cells originate (Fig. 4, *a-c*), human pulmonary fibroblasts (Fig. 4, *d-f*), HeLa cells (Fig. 4 *g-i*) and MDCK cells (Fig. 4 *j-l*). In the first two columns, cells were treated without or with 201-F for 1 h, whereas in the third column, cells were first treated for 1 h with 201-F, and then nocodazole was added into the culture medium for 15 min. Cells were then permeabilized in MT-stabilizing buffer and processed for indirect immunofluorescence labeling of α -tubulin. As shown in Fig. 4 (*b, e, h, k*), only a subset of tortuous MTs resisted 201-F treatment in each of the cell lines tested. 201-F exhibited similar effects in polarized epithelial cells (MDCK), nonpolarized epithelial cells (HeLa, Fao), or fibroblasts. Furthermore, as shown above in WIF-B cells, 201-F-resistant MTs were actually stable as they resisted nocodazole-induced depolymerization (Fig. 4 *c, f, i, l*).

Dynamic MTs Mediate the Transport of Secretory Proteins

Pulse-chase experiments were used to study the role of dynamic and stable MTs in the transport of both albumin (Cassio et al., 1991; Shanks et al., 1994) and AAT, which are constitutively secreted by WIF-B cells.

Radiolabeling of newly synthesized proteins followed by a 1-h chase in the presence of increasing concentrations of 201-F showed that the drug inhibited albumin and AAT

secretion in a concentration-dependent manner (Fig. 5 *a*). After 1 h of chase, the mean inhibition reached 50% with 25 μ M 201-F. Secretion inhibition was not due to a protein synthesis impairment, as incorporation of radiolabeled methionine and cysteine into albumin, AAT, and total proteins was identical in control and 201-F-treated cells (data not shown). It should also be pointed out that, in 201-F-treated cells, secreted AAT seemed normally glycosylated as the secreted fraction exhibited a molecular mass identical to that of AAT secreted by control cells (Fig. 5 *a*). The effect of 201-F on the secretion kinetics of albumin and AAT was then studied. As shown in Fig. 5 *b*, secretion kinetics of both proteins were severely retarded in treated cells. The inhibitory effect of 201-F resulted in a twofold increase in the transit times for albumin and AAT secretion as compared with control cells. After a 4-h chase period, the secretion yield of albumin and AAT reached 85–90% in both control and treated cells (data not shown).

Because IQ, from which 201-F originates, is known to disrupt the Golgi complex and inhibit transport vesicle budding *in vitro*, we tested whether the effects of 201-F on protein secretion resulted from its action on dynamic MTs or from a side effect that might have altered the integrity or the function of the Golgi complex.

To test these hypotheses, we first analyzed the effects of 201-F on the structure of the Golgi by means of immunofluorescence and electron microscopy. The Golgi complex was visualized using antibodies to rat mannosidase II

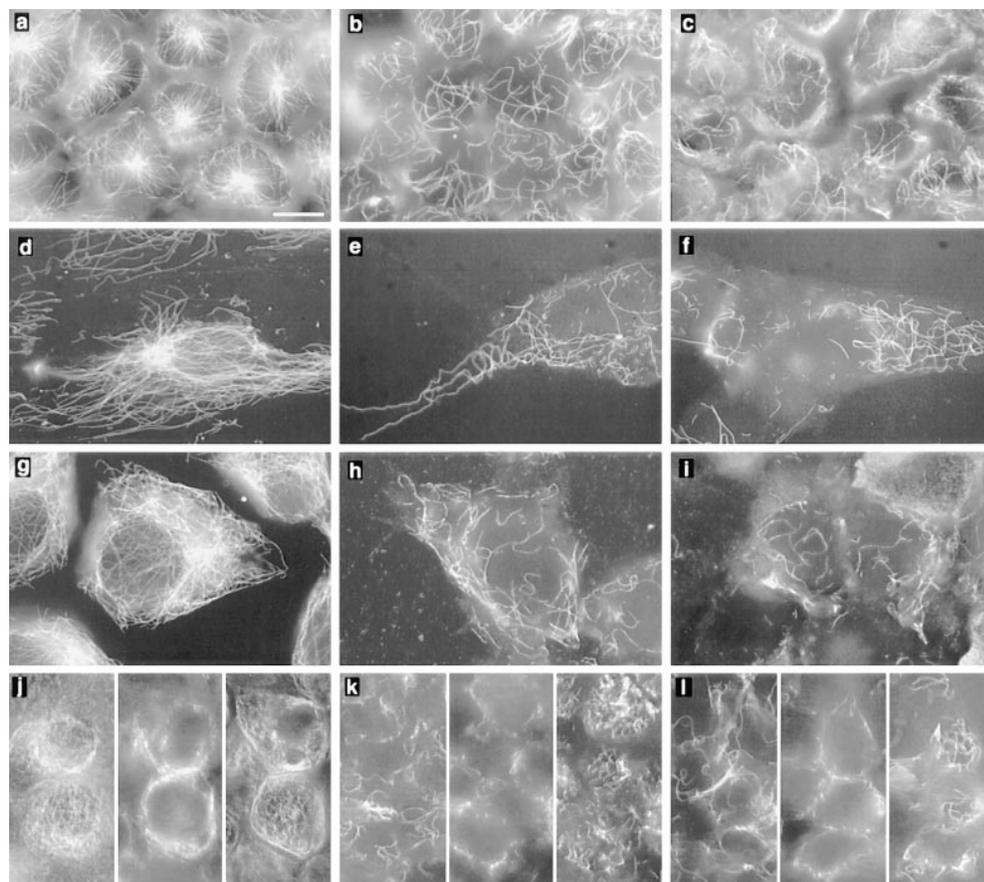


Figure 4. Dynamic MTs are selectively depolymerized by 201-F treatment in fibroblastic and epithelial cells. Fao rat hepatoma cells (*a-c*), human pulmonary fibroblasts (*d-f*), HeLa cells (*g-i*), and MDCK cells (*j-l*) were treated without (*a, d, g, j*) or with 25 μ M 201-F for 1 h (*b-c, e-f, h-i, k-l*). In some cases, 10 μ M nocodazole was added into the culture medium for 15 min at the end of the 1-h 201-F treatment (*c, f, i, l*). After three washes with MT-stabilizing buffer, soluble tubulin was extracted with warm MT-stabilizing buffer containing 0.02% Triton X-100 (37°C, 4 \times for 1 min). After fixation (methanol at -20° C for 5 min), cells were processed for immunofluorescence labeling of α -tubulin and examined in a fluorescence microscope. For MDCK cells, three optical sections of the same cells were taken near the basal domain (*left*), across the lateral domain (*middle*) and near the apical domain (*right*) in each panel (*j-l*). Bar, 10 μ m.

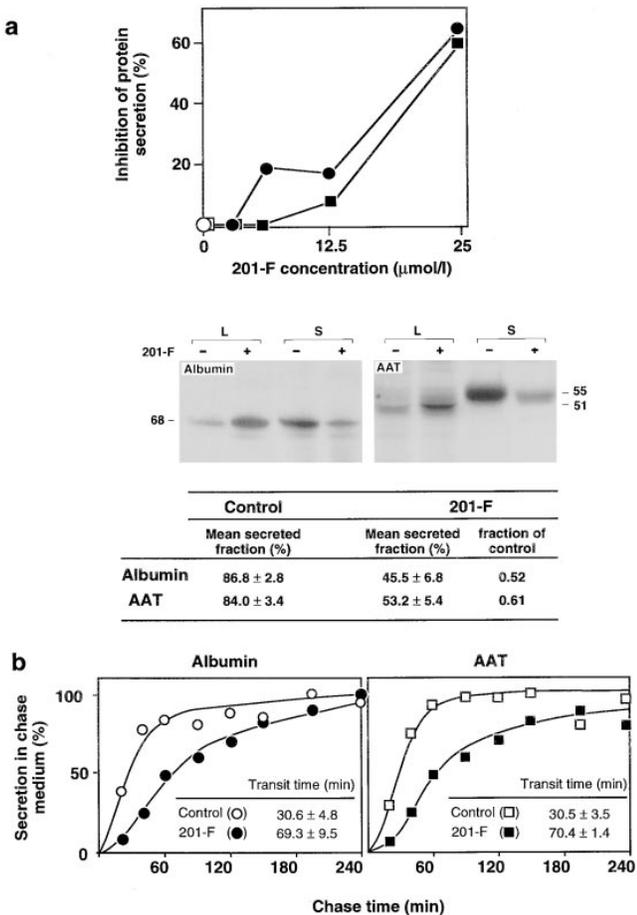


Figure 5. 201-F inhibits secretion of albumin and AAT in WIF-B cells. Cells were depleted of methionine and cysteine for 30 min, and then newly synthesized proteins were radiolabeled for 15 min with [^{35}S]methionine and cysteine, and were chased for various times. When appropriate, 25 μM 201-F was added into the depletion, pulse, and chase media. Albumin (circles) and AAT (squares) were immunoprecipitated from cell lysates and chase supernatants, and were quantified by SDS-PAGE fluorography and densitometry. Secretion is expressed as the ratio of secreted protein to total (secreted plus intracellular) immunoprecipitated protein to normalize for the variations in the incorporation of [^{35}S]methionine and cysteine. Secretion inhibition was measured relative to control values at the last chase time. (a) Effect of 201-F on albumin and AAT secretion after 1 h of chase. In the upper graph, the concentration-dependent effect of 201-F is shown. The lower part of the panel summarizes the data (mean secreted fractions \pm SEM) from four (AAT) to eight experiments (albumin), and show a sample SDS-PAGE of albumin and AAT immunoprecipitated from cell lysates (L) and chase supernatants (S). (b) Secretion kinetics of albumin and AAT in control (open symbols) and 201-F-treated cells (closed symbols). Kinetic data are fitted to a three-compartment model for secretion by a nonlinear least-square method. Transit times are the sum of the reciprocals of the two rate constants computed from experimental data; they are the mean \pm SEM of three independent determinations.

(Man II). The Golgi was also readily visualized using antibodies raised against rat serum albumin, which is highly concentrated in the organelle (Cassio et al., 1991; Shanks et al., 1994). In double-labeling experiments, the two markers colocalized in control cells and labeled a region

between nuclei and bile canaliculi (Fig. 6, a and b). In cells treated with 201-F, the Golgi complex was disrupted, and both markers were found in organelle fragments that scattered into the cytoplasm while a fraction remained in the original location by the apical membrane (Fig. 6, c and d). At the electron microscopy level, individual Golgi stacks were still identified, and appeared structurally normal except for some swelling of the cisternae (Fig. 7). Such a conservation of a stacked organization was evocative of an MT-dependent disruption of the organelle (Rogalski et al., 1984). Therefore, putative involvement of MT depolymerization in the disruption process was verified in experiments where MTs were stabilized with taxol before 201-F treatment. In these conditions, Golgi fragmentation was markedly inhibited (Fig. 6, e–l), indicating that the effect of 201-F resulted from its action on MTs as shown previously with other MT-depolymerizing drugs (Thyberg et al., 1980; Pavelka and Ellinger, 1983; Wehland and Willingham, 1983; Rogalski and Singer, 1984).

To study the involvement of Golgi fragmentation in the secretory effects of 201-F, we devised experiments in which WIF-B cells exhibited a normal MT network, but a disrupted Golgi complex. A way to achieve such conditions was found that involved depolymerization of the MT network during a 1.5-h treatment with nocodazole to fragment the Golgi complex. Nocodazole was then washed out to allow reconstitution of the MT network. As shown in Fig. 8 a, double labeling experiments in which we followed the time courses of tubulin polymerization and Golgi compaction after such nocodazole treatment and washout showed that the MT network was rebuilt within 20 min after nocodazole removal, whereas the Golgi remained disrupted for at least 1.5 h. Pulse–chase experiments were performed in the time window provided by the differential kinetics of MT repolymerization and Golgi compaction. Cells were pretreated for 1.5 h with nocodazole. After drug removal, newly synthesized proteins were pulse-labeled and chased for 1 h without nocodazole. In such conditions (Fig. 8 b, column 2) albumin secretion was not inhibited, as compared with control cells (Fig. 8 b, column 1). This result indicates that Golgi fragmentation was not responsible for inhibition of albumin secretion observed when dynamic MTs were depolymerized in 1-h pulse–chase experiments in the presence of 201-F (Fig. 8 b, column 5). As proposed by Cole et al. (1996), an alternative hypothesis would be that, during nocodazole treatment, the Golgi fragments relocalize near peripheral ER exit sites to rescue part of the secretory processes via an MT-independent ER-to-Golgi transport step. When examining the time-dependent effect of nocodazole on albumin secretion for time periods ranging from 30 min to 12 h, we observed a twofold decrease in the inhibitory effect of the drug after 4 h of treatment (Fig. 8 c). Such an evolution of the inhibition curve might be a good reflection of the relocalization phenomenon. This observation would also mean that, within the 1.5-h treatment that was used in the nocodazole washout experiments, the relocalization process was far from being complete, therefore validating our former conclusion that protein secretion was not inhibited by Golgi fragmentation.

To rule out the possibility that 201-F might also have affected secretion independently from its effect on dynamic

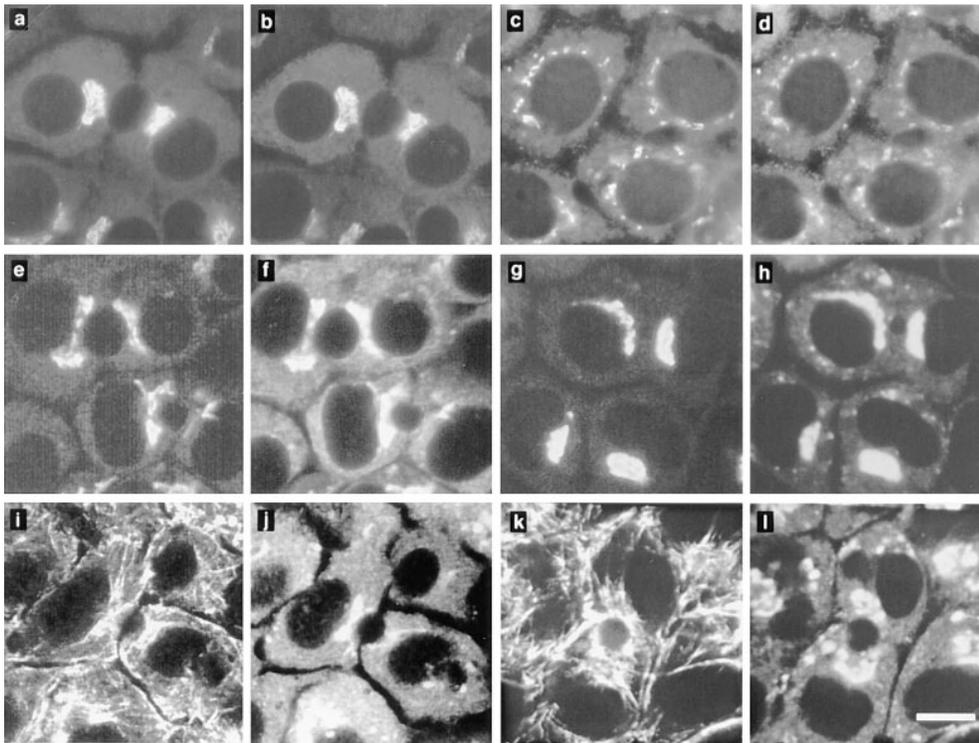


Figure 6. 201-F causes MT-dependent fragmentation of the Golgi complex. Cells were treated without (*a, b*) or with (*c, d*) 25 μ M 201-F for 1 h, fixed, and processed for double immunofluorescence labeling using antibodies to Man II (*a, c*) and secretory albumin (*b, d*). In a second set of experiments, cells were treated with 10 μ M taxol for 2 h in serum-free culture medium to stabilize MTs (*e, f, i, j*), or with taxol alone for 1 h and then with a mixture of taxol and 201-F for one more hour (*g, h, k, l*). After fixation, cells were subjected to double immunofluorescence labeling of Man II (*e, g*) and albumin (*f, h*), or that of α -tubulin (*i, k*) and albumin (*j, l*). All samples were examined in a confocal microscope. Bar, 10 μ m.

MTs, we performed experiments in which we combined nocodazole and 201-F treatments as follows: cells were pretreated for 4 h with nocodazole to get a residual inhibitory effect on secretion. 201-F was then added into the culture medium 30 min before protein radiolabeling, and a 1-h chase was performed in the presence of both nocodazole and 201-F (Fig. 8 *b*, column 4). The control experiment consisted of a 4-h nocodazole pretreatment followed by a 1-h pulse-chase in the presence of nocodazole (Fig. 8 *b*, column 3). The fact that 201-F addition did not increase the inhibitory effect of nocodazole on albumin secretion means that 201-F effect on secretion did not involve an MT-independent process. Otherwise, 201-F addition to nocodazole would have inhibited secretion to the same extent as 201-F alone (Fig. 8 *b*, columns 4 and 5, respectively).

This first set of experiments showed that 201-F-sensitive dynamic MTs were strongly involved in carrying secretory proteins to the cell surface. Moreover, protein secretion was inhibited to the same extent when cellular content of stable MTs was preserved with 201-F (Fig. 8 *b*, column 5) or lost by 90% with a 1-h nocodazole treatment (Fig. 8 *b*, column 6; see also Fig. 2). This observation means that if the structure and function of stable MTs was maintained in 201-F-treated cells, stable MTs did not participate in the transport of secretory proteins to the cell surface. Another argument that also indicates that the effects of 201-F on secretion are specifically due to dynamic MT depolymerization arises from observations we performed in taxol-treated cells. As shown previously in rat hepatocytes (Oda and Ikehara, 1982), treatment of WIF-B cells with 10 μ M taxol inhibited protein secretion to the same extent as 10 μ M nocodazole in 1-h pulse-chase experiments (Fig. 8 *b*, columns 7 and 6, respectively). This observation might

indicate that stabilization of dynamic MTs by taxol altered their involvement in the secretory process, therefore reinforcing our previous conclusion that stable MTs did not participate in protein secretion. In such conditions, the fact that a combination of taxol and 201-F (Fig. 8 *b*, column 8) inhibited secretion to the same extent as taxol alone (Fig. 8 *b*, column 7) also argues against any MT-independent effect of 201-F on secretion.

Stable MTs but not Dynamic MTs Participate in the Basolateral Transport of Membrane Proteins to the Cell Surface

In the following experiments, we studied basolateral expression of two membrane markers: B1 and B10 (Maurice et al., 1985; Maurice et al., 1988). B1 is expressed at the basolateral surface of hepatocytes, while B10, an alkaline phosphodiesterase (Scott et al., 1997), is a marker of bile canaliculi and undergoes indirect addressing via the basolateral membrane in hepatocytes (Maurice et al., 1994) and in WIF-B cells.

Unlike their well-known role in secretory protein transport, MT involvement in basolateral targeting of membrane proteins remains controversial. As suggested by Lafont et al. (1994), previous attempts might have failed because of significant resistance of MTs to depolymerizing drugs, thereby suggesting that stable MTs are good candidates for mediating this step of protein trafficking. Establishing the conditions in which all the MTs were disassembled was thus a prerequisite to perform such a study. These conditions were obtained using colchicine or nocodazole for 10 h. In the following experiments, colchicine was preferred to nocodazole because of its irreversible effect on MTs. As shown in Fig. 9 *a* (in which cells were per-

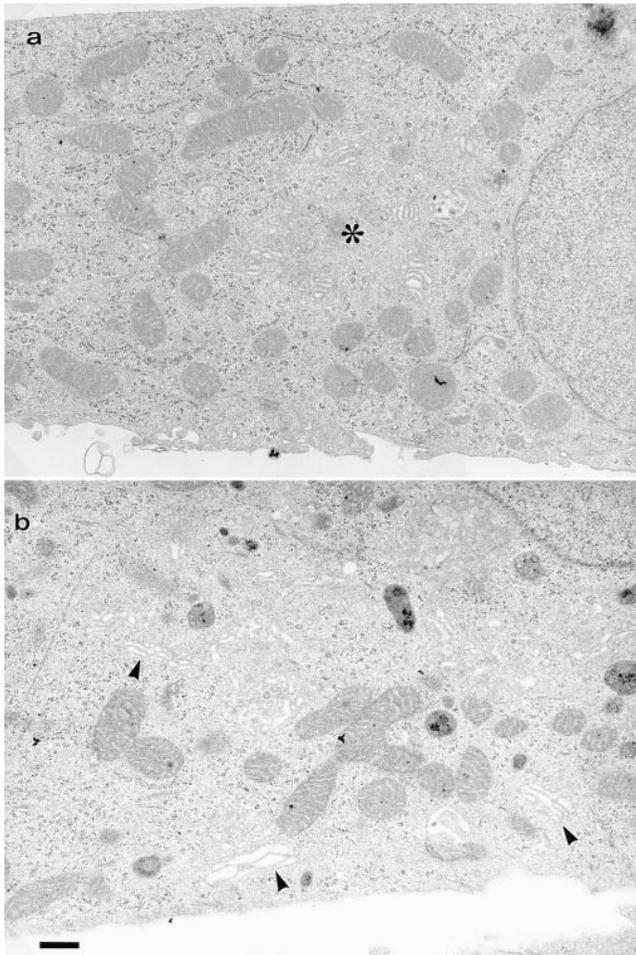


Figure 7. In 201-F-treated cells, the Golgi fragments are composed of stacked cisternae. The Golgi fragments were visualized by electron microscopy in control cells (*a*) or after cells were treated for 60 min with 201-F (*b*). Cells were fixed with 3% glutaraldehyde in sodium phosphate buffer, post-fixed in 1% osmic acid, embedded in Epon, and ultrathin sections were counterstained with uranyl acetate and lead citrate. In a typical control cell, the Golgi complex was observed as a condensed organelle (*asterix*) located close to the nucleus. 201-F-treated cells exhibited Golgi fragments with stacked cisternae (*arrowheads*). Bar, 0.5 μm .

meabilized in an MT-stabilizing buffer to minimize the cytoplasmic background fluorescence), a few MTs resisted depolymerization within the first 2 h of treatment. Small MT fragments were still observed after 4 h, but complete breakdown was achieved after a 10-h treatment.

To test whether dynamically unstable MTs are involved in the transport of B1 and B10 to the basolateral surface, cells were pretreated with 201-F, and pulse-chase experiments were performed in the presence of the drug. These experiments were followed by a step of cell surface biotinylation. Even though tight junctions of WIF-B cells are permeable to the biotinylation reagent (Ihrke et al., 1993), limiting the chase time to 1 h ensured that most B10 protein was still expressed at the basolateral surface and was not undergoing transcytotic transport to the apical membrane (Maurice et al., 1994). Our experiments showed that after 201-F treatment, B1 and B10 proteins were normally

inserted into the plasma membrane (Fig. 9 *b*), indicating that dynamic MTs did not participate in the basolateral transport of these two proteins.

By contrast, complete disassembly of dynamic and stable MTs by 10-h colchicine treatment inhibited basolateral membrane expression of both B1 and B10 proteins (Fig. 9 *b*). This set of experiments suggested that the transport of membrane proteins to the basolateral domain of WIF-B cells involved stable MTs. This result would also indicate that, as hypothesized earlier, 201-F did not impair the function of stable MTs in terms of protein transport, and would therefore validate our conclusion about the lack of involvement of stable MTs in the transport of secretory proteins to the cell surface.

Dynamic MTs Mediate the Transcytosis of B10 and DPP IV

Apical proteins undergo indirect addressing via the basolateral membrane in both hepatocytes and WIF-B cells (Maurice et al., 1994; Ihrke et al., 1998). The transcytotic transport of apical proteins in hepatocytes has already been shown to depend on the integrity of the MT network (Hemery et al., 1996). To study the functional specialization of MTs in transcytosis, newly synthesized B10 and DPP IV transiently expressed at the basolateral membrane were continuously tagged by adding specific antibodies to the culture medium for 2 h in control and 201-F-treated cells. In control cells, both proteins were highly concentrated in the apical membranes (Fig. 10, *a* and *b*). When cells were treated with 201-F, transcytosis was partly inhibited with a reduction in the relative fluorescence in apical membranes and an increase in basolateral labeling of both proteins. A significant amount of both proteins was also observed inside the cell, suggesting that 201-F did not inhibit the internalization from the basolateral membrane (Fig. 10, *c* and *d*). These experiments indicate that dynamic MTs were necessary for transcytotic transport of apical proteins, and that stable MTs unaffected by 201-F treatment could not achieve normal transcytosis from the basolateral to the apical surface. When cells were treated overnight with colchicine, bile canaliculi were very difficult to identify morphologically (not shown), as they tended to collapse (Durand-Schneider et al., 1991). Conversely, treatment for 10 h with nocodazole allowed morphological identification of bile canaliculi. In such conditions, transcytosis of DPP IV and B10 was also partially inhibited, with immunofluorescence labeling on both basolateral and apical membranes and in a small intracellular fraction (Fig. 10, *e-f*). Thus, it seems that disassembly of the whole MT network did not inhibit transcytosis of B10 and DPP IV to a greater extent than selective disassembly of dynamic MTs, suggesting that stable MTs did not participate in transcytosis.

Discussion

We found that the magnesium salt of IQ, named 201-F, disrupted the Golgi complex and inhibited protein secretion in the polarized hepatic cell line WIF-B. 201-F selectively disassembled the highly dynamic network of MTs without affecting the function of stable MTs. The mechanism by which 201-F causes dynamic MT disassembly is

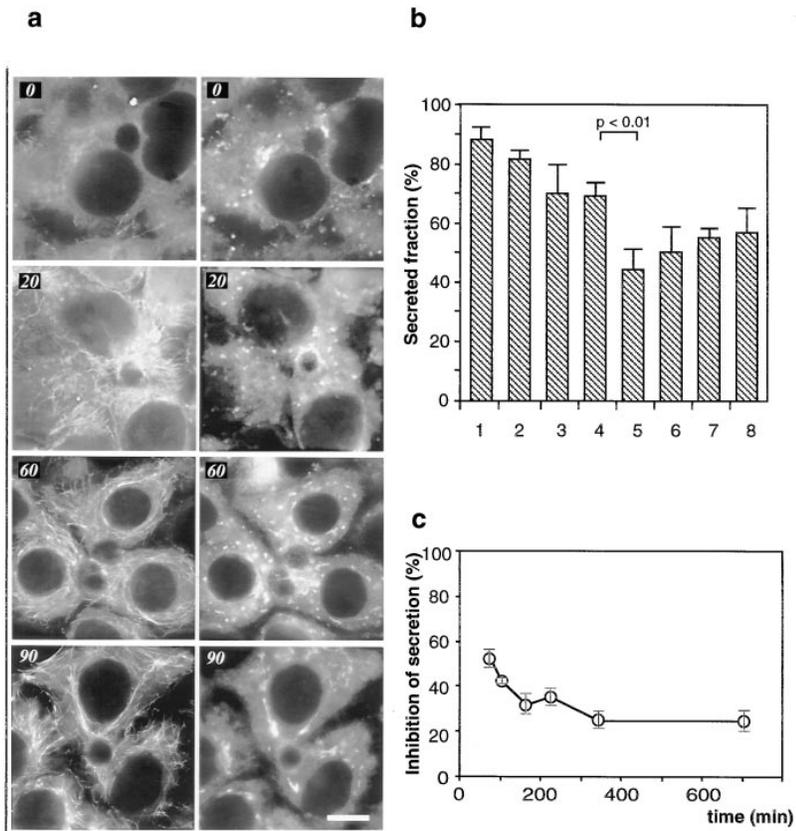


Figure 8. Neither Golgi fragmentation nor a direct effect of 201-F on Golgi membranes are responsible for the inhibitory effect of 201-F on protein secretion. In *a*, cells were treated for 1.5 h with 10 μ M nocodazole. Nocodazole was then washed out to allow reconstitution of the MT network and compaction of Golgi fragments. At various times ranging from 0 to 90 min after drug removal, cells were fixed, processed for double immunofluorescence labeling of α -tubulin (*left side*) and albumin (*right side*), and examined in a fluorescence microscope. Bar, 10 μ m. *b* shows the mean secreted fractions \pm SEM of albumin after 1-h pulse-chase experiments performed in the following conditions: (1) no treatment; (2) 10 μ M nocodazole pretreatment (1 h) and depletion of methionine and cysteine in the presence of nocodazole (30 min), radiolabeling (15 min), and chase (60 min) without nocodazole; (3) 10 μ M nocodazole pretreatment (4 h), depletion (30 min), pulse labeling (15 min), and chase periods (60 min) in the presence of nocodazole; (4) 10 μ M nocodazole pretreatment (4 h), adding 25 μ M 201-F during depletion (30 min), pulse labeling (15 min), and chase period (60 min); (5–7) Depletion (30 min), pulse labeling (15 min), and chase (60 min) in the presence of 25 μ M 201-F (5), 10 μ M nocodazole (6), or 10 μ M taxol (7); (8) 10 μ M taxol pretreatment (1 h), adding 25 μ M 201-F during the depletion (30 min), pulse labeling (15 min), and chase period (60 min). Data are the mean \pm SEM of eight (1, 5, 6), four (2), or three experiments (3, 4, 7, 8). Statistical

comparisons were performed using the Mann and Whitney test. Identical results were obtained with AAT (not shown). *c* shows the inhibition of albumin secretion measured during the time course of nocodazole treatment. Cells were pretreated with 10 μ M nocodazole for times ranging from 0 to 11 h before methionine and cysteine depletion and pulse-labeling with 35 S methionine and cysteine in the presence of the drug. Radiolabeled proteins were chased for 1 h in the presence of nocodazole, and then albumin was immunoprecipitated from chase supernatants and cell lysates. After measurement of the mean secreted fractions of albumin, secretion inhibition was calculated relative to control values. Data are the mean \pm SEM of three independent determinations.

not clear. However, in contrast to colchicine or nocodazole, 201-F acts indirectly on MTs. At concentrations up to 100 μ M, 201-F did not interfere with tubulin assembly or disassembly *in vitro* (D. Guénard, personal communication). In addition, the effect of 201-F did not involve a sustained increase in cytoplasmic calcium levels (C. Poüs and L. Combettes, unpublished results). We are currently investigating the possibility that 201-F affects tubulin phosphorylation (Mac Rae, 1997), or that it interferes with the phosphorylation of structural or motor microtubule-associated proteins (MAPs) and/or their binding to MTs (Hirokawa, 1994). The key property of 201-F was its ability to disassemble dynamic MTs and reproducibly and durably preserve the stability of acetylated MTs. As judged by the amount of cellular MTs that resisted 201-F treatment and the increase we observed in acetylation of cellular MTs, 201-F might also moderately increase the stable MT population. Even when used at high concentrations for several hours, 201-F did not cause disassembly of stable MTs in contrast to nocodazole. This property was used to study the specialized functions of stable and dynamic MTs in mediating various steps of vesicular protein transport.

Our results show that transport of both secretory and membrane proteins involves distinct stability classes of

MTs. With regard to protein secretion, selective disassembly of highly dynamic MTs by 201-F caused a general slowdown of albumin and AAT secretion without accumulation in the ER, as the endoglycosidase-H sensitivity of AAT was identical in control and treated cells (T. Phung-Koskas, unpublished result). This result, together with the finding that in hepatic cells, stable MTs are the most probable carriers of albumin from the ER to the Golgi (Mizuno and Singer, 1994), would indicate that inhibition of protein secretion resulted from disassembly of dynamic MTs between the Golgi and the cell surface. In contrast, stable MTs that resisted 201-F treatment did not participate in the transport of secretory proteins to the cell surface, but mediated the transport of B1 and B10 to the basolateral membrane. These results are in keeping with previous data showing that secretory and membrane proteins are transported to the basolateral membrane of hepatocytes in distinct vesicular carriers (Saucan and Palade, 1994). Although transport of secretory proteins is inhibited by MT-acting drugs, membrane protein transport does not seem to involve MTs (De Almeida and Stow, 1991; Boll et al., 1991; Gilbert et al., 1991; Saucan and Palade, 1992). In contrast, two studies provided evidence that MTs are involved in the transport of membrane proteins to the baso-

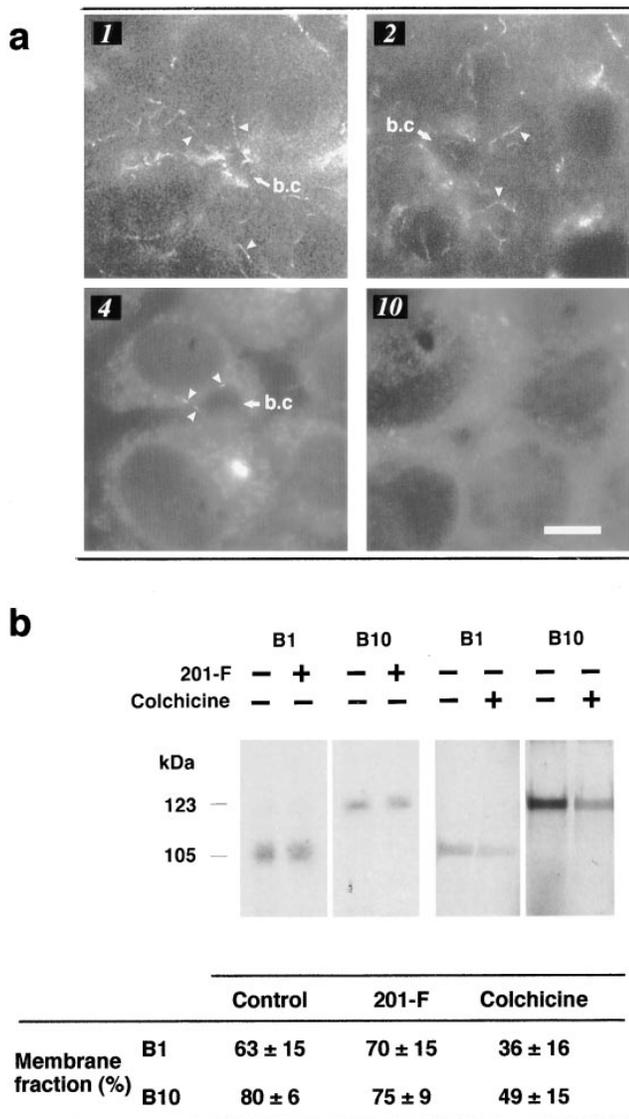


Figure 9. Stable MTs are involved in the transport of membrane proteins to the basolateral surface of WIF-B cells. In *a*, cells were treated for times ranging between 1 and 10 h with 10 μ M colchicine. To minimize background fluorescence, soluble tubulin was extracted by permeabilizing cells with 0.15% Triton X-100 in MT-stabilizing buffer (1 min, 37°C). Cells were then fixed, processed for immunofluorescence labeling of α -tubulin, and examined in a fluorescence microscope. Short MTs were still observed in cells treated for up to 4 h with colchicine (arrowheads), but could not be detected after 10 h of treatment. When present, bile canaliculi (*b.c.*) are identified. Bar, 10 μ M. *b* shows the effects of 1-h treatment with 201-F and 10-h treatment with colchicine on basolateral membrane expression of B1 and B10 proteins. Newly synthesized proteins were radiolabeled and chased for 1 h in the presence of 201-F or colchicine, and were then subjected to surface biotinylation. Total B1 and B10 were first immunoprecipitated from cell lysates, and then the biotinylated fractions were precipitated using streptavidin-agarose. Samples were analyzed by SDS-PAGE, fluorography and densitometry. Membrane fractions of B1 and B10 are the mean \pm SD of three determinations.

lateral surface: basolaterally targeted vesicles were shown to bind to MTs in vitro (Van der Sluijs et al., 1990), and an inhibitory effect of nocodazole was observed in streptolysin-O-perforated MDCK cells that provoked total MT disassembly (Lafont et al., 1994). The best explanation integrating our results and previous contradictory data is that stable MTs involved in the transport of membrane proteins resist depolymerizing drugs for periods that depend on the cell type, and can even exceed an entire interphase (Webster et al., 1987). Overnight colchicine treatment was sufficient to break down all the MTs in WIF-B cells and, in these conditions, we found that basolateral transport of B1 and B10 was inhibited.

With regard to transcytosis, the inhibitory effects of 201-F on apical expression of B10 and DPP IV are explained by the fact that transcytosis can be inhibited by MT-acting drugs in hepatocytes (Durand-Schneider et al., 1991; Hemery et al., 1996) and in other epithelial cells (Breitfield et al., 1990; Hunziker et al., 1990). In MDCK cells, the transcytotic step which has been shown to depend on MTs, corresponds to the movement from basolateral early endosomes to the apical endosome (Breitfield et al., 1990; Hunziker et al., 1990). Our observations are in agreement with these data, as a significant amount of immunolabeled B10 and DPP IV was found to accumulate inside 201-F-treated cells. We also observed a large increase in immunofluorescence at the basolateral membrane of treated cells. Breitfield et al. (1990) have also observed increased recycling of the pIgA-receptor at the basolateral membrane, and proposed that it might result from saturation of the transcytotic pathway sorting mechanism in basolateral endosomes. Whatever the mechanism of transcytosis inhibition, functional specialization of MTs is suggested by our data: the integrity of the dynamic MT network is necessary for transcytosis, but stable MTs did not seem to participate in the transcytotic transport process. Similar results were obtained in experiments performed in living WIF-B cells, in which the transcytosis of fluorescent aminopeptidase-N was arrested by 201-F or nocodazole treatments (M. Schrader and T.A. Schroer, personal communication). Such data would also indicate that stable MTs do not participate in transcytosis.

Although functional specialization of stable and dynamic MTs has already been explored in other fields of cell biology (see Nagasaki et al., 1994; Gurland and Gundersen, 1995; Tanaka et al., 1995), very few data on their respective roles in vesicular trafficking are available (Mizuno and Singer, 1994). Functional specialization has been demonstrated in the basolateral and apical trafficking pathways of MDCK cells, but was expressed in terms of molecular motor association, as kinesin and dynein were both involved in apical transport whereas only kinesin was involved in basolateral transport (Lafont et al., 1994). The molecular basis of MT specialization probably has a corresponding molecular specificity for interaction with transport vesicles. By analogy with the differential involvement of kinesin and dynein in apical and basolateral transport (Lafont et al., 1994), molecular motors may be good candidates to explain some of the functional differences we observed between stable and dynamic MTs. Other molecular specificities may also be involved in functional specialization, such as posttranslational maturation of α -tubulin

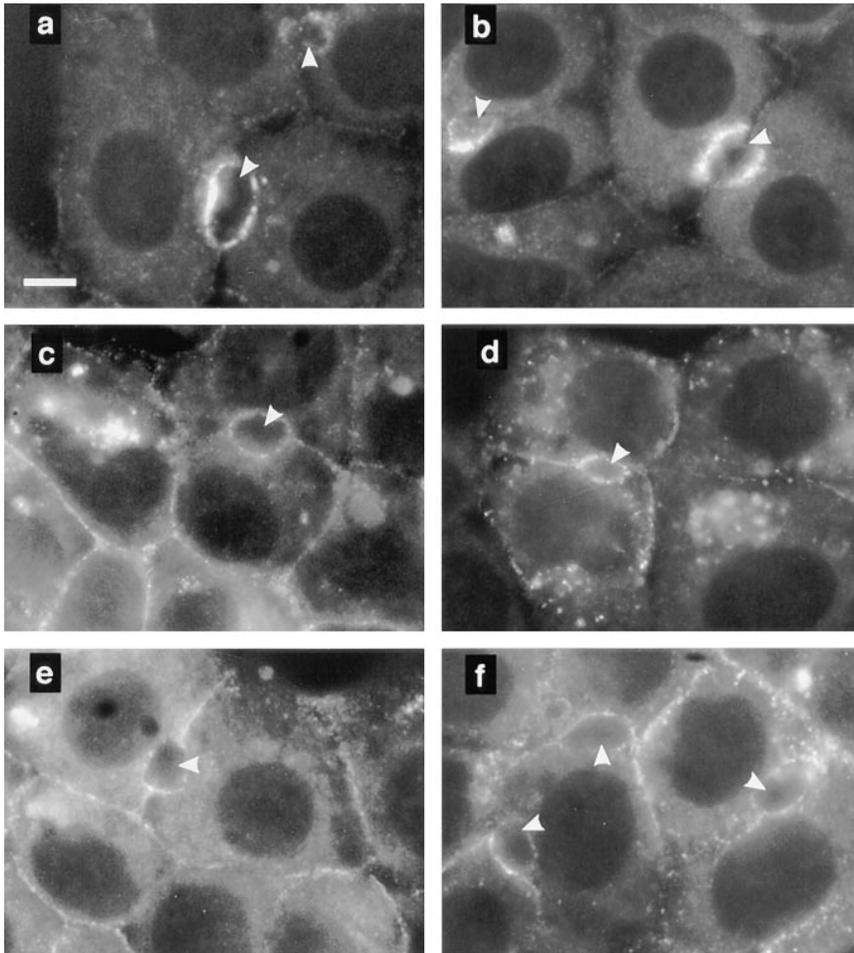


Figure 10. Dynamic MTs are involved in the transcytosis of B10 and DPP IV from the basolateral to apical surface of WIF-B cells. Control (*a, b*), 201-F-treated (2 h, 25 μ M; *c, d*) and nocodazole-treated cells (10 h, 10 μ M; *e, f*), were incubated at 37°C for 2 h with anti-DPP IV (*a, c, e*) or anti-B10 antibodies (*b, d, f*). After washing with PBS, cells were fixed, and the immune complexes were revealed using FITC-labeled secondary antibody. Cells were examined in a fluorescence microscope. Bar, 10 μ m.

and/or variable patterns of MAPs bound to MTs. These hypotheses are not exclusive as *in vitro* data suggest that kinesin binds preferentially to detyrosinated MTs compared with tyrosinated MTs (Liao and Gundersen, 1996). Even though such data provide a few clues for understanding functional specialization, the actual scheme is probably not that simple as there may be deeper levels of specialization among dynamic and stable MT subsets depending on the nature of the structural and motor MAPs. There is increasing evidence that conventional kinesin and cytoplasmic dynein are not the only proteins involved in membrane traffic (Moore and Endow, 1996). It even appears that different motor proteins might be involved in the same transport steps (Goodson et al., 1997). In this context, 201-F may be an invaluable tool to study the functions of stable and highly dynamic MTs at the molecular level. Furthermore, given its original effect, 201-F may be of help in understanding the mechanisms underlying MT stabilization and the regulation of MT stability in living cells.

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References

- Bartles, J.R., H.M. Feracci, B. Stieger, and A.L. Hubbard. 1987. Biogenesis of the rat hepatocyte plasma membrane *in vivo*: comparison of the pathways taken by apical and basolateral proteins using subcellular fractionation. *J. Cell Biol.* 105:1241–1251.
- Biou, D., D. Monnet, D. Millet, J. Feger, and G. Durand. 1984. An immunochemical procedure to evaluate the degree of desialylation of alpha-1 acid glycoprotein in rat serum. *J. Immunol. Methods.* 74:267–271.
- Boll, W., J.S. Partin, A.I. Katz, M.J. Caplan, and J.D. Jamieson. 1991. Distinct pathways for basolateral targeting of membrane and secretory proteins in polarized epithelial cells. *Proc. Natl. Acad. Sci. USA.* 88:8592–8596.
- Breitfield, P.P., W.C. McKinnon, and K.E. Mostov. 1990. Effect of nocodazole on vesicular traffic to the apical and basolateral surfaces of polarized MDCK cells. *J. Cell Biol.* 111:2365–2373.
- Carlson, J., and J. Stenflo. 1982. The biosynthesis of rat alpha-1-antitrypsin. *J. Biol. Chem.* 257:12987–12994.
- Cassio, D., C. Hamon-Benais, M. Guerin, and O. Lecoq. 1991. Hybrid cell lines constitute a potential reservoir of polarized cells: isolation and study of highly differentiated hepatoma-derived hybrid cells able to form functional bile canaliculi *in vitro*. *J. Cell Biol.* 115:1397–1408.
- Cole, N.B., and J. Lippincott-Schwartz. 1995. Organization of organelles and membrane traffic by microtubules. *Curr. Opin. Cell Biol.* 7:55–64.
- Cole, N.B., N. Sciaky, A. Marotta, J. Song, and J. Lippincott-Schwartz. 1996. Golgi dispersal during microtubule disruption: regeneration of Golgi stacks at peripheral endoplasmic reticulum exit sites. *Mol. Biol. Cell.* 7:631–650.
- De Almeida, J.B., and J.L. Stow. 1991. Disruption of microtubules alters polarity of basement membrane proteoglycan secretion in epithelial cells. *Am. J. Physiol.* 260:C691–C700.
- Durand-Schneider, A.M., J.C. Bouanga, G. Feldmann, and M. Maurice. 1991. Microtubule disruption interferes with the structural and functional integrity of the apical pole in primary cultures of rat hepatocytes. *Eur. J. Cell Biol.* 56:260–268.
- Eddé, B., J. Rossier, J.P. Le Caer, E. Desbruyères, F. Gros, and P. Denoulet. 1990. Posttranslational germylation of α -tubulin. *Science.* 247:83–85.
- Gelfand, V.I., and A.D. Bershadsky. 1991. Microtubule dynamics: mechanism,

- regulation, and function. *Annu. Rev. Cell Biol.* 7:93–116.
- Gilbert, T., A. Le Bivic, A. Quaroni, and E. Rodriguez-Boulan. 1991. Microtubular organization and its involvement in the biogenetic pathways of plasma membrane proteins in Caco-2 intestinal epithelial cells. *J. Cell Biol.* 113:275–288.
- Goodson, H.V., C. Valetti, and T.E. Kreis. 1997. Motors and membrane traffic. *Curr. Opin. Cell Biol.* 9:18–28.
- Gurland G., and G.G. Gundersen. 1993. Protein phosphatase inhibitors induce the selective breakdown of stable microtubules in fibroblasts and epithelial cells. *Proc. Natl. Acad. Sci. USA.* 90:8827–8831.
- Gurland G., and G.G. Gundersen. 1995. Stable deetyrosinated microtubules function to localize vimentin intermediate filaments in fibroblasts. *J. Cell Biol.* 131:1275–1290.
- Hemery, I., A.M. Durand-Schneider, G. Feldmann, J.P. Vaerman, and M. Maurice. 1996. The transcytotic pathway of an apical plasma membrane protein (B10) in hepatocytes is similar to that of IgA and occurs via a tubular pericentriolar compartment. *J. Cell Sci.* 109:1215–1227.
- Hirokawa, N. 1994. Microtubule organization and dynamics dependent on microtubule-associated proteins. *Curr. Opin. Cell Biol.* 6:74–81.
- Hunziker, W., P. Male, and I. Mellman. 1990. Differential microtubule requirements for transcytosis in MDCK cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3515–3525.
- Ihrke, G., G.V. Martin, M.R. Shanks, M. Schrader, T.A. Schroer, and A.L. Hubbard. 1998. Apical plasma membrane proteins and endolyn-78 travel through a subapical compartment in polarized WIF-B hepatocytes. *J. Cell Biol.* 141:115–133.
- Ihrke, G., E.N. Neufeld, T. Meads, M.R. Shanks, D. Cassio, M. Laurent, T.A. Schroer, R.E. Pagano, and A.L. Hubbard. 1993. WIF-B cells: an in vitro model for studies of hepatocyte polarity. *J. Cell Biol.* 123:1761–1775.
- Khawaja S., G.G. Gundersen, and J.C. Bulinski. 1988. Enhanced stability of microtubules enriched in deetyrosinated tubulin is not a direct function of deetyrosination level. *J. Cell Biol.* 106:141–149.
- Kondracki, M.L., and M. Guyot. 1989. Biologically active quinone and hydroquinone sesquiterpenoids from the sponge *Smenospongia sp. Tetrahedron.* 45:1995–2004.
- Kreis, T.E. 1987. Microtubules containing deetyrosinated tubulin are less dynamic. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2597–2606.
- Lafont, F., J. Burkhardt, and K. Simons. 1994. Involvement of microtubule motors in basolateral and apical transport in kidney cells. *Nature.* 372:801–803.
- LeDizet, M., and G. Piperno. 1987. Identification of an acetylation site of *Chlamydomonas* α -tubulin. *Proc. Natl. Acad. Sci. USA.* 84:5720–5724.
- Liao and Gundersen. 1996. Kinesin preferentially binds to deetyrosinated microtubules compared with tyrosinated microtubules. *Mol. Biol. Cell.* 7(Suppl.):44a.
- Lippincott-Schwartz, J., J.G. Donaldson, A. Schweizer, E.G. Berger, H. Auri, L.C. Yuan, and R.D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggest an ER recycling pathway. *Cell.* 60:821–836.
- Mac Rae, T.H. 1997. Tubulin post-translational modifications. Enzymes and their mechanisms of action. *Eur. J. Biochem.* 244:265–278.
- Maurice, M., A.M. Durand-Schneider, M. Garbarz, and G. Feldmann. 1985. Characterization of rat hepatocyte plasma membrane domains by monoclonal antibodies. *Eur. J. Cell Biol.* 39:122–129.
- Maurice, M., E. Rogier, D. Cassio, and G. Feldmann. 1988. Formation of plasma membrane domains in rat hepatocytes and hepatoma cell lines in culture. *J. Cell Sci.* 90:79–92.
- Maurice, M., M.J. Schell, B. Lardeux, and A.L. Hubbard. 1994. Biosynthesis and intracellular transport of a bile canalicular plasma membrane protein: studies in vivo and in the perfused rat liver. *Hepatology.* 19:648–655.
- Misumi, Y., M. Sohda, K. Ohkubo, N. Takami, K. Oda, and Y. Ikehara. 1990. Molecular cloning and sequencing of the cDNA of rat alpha1-protease inhibitor and its expression in COS-1 cells. *J. Biochem.* 108:230–234.
- Mizuno, M., and S.J. Singer. 1994. A possible role for stable microtubules in intracellular transport from the endoplasmic reticulum to the Golgi apparatus. *J. Cell Sci.* 107:1321–1331.
- Moore, J.D., and S.A. Endow. 1996. Kinesin proteins: a phylum of motors for microtubule-based motility. *BioEssays.* 18:207–219.
- Nagasaki, T., G. Liao, and G.G. Gundersen. 1994. Isolated plasma membranes induce the loss of oriented deetyrosinated microtubules and other contact inhibition-like responses in migrating NRK cells. *J. Cell Sci.* 107:3413–3423.
- Oda, K., and Y. Ikehara. 1982. Taxol, a potent promoter of microtubule assembly, inhibits secretion of plasma proteins in cultured rat hepatocytes. *Biochem. Biophys. Res. Commun.* 107:561–567.
- Pavelka, M., and A. Ellinger. 1983. Effect of colchicine on the Golgi complex of rat pancreatic acinar cells. *J. Cell Biol.* 97:737–748.
- Redeker, V., N. Leveilliers, J.M. Schmitter, J.P. Le Caer, J. Rossier, A. Adoutte, and M.H. Bré. 1994. Polyglycylation of tubulin: a posttranslational modification in axonemal tubulin. *Science.* 266:1688–1691.
- Rogalski, A.A., J.E. Bergmann, and S.J. Singer. 1984. Effect of microtubule assembly status on the intracellular processing and surface expression of an integral membrane protein of the plasma membrane. *J. Cell Biol.* 99:1101–1109.
- Rogalski, A.A., and S.J. Singer. 1984. Associations of elements of the Golgi apparatus with microtubules. *J. Cell Biol.* 99:1092–1100.
- Saucan, L., and G.E. Palade. 1992. Differential effects on the transport of membrane and secretory proteins in rat hepatocytes in vivo: bipolar secretion of albumin. *Hepatology.* 15:714–721.
- Saucan, L., and G.E. Palade. 1994. Membrane and secretory proteins are transported from the Golgi complex to the sinusoidal plasmalemma of hepatocytes by distinct vesicular carriers. *J. Cell Biol.* 125:733–741.
- Scott, L.J., D. Delautier, N.R. Meerson, G. Trugnan, J.W. Goding, and M. Maurice. 1997. Biochemical and molecular identification of distinct forms of alkaline phosphodiesterase I expressed on the apical and basolateral plasma membrane surfaces of rat hepatocytes. *Hepatology.* 25:995–1002.
- Shanks, M.R., D. Cassio, O. Lecoq, and A.L. Hubbard. 1994. An improved polarized rat hepatoma hybrid cell line: generation and comparison to its hepatoma relatives and hepatocytes in vivo. *J. Cell Sci.* 107:813–824.
- Takizawa, P.A., J.K. Yucel, B. Veit, D.J. Faulkner, T. Deerinck, G. Soto, M. Elisman, and V. Malhotra. 1993. Complete vesiculation of golgi membranes and inhibition of protein transport by a novel sea sponge metabolite, Ili-maquinone. *Cell.* 73:1079–1090.
- Tanaka, E., T. Ho, and M.W. Kirschner. 1995. The role of microtubule dynamics in growth cone motility and axonal growth. *J. Cell Biol.* 128:139–155.
- Thyberg, J., A. Piasek, and S. Moskalewski. 1980. Effects of colchicine on the Golgi complex and GERL of cultured rat peritoneal macrophages and epiphyseal chondrocytes. *J. Cell Sci.* 45:41–58.
- Van der Sluijs, P., M.K. Bennett, C. Antony, K. Simons, and T.E. Kreis. 1990. Binding of exocytic vesicles from MDCK cells to microtubules in vitro. *J. Cell Sci.* 95:545–553.
- Veit, B., J.K. Yucel, and V. Malhotra. 1993. Microtubule independent vesiculation of Golgi membranes and the reassembly of vesicles into golgi stacks. *J. Cell Biol.* 122:1197–1206.
- Webster, D.R., G.G. Gundersen, J.C. Bulinski, and G.G. Borisy. 1987. Differential turnover of tyrosinated and deetyrosinated microtubules. *Proc. Natl. Acad. Sci. USA.* 84:9040–9044.
- Webster, D.R., and G.G. Borisy. 1989. Microtubules are acetylated in domains that turn over slowly. *J. Cell Sci.* 92:57–65.
- Wehland, J., and M.C. Willingham. 1983. A rat monoclonal antibody reacting with the tyrosinated form of α -tubulin. II. Effects on cell movement, organization of microtubules and intermediate filaments, and arrangement of Golgi elements. *J. Cell Biol.* 97:1476–1490.