

Intrinsic Microtubule Stability in Interphase Cells

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Abstract. Interphase microtubule arrays are dynamic in intact cells under normal conditions and for this reason they are currently assumed to be composed of polymers that are intrinsically labile, with dynamics that correspond to the behavior of microtubules assembled in vitro from purified tubulin preparations. Here, we propose that this apparent lability is due to the activity of regulatory effectors that modify otherwise stable polymers in the living cell. We demonstrate that there is an intrinsic stability in the microtubule network in a variety of fibroblast and epithelial cells. In the absence of regulatory factors, fibroblast cell interphase microtubules are for the most part resistant

to cold temperature exposure, to dilution-induced disassembly and to nocodazole-induced disassembly. In epithelial cells, microtubules are cold-labile, but otherwise similar in behavior to polymers observed in fibroblast cells. Factors that regulate stability of microtubules appear to include Ca^{2+} and the p34^{cdc2} protein kinase. Indeed, this kinase induced complete destabilization of microtubules when applied to lysed cells, while a variety of other protein kinases were ineffective. This suggests that p34^{cdc2}, or a kinase of similar specificity, may phosphorylate and inactivate microtubule-associated proteins, thereby conferring lability to otherwise lengthwise stabilized microtubules.

MICROTUBULES form dynamic arrays in interphase cells. Recent studies using tubulin analogs have shown that interphase microtubules readily exchange subunits with soluble pools of tubulin (Saxton et al., 1984; Soltys and Borisy, 1985; Schulze and Kirschner, 1986; Sammak et al., 1987; Sammak and Borisy, 1988; Schulze and Kirschner, 1988; Shelden and Wadsworth, 1993) and direct observations of individual polymers in intact cells have shown length fluctuations that resemble the dynamic instability observed in vitro (Cassimeris et al., 1988; Sammak and Borisy, 1988; Schulze and Kirschner, 1988; Shelden and Wadsworth, 1993). Therefore, it is currently assumed that interphase microtubule networks are composed of polymers that are intrinsically dynamic, similar to microtubules assembled from pure tubulin in vitro. Such microtubules also show spontaneous and rapid subunit exchange with unassembled tubulin at steady state, and dynamic instability (Mitchison and Kirschner, 1984; Farrell et al., 1987). In vitro, these dynamic properties are tempered, but not suppressed, by associated proteins, microtubule-associated proteins (MAPs)¹, such as MAP1, MAP2, MAP4, and tau proteins (Pryer et al., 1992). Such proteins are assumed to

account for the apparent local modulations of microtubule dynamics observed in intact cells (Sammak and Borisy, 1988; Schulze and Kirschner, 1988).

However, the foregoing view that interphase microtubules resemble polymers composed of pure tubulin or MAP-containing microtubules is difficult to reconcile with observations which suggest that many interphase microtubules are stable. Certain cells have been found to contain significant populations of cold-stable microtubules, and it is well documented that most microtubules remain stable upon dilution after disruption of interphase cells in large volumes of detergent-containing buffers. By contrast, microtubules composed of pure tubulin or MAP-containing microtubules disassemble rapidly in vitro upon exposure to cold temperature or if suspensions are diluted with large volumes of buffer (Dustin, 1984). Therefore, the dynamic properties of cytoplasmic microtubules may differ significantly from the properties of microtubules composed of pure tubulin or of MAP-containing tubulin preparations in vitro.

Reports of cell types containing abundant subsets of cold-resistant interphase microtubules can be found in the literature (Bershadsky et al., 1979; Moskalewski et al., 1980; Dustin, 1984). In vitro, such polymers represent an extreme example of non dynamic polymers. They can be isolated as stable polymers of fixed length (Webb and Wilson, 1980). They do not depolymerize upon dilution at physiological temperature (Job and Margolis, 1984; Margolis et al., 1990) and they do not disassemble in response to depolymerizing drugs (Job et al., 1981). The presence of such cold-stable

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1. *Abbreviations used in this paper:* cdks, cyclin-dependent kinases; MAPs, microtubule-associated proteins; REF, rat embryo fibroblast.

microtubules in cycling cells seems poorly compatible with the well-documented dynamic behavior of interphase microtubule networks and has apparently been regarded as a curiosity, possibly artifactual (Bershadsky et al., 1979). But, such a view is not supported by studies performed in other systems which indicate that cold stability is conferred to microtubules by specific associated proteins (Margolis et al., 1990; Multigner et al., 1992).

When cells are lysed rapidly in large volumes of Pipes-EGTA-based buffers containing detergents, cytoplasmic microtubules appear to remain intact (Bershadsky et al., 1978; Solomon et al., 1979; Duerr et al., 1981; Schliwa et al., 1981; Deery et al., 1984; Saxton et al., 1984; Soltys and Borisy, 1985). Such resistance to dilution-induced depolymerization is apparently universal, occurring even with cells known to contain predominantly cold-labile microtubule networks (Duerr et al., 1981). Resistance to dilution-induced depolymerization is currently ascribed to a stabilizing effect of Pipes-EGTA-based buffers. However, dynamic instability was described in the same Pipes-EGTA-based buffers (Kristofferson et al., 1986). Therefore, resistance to dilution-induced depolymerization after cell-lysis could be an intrinsic property of interphase microtubules, induced by specific microtubule-stabilizing effectors. Cold-labile but dilution-stable polymers have not yet been isolated *in vitro*. Therefore, data on the dynamic behavior of such microtubules are lacking. However, such polymers would be expected to behave exactly as cold-stable microtubules at physiological temperature, showing similar absence of dynamic instability behavior and lack of response to depolymerizing drugs. Therefore, the presence in abundance of dilution-stable polymers in cells would raise the same paradox with regard to the apparent interphase microtubule dynamics as that of cold-stable microtubules.

The purpose of the present study was to establish the basic stability properties of microtubules in interphase cells. We find that microtubule cold stability and/or microtubule resistance to dilution-induced disassembly do not result from artifacts. Instead such stability is an intrinsic property of interphase microtubules throughout their length. We propose that microtubule instability in cells is largely induced by regulatory factors which include calcium and protein kinases related to the p34^{cdc2} kinase. The dynamic behavior of interphase networks and their sensitivity to depolymerizing drugs may depend on the activity of such regulatory factors acting on a template of stable polymers.

Materials and Methods

Materials

Okadaic acid was obtained from Moana Bioproducts, Hawaii. Nocodazole was purchased from Aldrich, Strasbourg, France; sodium azide from Merck, Darmstadt, RFA; Aquamount from BDH, UK; culture media from Boehringer, Houston, TX and other drugs and reagents from Sigma Chem. Co., St. Louis, MO. Nocodazole, PMA, and calcium ionophore A23187 were dissolved in DMSO at concentrations of 5 mM, 4 mM, and 4 mM, respectively. Forskolin was dissolved at concentration of 10 mM in ethanol, okadaic acid at concentration of 0.1 mM in dimethylformamide, polyamines (spermidine, spermine, putrescine) at concentration of 10 mM in Tris 10 mM, pH 7.2. These stock solutions were stored at -20°C . Sodium orthovanadate and sodium azide were dissolved at concentrations of, respectively, 100 mM in distilled water and 20 mM in PBS/0.8 mM $\text{MgCl}_2/0.7$ mM CaCl_2 , pH 7.4, just before use. Protease inhibitors were used as a

cocktail including 0.5 $\mu\text{g}/\text{ml}$ leupeptin, 1 μM pepstatin A, and 200 μM PMSF (final concentrations). MAP kinase and the catalytic subunit from c-AMP dependent kinase were kindly provided by Drs. O. Haccard (Centre National de la Recherche Scientifique URA 1449, Paris, France) and I. Vilgrain (Centre d'Etudes Nucléaires de Grenoble, Grenoble, France), respectively. Ca^{2+} /calmodulin dependent kinase and casein kinase II were generous gifts from Dr. C. Cochet (Centre d'Etudes Nucléaires de Grenoble, Grenoble, France). The protein kinase p34^{cdc2}-cyclin B was purified according to Labbé et al. (1991).

Cells

HeLa, Y1, B16, and NIH/3T3 cells were generous gifts from Drs. V. Chevrier, C. Blanc-Brude, D. Rousseau (Centre d'Etudes Nucléaires de Grenoble, Grenoble, France), and R. L. Margolis (Institut de Biologie Structurale, Grenoble, France), respectively. Rat2 cells (ATCC CRL 1764) and MDBK cells (ATCC CCL 22) were purchased from Amer. Type Culture Collection, Rockville, MD. BAC cells were primary cultures from bovine adrenal cortex and were kindly provided by Dr. C. Blanc-Brude (Centre d'Etudes Nucléaires de Grenoble, Grenoble, France). Rat Embryo Fibroblasts (REFs), transformed with Ha-*ras* and a thermosensitive mutant of p53, clone 6, (Pinhasi-Kimhi et al., 1986; Michalovitz et al., 1990) was a generous gift from Dr. M. Oren (WIS, Rehovot, Israel). Rat2, B16, and transformed REF cells were grown in DME complemented with 5% FCS, and NIH/3T3 cells in the same medium with 10% FCS. Y1 and BAC cells were grown in Ham's F-10 medium plus 10% FCS. MDBK and HeLa cells were cultured in RPMI 1640 medium containing 10% FCS. Each culture medium was supplemented with 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin and all cell types were incubated at 37°C in a humidified incubator with 6% CO_2 .

Treatments of Intact Cells

Unless noted otherwise, drug treatments involving intact cells were performed at 37°C . Drugs were added to growth medium except with sodium azide and calcium ionophore A23187 (see below). Cells were treated for 1 or 2 h with 0.1 μM or 1 μM okadaic acid, 1 mM sodium orthovanadate, 100 μM polyamines, 20 μM forskolin, and 0.1 μM PMA. Cells were treated with 1 μM nocodazole for time durations ranging from 5 min to several hours. Calcium ionophore A23187 (10 μM) was added either to the growth medium or after replacement of the medium by one of the following media: RPMI, DME, or DME in which calcium concentration has been raised to 5 mM. These latter media were minus FCS. Controls were performed by adding 0.25% DMSO in the different media. Treatment durations ranged from 30 s to 1 h. For sodium azide treatment, culture medium was removed, cells were washed with PBS supplemented with 0.8 mM MgCl_2 and 0.7 mM CaCl_2 (PBS-Mg-Ca), and then were incubated for 1–2 h in PBS-Mg-Ca (control cells) or in PBS-Mg-Ca containing 20 mM sodium azide (azide-treated cells). For exposure to cold temperature, Petri dishes containing cells on glass coverslips were cooled to 0°C by placing the dishes on ice. Cells on coverslips were then fixed at desired times and prepared for immunofluorescence microscopy.

Cell Lysis

Unless otherwise indicated, cells grown on coverslips were immersed at the desired temperatures in 100 ml lysis buffer composed of 80 mM Pipes-KOH, pH 6.8, 1 mM MgCl_2 , 1 mM EGTA, 0.5% Triton X-100 (vol/vol), and 10% glycerol (vol/vol), containing protease inhibitors.

Treatments of Lysed Cells

To determine the resistance of microtubules to dilution-induced disassembly, cells on coverslips were either incubated directly in 100 ml of lysis buffer at 30°C for desired times, or cells on coverslips were lysed (1 min in 100 ml of lysis buffer at 30°C) followed by three 1-min washes with 100 ml vol of lysis buffer at 30°C , and then incubated at 30°C for the desired times. To determine the resistance of microtubules to cold-temperature-disassembly, cells on coverslips were either incubated directly in 100 ml of lysis buffer at 0°C for desired times, or cells on coverslips were lysed (1 min in 100 ml of lysis buffer at 0°C) followed by three 1-min washes with 100 ml vol of lysis buffer at 0°C , and then incubated for the desired times. To analyze the effects of Ca^{2+} on microtubules in lysed cells, cells on coverslips either were lysed directly in lysis buffer at the desired temperature containing 1.1 mM or 5 mM CaCl_2 , or were lysed for 1 min in lysis buffer

without CaCl₂ followed by three 1-min washes with 100 ml vol of lysis buffer (no CaCl₂), and then incubated for the desired times in lysis buffer containing CaCl₂. Because the lysis buffer contains 1 mM EGTA, final free Ca²⁺ concentrations for 1.1 mM or 5 mM added CaCl₂ were 0.1 mM and 4 mM, respectively. To determine the effects of nocodazole on microtubules in lysed cells, cells on coverslips were lysed (1 min in 100 ml of lysis buffer at 30°C), washed once with 100 ml of lysis buffer at 30°C, and then incubated for 30 min with 30 μl of lysis buffer at 30°C containing 5 μM nocodazole. Incubation of lysed cells with protein kinases was performed by initially lysing and washing cells on coverslips at room temperature as described above. Protein kinases in the appropriate buffer (see below) were added to lysed cells for 20 min (room temperature). Resistance of microtubules to cold-temperature disassembly or to dilution-induced disassembly was determined as described above. Before use in experiments, the activities of p34^{cdc2}-cyclin B kinase, MAP-kinase, the catalytic subunit of c-AMP dependent kinase, Ca²⁺/calmodulin dependent kinase and casein kinase II were verified with lysine-rich histone (type III-S from Sigma Chem. Co.), myelin basic protein, histone type II-A, arginine-rich histone (type VIII-S from Sigma Chem. Co.), and casein, respectively. Final volumic activities of protein kinases used during incubation were: p34^{cdc2}-cyclin B, 20 pmole/min/μl; MAP kinase, 36 pmole/min/μl; catalytic subunit from c-AMP dependent kinase 10 pmole/min/μl; Ca²⁺/calmodulin dependent kinase, 2.5 pmole/min/μl; casein kinase II, 30 pmole/min/μl. Incubation buffers were composed of lysis buffer containing 1 mM ATP; and 30 mM NaCl, 10 mM MgCl₂ for p34^{cdc2}-cyclin B; 10 mM MgCl₂ for MAP kinase; 5 mM MgCl₂ for catalytic subunit of c-AMP dependent kinase and casein kinase II; 5 mM MgCl₂, 0.1 mM calmodulin and 0.9 mM calcium in the case of Ca²⁺-calmodulin dependent kinase. For sonication experiments, lysed cells on coverslips were placed horizontally on a plexiglass support at the bottom of a tube. Five milliliters of lysis buffer were added and lysed cells were sonicated for 3 s at half-maximal power (equipment Sonimasse S20, Annemasse, France). Coverslips either were fixed immediately in methanol, or incubated at 0 or 30°C for 5 min to 1 h before fixation.

Microtubule Nucleation on Cellular Centrosomes

NIH/3T3 cells grown on coverslips were lysed in lysis buffer at 0°C containing 5 mM CaCl₂ (1 min), and then incubated for 10 min in another bath of the same buffer at 0°C. This procedure induced complete microtubule disassembly. Coverslips were then sequentially washed for several seconds in 100 ml of lysis buffer and in 100 ml of 80 mM Pipes-KOH, pH 6.8, 1 mM EGTA, 1 mM MgCl₂ (nucleation buffer). Coverslips were then individually incubated with 50 μl of a mixture containing 3.5 mg/ml tubulin, 5 mM GTP, 5% glycerol in nucleation buffer. After 20 min of incubation at 37°C, nucleated microtubules either were directly cross-linked or were tested for resistance to cold-temperature disassembly, for resistance to dilution-induced disassembly (see above) or for Ca²⁺ effects before cross-linking. For a good preservation of asters during subsequent washing procedures, asters were cross-linked. Cross-linking of nucleated microtubules was carried out by immersion of coverslips for 3 min in 2 ml of nucleation buffer at room temperature containing 0.5% 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, 0.1 mM GTP and 10% glycerol. Coverslips were subsequently washed in 100 ml nucleation buffer supplemented with 10% glycerol and fixed in methanol for immunofluorescence.

Immunofluorescence Microscopy

Cells grown for 1–4 d on glass coverslips were either directly processed for immunofluorescence or exposed to the various treatments described above before further processing. Cells were fixed for 6 min in anhydrous methanol at –20°C and were subsequently washed in PBS/0.1% Tween 20 (vol/vol). For tubulin staining, they were sequentially incubated with anti-β tubulin mAb TUB 2.1 (1:100, vol/vol, in PBS containing 0.1% BSA), and then with FITC-conjugated rabbit anti-mouse antibody (1:50, vol/vol, in PBS/0.1% BSA). Nuclei were stained using HOECHST 33258 at 10 μg/ml in PBS containing 0.1% BSA. Coverslips were mounted in Aquamount and observed using a Zeiss Axioskop microscope (Zeiss, RFA).

Preparation of Dimer and Polymer Tubulin Fractions from Intact Cells

Cells were grown on 100-mm Petri dishes until they reached confluence. They were then directly extracted or cold treated before extraction. For preparation of dimer tubulin fractions, cells were washed twice with 5 ml PBS at 30°C or at 0°C in the case of cold-treated cells, PBS was removed,

and cells were extracted for 3 min in 500 μl lysis buffer, at the appropriate temperature. The extract was collected and immediately boiled for 3 min in 2.3% SDS, 125 mM Tris-HCl, 10% (vol/vol) glycerol, 1% (vol/vol) 2-mercaptoethanol, and analyzed by immunoblotting. For polymer fraction preparations, cells were washed two times with 5 ml PBS at the appropriate temperature, PBS was removed and cells were extracted for 3 min with 10 ml of lysis buffer. After a brief washing with 5–10 ml of lysis buffer, 500 μl of 1% SDS in water was added, cells were scraped, vigorously sonicated for 1 min, and boiled 3 min as described above and analyzed by immunoblotting.

Preparation of Polymer Fractions from Lysed Cells

Cells were grown on 100-mm Petri dishes until they reached confluence. After PBS washings, they were lysed in 10 ml of lysis buffer at 0 or 30°C and incubated in the same buffer for 30 min. They were further washed briefly with 10 ml of lysis buffer, and then extracted with 500 μl of 1% SDS in water. Further processing was as above.

Western Immunoblotting

SDS-PAGE of cell extracts was performed according to Laemmli (1970). Afterwards, proteins were transferred on nitrocellulose membrane according to Towbin et al. (1979) and the membrane was saturated in PBS/0.1% Tween 20 for 30 min. The membrane was then incubated for 45 min with anti-β tubulin TUB2.1 antibody diluted to 1:2,000 in PBS/0.1% Tween 20 containing 100 μg/ml heparin. After three washes in PBS/0.1% Tween 20, the membrane was incubated with HRP-coupled anti-mouse antibody from Cappel (1:5,000 in PBS/0.1% Tween 20 plus heparin), washed three times as above, and subsequently developed using an ECL kit from Amersham, UK.

Analysis of p34^{cdc2}-Cyclin B Substrates

NIH/3T3 and HeLa cells were grown on 35-mm Petri dishes until they reached confluence. Culture medium was removed, cells were lysed at room temperature in 2 ml of lysis buffer (1 min) and subsequently washed in 2 ml of the same buffer. The phosphorylation reaction was performed in 100 μl of lysis buffer containing 30 mM NaCl, 10 mM MgCl₂, 1 mM ATP, 20 μCi γ³²P-ATP, in the presence or absence of p34^{cdc2}-cyclin B. After 20 min of incubation at room temperature, solubilized proteins in the incubation buffer were collected, precipitated with TCA, and pelleted. The pellet was washed twice with large volumes of acetone at –20°C, solubilized in 1% SDS. Further processing was as described for polymer fraction preparation. Aliquots were then run on 8% and 12% SDS polyacrylamide gels, and gels were dried and autoradiographed using hyperfilm-MP (Amersham, UK).

Results

Cold-Stable Cytoplasmic Microtubules in Intact Cells

To know whether the occurrence of populations of cold-stable microtubules in interphase cell was exceptional or if it had generality, a series of different cell types were tested for the presence of such cold-resistant microtubules. These included five cell types of epithelial origin or with epithelial-like morphology: HeLa cells (human cervical carcinoma cells), Y1 cells (murine adrenocortical cells), BAC cells (normal bovine adrenal cortex cells), MDBK cells (Madin-Darby bovine kidney cells), and B16 cells (murine melanoma cells); and three cell lines from fibroblast origin: NIH/3T3 cells (murine fibroblasts), RAT2 cells (rat fibroblasts), transformed REF cells (REF transformed with Ha-ras and a thermosensitive mutant of p53).

Cells were exposed to the cold and the course of microtubule disassembly was monitored at different time points using immunostaining with an anti-β tubulin antibody. In epithelial cell types, polymer disassembly was essentially complete after 30 min of cold exposure, while most microtubules survived this treatment in other cell types. Results were es-

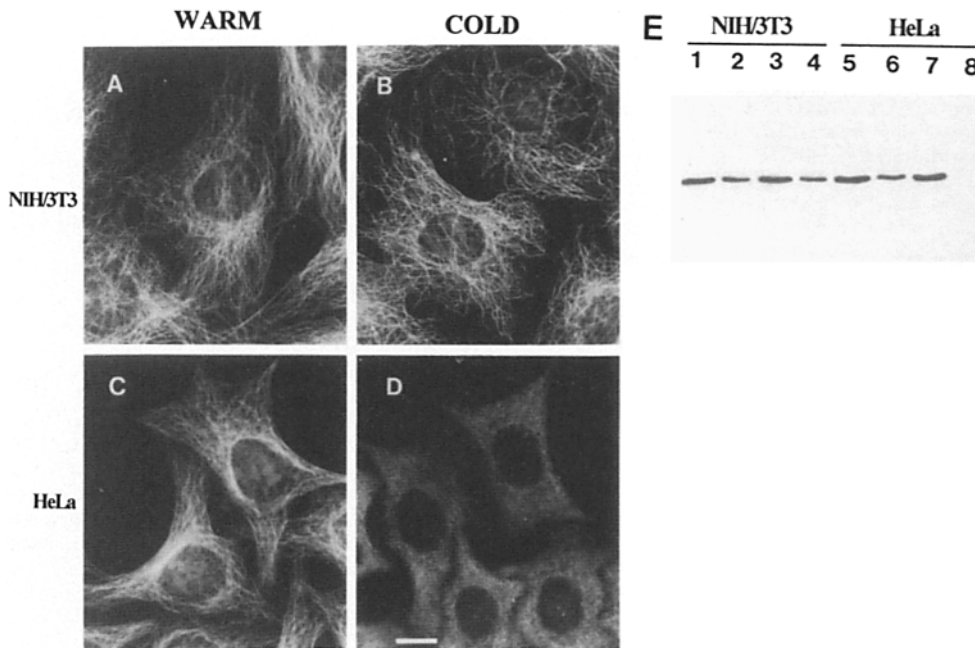


Figure 1. Microtubule cold stability in NIH/3T3 and HeLa cells. (A–D) Immunostaining of interphase cells with a mAb directed against β -tubulin, before (A and C) or after (B and D) 30 min exposure to cold temperature. (A and B) NIH/3T3 cells; (C and D) HeLa cells. NIH/3T3 cells showed extensive arrays of cold-stable microtubules, in contrast with HeLa cells which mostly contained cold-labile polymers. Bar, 10 μ m. (E) Immunoblot analysis of dimer and polymer tubulin fractions in NIH/3T3 cells (lanes 1–4) and HeLa cells (lanes 5–8), before (1, 2, 5, and 6) or after (3, 4, 7, and 8) exposure to cold temperature. Dimer and polymer fractions were prepared and analyzed on immunoblots using an anti- β tubulin mAb. Equal aliquots (20 μ l) of each protein fraction were run into the gel. Lanes 1, 3, 5, and 7 correspond to dimer fractions. Lanes 2, 4, 6, and 8 correspond to polymer fractions.

essentially the same for longer periods of cold exposure (up to at least 3 h), although cold-resistant polymers tend to become curly (not shown). These results are illustrated in the case of NIH/3T3 cells and HeLa cells which were representative of fibroblast and epithelial cells, respectively (Fig. 1). Western immunoblot analysis confirmed the immunofluorescence data (Fig. 1 E). Pools of free or polymerized tubulin were differentially extracted from these cells with or without prior exposure to cold temperature. Protein fractions were immunoblotted using an anti- β tubulin antibody as a probe. Cold exposure had no significant effect on the amount of polymerized tubulin in NIH/3T3 cells (Fig. 1 E, lane 4). In contrast, polymerized tubulin was undetectable in HeLa cells exposed to cold temperature before extraction (Fig. 1 E, lane 8).

We have made extensive attempts to induce either the formation of cold-stable microtubules in HeLa cells or of labile microtubules in NIH/3T3 cells. In particular, we applied various drugs known to affect protein kinase or protein phosphatase activities. These included forskolin, which indirectly activates c-AMP dependent protein kinase, PMA, an activator of protein kinase C, polyamines which stimulate casein kinase II, and the phosphatase inhibitors vanadate and okadaic acid. Results were invariably negative: cells retained their microtubule cold stability properties in all instances (not shown).

Microtubule Cold Stability in Lysed Cells

Microtubule cold stability in intact cells could reflect influences of the intracellular environment or it could be an intrinsic property of the polymers. To test which of these

mechanisms was correct, cells were rapidly lysed and microtubule cold stability was compared in intact and lysed cells. For cell lysis, we used a Pipes-based buffer adapted from Bré et al. (1991) containing 10% glycerol (lysis buffer). If microtubule stability were dependent on interactions with the intracellular environment, one would expect to find no correlation between microtubule stability in intact and in lysed cells. If, on the contrary, microtubule stability were an intrinsic property of these polymers, one would expect microtubules to have a similar behavior in intact and lysed cells.

When HeLa or NIH/3T3 cells were lysed in a large volume of lysis buffer preequilibrated at 30°C, exposed microtubule networks remained apparently intact for at least 30 min (Fig. 2, A and C). When the same cells were lysed in lysis buffer preequilibrated at 0°C, HeLa cell microtubules mostly depolymerized (Fig. 2 D) while NIH/3T3 cell microtubules did not (Fig. 2 B). The small population of cold-stable microtubules visible in lysed HeLa cells (Fig. 2 D) could also be detected in cold-exposed intact cells briefly extracted before fixation, in order to remove the background of depolymerized tubulin (see inset of Fig. 9 D). Immunoblot analysis confirmed these results (Fig. 2 E). It showed no detectable difference in the amount of polymerized tubulin contained in lysed NIH/3T3 cells incubated for 30 min at 30 or at 0°C (Fig. 2 E, lanes 1 and 2). In contrast, cold exposure of lysed HeLa cells left no detectable amount of polymerized tubulin (Fig. 2 E, lane 4).

These experiments were repeated using the other cell types described in the first section. In all cases, they showed an absolute correlation of microtubule cold stability properties in intact and lysed cells, strongly suggesting that micro-

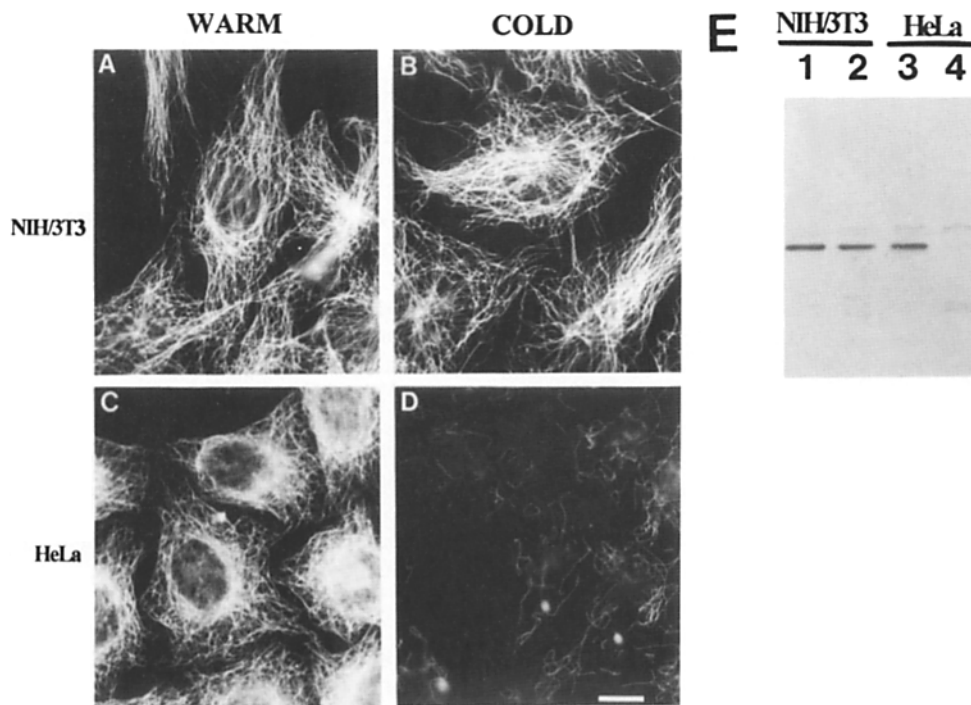


Figure 2. Microtubule cold stability in lysed NIH/3T3 and HeLa cells. (A–D) Immunostaining of lysed NIH/3T3 (A and B) and HeLa (C and D) cells with an anti- β tubulin mAb. Cells were lysed at 0 or 30°C and further incubated for 30 min in the same conditions, before immunostaining. (A and C) Cells lysed and incubated at 30°C; (B and D) cells lysed and incubated at 0°C. In lysed cells, microtubules retained the cold stability properties observed in the original intact cells. Bar, 10 μ m. (E) Immunoblot analysis of polymer tubulin fractions in NIH/3T3 cells (Lanes 1 and 2) and HeLa cells (lanes 3 and 4) lysed and incubated either at 30°C (1 and 3) or 0°C (2 and 4) before extraction. Polymer fractions were prepared and immunoblotted using an anti- β -tubulin mAb. Equal aliquots (20 μ l) of each protein fraction were run into the gel.

tubule cold stability in intact cells reflected an intrinsic property of these polymers.

Mechanisms of Microtubule Cold Stabilization

Two possible mechanisms might account for microtubule

cold stability in lysed cells: it might be due either to lengthwise stabilization or to endwise protection of otherwise labile polymers. Lysed cell microtubules were fragmented using sonication in order to test which of these mechanisms was correct. If entire polymers were stable, introduction of

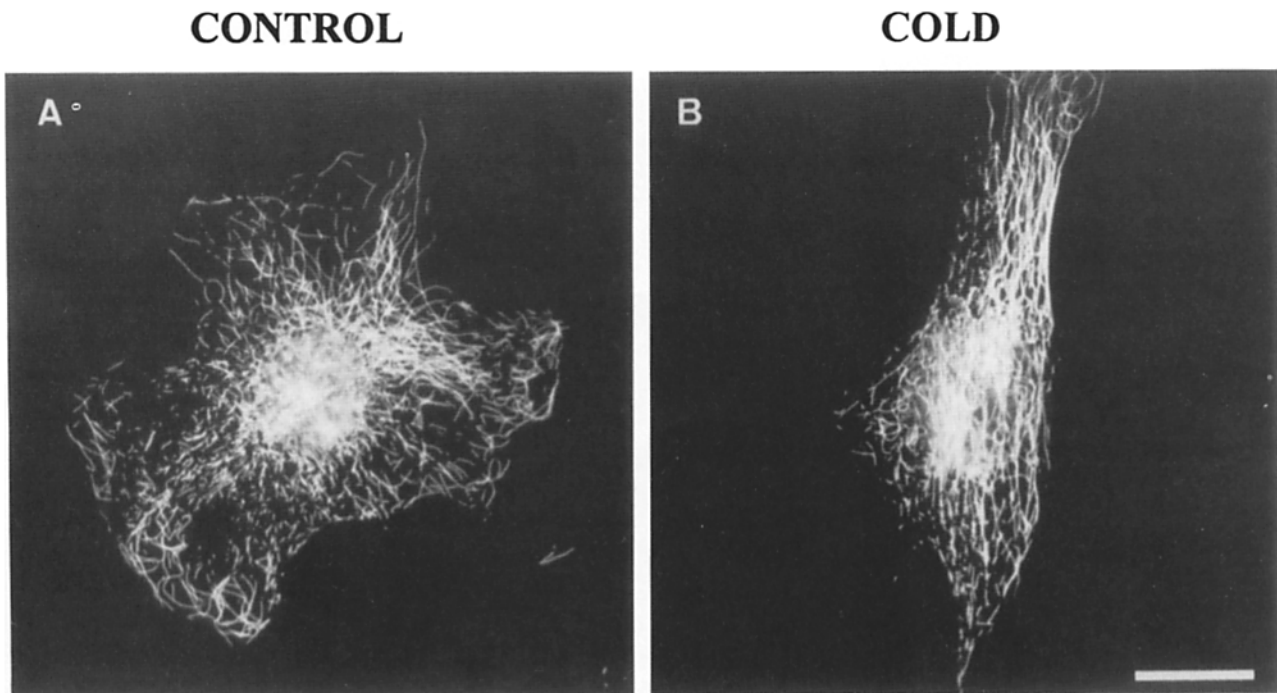


Figure 3. Microtubule cold stability in lysed NIH/3T3 cells after polymer fragmentation by sonication. Lysed NIH/3T3 cells were sonicated and immunostained with an anti- β tubulin mAb immediately after sonication (A) or after 30 min of cold exposure (B). Fragments retained the cold stability properties of the intact microtubules. Bar, 15 μ m.

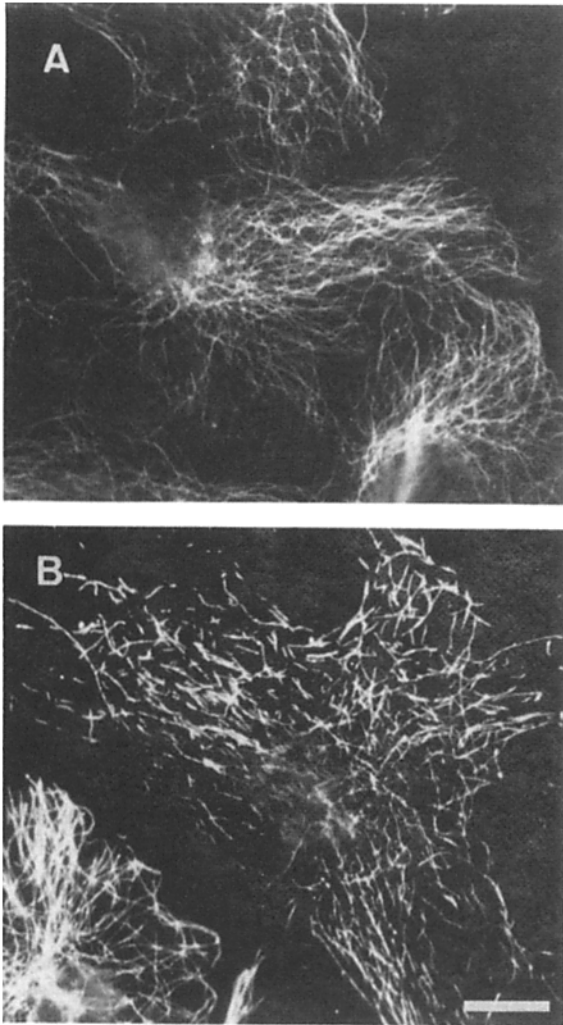


Figure 4. Microtubule severing in intact NIH/3T3 cells during calcium ionophore treatment. Cells were exposed to extracellular Ca^{2+} for 10 min in the absence (A) or presence (B) of calcium ionophore A23187, before fixation and anti- β tubulin immunostaining. In the presence of the calcium ionophore A23187, extensive microtubule fragmentation was evident in many cells. Bar, 10 μm .

new ends should not affect polymer stability. If, however, end caps did protect an otherwise cold-labile polymer from disassembly, fragmentation should introduce unprotected new ends and result in rapid disassembly.

That sonication generated new free ends was evident from inspection of the fragmented cytoskeletons: multiple breakages occurred along microtubules, and most fragments were no longer attached to centrosomes (Fig. 3 A). Nevertheless, microtubule fragments retained the cold stability of the original polymers (Fig. 3 B). These observations strongly suggest that microtubules are stabilized along their whole length as previously observed in the case of other cold-stable polymers (Job and Margolis, 1984; Multigner et al., 1992).

Regulations of Microtubule Cold Stability

Our results suggest that microtubule cold stability in lysed cells does not result from experimental artifacts but that it reflects the lengthwise association of these polymers with un-

known physiological effectors. As a consequence, one would expect microtubule cold stability in lysed cells to be affected by regulatory systems also operative *in vivo*. In this section, we have started to investigate the effects of Ca^{2+} and of protein kinases.

Calcium Effects

It has been shown previously (Schliwa et al., 1981; Deery et al., 1984; Cyr, 1991) that lysed cell microtubules are disassembled by free Ca^{2+} concentrations above 0.1 mM. At lower concentrations, Ca^{2+} induced apparent microtubule fragmentation (Deery et al., 1984).

Ca^{2+} -dependent fragmentation of microtubules does not appear to be an artifact of lysed cell systems, since it can be observed in both HeLa and NIH/3T3 whole cells treated with the Ca^{2+} -ionophore A23187 (Fig. 4). We tested to determine whether this effect results from a direct effect of calcium on microtubules or from the action of a microtubule-associated severing activity. When lysed NIH/3T3 cells were extensively washed in lysis buffer before Ca^{2+} exposure, no microtubule fragmentation was observed, indicating that fragmentation depends on the association of a specific severing activity with microtubules, and not on a direct action of Ca^{2+} on these polymers (Fig. 5 C). This loss of severing activity after washing was temperature dependent, occurring more extensively and more rapidly at 37°C than at 30°C: three washes of one minute each were sufficient at 37°C (Fig. 5 C), but not at 30°C (not shown). In contrast, the Ca^{2+} -dependent severing activity was retained in lysed cells that were not extensively washed with lysis buffer (Fig. 5 A). We examined the temperature dependence of the Ca^{2+} -dependent fragmentation process. If Ca^{2+} -induced fragmentation were due to a depolymerizing effect of calcium, fragmentation should be more rapid at cold temperature (Webb and Wilson, 1980; Job et al., 1981). On the other hand, if fragmentation actually involves a severing protein, one would expect it to be slower at cold temperature, a condition that slows down most biochemical reactions. When lysed NIH/3T3 cells were exposed to 0.1 mM Ca^{2+} for one minute, extensive fragmentation occurred at 37°C (Fig. 5 A), but no fragmentation was observed at 0°C (Fig. 5 B). These results indicate that microtubule fragmentation and microtubule depolymerization are distinct processes.

Finally, we compared the cold stability of Ca^{2+} -generated fragments to the cold stability properties of the intact polymers. As in sonication experiments, fragments retained the stability properties of the intact microtubules (not shown).

Effect of Protein Kinases

Microtubule dynamics are thought to be regulated by various protein kinases. To examine their effect on the stability of lysed cell microtubules, lysed cells were incubated at room temperature with ATP- Mg^{2+} alone or in the presence of added purified protein kinases. They were subsequently placed in lysis buffer at 30 or 0°C and examined 30 min later. We found no effect of ATP- Mg^{2+} alone (Fig. 6 A, inset). Similarly, in our assay conditions, c-AMP dependent protein kinase, MAP kinase, casein kinase II, and Ca^{2+} -calmodulin dependent protein kinase were inactive (not shown).

On the contrary, the kinase p34^{cdc2} had a spectacular effect, inducing complete microtubule destabilization. Not

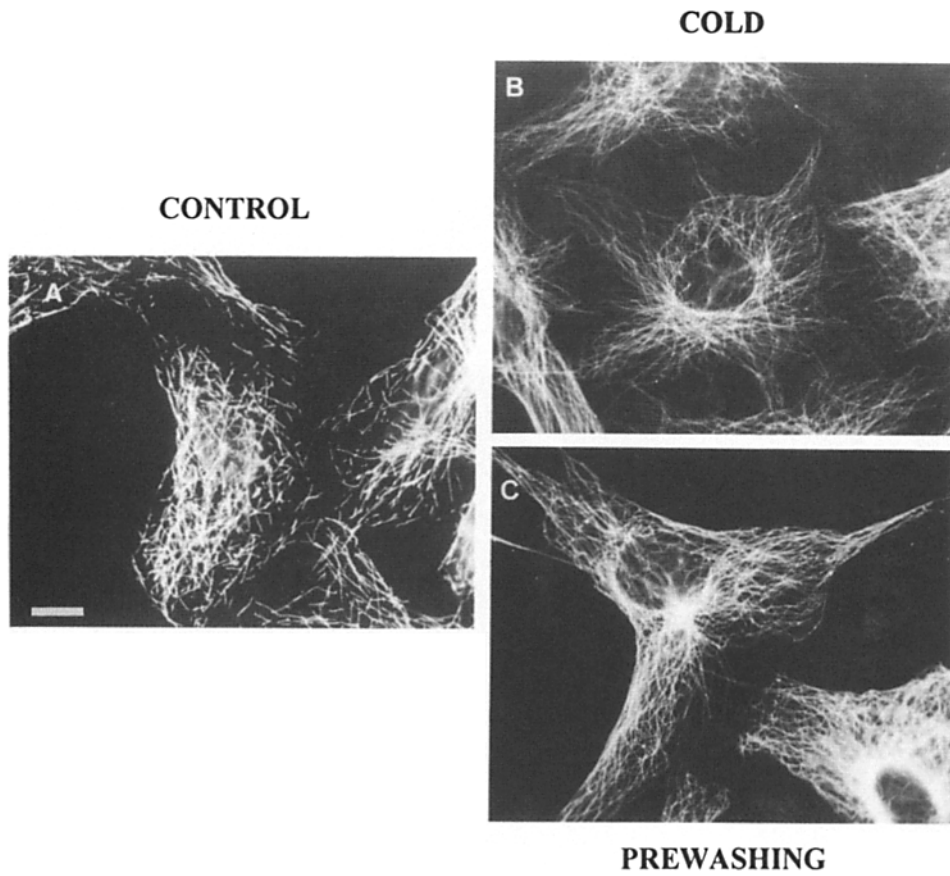


Figure 5. Calcium-dependent microtubule severing in lysed NIH/3T3 cells: effects of temperature and of prewashing. NIH/3T3 cells were lysed and treated with 0.1 mM Ca^{2+} in the various conditions described below, before fixation and immunostaining with anti- β tubulin mAb. (A) lysed cells Ca^{2+} -treated at 37°C for 1 min; (B) lysed cells Ca^{2+} -treated at 0°C for 1 min; (C) lysed cells prewashed (3×1 min) at 37°C in the absence of Ca^{2+} , and then Ca^{2+} -treated at the same temperature for 1 min. Cold temperature and prewashes inhibited Ca^{2+} -induced fragmentation. Bar, 10 μm .

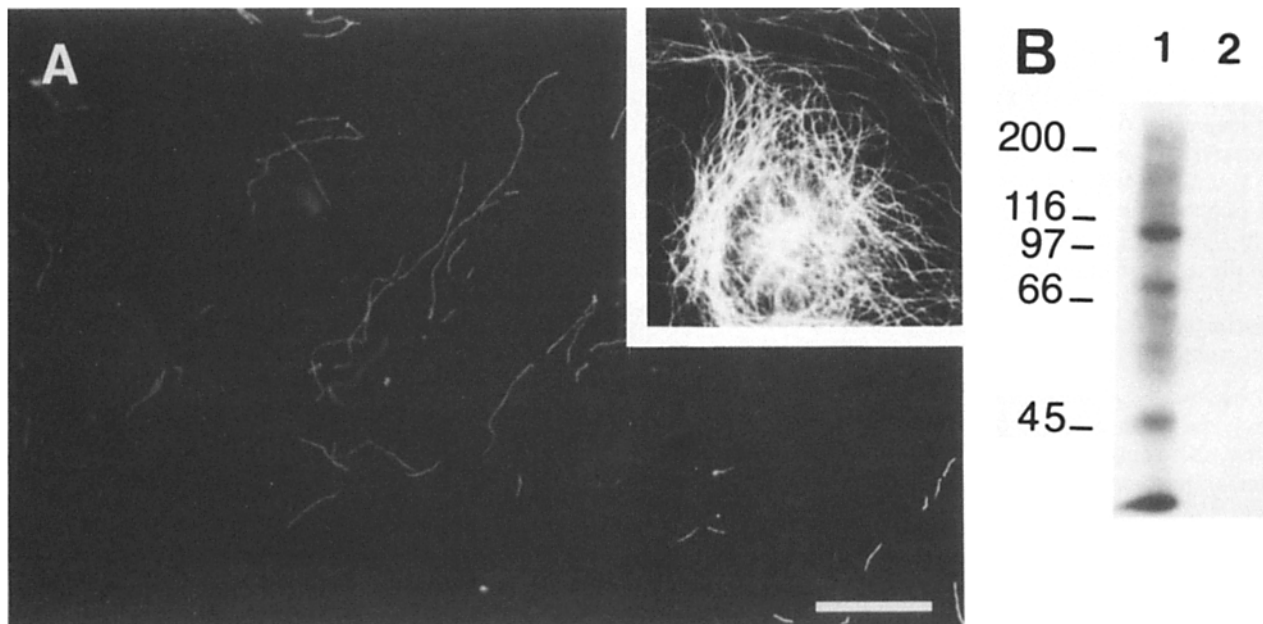


Figure 6. Effect of p34^{cdc2}-cyclin B on lysed NIH/3T3 cell microtubules. NIH/3T3 cells were lysed, washed, and treated either with p34^{cdc2}-cyclin B (A) or with kinase buffer alone (*inset*). Microtubules were subsequently tested for resistance to dilution at 30°C, and then fixed for anti- β tubulin immunostaining. Results showed a complete destabilization of interphase networks in the presence of p34^{cdc2}-cyclin B. Bar, 15 μm . (B) Phosphorylated substrates of p34^{cdc2}-cyclin B in lysed NIH/3T3 cells. Cells were lysed, washed, and incubated with p34^{cdc2}-cyclin B (lane 1) or with kinase buffer (lane 2) in the presence of γ -³²P-ATP. Soluble fractions were then collected and analyzed by SDS-PAGE and autoradiography. Positions of *Mr* standards are indicated, $\times 10$.

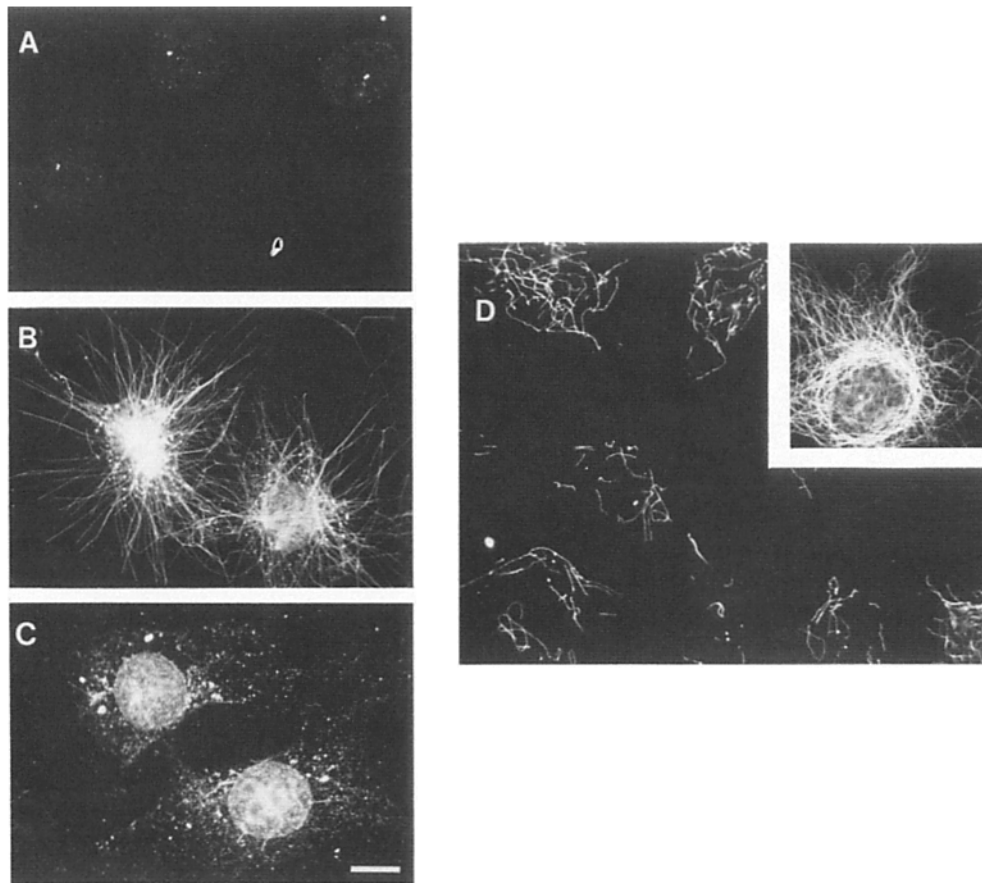


Figure 7. Evidence that microtubule resistance to dilution-induced disassembly in HeLa cells does not result from a stabilizing effect of the lysis buffer. (A–C) Microtubules in lysed NIH/3T3 cells were solubilized, as described in Materials and Methods. Asters of pure tubulin microtubules were grown on the remaining centrosomes and further tested for resistance to dilution at 30°C. Patterns were analyzed by immunofluorescence using an anti- β tubulin mAb. (A) Lysed NIH/3T3 cells after microtubule solubilization. Only centrosomes were stained. (B) Asters formed of pure tubulin microtubules. (C) Same as in B after 15 min of dilution in the lysis buffer at 30°C. Pure tubulin asters rapidly depolymerized after dilution in lysis buffer. (D) HeLa cells were lysed, washed, and treated either with p34^{cdc2}-cyclin B (D) or with kinase buffer alone (inset). Microtubules were subsequently tested for resistance to dilution at 30°C, and then fixed for anti- β tubulin immunostaining. Results showed a complete destabilization of interphase networks in the presence of p34^{cdc2}-cyclin B. Bar, 10 μ m.

only did microtubules become cold-labile (not shown) but they even became labile to dilution at 30°C (Fig. 6 A). When phosphorylation was performed in the presence of γ^{32} P-ATP, autoradiography of solubilized fractions showed absence of phosphorylation in the absence of p34^{cdc2} (Fig. 6 B, lane 2) and a limited number of phosphorylated substrates in the presence of the kinase (Fig. 6 B, lane D).

Microtubule Resistance to Dilution-induced Depolymerization in HeLa Cells

Cell lysis induced a considerable dilution of the intracellular medium and yet, in all cell types, interphase microtubule arrays remain apparently intact. Such resistance to dilution is natural in the case of cold-stable microtubules (Webb and Wilson, 1980; Job and Margolis, 1984) but it poses problems in the case of cold-labile microtubules as HeLa cell microtubules (see Introduction). It could result from a specific property of interphase microtubules or from experimental artifacts, such as an incomplete removal of free tubulin-GTP during cell lysis or a stabilizing effect of lysis buffer.

To ascertain that not enough tubulin-GTP was left in lysed cells to permit assembly, HeLa cells were exposed to cold before lysis, lysed, and then rewarmed in lysis buffer. No repolymerization was observed (not shown). A possible

stabilizing effect of lysis buffer was tested by examination of the behavior of pure tubulin microtubules placed under our experimental conditions. Microtubules from lysed cells were fully dissolved using cold temperature exposure and high Ca²⁺ concentrations (Fig. 7 A). Pure tubulin microtubules were then assembled onto the remaining centrosomes. Large asters were formed (Fig. 7 B) and further challenged for resistance to dilution. Results showed that they disappeared within 15 min after dilution in lysis buffer (Fig. 7 C). We also examined the disassembly kinetics of MAP-containing microtubules from bovine brain. Such microtubules depolymerized within 15 min after dilution in lysis buffer (not shown). We conclude that microtubule resistance to dilution in lysis buffer is a specific property of interphase polymers which is not shown by pure tubulin microtubules or MAP-containing microtubules.

We then tested on HeLa cells the effects of sonication, of Ca²⁺, and of protein kinases after the same procedures as the one used in the case of NIH/3T3 cells with the exception that microtubule resistance to dilution and not to cold temperature exposure was used as a test of stability. Results were superimposable: resistance to dilution was conserved by microtubule fragments after sonication, and the effects of Ca²⁺ (not shown) and of protein kinases (Fig. 7 D) were the same. This suggests that microtubule resistance to dilution

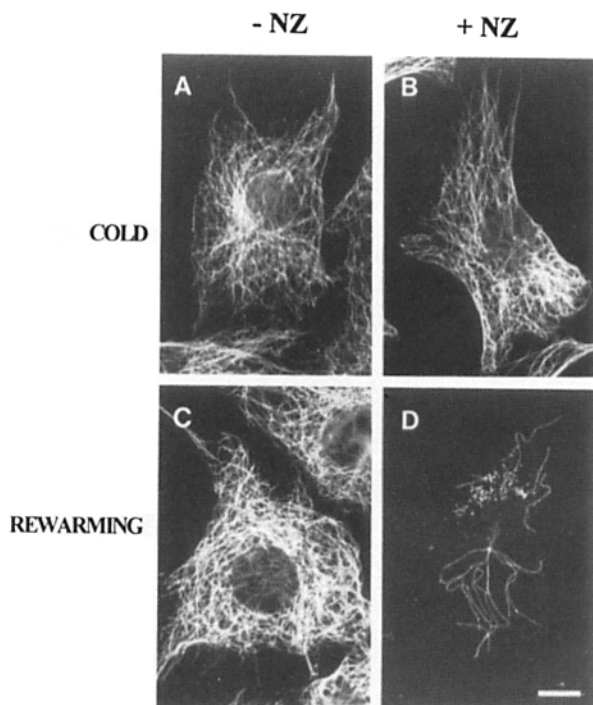


Figure 8. Effect of cold temperature on nocodazole (NZ) sensitivity of cytoplasmic microtubules. NIH/3T3 cells were exposed to cold temperature for 30 min, and then submitted to various treatments, lysed for 1 min to remove the background of depolymerized tubulin, and further processed for anti- β tubulin immunostaining. Treatments were (A) none; (B) cells treated with 1 μ M nocodazole for 30 min at 0°C; (C) cells rewarmed in culture medium at 30°C for 30 min; and (D) cells rewarmed and treated simultaneously with 1 μ M nocodazole for 30 min at 30°C. Microtubules were insensitive to nocodazole at 0°C, but they rapidly recovered drug sensitivity upon rewarming. Bar, 10 μ m.

in HeLa cells and microtubule cold stability in fibroblast cells are controlled by related effectors.

Nocodazole Effects on Interphase Microtubules

Interphase microtubules in cells are known to rapidly disassemble upon nocodazole exposure. According to current views, the sensitivity of interphase microtubules to depolymerizing drugs is an immediate consequence of their physicochemical properties: pure tubulin microtubules or MAP-containing microtubules are known to be sensitive to depolymerizing drugs *in vitro*. But the results described above support the view that in the absence of specific cell regulation, in all cell types, interphase microtubules are for the most part dynamically inactive. This implies that their drug sensitivity should be conditional to the presence and activity of cellular regulatory systems such as protein kinases and this possibility is tested in the experiments described below.

In both intact NIH/3T3 cells and HeLa cells, cytoplasmic microtubules rapidly depolymerized upon exposure to nocodazole (not shown). In contrast, they were insensitive to nocodazole after cell lysis, a procedure which eliminates ATP, and most protein kinases (not shown). Protein kinases are temperature- and ATP-dependent. Therefore in intact

cells, microtubules should show drug resistance at low temperature and recover drug sensitivity upon rewarming, and they should become drug resistant after ATP depletion. As shown in Fig. 8, in NIH/3T3 cells placed at 0°C, microtubules are nocodazole resistant (Fig. 8 B). The same polymers rapidly disassemble upon rewarming (Fig. 8 D). It is known that metabolic inhibitors such as azide suppress drug sensitivity in cells (Moskalewski et al., 1980; Bershadsky and Gelfand, 1981; De Brabander et al., 1981). These agents induce an ATP depletion while leaving enough GTP to permit tubulin assembly (De Brabander et al., 1981). We have repeated these experiments in the case of NIH/3T3 cells and HeLa cells with concomitant examination of microtubule stability toward cold temperature and dilution. After azide treatment, microtubules became nocodazole resistant in both cell types (Fig. 9, A and B) and this occurred without detectable changes in their stability behavior. Microtubules remained cold-stable in NIH/3T3 cells and cold-labile in HeLa cells (Fig. 9, C and D). In lysed cells treated with azide before lysis, microtubule resistance to dilution or microtubule reactivity to Ca^{2+} were not significantly modified (not shown).

Taken together, these results strongly support the view that the drug sensitivity of interphase microtubules in intact cells depends on specific regulatory factors acting on stabilized, nocodazole resistant, polymers. The suppressive effect of metabolic inhibitors on microtubule sensitivity to depolymerizing drugs has not been easy to explain under the hypothesis of polymers behaving as pure tubulin or MAP-containing microtubules. The present study suggests that these agents simply make evident, in intact cells, the intrinsic non dynamic state of interphase microtubules, as was revealed above using cell lysis and dilution of soluble components.

Discussion

The present work provides experimental support to the view that interphase microtubules are intrinsically stable, and that microtubule instability is conferred to these otherwise stable microtubules in the living cell by regulatory mechanisms that may involve Ca^{2+} - and phosphorylation-dependent events. In the fibroblast cell lines we examined, most interphase microtubules were cold-stable. Microtubule cold stability has also been previously observed in cycling cells of neuronal origin (Margolis et al., 1990) and in glial cells (unpublished results). In contrast, the epithelial cells we examined were essentially devoid of cold-stable polymers. If further work shows that cold lability is a general feature of epithelial cells, it might indicate a specific property related to epithelial differentiation. However, this difference in stability is not absolute since a few cold-stable polymers were observed in epithelial cells and since other cell types generally contain a subset of cold-labile polymers.

These differences in the degree of cold stability between cell types were experimentally useful to establish that, as in the case of brain or flagellar cold-stable microtubules, cytoplasmic microtubule stabilization did not require the integrity of cellular structures but that, instead, it reflected the association of these polymers with specific effectors (Job et al., 1982; Multigner et al., 1992). This was shown by the excellent correlation that we found between microtubule behavior toward cold in intact and lysed cells. This correlation

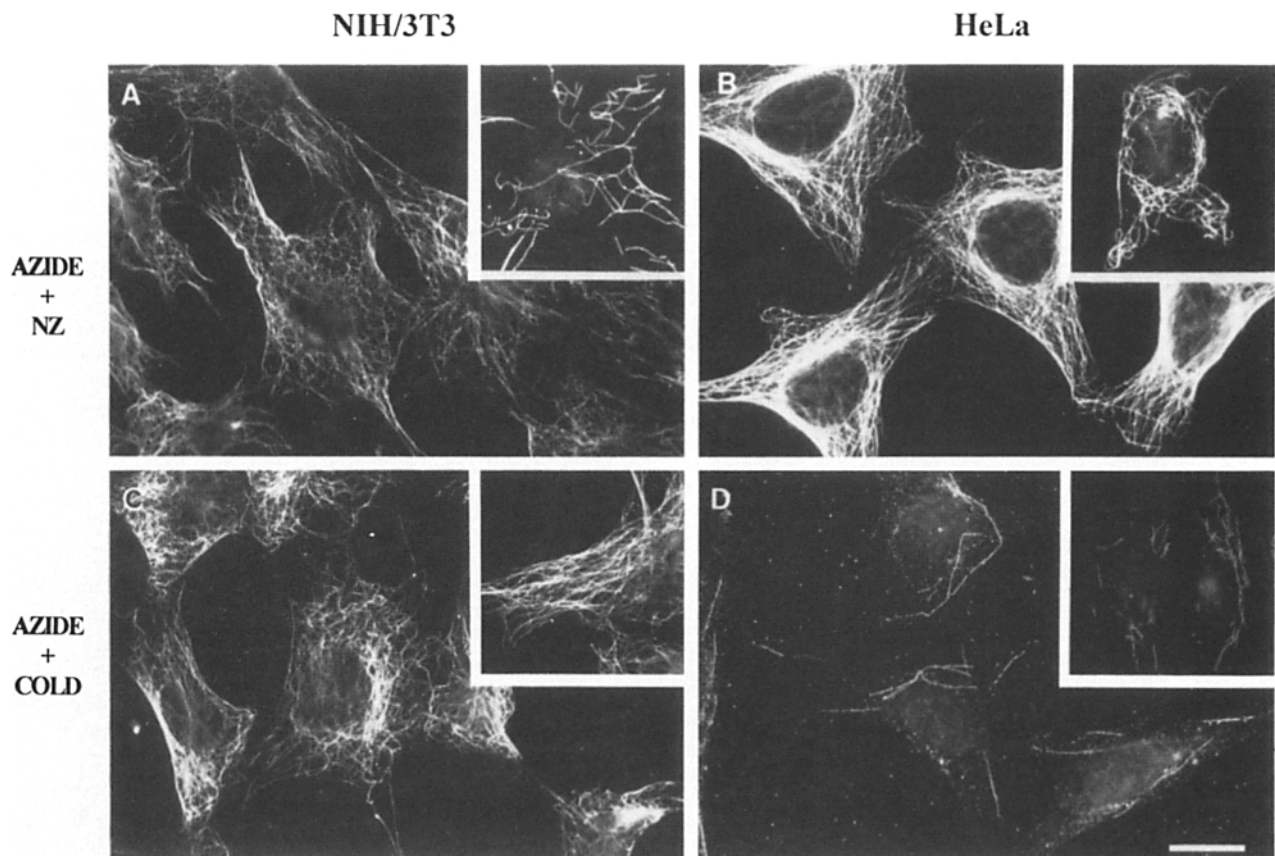


Figure 9. Sodium azide treatment of cells: effects on microtubule nocodazole (NZ) sensitivity and on microtubule cold stability. NIH/3T3 cells (A and C) and HeLa cells (B and D) were treated for 1 h at 37°C with 20 mM sodium azide, and then submitted to various other treatments, lysed for 1 min to remove tubulin background, and further processed for immunostaining using an anti- β tubulin antibody. Treatments were (A and B) cells treated with 1 μ M nocodazole for 15 min at room temperature in the presence of sodium azide; and (C and D) cells exposed to cold temperature for 30 min in the presence of sodium azide. (Insets) Control experiments in which azide was omitted in the incubation buffer. Sodium azide treatment suppressed nocodazole sensitivity of microtubules in both cell types without apparent modifications of microtubule cold stability properties. Bar, 15 μ m.

also ruled out many possible artifacts. For instance, if microtubule stability were triggered by artifactual association of non specific factors during cell disruption, this correlation would not be expected, and, if microtubule cold stability in intact cells was generated by non-physiological perturbations during cold exposure, one would not find cold-stable polymers in lysed cells not cold-treated before lysis.

We examined the effect of dilution of soluble components and the effect of nocodazole on microtubules in lysed cells. Microtubules from fibroblast cells remained stable under such conditions. This result was expected since cold-stable microtubules resist dilution and nocodazole-induced depolymerization *in vitro* (Job et al., 1981; Job and Margolis, 1984; Margolis et al., 1990). Microtubules also remain stable in epithelial cells, and this stability is not due to stabilization effects of buffers. Such stability contrasts with the well-known instability of microtubules assembled from pure tubulin and from MAP-containing preparations *in vitro* and suggests that microtubules assembled *in vitro* lack a component that confers stability to interphase polymers.

Although we did not characterize the component that confers stability to interphase microtubules in cycling cells, we suspect that at least in fibroblast cells, it could be an analog

of neuronal STOP protein (Margolis et al., 1986). This protein can protect microtubules against cold temperature disassembly, it can stabilize microtubule lengthwise by adsorption onto the polymer surface, its effect can be abolished through interaction with Ca^{2+} -calmodulin, and presumably by protein phosphorylation (Job et al., 1981, 1983; Pirolet et al., 1992). In fact, a polyclonal antibody directed against the whole neuronal STOP protein and an anti-peptide polyclonal antibody directed against part of the recently microsequenced protein both recognize a major 100-kD antigen in those cells which contain cold-stable microtubules (unpublished results and Margolis, R. L., personal communication). We also determined that such MAPs as MAP2 or tau proteins did not confer dilution resistance to pure tubulin microtubules in our cell lysis buffer (not shown).

To substantiate the view that cell lysis and dilution of soluble components reveal the intrinsic stability of interphase microtubules, we verified that stable microtubules in lysed cells were not different from intracellular microtubules with regard to the action of Ca^{2+} . In lysed cells, Ca^{2+} induces microtubule disassembly at millimolar concentrations and microtubule fragmentation at lower concentrations (Schliwa et al., 1981; Deery et al., 1984; Cyr, 1991). Both effects were

also observed in intact cells microinjected with Ca²⁺-calmodulin (Keith et al., 1983) or exposed to a calcium ionophore (Fuller and Brinkley, 1976; Schliwa et al., 1976; and our results). Microtubule fragmentation might involve a Ca²⁺-regulated severing protein, perhaps similar to that described in Protozoa (Schliwa, 1976; Febvre-Chevalier and Febvre, 1992).

Microtubules in lysed cells were also exposed to various protein kinases. Multiple protein kinases are known to phosphorylate MAPs and to regulate their effect (for a review, see Wiche, 1989). In some systems, MAP kinase (Gotoh et al., 1991) and okadaic acid (Eriksson et al., 1992; Gliksman et al., 1992) were found to cause profound modification of microtubule dynamics. Combining experiments on intact and lysed cells, we have tested the potential effects of c-AMP dependent protein kinase, protein kinase C, casein kinase II, MAP kinase, Ca²⁺-calmodulin dependent protein kinase, and phosphatase inhibitors. None of these agents had a significant effect on microtubule stability. In contrast, the p34^{cdc2} protein kinase induced complete destabilization of cold-stable and dilution-resistant microtubules in lysed cells. It has been previously demonstrated that in intact cells, microinjection of p34^{cdc2} induces extensive dissolution of interphase networks (Lamb et al., 1990) and that addition of p34^{cdc2} to *Xenopus* egg extracts causes profound changes in microtubule dynamics (Verde et al., 1990). In the present work we find that p34^{cdc2} affects stable microtubules in a system that is simpler than intact cells or *Xenopus* egg extracts. The absence of detectable protein phosphorylation in control experiments in which p34^{cdc2} was omitted indicated that most endogenous protein kinases were eliminated during cell wash and this suggests that p34^{cdc2} may have acted in these experiments through direct phosphorylation of a microtubule-associated effector. Recent work has suggested that p34^{cdc2} could also affect microtubule behavior through activation of microtubule-severing activities (Vale, 1991; Faruki et al., 1992; Shiina et al., 1992). In lysed cells, no microtubule fragmentation was observed upon exposure to p34^{cdc2}, so the possibility of such an effect seems unlikely. Our work is in progress to identify the microtubule-stabilizing protein whose activity is abolished in vitro upon phosphorylation by p34^{cdc2}.

The specific effect of the p34^{cdc2} kinase on microtubule stability in lysed cells substantiates the view that microtubules in lysed cells are stabilized by physiological effectors. Such an effect may also be representative of the mechanisms which confer instability to intrinsically stable microtubules in cycling cells. Conditions that presumably inhibit protein kinases, such as cold temperature or ATP-depletion also suppress microtubule sensitivity to nocodazole in intact interphase cells and we propose that under such conditions, the intrinsic stability properties of interphase microtubules are made evident.

However, in the present study, experiments were performed using cyclin B-cdc2 kinase, which activates only at the G2 to M phase transition of the cell cycle. Obviously, this kinase cannot confer instability to microtubules in G1 cells. A variety of cyclin-dependent kinases (cdks) have been shown or are believed to be activated at various times of G1 phase (see Meyerson et al., 1992 for a review). The substrate specificity of the various cdks has not been investigated in detail, but at least some of them (cdk2, cdk3, and cdk4) ap-

pear to share in common with cdc2 the same substrate specificity at least in vitro. Moreover, cyclin A-cdc2 kinase and cyclin B-cdc2 kinase have similar substrate specificity, at least in vitro (Lorca et al., 1992), suggesting that the cyclin subunit may serve to target the catalytic subunit or specify its time of activation rather than directly modulate its substrate specificity. Therefore, it is reasonable to assume that the cyclin B-cdc2 kinase preparation used in the present experiments may have mimicked a cdk of similar consensus sequence acting at interphase to destabilize microtubules in cycling cells. Further work will be required to identify this putative kinase.

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