Stepwise Reconstitution of Interphase Microtubule Dynamics in Permeabilized Cells and Comparison to Dynamic Mechanisms in Intact Cells

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Abstract. Microtubules in permeabilized cells are devoid of dynamic activity and are insensitive to depolymerizing drugs such as nocodazole. Using this model system we have established conditions for stepwise reconstitution of microtubule dynamics in permeabilized interphase cells when supplemented with various cell extracts. When permeabilized cells are supplemented with mammalian cell extracts in the presence of protein phosphatase inhibitors, microtubules become sensitive to nocodazole. Depolymerization induced by nocodazole proceeds from microtubule plus ends, whereas microtubule minus ends remain inactive. Such nocodazole-sensitive microtubules do not exhibit subunit turnover. By contrast, when permeabilized cells are supplemented with *Xenopus* egg extracts, microtubules actively turn over. This involves continuous creation of free microtubule minus ends through microtubule fragmentation. Newly created minus ends apparently serve as sites of microtubule depolymerization, while net microtubule polymerization occurs at microtubule plus ends. We provide evidence that similar microtubule fragmentation and minus end–directed disassembly occur at the whole-cell level in intact cells. These data suggest that microtubule dynamics resembling dynamics observed in vivo can be reconstituted in permeabilized cells. This model system should provide means for in vitro assays to identify molecules important in regulating microtubule dynamics. Furthermore, our data support recent work suggesting that microtubule treadmilling is an important mechanism of microtubule turnover.

Key words: microtubule • turnover • reconstitution • permeabilized cells • treadmilling

Many essential cellular functions such as cell morphogenesis and organelle transport in interphase cells rely on organized microtubule arrays. Microtubule polarity and dynamics are therefore central to the maintenance of cell function. In most animal cells, microtubules are nucleated on centrosomes and have ordered polarity. The slow-growing (minus) ends of microtubules are oriented toward the centrosome, whereas the rapidly growing (plus) ends are distal to the centrosome (Heidemann and McIntosh, 1980; Euteneuer and McIntosh, 1981). As a consequence of the dynamic behavior of microtubule ends, tubulin dimers exchange rapidly with polymers, and the entire microtubule network turns over within minutes (Saxton et al., 1984; Schulze and Kirschner, 1986). To a large extent, investigations of the mechanism of microtubule turnover in vivo have been based on in vitro observations showing that microtubules have intrinsic dynamic activity.

Microtubules assembled from purified tubulin preparations in vitro show both spontaneous length fluctuations (Mitchison and Kirschner, 1984; Horio and Hotani, 1986) and treadmilling behavior (Margolis and Wilson, 1978; Margolis and Wilson, 1981). In living cells, dynamic instability generates polymer growth and shrinkage at free peripheral microtubule plus ends (Sammak et al., 1987; Sammak and Borisy, 1988; Schulze and Kirschner, 1988; Cassimeris et al., 1988). In interphase cells such length excursions are limited by apparent regulatory factors (Sammak and Borisy, 1988; Schulze and Kirschner, 1988), and the observed rate of turnover may be substantially more

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rapid than can be explained by the dynamic instability of microtubule plus ends (Shelden and Wadsworth, 1993; Gliksman et al., 1993).

This apparent discrepancy between observed individual microtubule dynamics and the rate of microtubule turnover has been at least partially resolved by the recent discovery of microtubule treadmilling in interphase arrays in vivo (Rodionov and Borisy, 1997). Such treadmilling behavior requires microtubule minus ends to be free for subunit exchange. It has been shown that many microtubule minus ends are free in the cytoplasm rather than embedded in the pericentriolar material (Keating et al., 1997), apparently as a result of microtubule release from the centrosome. Free microtubule minus ends can also occur elsewhere in the cytoplasm (Vorobjev et al., 1997; Yvon and Wadsworth, 1997), and can be produced by microtubule breakage in the periphery of motile cells (Waterman-Storer and Salmon, 1997). These newly created microtubule minus ends can function as sites of polymer disassembly while concomitant polymer assembly may occur on microtubule plus ends (Keating et al., 1997; Waterman-Storer and Salmon, 1997). As a result, microtubule turnover can be a polarized process, yielding a continuous flow of microtubules from the center toward the periphery of the cell. Frequently, minus end polymer disassembly and plus end polymer assembly occur at similar rates on individual microtubules, creating polymers that are stable in length while constantly treadmilling (Rodionov and Borisy, 1997). The overall picture that emerges from these studies is one of complexity. Microtubule networks once thought to be strictly centered on centrosomes now appear to be subject to fragmentation, and microtubule turnover must involve complex control of both microtubule ends.

Identification of molecules important in regulating microtubule dynamics would be facilitated by the design of an assay system that would allow stepwise analysis of microtubule dynamics. In the work described here, we have developed an in vitro system in which permeabilized cells are supplemented with various cell extracts in order to reconstitute the dynamics of their microtubule networks.

We have previously shown that in permeabilized mammalian culture cells, microtubules remain quantitatively assembled, but immediately shift from a normal dynamic state to an entirely inert state (Lieuvin et al., 1994). Microtubules in interphase cells are insensitive to depolymerizing conditions such as dilution of the cell-soluble components or exposure to the assembly inhibitor nocodazole. Inactivation of microtubule dynamics is apparently the result of removal of soluble factors such as protein kinases during the process of permeabilization. The inactive polymers of permeabilized cells offer a starting system to permit stepwise reconstitution of microtubule turnover. Since cell permeabilization eliminates soluble cell fractions, we began by testing the ability of soluble cell extracts to reconstitute at least some aspects of microtubule dynamic activity.

We find that addback of soluble cell extracts to permeabilized cells reconstitutes microtubule sensitivity to nocodazole. This restoration of sensitivity only involves microtubule plus ends, and is insufficient to induce detectable microtubule turnover. These results define an intermediate stage of microtubule activation, but also show that soluble cell extracts lack essential activities involved in generating microtubule turnover. We find that such essential activities are apparently present in *Xenopus* egg extracts. Addback of interphase *Xenopus* egg extracts to permeabilized cells reconstitutes rapid polymer turnover. During turnover induced by *Xenopus* extracts, disassembly of the interphase microtubule network mainly proceeds from microtubule minus ends that are created by continuous microtubule fragmentation. We provide evidence that similar microtubule fragmentation and minus end–directed disassembly occurs in intact fibroblastic cells. These results show that at least one type of microtubule dynamics resembling in vivo microtubule dynamics can be reconstituted in vitro. Such a permeable cell system should provide a powerful assay to study microtubule regulation. In addition, our results support recent observations of treadmilling as an important mechanism of microtubule turnover (Rodionov and Borisy, 1997).

Materials and Methods

Materials

Nocodazole was purchased from Sigma Aldrich (Strasbourg, France), and colchicine was obtained from Merck (Darmstadt, Germany). These drugs were dissolved in 100% ethanol to prepare stock solutions, nocodazole at a concentration of 10 mM, and colchicine at a concentration of 100 mM. The stock solutions were stored at −20°C.

Culture media, sera, antibiotics, and okadaic acid were purchased from Gibco Laboratories (Grand Island, NY); Aquamount was obtained from BDH (Pool, England), and fibronectin was obtained from Calbiochem Corp. (San Diego, CA).

The rat monoclonal antibody YL1/2 that binds the tyrosinated form of α-tubulin (Tyr-tubulin) was a generous gift from Dr J.V. Kilmartin (Cambridge, England). Rabbit polyclonal antibody directed against detyrosinated (Glu-tubulin) was kindly provided by Dr. L. Paturle-Lafanechère (DBMS/CS, CEA, Grenoble, France). The nonimmune rabbit IgGs were purchased from Cappel Organon Teknika Co. (Durham, NC). Other antibodies used in the present study were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells

NIH/3T3 cells were grown in DME complemented with 10% FBS. Cells lacking tubulin tyrosine ligase, subcloned from a parental NIH3T3 line (TTL^- cells, clone A607), were a generous gift of Dr. L. Lafanechère (Lafanechère et al., 1998). These cells were grown in DME complemented with 10% Donner calf serum and 1% FCS. Each culture medium was supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. All cell types were incubated at 37°C in a humidified incubator in a 6% CO_2 environment.

Extracts

To obtain mammalian cell extracts, cells were grown on tissue culture flasks with surface areas of 175 cm^2 (Greiner Labortechnik, Frickenhausen, Germany) until they reached 70% confluence. The culture medium was removed and cells were washed in PBS composed of 137 mM NaCl, 8 mM sodium phosphate, 1.5 mM potassium phosphate, 2.7 mM KCl, pH 7.4. Cells were trypsinized at 37°C for 3 min, and the reaction was stopped by adding culture medium. Cells were then collected by centrifugation, (10 min, 37°C, 300 *g*), and the pellets were resuspended in PBS and recentrifuged (10 min, 37°C, 300 *g*). Finally, cells were resuspended in permeabilization buffer (vol/vol) composed of 80 mM Pipes-KOH, pH 6.75, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100 (vol/vol), and 10% glycerol (vol/vol) supplemented with 5 mM $CaCl_2$. These cells were incubated for 10 min at 4°C. After treatment, EGTA was added to a final concentration of 10 mM, and the extracts were centrifuged (200,000 *g*, 15 min, 4°C) in a Beckman TLA-100 rotor. The supernatants were immediately frozen

in liquid nitrogen, and were then stored at −80°C for further use. Typically, the total protein concentration in soluble cell extracts was ~20 mg/ml. The tubulin concentration, as estimated on comparative immunoblots using pure tubulin as a standard, was ~10 μM.

Interphase *Xenopus* egg extracts were prepared 40 min after parthenogenetic activation by electric shock according to the method of Murray and Kirschner (1989). Total protein and tubulin concentrations were ~45 mg/ml and 20 μM, respectively.

Protein Preparation

Phosphocellulose-purified tubulin was prepared from fresh beef brain as described elsewhere (Saoudi et al., 1995). Detyrosinated tubulin (Glu-tubulin) was prepared by carboxypeptidase A digestion of pure tyrosinated tubulin as described in Paturle et al. (1989).

For preparation of colchicine-tubulin dimer (CD)[1] complexes, phosphocellulose-purified tubulin (100 μM) was incubated with 1 mM colchicine for 30 min at 30°C in the presence of 1 mM free GTP. Unbound colchicine was removed by gel filtration at 4°C using Biogel P6 (BioRad, Hercules, CA) spin columns. The gel-filtered colchicine-tubulin complexes were immediately used for further experiments.

Tubulin tyrosine ligase (TTL) was expressed in insect cells using the Baculovirus Expression VectorSystem (PharMingen, San Diego, CA) according manufacturer's instructions. Complete TTL cDNA from pig (Ersfeld et al., 1993) was inserted into the baculovirus transfer vector pVL 1392 (PharMingen). Sf9 cells (PharMingen) were cotransfected with Baculo Gold™ DNA (PharMingen) and the recombinant TTL baculovirus transfer vector DNA. Plaque-purified virus was amplified in Sf9 cells to produce a high-titer stock. For production of recombinant TTL, Sf9 cells were grown in spinner culture bottles, infected with amplified virus, and grown for up to 4 d using TC 100 medium (Gibco Laboratories, Grand Island, NY) complemented with 10% FCS and 2 mM glutamine. Infected cells were harvested by centrifugation, and cell pellets were stored at−80°C. TTL was further purified using immunoaffinity chromatography as previously described (Paturle et al., 1989).

Reconstitution Experiments

To prevent cellular detachment after permeabilization, cells were grown for 1–2 d on fibronectin-coated coverslips prepared as previously described (Crowley and Horwitz, 1995). For cell permeabilization, cells on coverslips were immersed for 1 min at 34°C in 100 ml of permeabilization buffer. The permeabilized cells were washed with 100 ml of permeabilization buffer for 1 min at 34°C, and were then incubated with extracts under various conditions as indicated in the figure legends, and finally processed for immunofluorescence (see below).

Microinjection

Cells grown on glass coverslips were injected using a 5171 micromanipulator and a 5246 transjector (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany).

For microinjection of CD complexes, CD complexes were diluted to a concentration of 25 μM in PME buffer (100 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.65). Before microinjection, the solution was clarified by centrifugation in a Beckman TLA-100 rotor (100,000 *g*, 4°C, 10 min). The supernatant was then supplemented with nonimmune rabbit IgGs (5 mg/ml) to detect injected cells. The protein mixture was then loaded into injection needles and was injected into NIH3T3 cells.

Purified tubulin tyrosine ligase was injected into TTL^- cells at a concentration of 6 mg/ml. After various periods of time as indicated in the figure legends, cells were processed for immunofluorescence microscopy (see below).

Immunofluorescence Microscopy

Reconstituted or intact cells were fixed on coverslips for 6 min in anhydrous methanol at −20°C and subsequently washed in PBS/0.1% Tween-20 (vol/vol). Cells were sequentially incubated with different primary antibodies (1/1,000 in PBS/0.1% BSA), and then with appropriate secondary antibodies (FITC-conjugated goat anti–rat or anti–rabbit 1/250, Cy3-conjugated goat anti–rat or anti–rabbit 1/1,000). Coverslips were mounted in Aquamount and observed using a Zeiss Axioskop microscope (Zeiss, Oberkochen, Germany).

Image Analysis

For image analysis and quantification of microtubule assembly, microtubule interphase networks were visualized by tubulin immunofluorescence. The fluorescent images were acquired with a charge-coupled device (CCD) camera (Princeton Instruments, Trenton, NJ), and were digitized using IP Lab Spectrum software (Signal Analytics Co., Vienna, VA). Images (~50 permeabilized cells for each condition) were displayed on a computer monitor (Sony Corp., Tokyo, Japan), and fluorescence intensity values of the pixels within delimited cells were integrated. Images were digitally rescaled by making the lowest nonzero value black (yielding a value of zero) and the highest pixel value white (yielding a value of 255). This technique (contrast stretching) enhances the appearance of images by using the full range of greyscale values to display each image. Then, a binary morphology of microtubular networks was computer-generated. Microtubule assembly was measured by taking the percent of positive pixels relative to the total number of pixels within the cell area.

Videomicroscopy

TTL^- cells were grown for 1–2 d on fibronectin-coated LAB-TEK chamber slides (Gibco Laboratories). Cells were then permeabilized as described above. Permeabilized cells were incubated for 15 min at room temperature with detyrosinated tubulin (Glu-tubulin) antibody (1/1,000 in permeabilization buffer). Cells were washed in permeabilization buffer and further incubated for 15 min at room temperature with secondary antibody (goat anti–rabbit Cy3, 1/1,000 in permeabilization buffer). *Xenopus* egg interphase extracts were then added to these antibody-treated cells. These cells were then observed using fluorescence optics and a CCD camera as described above. The camera was equipped with a video output connected to a video recorder (Sony Corp.). Video images were recorded in real time.

Results

Reconstitution of Microtubule Sensitivity to Nocodazole Action in Permeabilized NIH3T3 Cells

After cell permeabilization in Pipes Triton buffer, interphase microtubules remain intact, but shift to a dynamically inert state. One salient feature of this stable state is microtubule insensitivity to the depolymerizing drug nocodazole (Lieuvin et al., 1994). We have assayed nocodazole sensitivity to test the ability of soluble cytosolic extracts to reconstitute (at least partly) microtubule dynamics in permeabilized NIH3T3 cells. For this we have assayed nocodazole-induced depolymerization of microtubules in permeabilized cells supplemented with such extracts. Permeabilized cells were incubated with NIH3T3 cell extracts supplemented with 5 μM pure tubulin, and were then stained with tubulin antibody and examined by immunofluorescence microscopy. After adding cell extracts, interphase microtubule arrays conserved apparently normal morphology (Fig. 1 *A*) and remained insensitive to 20 μM nocodazole (Fig. 1 *B*). When cell extracts were supplemented with an ATP-regenerating system as described in Pirollet et al. (1987) and added to permeabilized cells, soluble tubulin from the extracts polymerized onto the preexisting interphase microtubules, as shown by the emergence of long microtubule extensions outward from the boundaries of the interphase arrays (Fig. 1 *C*). Adding 20 μM nocodazole to extracts precluded such mi-

1. *Abbreviations used in this paper*: CD, colchicine–tubulin dimer complexes; cdk, cyclin-dependent kinase; TTL, tubulin tyrosine ligase.

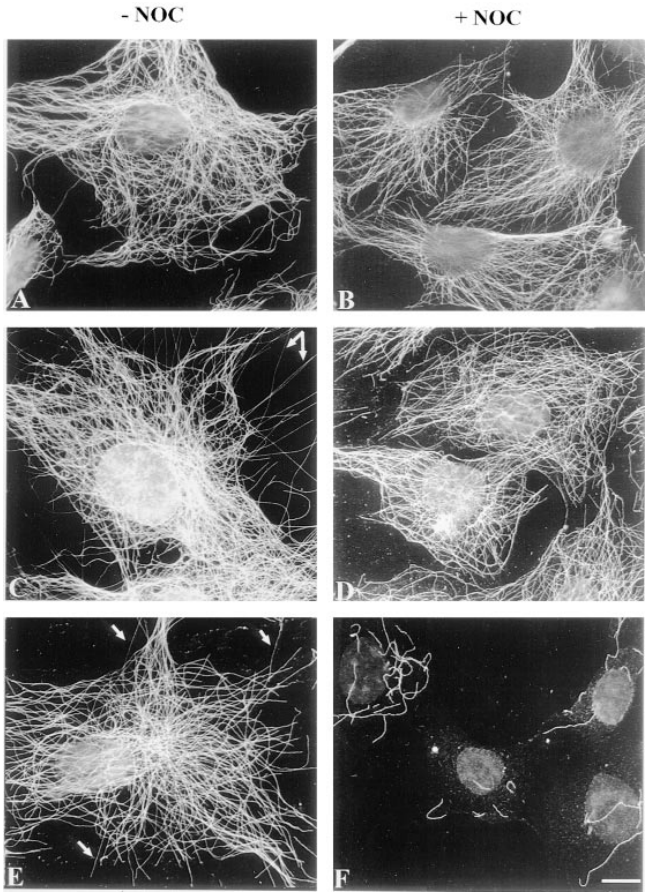

Figure 1. Reconstitution of microtubule sensitivity to nocodazole action in permeabilized NIH 3T3 cells. (*A–F*) Immunostaining of interphasic permeabilized NIH3T3 cells with tubulin antibody mAb YL1/2. Cells were permeabilized and then incubated for 30 min at 34°C with NIH3T3 cell extracts supplemented with 5 μM pure tubulin in the presence of: (*A*) no addition; (*B*) 20 μM nocodazole (*Noc*); (*C*) ATP-regenerating system; (*D*) ATP-regenerating system and 20 μM nocodazole; (*E*) ATP-regenerating system and 5 μM okadaic acid; and (*F*) ATP-regenerating system, 5 μM okadaic acid, and 20 μM nocodazole. Arrows show microtubule extensions arising from the polymerization of cell extract tubulin at the ends of interphase microtubules. Bar, 10 μm.

crotubule extensions, while the preexisting interphase microtubules remained insensitive to the drug (Fig. 1 *D*).

Since microtubule stability in permeabilized cells is probably related to the phosphorylation state of microtubule-stabilizing proteins (Lieuvin et al., 1994), we tested the effect on microtubule drug stability of adding back cell extracts supplemented with both an ATP-regenerating system and the protein phosphatase inhibitor okadaic acid. When permeabilized cells were incubated with such supplemented extracts (OA cell extracts), tubulin from the extracts still polymerized from the ends of interphase microtubules (Fig. 1 *E*). However, adding nocodazole to extracts under the same conditions induced almost complete disassembly of the original interphase microtubule network. In the presence of this drug combination, only centrioles and a few surviving polymers were evident (Fig. 1 *F*). Similar results were observed using microcystin instead of okadaic acid as the protein phosphatase inhibitor. In both cases extensive microtubule depolymerization occurred after 15 min of cell exposure to nocodazole, and was almost complete after 30 min in the drug (data not shown). Control experiments showed absence of effect of okadaic acid in the absence of an ATP regenerating system (data not shown).

These results show that the sensitivity of the interphase microtubule array to nocodazole can be restored in permeabilized cells by adding soluble cell extracts (OA cell extracts) under conditions that simultaneously favor protein phosphorylation (ATP regenerating system) and inhibit protein dephosphorylation (protein phosphatase inhibitors).

Nocodazole Induces Microtubule Depolymerization Through Inactivation of Tubulin Dimers

Although the precise mechanism by which nocodazole blocks tubulin polymerization remains unclear, the simplest possibility is that nocodazole associates with tubulin dimers and renders them incompetent for assembly (Vasquez et al., 1997). To test this possibility, permeabilized NIH3T3 cells were reconstituted with OA cell extracts (Fig. 2 *A*) in the presence of either 20 μM nocodazole or of a mixture containing both 20 μM nocodazole and 20 μM tubulin dimers. Adding the drug alone induced extensive interphase microtubule depolymerization in the reconstituted cells (Fig. 2 *B*). In contrast, microtubules in cells incubated with OA cell extracts supplemented with stoichiometrically balanced amounts of nocodazole and tubulin remained intact (Fig. 2 *C*). These results indicate that no-

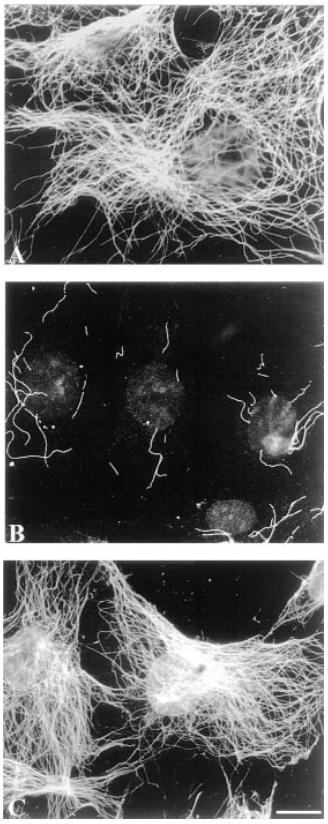

Figure 2. Analysis of the mechanism of nocodazole action. (*A–C*) Immunostaining of interphasic permeabilized NIH3T3 cells with tubulin antibody mAb YL1/2. Cells were permeabilized and then incubated for 30 min at 34°C with NIH3T3 cells extracts supplemented with 5 μM pure tubulin in the presence of: (*A*) ATP-regenerating system and 5 μM okadaic acid; (*B*) ATP-regenerating system, 5 μM okadaic acid and 20 μM nocodazole; and (*C*) ATP-regenerating system, 5 μM okadaic acid, 20 μM nocodazole, and 20 μM tubulin. Bar, 10 μm.

codazole induces microtubule depolymerization by decreasing the concentration of assembly-competent tubulin dimers, and that nocodazole–tubulin complexes have no detectable depolymerizing effect. We conclude that in the presence of nocodazole, microtubule disassembly is driven by the intrinsic disassembly properties of microtubule ends.

Comparison of Colchicine and Nocodazole Effects in Reconstituted Permeabilized Cells Shows that Nocodazole-induced Disassembly Proceeds from Microtubule Plus Ends

Nocodazole-sensitive microtubules in reconstituted permeabilized cells might be disassembling at their plus ends, their minus ends, or at both ends. We used colchicine as a probe to distinguish between these possibilities, and thus determine the relative contributions of microtubule plus ends and of microtubule minus ends in the observed nocodazole-induced disassembly. Colchicine forms stable complexes with free tubulin dimers (CD complexes; Margolis and Wilson, 1977). These CD complexes apparently cap and stabilize microtubule plus ends while allowing minus end–directed microtubule disassembly to continue unimpeded (Margolis and Wilson, 1977; Margolis et al., 1980; Farrell and Wilson, 1984). Therefore, CD complex addition to cell extracts was expected to inhibit plus end–dependent microtubule assembly/disassembly events and to allow a specific assay of microtubule minus end properties. We tested to see whether plus end capping by CD complex addition occurred in reconstituted permeabilized cells as in purified microtubule preparations. For this, permeabilized cells were incubated with isolated CD complexes, washed, and then incubated with cell extracts (Fig. 3). Assembly of tubulin from the extract onto the peripheral ends of such CD-treated polymers was then monitored. For these experiments we used cells and extracts containing distinct tubulin isotypes (Fig. 3). Permeabilized NIH3T3 cells were incubated with extracts from TTL^- cells supplemented with 5 μM purified Glu-tubulin and an ATP regenerating system. TTL^- cells are devoid of a tubulin-modifying enzyme, tubulin tyrosine ligase (Ersfeld et al., 1993), which normally maintains a tyrosine residue on the COOH terminus of α-tubulin (MacRae, 1997). Despite the lack of TTL, microtubule turnover is apparently normal in TTL^- cells (Lafanechère et al., 1998). The tubulin in TTL^- cells is extensively detyrosinated (Lafanechère et al., 1998), and contains a COOH-terminal glutamate residue (Glu-tubulin). In contrast, the microtubule array in the permeabilized NIH3T3 cells consisted largely of tyrosinated tubulin (Tyr-tubulin). Isoform-specific antibodies clearly distinguish between Tyr-tubulin microtubules and Glu-tubulin microtubules (Gundersen et al., 1984). Using this system, we found that the minimum concentration of CD complex able to inhibit extension of microtubules in the permeabilized cells was 50 nM (data not shown). Further experiments were run using either 5 μM free colchicine or 5 μM CD complex (100× excess colchicine compared with the minimum inhibitory concentration).

When permeabilized cells were treated with CD complex before incubation with TTL^- cell extracts, no polymerization of tubulin at interphase microtubule ends occurred (Fig. 3, *C* and *D*), suggesting that CD complexes had stably poisoned microtubule assembly ends. In control experiments, permeabilized cells were exposed to a mixture of 5 μM BSA and 5 μM colchicine before incubation with cell extracts. Under these conditions, no detectable inhibition of the assembly of tubulin from the cell extracts

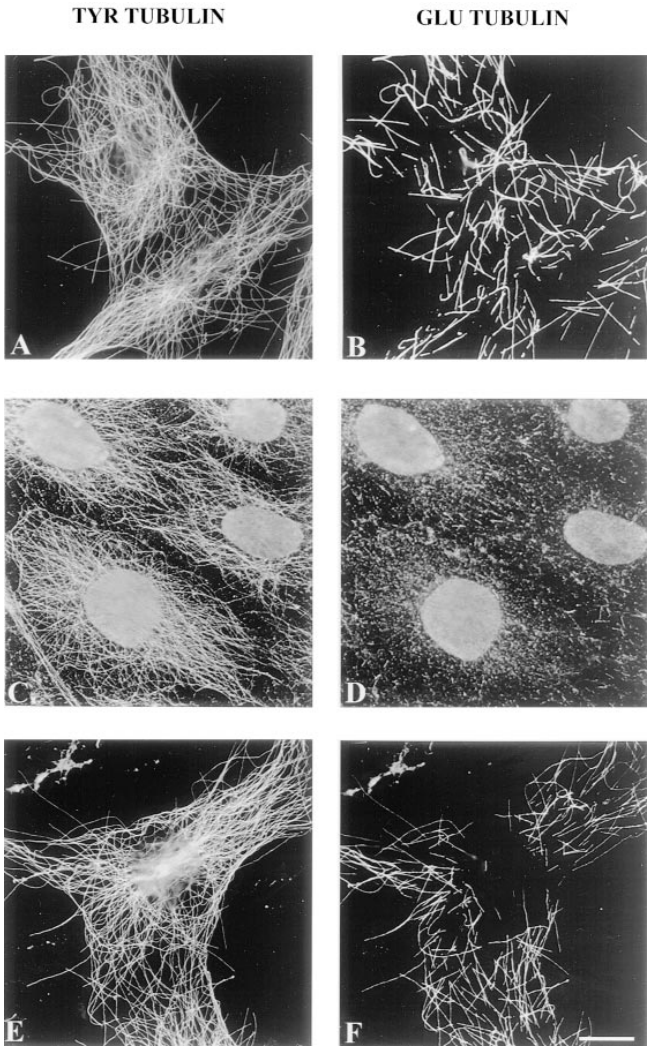

Figure 3. Persistent poisoning of microtubule assembly by CD-complex in reconstituted permeabilized cells. (*A–F*) Double immunostaining of permeabilized NIH3T3 cells using Tyr-tubulin antibody YL1/2, and Glu-tubulin antibody. Permeabilized NIH3T3 cells were incubated for 5 min at 34°C with TTL^- cell extracts supplemented with ATP-regenerating system, and with 5 μM purified Glu-tubulin after a 15-min preincubation at the same temperature under the following conditions: (*A–B*) permeabilization buffer alone; (*C–D*) 5 μM CD complex; (*E–F*) 5 μM colchicine plus 5 μM BSA. In the absence of cell preincubation with CD complex, the tubulin from extracts formed microtubule extensions (*B* and *F*) whose formation is extensively inhibited in cells preincubated with CD complexes (*C* and *D*). Note that these extensions are stained by Tyr-tubulin antibody due to the presence of low levels of Tyr-tubulin in TTL^- cell extracts (*A* and *E*), and that inhibition of tubulin assembly by CD complex induces some background staining, including nonspecific nuclear staining (*C* and *D*). Bar, 10 μm.

occurred (Fig. 3, *E* and *F*). These results ruled out the possibility that the persistent poisoning of microtubule assembly after incubation with CD complexes was due to contamination of cell extracts with remaining free colchicine. These results strongly suggest that CD complexes cap interphase microtubule plus ends in reconstituted permeabilized cells, as previously observed in purified microtubule preparations (Margolis and Wilson, 1977; Skoufias and Wilson, 1992).

We then used CD complexes to test microtubule minus end activity in permeabilized cells supplemented with OA cell extracts. When permeabilized cells were incubated with such extracts in the presence of 5 μM CD complex, microtubule disassembly was minimal (Fig. 4, *A* and *B*). These results suggested that microtubule minus ends in permeabilized cells did not noticeably activate in the presence of OA cell extracts. However, it remained an alternative formal possibility that CD complexes also stabilized microtubule minus ends in cell extracts. To test this possibility, microtubules were assembled in vitro by incubation of cell extracts at 30°C. These free microtubules were then incubated with 5 μM CD complex. After 30 min, the free microtubules had depolymerized, showing that CD complexes did not stabilize the minus ends of free microtubules to disassembly (Fig. 4, *C* and *D*).

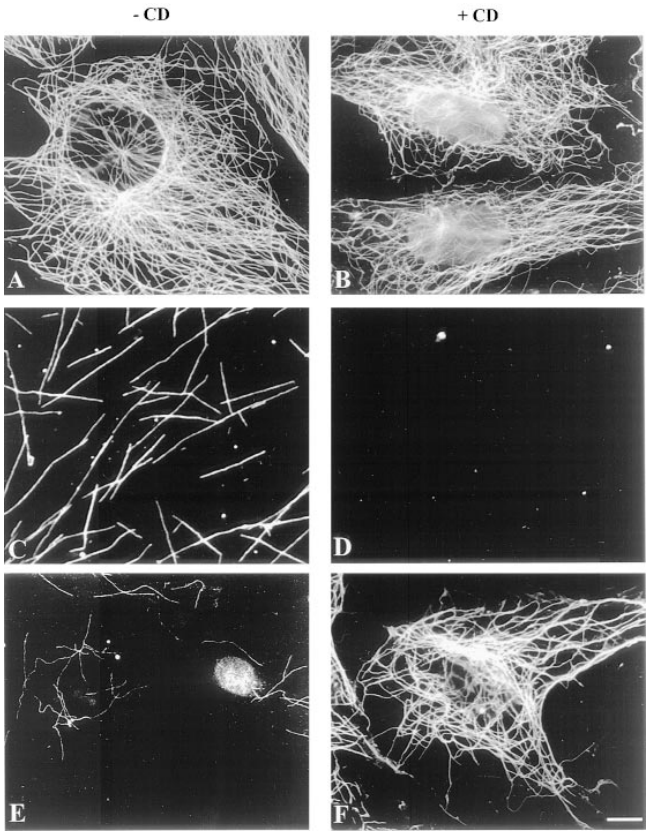

Figure 4. Inhibition of nocodazole action in the presence of added CD complex. (*A–B*) Immunostaining of interphasic permeabilized NIH3T3 cells with tubulin antibody mAb YL1/2. Cells were permeabilized and then incubated for 30 min at 34°C with NIH3T3 cell extracts supplemented with 5 μM pure tubulin, ATP-regenerating system and 5 μM okadaic acid in the absence of CD complex (*A*), or in the presence of 5 μM CD complex (*B*). (*C–D*) Immunofluorescence analysis of the microtubule content of control (*C*) or CD complex–supplemented (*D*) cell extracts. Cell extracts supplemented with 10 μM tubulin and ATP-regenerating system were incubated for 30 min at 30°C. Aliquots were then incubated for 30 min in the absence (*C*) or presence of 5 μM CD complex (*D*). Microtubules were then cross-linked, spun onto coverslips, and immunostained as described in Saoudi et al. (1995). (*E–F*) Immunostaining of interphase-permeabilized NIH3T3 cells with tubulin antibody mAb YL1/2. Cells were permeabilized and then incubated for 30 min at 34°C with NIH3T3 cell extracts supplemented with 5 μM pure tubulin, ATP-regenerating system, 5 μM okadaic acid, and 20 μM nocodazole in the absence of CD complex (*E*) or the presence of 5 μM CD complex (*F*). Bar, 10 μm.

These results suggested that nocodazole induces plus end–directed microtubule disassembly in the absence of minus-end dynamics. In this case one would expect to observe inhibition of nocodazole action in the presence of added CD complex. To test this possibility, permeabilized cells were supplemented with OA cell extracts containing either nocodazole alone or a mixture of nocodazole and CD complex. Microtubule arrays in nocodazole-treated cells exhibited complete microtubule disassembly, whereas adding CD complex together with nocodazole resulted in marked inhibition of microtubule disassembly (Fig. 4, *E* and *F*). In control experiments, permeabilized cells were reconstituted with OA cell extracts that were supplemented with a mixture of nocodazole and free colchicine. In this case, no detectable inhibition of nocodazole-induced depolymerization was observed (data not shown) showing the specificity of the activity of the CD complex as compared with free colchicine.

Taken together, these results suggest that in permeabilized cells supplemented with OA cell extracts, the observed reconstitution of microtubule sensitivity to nocodazole involves cell regulatory factors that affect microtubule plus ends, whereas microtubule minus ends remain inactive.

The Nocodazole-sensitive Microtubules in Permeabilized Cells Reconstituted with OA Cell Extracts Do Not Show Significant Subunit Turnover

We tested to see whether microtubule plus end dynamics in permeabilized cells reconstituted with OA cell extracts were sufficient to induce complete subunit turnover in the microtubule array. For this test, we incubated permeabilized NIH3T3 cells with interphase OA cell extracts from TTL^- cells and assayed the progressive replacement of the original interphase Tyr-microtubule array by Glu-microtubules composed of tubulin from the extracts. To optimize conditions for visualization of possible microtubule turnover through plus end excursions, cell extracts were supplemented with various concentrations of pure tubulin (1–10 μM). We find addition of 1 μM tubulin to cell extracts to be just sufficient to promote limited tubulin assembly on interphase microtubule ends, but not sufficient to sustain continuous microtubule growth during the time course of the experiment. Since dynamic instability is inhibited in growing microtubules (Carlier, 1991), this protocol was designed to optimize possible plus end excursions. After different periods of incubation in the presence of OA cell extracts, permeabilized cells were fixed and double-labeled with Glu- and Tyr-tubulin antibodies.

Results showed that after a 30-min incubation, the interphase networks remained almost entirely composed of Tyr-tubulin (Fig. 5 *A*). Quantification of the amount of Tyr-tubulin microtubules left in interphase microtubule arrays during the time course of the experiment showed only a slight decrease in fluorescence signal (Fig. 5 *D*). Direct observation of cells suggested that this decrease could be accounted for by some diffuse detyrosination of the interphase network, which may have resulted from residual carboxypeptidase activity present in the cell extract. The Glu-tubulin from the extracts formed short tails on the ends of interphase microtubules, but did not invade the interphase microtubule array, revealing the apparent absence of wholesale microtubule turnover under these conditions (Fig. 5 *B*). The Glu-tubulin tails were first evident after 5 min of incubation, and thereafter their average length remained apparently constant (data not shown). These tails may represent length fluctuations on Tyr-microtubule plus ends. If there are fluctuations, our results show that such putative microtubule plus end excursions are not of sufficient amplitude to induce disassembly of the initial microtubule network. As a control of the activity of OA cell extracts, cells were incubated under identical conditions, but in the presence of nocodazole. In the presence of nocodazole, microtubule arrays completely depolymerized during 30 min of incubation (Fig. 5 *C*). We also performed turnover experiments using higher tubulin concentrations. When a high concentration of tubulin was used, long tails formed without invasion into the body of the initial array (data not shown). In the absence of added tubulin, results varied with the extract used. Results were either identical to the results shown in Fig. 5, or original interphase microtubules disassembled during the time course of the experiments, and no polymerization of the Glu-tubulin derived from extracts was observed (data not shown). This variability presumably reflected small variations in the tubulin content of the different extracts. We conclude that adding OA cell extracts to permeabilized interphase cells induces an intermediate state of microtubule activation. Only microtubule plus ends are affected. The observed activation of microtubule plus ends is sufficient to induce microtubule sensitivity to nocodazole, but not sufficient to induce significant microtubule turnover. Obviously, OA cell extracts lack important regulatory activities which are indispensable for the induction of microtubule turnover.

Adding Xenopus Egg Extracts to Permeabilized Cells Induces Rapid Microtubule Turnover Involving Microtubule Fragmentation and Microtubule Disassembly at Minus Ends

Before initiating a systematic search for additional factors that may reconstitute microtubule turnover in permeabilized cells supplemented with OA cell extracts, we needed to determine whether such reconstitution was actually possible using other cell extracts. While mammalian cell extracts may lack some activities present in intact cells, *Xenopus* egg extracts are generally competent to reconstitute complex cell functions (Murray and Kirschner, 1989; Murray et al., 1989; Belmont et al., 1990; Verde et al., 1990). To assay the ability of *Xenopus* egg extracts to reconstitute interphase microtubule turnover, we used permeabilized TTL$^-$ cells and *Xenopus* egg extracts converted to interphase extracts according to Murray and Kirschner (1989). The original microtubule arrays in these

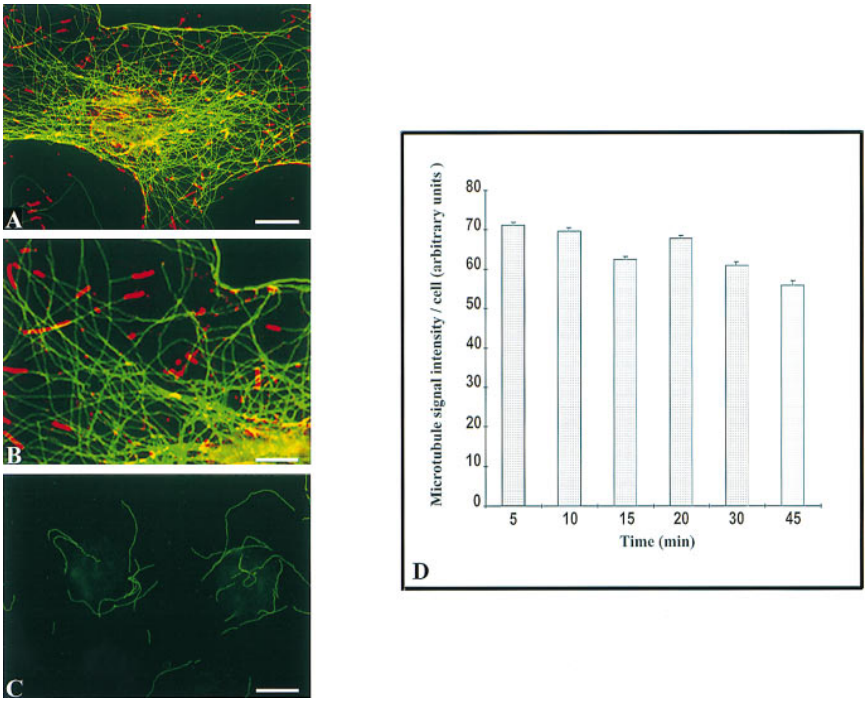

Figure 5. Assay of microtubule turnover in permeabilized NIH3T3 cells reconstituted with mammalian interphase cell extracts. Permeabilized NIH 3T3 cells were incubated at 34°C with TTL$^-$ cell extracts containing 1 μM purified Glu-tubulin, ATP-regenerating system, and 5 μM okadaic acid. (*A–B*) Double immunostaining of interphase microtubule arrays in permeabilized cells with Tyr-tubulin monoclonal antibody YL1/2 (*green*) and Glu-tubulin antibody (*red*) after a 30-min incubation with cell extracts. (*A*) The tubulin from the extract forms short tails on preexisting interphase microtubules, but fails to invade the interphase network. (*B*) 2× enlargement of a peripheral zone of the cell showing the Glu-tubulin tails at the ends of Tyr-microtubules. (*C*) Immunostaining of interphase permeabilized NIH3T3 cells with tubulin antibody mAb YL1/2. Cell extracts were supplemented with 20 μM nocodazole; this control experiment shows that microtubules were nocodazole-sensitive during microtubule turnover assay. Bars: (*A* and *C*) 10 μm; (*B*) 5 μm. (*D*) Quantitative analysis of microtubule turnover. Cells were incubated with TTL$^-$ cell extracts as described in *A*. At the indicated time points, cells were fixed and stained with Tyr-tubulin antibody, and the amount of Tyr-tubulin in interphase microtubule networks was quantified as described in Materials and Methods.

permeabilized cells were therefore composed of Glu-tubulin, whereas *Xenopus* egg extracts contained Tyr-tubulin. The complete distinction between the tubulin molecules comprising the permeabilized cell interphase networks and the tubulin derived from the *Xenopus* egg extracts was crucial to successful analysis of the experiments reported below.

Microtubule turnover involves disassembly events (permitting original microtubule interphase networks to disappear) as well as assembly events (permitting the concomitant growth of extensive microtubule arrays from the tubulin of the soluble pool). In classical microtubule turnover experiments using microinjection of fluorescent tubulin into cells (Schulze and Kirschner, 1986), the disassembly of the original interphase network cannot be followed. After introducting fluorescent tubulin, only net growth events and total tubulin assembly can be scored. In contrast, the methodology in the present study permits us to monitor disassembly of the original interphase microtubules by following the Glu-tubulin staining. Simultaneously, we can monitor microtubule assembly in the Tyr-tubulin channel.

When permeabilized cells were incubated with *Xenopus* egg extracts and double-stained with Glu- and Tyr-tubulin antibodies, the Glu-tubulin network (representing the original interphase microtubule network) showed dramatic disassembly during a 15-min incubation (Fig. 6, *A*, *C*, and *E*), and was replaced by an extensive array of Tyr-tubulin microtubules. Since Tyr-tubulin can only be generated on unassembled dimers (MacRae, 1997), this array must have derived from the soluble tubulin pool, not from tyrosination of polymeric Glu-tubulin. These newly formed Tyr-microtubules assembled both on the ends of preexisting microtubules and at centrosomes (Fig. 6, *B*, *D*, and *F*), as in classical turnover experiments performed in intact cells (Schulze and Kirschner, 1986). These results demonstrate that reconstitution of rapid microtubule turnover in permeabilized cells is possible when they are supplemented with *Xenopus* egg extracts. Microtubule turnover in such permeabilized cells apparently involved microtubule fragmentation: at intermediate stages during microtubule turnover involving the disassembly of the original Glu-microtubule network, Glu-microtubule fragments were visible (Fig. 6 *C*). This apparent microtubule fragmentation suggested that, in contrast with OA cell extracts that only effect microtubule plus ends, *Xenopus* egg extracts may contain a severing activity, perhaps katanin (McNally and Vale, 1993), able to induce microtubule fragmentation and creation of active microtubule minus ends. In the presence of such activity, microtubule turnover could be (for a large part) induced by concomitant minus end–directed microtubule disassembly and plus end microtubule assembly. Similar severing-dependent turnover has been recently described in intact cells (Keating et al., 1997; Waterman-Storer and Salmon, 1997). However, before such a conclusion could be reached, one had to eliminate the possibility of artefactual microtubule breakage during experimental manipulation of the permeabilized cells, and also show that microtubule minus ends were truly the main sites of microtubule disassembly during microtubule turnover.

We used time-lapse microscopy to monitor the fate of

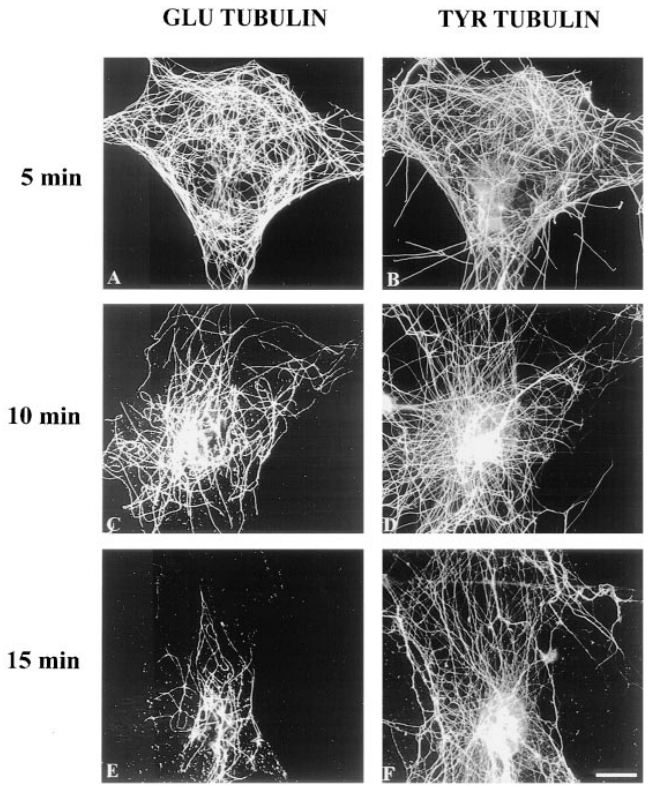

Figure 6. Reconstitution of microtubule turnover in permeabilized cells supplemented with *Xenopus* egg extracts. Permeabilized TTL$^-$ cells were incubated at 34°C with interphase *Xenopus* egg extracts for the indicated periods of time. Cells were then double-stained using Glu-tubulin antibody (*A*, *C*, and *E*) and Tyr-tubulin mAb YL1/2 (*B*, *D*, and *F*). Within 15 min, most of the original interphase microtubules had depolymerized. At intermediate stages of microtubule turnover, Glu-microtubule fragments, apparently detached from the centrosome, were visible (*C*). The Tyr-tubulin from the soluble tubulin pool initially formed tails and then invaded the whole interphase network (*B*, *D*, and *F*). Image superposition showed that microtubule tails grew on the ends of preexisting microtubules (data not shown). Bar, 10 μm.

Glu-microtubules in permeabilized cells supplemented with *Xenopus* egg extracts. Time-lapse microscopy permitted continuous observation in the absence of possible artefactual mechanical breakage of microtubules due to experimental manipulation. For this assay, the original interphase microtubules in permeabilized TTL$^-$ cells were labeled with Glu-tubulin antibody without fixation, and then the permeabilized interphase cells were incubated with interphase *Xenopus* egg extracts. The pathway of disassembly of the original interphase microtubules was monitored either by taking successive pictures or by continuous videomicroscopy recording of the same cell during the course of incubation.

Three successive images of the same cell examined over a period of 5 min are shown in Fig. 7 *A*. Fragmentation of the original Glu-microtubules was evident in successive frames. Videomicroscopy images of interphase microtubules over a 20-s period in another cell showed the appearance of several sites of apparent microtubule severing

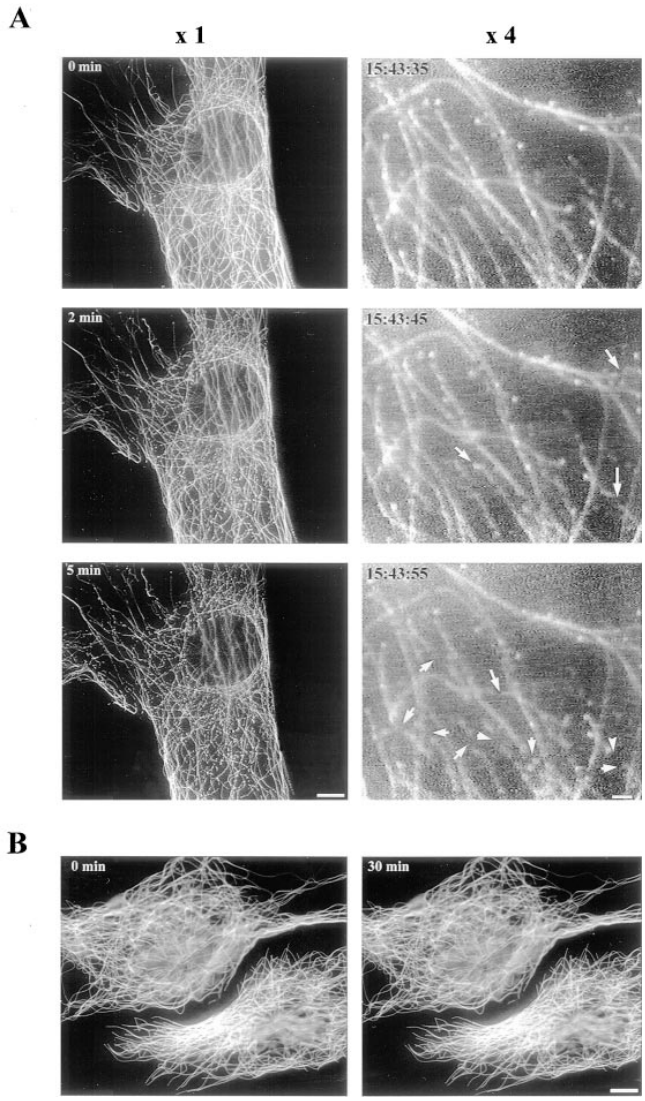

Figure 7. Reconstitution of microtubule turnover in permeabilized cells involves disassembly of fragmented microtubules. TTL⁻ permeabilized cells were labeled with Glu-tubulin antibody and secondary Cy 3 goat anti–rabbit IgG antibody in warm permeabilization buffer without cell fixation. Cells were washed with the same buffer, and then the antibody-labeled cells were incubated with interphase *Xenopus* egg extracts (*A*) or with NIH3T3 cell extracts supplemented with 1 μM purified Tyr-tubulin, ATP-regenerating system, and 5 μM okadaic acid (*B*). (*A*; *left*) Successive images of the same cell were taken at the indicated time points to visualize the pathway of disassembly of the original interphase Glu-microtubules in the presence of *Xenopus* egg extracts. Images showed microtubule fragmentation and segmental disassembly. Bar, 5 μm. The zero time point corresponds to the beginning of observation and not to the beginning of incubation (~5-min difference). Therefore, this time point differs from the zero time point in Fig. 6 (fixed cell experiments). (*A*; *right*) Videomicroscopic images (4× enlarged) of disassembling original Glu-interphase microtubules. A randomly selected part of a permeabilized TTL⁻ cell labeled with Glu-antibody and reconstituted with *Xenopus* egg extracts was observed using videomicroscopy over a period of 20 s. Successive images showed apparent microtubule breakage followed by variable extents of microtubule depolymerization. Arrows show microtubule breakage events. Zero time point is as above. Bar, 100 nm. (*B*) Control experiment showing apparent absence of microtubule severing in permeabilized TTL⁻ cells incubated with soluble NIH3T3 cell extracts. Bar, 5 μm.

within this time course (Fig. 7 *A*). Control experiments were run to eliminate the possibility that the observed images of microtubule severing may result from artefacts such as unequal microtubule decoration with antibody or segmental dissociation of the antibody. For this, permeabilized TTL⁻ cells were supplemented with OA cell extracts instead of *Xenopus* egg extracts, and were examined using time-lapse videomicroscopy as above. As shown above, neither microtubule turnover nor microtubule severing is detected in permeabilized cells supplemented with OA cell extracts. Accordingly, no images of microtubule severing occurred during video microscopy examination of such cells (Fig. 7 *B*).

Time-lapse microscopy did not have enough resolution to determine whether severed microtubules disassembled from their plus ends or from their minus ends, or from both ends during microtubule turnover. We used CD complexes to discriminate between these different possibilities. Interpretation relies on the hypothesis that CD complexes interact with cytoplasmic microtubules of permeabilized cells supplemented with *Xenopus* egg extracts in a manner similar to that observed on microtubules in cells supplemented with mammalian cell extracts. Given the essential irreversibility of tubulin–colchicine interactions (Margolis et al., 1977) and the identical activity of CD complexes both in purified systems and in cell extracts (see above), the alternative possibility, that CD complexes have a different activity in the presence of *Xenopus* egg extracts, is quite remote. As shown above, CD complexes block microtubule plus ends. Adding CD complexes to *Xenopus* egg extracts and subsequent monitoring of microtubule disassembly therefore assays minus end–directed microtubule disassembly. When permeabilized cells were incubated in the presence of added CD complex, Glu-microtubules exhibited rapid and complete depolymerization (Fig. 8 *A*). The time course of Glu-microtubule disassembly during exposure to CD complex was similar to the time course of Glu-microtubule disassembly during microtubule turnover. Rapid microtubule disassembly also occurred in time-lapse videomicroscopy experiments involving antibody-decorated Glu-microtubules (Fig. 8 *B*), suggesting absence of influence of antibody decoration on microtubule turnover. In these video microscopy experiments, Glu-microtubule fragmentation was evident. These results are in striking contrast to those above (Fig. 3), where only microtubule plus ends were affected. Taken together, these results suggest that microtubule minus ends are the principal sites of microtubule depolymerization during the microtubule turnover observed in permeabilized cells supplemented with *Xenopus* egg extracts. Thus, such turnover is the result of net minus end depolymerization balanced by net polymerization at microtubule plus ends, with new assembly at centrosomes.

Microtubule Turnover in Intact Cells

Xenopus egg extracts reconstitute active microtubule turnover in permeabilized cells. As the addition of *Xenopus* egg extracts to permeabilized NIH3T3 cells creates an interaction between two heterologous systems, one may reasonably question whether conclusions drawn from such experiments can be extrapolated to intact cells. Addition-

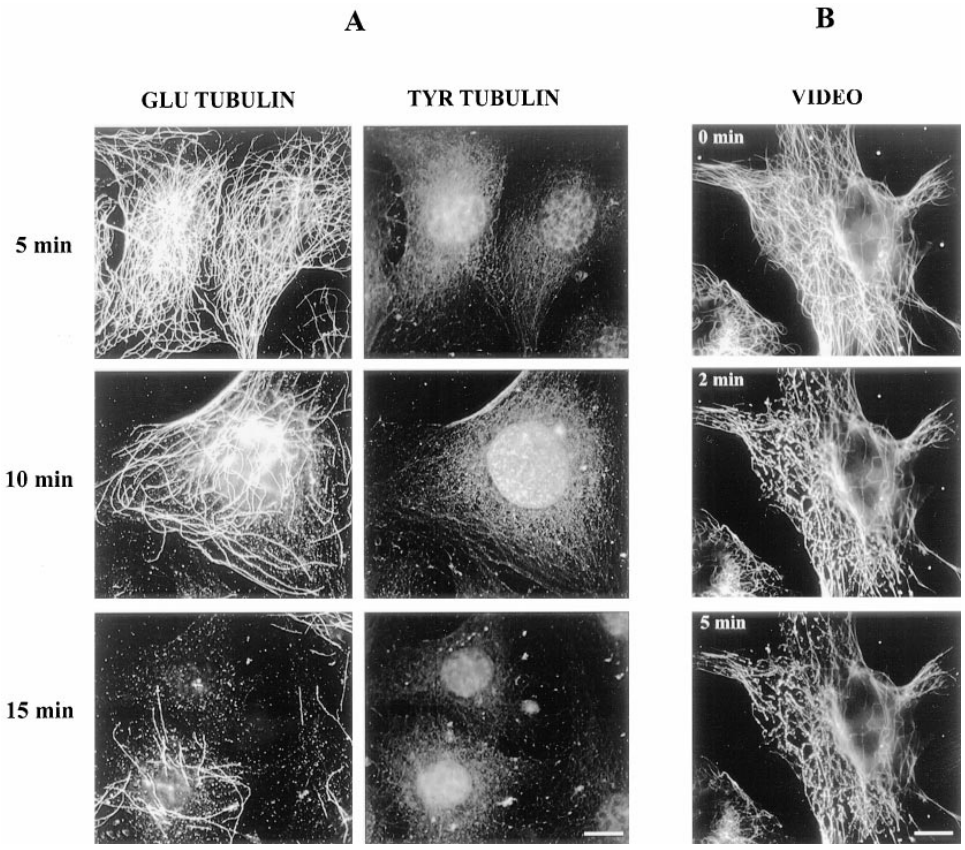

Figure 8. Microtubule turnover in permeabilized cells reconstituted with Xenopus egg extracts involves minus end directed depolymerization. (*A*) Permeabilized TTL⁻ cells were incubated at 34°C with *Xenopus* interphase egg extracts supplemented with 5 μM CD complex. At the indicated times, cells were double-labeled using Glu-tubulin antibody (*left*) and Tyr-tubulin monoclonal antibody YL1/2 (*right*). Within 15 min, most interphase microtubules had depolymerized. No polymerization of the tubulin from the extracts was observed in the presence of CD complex (*right*), showing blockage of microtubule assembly by CD complexes. Bar, 10 μm. (*B*) Videomicroscopy images of disassembling original interphase Glu-microtubules. Interphase microtubules of TTL⁻ permeabilized cells were labeled with Glu-tubulin antibody and secondary Cy3 conjugated goat anti–rabbit IgG antibody in warm permeabilization buffer without cell fixation. Cells were washed with the same buffer, and then the antibody-labeled cells were incubated with interphase *Xenopus* egg extracts supplemented with 5 μM CD complex. The pattern and kinetics of Glu-microtubule disassembly were similar to the ones observed during microtubule turnover (see Fig. 7 *A*). This result suggests that disassembly proceeds mainly from microtubule minus ends, during turnover (see text). Bar, 10 μm.

ally, in the videomicroscopy experiments reported above, microtubule turnover was tested in the presence of Glu-tubulin antibody. Although results were at least qualitatively similar in the presence (time-lapse microscopy) or absence (fixed cell experiments) of antibody, antibody decoration of microtubules may modify microtubule behavior. Therefore, we tested whether we could transpose the methods used in vitro (e.g., antigenic separation of assembling and disassembling polymers) to intact cells, and thus test at the whole cell level the validity of the conclusions drawn from in vitro experiments.

To achieve antigenic separation of polymer and dimer tubulin at time zero in intact cells, TTL⁻ cells were microinjected with concentrated tubulin-tyrosine-ligase (TTL). TTL only tyrosinates tubulin dimers, not microtubules (MacRae, 1997). We have verified, using double Glu/Tyr-tubulin antibody labeling of microinjected cells, that within 1 min after TTL microinjection, free tubulin dimers were composed of Tyr-tubulin while interphase microtubules remained detyrosinated (data not shown).

Using this protocol, we found that microtubule turnover was complete within 15 min (Fig. 9). At all steps of microtubule turnover, the Glu-microtubules (representing the remnants of the original interphase microtubule network) and the Tyr-microtubules (representing the newly assembled polymers) formed two distinct arrays, illustrating complete antigenic segregation of the initial polymer and dimer tubulin pools achieved by TTL microinjection. The pattern of Tyr-tubulin assembly (Fig. 9, *A*, *C*, *E*, and *G*) was identical to that of fluorescent tubulin in classical microinjection experiments (Schulze and Kirschner, 1986). After 1 min, Tyr-tubulin tails were formed on preexisting microtubules, and new Tyr-polymers were also nucleated at the centrosome. At later steps of microtubule turnover, Tyr-microtubules formed regular radial arrays of microtubules arising from the centrosomes, and finally invaded the whole interphase network. Most interestingly, during microtubule turnover, the original Glu-microtubule array disappeared in a centrifugal manner. Glu-microtubules evolved toward a set of microtubule fragments mainly located in the periphery of the cell, and often clearly not connected to the centrosome. The difference between the centrosome centered Tyr-microtubule network and the peripheral Glu-microtubule network in the same cell was clear-cut (Fig. 9, *C* and *D*). These results suggest that during microtubule turnover, microtubules initially nucleated on centrosomes detach from centrosomes, and through apparent minus end–directed disassembly evolved toward fragments in the cell periphery. This was the behavior expected, provided microtubule fragmentation and treadmilling is an important mechanism of microtubule turnover in intact cells. Microtubule severing was less evident

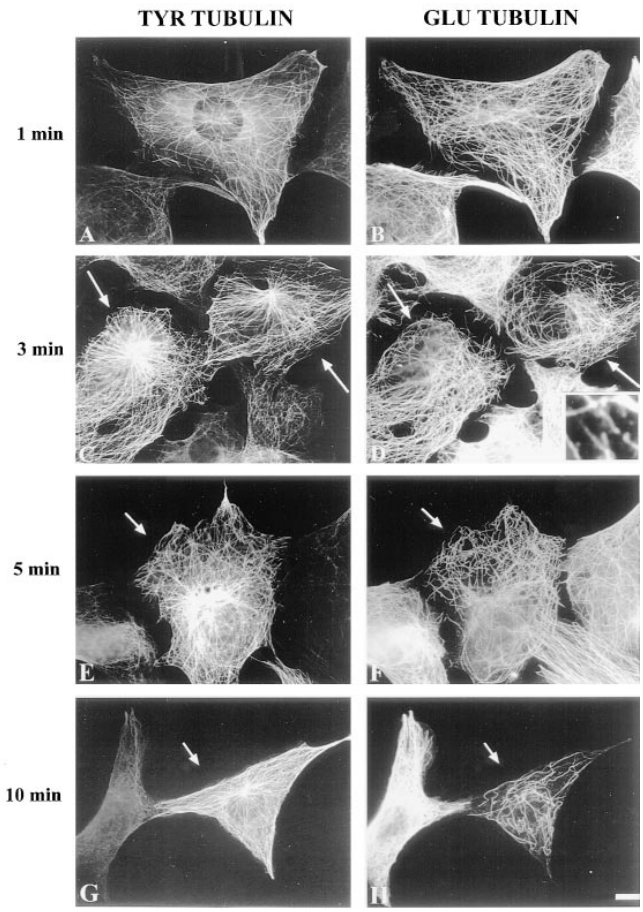

Figure 9. Microtubule turnover in intact cells. TTL⁻ cells were microinjected with pure TTL. At the indicated time points after microinjection, cells were fixed and double-labeled using Tyr-tubulin monoclonal antibody YL1/2 (*A*, *C*, *E*, and *G*) and Glu-tubulin antibody (*B*, *D*, *F*, and *H*). Arrows indicate microinjected cells. During microtubule turnover, Tyr-microtubules formed radial arrays arising from centrosomes (*C*, *E*, and *G*), whereas Glu-microtubules appeared as fragments detached from centrosomes not centered as Tyr-microtubules (*D*, *F*, and *H*). (*D*, *insert*) Image of a severed microtubule (5×). Bar, 10 μm.

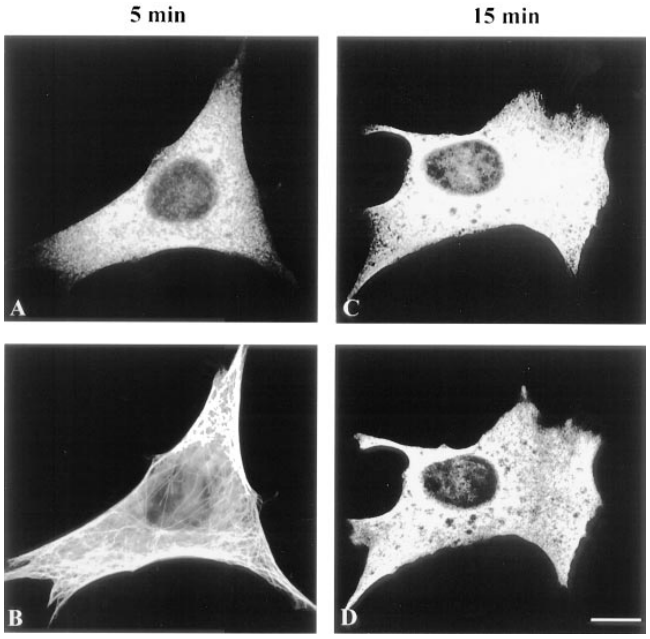

Figure 10. Microinjection of CD complex in intact cells induces rapid microtubule depolymerization. NIH3T3 cells were injected with a mixture of nonreactive rabbit IgGs and with 25 μM CD complex. Cells were double-labeled using goat rabbit IgG antibody (*A* and *C*) and Tyr-tubulin monoclonal antibody YL1/2 (*B* and *D*). After 5 min, background staining of depolymerized free tubulin was evident in microinjected cells (*A* and *B*), and microtubule depolymerization was complete after 15 min (*C* and *D*). Bar, 10 μm.

and extensive in intact injected cells than in permeabilized cells reconstituted with *Xenopus* egg extracts. This result may be due to the centrosomal localization of severing activity in intact cells (Hartman et al., 1998), while severing activity must be disperse in *Xenopus* egg extracts. However, upon close examination, images of microtubule severing, as shown in the to Fig. 9 *D* (*insert*), could routinely be found throughout the cells in the course of these experiments.

We also used the CD complex plus end block to assay the activity of microtubule minus ends selectively in intact cells as shown above in reconstitution experiments. When cells were microinjected with preformed CD complex, a background of disassembled tubulin was evident after 5 min, and microtubule disassembly was complete within 15 min (Fig. 10). This result suggests that minus end–directed microtubule disassembly does indeed occur in intact cells, and that minus end–directed disassembly is rapid enough to account for the observed rate of microtubule turnover. Taken together, these results suggest that microtubule turnover occurs through similar mechanisms in intact cells and in permeabilized cells supplemented with *Xenopus* egg extracts.

Discussion

Microtubules in intact cells are sensitive to depolymerizing drugs such as nocodazole and colchicine, and show rapid subunit turnover. These properties are lost after cell lysis and dilution of the soluble cell components in large volumes of buffer (Lieuvin et al., 1994). This study provides evidence that stepwise reconstitution of both microtubule sensitivity to drugs and of microtubule turnover can be achieved in permeabilized cells supplemented with various cell extracts. The conclusions of this study are restricted to interphase fibroblast cells. Although the basic mechanisms of microtubule turnover are thought to be general, microtubule turnover also shows cell cycle–specific and cell type–specific variations. Microtubules are considerably more dynamic in mitotic cells than in interphase cells (Saxton et al., 1984; Belmont et al., 1990). Microtubules are also more sensitive to nocodazole (Wadsworth and McGrail, 1990), and are more dynamic in fibroblasts (Shelden and Wadsworth, 1993) than in epithelial cells. Differences in the dynamic instability behavior of microtubules between these cell types have been demonstrated (Shelden and Wadsworth, 1993). Free microtubule minus ends can be

created by polymer breakage in motile cells where microtubules are subjected to bending forces (Waterman-Storer and Salmon, 1997). Furthermore, microtubule turnover is considerably slower in neuronal cells compared with cycling cells (Lim et al., 1989; Takeda et al., 1995; Li and Black, 1996). Many of these cell type–specific variations may be due to the existence of tissue-specific microtubule-associated proteins (Hirokawa, 1994; Denarier et al., 1998; Guillaud et al., 1998) and/or of tissue-specific regulations. Further work should reveal whether cell cycle– and tissue-dependent variations in microtubule turnover can be reproduced in a reconstituted cell system. Such work will be necessary to assess the generality of our observations, and may help identify molecules and mechanisms involved in the regulation of microtubule turnover.

Stepwise Reconstitution of Microtubule Dynamics in Permeabilized Cells

It has previously been demonstrated that the interphase microtubule array remains apparently intact, but is completely devoid of dynamic activity after cell permeabilization (Lieuvin et al., 1994). This inert status probably represents a physiological ground state for interphase microtubules. Such prior evidence had led us to conclude that microtubule dynamics thus probably result from activation of stabilized polymers by soluble factors, including protein-kinases (Lieuvin et al., 1994).

Here we have demonstrated that addback of soluble fibroblast cell extracts or *Xenopus* egg extracts to interphase permeabilized cells restores microtubule sensitivity to depolymerizing drugs and at least one type of microtubule turnover. The response, however, is condition-dependent, and varies with the extract used. Under conditions that favor protein phosphorylation, interphase fibroblast cell extracts restore microtubule sensitivity to nocodazole through activation of plus end dynamics, but do not trigger microtubule minus end dynamics. In the presence of cell extracts, plus end dynamics are not sufficient to generate detectable microtubule turnover. It is possible that adding factors such as catastrophe factor to cell extracts could induce microtubule turnover. In any case, this study suggests the existence of cellular factors that selectively affect microtubule plus end activity without an apparent effect on microtubule minus ends. Permeabilized cells reconstituted with cell extracts provide an experimental system that should facilitate identification of these factors. Some of these factors are undoubtedly protein kinases. The requirement for protein phosphatase inhibitors to reconstitute microtubule sensitivity to nocodazole, demonstrated in the present study, substantiates previous evidence that such protein kinases are centrally involved in regulating microtubule dynamic activity. What could be the nature of these protein kinases? A previous study (Lieuvin et al., 1994) suggested that they may be related to cyclin-dependent kinases (cdks). In agreement with this hypothesis we have observed that inhibitory peptides, mimicking the cdk-dependent consensus phosphorylation site, could block nocodazole-induced microtubule depolymerization in reconstituted permeabilized cells (data not shown). However, our efforts to identify the responsible cdks have not been successful to date. Therefore, although the protein kinase may be a member of or related to the cdk family, the identity of the activating protein kinase remains to be determined.

Microtubule dynamic activity generating rapid turnover of the interphase microtubule array is induced by adding *Xenopus* egg extracts to permeabilized cells. In this case, microtubule severing occurs, and the free microtubule minus ends serve as sites of microtubule depolymerization while tubulin polymerization occurs both at microtubule plus end and at centrosomes. Such a behavior is consistent with recent observations of microtubule turnover through microtubule fragmentation and treadmilling in intact cells (Rodionov and Borisy, 1997). Microtubule severing in cells incubated with *Xenopus* egg extracts is probably caused by katanin (McNally and Vale, 1993). In reconstituted systems, microtubule severing occurs at apparently random sites. Recent work suggests that the situation is different in intact cells. In this case, severing activity is probably targeted to centrosomes (Hartman et al., 1998). Microtubule-severing activity may not be sufficient to induce microtubule depolymerization at minus ends. Previous work has shown that once created by microtubule severing, microtubule minus ends may remain stable for a period of time before entering a phase of depolymerization (Keating et al., 1997; Waterman-Storer and Salmon, 1997). It has been proposed that the minus end–stabilizing agent may be a γ tubulin complex (Moritz et al., 1995; Keating et al., 1997). The nature of the activating factors remains elusive. The availability of in vitro reconstituted cell systems should provide means to test the functional importance of defined regulatory mechanisms using in vitro assays. For instance, one could deplete cell extracts of γ tubulin and/or supplement such extracts with katanin and determine if these changes affect the ability of the extract to induce microtubule turnover. Furthermore, it will be interesting to determine whether depletion of katanin, cdks, or tubulin effects the activity of *Xenopus* egg extracts.

Microtubule Turnover in Intact Cells

In this study we have taken advantage of the availability of TTL^- cells and of the enzymatic properties of tubulin tyrosine ligase to achieve complete segregation of the initially free and initially polymeric tubulin pools in intact cells. Normally, mammalian cells contain microtubule arrays in which the tubulin is modified by enzymatic addition of tyrosine to the COOH terminus of the tubulin α-subunit (MacRae, 1997). TTL^- cells lack tubulin tyrosine ligase, and therefore contain Glu-tubulin networks in which the tubulin α-subunit COOH terminal Tyr residue is absent (Lafanechère et al., 1998). As the TTL cannot add tyrosine to tubulin in microtubule arrays (MacRae, 1997), preexisting arrays remain antigenically marked as long as they persist. Microinjection of TTL into TTL^- cells thus permits a clear distinction of newly formed from preexisting polymers. Examination of the relative organization of the Tyr- and Glu-microtubule networks in the same cells after TTL microinjection has thus yielded a comprehensive view of the organization of newly formed microtubules (Tyr-microtubules), and of their subsequent fate during microtubule turnover (Glu-microtubules). This observation has permitted a determination at the whole cell

level whether treadmilling was of significance for microtubule turnover compared with dynamic instability. Dynamic instability induces progressive shortening of the initial interphase microtubule array of interphase cells from the peripheral microtubule plus ends. If dynamic instability were the dominant mechanism of microtubule turnover in intact TTL^- cells, the disassembly of original interphase microtubule network would be centripetal. In contrast, with treadmilling, preexisting microtubules will detach from centrosomes, and microtubule remnants will be localized mainly in the cell periphery. The results of such experiments are striking. Tyr-microtubules formed regular arrays of centrosome-nucleated polymers while, in the same cells, Glu-microtubules appear after a few minutes as fragments detached from centrosomes and mainly located at the cell periphery. Such images have given a global view of the mechanism of microtubule turnover at the whole cell level, and they present evidence that microtubule treadmilling is an important mechanism of microtubule turnover in intact TTL^- cells.

Microtubule fragmentation is evident in intact cells, but is less extensive than in reconstituted permeabilized cells. It is possible that microtubule severing is localized in intact cells, while severing activity is disperse in cell-free extracts, where severing enzymes may be comparatively delocalized (see above).

Although microtubule severing activity, due to the enzyme katanin, was first discovered and characterized in *Xenopus* egg extracts (Vale, 1991; McNally and Vale, 1993), severing activity must also be present in mammalian cells. We have previously demonstrated a microtubule-severing activity in such cells (Lieuvin et al., 1994) that differed from katanin by requiring calcium for activity. It has also been demonstrated that elongation factor 1 alpha has microtubule-severing activity both in vitro and in vivo (Shiina et al., 1994). The meaning of these observations has remained somewhat elusive in the absence of a well-defined role for microtubule severing. It now appears that microtubule severing is central to microtubule physiology in interphase arrays. Obviously it is now important to characterize fully the factors involved in microtubule severing, and to elucidate the corresponding regulatory pathways.

Colchicine and Nocodazole as Probes of Microtubule Dynamics

In this study we have made extensive use of nocodazole and of colchicine as specific probes of different microtubule properties. We have shown that nocodazole acts by inactivating free tubulin dimers, and can induce microtubule depolymerization from plus ends in the presence of stable minus ends. Moreover, the use of permeabilized cells has allowed us to test directly the mechanism of action of colchicine. Our results have yielded a clear-cut demonstration of the kinetic blockage of the microtubule plus end by CD complexes that had previously been deduced from studies on purified microtubule preparations (Margolis and Wilson, 1977; Margolis et al., 1980; Farrell and Wilson, 1984; Skoufias and Wilson, 1992). This demonstration allowed separate analysis of microtubule minus end properties, presumably independent of dynamic activity at microtubule plus ends. We believe that the nocodazole/CD complex test described here, applied to both reconstituted and intact cells under various conditions, should be most helpful in permitting us to further decipher microtubule regulation.

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