Control of Microtubule Nucleation and Stability in Madin–Darby Canine Kidney Cells: The Occurrence of Noncentrosomal, Stable Detyrosinated Microtubules

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Abstract. The microtubule-nucleating activity of centrosomes was analyzed in fibroblastic (Vero) and in epithelial cells (PtK$_2$, Madin–Darby canine kidney [MDCK]) by double-immunofluorescence labeling with anti–centrosome and antitubulin antibodies. Most of the microtubules emanated from the centrosomes in Vero cells, whereas the microtubule network of MDCK cells appeared to be noncentrosome nucleated and randomly organized. The pattern of microtubule organization in PtK$_2$ cells was intermediate to the patterns observed in the typical fibroblastic and epithelial cells. The two centriole cylinders were tightly associated and located close to the nucleus in Vero and PtK$_2$ cells. In MDCK cells, however, they were clearly separated and electron microscopy revealed that they nucleated only a few microtubules.

The stability of centrosomal and noncentrosomal microtubules was examined by treatment of these different cell lines with various concentrations of nocodazole. 1.6 μM nocodazole induced an almost complete depolymerization of microtubules in Vero cells; some centrosome nucleated microtubules remained in PtK$_2$ cells, while many noncentrosomal microtubules resisted that treatment in MDCK cells.

Centrosomal and noncentrosomal microtubules regrew in MDCK cells with similar kinetics after release from complete disassembly by high concentrations of nocodazole (33 μM). During regrowth, centrosomal microtubules became resistant to 1.6 μM nocodazole before the noncentrosomal ones, although the latter eventually predominate. We suggest that in MDCK cells, microtubules grow and shrink as proposed by the dynamic instability model but the presence of factors prevents them from complete depolymerization. This creates seeds for reelongation that compete with nucleation off the centrosome. By using specific antibodies, we have shown that the abundant subset of nocodazole-resistant microtubules in MDCK cells contained detyrosinated α-tubulin (glu tubulin). On the other hand, the first microtubules to regrow after nocodazole removal contained only tyrosinated tubulin. Glu-tubulin became detectable only after 30 min of microtubule regrowth. This strongly supports the hypothesis that α-tubulin detyrosination occurs primarily on "long lived" microtubules and is not the cause of the stabilization process. This is also supported by the increased amount of glu-tubulin that we found in taxol-treated cells.

S EVERAL lines of evidence support the view that microtubules are involved in cytoplasmic organization and orientation of intracellular transport. Together with microtubule-organizing centers (MTOCs)[1] they are thought to determine the axis of polarity in many cells (8, 24, 32). Although it is known that microtubules are polymers in a dynamic equilibrium with a pool of soluble subunits (17), the intrinsic dynamic characteristics of pure microtubules have been studied only recently in vitro and called dynamic instability (16, 20, 25, 26). Microinjection of labeled tubulin or microtubule-associated proteins (MAPs) into living cells has also shown that most cytoplasmic microtubules in fibroblast

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1. *Abbreviations used in this paper:* glu-tubulin, detyrosinated α-tubulin; MAP, microtubule-associated protein; MTOC, microtubule-organizing center; tyr-tubulin, tyrosinated α-tubulin.

cells turn over with a half-life of ∼10 min (30, 31, 34). A smaller fraction of the microtubules is very stable with a half-life of several hours (34). The dynamic instability of microtubules has several potentially important implications for understanding how different subclasses of microtubule networks are generated during the cell cycle and cell differentiation. Kirschner and Mitchison propose that during morphogenetic processes (polarization or any sort of cell shape remodeling) microtubules, although globally dynamic, become more stable in a restricted area of the cell, thereby providing a basis for cytoplasmic asymmetry (20). This selective stabilization hypothesis predicts that: (*a*) microtubules with different stability can coexist in a given cell; (*b*) microtubule-stabilizing factors could be distributed nonhomogeneously in the cytoplasm; and (*c*) external signals

could influence the regional distribution of microtubule-stabilizing factors.

Another important level of control over microtubule organization concerns the mechanism of polymer nucleation in vivo. It is currently assumed that radial arrays of microtubules are nucleated off the centrosome by the pericentriolar material (2–4, 11, 12, 32). However, the pericentriolar material sometimes dissociates from the centrioles and other microtubule patterns are then generated (37). Moreover, spontaneous nucleation could occur in the cytoplasm if the tubulin concentration were high enough or if microtubule-stabilizing factors were present (7, 19). The proportion and dynamics of centrosome-nucleated microtubules may therefore be affected by the number and activity of other potential MTOCs, active tubulin concentration, and microtubule-stabilizing factors.

In this paper we compare the microtubule patterns in fibroblasts (Vero cells), epithelial-like (PtK$_2$), and epithelial cells (MDCK) as well as the relative stability of microtubules in these cell lines. The relationship between microtubule resistance to nocodazole, age, and α-tubulin detyrosination has also been investigated. We show that stable noncentrosomal microtubules exist in MDCK cells. We discuss the mechanism of generation and stabilization of these microtubules, and we present experimental evidence supporting the general hypothesis that α-tubulin is detyrosinated when associated with long-lived microtubules (13, 14, 21).

Materials and Methods

Cells

Madin–Darby canine kidney epithelial cells (MDCK) strain II were grown in Eagle's minimal essential medium with Eagle's salts supplemented with 10 mM Hepes pH 7.3, 1% L-glutamine, 5% FCS, penicillin (110 U/ml), and streptomycin (100 µg/ml). African green monkey kidney cells (Vero) and Potoroo kidney epithelial cells (PtK$_2$) were grown as previously described (22). The cells were seeded on glass coverslips and incubated in a humidified atmosphere equilibrated with 5% CO$_2$ in air at 37°C.

Drug Treatments

Cells were seeded at a density that normally allows confluency between day 2 and 3 (36). All the experiments described in this paper were carried out on subconfluent MDCK cells taken 24 h after seeding. Preliminary experiments indicated that the nocodazole concentration required for intermediate depolymerization in MDCK cells was 1.6 µM. To induce an almost total depolymerization, a concentration of 33 µM was required for at least 3 h.

Nocodazole (Sigma Chemical GmbH, Deisenhofen, Federal Republic of Germany) was kept as a stock solution in DMSO at −20°C (33 mM) and diluted in culture medium at the appropriate concentration just before use. Experimental protocols are described in the figure legends. Cells were treated at 37°C with taxol at 5 µM using a stock solution in DMSO (10 mM) kept at −20°C. (Taxol was supplied by Dr. M. Suffness, Natural Product Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.) Experimental protocols are described in the figure legends.

Antibodies

Affinity-purified rabbit anti–glu tubulin antibodies was obtained by immunizing rabbits with synthetic peptide containing the 12 carboxy-terminal amino acid of α-tubulin without the last tyrosine. The monoclonal anti–tyr tubulin was obtained by immunizing mice with the carboxy-terminal 13 amino acid of α-tubulin (including the last tyrosine residue). The preparation and complete characterization of these antibodies will be described elsewhere (22a). Each antibody was preabsorbed with the other peptide before use in order to eliminate any possible cross-reaction. The monoclonal anti–β-tubulin was from Amersham Buchler GmbH, Braunschweig, FRG.

Human anti–centrosome antibodies (5051) are a gift of T. Mitchison and M. Kirschner (26).

Immunofluorescence

All the results presented here have been obtained on cells preextracted for 10 s in microtubule-stabilizing medium (80 mM K-Pipes pH 6.8, 5 mM EGTA, 1 mM MgCl$_2$, 0.5% Triton X-100) and fixed in methanol at −20°C for 5 min as previously described (19). In control experiments, cells were fixed directly in methanol at −20°C. These and other fixation procedures involving the use of glutaraldehyde combined with extraction buffer gave the same results. Preextraction is useful because the background due to free tubulin subunits is eliminated, making the images sharper. Immunofluorescence was carried out as previously described (19) including 0.1% Triton X-100 in the PBS washes. Coverslips were covered with 20 µl rabbit anti–glu-tubulin (1:10 dilution) for 10 min. 20 µl of a monoclonal anti–tyr-tubulin (1:400) were added for a further 10 min incubation. Directly mixing the two antibodies gave similar results. After three washes (5 min each), the coverslips were covered for 10 min with 20 µl of fluorescein-labeled goat anti–rabbit and Texas Red–labeled goat anti–mouse antibodies (1:100 dilution; Dianova GmBH, Hamburg, FRG). After further washes, the coverslips were cleaned quickly in ethanol and mounted in Mowiol.

In some experiments, cells were double stained with a monoclonal mouse anti–β-tubulin (1:500 dilution) and a human anti–centrosome antibody (1:100). In this case, secondary antibodies were a goat anti–mouse labeled with fluorescein (1:50) and a goat anti–human labeled with Texas Red (1:100; Dianova GmBH). All antibodies were diluted in PBS containing 0.1% Triton X-100, 3% BSA, and 0.1% sodium azide.

Immunogold Labeling for Electron Microscopy

Cells grown on plastic coverslips were briefly washed in PBS, preextracted with the stabilizing medium, and fixed with 0.3% glutaraldehyde in the same medium at 37°C for 10 min. Free aldehyde groups were titrated by sodium borohydride (1 mg/ml in PBS) for 7 min. After washing in PBS, the coverslips were covered with a polyclonal rabbit anti–tubulin antibody for 30 min and washed five times (15 min each). The coverslips were then incubated for 30 min with gold-labeled protein A (8 nm size). After an overnight wash in PBS containing 0.1% Triton X-100, the cells were postfixed in 1% glutaraldehyde in 80 mM K-Pipes pH 6.8, 5 mM EGTA, and 1 mM MgCl$_2$. The specimens were then processed for electron microscopy and embedded in Epon. Serial thick (0.15–0.25 µm) and thin sections were cut and observed in a Philips 400 electron microscope.

Semiquantitative Analysis of Tyrosinated and Detyrosinated Tubulin by Immunoblotting

Cells were seeded on 3-cm petri dishes so as to reach confluency 24 h later when they were treated with nocodazole or taxol as described in the figure legends. At the desired time, the cells were lysed directly in hot Laemmli's sample buffer (0.5 ml) (23a). Care was taken to recover the whole cell layer from each dish. Samples were further treated at 95°C for 5 min, sonicated for 3 min in a water bath sonicator, spun 5 min in a microfuge (Eppendorf; Brinkmann Instruments Inc., Westbury, NY), divided into aliquots of 50 µl each, and kept frozen at −70°C until use. 5 µl of each sample were applied on 10% Laemmli's polyacrylamide minigels, or 50 µl on normal 10% polyacrylamide gels. After transfer onto nitrocellulose according to Towbin et al. (39), tyrosinated and detyrosinated tubulins were detected with the monoclonal anti–tyr-antibody (1:6,000) and the polyclonal anti–glu antibody (1:1,000). Peroxidase-labeled rabbit anti–mouse (1:1,000) and goat anti–rabbit (1:1,000) from Tag., Inc., (Burlingame, CA) were used as secondary antibodies. Diaminobenzidine (5 mg/ml in 50 mM Tris-HCl pH 7.6, 0.01% H$_2$O$_2$) was used as the substrate for the peroxidase reaction. The reaction was carefully controlled and allowed to proceed for the same time for a given set of blots (1–3 min). The reaction was arrested by a quick wash in distilled water followed by a wash in 5% TCA.

Carboxypeptidase A Treatment of Cell Lysates

After trypsinization, 1-d-old MDCK cells were washed twice with 50 mM NaCl, 50 mM Tris-HCL pH 7.2, and 1 mM EDTA at 4°C. An aliquot of cells was resuspended directly in hot Laemmli's sample buffer at 2 × 10^6 cells/ml and boiled for 5 min. Another aliquot was resuspended, at 4°C, in 50 mM K-Pipes pH 6.9, 1 mM EGTA, and 0.1% Triton at 4 × 10^6 cells/ml. This aliquot was then treated for 20 min at 37°C with 0.25 µg/ml

of carboxypeptidase A (phenylmethylsulfonyl fluoride treated; Serva Co., Heidelberg, FRG) prepared as previously described (38). The reaction was stopped by adding an equal volume of hot sample buffer to the reaction mixture and further boiling for 5 min. 10^5-cell equivalents (50 µl) were loaded on 10% polyacrylamide Laemmli's gels and analyzed by immunoblotting.

Results

### Centrosomes Are Split and Not Associated with the Nucleus in Subconfluent MDCK Cells

In most fibroblastic cells, the two centriole cylinders are tightly associated and bound to the nucleus (1, 2, 10, 27). By immunofluorescence microscopy, using scleroderma human anti–centrosome serum (42), they appeared as one bright dot or two smaller dots tightly associated with each other both in Vero and PtK₂ cells (Fig. 1 a). In subconfluent MDCK cells they were separated, sometimes by distances as large as 10 µm (Fig. 1 b). No specific association with the nucleus was found by electron microscopy (Fig. 2 a).

Centrosomes of MDCK Cells Are Not Preferred Sites of Nucleation

The aspect of the microtubule network in MDCK, Vero, and PtK₂ cells stained with a monoclonal anti–β-tubulin antibody is shown in Fig. 3. The microtubule network was clearly centered on the centrosome in most Vero cells. This was less obvious in PtK₂ cells. In MDCK cells, the microtubule network was randomly organized. There was no obvious correlation between the position of the split centrosome and the microtubule pattern. The cells shown in Fig. 3 have been double labeled with the centrosome antibody (data not shown). The positions of centrosomes are shown by arrows. We have observed serial thick sections of these cells by electron microscopy after immunogold staining of the microtubules. Between 0 and 15 microtubules were found to radiate from the pericentriolar area in 12 pairs of centrioles observed. Fig. 2 b shows one of these sections in which only 2–3 microtubules were found to radiate from the centrioles.

Most of the Noncentrosomal Microtubules Are Stable

To determine the relative stability of the noncentrosomal microtubules in MDCK cells, we treated the cells with a concentration of nocodazole (1.6 µM) known to depolymerize almost all microtubules in a few minutes in L₉₂₉ cells (19). This actually induced an almost complete depolymerization in Vero cells (Fig. 3) fixed 30 min after addition of the drug. Only occasional, long and curly stable microtubules remained, usually deriving from the centrosomes. In PtK₂ cells, many more similar microtubules were left, all of them emanating from the centrosome area (Fig. 3). In MDCK cells, a large number of extremely tortuous microtubules persisted 30 min after addition of the drug (Fig. 3). Most of these microtubules apparently did not radiate from the centrosomes (Fig. 3, arrows). Upon prolonged incubation in the same drug concentration, the stable microtubules found in all three cell lines progressively disappeared. Short microtubules started to grow and elongate off the centrosomes between 1 and 4 h after addition of this concentration of the drug. The strongest regrowth was observed in PtK₂ cells. At higher nocodazole concentration (33 µM, not shown), the kinetics of depolymerization was essentially the same al-
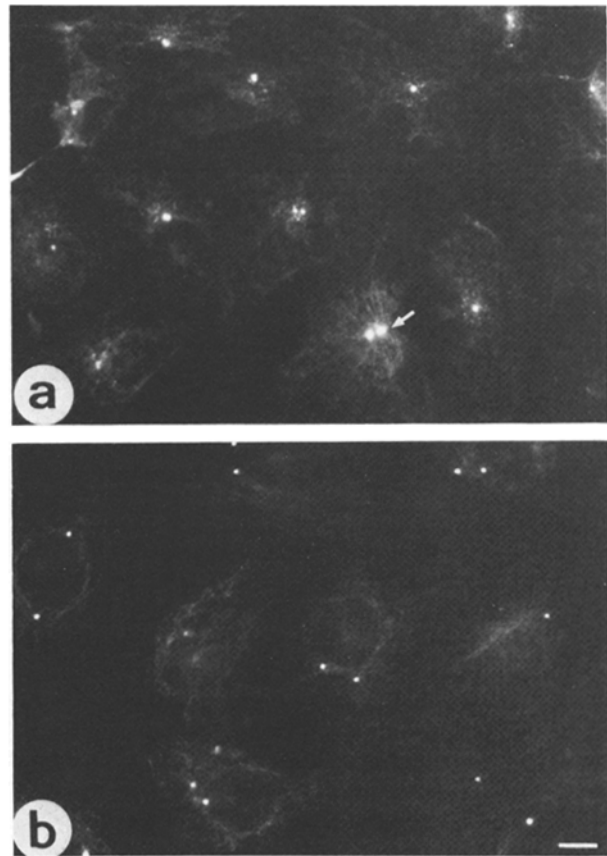

Figure 1. Centrosome splitting in subconfluent MDCK cells. (a) Centrosomes detected by the human anti–centrosome antibody (diluted 1:100) are visible as one large dot or two closely associated smaller dots in Vero cells. The brighter dots correspond to a centrosome in early prophase (arrow). (b) Each MDCK cell contains two individual structures decorated by the same antibody and sometimes separated by a large distance. Bar, 5 µm.

though fewer microtubules remained. Microtubule regrowth from centrosomes was never observed after 4 h of treatment.

These results show that by adding nocodazole at an intermediate concentration it is possible to induce a shift from a mainly noncentrosomal pattern to an exclusively centrosomal pattern of microtubules in MDCK cells. A large fraction of the microtubules in MDCK cells does disappear after nocodazole addition. Therefore, the population of stable microtubules coexist with more labile microtubules. The ratio of stable versus unstable microtubules seems to be high in MDCK, intermediate in PtK₂, and very low in Vero cells. Inversely, the ratio of centrosomal versus noncentrosomal microtubules seems to be low in MDCK, intermediate in PtK₂, and very high in Vero cells.

Most Stable Microtubules Contain Detyrosinated Tubulin

It has been recently reported that a specific class of long sinuous microtubules is enriched in the posttranslationally modified form of α-tubulin, the detyrosinated α-tubulin (glutubulin) (15). A monoclonal anti–tyrosinated tubulin (anti-tyr) and an affinity-purified rabbit anti–detyrosinated tubulin (anti-glu) have been prepared. These antibodies have been
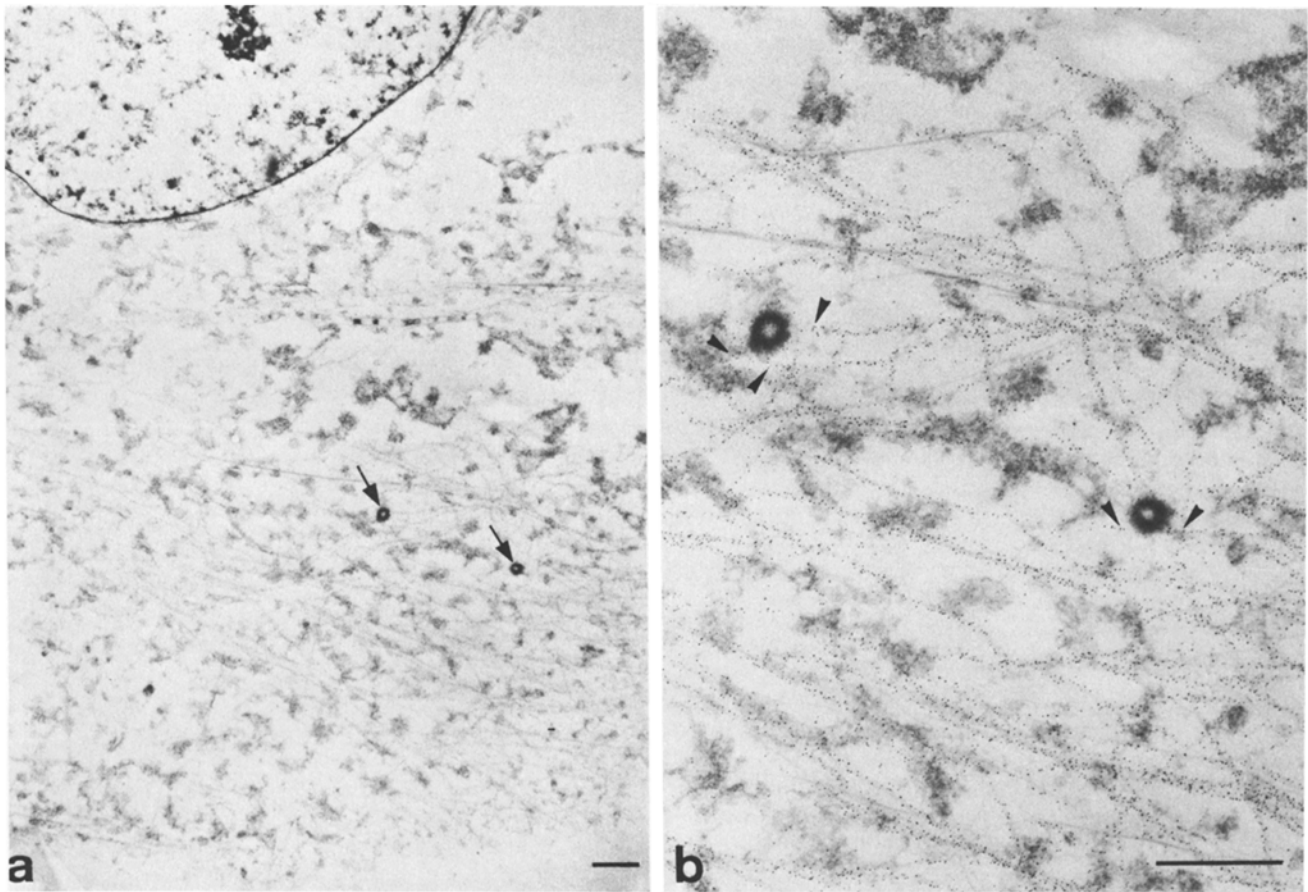
Figure 2. Thick section of MDCK cells observed by electron microscopy after immunogold staining of microtubules. Subconfluent MDCK cells grown on plastic were immunostained for tubulin as described in Materials and Methods and thick sections screened for centrioles. (*a*) Two centrioles (*arrows*) ∼3 μm apart from each other, not associated with the nucleus, are visible. (*b*) Higher magnification of the centrioles shown in *a*. Among all the microtubules decorated by gold particles, only a few actually emanate from the centrioles (*arrowheads*) Bars, 1 μm.

raised against the synthetic carboxy-terminal peptides of α-tubulin and found to be highly specific for each form of α-tubulin (22a).

The microtubule pattern revealed by the anti–tyr antibody (tyr-microtubules) in MDCK cells was similar to the pattern obtained after staining with the monoclonal anti–β-tubulin (Fig. 4). The anti–glu antibodies stained a distinct subset of long, wavy microtubules as previously reported for TC₇ cells (glu-microtubules; Fig. 4, *b* and *d*) (15). However, MDCK cells appeared to contain many more of these microtubules. Most of the microtubules remaining 30 min after addition of nocodazole (1.6 μM) were decorated by the glu-antibodies, although to a variable extent (Fig. 4 *d*). These microtubules were also decorated by the tyr-antibody but very few microtubules were stained only by the tyr-antibody (Fig. 4 *c*). We will call "glu" any microtubule that is clearly stained by the glu-antibody. This does not mean that it is not detected by the tyr-antibody, inasmuch as some of them being more strongly labeled by this antibody than by the glu-antibody. The number of glu-microtubules slowly decreased with time of incubation in the drug. The short centrosomal microtubules regrowing slowly between 1 and 4 h were first exclusively stained by the anti–tyr-antibody and became stained by the glu-antibody at 4 h, in the vicinity of

the centrosome (Fig. 4 *f*). At this time point, occasional, long glu-microtubules were found associated with the centrosomes. These were probably "old microtubules".

One End of Noncentrosomal Glu-Microtubules Can Be Exclusively Composed of Tyr-Tubulin

At the periphery of MDCK cells we occasionally found glu-microtubules with ends exclusively stained by the tyr-antibody. Unfortunately, microtubule density hampered our attempts to determine what happened at the other end of these microtubules. It was easy, however, to observe microtubules double stained with tyr- and glu-antibodies over their entire length in cells treated with nocodazole for 1 and 2 h since only a few microtubules remained (Fig. 5). 1 h after addition of nocodazole (1.6 μM), very few glu-microtubules contained ends stained only by the tyr-antibody (not shown). However, at 2 h, when tyr-microtubules start to appear around the centrosomes, virtually all the remaining noncentrosomal glu-microtubules had only one end exclusively stained by the tyr-antibody (Fig. 5, *a* and *b*). This staining was often much brighter than over the rest of the microtubules and was rarely observed on the glu-microtubules of cells treated with 33 μM nocodazole (Fig. 5, *c* and *d*). This concentration prevented the regrowth of tyr-microtubules
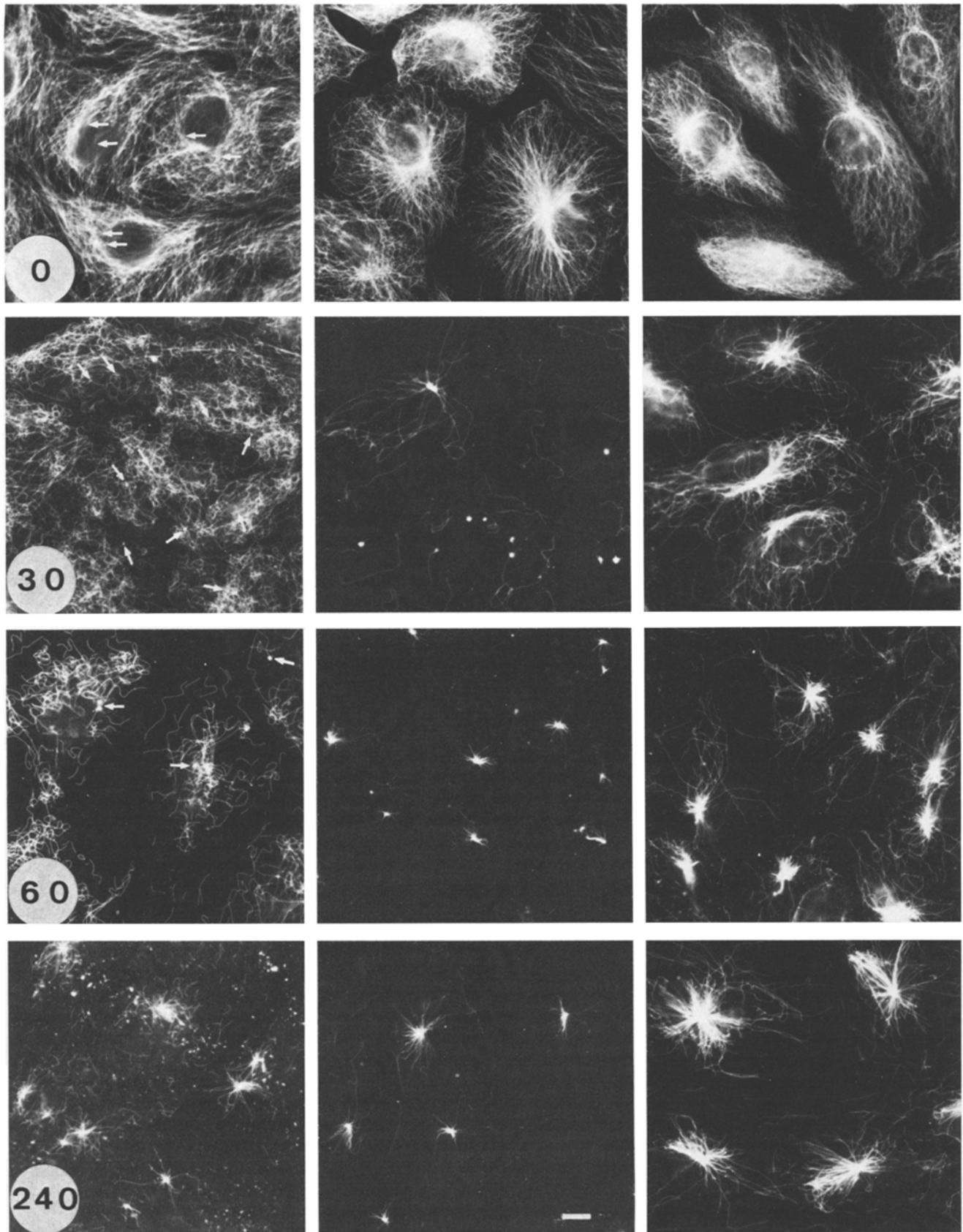
Figure 3. Microtubule pattern and stability in MDCK, Vero, and PtK2 cells. MDCK (*left*), Vero (*center*), and PtK (*right*) cells were stained by double immunofluorescence for microtubules and centrosomes as described in Materials and Methods. A mouse monoclonal anti–β-tubulin was used for microtubule staining. The centrosome staining is not shown, but the position of each centrosome is indicated when not obvious (*arrows*). Cells were fixed before or after various times of incubation in 1.6 μM nocodazole. Numbers (*left*) indicate the time in minutes of incubation of each cell line in nocodazole.
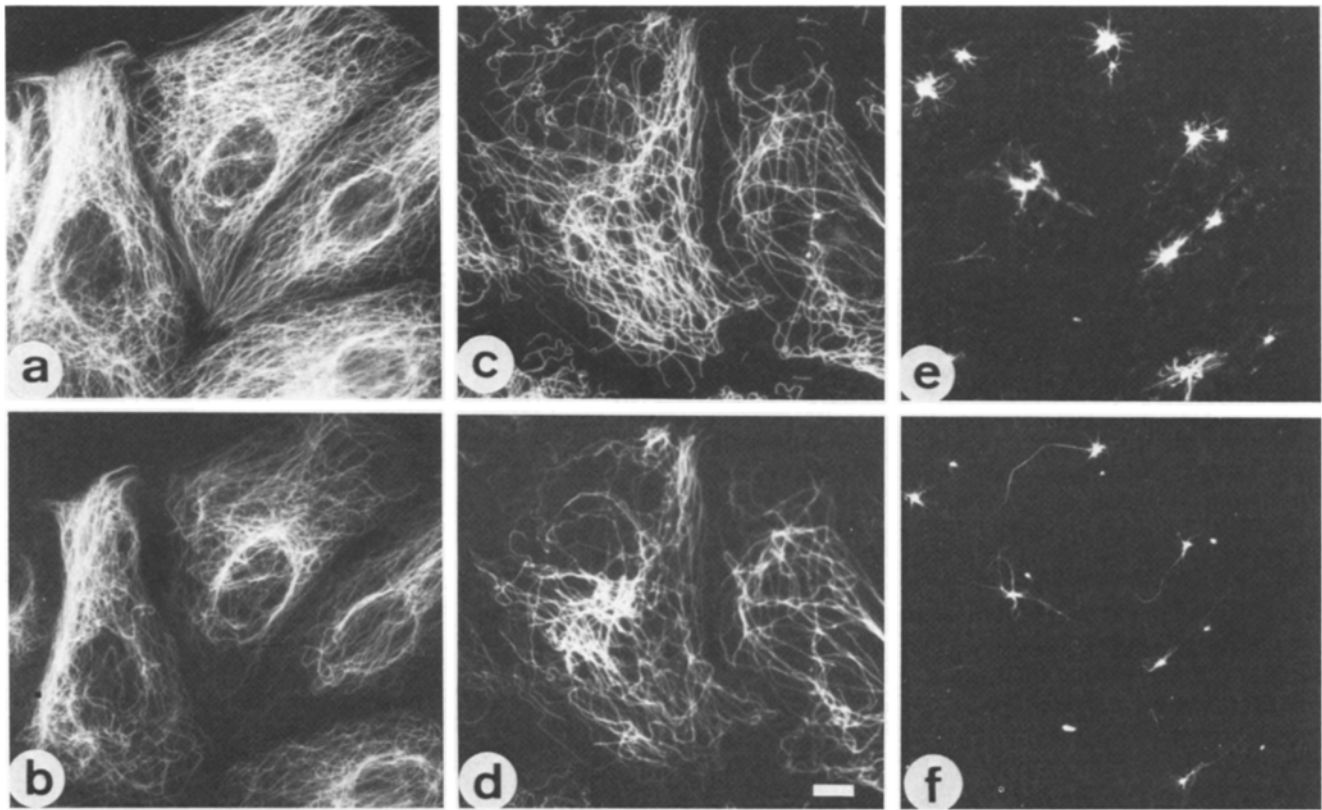
Figure 4. Stable microtubules in MDCK cells contain detyrosinated α-tubulin. MDCK cells were fixed before (*a* and *b*), 30 min (*c* and *d*), and 4 h (*e* and *f*) after incubation in 1.6 μM nocodazole. Double immunostaining was performed with anti–tyr- (*a, c,* and *e*) and anti–glu- (*b, d,* and *f*) antibodies. Secondary antibodies were labeled with rhodamine (*a, c,* and *e*) and fluorescein (*b, d,* and *f*) (Bar, 5 μM).

from the centrosomes during the treatment. In this case, however, different staining intensities were occasionally observed with the glu-antibodies along the length of a given microtubule (Fig. 5 *d*). It seems that newly formed microtubules or microtubule fragments contain mainly tyrosinated α-tubulin.

Microtubule Network Reassembly Precedes Tubulin Detyrosination after Nocodazole Removal

To investigate further the relationship between microtubule polymerization and detyrosination of α-tubulin in vivo, we examined the kinetics of tyr- and glu-microtubule regrowth after nocodazole reversal (Fig. 6). After 4 h of incubation in 33 μM nocodazole, most microtubules were depolymerized. Only a few, long centrosomal microtubules containing glu-tubulin remained (Fig. 6). Centrioles could be identified with the glu-antibodies, which seemed to strongly stain the centrioles (Fig. 6). Interestingly, the tyr-antibody stained numerous dots, scattered in the cytoplasm, which were not stained by the glu-antibodies. This was always found and does not seem to be an artifact of fixation. Indeed, these dots were also detected by the anti–β-tubulin in MDCK cells but not in other cells (Fig. 3).

3 min after nocodazole removal, many short microtubules were detected by the tyr-antibody, growing as small asters from the centrosomes as small foci or as short individual segments scattered throughout the cytoplasm (Fig. 6). With the exception of long (probably old) microtubules, none of the short (newly formed) microtubules were stained with the glu-

antibodies (Fig. 6). Microtubules detected by the tyr-antibody elongated, and at 30 min an apparently normal microtubule pattern was reformed. It was only then that some microtubules became reactive to the glu-antibodies, and only in a fraction of the cell population. A glu-microtubule pattern comparable to what was found in the normal population was observed at 1 h after nocodazole removal. The ratio of noncentrosomal to centrosomal microtubules increases with time after nocodazole removal.

Kinetics of Centrosomal and Noncentrosomal Microtubule Stabilization during Regrowth after Nocodazole Removal

The low abundance of centrosomal microtubules at steady state in MDCK cells is very surprising in view of the large amount of microtubules regrowing from centrosomes at early time points after nocodazole removal. This means that noncentrosomal microtubules eventually predominate in this cell line. This is different than what has been found in fibroblastic cells where centrosomal microtubules predominate. To investigate the potential correlation between the appearance of noncentrosomal microtubules and polymer stabilization, we incubated cells for 4 h in 33 μM nocodazole, then removed the drug, and at various times during microtubule regrowth we further added nocodazole at 1.6 or 33 μM for 30 min. The cells were then fixed and double stained with the anti–glu and anti–tyr antibodies (Fig. 7). Only tyr-microtubules are shown. Long centrosomal and short non-
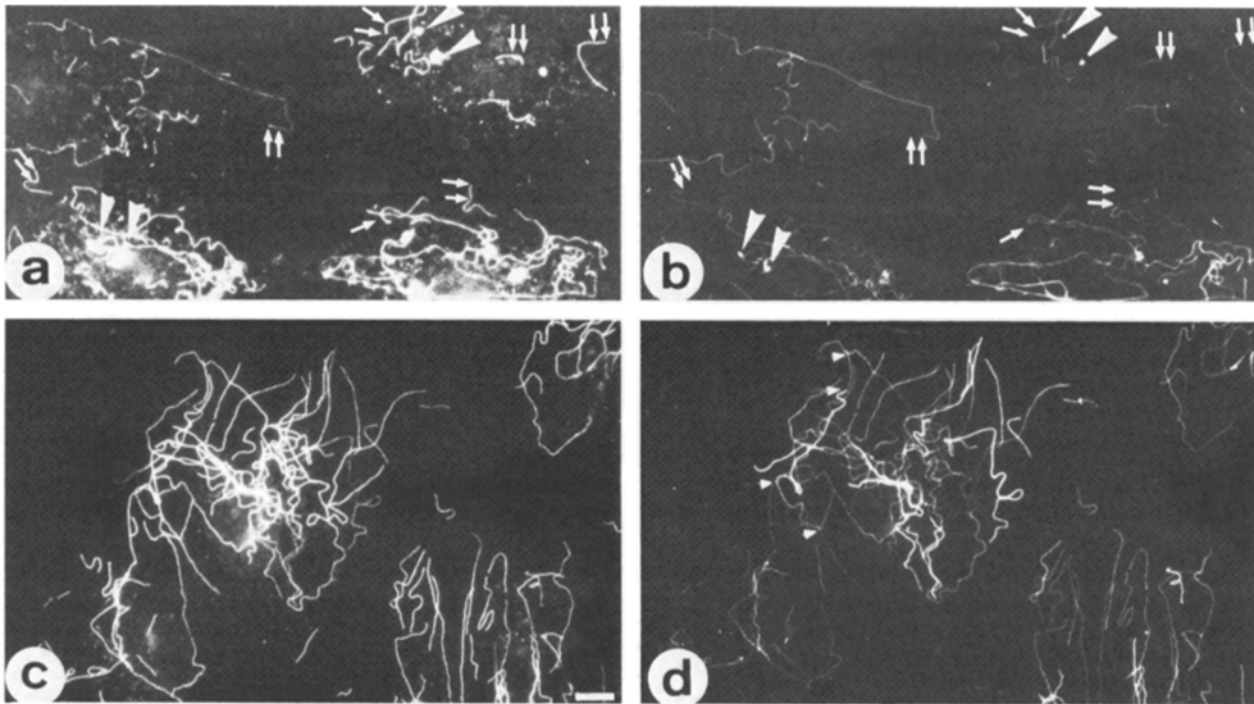
Figure 5. In MDCK cells glu-microtubule ends are exclusively stained by the tyr-antibody during depolymerization in 1.6 μM nocodazole. 2 h after the addition of nocodazole at 1.6 μM (*a* and *b*) and 1 h after addition of nocodazole at 33 μM (*c* and *d*) MDCK cells were fixed and double stained with anti–tyr- (*a* and *c*) and anti–glu- (*b* and *d*) antibodies. (*Arrows*) Microtubule ends exclusively stained by the anti–tyr-antibody. (*Large arrowheads*) Centrosomes. (*Small arrowheads*) Ends weakly labeled by the anti–glu-antibody in a microtubule that is strongly stained. The large spots produced by the anti–tyr-antibody around centrosomes are in fact numerous, short microtubules not resolved on the picture. Bar, 5 μm.

centrosomal microtubules were found to be resistant to 1.6 μM nocodazole 15 min after release from the first drug treatment (Fig. 7 *a*). They were not stained by the glu-antibodies (not shown). Long noncentrosomal microtubules became resistant to this drug concentration only after 30 min of regrowth. They were predominant by 1 h (Fig. 7, *b* and *c*). This coincides with the first appearance of microtubules strongly stained by the glu-antibodies (Fig. 6). The use of 1.6 μM nocodazole for 30 min as a test for the presence of relatively stable microtubules is fully valid since this treatment induces a complete depolymerization in fibroblasts. However, we were concerned that the "stable" centrosomal microtubules observed after 15 min of regrowth arose by elongation during the further 30 min of treatment in 1.6 μM nocodazole. To eliminate this possibility, we repeated the kinetics of polymer stabilization during regrowth by using a high concentration of nocodazole (33 μM) (Fig. 7, *d–h*). The result was striking: many centrosomal microtubules were stabilized immediately after being nucleated (as soon as 3 min; Fig. 7 *d*). Moreover, the length of stabilized centrosomal microtubules increased with time of regrowth while their number seemed to drop. Stable, short noncentrosomal microtubules arose between 8 and 15 min. Long stable noncentrosomal microtubules were much less abundant at 30 and 60 min than in cells treated with 1.6 μM nocodazole (Fig. 7, *b*, *c*, *g*, and *h*).

We conclude from these experiments that: (*a*) Some centrosomal microtubules are stabilized immediately after being nucleated. (*b*) The number of stable centrosomal microtubules drops and their length increases with time of regrowth.

(*c*) Noncentrosomal microtubules are stabilized later than centrosomal microtubules. (*d*) Many of the noncentrosomal microtubules, which are stable in 1.6 μM, are unstable in 33 μM nocodazole. (*e*) Microtubule stabilization is reversible since the pattern of stable microtubules evolves with time of regrowth. (*f*) Tubulin detyrosination occurs after partial microtubule stabilization.

## Microtubule Stabilization by Taxol Promotes Tubulin Detyrosination

The results reported above strongly suggest that detyrosination of α-tubulin occurs primarily on stabilized polymer in vivo. To strengthen this correlation, we examined the microtubule staining with the glu-antibodies in cells treated with taxol, a drug known to reduce microtubule dynamics (Fig. 8). There was a complex rearrangement of the microtubule network over the first 2 h of incubation in taxol. No obvious increase in the number of glu-microtubules could be detected during the first 30 min of incubation. By 2 h, however, while the microtubule pattern seemed to be relatively stable, most microtubules appeared to be stained by the glu-antibodies.

Amount of Glu-Tubulin Is Decreased in Nocodazole and Increased in Taxol-treated Cells

The previous set of experiments clearly shows that newly formed microtubules are mostly composed of tyrosinated tubulin. On the other hand, stable microtubules or taxol-stabilized microtubules are clearly enriched in glu-tubulin. This strongly suggests that most of the free tubulin is tyrosi-
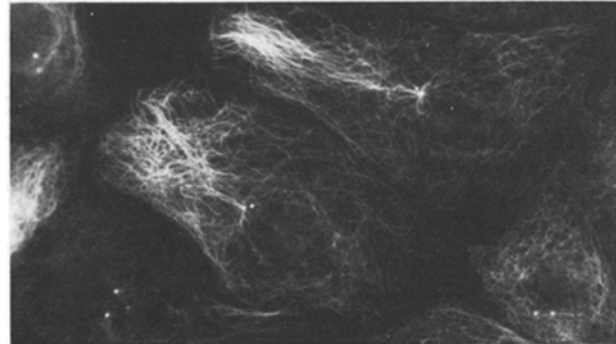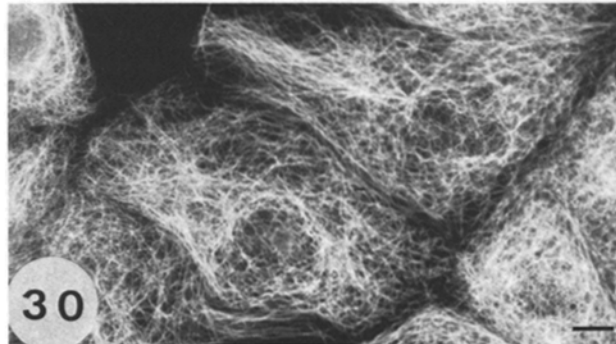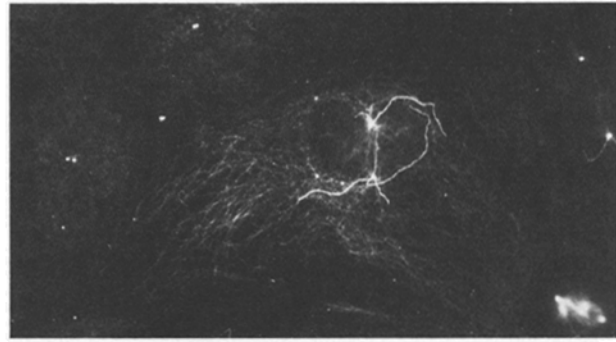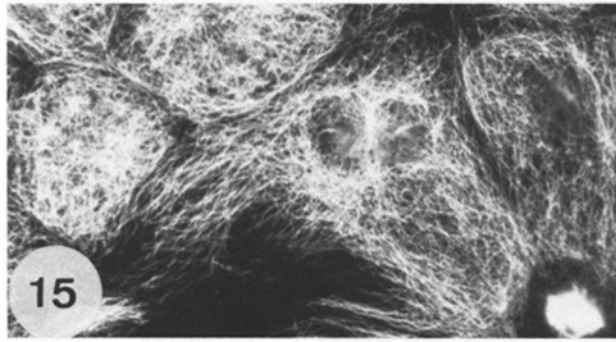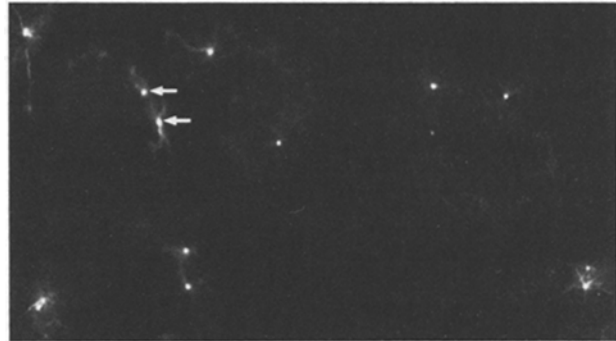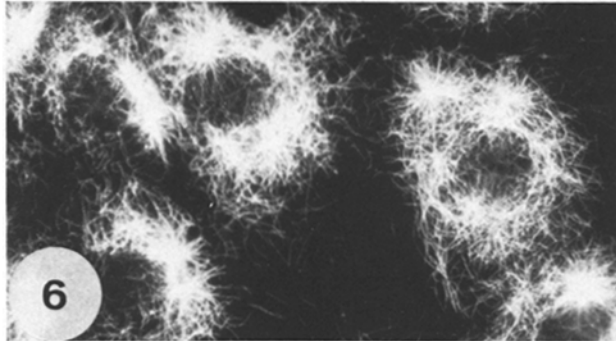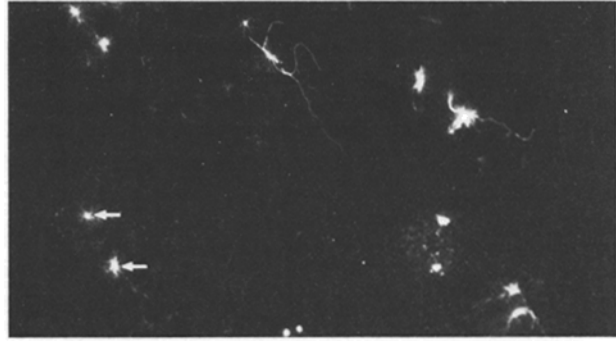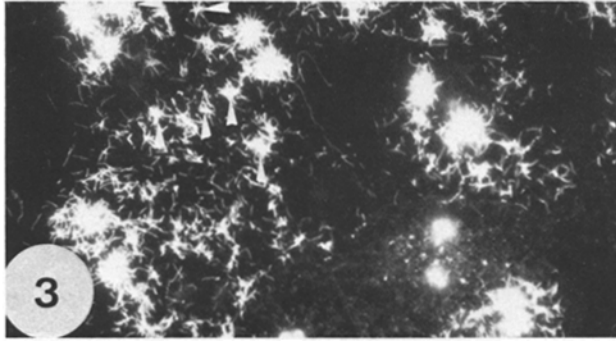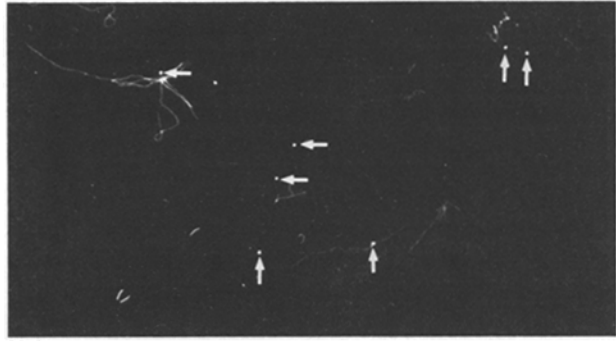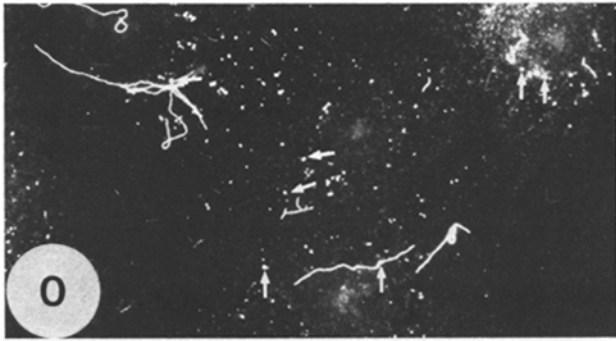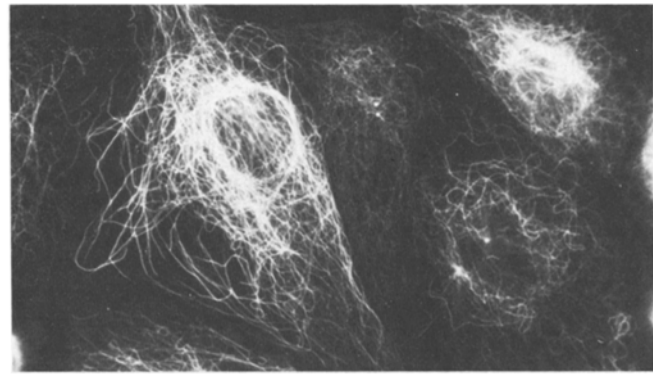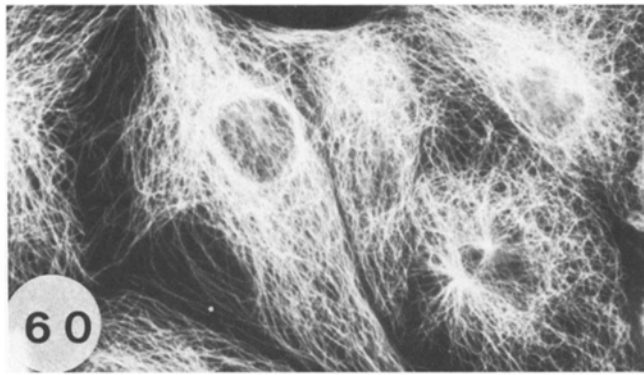
Figure 6. Kinetics of tyr- and glu-microtubule regrowth in MDCK cells after nocodazole removal. MDCK cells were treated for 4 h with 33 μM nocodazole. The drug was removed, the cells were fixed at the time (in minutes) indicated on the left of the figure, and double stained for tyr- (*left*) and glu- (*right*) α-tubulin. (*Arrows*) Centrosomes; (*arrowheads*) noncentrosomal microtubule foci. Bar, 5 μm.

nated while detyrosination occurs on the polymer. If this were the case, most of the cellular tubulin in nocodazole-treated cells should be tyrosinated (since it is soluble) while in taxol-treated cells, tubulin should be enriched in the glu-form. To test this possibility, we carried out immunoblots with the anti–glu and anti–tyr-antibodies on total extracts of cells after nocodazole and taxol treatments (Fig. 9). The specificity of the antibodies for tyrosinated and detyrosinated tubulin has been checked on immunoblots made with total cell extracts before and after treatment with carboxypeptidase A (Fig. 9 *a*). The reactivity of the tyr-antibody was decreased (Fig. 9 *a*, lane *2*) and the reactivity of the glu-antibodies dramatically increased (lane *4*) after action of carboxypeptidase A. Cells incubated for 4 h in 33 μM nocodazole and lysed directly in sample buffer contained a very low amount of glu-tubulin. Glu-tubulin became detectable between 15 and 30 min after nocodazole removal. The amount of tyr-tubulin remained constant throughout this period, demonstrating that the lack of detection of glu-tubulin after nocodazole treatment is not due to a different amount of total tubulin present in these samples (Fig. 9 *b*). In cells treated with taxol, the amount of glu-tubulin increased progressively with the time of treatment. Here, the amount of tyr-tubulin remained constant or dropped slightly at longer times of incubation in taxol (Fig. 9 *c*).

Discussion

Centrosome and Centrioles in Subconfluent MDCK Cells

The term centrosome in mammalian cells usually refers to the complex formed by the two centriole cylinders and the pericentriolar material that nucleates microtubules (2). We have found that in subconfluent MDCK cells, the two cylinders are largely independent from each other and from the nucleus. A detailed study is underway. This unusual condition of the centrosome has been found in other situations (32, 33, 35). In MDCK cells, the two cylinders migrate up to the apical and central part of the cell at a relatively constant distance from each other during cell polarization (Simons, K., M. H. Bré, and E. Karsenti, unpublished data). This provides us with a unique model to study the control of centrioles and pericentriolar material location during cell

differentiation and polarization. Several examples suggest, indeed, that centrioles are relocated or eliminated during cell differentiation (2, 8, 9, 32, 37, 40, 41, 45).

Origin and Persistance of Noncentrosomal Microtubules in MDCK Cells

The microtubule pattern in MDCK cells appears to be much more disorganized than in Vero and PtK₂ cells, where most microtubules originate at the centrosome. One could argue that this is due to the fact that in MDCK cells the two centrioles are separated. Indeed, by being nucleated in two different locations, the microtubules could form a network that would appear less organized. However, in the electron microscope, the centrioles were found to nucleate only a few microtubules. Therefore, in MDCK cells, many microtubules are generated by a mechanism other than nucleation by the centrosome. One possibility would be that the microtubule-nucleating material that is associated with the centrioles in fibroblasts is more dispersed in MDCK cells. The balance between centrosomal and noncentrosomal microtubules could also be controlled by modulating the dynamics of microtubule assembly and disassembly. Mitchison and Kirschner have shown that microtubule asters formed on purified centrosomes in vitro are very dynamic (25). Microtubules grow and then start to depolymerize catastrophically with a probability that increases with decreasing concentrations of tubulin. The nucleation sites left unoccupied on the centrosome can then nucleate new microtubules below the steady state tubulin concentration. This behavior in vitro may explain why centrosomal microtubules predominate in some cell lines. Indeed, during microtubule regrowth after removal of depolymerizing drugs like nocodazole, both centrosomal and noncentrosomal microtubules appear in the cytoplasm of these cells. However, the noncentrosomal microtubules eventually disappear and at equilibrium a centrosome-nucleated aster of microtubules predominates. In the context of the dynamic instability model, this behavior is easy to explain: Upon removal of nocodazole, the free tubulin concentration in the cell is very high. Spontaneous nucleation will be frequent and compete efficiently with centrosome nucleation. However, with time, more polymer assembles and since the cell is a closed system the free tubulin concentration drops. A steady state situation is reached where the tubulin concentration is too low to allow spontane-
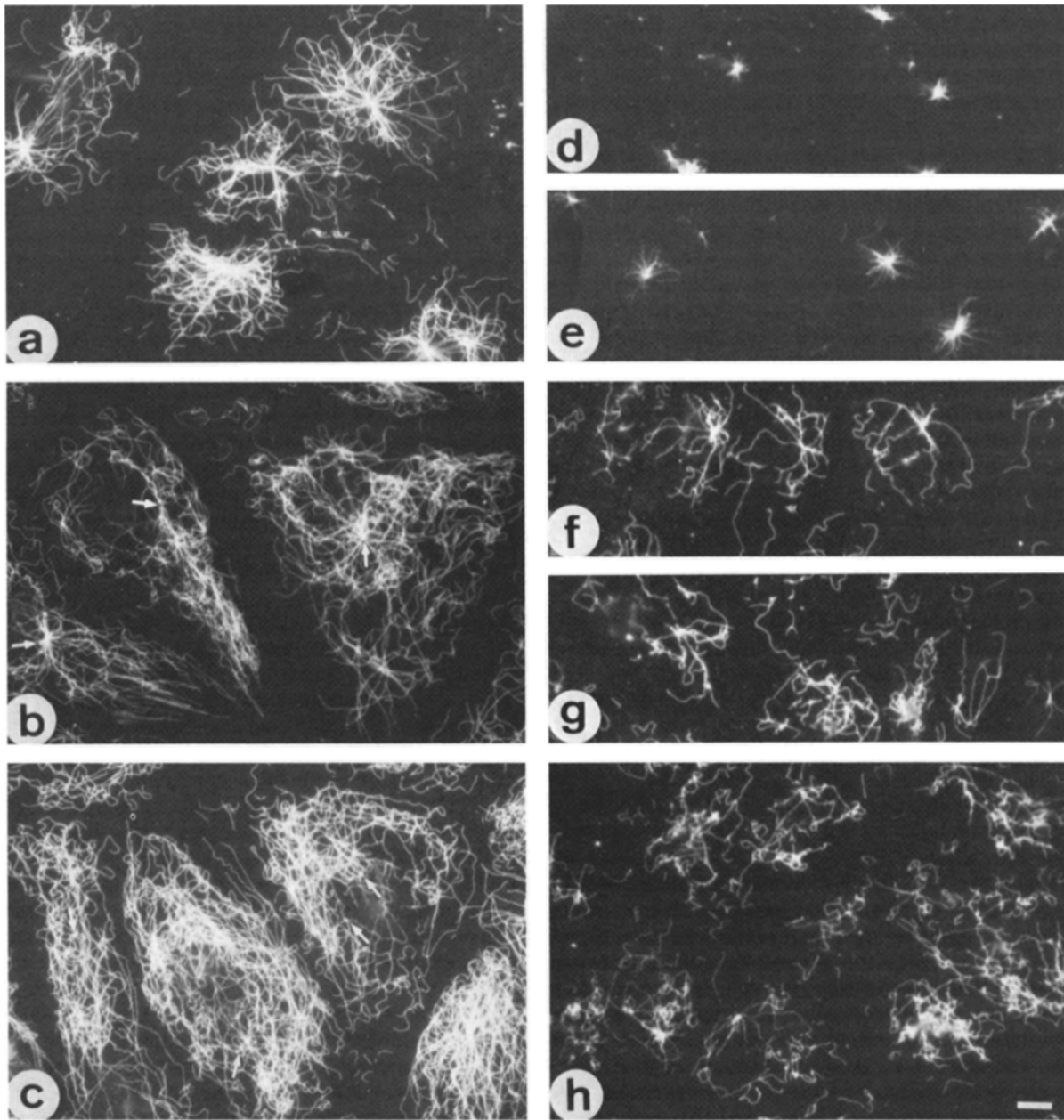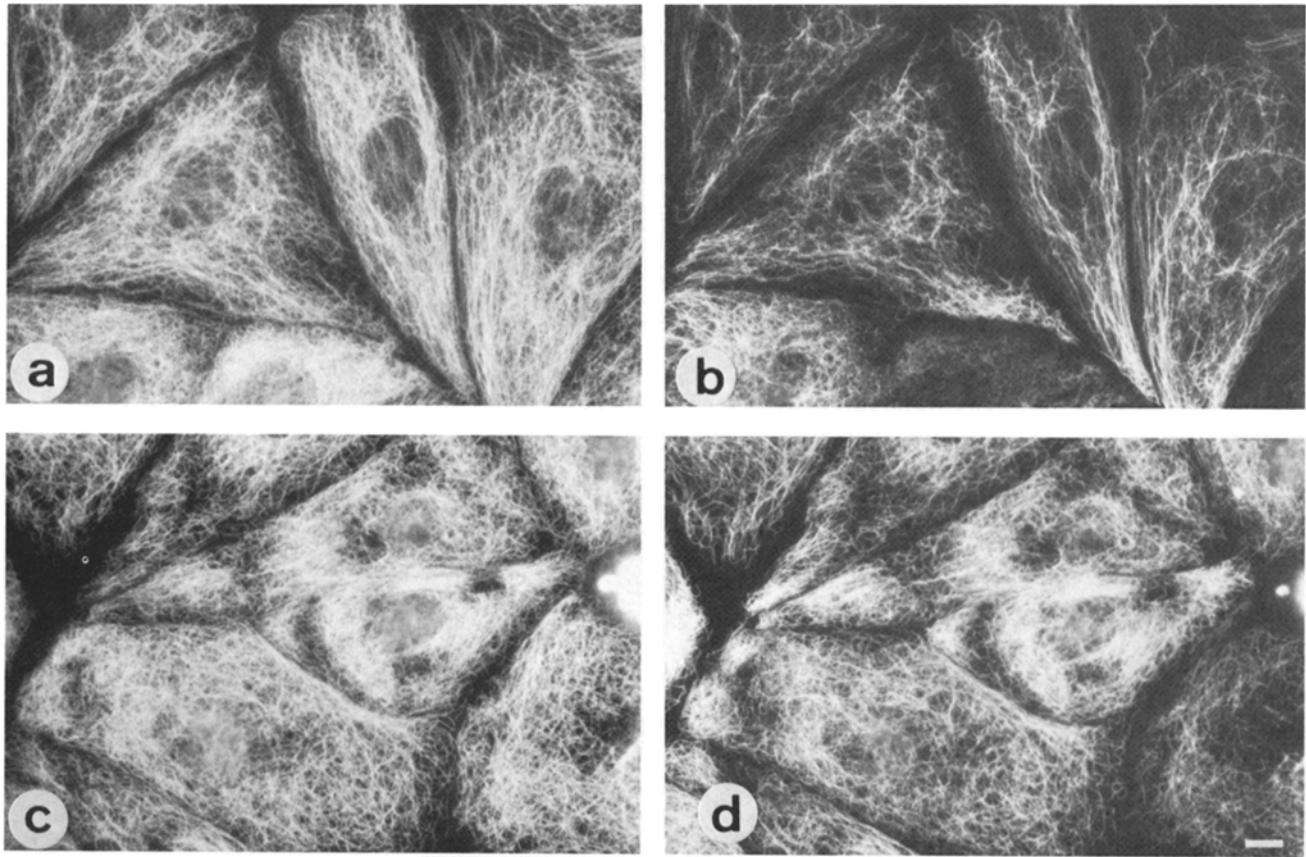
Figure 7. Kinetics of microtubule stabilization after nocodazole removal. MDCK cells were treated for 4 h with 33 µM nocodazole. The drug-containing medium was then removed and the cells were quickly washed three times and incubated with fresh, warm medium. After 15, 30, and 60 min of regrowth, nocodazole (1.6 µM) was further added for 30 min, and the cells fixed (*a–c*). After 3, 8, 15, 30, and 60 min of regrowth, nocodazole (33 µM) was further added and the cells fixed (*d–h*). Double immunofluorescence with anti–tyr- and anti–glu- antibodies was carried out. Only the tyr staining is shown. The positions of centrosomes are indicated (*arrows*). Bar, 5 µm.

ous nucleation and therefore only centrosome-nucleated microtubules remain (25). This situation implies that the centrosomal aster is a very dynamic structure and one would expect it to disappear quickly in response to nocodazole. This is what was found for L$_{929}$ cells and we report a similar situation for Vero cells. In PtK$_2$ cells, many centrosomal microtubules are apparently stable since they are resistant to a similar treatment.

In MDCK cells, the situation is different. Most stable microtubules (which are numerous) appear to be noncentrosomal. In a way microtubules behave naturally in these cells as they do in other cells (PtK$_2$, 3T3, human fibroblasts) that

have been treated with taxol. De Brabander et al. (6) have shown that taxol induces spontaneous microtubule assembly in the cytoplasm of interphase cells without spatial relation to the centrosome. Moreover, the preexisting centrosomal microtubules gradually disappear. This effect was explained by the properties of taxol that decrease the critical concentration for tubulin polymerization and increase microtubule stability. Therefore, it seems that MDCK cells contain factors capable of stabilizing microtubules as taxol does. These may be specific MAPs. The exact molecular mechanism by which such factors would function is still unclear. Brain MAPs, for example, bind quickly and tightly to microtubules

Figure 8. Stabilization of microtubules by taxol increases the proportion of detyrosinated microtubules. MDCK cells were treated with taxol (5 μM) for 30 min (*a* and *b*) and 2 h (*c* and *d*). After fixation the cells were processed for double immunofluorescence with the anti–tyr- (*a* and *c*) and anti–glu- (*b* and *d*) antibodies. Bar, 5 μm.

in vitro, leading to a rapid and almost irreversible stabilization of microtubules (18). The presence of such molecules in saturating amounts would not explain the progressive predominance of noncentrosomal microtubules in MDCK cells during regrowth after nocodazole removal. However, Job et al. (18) discuss the possibility that MAPs present in nonsaturating amounts could bind in discrete sites along microtubules because of their fast association rate. They argue that microtubule stability subclasses could be generated in this way. Moreover, they suggest that a given microtubule could have different levels of stability along its length depending on the amount of MAPs bound locally. The presence of limiting concentrations of MAPs in MDCK cells could explain the progressive predominance of noncentrosomal microtubules according to the following mechanism: Initially after nocodazole removal, numerous short microtubules are nucleated in the cytoplasm. Both on free and centrosome-nucleated microtubules, very short segments are stabilized by the binding of MAPs. The existence of such stable segments along noncentrosomal microtubules would prevent them from complete depolymerization when the steady state free tubulin concentration is approached. Such segments could then act as seeds for polymer elongation below the free tubulin concentration required for spontaneous nucleation. If many stable seeds exist, they will compete with the nucleation activity of the centrosome.

We find that stable centrosomal microtubules arise early and before the noncentrosomal ones during regrowth. This probably occurs because they are capped at one end by the centrosome. This indeed increases the probability of stabilization of a visible microtubule stretch. Interestingly, during regrowth, the length of stable centrosomal microtubules increases in parallel with microtubule elongation. While this occurs the number of stable centrosomal microtubules drops. This behavior fits well with an initial rapid and reversible stabilization due to soluble factors, followed by their random redistribution. Short, stable noncentrosomal microtubules become visible after 8–15 min of regrowth; this occurs when they happen to bind stabilizing factors in two different points. In other words, a stable microtubule could be composed of a long nonstabilized region flanked by two short stabilized segments. The probability for this to occur increases with the length of the microtubule and with its life span.

This mechanism would produce microtubules with segments of different ages as previously suggested (18) and with dynamic extremities. Several observations argue in favor of this idea: (*a*) We found microtubules strongly stained by the glu-antibodies in their middle part with a weaker staining on each side. Since we assume that the extent of α-tubulin detyrosination is representative of the microtubule age, this suggests that the microtubule ends are not as "old" as the rest of the microtubule. (*b*) After 1 h of microtubule depolymerization in the presence of 1.6 μM nocodazole, tyr-microtubules regrow slowly from the centrosomes. In the same time, short microtubule segments exclusively stained by the tyr-antibody appear at one end of the remaining noncen-
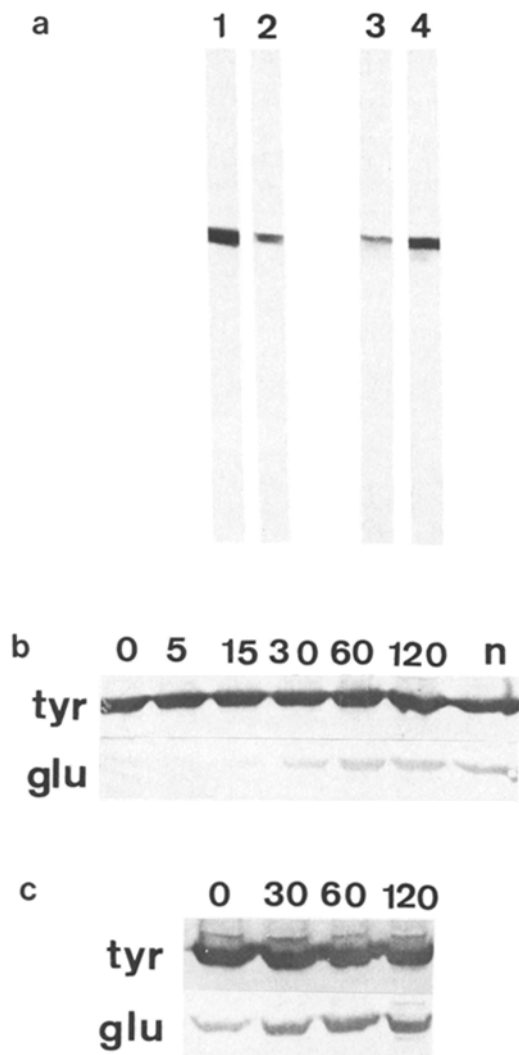
Figure 9. Immunoblot analysis of tyrosinated and detyrosinated tubulin in MDCK cell lysates. (a) Cell lysates were prepared and treated by carboxypeptidase A as described in Materials and Methods. The proteins were run on a 10% Laemmli gel, transferred to nitrocellulose, and stained with the anti–tyr (lanes 1 and 2) or anti–glu (lanes 3 and 4) antibodies. Carboxypeptidase-treated samples are in lanes 2 and 4. (b) Cells were treated with 33 μM nocodazole for 4 h, lysed in hot gel sample buffer at the indicated times (in minutes) after nocodazole removal, and loaded on one 10% Laemmli minigel in duplicate. n, cells not treated by nocodazole. After transfer, the nitrocellulose sheet was split in two parts: one-half was stained with the anti–tyr antibody (tyr) and the other half with the anti–glu antibody (glu). (c) Cells were treated with 5 μM taxol, lysed in hot gel sample buffer at the indicated times (in minutes), and loaded on one 10% Laemmli minigel in duplicate. After transfer, the nitrocellulose sheet was split in two parts: one-half was stained with the tyr-antibody (tyr) and the other with the glu-antibody (glu). In all cases, the secondary antibody was labeled with peroxidase. See Materials and Methods for details.

trosomal glu-microtubules. These are probably newly poly-merized segments. Since most noncentrosomal microtubules have disappeared by 4 h, we must conclude that during depo-lymerization in the presence of 1.6 μM nocodazole they grow and shrink (the overall shrinking rate being higher than the growth rate). The tyr-containing end might be the active end (+ end) in these microtubules.

The regrowth of small centrosomal asters from centrosomes after 4 h of incubation in 1.6 μM nocodazole shows that some active tubulin remains under such conditions. The absence of noncentrosomal microtubules in the same conditions suggests that the mechanism of microtubule stabilization is different from the mechanism of nucleation by the centrosome. The centrosome probably caps irreversibly the "minus" ends of microtubules, while stabilizing factors bind along the microtubule wall. Therefore, noncentrosomal microtubules having two ends free would be more sensitive to very low tubulin concentrations. This interpretation is also supported by the observation that stable centrosomal microtubules resist equally well to 1.6 and 33 μM nocodazole, while many noncentrosomal microtubules do not (Fig. 7).

Although the model discussed above for the generation of noncentrosomal microtubules fits well with our experimental observations, other explanations could be proposed: Noncentrosomal microtubules could be stabilized by local interactions with insoluble structures such as intermediate filaments or membrane sites for example (41). Some stable noncentrosomal microtubules could also arise by falling off the centrosome (43).

Microtubule Stability and α-Tubulin Detyrosination

It has been shown recently that cells contain two classes of microtubules that can be distinguished by antibodies directed against the tyrosinated and detyrosinated forms of α-tubulin (15). The most abundant class of microtubules contains mainly tyrosinated α-tubulin. A less abundant class in many cells is composed of long sinuous microtubules containing considerable amounts of detyrosinated α-tubulin. As recently shown, there seems to be a continuum in the ratio of tyrosinated and detyrosinated α-tubulin in all microtubules (13). It has been suggested that the microtubules strongly stained by the anti–glu antibodies are long lived, stable microtubules (13, 21). In the present paper we have accumulated evidence that this is indeed the case. In fact, it is likely that the turnover of the caboxy-terminal tyrosine of α-tubulin is tightly coupled to microtubule dynamics as proposed by Thompson (38). In vivo as in vitro (28, 29), the tubulin tyrosin ligase seems to function only on the dimer. In vitro the tubulin carboxypeptidase appears to function preferentially on the polymer (23). If in vivo the carboxypeptidase functions on the dimer, we must assume that this reaction is much slower than the tyrosination reaction since after microtubule depolymerization by nocodazole we detect almost no detyrosinated α-tubulin on immunoblots of total cell lysates. Moreover, three lines of evidence suggest that tubulin detyrosination occurs only on long-lived stabilized microtubules: (a) After nocodazole release, the first microtubules to regrow are tyrosinated. (b) The first detyrosinated microtubules appear after 30 min of regrowth while stable microtubules already exist 3 min after nocodazole removal. This time lag between microtubule polymerization and the appearance of the first detyrosinated microtubules is in agreement with results obtained in vitro (5). (c) Stabilization of microtubules with taxol leads to an increase in the number of detyrosinated microtubules and also to an increased amount of total detyrosinated tubulin.

This shows that anti–glu antibodies are good markers of long-lived microtubules and raises intriguing questions as to how the tubulin carboxypeptidase functions. In any case,

our results demonstrate that detyrosination is not the cause of reversible microtubule stabilization. Rather, microtubule stabilization appears to be a prerequisite to tubulin detyrosination, at least in MDCK cells. This may mean that the tubulin carboxypeptidase functions slowly. If a microtubule is short lived, the proportion of detyrosinated subunits may remain too low to be visualized by immunofluorescence. The amount of detyrosinated tubulin in a given microtubule will depend on its age. This may explain why nocodazole-resistant (stable) microtubules contain variable amounts of detyrosinated tubulin (Figs. 4 and 5). Indeed, the age of these stable microtubules could vary broadly. The sort of microtubule stabilization we term "reversible" in this discussion could be better named "short term". It is possible that detyrosination plays a direct or indirect role in the mechanisms involved in "long term" microtubule stabilization.

The respective functions of dynamic and stable microtubules are unknown. Our results would imply that in an interphase cell at steady state many dynamic microtubules are centrosome nucleated. Since the centrosome could be located in various places in the cell, it is tempting to speculate that together with its dynamic aster of microtubules, it constitutes a functional entity that can concentrate or keep concentrated specific cytoplasmic organelles in a given area of the cell. Indeed, by sending out new microtubules continuously and at random the centrosome can probe constantly the cytoplasmic space. The stable noncentrosomal microtubules could fulfill other transport or structural functions. It would be interesting to look for such microtubules in various cell systems undergoing specific morphogenetic rearrangements. They might play a scaffolding role as it seems to be the case for microtubules during myogenesis (44). The function of α-tubulin detyrosination is still unclear. The specific coupling that seems to exist between microtubule stabilization and detyrosination strongly suggests that this posttranslational modification plays an important functional role.

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