Non-centrosomal microtubule formation and measurement of minus end microtubule dynamics in A498 cells

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SUMMARY

Experiments performed on a cell line (A498) derived from a human kidney carcinoma revealed non-centrosomal microtubules in the peripheral lamella of many cells. These short microtubules were observed in glutaraldehyde-fixed cells by indirect immunofluorescence, and in live cells injected with rhodamine-labeled tubulin. The non-centrosomal microtubules were observed to form de novo in living cells, and their complete disassembly was also observed. Low-light-level fluorescence microscopy, coupled to imaging software, was utilized to record and measure the dynamic behavior of both ends of the non-centrosomal microtubules in these cells. For each, the plus end was differentiated from the minus end using the ratio of their transition frequencies and by measuring total assembly at each end. For comparative purposes, dynamics of the plus ends of centrosomally nucleated microtubules were also analyzed in this cell line. Our data reveal several striking differences between the plus and minus ends. The average pause duration was nearly 4-fold higher at the minus ends; the percentage of time spent in pause was 92% at the minus ends, compared to 55% at plus ends. Dynamicity was decreased 4-fold at the minus ends, and the average number of events per minute was reduced from 7.0 at the plus end to 1.5 at the minus ends. The minus ends also showed a 6-fold decrease in frequency of catastrophe over the plus ends. These data demonstrate that in living cells, microtubules can form at sites distant from the perinuclear microtubule organizing center, and once formed, non-centrosomal microtubules can persist for relatively long periods.

Key words: Microtubule dynamics, Minus end, Non-centrosomal microtubule

INTRODUCTION

Microtubules are cytoskeletal polymers composed of α and β tubulin heterodimers; this arrangement produces an inherent polarity in their structure, resulting in differences in their ends. In most cultured interphase animal cells, the minus ends of the microtubules are embedded in the perinuclear microtubule organizing center (MTOC) or centrosome, while the plus ends extend out in a radial array toward the periphery of the cell. The MTOC, or centrosome, is a major site of microtubule nucleation in cells, and isolated MTOCs can nucleate microtubule assembly under conditions where spontaneous microtubule formation does not occur (Mitchison and Kirschner, 1984b). In many cell types, however, a perinuclear MTOC and a radial arrangement of microtubules is not observed. For example, in polarized epithelial cells, the microtubule organizing material is dispersed across the apical region of the cell and an array of parallel microtubules extends from it toward the basal end of the cell (Bacallao et al., 1989; Bre et al., 1987). A reorganization of the microtubule nucleating material, from a conventional pericentriolar localization to one on the nuclear surface, is seen in multinucleate myotubes following myoblast fusion (Tassin et al., 1985). In higher plant cells, a well defined MTOC is absent; instead, the surface of the nucleus appears to be a major site of nucleation (Lambert, 1993). Plant cell microtubules can also assemble at sites other than the nuclear surface, consistent with the idea of a dispersed or flexible centrosome in these cells (Mazia, 1987). In other cases, the MTOC may function to nucleate microtubules, but the microtubules are subsequently released and rearranged. Perhaps the best example of this is in neuronal cells, where recent work indicates that axonal and dendritic microtubules are nucleated and assembled at the cell body’s MTOC, and then transported into the developing processes (reviewed by Baas, 1997; Sharp et al., 1995). This results in numerous microtubules in the axon that are no longer associated with the MTOC, and thus have two ‘free’ ends. In another example, that of nucleated erythrocytes, the microtubule array consists of a peripheral, hoop-like bundle of microtubules called the marginal band. These microtubules originate in association with a conventional MTOC and are subsequently released and rearranged into the characteristic peripheral bundle (Cohen, 1991).

Individual microtubule behavior is described by the model of dynamic instability, whereby stochastic transitions between assembly and rapid disassembly occur (Caplow, 1992; Erickson and O’Brien, 1992; Mitchison and Kirschner, 1984a). Although it is rarely observed with purified tubulin, an attenuated or paused state, where no loss or addition of tubulin subunits can be detected, has been observed in vivo (Shelden and Wadsworth, 1993; Walker et al., 1988). Dynamics of microtubule plus ends have been measured in vitro and in vivo by
various groups (Cassimeris et al., 1988; Sammak and Borisy, 1988; Schulze and Kirschner, 1986; Shelden and Wadsworth, 1993; Walker et al., 1988); the data clearly demonstrate that the dynamic behavior of microtubule plus ends is regulated throughout the cell cycle and is cell type specific (Belmont et al., 1990; Shelden and Wadsworth, 1993). Although the dynamic behavior of microtubule minus ends has been characterized in in vitro experiments (Kowalski and Williams, 1993b; Panda et al., 1996; Vasquez et al., 1994; Walker et al., 1989), the usual inaccessible position of minus ends in the centrosome has largely prevented previous measurement of dynamic instability parameters in vivo (Rodionov and Borisy, 1997).

In addition to dynamic instability behavior, microtubules also exhibit opposite-end assembly-disassembly behavior, or treadmilling (Margolis and Wilson, 1981). Recently, treadmilling of individual microtubules has been observed in experimentally produced cytoplasmic fragments which lack a conventional MTOC (Rodionov and Borisy, 1997). In addition, photoactivation experiments show the poleward movement of marked spots on spindle microtubules, a phenomenon termed poleward flux (Mitchison, 1989). For flux to occur, the loss and gain of tubulin subunits at opposite microtubule ends must be coupled to translocation of the entire microtubule lattice (Mitchison and Sawin, 1990). The demonstration of treadmilling in vivo and the observation of flux, both in vitro (Sawin and Mitchison, 1991) and in vivo (Mitchison, 1989; Mitchison and Salmon, 1992), directly demonstrate that subunits exchange at microtubule minus ends under some circumstances.

In the experiments reported here, we have observed the formation of individual, non-centrosomal microtubules in the peripheral lamellae of a human kidney carcinoma cell line. Our results demonstrate that individual microtubules can form at sites distant from the MTOC and that once formed, these microtubules can persist for many minutes. Since these microtubules are observed in an area of the cell that does not have a dense microtubule population, we were able to directly measure the behavior of both ends of these microtubules. Our data demonstrate that one end of the non-centrosomal microtubules was active, undergoing the process of dynamic instability. The other end was extremely stable, spending an average of 94% of the time in a state where no changes in length could be detected. The centrosomal and non-centrosomal microtubule plus ends in these cells were less dynamic than microtubules previously measured in various cells, supporting the view that microtubule stability may contribute to the persistence of non-centrosomal microtubules in these cells.

**MATERIALS AND METHODS**

**Materials**

All materials for cell culture were obtained from Gibco-BRL, Gaithersburg, MD, with the exception of fetal calf serum, which was obtained from Hyclone Laboratories, Inc., Logan, UT. A498 cells were the kind gift of Drs Mary Ann Jordan and Leslie Wilson, University of California, Santa Barbara. Unless otherwise noted, all other chemicals were obtained from Sigma Chemical Company, St Louis, MO.

**Cell culture and microinjection**

All cells were grown in 10% fetal calf serum and antibiotics, with 5% CO2 at 37°C. A498 and Ptk-1 cells were grown in 90% MEM, supplemented with 1.0 mM sodium pyruvate; A431 and BSC-1 cells were grown in 90% DME; CaOV-3 cells were grown in 90% DME, supplemented with 4.5 g/l glucose; CHO cells were grown in 90% Ham’s F-12. For use in experiments, cells were plated on etched glass coverslips (Bellco Glass Co., Vineland, NJ) 24-72 hours before use. For microinjection and subsequent observation, cells were transferred to medium containing 10 mM Hepes buffer, pH 7.3, lacking bicarbonate. Pressure microinjection was performed on a Zeiss IM-35 inverted microscope using a x32 phase objective lens, an Eppendorf 5242 microinjector and a Narishige micromanipulator. Needles were pulled from Omega Dot capillary glass tubes (Friedrich and Dimmock, Millville, NJ) on a Brown-Flaming P-80 micropipette puller. Rhodamine tubulin was microinjected at a needle concentration of 2.2 mg/ml; for co-injection experiments, fluorescent dextran (Molecular Probes, Eugene, Oregon) and tubulin were injected at needle concentrations of 0.1 mg/ml and 3.3 mg/ml, respectively. Solutions to be microinjected were centrifuged in an Eppendorf 5415C centrifuge at 14,000 rpm for 15 minutes immediately prior to injection, and the supernatant was transferred to a clean tube. Following injection, the cells were returned to a 37°C incubator for at least 2 hours to allow incorporation of the labeled tubulin into microtubules.

**Immunofluorescence**

Immunofluorescence localization of microtubules was performed on cells fixed in 0.25% glutaraldehyde in PBS for 1 minute, followed by brief lysis (1 minute) in a solution containing 0.5% Triton X-100, 1 mM MgSO4, 5 mM EGTA, 80 mM Pipes, pH 6.8, with additional fixation in 0.25% glutaraldehyde for 5 minutes. Cells were rinsed in PBS containing 0.1% Tween-20 and 0.02% Azide (PBS-Tw-Az) and then incubated for 1 hour at 37°C in a monoclonal anti-α-tubulin antibody (DM1a; 1:100 dilution in PBS-Tw-Az with 1% BSA). Cells were then rinsed in PBS-Tw-Az and incubated in affinity purified, fluorescein-conjugated, goat anti-mouse secondary antibodies (Organon Teknika, Durham, NC; 1:50 dilution in PBS-Tw-Az with 1% BSA) for 30-45 minutes at room temperature. Stained cells were mounted in 90% glycerol, containing 0.1% p-phenylene diamine, and sealed with nail polish. Immunofluorescent preparations were observed and photographed on a Zeiss Axiovert microscope using a x63 neofluor objective lens and TMax 400 film. To obtain images for reproduction, 35mm negatives were digitized using a Sprint Scan-35 negative scanner and Adobe Photoshop software running on a Macintosh IIfx and printed using a Tektronix Phaser 440 printer.

**Preparation of rhodamine-labeled tubulin**

Rhodamine-labeled tubulin was prepared as described previously (Shelden and Wadsworth, 1993). Briefly, phosphocellulose purified porcine brain tubulin (Sloboda et al., 1976) was assembled in a buffer containing 100 mM Pipes, 10 mM MgSO4, 2 mM EGTA, 4 M glycerol, and 1 mM GTP, pH 6.9, and then incubated for 10 minutes at 37°C with a 40-fold molar excess of 5,6-carboxy succinimidyl ester of rhodamine (Molecular Probes, Eugene, OR). The fluorescent microtubules were collected by centrifugation (100,000 g for 2 hours) through a 40% sucrose cushion. The resulting pellet was resuspended in a buffer containing 1 M glutamate, 1 mM EGTA and 0.5 mM MgSO4, at pH 6.9, and disassembled on ice for 30 minutes. The protein was centrifuged for 45 minutes at 100,000 g, and the supernatant was retained. The supernatant was then carried through 2 cycles of temperature-dependent assembly and disassembly. The final pellet was resuspended in injection buffer (20 mM glutamate, pH 7.0, 0.5 mM MgSO4, and 1 mM EGTA) and stored at ~80°C in small aliquots.

**Low-light level fluorescence microscopy and data analysis**

Images of rhodamine-labeled microtubules in the injected cells were obtained as previously described, using a x100 1.3 NA apochromat lens (Shelden and Wadsworth, 1993). A Dage ISIT video camera, operated at maximum gain, was used to collect images, which were digitized using a Perceptrics PixelPipeline card in a Macintosh Quadra 950 running BDS Image (Oncor Inc., Gaithersburg, MD) software. Thirty-two frame averages were collected at 2 second intervals, stored on a Perceptrics PixelBuffer store card, and subsequently transferred...
to optical discs. For coverslips to be immunostained for total tubulin following live cell imaging, only one fluorescence image was collected. In order to facilitate re-location of the same cell following staining, a phase image was also collected using a Dage STT video camera. Coverslips were fixed within 2 minutes of fluorescence image collection and stained as described above under Immunocytochemistry. The previously imaged cell was identified and a second set of phase and fluorescence images, to verify the location and existence of the non-centrosomal microtubule, was collected as described above.

Quantification of microtubule dynamic behavior was performed using laboratory-written software (Shelden and Wadsworth, 1993). The position of an individual microtubule in a sequence of images was marked using a mouse-driven cursor; the position information was used to create a microtubule ‘life history’ plot using the Cricket Graph program (Cricket Software, Malvern, PA). Phases of growth, shortening and pause were marked on the plots; additional in-house software was used to determine the duration, distance, and rate of growth and shortening events and the duration of pause events. Due to the detection limits of this method, only changes of greater than 0.5 µm were considered growth or shortening events; we estimate that the initial bright dot during non-centrosomal microtubule formation must be approximately 0.5 µm (or 800 tubulin dimers) to be detected. Data for each microtubule was entered into a spreadsheet (Kaleidagraph, Synergy Software, Reading, PA) for statistical analysis. The frequency of catastrophe was determined by dividing the sum of growth to shortening and pause to shortening transitions, by either the sum of the distance grown or the sum of the time in growth and pause. The frequency of rescue was determined by dividing the sum of shortening to growth and shortening to pause transitions, by the time spent shortening or the distance shortened. Dynam- icity was calculated by dividing the sum of the total length grown and shortened, by the time of observation of the particular microtubule.

To correlate the amount of unpolymerized fluorescent tubulin with cell thickness, images of cells co-injected with fluorescein dextran and rhodamine tubulin were collected. The thicker edge and the flatter area adjacent to it were compared using the ratios of pixel intensity per unit area between the two regions. These were determined by selecting a portion of both regions of the cell with the cursor and measuring the fluorescence intensity in the same regions of both the fluorescein and rhodamine images. The ratio of the rhodamine to fluorescein intensity was then determined and compared.

RESULTS

Morphology of A498 cells and formation of non-centrosomal microtubules

The morphology of A498 cells is shown in Fig. 1. Many cells have an unusual morphology in the form of phase dense regions in the peripheral lamellae (Fig. 1A), which are transient, forming and dispersing over time. A498 cells have what appears to be a conventional MTOC, as determined by immunofluorescence staining for pericentrin (data not shown), and a normal, though dense, radial array of microtubules. We have observed non-centrosomal microtubules of varying lengths both in the periphery of living cells which had been microinjected with fluorescently labeled tubulin (Fig. 1C-E) and in uninjected, glutaraldehyde-fixed cells by indirect immunofluorescence (Fig. 1B), thus eliminating the possibility that their formation was an artifact of tubulin microinjection. In both preparations, a rim of soluble tubulin fluorescence was frequently observed at the cell periphery (Fig. 1E). To determine whether this was caused simply by an increase in cell thickness in that region, or by some specific compartmentalization phenomenon, fluorescein-labeled dextran was co-injected with rhodamine-labeled tubulin. The dextran diffuses throughout the cytosol, providing a marker for cytoplasmic volume. The ratios between the intensities of dextran and tubulin in the thick lamella and in the area proximal to it were compared. Since the ratio was not statistically different between the two regions, the increased lamellar brightness must be due to increased volume in that area (data not shown). Non-centrosomal microtubules are often observed in these peripheral regions.

We have observed the formation of non-centrosomal microtubules in living cells injected with rhodamine-labeled tubulin. Forming non-centrosomal microtubules (Fig. 2) usually appear as bright spots, and elongation occurs unidirectionally from that point. It is interesting to note that non-centrosomal microtubule disassembly (Fig. 2A) occurs in that manner also, from one end back toward the other, rather than from both ends toward the center. The possibility that a segment of a longer microtubule coursing in and out of the plane of focus was mistaken for non-centrosomal microtubule formation was eliminated by through-focusing in the region where de novo microtubule formation was observed. Because some cells contain stable populations of microtubules that turn over very slowly (Webster and Borisy, 1989), it was necessary to confirm that these are truly non-centrosomal microtubules, and not just fluorescent segments assembled onto the ends of microtubules that had not incorporated rhodamine-tubulin. In order to do this, live cells were microinjected with rhodamine-labeled tubulin and allowed to recover for at least 2.5 hours before an

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**Fig. 1.** Morphology of A498 cells. (A) Phase-contrast light microscopy of live cells; many cells have phase-dense peripheral lamella. (B) Immunofluorescence observation of glutaraldehyde-fixed cells stained with an antibody to α-tubulin. (C-E) Low light level fluorescence microscopy of live cells injected with rhodamine tubulin. In many cells, the microtubule array does not extend all the way to the cell edge. Non-centrosomal microtubules of varying lengths can be observed at the cell periphery of both fixed and living cells (B-E). The thicker edge in (E) is brighter due to an increased amount of soluble fluorescent tubulin. Bars: 50 µm (A); 20 µm (B); 2 µm (C-E).
image of a non-centrosomal microtubule was collected. Following fixation and immunofluorescence staining for tubulin, the same microtubule was located and imaged again. The results are shown in Fig. 3. In no case was a “free” microtubule in a live cell proven, by immunocytochemistry, to be part of a longer microtubule (n=6).

Non-centrosomal microtubule formation was relatively infrequent: in 135 minutes of observation, 19 instances were observed, a frequency of one formation per 7.1 minutes. Interestingly, complete disassembly of non-centrosomal microtubules was twice as rare as their formation. It occurred only 9 times, a frequency of one disassembly per 15 minutes of observation. So once formed, non-centrosomal microtubules are persistent; we have seen them persist for as long as 17 minutes (data not shown). This is not surprising, in view of the fact that they are present in A498 cells more often than not, but we rarely observe their formation. During observation of this cell line for this and other studies, formation of non-centrosomal microtubules by severing of longer microtubules has been observed only once. Therefore, it is unlikely that this is the primary mechanism for their formation. Finally, non-centrosomal microtubules could arise by transport, to the cell periphery, of microtubules which were nucleated at and released from the MTOC. We did not observe either centrifugal or centripetal motion of non-centrosomal microtubules in these experiments, making transport unlikely as the mode by which these microtubules arise.

We estimate that these short non-centrosomal microtubules exist in 65% of A498 cells at any given time. This value is likely an underestimate because we cannot determine if non-centrosomal microtubules are present in the central regions of the cell, where the microtubule density is high. We have also examined other cell lines for the presence of non-centrosomal microtubules using preparations of glutaraldehyde-fixed cells.
stained with anti-tubulin antibodies and living cells injected with rhodamine-labeled tubulin. Non-centrosomal microtubules have been observed in A431, BSC-1, CaOV-3, CHO and PtK1 cells at varying frequencies, but in all cases, less commonly than in A498 cells (data not shown). The occurrence and certainly the observation of non-centrosomal microtubules may be related to cell morphology. They are more frequently observed in cells characterized by a spread, flattened periphery containing a sparse microtubule array.

Dynamics of non-centrosomal microtubules in vivo
Since both ends of non-centrosomal microtubules were accessible, we were able to study dynamic behavior at the plus and minus ends of the same microtubule, which allowed a distinction to be made between the two so that the populations could be compared. The position of each end of non-centrosomal microtubules was tracked using a mouse-driven cursor, and plotted versus time (Figs 4, 5). In 75% of the microtubules analyzed, one end had no growth or shortening events at all. (An event is defined as an excursion greater than or equal to 0.5 μm.) In the remainder of the cases, one end had significantly fewer transitions between growth and shortening or vice versa, than the other. For each microtubule, the ratio of the transition frequencies was used to differentiate the active end from the inactive end. We also determined the total growth at each end of the non-centrosomal microtubules: in each case, the end

![Fig. 4. Dynamic behavior of individual non-centrosomal microtubules. Images were collected at 2 second intervals; elapsed time, in seconds, is indicated in the corner of each panel. The asterisks were placed at the same pixel location in each panel to show the stability of one end of the non-centrosomal microtubule. The other end undergoes dynamic changes: in both A and B, it grows between the first and second panels, then shortens between the second and third panels, and grows again between the third and last panels. Partial closure of the field diaphragm is responsible for the dark areas on the left side of the panels in B. Bar, 2 μm.](image-url)
that showed greater total growth was also the active end, as defined above. Over a 33-minute period of measurement, the average growth per microtubule at the active end was 1.5 μm, while that at the inactive end was 0.2 μm. Thus, on the basis of transition frequencies and net growth, we designated the active ends the plus ends, and the inactive ends the minus ends (Bergen and Borisy, 1980; Horio and Hotani, 1986). It should be noted that, in some cases, our limit of resolution may preclude distinction between minus end growth or shortening and slight translocation of the microtubule as a whole. Dynamics of the plus ends of radially extending microtubules, which were most likely centrosomally nucleated, were also analyzed in this cell line and compared with the dynamics of the plus ends of non-centrosomal microtubules. The two sets of data correspond closely, reinforcing the correctness of our original classification of the plus and minus ends (Fig. 6 and Table 1). It should be noted that in most cases, the non-centrosomal microtubules used for dynamic analysis were present at the start of image acquisition, and their formation was not observed.

Fig. 5 is a representation of the types of behavior observed at both ends of non-centrosomal microtubules. In many cases, the plus end was very active, while the minus end was completely inactive (Fig. 5A). Some minus ends did undergo events (Fig. 5B), but could still be distinguished from the plus end based on the frequency of transitions. Occasionally, a plus end displayed relatively little dynamic behavior, however, its minus end experienced no transitions at all (Fig. 5C). The low number of events observed at one end of each non-centrosomal microtubule is highlighted in Fig. 7. The total number of growth events at the plus ends was 52, compared with 7 at the minus ends; there were only 9 shortening events at the minus ends, as opposed to 52 at the plus ends.

As Table 1 indicates, dynamic analysis revealed striking differences between the plus and minus ends in several parameters. The pause duration was nearly 4-fold higher at the minus ends; and consequently, the percentage of time spent in pause...
increased to 94% at the minus ends, compared to 58% at the plus ends. Also, the dynamicity was decreased by a factor of 8 at the minus ends; and, the average number of events per microtubule was reduced from 6.4 at the plus ends to 1.1 at the minus ends. These parameters reflect the very stable nature of the minus ends. Perhaps the most intriguing result was the nearly 7-fold decrease in catastrophe frequency at the minus ends, compared with the plus ends. Although greatly suppressed at the minus ends, when a catastrophe did occur, it was rescued with essentially the same frequency as at the plus ends. It should be noted that the rescue frequency at the minus ends was calculated on the basis of a small number of events since catastrophe was so rare.

Predictably, there were no statistically significant differences in the rates of growth and shortening between the centrosomal and non-centrosomal microtubule plus ends in A498 cells (Table 2 and Fig. 6). The non-centrosomal microtubule plus and minus ends also showed no significant rate differences (Table 2 and Fig. 7), a result corroborated by previous in vitro studies which showed notable overlap in the growth and shortening rate distributions of plus and minus ends (Kowalski and Williams, 1993b). Comparison of the growth and shortening rates of A498 microtubules with those of other cell lines (Table 2) highlights the variability in the parameters of microtubule dynamic instability from one cell type to another. The microtubule growth rate in A498 cells falls between that in untreated BSC-1 cells and that in MAP2-injected BSC-1 cells, and is slower than in PtK1 or CHO cells. The rate of shortening measured in A498 cells is slower than in any of the other cell types, even those microinjected with MAP2.

### DISCUSSION

Our data clearly demonstrate that non-centrosomal microtubules can form in the peripheral lamella of A498 cells. Once formed, these microtubules persist, allowing the dynamic behavior of each end of the microtubule to be analyzed. We find that the minus end of these microtubules is extraordinarily stable: in the majority of cases, it did not grow or shorten at all; the catastrophe frequency was remarkably low; and, every catastrophe we observed was rescued.

### Comparison of minus end dynamic Instability in vitro and in vivo

The dynamic instability behavior of microtubule plus and minus ends has been measured previously in vitro, using purified brain tubulin (Panda et al., 1996; Vasquez et al., 1994; Walker et al., 1988). The data reveal differences in the dynamic behavior at the two microtubule ends: at the minus end, the association and dissociation rate constants are lower and the rescue frequency is higher than at the plus end, at all concentrations of tubulin examined. The frequency of catastrophe is greater at the plus end at low tubulin concentrations, but this difference is negligible at higher tubulin levels (Walker et al., 1988). Because these experiments are performed using purified tubulin, the differences between the plus and minus ends cannot be attributed to regulatory molecules in the preparation. Rather, these differences must result from the orientation of tubulin dimers in the microtubule lattice (Mandelkow and Mandelkow, 1989). Tubulin dimers contain one non-exchangeable GTP on the α subunit, and an exchangeable GTP, which is hydrolyzed subsequent to incorporation into the microtubule lattice, on the β subunit (reviewed by Caplow, 1992).

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<th>Table 1. Microtubule dynamic instability parameters in A498 cells*</th>
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<tr>
<td>Pause duration (seconds)</td>
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<td>% Time shortening</td>
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<td>Average events/MT</td>
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<td>Dynamicity (dimers/second)</td>
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<td>Rescue frequency (second)</td>
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<td>Catastrophe frequency (second)</td>
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<td># of MTs analyzed</td>
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<td># of cells analyzed</td>
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*Standard deviations are not reported because these values were calculated for a population of microtubules, rather than on an individual basis.

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<table>
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<tr>
<th>Table 2. Microtubule growth and shortening rates</th>
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<tr>
<td>Growth rate (μm/minute)</td>
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<td>-------------------------</td>
</tr>
<tr>
<td>A498 centrosomal plus ends</td>
</tr>
<tr>
<td>A498 non-centrosomal plus ends</td>
</tr>
<tr>
<td>A498 non-centrosomal minus ends</td>
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<tr>
<td>PtK1 plus ends*</td>
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<td>CHO plus ends*</td>
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<td>BSC-1 plus ends†</td>
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<td>BSC-1 + 1.0 mg/ml MAP2†</td>
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†From Dhamodharan and Wadsworth (1995).
immunofluorescence studies, using an antibody to the N-terminus of α-tubulin (Fan et al., 1996), confirm previous results showing that β-tubulin, with its exchangeable GTP, is exposed at the plus end of the microtubule (Mitchison, 1993). Given this orientation of the dimer within the microtubule lattice, the enhanced stability of minus ends, relative to plus ends, could be the result of having a non-hydrolyzable GTP in the terminal position. The hypothesis that differences in the structure of the two microtubule ends contribute to differences in their behavior is supported by experiments in which new minus and plus ends are created by severing microtubules with a u.v. microbeam. Newly created minus ends are initially stable, and then grow slowly; newly created plus ends, which lack a GTP cap, rapidly disassemble (Walker et al., 1989).

The behavior of the minus ends we have measured in vivo can be compared with previous observations of minus end behavior in vitro. In A498 cells, the catastrophe frequency is 6-fold higher at the plus end than at the minus end, a difference greater than that measured using purified tubulin in vitro (Panda et al., 1996; Vasquez et al., 1994; Walker et al., 1988). Interestingly, the catastrophe frequency of the minus ends in vivo, while greatly suppressed relative to that of the plus ends, is actually greater than that reported for minus ends in several in vitro studies (Panda et al., 1996; Vasquez et al., 1994; Walker et al., 1988). Regardless of the exact values, the minus ends in this study are highly stable, especially when compared with the plus ends. This stability probably results from the inherent structural differences, as discussed above, which could directly modulate the kinetic behavior of the two ends. Differences in the structure of the two ends of the microtubule could also affect microtubule behavior indirectly, by modulating the interactions of regulatory molecules with microtubule ends. In support of this view, several molecules are now known to differentially affect the two microtubule ends: XMAP stimulates microtubule turnover by increasing the association and dissociation rate constants at the plus end (Vasquez et al., 1994), XKCM1 stimulates the frequency of catastrophe at the plus end (Walczak et al., 1996), and Kar3 stimulates disassembly at the minus end (Endow et al., 1994; Saunders et al., 1997). Recent studies also demonstrate that the drug vinblastine differentially affects the two ends, stabilizing the plus end while increasing turnover at the minus end (Panda et al., 1996). Cellular molecules that stimulate catastrophe transitions at the plus end could contribute to the differences that we observe in vivo in the behavior of the plus and minus ends. Such a scenario would be consistent with the observation that the rescue frequency is comparable at the two microtubule ends, while the catastrophe frequency is different.

Although minus end events were uncommon, when non-centrosomal microtubules underwent events, those events were statistically indistinguishable in rate, duration and distance (data not shown) at the plus and minus ends. The release or destabilization of a minus end dimer, possibly caused by a Kar3-like factor or loss of a cap, would result in dynamics which would then presumably be regulated by the MAPs bound along the microtubule wall, the same molecules which influence plus end dynamics.

Comparison of minus end behavior with previous in vivo observations
Historically, the behavior of minus ends has been difficult to study in vivo. Typically, a high density of minus ends is associated with the MTOC, making imaging of individual minus ends in living cells extremely difficult. Electron microscopic analysis has shown that minus ends are sometimes found at sites distant from the MTOC: in neuronal cells, minus ends are found along the length of the axon (Yu et al., 1993) and in mammalian spindles, the minus ends of interpolar microtubules are located throughout the spindle region (Mastronarde et al., 1993). There have also been reports in cultured cells of microtubule minus ends not associated with the centrosome in the interphase array (Bre et al., 1987). In all of these cases, the density of microtubules precludes the analysis of individual minus end dynamic behavior in vivo. Recent work in cultured mammalian cells demonstrated the release of microtubule minus ends from the MTOC, and their subsequent behavior in that context (Keating, 1997).

One approach to analyzing microtubule minus ends in vivo has been to create new ends using u.v. irradiation or mechanical cutting (Nicklas et al., 1989; Spurck et al., 1990; Tao et al., 1988; Wilson and Forer, 1988). In most cases, the newly created minus ends are stable, neither growing nor shortening detectably. However, in the case of crane fly spermatocyte meiotic spindles, poleward motion of the irradiated region has been observed; this could result from minus end growth (Wilson and Forer, 1988). One explanation for the behavior of minus ends in this and other studies is that, due to the structure of the minus end, the transition to rapid shortening is kinetically unfavorable; therefore, these ends either grow slowly or remain in an attenuated state, during which no growth or shortening is detectable (Toso et al., 1993). Alternatively, new minus ends might be rapidly and efficiently capped by molecules that reduce the likelihood of catastrophe.

Additional information regarding the dynamic behavior of minus ends in living cells comes from analysis of flux and treadmilling of microtubules (Mitchison, 1989; Rodionov and Borisy, 1997; Sawin and Mitchison, 1991, 1994; Keating, 1997). Fluorescent marks, created on spindle microtubules by photoactivation of caged tubulin, are observed to move poleward at a rate of about 0.5 μm/minute; this concurrent plus end subunit assembly, minus end subunit disassembly, and translocation poleward of the entire microtubule lattice has been termed poleward flux (Mitchison, 1989). In fragments of interphase cells lacking centrosomes, microtubules with two free ends are observed; photobleaching experiments demonstrate that the apparent cytoplasmic translocation of these microtubules results from the coordinated gain and loss of subunits at the two ends (Rodionov and Borisy, 1997). Experiments in which a laser-bleached spot was created on microtubules released from the MTOC revealed stable or shortening minus ends, in conjunction with microtubule translocation (Keating, 1997). In all of these cases, net loss of subunits occurs at the minus end. Specific regulatory components, for example Kar3-like factors, might be required to facilitate subunit turnover at minus ends (Saunders et al., 1997). Alternatively, minus end capping factors could be released, resulting in minus end turnover. We have observed no translocation of the non-centrosomal microtubules through the cytoplasm, as might be expected for treadmilling, and the dynamic behavior of the inactive end was not coordinated with the opposite, active end. Thus, if treadmilling is modulated by specific non-tubulin factors, either these are not active in A498 cells, or the ends are inaccessible to them.
Stability of microtubules in A498 cells and persistence of non-centrosomal microtubules

The data reported here show that the plus ends of both non-centrosomal and centrosomal microtubules in A498 cells are considerably more stable than the plus ends of microtubules in other cell lines. For example, the rescue frequency is 58% higher and the catastrophe frequency is 56% lower at the plus ends in A498 cells than in a non-cancerous kidney cell line, BSC-1 (Dhamodharan and Wadsworth, 1995). In addition, the elongation rate of microtubules in A498 cells is slower than in any other cell line studied, with the exception of MAP2-injected BSC-1 cells, and the shortening rate is the lowest overall (Table 2). One possible explanation for the remarkable stability of microtubules in A498 cells is that high levels of stabilizing MAPs contribute to the low level of microtubule dynamics in these cells. Neuronal MAPs, MAP-2 and tau, suppress microtubule dynamic instability by promoting rescue and decreasing catastrophe transitions (Drechsel et al., 1992; Kowalski and Williams, 1993a; Pryer et al., 1992). Recent in vitro experiments show that the major non-neuronal MAP, MAP-4, increases rescue transitions (Ookata et al., 1995). Alternatively, it has been shown that tubulin isotype composition plays a role in microtubule dynamics (Panda et al., 1994). Perhaps A498 cells express an isotype of α or β tubulin which confers unusual stability on their microtubules. Regardless of its cause, the low level of microtubule dynamic behavior in these cells almost certainly contributes to the persistence of non-centrosomal microtubules. For example, following a catastrophe, the disassembly rate is slow and the rescue frequency is comparatively high, so more often than not, the microtubule is only partially disassembled.

Origin of non-centrosomal microtubules

A compelling question to emerge from this study is how and why do non-centrosomal microtubules form in A498 cells? It seems likely that their formation is the result of an alteration in some aspect(s) of the usual regulation of microtubule nucleation and/or dynamics. One possible explanation of non-centrosomal microtubule formation in A498 cells is that centrosomal components with nucleating activity exist outside of the MTOC. Exciting new work has identified a microtubule nucleation complex, termed γ-tubulin ring complex (γTuRC), which contains γ-tubulin, α and β tubulin, and at least four other proteins (Moritz et al., 1995; Zheng et al., 1995). An intriguing hypothesis is that A498 cells have active γTuRCs which are dissociated from the centrosome and could act to nucleate non-centrosomal microtubules. Two recent pieces of work support this hypothesis. First, Shu et al. (1995) report a transient association of γ-tubulin with the minus ends of midbody microtubules, and suggest that γ-tubulin acts to nucleate microtubules at sites distant from the centrosome. Second, in extracts of two human cell lines, most of the γ-tubulin exists in the cytosolic, rather than centrosomal, fractions (Moudjou et al., 1996; Stearns et al., 1991; Zheng et al., 1991). However, much of this soluble pool is associated with the chaperone Tcp1, and there is no indication that these γ-tubulin-containing particles are actually functional nucleators (Moudjou et al., 1996). Finally, it should be noted that a nucleating complex, such as γTuRC, could potentially act as a minus end cap which, when associated with the microtubule end, could act to suppress addition or loss of subunits.

An alternative explanation for the formation of non-centrosomal microtubules is that tubulin or assembly-promoting MAPs are over-expressed in these cells, decreasing the critical concentration for spontaneous microtubule assembly (Murphy et al., 1977). If this were the case, centrosomal polymerization would still be favored, due to the presence of nucleation sites in the pericentriolar material, but non-centrosomal polymerization could occur. Non-centrosomal microtubule formation has been previously observed in cases when the tubulin concentration is above that which is critical for spontaneous polymerization: following release from drug induced disassembly (Bre et al., 1987), and in cytoplasts lacking an MTOC (Karsenti et al., 1984). Additional data on the soluble tubulin concentration, the ratio of unpolymerized to polymerized tubulin, and/or the concentration of MAPs in these cells is necessary to draw any conclusions in this regard. Finally, the possible lack of certain regulatory molecules could also contribute to spontaneous microtubule assembly. Recent work in sea urchin eggs has identified an activity which blocks assembly at microtubule minus ends (Spittle and Cassimeris, 1996). The authors hypothesize that a tubulin dimer-binding protein is present in vivo and functions to prevent spontaneous polymerization. If cells were deficient in such a factor, non-centrosomal microtubules could form freely; this might be the case with A498 cells.

The extremely slow dynamic turnover of microtubules in A498 cells suggests another interpretation of why they are found in these cells. In this scenario, the inherent stability of microtubules in these cells might contribute to spontaneous formation of non-centrosomal microtubules. This is consistent with the observations that taxol, which suppresses the dynamics of microtubules, induces the formation of non-centrosomal microtubules in cells (De Brabander et al., 1981). Once formed, the low level of dynamic turnover would increase the probability that they persist. In fact, non-centrosomal microtubules appear to arise, at some frequency, in diverse cells; in most cases however, dynamic instability probably ensures their rapid disappearance, resulting in less persistent and fewer detectable non-centrosomal microtubules. Thus, the parameters of dynamic instability in A498 cells might increase both the probability that a stable nucleus of a microtubule might form and that it might persist in the cytoplasm.

Regardless of the mechanism by which the non-centrosomal microtubules form, their formation does not induce a detectable disruption of the nucleated array and both the non-centrosomal and centrosomal microtubules co-exist in the same cytoplasm. Our data, and recent observations of microtubule treadmilling in cytoplasmic fragments which lack an MTOC (Rodionov and Borisov, 1997), strongly suggest that the type of microtubule dynamic turnover which predominates in a given array can be modulated by alterations in the quantity or activity of nucleating factors, of tubulin, and of regulatory factors such as MAPs.

Conclusion

In conclusion, we have observed and quantitated the dynamic behavior of microtubule minus ends in vivo: the data directly demonstrate that individual minus ends in vivo are remarkably stable and suggest that their stability is intrinsic to their structure, possibly due to a ubiquitous GTP cap. Further, we
have observed the de novo formation of microtubules at sites distant from the centrosome. This work has served to challenge the traditional doctrine that microtubules are nucleated solely at MTOCs and offers an example of a case in which that is not so.

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